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Squamous Cell Carcinoma

Edited by Xiaoming Li



SQUAMOUS CELL CARCINOMA

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Meet the editor



Xiaoming Li, MD, PhD is now Professor and Director of Department of Otolaryngology Head & Neck Surgery, Bethune International Peace Hospital, China. He is now the Head of Surgical Head and Neck Oncology Section, Chinese Otolaryngology Head and Neck Surgery Society. His special interest in clinic is surgical management of head and neck cancers including tumor resection and one-stage reconstruction. His expertise in surgical salvage for advanced recurrent head and squamous cell carcinomas (HNSCC) is recognized in that he gave two special talks in round table session on reconstructive surgery for HNSCC and one keynote lecture on salvage surgery of recurrent HNSCC in recent two IFOS world congresses. Over the years, he conducted a series of studies on the clinopathologic features of HNSCC, defining some important factors for predicting metastasis and recurrence. He also devotes himself to in-depth investigation on molecular aspects of tumor invasion and progression, and therapeutic resistance of HNSCC. He was a research scientist from 1999 to 2002 in the National Cancer Institute (NCI), NIH, USA. He was visiting professor of several universities in the world. He published a paper in *Nature* in 2003 for definition of a new pathway on TNF receptor signaling. His research work is now focusing on genetic manipulations of resistance of HNSCC to chemoradiation by targeting some genes and cell populations such as cancer stem cells.

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Preface

Squamous cell carcinoma (SCC) represents a common malignancy of mankind. It originates in the squamous epithelia covering the external surface of the body or lining the inner surface of lumen of different body systems. Because of the diversity in the location of the primary tumors, patients may present with distinct clinicopathologic manifestations, making it necessary to manage them individually and multidisciplinary. Even with marked advances in the understanding of the disease and treatment modalities, there are still many difficult problems to solve and unclear questions to address.

This book is unique in that it supplies an in-depth discussion of a series of interesting topics on SCC. Firstly, the features and management of some specific SCC is discussed to give the readers the general principles in dealing with these uncommon and sophisticated conditions. Some new concepts in adjuvant therapy including neoadjuvant therapy and gold nanoparticle-based photo dynamic therapy are introduced, the latter of which shows a great promise in the treatment of SCC. Secondly, a detailed discussion of molecular aspects of tumor invasion and progression in SCC is provided with the emphasis on the roles of some important factors. The role of tumor microenvironment in head and neck SCC is specifically discussed, which is comprised of cellular and non-cellular components. Thirdly, as a relatively new concept, the roles of cancer stem cells (CSC) in cancer therapy of SCC are described. Molecular mechanisms involving therapeutic resistance and new therapeutic strategies targeting CSC are discussed in detail. Finally, other aspects concerning SCC are also included, which involve the assessment, genetic manipulation and its possible clinical implications for the treatment of SCC. It is really our hope that the book can point to some new areas deserved for further investigation on SCC, and much progress in the understanding and treatment of the disease be made in the near future.

To be frank, it is really such an extensive topic to discuss about SCC. Moreover, within a book containing limited chapters, we are definitely unable to cover nearly all related issues on SCC. Our intent in writing the book "Squamous Cell Carcinoma" is to provide the readers with not very comprehensive but exclusive presentations on some special issues in the recent advances in the study of SCC. Perhaps, many important parts concerning diagnosis and treatment of SCC have been missed, which seems to be our regret for this book.

I wish to give special thanks to all contributors of the book around the world. Without the great efforts and excellent work they made, the publication of the book would not have been realized. I finally express my love and thanks to my wife and other family members for their constant understanding, support, love and patience in helping such a project to a final success.

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Part 1

Features and Management of Some Specific Squamous Cell Carcinomas

Metastasis of Head and Neck Squamous Cell Carcinoma

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and accounts for approximately 650,000 new diagnoses and 350,000 cancer deaths every year (Parkin, et al., 2005). In the United States, HNSCC accounts for approximately 5% of all cancer cases diagnosed per year. Even with significant advances in operative skills such as reconstructive microvascular free tissue transfer, and in adjuvant therapies such as hyperfractionated radiotherapy and concomitant chemoradiation, the 5-year survival of the HNSCC has not been markedly improved in the past three decades. The number of annually diagnosed cases amounts to over 42,000 individuals and results in more than 12,000 deaths per year in the United States. As is known, HNSCC is a locoregional disease notoriously for regional and distant metastases, representing the leading cause of death in HNSCC patients. Although surgical resection of isolated metastases is beneficial for some patients, the overall efficacy of surgery, chemotherapy or radiotherapy is still limited. The main reason for the poor 5-year survival may be that the most important prognostic factors for these patients are not only local control, but regional and distant metastases as well.

2. Development of metastasis

Metastasis is defined as the spread of disease from one organ or part to another not directly connected with it through the blood, lymph, or serosal surfaces. Recent investigations disclose the mysterious aspects of the cancer metastasis. The development of metastasis of tumor is a multi-step process, in which multiple genes participate in and play different roles. With regard to the regional lymph node metastasis, several important gene proteins related to microvascular angiogenesis and lymphogenesis function as promoters of regional lymph node metastasis. For the regional metastasis to occur, it is necessary for tumor cells to enter the microvessels to gain the pathway to the lymphatic channels. After entering the tumor-draining lymphatic channels, the tumor cells migrate to the regional lymph nodes in the neck, in which they settle and form the foci of micrometastasis. In the event of distant metastasis, several processes determine the tumor spreading to other organ systems, including angiogenesis, tumor invasion into local stroma and vascular system, circulation of tumor cells, arrest of tumor cells at distant site, and colony formation at secondary site. It is obvious that tumor invasion into stroma and vascular system is a prerequisite to the

development of distant metastasis, which involves attachment of tumor to the basement membrane, degradation of extracellular matrix components, migration of malignant cells to the stroma and ultimate invasion to the surrounding blood vessels or lymphatic channels. Recently, results from several studies indicate that the disseminated cancer cells alter their adjacent stroma into a “metastatic” microenvironment that is similar to the primary tumor microenvironment in which they can survive and proliferate. A better understanding of the gene expression pattern and molecular biologic mechanisms of metastasis in HNSCC may be beneficial for exploration of new effective therapies to prevent the development of metastasis and to improve the survival of these patients.

2.1 Genes involved in metastasis

Development of metastatic carcinoma is associated with masses of molecules involved in cell adhesion, migration, and invasion in HNSCC. Further insight into the molecular basis of metastasis in HNSCC could lead to advances in screening, diagnosis, and treatment with improved clinical outcome. Multiple gene products are involved in angiogenesis, all of which have been demonstrated to be critical for regulating angiogenic phenotype. This has raised the need for comprehensive analysis of the angiogenic phenotype using microarray analysis and global proteomic approaches. Complex interplay between positive and negative regulators determines the degree of neovascularization in and around the tumor. And now emerging evidence suggests that the lymphangiogenic factors may also play important roles in lymph node metastasis in many cancers.

2.1.1 Vascular endothelial growth factor (VEGF)

As a key regulator of angiogenesis, the role of VEGF has been extensively studied. Tumor cells enter the circulation by penetration through proliferating capillaries that have fragmented basement membrane. Further progress in this multi-step cascade is controlled by the positive and negative regulators of angiogenesis. Recent studies have shown that VEGF receptor-expressing cells from the bone marrow arrive at a specific site of future metastasis even prior to arrival of metastatic cells (Ellis, 2008). First and foremost, VEGF is a highly potent angiogenic agent that acts to increase vessel permeability and enhance endothelial cell growth, proliferation, migration and differentiation (Johnstone & Logan, 2007). In addition, VEGF promotes angiogenesis in many different tumor types. VEGF levels may affect tumor growth, metastatic potential, and response to radiotherapy. VEGF expression may prove to be an important prognostic factor in head and neck cancer (Smith, et al., 2000); VEGF positivity is the most significant predictor of poor prognosis. Accordingly, the potent role of VEGF in angiogenesis has spurred interest in using this molecule as a therapeutic target in antiangiogenic therapy.

2.1.2 Matrix metalloproteinases (MMP)

As is known, MMP has the ability to degrade connective tissues such as the basement membrane, which is a crucial step in the initiation of metastatic process, thus serving as a positive regulator of metastasis. Expression levels of molecules involved in tissue remodeling and extracellular matrix (ECM) adhesion, especially *MMP-1* and *integrin-3*, can provide an accurate biomarker system for predicting the risk of cervical lymph node

metastasis in oral squamous cell carcinoma (Nagata, et al., 2003). In order to breach the basement membrane and invade the connective tissue stroma, HNSCC must produce enzymes capable of degrading the extracellular matrix. General classes of these proteolytic molecules include MMPs, named for their dependence on Zn^{2+} as a catalyst, and the plasminogen activators. The MMPs are a large group of secreted proteinases that require zinc for catalytic activity. MMP-2 and MMP-9 are the largest members of this gene family. They are able to degrade connective tissue, among other substrates, the basement membrane collagen, which appears to be very crucial in tumor cell invasion and in the process of metastasis.

The association of the expression of MMP-9 and MMP-2 with mode of tumor invasion and nodal involvement has previously been found in squamous cell carcinoma, and recently its utility has been proven in oral cancers (Miyajima, et al., 1995, Patel, et al., 2007). However, some studies have shown that the activation of MMP-2 was more prominent as compared with MMP-9 in malignant oral SCCs. Elevated activation ratio of MMP-2 has also correlated significantly with lymph node metastasis in oral SCCs. Accordingly, MMP-2 was considered by some investigators as more selective molecular marker for prediction of metastatic potentials of oral SCCs (Patel, et al., 2007). Certain other studies have shown results favoring the use of MMP-9 as a prognostic indicator (Ruokolainen, et al., 2004). Association between MMP-9 and vascular endothelial growth factor expression or micro vessel density has been found in head and neck carcinoma (Riedel, et al., 2000). The summary of MMPs produced by HNSCC is illustrated in table 1.

MMP	Name	Substrate
Collagenases		
MMP-1	Interstitial collagenase	Collagens I, II, III, V, IX Collagens I, II, III, V, IX
MMP-8	Neutrophil collagenase	Elastin
MMP-12		Collagen III
MMP-13	Metalloelastase Collagenase3	
Stromelysins		
MMP-3	Stromelysin 1	Proteoglycans, collagen IV, gelatins
MMP-7	Matrilysin	Fibronectin, collagen IV
MMP-10	Stromelysin 2	Proteoglycans, collagen IV, gelatins
MMP-11	Stromelysin 3	Laminin and fibronectin
Gelatinases		
MMP-2	Gelatinase A	Gelatin, collagens IV and V
MMP-9	Gelatinase B	Gelatin, collagens IV and V

Table 1. MMP produced by HNSCC

2.1.3 Endostatin

Endostatin exhibits specific inhibitory action on the proliferating endothelial cells of newly formed blood vessels, representing one of the better defined and most potent negative

regulators of angiogenesis (O'Reilly, et al., 1997). Earlier studies have shown that plasma levels of endostatin in patients with HNSCC have been associated with histologic grade, recurrence, and survival rate (Homer, et al., 2002). However, the immunohistochemical expression of endostatin and collagen XVIII in SCC tissues and their significance for the growth and metastatic potential of these tumors have not been widely studied. In a recent study, the levels of endostatin were lower in the primary tumors of cases with multiple metastatic lymph nodes compared with non metastatic tumors. The differences in endostatin expression between these tumors corresponded well with the levels of collagen XVIII, suggesting that the reduction in endostatin expression in the node positive group is because of decreases in the production of the precursor molecule collagen XVIII. On the other hand, these results contradict with those of Homer *et al* (Homer, et al., 2002), who observed a positive trend between higher levels of endostatin and nodal metastasis and an association between increased endostatin expression and higher tumor grade, recurrence, and death in patients with HNSCC. The authors attributed this discrepancy to differences in methods used, as these investigators measured the circulating levels of endostatin, whereas this study assessed the levels in tissue samples (Nikitakis, et al., 2003).

2.1.4 Others

E-cadherin is an important molecule that promotes cell to cell adhesion which serves as a positive regulator of metastasis. Low expression of E-cadherin should be considered as a high-risk group for late cervical metastasis when a wait-and-see policy for the neck is adopted (Lim, et al., 2004). The plasminogen activators (PAs) are another class of proteases that have been confirmed to play important parts in invasion and metastasis of HNSCC. PAs are neutral serine proteases which catalyze the synthesis of plasmin from plasminogen. Plasmin is a fibrinolytic enzyme, also active in degrading type IV collagen and laminin.

2.2 Molecular pathologic changes during development of metastasis

HNSCC will progress from carcinoma in situ, to microinvasive carcinoma, to an invasive tumor with stromal invasion, and to a deeply invasive tumor with lymphatic metastasis. The essential element in the transition from carcinoma in situ or preinvasive to invasive carcinoma is the destruction of the underlying basement membrane. A reasonable interpretation of these studies is that increased degradation of basement membrane correlates with increased invasion and metastasis. Adherence to the basement membrane and extracellular matrix components is another method by which tumor cells can facilitate local invasion and metastasis. Alterations in tumor cell adherence and the expression of these cell surface ligands may facilitate invasion, metastasis, and neovascularization.

2.2.1 Detachment and migration of tumor cells

Essential characteristics of cancer are the ability to invade surrounding tissues and metastasize to regional and distant sites. The events attendant to local invasion by an epithelial tumor include loss of adhesion to surrounding tumor cells and basement membrane, production of enzymes and mediators which facilitate the incursion of malignant cells into the subjacent connective tissue. Therefore, the late stages of cancer involve progressive tumor invasion and metastasis, which are the stages that ultimately affect vital functions and cause death in patients. Many important histopathologic and

molecular events associated with tumor progression and metastasis. Development of invasive carcinoma is associated with focal dissolution of the basement membrane and extracellular matrix (ECM), detachment, and migration of cells into the submucosal tissue. HNSCCs that exhibit a streaming pattern of small clusters of cells through the ECM are associated with more aggressive behavior and poor prognosis. HNSCCs exhibit alterations in expression of a repertoire of cell adhesion molecules and ECM substances that function in attachment and migration.

2.2.2 Angiogenesis

In 1972, Folkman first articulated the hypothesis that tumor growth was angiogenesis-dependent. Characteristics of prevascular tumors include a linear growth phase, absence of intratumoral vessels, and size limited to $< 1 \text{ mm}^3$. Once tumors become vascularized, obtaining nutrients and exchanging metabolic waste products with the host become more efficient and the growth properties of the tumor change. Characteristics of tumors in the 'vascular phase' are histological demonstration of intratumor capillary networks, $\text{size} > 1 \text{ mm}^3$, and an exponential growth phase (Folkman, 1990, 1992). Tumor progression to a size that becomes visible and has an effect on adjacent structures requires an increase in supply of oxygen and nutrients and removal of waste, which implies that new blood vessel formation is critical in cancer progression (Folkman, 1996). Enlargement of tumors to a size beyond 0.5 cm exceeds the range for diffusion of oxygen from existing vessels and necessitates new blood vessel formation, called neovascularization. Angiogenesis is increased in various human cancers, including HNSCCs, and correlates with tumor progression and metastasis. Vascular endothelial growth factor (VEGF) has been shown to be a key regulator of angiogenesis.

The ability to stimulate new blood vessel growth (neovascularization or angiogenesis) is an integral part of organogenesis, reproduction, and wound healing and repair, and in this context it is short term and self-limiting. Pathologic angiogenesis is not autoregulated and results from alterations in growth control, which are parts of particular disease processes. However, the ability of a tumor to stimulate an angiogenic response should directly determine the capability of a tumor to metastasize and ultimately kill the host. The evidence regarding microvessel density as a predictor of nodal metastasis, or response to treatment in HNSCC remains conflicting, furthermore initially good correlations between microvessel density and outcome recently being challenged. Tumors invade local connective tissues by the production of proteinases and the expression of cell surface markers which facilitate attachment to components of the extracellular matrix. Tumor size is limited by the diffusion of nutrients from adjacent blood vessels, however, tumors circumvent this limitation by recruiting host capillaries to form an intratumor blood supply.

2.2.3 Lymphoangiogenesis

Lymphangiogenesis is associated with locoregional disease recurrence in early-stage oral carcinoma (Munoz-Guerra, et al., 2004). The presence of intratumoral lymphangiogenesis is a useful discriminator in predicting the outcome of patients with absence of lymph node metastasis. Various studies have stressed on the impact of tumor thickness as a significant factor that had predictive value for local disease recurrence, survival and neck metastasis. The rationale was that the depth of invasion would determine proximity to blood and lymphatic vessels and facilitate the ability of the tumor to expand. In most cases, metastasis

in squamous cell carcinoma occurs via the lymphatic vessels and dilation of lymphatic vessels is frequently found in oral tumors with lymph node involvement. However, the influence of intratumoral or peritumoral lymphangiogenesis on squamous cell carcinoma of the oral cavity is still controversial.

Several markers have been utilized in the study of lymphangiogenesis. The main disadvantage of this method is that it relies on quantitative rather than qualitative differences between lymphatic and blood vessels and therefore requires a certain amount of subjective interpretation. In addition, most antibodies used react with both blood vessels and lymph vessels (Hannen & Riediger, 2004). Some of these studies have correlated the presence of VEGF-C in the tumor cells with an increased likelihood of lymph node metastasis in oral SCC, which seems promising (Kishimoto, et al., 2003, Shintani, et al., 2004, Warburton, et al., 2007). An association between lymphangiogenic growth factors, intralymphatic growth and tumor metastasis has been suggested. However, the role of intratumoral lymphangiogenesis in the progression of squamous cell carcinomas has not been studied. Tumor invasion of capillaries and lymphatics leads to dissemination of tumors and the establishment of histologically identical tumors at secondary sites.

2.2.4 Cellular components of tumor microenvironment

It has been shown that during progression, squamous cell carcinomas undergo additional changes needed for growth and metastasis that depend on the host (Chen, et al., 1997). Inflammatory cells infiltrating squamous cell carcinomas are one of the host components that promote growth and metastasis. New vessel formation is commonly associated with an increase in inflammatory cells. Growth of the tumor epithelia and angiogenesis is also accompanied by increased infiltration of inflammatory cells and proliferation of fibrous stroma. These inflammatory cells bear a stem cell marker called CD34 and appear to differentiate into granulocytes and endothelial cells that form new blood vessels. Granulocytes have been found to promote growth and metastasis. Granulocytes from the host can release growth factors and proteases that stimulate growth and invasion of tumor cells. Several studies have suggested that tumor cells capable of inducing host inflammatory and stromal cell responses grow, invade, and metastasize more rapidly.

Squamous cell carcinomas also induce proliferation of stromal fibroblasts. Fibroblasts also secrete factors and ECM substances that can promote growth. The establishment of metastases requires cell arrest and vessel formation in a new location. HNSCC shows a predilection for metastases to the lymphatics, lungs, liver, and bone marrow, suggesting that the cells and substrate of the reticuloendothelial system provide a favorable environment for arrest and formation of squamous cell carcinoma metastases. Non-malignant cells within the tumor microenvironment also play important roles in modulating tumor progression and metastases. Functional studies have identified several tumor-promoting functions for macrophages in primary tumors. These include promotion of angiogenesis, tumor cell invasion, migration and intravasation.

Local tissue invasion and migration into the subjacent connective tissue matrix by HNSCC are dependent of the production of cell surface molecules, enzymes and motility factors. In addition to the production of these locally active molecules, HNSCC produces growth factors or cytokines which target other cell types. Cytokines are low molecular-weight

proteins which affect cell-cell communication and signal cellular proliferation, differentiation, activation, and migration.

3. Biological processes of the metastatic cascade

Tumor metastasis is ultimately the result of an imbalance between forces favoring and opposing the development of secondary tumors. The first steps in the development of distant metastases involve (1) the initiation of the primary tumor in a genetically susceptible host, (2) the promotion and progression of malignant cell gene mutations favoring clone expansion, and (3) uncontrolled proliferation of these malignant clones of cells due to the actions of autocrine growth factors and growth factor receptors.

The risk of distant spread is related to primary tumor site, its local and regional extension, and the phenotype (Li, et al. 2009). Distant metastases are particularly important in supraglottic laryngeal and pharyngeal cancers (Buckley, 2000). Factors which favor the development of metastases include the primary tumor's ability to activate oncogenes, downregulate tumor suppressor genes, express cell-surface adhesion molecules, synthesize and respond to autocrine and paracrine growth and motility factors, secrete proteases, and produce angiogenic and immunosuppressive cytokines. Factors opposing the development of metastases include activated tumor suppressor and antimetastasis genes, enhanced host immune responses, synthesis of protease and angiogenesis inhibitors by both the tumor and the host, and anatomic and structural barriers. All of these phenomena are the result of multiple gene mutations culminating in the development of secondary tumors at distant sites.

Fidler and colleagues (Fidler & Hart, 1982) articulated the principal of tumor heterogeneity, which is now widely accepted. The development of local-regional and distant metastases begins with the initiation of the primary tumor and ends with the establishment of metastatic clones throughout the host. Several processes including the differential expression of cell adhesion molecules, release of metalloproteinases, and angiogenesis occur at multiple points in the metastatic cascade. This cascade involves an sequential process including tumor invasion into local stroma and vascular system, circulation of tumor cell and arrest at the distant site, and clonal formation at secondary site.

3.1 Invasion into local stroma and vascular system

The process of tumor invasion involves attachment of the tumor to the basement membrane, degradation of extracellular matrix components, and migration of the malignant cells into the surrounding stroma. We will refer again the process when we consider the establishment of the tumor at a secondary (distant) site.

3.2 Circulation of the tumor and arrest at the distant site

Metastatic tumors, regardless of how they exist in the circulation, will establish distant metastases either by mechanical impaction or attachment to the endothelial cell surfaces. Mechanical impaction of the tumor/lymphocyte/platelet emboli will occur when the diameter of the embolus approaches that of the vessel. The tumor will then adhere to the lumen surface of endothelial cells and begin to grow. The second mechanism is the

attachment of single tumor cells to the exposed basement membrane on the subendothelial side of the capillary lumen.

3.3 Colony formation at the secondary site

The common theoretical mechanisms exist for determining the locations of distant metastases were first articulated by Fidler as the 'seed and soil hypothesis' of tumor metastasis. These mechanisms include: (1) tumor metastasis equally to all organs, but preferentially only grow in locations which provide appropriate growth factors (soil); (2) circulating tumor cells have receptors specific for the endothelial cells of only certain target organs (seed), and (3) circulating tumors have receptors for specific chemotactic factors produced by the target organ. These factors result in the preferential attraction of the tumors to the target organ (seed soil) (Markus, 1988).

4. Lymph node metastasis of HNSCC

The status of the regional lymphatics is one of the most important prognostic indicators in patients with head and neck cancer. HNSCCs that are localized to the primary site without regional lymph node metastasis have excellent cure rates with either surgery or radiation therapy. The presence of regional metastases results in cure rates that are approximately half of those obtainable if metastasis to the regional lymphatics is not present. Thus the treatment of the neck has become one of the most actively debated topics in the field of head and neck oncology.

4.1 Patterns

The primary sites for HNSCC are mainly in the oral cavity, oropharynx, hypopharynx and larynx. In 1972, Lindberg published the location of nodal metastases in patients with squamous carcinoma of the upper aerodigestive tract as determined by clinical examination (Lindberg, 1972). This review consisted of 2,044 previously untreated patients with HNSCC. The presence of nodal metastasis and its location was assessed and correlated with the location and stage of the tumor at the primary site. Primary sites were divided into oral tongue, floor of mouth, retromolar trigone/anterior faucial pillar, soft palate, tonsillar fossa, base of tongue, oropharyngeal walls, supraglottic larynx, hypopharynx and nasopharynx. Fifty-seven percent of patients presented with clinical evidence of metastasis in the cervical nodes. Lindberg showed that for lesions of the oral tongue, floor of mouth, retromolar trigone/anterior faucial arch and soft palate, the incidence of cervical nodal metastasis increased with the size of the primary tumor. However, the incidence of nodal metastasis did not correlate with the size of the primary in tumors of the tonsillar fossa, base of tongue, supraglottic larynx, and hypopharynx.

Clinicopathological studies on the specimens from surgical removal of primary tumors and the associated treatment neck dissection tissues revealed patterns and impacting factors of cervical lymph node metastasis in HNSCC (Li, et al., 1996). For oral cavity cancers, the most common neck regions for neck node metastasis are level I to level III. Whereas, cervical lymph node metastasis from the cancers of oropharynx, hypopharynx and larynx are most frequently found in level II to level IV. Lindberg demonstrated that squamous cell carcinomas of the upper aerodigestive tract tend to metastasize to the neck in a predictable

pattern. By far, the most common site of metastasis by all tumors is to the ipsilateral level II nodes. Tumors that lie within the oral cavity anterior to the circumvallate papillae have a propensity to metastasize to levels I through III, with levels IV and V seldom involved. Tumors of the oropharynx have a low propensity to metastasize to level I; metastasis is most common to level II with decreasing incidence of metastasis in levels III and IV. These tumors have a higher rate of metastases to level V than oral cavity tumors but the rate is still low. Tumors of the supraglottic larynx and hypopharynx rarely metastasize to level I, again metastases were most common to level II with a decreasing incidence in levels III and IV and metastases to level V were infrequent. Contralateral metastases were uncommon in cancers of the floor of mouth, oral tongue, hypopharynx, and retromolar trigone/anterior faucial arch. In contrast, tumors of base of tongue, oropharyngeal walls, soft palate, supraglottic larynx, and tonsil have substantial rates of contralateral metastases.

Lindberg's data clearly showed that in cases of squamous cell carcinoma of the upper aerodigestive tract, with the exception of nasopharyngeal carcinoma, nodal metastasis occurs in a predictable pattern and it may, in certain instances, be sound to exclude dissection of the level V lymph nodes. However, this study provides only information on clinically positive nodal metastasis—it provides no information on the incidence and location of occult nodal metastasis. Such information on microscopic metastasis can only be obtained from a surgical specimen. Byers and colleagues published one such study (Byers, et al., 1988) in 1988. They examined the specimens of 428 patients undergoing 648 modified neck dissections and correlated the location of the pathologically positive lymph nodes with the primary site. The majority of these neck dissections were selective neck dissections and therefore not all of the lymph node levels at risk were examined in each patient. This study essentially confirms the clinical data of Lindberg (Lindberg, 1972) that lesions anterior to the circumvallate papillae are most likely to metastasize to lymph nodes levels I through III and lesions within the hypopharynx and larynx to levels II through IV. It must be pointed out, however, that the majority of these dissections were less than comprehensive and therefore the low incidence of metastasis to certain nodal levels may simply reflect the lack of sampling of those levels.

In order to fully assess all the lymph node levels at risk for a particular primary site, surgical specimens should include all lymph node levels (comprehensive neck dissection). Just such information is provided in a series of studies by Shah and colleagues, (Candela, et al., 1990, Candela, et al., 1990, Shah, et al., 1990) which involved 1,081 previously untreated patients who underwent 1,119 classic RNDs for squamous carcinoma of the upper aerodigestive tract. The operations consisted of 343 elective RND in the clinically N0 setting and 776 therapeutic RND in the clinically N+ setting. In patients with primary tumors of the oral cavity undergoing therapeutic RND, the majority of metastatic nodes were located in levels I to III; level IV was involved in 20 percent of specimens and level V in only 4 percent. In those with primary oropharyngeal tumors, the majority of metastases were located in levels II to IV; levels I and V were involved in 17 percent and 11 percent of the specimens respectively. Therapeutic neck dissection in hypopharyngeal tumors showed that the majority of metastases were located in levels II to IV, while levels I and V were involved in 10 percent and 11 percent of the specimens, respectively. Primary tumors of the larynx metastasized to levels II through IV with levels I and V being involved in 8 percent and 5 percent of the specimens, respectively.

In the setting of elective RND in patients with primary tumors of the oral cavity, the majority of metastases were located in levels I to III; levels IV and V were involved in 9 percent and 2 percent of the specimens, respectively. In patients with primary tumors located in the oropharynx, the majority of metastases were located in levels II to IV; levels I and V were involved in 7 percent of the specimens. Patients with tumors of the hypopharynx undergoing elective RND had the majority of metastases in levels II to IV, while levels I and V were not involved in any of the specimens. Primary tumors of the larynx metastasized primarily to levels II through IV, while levels I and V were involved in 14 percent and 7 percent of the specimens, respectively. O'Brien et al. (O'Brien, et al., 2000) found occult metastatic disease in 30% of patients, and Lim et al (Lim, et al., 2006, Lim, et al., 2006) found it in 28%.

The question of metastasis to level V was addressed by another study by Davidson and colleagues. (Davidson, et al., 1993) They examined the specimens of 1,123 patients undergoing 1,277 RNDs and found metastases to level V in only 3 percent of patients. Level V metastases were highest in patients with hypopharyngeal and oropharyngeal primary sites (7% and 6% respectively). Only 3 of the 40 patients with level V metastases had these in the face of a clinical N0 stage. They concluded that the incidence of metastases to level V was small in general, and extremely unlikely in the clinically N0 patient.

4.2 Risk factors

Clinicopathologic factors associated with the development of cervical lymph node metastasis have been well studied for other locations like tongue, mouth floor, and cheek, in particular concerning tumor size (in tongue carcinoma ≥ 3 mm), tumor depth (≥ 4 mm in tongue carcinoma), differentiation, mode of invasion, microvascular invasion, and histologic grade of malignancy (Kurokawa, et al., 2002, Sparano, et al., 2004, Wallwork, et al., 2007). The presence or absence of lymph node metastasis is a major prognostic factor for survival in patients with negative cervical lymph nodes (Hiratsuka, et al., 1997). A high incidence (20–30%) of cervical metastasis of cancer in the tongue/mouth floor has been well studied (Kurokawa, et al., 2002, Sparano, et al., 2004, Wallwork, et al., 2007). But very few studies have been performed concerning squamous cell carcinoma of the maxilla (Simental, et al., 2006). Sparano et al (Sparano, et al., 2004) and Kruse et al (Kruse & Gratz, 2009) reveal that the higher the grading, the higher the risk of cervical metastasis. Therefore, regarding the proportion of late cervical metastasis, the question arises whether an elective neck dissection should be provided in early-stage squamous cell carcinoma. Capote et al. (Capote, et al., 2007) reported that in pT1N0 and pT2N0 oral squamous cell carcinoma, neck dissection therapy was a significant prognostic factor for recurrence and survival. Therefore, tumor size, tumor depth, and differentiation should be taken into consideration for the planning of neck dissection for squamous cell carcinoma of the upper jaw. Also the mode of invasion plays an important role in therapy planning because in certain localizations like the palate, the tumor does not need to invade very deeply before reaching the bone.

4.3 Prognostic factors

As an independent prognostic factor, cervical lymph node metastasis has a great impact on disease-free and overall survival of patients with HNSCC. Among various

clinicopathological factors, the most important prognostic factors are pN+, numbers of positive node (more than 3 positive nodes), lower level of invasion, and especially the extracapsular nodal spread (ECS) (Di, et al., 2009). A review of literature reveals the impacts of clinicopathological factors on neck recurrence. For example, if residual disease after neck dissection, 2 or more pathologic lymph nodes, extracapsular spread (ECS), more than 3 cm-diameter pathologic lymph node and invasion of soft tissue are found in neck dissection specimens, the risk of neck recurrence is considered to be high (Li, et al., 2009). Since treatment neck dissections for HNSCC vary from selective neck dissection (SND) to radical neck dissection (RND), it is necessary to analyze the clinicopathologic risk factors for regional recurrence in a group of positive-node patients treated with such neck dissections and postoperative radiotherapy (PORT) in order to determine which patients need further adjuvant therapy and further short-interval follow-up. However, the majority of the literatures draw these conclusions in the absence of adjuvant radiotherapy. Furthermore, some reports show that these factors have no statistical significance in predicting regional failure following neck dissection and adjuvant PORT. It is now well established that the development of cervical metastases, in particular those with extranodal extension of tumor, negatively impacts both regional control and survival of patients with laryngeal carcinoma (Myers & Fagan, 1999).

4.4 Modern concepts in management of cervical lymph node metastasis in HNSCC

The lymphatic system of the head and neck is complicated (Fisch, 1964). An extensive analysis of 2044 medical records of patients with HNSCC who had not received prior treatment led Lindberg to divide nine lymph node regions on each side and, additionally, the parited lymph nodes (Lindberg, 1972). The lymph fluid of the upper aerodigestive tract is drained via about 300 regional cervical lymph nodes, which are divided according to the current classification established by Robbins (Robbins, et al., 2002) into nine lymph node levels (level I–VI). It is well understood that an incomplete surgical resection margin is the most important single factor for tumor recurrence, which is determined not only by the experience of the surgeon but also by the limitation of surgical excision. For example, if multiple nodes or ECS of neck diseases are present, it may be difficult to obtain adequate surgical resection margins or to resect all metastatic lymph nodes in the neck. Recurrence in the neck is more likely to occur in patients with these neck situations. For this reason, it is widely accepted that ECS is a marker for biologically aggressive disease and patients with HNSCC who have evidence of ECS need aggressive multimodality therapies including surgery, PORT and, even chemotherapy.

4.4.1 Detection of lymph node metastasis

Most tumors of the head and neck initially metastasize to the regional lymph nodes. The presence of cervical metastases is the most significant oncological factor in the prognosis of HNSCC (SCC) because early detection and treatment may prevent distant metastases (Gray, et al., 2000). The assessment of cervical lymph nodes is known to be extremely difficult clinically. Despite recent advances in the fields of radio diagnosis, its utility to detect occult neck metastasis still lacks considerable power. Owing to the high number of undersized lymph node metastases, the non-invasive neck staging methods are limited to a maximum accuracy of 76% (Stuckensen, et al., 2000). Pre-surgical staging of the neck has become more

complex over the years. Clinical assessment of the neck by palpation, while providing critical information, is inadequate in its sensitivity for detecting metastatic disease to the cervical nodes. Error rates as high as 40 percent have been reported when physical examination alone is used to evaluate the neck (Teichgraeber & Clairmont, 1984). Patient factors such as a short, obese neck, as well as prior irradiation play a role in decreasing the accuracy of this technique. Clearly, radiologic assessment of the neck adds to the sensitivity and specificity of preoperative neck evaluation.

4.4.1.1 Comouterized tomography (CT) and magnetic resonance imaging (MRI)

Computerized tomography (CT) and magnetic resonance imaging (MRI) have become the workhorses of imaging modalities in HNSCC. Size criteria are frequently used as indicators of metastatic involvement. Other features such as central necrosis or ring-enhancement aid in specificity but are relatively infrequent findings. Generally, a subdigastric node measuring > 15 mm, a submandibular node > 12 mm, and other nodes > 10 mm are suspicious for involvement. Using criteria such as these, the accuracy of detecting neck disease approaches 90 percent (John, et al., 1993). Size, however, is certainly not pathognomonic for cancerous involvement of lymph nodes. Even in the patient with an identified squamous cell carcinoma of the upper aerodigestive tract, a myriad of alternative causes of enlarged lymph nodes exist. Further, microscopic foci of disease may exist in nodes of normal size. As CT or MRI is often employed to evaluate the primary lesion, inclusion of the neck in the area of study incurs nominal additional expense and no morbidity. Although CT and MRI provide excellent anatomic detail and are the current modalities of choice, they provide little information on the biology of the lymph node.

4.4.1.2 Positron emssion tomography (PET)

Several studies have evaluated fluorodeoxyglucose (FDG) PET in this setting, attempting to identify the patients who need neck dissection. In 3 studies totaling 48 patients, in which a sentinel node biopsy with immunohistochemistry was used as the gold standard, the detection rate of PET was between 0% and 30%, making PET an unreliable modality in this clinical setting (Civantos, et al., 2003, Stoeckli, et al., 2002). This is not unexpected, given that 40% of cervical nodal metastases are less than 1 cm in size and PET detection rate for nodes less than 1 cm is reported at 71% (Menda & Graham, 2005). Numerous promising pilot studies have evaluated sentinel node biopsy (SNB), up to 16% patients required additional immunohistochemistry (IHC) on the sentinel nodes to detect metastasis (Civantos, et al., 2006). Owing to these inadequacies in detection of occult nodal metastasis, surgical dissection and serial histologic examination are the currently accepted "yardsticks".

Another problem that should be considered seems to be the detection of micrometastasis. The assessment of the status of cervical lymph nodes is difficult, and therefore a treatment of patients with a clinical stage N0 neck is controversial. In most studies, the use of CT has an error rate ranging from 7.5 to 19% (van den Brekel, et al., 1990). In the late 1990s, the PET using F-18 FDG, a functional imaging methodology that provides information about tissue glucose metabolism, was applied. Consequently, a high FDG accumulation is manifested on PET images, but inflammation also reveals an increased FDG uptake and can lead to false-positive results. On the other hand, low tumor metabolic activity, the presence of small lesions, and hypoglycemia can lead to false-negative results (Murakami, et al., 2007). Concerning cervical lymph nodes, Ng et al. (Ng, et al., 2005) reported that sensitivity and

specificity of PET images were 75% and 93%, respectively. Sigg et al. (Sigg, et al., 2003) reported a sensitivity of 93% and a specificity of 100%. PET together with CT images showed a 15% increase in the accurate identification of nodal staging over using the PET images alone (Jeong, et al., 2007). PET/CT seems to have a higher sensitivity and specificity for detecting lymph node metastasis (Leong, et al., 2006, Wild, et al., 2006).

4.4.1.3 Ultrasound

Due to its non-invasiveness and affordability, ultrasound (US) has been investigated as a potential tool in evaluating neck disease. Factors such as size, irregular margins, and echo characteristics of lymph nodes have been shown to have predictive value in assessing involved nodes. The overall sensitivity of this approach, however, is limited due to the operator-dependant nature of ultrasound. (John, et al., 1993) Some authors have proposed ultrasound in combination with ultrasound-guided fine needle aspiration as an approach to diagnosis. Takes and colleagues (Takes, et al., 1998) examined, with ultrasonography, 64 necks staged N0 based on physical examination. Those with nodes greater than 5 mm in size underwent ultrasound-guided needle biopsy. Results were further verified with histopathologic examination and the findings compared with CT of the neck for detection of involved nodes. They found a 48 percent sensitivity, 100 percent specificity, and 79 percent accuracy for ultrasound versus 54, 92, and 77 percent respectively for CT. These results demonstrate that, in experienced hands, ultrasound can be a useful tool. Its widespread application, however, is limited by the technical expertise required for accurate interpretation.

4.4.2 Management

4.4.2.1 General principles

The type, grade, site and stage of the primary tumor determine the risk of cervical metastases and hence the type of treatment modality. Treatment of the neck in patients with clinical evidence of nodal metastasis has traditionally been surgical. In recent decades, this has been extended to include a combination of surgery and radiation therapy. The role of chemotherapy in the management of neck disease remains controversial and is currently being actively investigated. In oral tongue carcinoma, the risk of neck metastasis is significantly associated also with the depth of tumor invasion (Pentenero, et al., 2005). The patterns of spread of cancer to cervical lymph nodes are predictable, based on the anatomical location of the primary tumor. Therefore, in the absence of clinical evidence of neck disease, the pathological features of the primary tumor along with its site of origin and clinical T stage are used to stratify the risk of positive neck metastases and, consequently, the need for a neck dissection. When the risk for positive neck lymph nodes exceeds 15–20%, elective neck dissection is indicated – not only as treatment but also to evaluate the need for adjuvant therapy. A selective neck dissection, directed to the basins at risk for lymphatic spread, is commonly used for this purpose. The presence of palpable neck disease mandates comprehensive clearance of the lymphatic basins in the neck. Radical neck dissection was considered the primary modality for treatment of HNSCC with clinical evidence of cervical metastases. However, sacrificing vital structures during radical neck dissection causes severe disabilities in patients and a markedly reduced quality of life. Advances in the anatomic elucidation of the neck, enhanced understanding of the biological behavior of

tumors, and improved surgical methods have contributed to the emergence of the functional neck dissection technique, resulting in excellent survival and functional outcome (Shah & Gil, 2009). Exact knowledge of the anatomy of the neck and its adjacent structures and the risk and location of common cervical metastases is essential for the operative treatment of HNSCC.

Although primary tumor control is achievable in early tumors with minimally invasive surgery, such as transoral or robot-assisted procedures, the management of the neck is still an important consideration in the treatment of HNSCC. Surgical management of the neck in patients with pharyngeal cancers does not usually involve a dissection of the retropharyngeal lymph node (RPLNs). Neck dissections do not routinely address RPLNs, creating a potential for recurrence in the retropharynx and the need to address this nodal basin with radiotherapy (Tauzin, et al., 2010). Treatment of the neck in patients with clinical evidence of nodal metastasis has traditionally been surgical. In recent decades this has been extended to include a combination of surgery and radiation therapy. The role of chemotherapy in the management of neck disease remains controversial and is currently being actively investigated.

4.4.2.2 Adjuvant therapy

Although the practical value of postoperative radiotherapy (PORT) for improving survival in HNSCC is well acknowledged, it remains controversial whether this postoperative radiotherapy, an adjuvant treatment, could prevent recurrence in the neck in patients having ECS. Smeele et al. found that PORT dose of 62.5 Gy and more could increase neck control rates in patients with ECS treated with surgery and PORT for HNSCC. Peters et al. demonstrated that metastatic lymph nodes with ECS were adequately controlled by PORT at dosage of 63 Gy or more (Peters, et al., 1993). However, Prim et al. reported that the 3-year recurrence rates in the neck were 10.7% in patients without ECS and 49.6% with ECS in squamous cell carcinoma of the larynx with pathologically proven lymph node metastasis, and PORT did not appear to improve the outcome (Prim, et al., 1999). Shingaki et al. found that PORT did not decrease the rate of neck recurrence in patients of oral cavity carcinomas with ECS (Shingaki, et al., 2003). These findings suggest that the exact value of PORT in controlling neck recurrence needs to be further documented and recommended for adjuvant chemotherapy. Our findings suggest that the presence of ECS remains a determined risk factor for neck recurrence after surgery and adjuvant PORT in N⁺ patients with HNSCC. There were no significant risk factors associated with regional failure in ECS⁻ group. Except for PORT, no additional adjuvant therapy is required for N⁺ patients without ECS. However, more adjuvant therapies are to be considered after PORT in patients with ECS for the purpose of a more effective neck control.

4.4.2.3 Scenario in the management of an N0 neck

Due to the fact that the prognosis of patients suffering from squamous cell carcinoma of the upper aerodigestive tract depends significantly on the presence or absence of lymph node metastasis, the question of detecting clinically occult lymph node metastases is still important concerning the management of the clinical N0 neck. The published rate of lymph node metastasis depends on the location of the primary tumor, with values from 12% to over 50% (median, 33%) (Hosal, et al., 2000). Numerous authors favor elective treatment of the lymphatic region (neck dissection) if the presence of occult lymph node metastasis can

be expected with a probability of 20% or more. However, other authors prefer to adopt a “wait and see” strategy, although this requires both great compliance from the patient and great expertise on the part of the responsible physician to identify metastasis early. Another argument in favor of elective neck dissection versus a “wait-and-see” strategy is the significant deterioration of the survival rate when neck dissection is due after clinical disease is detected (Godden, et al., 2002).

Regarding the current scenario in the management of an N0 neck, there are presently three policies advocated, which include elective neck irradiation, prophylactic neck dissection or close observation. The choice of therapy often takes into consideration T stage, site of primary, grade, compliance for follow-up, or the probability for occult metastasis [$>20\%$]. Treatment of the neck, even when included with the primary treatment, often confers additional costs, morbidity and prolonged treatment time to the patient. Most often, a single modality treatment is used to treat the primary site and neck. The choice of which is dictated by the treatment of the primary site. There is no conclusive evidence to show if this elective neck treatment approaches contribute to improved overall survival for the patients with HNSCC and clinically negative neck.

4.4.2.4 Sentinel node and elective neck dissection

The elective treatment of the regional lymphatic drainage can generally be performed either surgically or radiotherapeutically. The choice of one of these procedures generally depends on the therapy of the primary tumor. An advantage of elective neck dissection over radiotherapy is that the histological examination of the neck dissection specimen can give important information for deciding therapy, as well as about the prognosis. Thus, the sentinel node concept for squamous cell carcinomas of the upper aerodigestive tract is quite appealing. Furthermore, limits and pitfalls of SLNs for HNSCC discussed elsewhere illustrate that an advanced intranodal tumor growth with extracapsular metastatic spread, leads to a significant reduction of the radiotracer uptake (Dunne, et al., 2001). Even small, clinically unsuspected lymph nodes may reveal extracapsular tumor growth with resulting lack of radiopharmakon accumulation (Coatesworth & MacLennan, 2002). The dominating metastatic region of pharyngeal and laryngeal carcinomas is mainly level II and less commonly, level III. Carcinomas of the anterior oral cavity drain mostly into level I and less commonly into level II. Accordingly, neck dissection of these lymph node levels can be expected to include the majority of clinically occult metastases. With this background, it must still be clarified whether the intraoperative identification of the radiolabeled SLN is appropriate to reduce the extent of selective neck dissection in the suspected N0 neck, or whether neck dissection can be completely avoided in the case of histologically-proven tumor-free SLN. Opponents of such a procedure argue that selective neck dissection already has a morbidity that must be considered. Supporters of sentinel lymphadenectomy stress both protecting the intact, i.e. non-metastatic, cervical lymph node systems and reducing the extent of surgery. Scarring contractures, paresthesia, and persisting lymph edemas can be reduced by a selective SLN dissection. Current research aims to optimize surgical access to the SLNs. The first results on endoscopically performed selective lymphadenectomy led to the assumption that this method of lymph node dissection could achieve some significance in the therapy of the clinical N0 neck, provided that it is based on the SLN concept (Werner, et al., 2004).

However, the techniques would have to be optimized. Furthermore, prospectively collected data should be gathered and analyzed. Within such an investigation, it would make sense to examine frozen sections of the excised lymph node. Depending on the histopathological result, a surgical resection of the lymphatic drainage in the form of a selective neck dissection could then be indicated. At present, the technical diversity and importance of endoscopic lymphadenectomy in the neck shows scientific and clinical potential. The question about the significance of the procedure, however, can not yet be answered conclusively.

5. Distant metastasis

Generally, distant metastasis is defined as tumor spread to other organ systems from its primary site. As a relatively rare but clinically relevant event, the development of distant metastasis is usually difficult to predict in clinic, especially when initial treatment planning is made.

5.1 Clinicopathological features of distant metastasis in HNSCC

5.1.1 The incidence and common sites of distant metastasis in HNSCC

Alavi et al. (Alavi, et al., 1999) reviewed 342 patients with mucosal HNSCC, and 47 (13.7%) had distant metastases. Five patients (1.5%) had metastases to infraclavicular lymph nodes (axilla, inguinal and presternal). The clinical detection of metastatic foci occurs in 10% to 30% of cases, whereas autopsy studies yield an incidence of about 50% of cases with metastases below the clavicle (Amer, et al., 1979, Dennington, et al., 1980). Clinical data in recently reported studies indicates an incidence of 4% to 23.8%, whereas autopsy data documents that 12% to 57% of cases had disseminated disease (Dennington, et al., 1980). Merino and associates (Merino, et al., 1977) in an analysis of 546 of 5019 untreated patients with squamous carcinoma of the upper respiratory tract who completed curative treatments, found clinically manifested metastases below the clavicle in 10.9% of the cases. The risk of subpathological distant metastases has also to be considered. New and highly sensitive investigations (immunohistochemistry, molecular analysis and FDG-PET/CT) and serial sectioning of nonregional lymph nodes and at risk organs may increase the detection of distant micrometastases in head and neck cancer patients. Probably the different reported incidence depends on the selection criteria of screening for distant metastases and the characteristics of the patients included (Leon, et al., 2000).

The lungs, bones (especially the vertebrae, ribs, and skull) and the liver are the most common sites of hematogenous distant metastases from HNSCC (Gowen & Desuto-Nagy, 1963). During the follow-up period after the initial treatment, 6.2% of the patients were diagnosed of having distant metastasis. The most common sites of distant metastasis were the lungs (58%) and the bones (22%). The lung is clearly the most common site of distant spread. The incidence of pulmonary metastases is also high in patients who present with extensive soft tissue extension of the primary or metastatic regional nodal disease. Holsinger et al, from the Anderson Cancer Center in Houston, provided a panel of clinical and histopathological predictors that may identify patients at the greatest risk for development of distant metastases in HNSCC (Holsinger, et al., 2000). In their study, the 5-year incidence of distant metastasis was 15.1 % (94/622). Pulmonary metastases were most commonly

found: 65.9% to the lung, 4.2% to the mediastinum, 2.1 % to the pleura. Metastases to bone (22.3%) and to the liver (9.5%) were the next most commonly encountered. Thirty (31.9%) patients with distant metastases presented with more than one metastatic site. Lung was the most common site for solitary metastasis. The most common site for bony metastasis was the spine (12.7%), followed by skull (4.2%), rib (3.1 %), and axial bones (femur, humerus; 2.1 %). More than half of patients with osseous metastases presented with multiple sites. The patients who present with jugular vein invasion or extensive soft tissue disease in the neck clearly have a high incidence of pulmonary metastases. Other less common sites of metastases include the mediastinum, adrenal gland, brain, pericardium, kidney, and thyroid gland (Troell & Terris, 1995).

5.1.2 Risk factors

Taken into consideration to be relatively important factors in clinic, clinical T stage, N stage, tumor site, tumor thickness, differentiation, pattern of invasion, vascular and/or lymphatic invasion, bone and/or cartilage invasion, perineural invasion, and lymph nodal status have been reported to be associated with distant metastasis in HNSCC. However, the conclusions concerning the role of each independent factor differ among the various authors. In a recent study, we successfully demonstrated that primary tumor site, level of tumor invasion and numbers of levels with positive lymph node are closely related to the occurrence of distant metastasis in HNSCC (Li, et al., 2009).

The incidence of metastases is influenced by T and N stage, as well as control of the primary lesion. As local and regional control of head and neck cancer has improved, distant metastases have become an increasingly common cause of death. (Vikram, et al., 1984) The disturbance of the lymphatic system in the cervical region resulting from radiotherapy or neck dissection can result in alternative pathways of lymphatic drainage. These newly formed pathways of drainage can ultimately result in lymphatic dissemination of head and neck cancers to sites below the clavicles. Metastasis from head and neck carcinomas to infraclavicular lymph nodes has been reported very infrequently in the literature (Nelson & Sisk, 1994). Recognition of this phenomenon is crucial in the evaluation of patients with recurrent head and neck cancer, especially when salvage surgery is entertained.

The incidence of distant metastases is directly related to the clinical stage of the tumor, with high incidence of distant metastases in stage IV tumors, particularly in patients who present with advanced nodal disease. The distant metastasis ratio was much higher in patients with T3 to T4, N2 to N3 lesions who received postoperative radiotherapy. It is reported that locally extensive lesions T3 and T4 are most likely to metastasize and that nodal involvement is also associated with increased risk of distant spread. Lesions arising in the larynx and hypopharynx have a greater predilection to metastasize than oral lesions, although true vocal cord lesions infrequently metastasize as demonstrated by Snow and coworkers (Snow, et al., 1980). In data of Merino (Merino, et al., 1977), 8% of all patients who had local control developed metastases, while 23% of those with T3 to T4 lesions had local control and developed distant spread.

The incidence of pulmonary metastases is extremely high in patients who present with bilateral N3 disease. Disease stage showed a striking correlation with the risk for distant metastases (as follows): stage I, 1 %; stage II, 14%; stage III, 15%; stage IV, 20% ($p < 0.0003$).

Advanced disease (T stage > 3 and N stage > 2a) was significantly correlated statistically with the development of distant metastases ($p < 0.003$). The authors found that certain clinical features (extent of cervical metastasis or N stage) and histopathologic data (evidence of lymphatic or vascular invasion and extension beyond the confines of the lymph node) are associated with significantly increased rates of distant metastases.

Spector (Spector, 2001) report a retrospective tumor registry analysis of patients with HNSCC of the larynx and hypopharynx who were treated with curative intent between January 1971 and December 1991. In 2,550 patients, the mean age, sex and tumor differentiation did not affect the incidence of distant metastases. The overall incidence of distant metastases was 8.5% (217/2,550 patients) with the following distribution: glottis 4.4%, supraglottis 3.6%, subglottis 14%, aryepiglottic fold 16%, pyriform sinus 17% and posterior hypopharynx 17.6%. The overall 5-year disease-specific survival for distant metastases was 6.4%. Distant metastases were related to advanced local disease (T3 + T4), lymph node metastases at presentation (N+), tumor location (hypopharynx) and locoregional tumor recurrence ($p = 0.028$). A meta-analysis of variables which predispose to a higher incidence of distant metastases indicate that tumor location (hypopharynx > larynx), advanced primary disease (T3 + T4), regional disease (N+), locoregional recurrences, and advanced regional metastases (N2 + N3) are statistically significant. The salvage rate for distant metastases was poor (6.4%) and significantly worse than the salvage rate for delayed regional node metastases (42%) or second primary malignancies (38%) ($p = 0.001$). The onset period of distant metastases was greatest between 1.5 and 6 years post initial treatment with a mean of ≤ 3.2 years.

Research for clinicopathological features of distant metastasis in HNSCC is of clinical implications in the diagnosis and treatment of the disease. Strong prognostic indicators that predict development of distant metastases are the presence and number of lymph node metastases in the neck, and extranodal spread. Once distant metastases are detected, patients have a very poor prognosis. The time interval between the diagnosis of distant metastasis and death is less than 2 years in greater than 90% of such cases.

5.1.3 Retrograde dissemination

Alvarez reported a retrospective study of 633 patients with HNSCC to describe the clinical characteristics of the distant metastasis. During the follow-up period after the initial treatment, 6.2% of the patients were diagnosed of having distant metastasis (Alvarez Marcos, et al., 2006). The site of primary tumor was hypopharynx in 14.4%, unknown origin in 11.8% and oropharynx in 8.5%. Three year overall survival in patients with distant metastasis was 2.5% (versus 49.5% in the control group).

Nonregional lymph node dissemination should be classified as distant metastasis but axillary and mediastinal metastases can be part of a regional dissemination of HNSCC. Metastases to lymph nodes of the upper mediastinum are very common among patients with subglottic, hypopharynx and thyroid carcinomas. Axillary metastases are found at autopsy in 2–9% of the patients who died of HNSCC and are frequently associated with skin implantation in aggressive recurrent head and neck carcinomas. The possible explanations for this location of metastasis were retrograde dissemination due to lymph system blockage, further tumor dissemination after a parastomal recurrence, hematogenous dissemination, and metastasis from a second primary tumor (Kowalski, 2001).

5.2 Diagnose of distant metastasis in HNSCC

5.2.1 Schemes for screening

Because distant metastasis has an important impact on survival, early detection of this unfavorable status in HNSCC is substantial for therapeutic strategy regulation. The metastatic workup for patients with head and neck cancer frequently includes examination of the cervical lymph nodes as well as chest radiography, liver function tests, and a serum calcium level determination. This evaluation may fail to detect metastases to distant lymph nodes in patients who present with recurrent or second primary cancers after previous therapy that has affected the cervical lymphatics. A predictable pattern of lymphatic metastasis based on tumor histology and site of origin has also been well documented for most cancers that arise in the head and neck region.

The diagnostic and screening procedures used for distant metastasis in HNSCCs are sometimes equivalent and sometimes complementary. The available methods for the assessment of tumor status include: (1) conventional radiographs (X-rays); (2) sectional imaging - CT, magnetic resonance imaging (MRI), positron emission tomography (PET); (3) ultrasound and ultrasound-guided fine needle biopsy; (4) radionuclide scanning; (5) endoscopic examination and (6) histological and cytological investigations - conventional histology, semiserial sections, immunohistochemistry, molecular analysis and techniques of cell culture.

As the lungs, bones and the liver are the most common sites of distant metastases from HNSCC, routine examination about these organs should be performed for high risk patients of metastasis in HNSCC. The prevalence of metastases at autopsy (37-57%) is much higher than in clinical studies (4-26%) (Leon, et al., 2000). This suggests that distant metastases in head and neck cancer are often asymptomatic, which raises the question of screening. Any investigations used for screening need to be sensitive, highly specific, inexpensive, noninvasive and readily available (Troell & Terris, 1995). In the absence of useful screening tests, metastases are usually detected by specific investigation of suspicious symptoms. Plain X-rays, computed tomography (CT) and bone scanning are the most frequently used investigations.

Chest CT is recommended for high-risk patients, especially during the follow-up period. Intensified evaluation and management are mandatory for indeterminate small solitary pulmonary nodules because of the high rate of malignant neoplasms (Hsu, et al., 2008). Otherwise, cross-sectional imaging with CT and MR imaging is commonly used for tumor metastasis detection.

Recently, PET using the radiotracer ^{18}F FDG is widely used to evaluate patients with HNSCC. The combined technique, PET/CT, provides anatomic and functional information and is useful for identification of an unknown primary tumor, detection of distant metastasis, establishing radiation-therapy planning, assessing therapy response, and long-term surveillance for recurrence. Positron emission tomography-computed tomography with fluorodeoxyglucose F18 (FDG-PET/CT) is widely used to evaluate patients with HNSCC. PET/CT can provide early, accurate detection of bone metastases from HNSCC and to determine the impact of detecting occult bone metastases on patient care. Use of FDG-PET/CT in restaging HNSCC allows for detection of occult lung, liver and bone metastases, and this early detection frequently influences therapeutic decision making (Basu, et al., 2007).

5.2.2 Confirmation of miscellaneous distant metastases

5.2.2.1 Pulmonary metastasis

Chest computed tomography (CT) scan is clearly more sensitive in identifying and localizing pulmonary metastasis than plain chest radiography, which can serve as a useful screening tool.

Treatment of pulmonary metastases requires some evaluations concerning control of the primary site and regional lymph nodes, and the general physiological and mental condition of the patients as well as the patient's willingness to be treated. In addition, to determine the optimal treatment, especially when considering surgical treatment, it is necessary to exclude other metastases and to precisely define the sequence after the surgery. Surgical excision is indicated as the optimal treatment for a solitary metastasis tumor of the lung when chemoimmunotherapy is ineffective. The patients who have jugular vein invasion or extensive soft tissue encroach in the neck clearly have a high incidence of pulmonary metastases.

5.2.2.2 Bone metastasis

Because metastasis to osseous tissue is the second most common presentation of distant metastases, bone scan is an important and sensitive test. However, because of its non specificity, CT-directed needle biopsies may be necessary to establish diagnosis.

5.2.2.3 Liver metastasis

Hematogenous spread to liver rarely occurs without evidence of pulmonary and bone disease. Although liver function tests may detect abnormality, elevation in liver enzymes ordinarily carries low sensitivity or specificity for liver involvement. Confirmation most often requires a diagnostic CT scan followed by ultrasound-guided needle biopsy.

5.2.2.4 Brain metastasis

Brain metastasis is a rare occurrence from head and neck cancer, it is particularly more probable in tumor involving the temporal bone simply because of its proximity to the cranial vault. CT scan and magnetic resonance imaging (MRI) provide the highest sensitivity of screening for intracranial disease well before neurological manifestations become apparent.

5.3 Management of distant metastasis in HNSCC

5.3.1 Prevention

It has been noted that, with the modern therapeutic regimens, the outcome of patients with distant metastasis from HNSCC remains dismal. Salvage therapy of metastesectomy or ionizing radiation is not sufficient to obtain a higher cure rate, when distant metastasis is at presence. It seems that optimal therapeutic strategies for distant metastasis may be adjuvant chemotherapy after surgery and postoperative radiotherapy at target groups of patients who are at high risk of developing distant metastasis. According to findings in our previous study (Li, et al., 2009), we propose that patients with multilevel nodal involvement in the neck, primary tumor localization at oropharynx, hypopharynx and larynx, and primary tumor invasion into muscle, bone or cartilage are at highest risk of developing distant

metastasis in HNSCC. Therefore, these subsets of patients with high risk factors should be considered for a more thorough evaluation for detecting distant metastasis and a more increasing utilization of adjuvant chemotherapy for preventing distant metastasis. However, it must be mentioned that screening for distant metastasis in the follow-up does little help in improving the outcome, since there is mostly no possibility of a curative intervention.

5.3.2 Surgery

Treatment of metastases is generally difficult. The difficulty seems to be caused by the low sensitivity of metastatic tumor cells to anticancer drugs and radiation. Surgery and radiotherapy are the main treatment modality of morning metastases for HNSCC. Metastasized regional lymph nodes are usually controlled by surgical removal (Shah & Andersen, 1994). However, surgical removal of distant metastatic tumors is usually not easy, especially in patients with multiple organ metastases. Because of these difficulties in conservative and surgical treatments, distant metastases are lethal in most patients.

Treatment planning for cases with axillary metastasis must take in consideration the likelihood of other regional recurrences and/or distant metastasis. Also, the presence of a second primary tumor must be ruled out. Whenever axilla is the only site of cancer recurrence, a standard axillary dissection must be considered. Upper mediastinal metastases from subglottic and hypopharyngeal cancer are managed by paratracheal and mediastinal dissection through the neck and postoperative radiotherapy (Kowalski, 2001).

Surgery is sometimes useful in the treatment of bone metastases. For example, pulmonary metastasectomy of isolated metastasis has been shown to be of benefit in selected patients (Wedman, et al., 1996). Surgery is sometimes useful in the treatment of bone metastases, although radiotherapy is the standard first-line treatment. In metastatic brain tumor, surgical resection should also be considered for patients with solitary brain metastasis and no extracranial disease or controlled extracranial disease. Whole-brain radiotherapy is routinely administered postoperatively (Hoegler, 1997). Although surgical removal of isolated solid metastatic tumors in liver is sometime carried out, adjuvant chemoradiation is the mainstream in the treatment modalities.

Occasionally, surgical resection of metastases is useful for metastases that do not respond to radiotherapy and in weight-bearing or high-stress areas (subtrochanteric region of the hip, mid-femoral diaphysis, mid-humeral metaphysis). Surgical stabilization can improve the remaining quality of life in these patients if it is carried out early enough (Sim, et al., 1992). A brief, fractionated course of radiotherapy is usually given postoperatively (Hoegler, 1997).

5.3.3 Chemotherapy

Head and neck cancer metastases are responsive to chemotherapy and the use of multiple agents may increase response rate. Unfortunately, neither single agent nor combinations of drugs have any significant impact on survival (de Mulder, 1999). The exception may be nasopharyngeal carcinoma, where platinum-based chemotherapy may increase survival even in the presence of distant metastases (Gebbia, et al., 1993). Chemotherapy also acting as a radiosensitizer, increases survival in advanced metastasis in HNSCC.

Neoadjuvant chemotherapy with the cisplatin and fluororacil (PF) regimen in HNSCC patients has no effect on locoregional relapse. However, it shows a small but significant

benefit in reducing distant metastasis and improving the overall survival (Su, et al., 2008). Many new chemotherapy ways are attempted in a broader sense of targeted therapy.

Multi-modality treatment or targeted therapy-containing management does not significantly improve overall survival.

Systemic chemotherapy management of extensive metastasis in HNSCC patients is a major concern. The drugs most commonly used clinically are the platin compounds (cisplatin and carboplatin), taxanes (docetaxel and paclitaxel), 5-FU, methotrexate, and ifosfamide. In an effort to improve response rates and, hopefully, survival time, combination chemotherapy needs to be developed.

5.3.4 Radiotherapy

Radiotherapy is the standard first-line treatment of bone metastases in HNSCC. Radiotherapy also has a role in the infrequent patients with brain metastases, especially for solitary brain metastasis with no extracranial diseases or have been controlled. It relieves clinical symptoms in 70-90% of patients (Hoegler, 1997). The use of stereotactic radiosurgical treatment remains to be defined. It is most often used to treat solitary metastases in previously irradiated patients.

Radiotherapy is unlikely to cure even solitary lung metastases. However, it may have a palliative role and increase survival when there are a limited number of foci, small metastases and locoregional control (Sugawara & Kaneta, 1983). Approximately 50% of patients with cancer develop bone metastases, although they are relatively unusual in head and neck cancer. They can cause pain and affect weight-bearing areas and consequently have a significant impact on quality of life. The role of radiotherapy in the palliation of bone metastases is well supported in the literature, with reported response rates of around 70-90% (Arcangeli, et al., 1998, Hoegler, 1997). The pain relief is complete in nearly half of the responders (Uppelschoten, et al., 1995) (Steenland, et al., 1999). If patients fail to respond to the first treatment, then they may respond to re-treatment.

5.3.5 Associated targeting therapy

Recently, molecular targeting biologicals with a different toxicity profile and hopefully less late damage to functionally important tissues may open new strategies in primary and adjuvant treatment of HNSCC. The principal strategies currently being used to design antiangiogenesis agents are aimed at blocking angiogenic factors (or enhancing negative regulators) or acting on endothelial cells to block cell surface receptors or prevent them from breaking down the surrounding matrix.

Besides cetuximab and other EGFR targeting mAbs, there are other receptors and non-receptor tyrosine kinase inhibitors, which might play an important role in the future treatment of HNSCC. Many investigations have been carried out to solve the problem of multi-drug resistance in HNSCC progenitor cells. Cetuximab, as an epidermal growth factor receptor-specific monoclonal antibody, plus radiation were shown to improve survival rate as compared to radiation treatment alone (Bonner, et al., 2006). However, one retrospective study suggests the duration of progression free survival and overall survival is shorter in patient receiving cetuximab plus radiation than those with cisplatin plus radiation (Pignon, et al., 2009).

It is postulated that VEGF targeted therapy has the potential to fulfill both anti-angiogenic and anti-tumorigenic functions (Tong, et al., 2008). As reported, CD44 certainly possesses a valid target for anti-cancer therapy. CD44 targeting members of several relevant pathways might be used to induce apoptosis or inhibit tumour angiogenesis and metastatic spread. Immunotherapy for HNSCC is a relatively new but promising therapeutic strategy. In HNSCC, immunotherapy has been implemented successfully in patients, especially in patients with end-stage metastasising disease, who had undergone a variety of other therapeutic modalities. Despite this fact, both clinical and translational trials with cytokines, monoclonal antibodies, and various kinds of other strategies have yielded promising results with little evidence of host toxicity. Future efforts will be focusing on finding ways to circumvent immune tolerance and overcome malignancy-related immune dysfunction to produce regimens with better efficacy.

6. References

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Advanced Squamous Cell Carcinoma of the Skin

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1. Introduction

The squamous cell carcinoma of the skin (SCCS) is one of the most common cancers around the world.(Ries, Melbert et al. 2007; 2008) It affects mostly the sun exposed areas of people with fair skin. The majority of cases are easily treatable by simple excision or radiotherapy with a good chance of achieving cure. Despite this, the aging population process associated to chronic ultraviolet radiation (U.V.) exposition is raising the SCCS incidence and consequently the number of patients with advanced tumors.(Staples, Elwood et al. 2006) This is a devastating presentation of the disease, where the lack of information occurs and even the professionals in the field are a few. Local disease progression, local and regional recurrence, lymph node or distant metastases are the focus of this review chapter. The characteristics of the tumors arising in the trunk and extremities are different from those in the head and neck, and they are described and discussed separately.

2. Advanced squamous cell carcinoma of the skin of the trunk and extremities

2.1 Definition

We consider as patients with advanced squamous cell carcinoma of the skin of the trunk and extremities, those with T3/T4 (tumor invading deep structures/axial eskeleton) or N1/2/3 (regional lymph node metastasis) tumors according the 7th UICC TNM classification(Sobin and Compton). Tumors arising from genital or anus are not considered.

2.2 Epidemiology, clinical presentation, diagnostic methods and defining a risk population

There are some clinical conditions associated to locally advanced disease. The patients are typically old, with risk conditions to skin carcinomas (chronic U.V. exposition and fair skin) and may have other local disease as burn scars, chronic skin ulcer and systemic pathologies related to immune system suppression (organ transplant receptors, hematopoietic disorders)(Cherpelis, Marcusen et al. 2002; Trakatelli, Ulrich et al. 2007). Low economic and educational status or difficult access to the health system may also play a role to the presentation of advanced cases, but not confirmed in studies.

The usual presentation is a patient with a long story of a “chronic ulcer” with many previous local treatments. Some present with pathological bone fractures or lymph node metastasis. (de Lima Vazquez, Sachetto et al. 2008)

TX Primary tumor cannot be assessed
T0 No evidence of primary tumor
Tis Carcinoma in situ
T1 Tumor ≤ 2 cm in greatest dimension with 2 high-risk features*
T2 Tumor > 2 cm in greatest dimension with or without one additional high-risk feature,* or any size with ≥ 2 high-risk features*
T3 Tumor with invasion of maxilla, mandible, orbit, or temporal bone
T4 Tumor with invasion of skeleton (axial or appendicular) or perineural invasion of skull base

*High-risk features include depth (> 2-mm thickness; Clark level ≥ IV); perineural invasion; location (primary site ear; primary site nonglabrous lip); and differentiation (poorly differentiated or undifferentiated).

Table 1. Definition of cutaneous squamous cell carcinoma tumor (T) staging system in 7th edition of American Joint Committee on Cancer

NX. Regional lymph nodes cannot be assessed
N0. No regional lymph node metastasis
N1. Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2. Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a. Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b. Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c. Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3. Metastasis in a lymph node more than 6 cm in greatest dimension

Table 2. Definition of cutaneous squamous cell carcinoma Nodes (N) staging system in 7th edition of American Joint Committee on Cancer

2.3 Factors related to prognosis

2.3.1 Clinical

The clinical factors classically related to prognosis are the tumor size and regional lymph node status (TNM stage). Many studies have confirmed both tumor size and local infiltration and presence and number of lymph node metastasis as the main prognosticator for advanced disease. The incidence of lymph node metastasis varies according to the population studied from 4.5 to 17% (North, Spellman et al. 1997; Cherpelis, Marcusen et al. 2002; Mullen, Feng et al. 2006). Other factors have been pointed out but the association is uncertain as

anatomic location and previous chronic disease locally on the skin (i.e. Marjolin ulcer). (Collins, Nickoonahand et al. 2004)

2.3.2 Pathological

A detailed histopathological descriptive classification considering all subtypes of squamous cell carcinoma of the skin was proposed by Cassarino (Cassarino, Derienzo et al. 2006), categorized tumors as low, intermediated and high risk, but it was not confirmed by well designed studies. The tumor length in millimeters (Breslow measurement) is associated with prognosis in some studies, but not confirmed (Breuninger, Black et al. 1990). The tumor grade, proposed by Broders and simplified to grade I, II and III (I well differentiated and III undifferentiated) is other controversial factor related to the prognosis, as well as the mitotic index and the Intratumoral lymphocytic infiltrate (ILI). In our retrospective study, the tumor grade was related to prognosis and the ILI was related to the lymph node metastasis. (de Lima Vazquez, Scapulatempo et al. 2011)

2.3.3 Molecular

With the advent of the molecular diagnosis, some authors looked at the relation of molecular changes and disease progression. Knowledge of the role of molecular markers in tumor progression and metastasis is limited. The tyrosine kinases Human Epidermal Receptor (HER) family (EGFR - Epidermal Growth Factor Receptor, HER-2, HER-3 and HER-4) are transmembrane glycoproteins related to cell proliferation, differentiation and apoptosis. Altered expression of the HER family is associated with several epithelial tumors such as breast carcinoma and esophageal squamous cell carcinoma. Small studies have also shown altered HER expression in localized squamous cell carcinoma when compared to normal skin. HER 2 expression in advanced CSCC of the trunk and extremities is not well studied and may be related to prognosis allowing the use of target therapies that block the HER pathway (Krahn, Leiter et al. 2001). E-cadherin is a transmembrane glycoprotein and it is a mediator of calcium-dependent cell-cell adhesion in normal cells. Reduced cell-cell adhesiveness is considered important in both early and late carcinogenesis. High E-cadherin expression in cell cytoplasm and low expression in the cell membrane is associated with tumor aggressiveness in different cancers. (Koseki, Aoki et al. 1999) Podoplanin is a membrane protein found on lymphatic vessel endothelium. Its function is poorly understood although it may govern endothelial motility and its absence in animal studies is associated with lymphedema and malformation of lymphatic vessels. (Schacht, Ramirez et al. 2003)

In our study with 55 patients with advanced cutaneous squamous cell carcinoma (CSCC) of the trunk and extremities, Primary tumor positivity was 25.5% for EGFR, 87.3% for HER-3 and 48.1% for HER-4. Metastases were positive for EGFR in 41.7%, for HER-3 in 83.3% and HER-4 in 43.5%. HER-2 was negative in all samples. Membrane E-cadherin and cytoplasmic E-cadherin were positive in 47.3% and 30.2% of primary tumors and 45.5% and 27.3% of metastases respectively. Podoplanin was positive in 41.8% of primary tumors and 41.7% of metastases. The hiperexpression of Podoplanin in the primary tumor was related to lower survival rates. The HER family and the E-cadherin were not related to prognosis. The HER-4 hiperexpression in the lymph node metastasis was associated to lower survival and showed that the HER family may play a role in the disease progression. (de Lima Vazquez, Scapulatempo et al. 2011)

2.4 The treatment modalities

2.4.1 Surgery

Surgery is the classic treatment for skin cancers and for advanced tumors it is still the most effective treatment. Unfortunately, in advanced cases amputations and extensive resections and dissections (i.e. extensive lymph node dissection with skin resection) are usual and have a high morbidity and sometimes mortality rate. Complex reconstructions with surgical flaps (figure 1) and other advanced techniques may be applied but local and clinical suboptimal conditions contraindicate them frequently. Local control is the goal, and the main objectives are to obtain clear margins and in case of lymph node metastasis, to clear completely the lymphatic chain (i.e. axilla or groin). Due the tumor characteristics of local and regional dissemination, aggressive approaches are indicated if clinical conditions are satisfactory. Recurrence rate vary in the literature achieving 50% {de Lima Vazquez, 2008}.

2.4.2 Radiation therapy

When surgery is not an option for advanced tumours, i.e - patient refusal, clinical adverse conditions - radiation may be applied, but with limited results. The main role of the radiation therapy is when incomplete resection occurs, and in the adjuvant setting, when tumour margins are not sufficient or after resection of bulky lymph node metastasis. Indications are personalised since there is no standard care with this method.

3. Head and neck tumors

3.1 Introduction

Squamous cell carcinoma accounts for 20% of non-melanoma cancers of the head and neck (Alam and Ratner 2001). In most cases, these tumors are cured with surgical treatment and / or radiotherapy, but a small portion of these patients had unfavorable outcomes with high rates of metastases and regional recurrence after treatment, which is associated with 20% of deaths from skin cancer (Alam and Ratner 2001). This more aggressive presentation is found in patients referred for high risk, which the literature has discussed the factors involved in this group (Veness 2007). In head and neck surgery, it is particularly associated with the presence of regional metastasis and invasiveness of the primary tumor. The latest edition of the UICC AJCC, published in 2010 (Edge and Compton 2010), showed major changes in the staging of nonmelanoma skin cancers, remarking lymph node staging aligned with the other sites of head and neck and including new factors for classification of the primary tumor.

This part of the chapter will present some specific features of the therapy of skin cancers of the head and neck that often overlap with those found in other regions of the body, but in advanced tumors may limit surgical treatment and carry a poor prognosis for these tumors.

3.2 Advanced tumors: Characteristics of primary tumor

The following factors define a high risk of metastasis and recurrence in skin cancers (Edge and Compton 2010):

Size of the primary tumor greater than 2 cm

Breslow tumor thickness greater than 2 mm, Clark level IV or greater

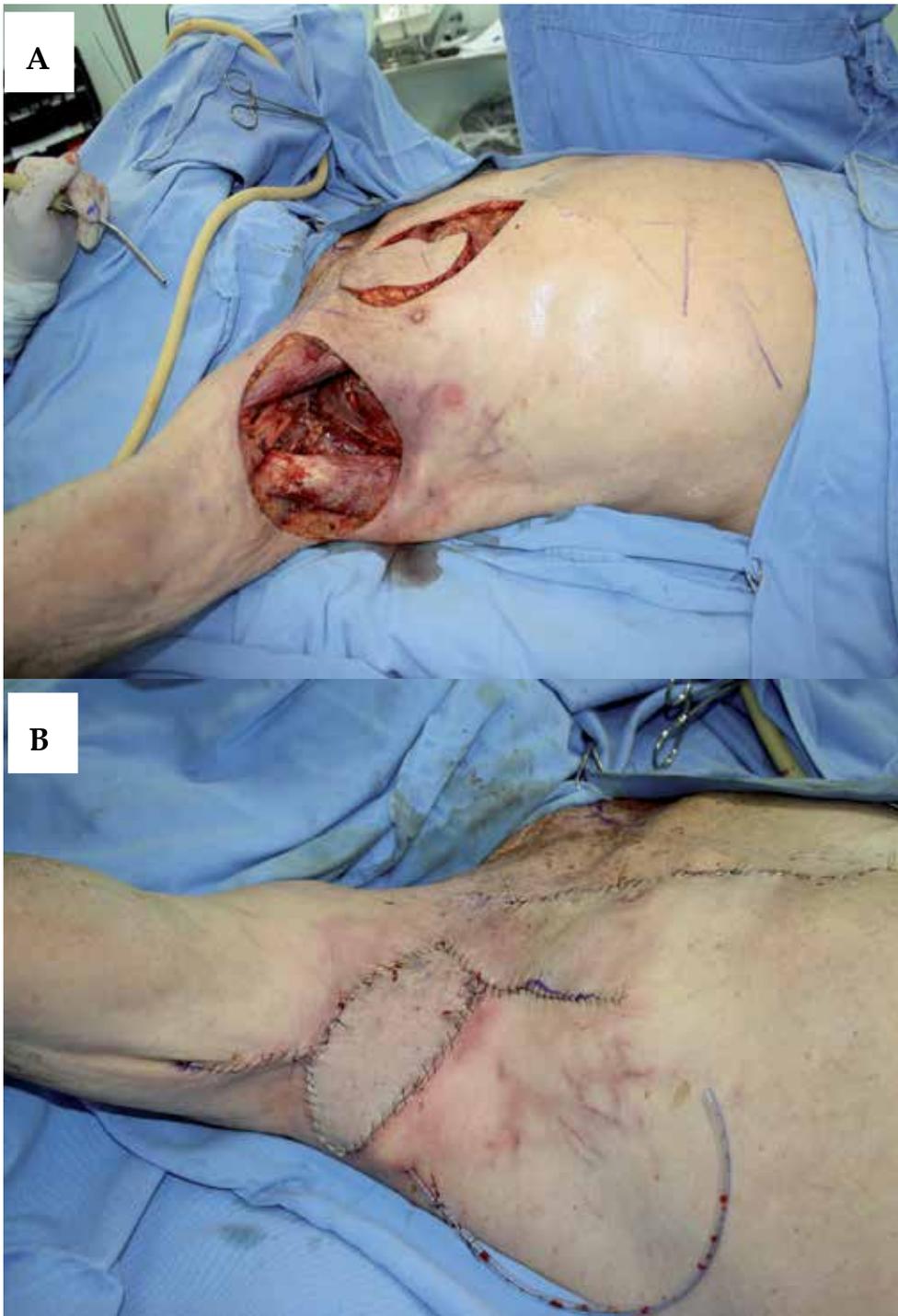


Fig. 1. A - Axilar lymphadenectomy with skin resection and miocutaneous flap prepared for the reconstruction. Fig. 1. B - Final aspect of the reconstruction

Perineural invasion (PNI)

Poor differentiation

Anatomic sites that which carry high risk for recurrence or metastasis

Immunocompromised state of the patient

These changes are based on data-derived, evidence-based medicine and some changed in the last edition of the AJCC and will be detailed below.

Tumor size: this parameter does not present a linear correlation of increased risk of metastasis and recurrence, according to tumor growth, however a limit of 2 cm is shown in several articles as a risk factor for locoregional recurrence. In the sixth edition of the AJCC edition, the limit of 5 cm separated the tumors in T2 and T3, however this limit did not have enough evidence to be sustained and was abolished in the seventh edition, being replaced by parameters more related to the invasiveness than the diameter of tumor (Farasat, Yu et al. 2011).

Tumor depth and PNI: Even the tumor thickness and depth of invasion are important risk factors to SCCHN metastasis and local recurrence (Farasat, Yu et al. 2011). So, in the last edition of AJCC, Breslow depth and Clark level were incorporated. These changes directly affected head and neck tumors that invade the facial bones or skull base, being classified as advanced tumors, with higher risk of metastases and local recurrence (Edge and Compton 2010).

Another factor was added is the perineural invasion of nerves at the base of the skull, which often restricts a craniofacial resection with clear margins and is associated with a worse prognosis. Although based on retrospective studies, PNI showed a higher association with tumors in the face, lower degree of differentiation, tumors larger than 2 cm and recurrent tumors (Leibovitch, Huilgol et al. 2005). There is evidence of an increased incidence of cervical lymphadenopathy and distant metastasis, along with significantly reduced survival in patients with tumors that showed PNI(Farasat, Yu et al. 2011).

A careful radiological preoperative assessment may reveal tumor invasion of branches of the trigeminal or facial nerve. The use of CT and MRI in invasive tumors of the skull base shows high accuracy in detecting perineural invasions, when correlated with intraoperative and pathological findings (Gandhi, Panizza et al. 2010).

Immunosuppression: Although not a specific factor that affects tumor staging, AJCC, in his last edition, highlights this as a risk factor for increased aggressiveness of skin tumors. Organ transplant recipients are 65 times greater risk of developing squamous cell carcinoma of the skin than the general population and have much more aggressive evolution.

Location of primary tumor: some anatomical sites are more associated with worse outcomes in head and neck. This can be seen in sites that drain to the parotid gland like external ear, temple, forehead and anterior scalp. The lower lip also has an increased risk of nodal metastasis.(Veness 2007)

Some series showed a poor outcome of cutaneous squamous cell carcinoma of external ear (Brantsch, Meisner et al. 2008; Turner, Morgan et al. 2009). Faustina et al found 24,3% of regional metastasis in 111 patients with squamous cell carcinoma of the eyelid and periocular region, advising close observation of parotid after the treatment of this site(Faustina, Diba et al. 2004)

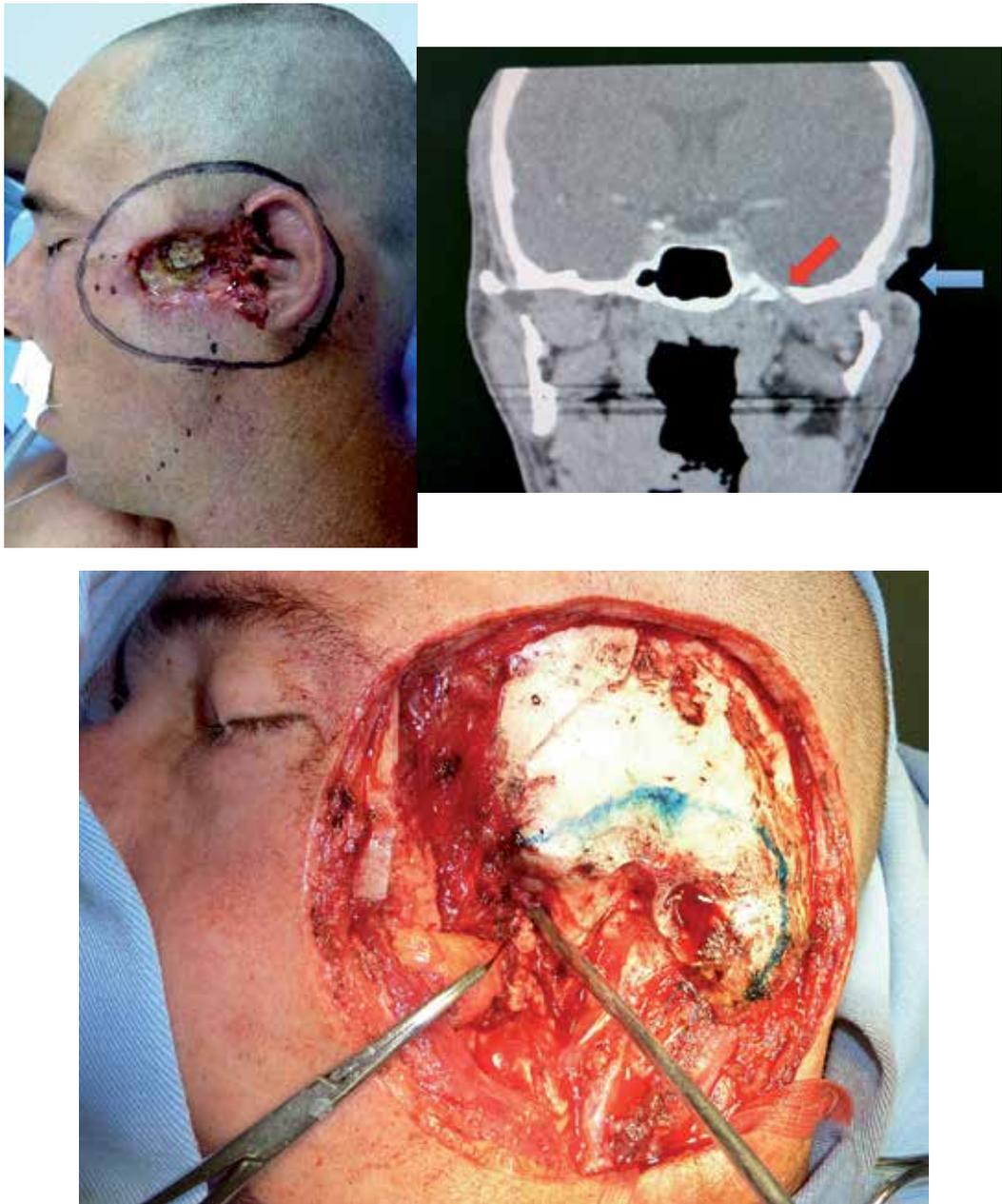


Fig. 2. A (On the left): Clinical aspect of the patient with invasive skin tumor and bone exposure of the zygomatic arc with installed facial nerve paralysis. Fig. 2. B (right): The red arrow reveals perineural invasion of V3 and the blue arrow shows the skin tumor of temporal region. Fig. 2. C: Surgical aspect after removal of skin tumor, with sacrifice of the facial nerve, zygomatic arc, ascending mandible and parotid gland. The blue line delimits the temporal bone resection by the neurosurgical team and the instruments pointing V3 at skull base, with perineural invasion

Histopathological differentiation grade: tumors less differentiated are associated with a more aggressive outcome. This item also has changed in the last edition of the AJCC, becoming one of the high risk factors, opposed to being a separate classification as in the sixth edition. (Farasat, Yu et al. 2011)

Lymph node metastases

SCCHN that develop nodal metastasis in the parotid gland or the neck is an aggressive disease and show poor outcomes. Some studies these prognosis revealing 5-years overall survival rates from 22 to 36% (Khurana, Mentis et al. 1995; Kraus, Carew et al. 1998).

Until the sixth edition of AJCC, the nodal staging of squamous carcinoma of skin only separate the presence of nodal metastasis or no. This classification was pointed by several authors as insufficient, what have already indicated the need of a new proposal for lymph nodes staging, including a P stage for parotid metastasis and stratification of nodal disease (O'Brien, McNeil et al. 2002; Ch'ng, Maitra et al. 2006). The seventh edition of AJCC incorporated some changes in nodal classification aligning with the staging of lymph nodes from others sites of head and neck but the P staging has not been implemented, because of the benefit of having subgroups of P and N stage is uncertain, but further research may demonstrate the need of this staging system (Palme, MacKay et al. 2007; Forest, Clark et al. 2010; O'Hara, Ferlito et al. 2010)

In a survey of tertiary treatment centers, about 5% of squamous cell carcinomas present skin present metastasis, usually to regional lymph nodes of the parotid and cervical level II. (2, 5). Sites such as cheek, ear, forehead, temple and lateral scalp are the most implicated in the onset of regional disease, which usually occurs on an average of 13 months of primary treatment, but may occur until 2 to 3 years later (Hong, Kriesel et al. 2005). The rate of regional metastases in head and neck can be between 10 and 20% when clinical and pathological characteristics of high-risk primary tumor are present.

When parotid metastasis with clinical negative neck are present, the risk of occult metastases in the cervical lymph nodes reaches 35-50%, which justifies elective neck dissection in the presence of parotid involvement(O'Hara, Ferlito et al. 2010). The data by Vauterin revealed when positive pathological neck is observed, level II is involved in 79% of cases and external jugular chain lymph nodes are particularly at risk, what should have not be forgot to be included in the neck dissection. (Vauterin, Veness et al. 2006).

Levels IV and V are only involved in massive lymph node disease to the neck, except in situations where the primary is located in the posterior scalp, in which the involvement of this code chain can be isolated. Metastases to the level I is present alone when the primary occurs in the anterior region of the face (O'Hara, Ferlito et al. 2010).

The radiological search of metastases to the parotid and neck should be performed only in patients at high risk, which can use CT, MRI and USG-guided FNA (O'Hara, Ferlito et al. 2010).

The use of sentinel lymph node in squamous cell carcinoma in head and neck is not yet defined and is not routinely used in cancer not melanoma, due to the low risk of nodal metastasis, but has potential for improved survival in patients at high risk (O'Hara, Ferlito et al. 2010). The sentinel lymph node study in the parotid region should be done with caution because it adds a possible morbidity due to the risk of facial nerve injury.

3.3 Treatment

3.3.1 Treatment of primary tumor

The treatment of advanced SCCHN tumors usually involves surgical resection and adjuvant radiotherapy. The goal of surgical treatment is tumor resection with clear margins. Tumors that fail to be cleared surgically often recur despite radiation. In contrast, high-risk SCCHN with clear surgical margins has documented excellent outcomes when compared to those with unreported margin status (local recurrence 5% vs. 8%, nodal metastasis 5% vs. 14%, distant metastases 1% vs. 7%, and disease-specific death 1% vs. 7%).

Skin squamous cell carcinoma with invasion of skull base are treated with craniofacial resections and have worse survival when compared to basal cell carcinomas. (Backous, DeMonte et al. 2005). Backous used as contraindication criteria for this type of resection encasement of the carotid artery or optic chiasm, cavernous sinus invasion or distant metastasis. Factors found to reduce survival are perineural invasion, intracranial extension with invasion of brain parenchyma and impossibility of adjuvant radiotherapy because of previous radiation.

Immunosuppressed patients should receive more aggressive surgical treatment and adjuvant radiotherapy should be strongly considered.

A multidisciplinary approach is recommended for the treatment of SCCHN, with combination of head and neck surgeon and plastic surgery, so that the reconstruction should not carry a limiting resection.

3.3.2 Treatment of regional metastasis

The published evidence suggests that the optimum treatment for metastatic SCCHN should be surgical resection with adjuvant radiotherapy. (O'Hara, Ferlito et al. 2010)

The most common site of metastases in the head and neck is the parotid gland. Usually when performing parotidectomy is associated the dissection of cervical levels I-III in negative neck and a radical neck dissection in positive necks (14, 18). This treatment option can save adjuvant irradiation in the pathologically negative neck restricting the field of radiation only to the parotid field, but another option is the radiation of the clinically negative neck.

In patients undergoing parotidectomy, Ebrahimi recommends selective neck dissection including level I to III for facial primaries, level II and III for anterior scalp and external ear primaries, and levels II to V for posterior scalp and neck primaries (Ebrahimi, Moncrieff et al. 2010). Isolated metastases of level V and primary region of the scalp or posterior suboccipital region, a posterior lateral neck dissection (II to V) is recommended. (O'Hara, Ferlito et al. 2010)

Cervical neck node disease without parotid involvement can be seen in 18 to 41% of patients. (Andruchow, Veness et al. 2006; Vauterin, Veness et al. 2006) In this situation, the recommendation is the treatment of the neck with classic or modified radical dissection associated with elective parotidectomy in primaries of anterior regions of scalp and lateral face (Barzilai, Greenberg et al. 2005; Jennings and Schmults 2010).



Fig. 3. A: Example of multidisciplinary approach: recurrent squamous cell carcinoma after local excision and radiation therapy. Facial nerve paralysis and intratemporal perineural nerve spread. Fig. 3. B: Surgical field of total parotidectomy and sacrifice of the facial nerve and ascending portion of mandible and neck dissection of levels I-III with sacrifice of sternocleidomastoid muscle. The blue line delimits the temporal bone resection by the neurosurgical team, with frozen sections of the nerve stump.

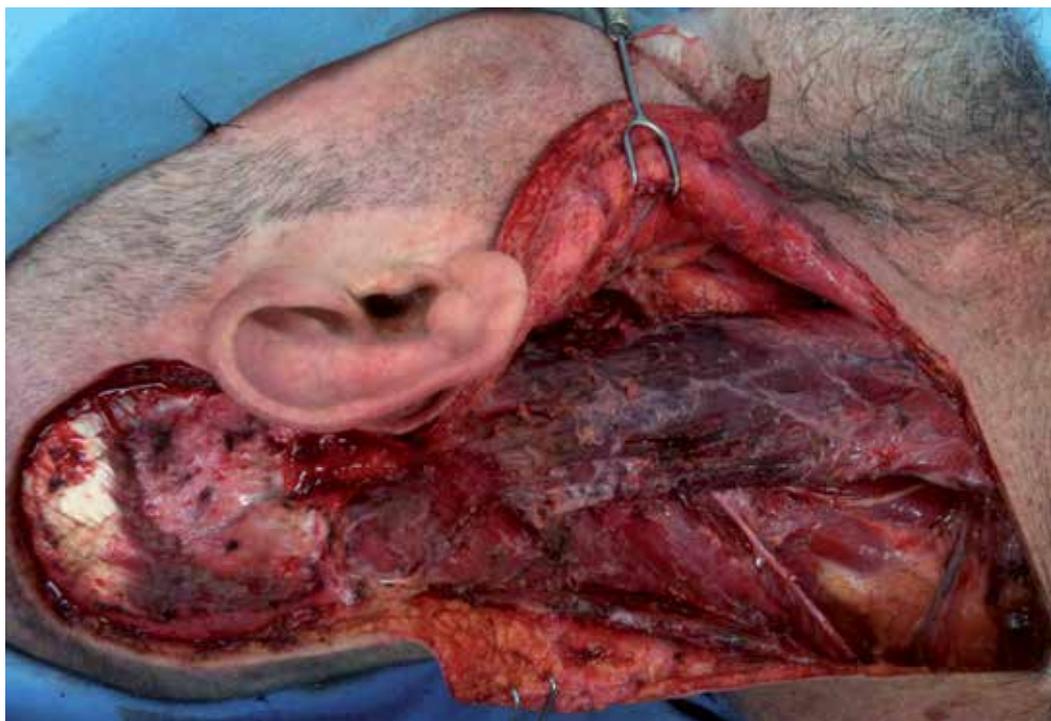


Fig. 4. Surgical treatment aspect of a posterior scalp skin tumor with posterior lateral neck dissection (cervical levels II-V), sparing the spinal accessory nerve

Although some anatomical regions have an increased risk of developing regional metastases, it is difficult to recommend elective neck dissection as a routine base, because of the low rate of nodal spread and high prevalence of these skin cancers. Others risk factors should be added to consider elective regional treatment like immunocompromised host, poorly differentiated grade (Veness 2007). Elective parotidectomy for patients without clinical or radiological evidence of metastasis of the neck or parotid is not recommended by most authors (Osborne, Shaw et al. 2008).

Intraparotid metastasis to lymph nodes may be attached to the facial nerve, which is at risk of sacrifice in some situations. All facial nerve not functioning in the preoperative evaluation or completely surrounded by tumor should be sacrificed but it should rarely be done when it has normal function before surgery. According to Iyer, surgical approaches to the parotid metastatic cancers shall, as far as possible, spare the facial nerve with normal function, even if that causes resection with microscopic involved margins and the need for adjuvant radiotherapy. Such strategies do not generate differences in the rates of recurrence and overall survival compared with patients undergoing resection with microscopically free margins. However, this study shows no statistical difference between groups, but shows a tendency to a worse local control and survival, which could have significance with a larger number of cases. In fact, free surgical margins greater than 5 mm are rarely obtained due to the proximity of parotidectomy metastasis with the facial nerve (Iyer, Clark et al. 2009).

Parotidectomy may be associated with resection of skin tumors of the parotid region and the series of Lai reveals perineural invasion of the facial nerve in 6 of the 23. Lai recommends

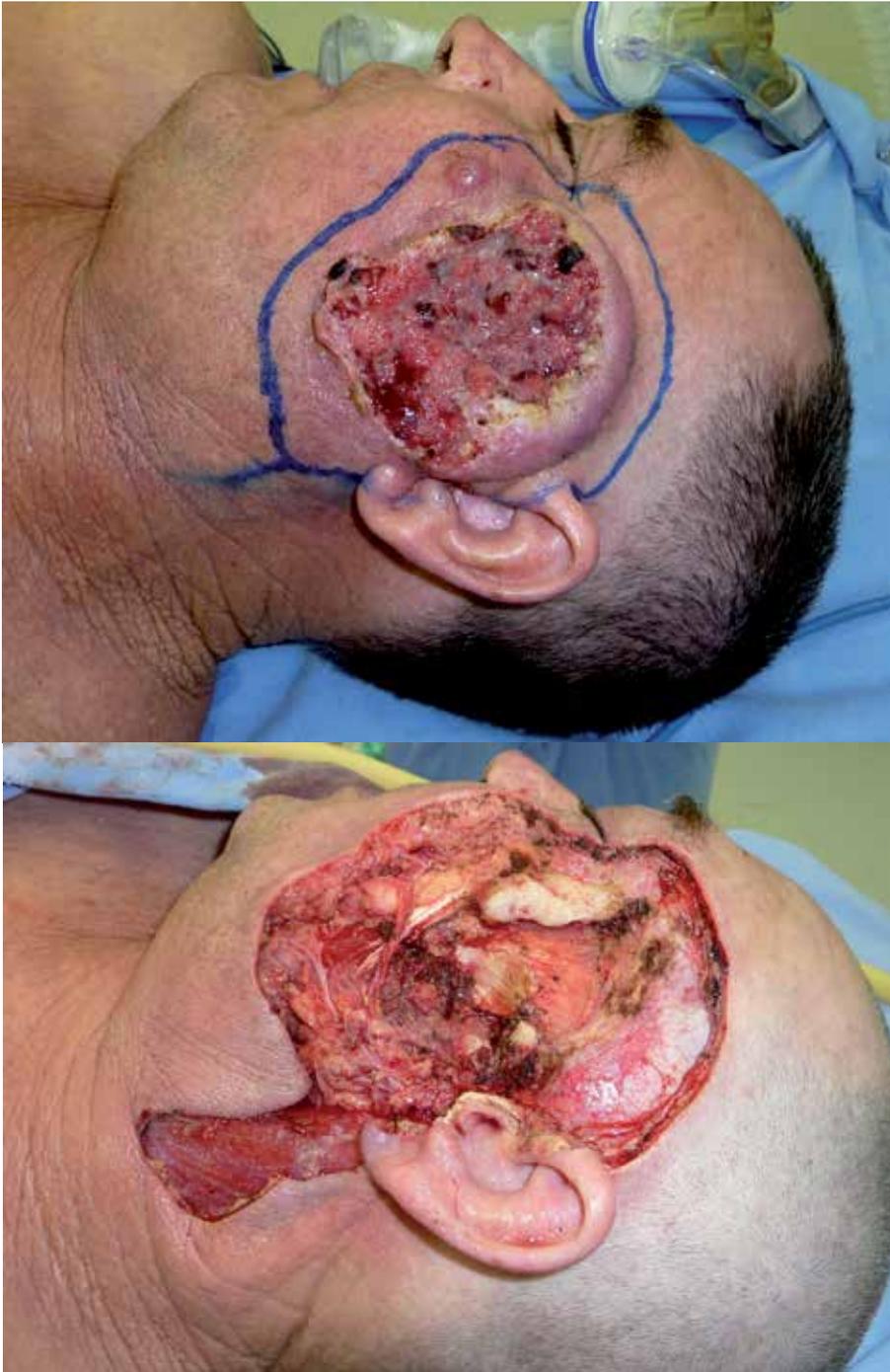


Fig. 5. A: Squamous cell carcinoma of skin of parotid region, with in transit metastasis. Fig. 5. B: Surgical field after resection including superficial parotidectomy and the zigomatic arc with sacrifice of the superior branch of facial nerve that was involved by the tumor.

parotidectomy depending on the depth of the primary lesion, especially in the preauricular region. Similarly, facial nerve dissection is necessary in superficial parotidectomy for nodal involvement or deep invasion from preauricular primary lesions. As the facial nerve is identified, areas of gross tumor involvement may necessitate partial or total facial nerve resection. When there is sacrifice of the facial nerve, some authors recommend frozen sections of the facial nerve stumps, to ensure free margins before a microsurgical nerve reconstruction (Lai, Weinstein et al. 2002).

3.3.3 Radiotherapy

Primary radiation for SCCHN can be an alternative treatment when the surgical defect causes challenging reconstructions and should be used in lesions with little invasiveness. Local tumor control in small lesions rivals that of surgical resection, even in recurrent disease. However, as T stage increases, local control decreases when compared to surgical excision. (Mendenhall, Amdur et al. 2009)

The treatment of neck or parotid metastasis, surgery and adjuvant radiotherapy should be done rather than radiation alone (Palme, O'Brien et al. 2003). Multiple studies have noted decreased disease-specific survival in patients treated with RT alone (delCharco, Mendenhall et al. 1998; Veness, Palme et al. 2003)

The use of adjuvant radiation should be strongly considered in incomplete excision or positive margins, perineural invasion, multiple nodal involvement and recurrent tumors. (Veness 2007)

4. Systemic therapy (for all tumors)

4.1 Adjuvant therapy for high risk SCCS

The risk of locoregional recurrence and regional or distant metastasis is the most important factor in determining the treatment for cutaneous SCC. In a large review of studies of SCC of the skin, lip and ear, between 1940 and 1992 it was observed that recurrence rates double from 7.4% to 15.2% for tumors greater than 2 cm in diameter, and that tumors less than 4 mm in depth are at low risk for metastasis compared with tumors deeper than 4 mm (6.7% and 45.7%, respectively). (Rowe, Carroll et al. 1992) Also, 30% of locally recurrent SCCS develop metastases. Long-term prognosis for metastatic disease is extremely poor. Ten-year survival rates are less than 20% for patients with regional lymph node involvement and less than 10% for patients with distant metastases. (Cherpelis, Marcusen et al. 2002).

Histopathologic features associated with an increased risk of local failure or metastasis include large lesion size, perineural invasion, and involvement beyond the subcutaneous tissue. (Clayman, Lee et al. 2005)]

Chemotherapy in the management of high-risk SCCS remains relatively unexplored. (Jennings and Schmults 2010) The role of retinoids, which are known to decrease new cancer formation, but do not alter the course of an existing tumor, as prophylactic agents in patients with diffuse actinic damage or recurrent CSCCs is well established, especially in organ transplant recipients (OTRs). (Harwood, Leedham-Green et al. 2005) Unfortunately, randomized trials of retinoids, either used alone for the adjuvant-treatment of established mucosal SCC of the head and neck (Toma, Bonelli et al. 2004) or in combination with interferon (Brewster, Lee et al. 2007) for established SCCS, have shown no benefit.

Many of the available agents with activity demonstrated in advanced SCCS, including EGFR inhibitors and oral capecitabine, are well tolerated with relatively low risks, and are potential candidates for adjuvant therapy in highest-risk cases. Further work remains to identify patient subsets likely to benefit from adjuvant chemotherapy and to define optimal regimens. Collaborative clinical trials are needed to establish standardized prognostic or treatment models to assist clinicians in most effectively identifying and managing patients at risk for poor outcomes. (LeBoeuf and Schmults 2011)

4.2 Systemic therapy for advanced SCCS

The use of systemic therapy is limited to patients with distant metastases or locally advanced disease that cannot be adequately managed with surgery or radiotherapy. Because of the rarity of metastatic squamous cell cancers of the skin, the approach to systemic treatment is based primarily upon isolated case reports, with only a few small case series.

Treatment of metastatic SCC may include systemic chemotherapy or treatment with biologic response modifiers. The efficacy of these methods has not been established.

Wollina and colleagues (Wollina, Hansel et al. 2005) reported 4 patients with advanced SCC of the skin who were treated with oral capecitabine and IFN subcutaneously, resulting in complete remission in 2 patients and partial response in the other 2. IFN may act synergistically to capecitabine by causing a forced accumulation of 5-FU in tumor cells as a result of stimulation of dThdPase. In another report the use of oral capecitabine alone for the treatment of 14 patients with advanced cutaneous SCC resulted in 2 partial remissions and 3 minimal remissions. (Cartei, Cartei et al. 2000)

Cisplatin-based combinations appear to be the most active regimens in the published experience. Most regimens that have been studied for the treatment of advanced SCCS were adapted from those used for squamous cell cancers arising in other sites. Sadek et al. reported on the treatment of 14 patients with advanced squamous cell carcinoma of the skin or lip with a combination of bolus cisplatin, plus a five-day infusion of bleomycin and 5-fluorouracil. (Sadek, Azli et al. 1990) Four complete and seven partial responses were observed and in seven patients, tumor regression permitted subsequent definitive local treatment with either surgery or radiation therapy.

Using a combination of cisplatin daily times four plus a four day continuous infusion of bleomycin to treat five patients with locally advanced disease of the head and neck (three squamous cell and two basal cell), Denic observed one complete response and 3 partial responses. (Denic 1999) One patient had disease progression.

Multiple targeted therapies are being developed for many malignancies, including those with squamous cell histologies. These may ultimately have utility in patients with advanced or metastatic non melanoma skin cancers. The primary targets of molecular inhibition in squamous cell carcinoma include the epidermal growth factor receptor (EGFR), the vascular endothelial growth factor (VEGF) and its receptor, and tyrosine kinase (TK). (O'Bryan and Ratner 2011) Molecular studies have demonstrated that these molecules are overexpressed in a subset of SCCS and may be associated with more aggressive clinical behavior. (Detmar, Velasco et al. 2000; Maubec, Duvillard et al. 2005; Ch'ng, Low et al. 2008)

Intracellular signal transduction mediated by the epidermal growth factor receptor (EGFR) has been one of the most studied pathways in carcinogenesis. The phosphorylation of EGFR activates multiple biological processes, including apoptosis, differentiation, cellular proliferation, motility, invasion, adhesion, DNA repair, and survival. EGFR is a transmembrane tyrosine kinase receptor involved in the proliferation and survival of many cancer cells and is one of the first molecular target against which monoclonal antibodies have been developed for cancer therapy. EGFR plays an important role in tumorigenesis of non melanoma skin cancer, especially metastatic squamous cell carcinoma, via mechanisms similar to those of other visceral tumors. (Khan, Alam et al. 2011)

Several case reports suggest that Cetuximab, a monoclonal antibody that targets the epidermal growth factor receptor (EGFR), has antitumor activity in patients with advanced squamous cell carcinoma of the skin. (Bauman, Eaton et al. 2007; Suen, Bressler et al. 2007; Arnold, Bruckner-Tuderman et al. 2009)

Maubec and colleagues reported the results of a phase II study that included 36 patients with advanced squamous cell carcinoma of the skin treated with cetuximab in the first line setting. (Maubec, Duvillard et al. 2010) In this study, cetuximab was administered on a weekly schedule (400 mg/m² on week 1 and then 250 mg/m² weekly). Eight partial and two complete responses were observed, and 21 had stable disease for an overall disease control rate of 69 percent. Furthermore, three patients were able to undergo complete resection of their tumor following systemic treatment with cetuximab. Similarly to what is reported in other malignancies, patients developing acneiform rash apparently had a better outcome.

The combination of cetuximab with chemotherapy is a promising approach. Association with platinum-based chemotherapy and 5-FU in patients with recurrent or metastatic SCC of the head and neck (SCCHN) has shown benefit in a large prospective randomized trial. (Vermorken, Mesia et al. 2008) In this study, 442 eligible patients with untreated recurrent or metastatic SCCHN were randomized to receive 5-FU with cisplatin or carboplatin, with or without cetuximab. The ORR was 36% versus 20% with and without cetuximab ($P = 0.001$). Survival increased from 7.4 to 10.1 months with the addition of cetuximab ($P = 0.04$), and progression-free survival increased from 3.3 to 5.6 months ($P \leq 0.0001$).

Other targeted EGFR inhibitors are also currently under investigation in clinical trials mainly for the treatment of SCCHN, including Panitumumab (Vectibix, Amgen, Thousand Oaks, CA) which is a fully human monoclonal antibody to EGFR. (Lacouture and Melosky 2007; AMGEN 2011; AMGEN 2011)

Additionally, many targeted molecular therapies to VEGF and VEGF TKs have proven efficacy in other malignancies. Research into their use for SCCHN is growing and these agents may also be useful for the treatment of SCCS. (Wang and Agulnik 2008) Bevacicumb (Avastin, Genentech, South San Francisco, CA) is a fully human monoclonal antibody against VEGF, and is being tested for recurrent and metastatic SCCHN in a phase 3 trial comparing chemotherapy alone versus chemotherapy plus bevacizumab. (Wang and Agulnik 2008) There is hope that the combination of EGFR and VEGF pathway inhibitors will provide an increased clinical benefit in such patients. This alternative is

being studied in an ongoing phase 2 trial, cetuximab, radiotherapy, and pemetrexed with or without bevacizumab is being tested in patients with locally advanced SCCHN. (Gold, Lee et al. 2009)

As mentioned earlier, the vast majority of studies focus on the use of molecular inhibitors in SCCHN. More research is needed, for the development of new treatment modalities and to establish their role in treating patients with advanced or aggressive CSCC.

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Sarcomatoid Squamous Cell Carcinoma

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1. Introduction

Although much has been characterized about squamous cell carcinoma of the lower female reproductive tract, there is limited knowledge and experience with cases demonstrating squamous cell carcinoma with sarcomatoid features. Sarcomatoid squamous cell carcinoma (SSCC) is mainly found in the upper aerodigestive tract with the larynx being the most common site in the head and neck region. (Otay et al., 2011) The esophagus and skin are other well known affected sites. In these sites, risk factors include, smoking, alcohol consumption, and previous irradiation of the head and neck region. (Otay et al., 2011) Risk factors for skin involvement include prolonged sun exposure and the effects of toxins and irradiation.

True sarcomas of the lower female genital tract arising in the vulva, vagina, and cervix, are extremely rare. They comprise only 1-2% of vulvar cancers, 2% of vaginal cancers (Temkin et.al,2007), 2-3% of cervical cancers, and 3% of endometrial cancers. They tend to be heterogeneous, rapidly progressive, with a high rate of recurrence and distant metastasis to organs such as liver and lungs. For the vulva and vagina, the main sites of occurrence are the labium majus and upper vagina, respectively. Leiomyosarcomas are the most common type of sarcoma in the female genital tract where several factors have been demonstrated to play a role in recurrence including tumor diameter, cytologic atypia, mitotic index, and infiltrating margins. Although there are no specific treatment guidelines, the mainstay of therapy has been surgical excision followed by chemotherapy. Neoadjuvant chemotherapy has been used in patients who have bulky tumors in which surgical debulking had not been optimal. Currently, some believe that neoadjuvant chemotherapy can also be used as primary therapy followed by surgical debulking regardless of tumor. It is thought that this treatment strategy can optimize surgical excision, giving rise to less morbidity, and a longer disease-free interval. (Temkin et.al,2007) The role of radiation therapy has been controversial as some believe that sarcomas themselves are induced by a history of radiation therapy or exposure. Nevertheless, there have been reports of radiation lengthening the time interval to local recurrence.

Sarcomatoid squamous cell carcinoma of the female genital tract has been reported in very small numbers. Human Papilloma Virus (HPV) has been known to be a major causal factor for the development of SCC. High risk subtypes of Human Papilloma Virus, 16 and 18, have been demonstrated in not only the squamous cell component of SSCC but also in the

sarcomatoid component. Making immunohistochemical assays of this cancer a hallmark to diagnosis to differentiate pure squamous cell carcinoma from a pure sarcoma.

Like pure sarcomas, Sarcomatoid Squamous Cell Carcinoma has a very poor prognosis, short disease-free intervals, and is diagnosed at later stages of disease. Even with optimal treatment and follow up, SSCC recur rapidly and metastasize to regions such as the peritoneum, kidney, and subcutaneous tissue. Most cases of SSCC of the female genitalia have been reported to arise from the cervix and vulva. There are currently 16 cases of cervical and vulvar SSCC reported in the English literature to date. (Tae-Wook Kong et. al., 2010 and Dong-Seok Choi et. al., 2006), with our current case of vulvar SSCC making up the 17th case.

SSCC of the Cervix Literature Review

Author	No. of Cases	Age	FIGO	Tumor Diameter(cm)	Primary treatment	Outcome after primary treatment	Survival (months)
Steeper et al (1983)	1	67	III	10	ERT	PD	2, DOD
Pang (1998)	2	65 61	N/A N/A	6 5	RAH+CRT RAH+CRT	PD RD	2, DOD 14, DOD
Rodrigues et al (2000)	1	39	IB2	6	ERT+RAH	RD	12, DOD
Brown et al (2003)	9	29 32 34 39 47 57 59 59 76	IB2 IB2 IB1 IB IIA IVA IVB IVB IIA	4 8 1,6 N/A 3 N/A N/A N/A 5	RAH+ERT ERT RAH+CRT ERT ERT ERT+exenteration TAH+CTx ERT ERT	RD CR CR RD CR RD RD RD CR	N/A 42, NED 5, NED N/A 40, NED N/A N/A N/A N/A 22, NED
Lin et al (2006)	1	31	N/A	6	RAH	CR	20, NED
Mohan et al (2008)	1	75	IIB	5,6	ERT	CR	10, NED
Kumar et al (2008)	1	54	IIIB	4	CRT	CR	6, NED
Tae-Wook King et al (2011)	1	26	IB1	2	LRH	CR	18, NED

Table 1. SSCC of the Cervix Reported Cases in the Literature (Tae-Wook King et al, 2010)

N/A= not available, ER= external pelvic radiation therapy, RAH= radical abdominal hysterectomy, CRT= chemoradiation therapy, TAH= total abdominal hysterectomy, LRH= laparoscopic radical hysterectomy, PD= progressive disease, RD= recurrent disease, CR= complete remission, DOD= dead of disease, NED= no evidence of disease

Authors	No	Age	FIGO stage	Histologic report	IF-LN	P-LN	Primary treatment	Adjuvant therapy	DFI	Survival
Way (1960)	6	-	-	Epithelioma of very unusual type	NA	NA	Surgical treatment	-	NA	No longer than 4.5 years
Gosling et al (1961)	2	-	-	Spindled SCC	NA	NA	Surgical treatment	-	NA	NA
Copas et al (1982)	1	54	III	Poorly differentiated spindle cell carcinoma	+	-	RV with bilateral groin and pelvic LND	CTx with RTx	1 month	2-3 months
Steeper et al (1983)	1	89	-	Pseudosarcomatous SCC	NA	NA	RTx	Simple vulvectomy	9 months	2.5 months
LiVolsi et al (1987)	2	-	-	Carcinoma with sarcomatoid features	NA	NA	NA	NA	NA	NA
Santeusanio et al (1991)	1	77	IV	Poorly differentiated carcinoma with sarcoma-like features	+	NA	RV with bilateral femoral inguinal LND	-	15 days	1 month
Parham et al (1991)	1	54	I	Mixed soft tissue sarcoma with atypical squamous cells	NA	NA	Local excision	-	3 years	More than 6 years
Cooper et al (2002)	1	73	III	Sarcomatoid SCC	+	NA	RV with bilateral inguinal LND	RTx	5 months	NA
Dong-Seok et al (2006)	1	43	II	Poorly differentiated SCC with extensive sarcomatoid features	-	NA	Radical local excision and bilateral groin LND	NA	NED	More than 2 years
Case Number Two Below	1	78	I	Poorly differentiated SSSC	-	-	Bilateral simple hemivulvectomy	NA	NED	Alive at present date

IF-LN= inguino-femoral lymph node metastasis, P-LN= pelvic lymph node metastasis, DFI= disease free interval, NA= not available, SCC= squamous cell carcinoma, NED= no evidence of disease, RV= radical vulvectomy, LND= lymph node dissection, CTx= chemotherapy, RTx= radiation therapy

Table 2. SSSC of the Vulva Reported Cases in the Literature (D.-S. Choi et al, 2006)

Four cases of vaginal SSSC have been reported, with our current case constituting the fifth.

Authors	Age	Site	FIGO Stage	Initial Diagnosis	Treatment	Follow up
Steeper et al (1983)	54	Fornices bilaterally	II	Malignant fibrous histiocytoma	8500 rads with F/U Adriamycin/vincristine DTIC	Died 11 months after diagnosis
	81	Lower 1/3 anterior, extending to labium minus	Positive right inguinal nodes	Carcinosarcoma	55mg of intravaginal radium, 9990 rad to vaginal surface, and 5075 rad to right pelvic lymph nodes	Free of recurrence 4 years after diagnosis
Motoyama et al (1989)	74	Middle 1/3 left lateral	II	Sarcomatous spindle cell tumor	7000 rad external radiation with F/U vincristine, actinomycin D, cyclophosphamide	Died 1 year after diagnosis with local recurrence and lung metastasis
Raptis et al (1993)	25	Upper 1/3 left lateral wall	II	Sarcoma	4600 CGw external radiation, and high dose brachytherapy 3x 800 cGw	No evidence of disease 6 months after diagnosis
Case number 1 below	67		III	Leiomyosarcoma	Radiation, Chemo	Metastatic disease at 2 years

Table 3. SSCC of the Vagina Reported Cases in the Literature (Raptis et al, 1993)

The low volume of cases poses a dilemma in that institutional encounters of such cases are sporadic and as such, there are no established guidelines for diagnosis or treatment. These cancers have traditionally been treated and staged similar to squamous cell carcinomas of their respective sites using the FIGO staging system. There has been reported success in long term survival rates of patients who present earlier in the disease and whose tumors could be fully resected. These presentations are rare, as patients tend to present with long standing bulky tumors and with metastasis.

2. Case report

Two cases of sarcomatoid squamous cell carcinoma, one of the vagina and one of the vulva, will be utilized as illustrative examples of this rare entity.

Case number one: 67 year old, Gravida 7 Para 6 woman status post total abdominal hysterectomy 13 years prior for reportedly benign fibroids, presented with profuse vaginal bleeding. Examination revealed a vaginal lesion which was biopsied, revealing a high-grade, malignant sarcomatoid neoplasm with no concurrent precursor lesion, such as an in-situ component. Pathologic assessment suggested that this was a sarcoma, specifically, a leiomyosarcoma, as evidence of smooth muscle differentiation was demonstrated by immunohistochemistry. However, with subsequent demonstration of pelvic sidewall and pelvic lymph node involvement, features not typically associated with sarcomas,

immunohistochemistry for p16 and in situ hybridization for high risk human Papilloma virus (HPV) subtypes was performed; this demonstrated that the tumor was, indeed, an HPV-driven malignancy compatible with sarcomatoid squamous cell carcinoma. Following a course of chemotherapy and radiation therapy, the patient presented two years later with deep vein thrombosis and obstructive uropathy, with subsequent biopsy demonstrating recurrent metastatic squamous cell carcinoma involving the ureter.

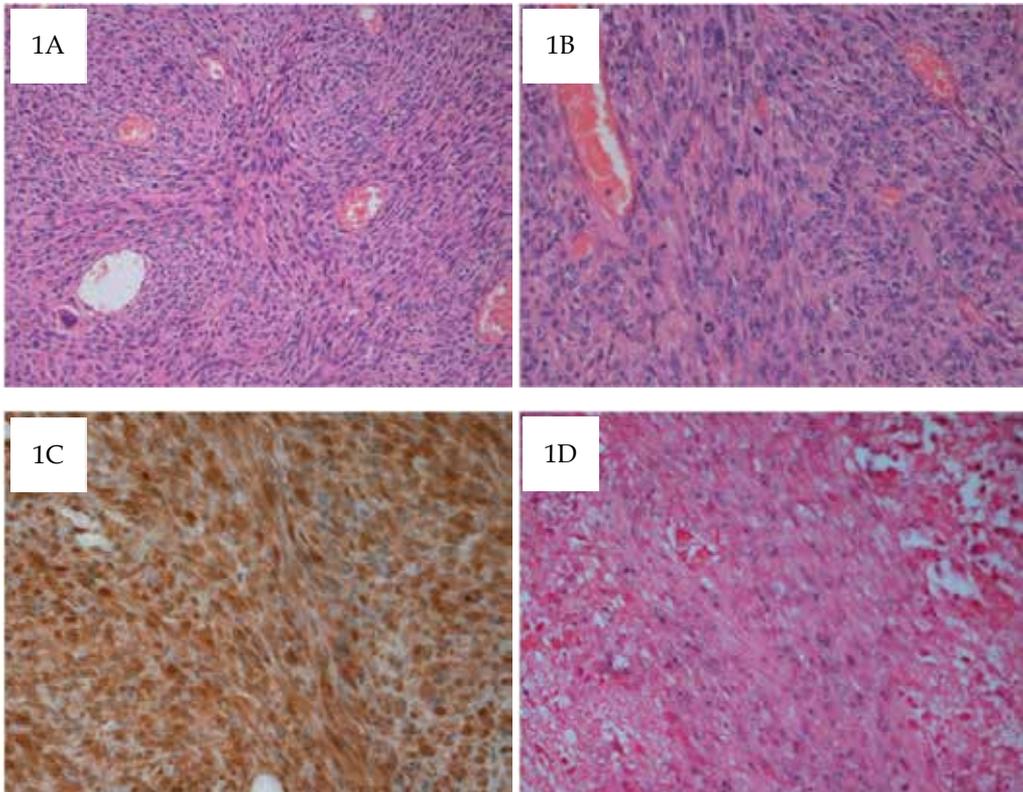


Fig. 1. Sarcomatoid squamous cell carcinoma of the vagina. (A) The lesion is characterized exclusively by spindled cells, architecturally arranged in fascicles. No in situ or conventional squamous cell morphology is apparent [200x original magnification]. (B) High-power magnification shows spindled cells with abundant mitotic figures [400x original magnification]. (C) Neoplastic cells show diffuse nuclear reactivity for p16, indicative of a HPV-driven tumor [400x original magnification]. (D) Chromogenic in situ hybridization for high-risk HPV subtypes demonstrates diffuse nuclear chromogenic labeling (in blue dot pattern) [400x original magnification].

Case number two: 78 year old Para 1 woman with long-standing vulvar irritation presented with a 4cm vulvar lesion. She underwent biopsies that demonstrated a high-grade vulvar intraepithelial lesion (VIN III, carcinoma in situ), and subsequent wide local excision showed involved margins. Four years later, she re-presented with a 4cm, enlarging vulva mass; excision of this mass revealed an invasive, poorly differentiated Sarcomatoid Squamous Cell Carcinoma (SSCC), again with positive margins. She subsequently underwent a bilateral simple hemivulvectomy with pathology demonstrating involvement of the urethral margin,

with other margins uninvolved. A metastatic evaluation was negative. Other comorbidities rendered the patient to be a poor candidate for additional surgical intervention.

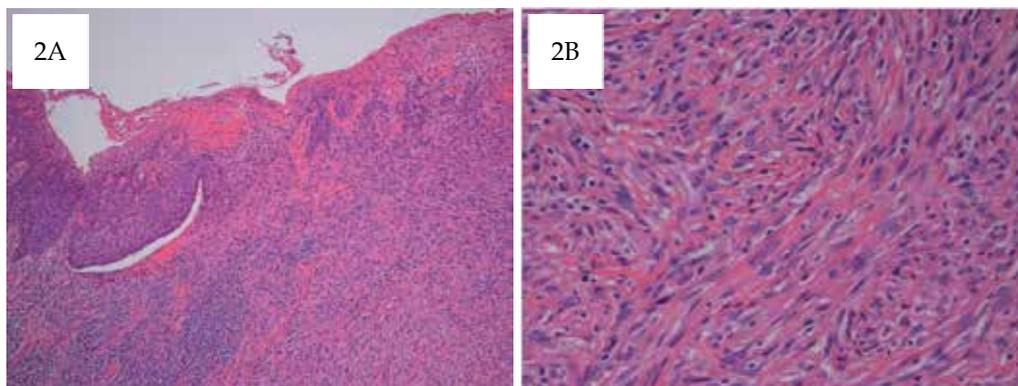


Fig. 2. (A) Sarcomatoid squamous cell carcinoma of the vulva. The lesion has an interface with an in situ component (left side of image), and transitions to a tumor of predominant spindled cell morphology (right side of image) [100x original magnification]. (B) High-power magnification shows spindle cell morphology in a fascicular architectural pattern, imparting a mesenchymal-like, sarcomatoid morphology [400x original magnification].

3. Incidence

The incidences of SSCC has not been well established but is deemed to be very low from the isolated published case reports, constituting, at most only 1-2% of all gynecologic malignancies overall. There have been more reported cases of vulvar and cervical SSCC than cases from the vagina. This trend is also seen in squamous cell carcinomas of the female genital tract, with cervix and vulvar being more common than vagina. In general, SSCC are observed in more frequency with advancing age, and there is a correlation between vaginal cancers in women who have undergone a hysterectomy for malignant disease. It would be interesting to see if this is also true in SSCC. A possibility for the low incidence of SSCC is that the sarcomatoid component is under recognized, or under reported as part of the pathology report.

4. Risk factors

Risk factors for SSCC of the female genital tract are assumed to be the same as risk factors for Squamous cell carcinoma. This is even more so if the belief that SSCC transforms from SCC. Risk factors include HPV infection, high risk sexual behavior, cigarette smoking, specific vitamin deficiencies, and immunosuppression.

Human Papillomavirus (HPV) is a well known causal factor for the development of squamous cell carcinoma of the vulva, vagina, cervix, anus, and oropharynx. It is the most frequently diagnosed sexually transmitted disease in the United States. HPV subtypes 16 and 18 in particular have a high known oncogenic potential and are thus called "high risk" subtypes, accounting for approximately 80% of cases of invasive cervical cancer. (Bereck and Hacker, 2010). HPV has been reported to affect roughly 20 million people in the U.S. and is the most frequently diagnosed STD. This number is expected to decrease as more

young 2A 2B Sarcomatoid Squamous Cell Carcinoma 7 women and men are being offered the Gardasil and Cervarix injections. These injections are FDA approved and are aimed at immunizing against some of the "high risk" human papillomavirus types that predispose to cancer and the "low risk" subtypes of human papillomavirus that cause genital warts. Despite relatively wide availability, less than 30% of patients receive the recommended three doses. (NCI Cancer Bulletin, 2011) Even though the rates of HPV infection decrease sharply after 30 years of age, older women are less likely to clear an infection with a high risk subtype of HPV. The older the patient at presentation the more likely the patient will have a more advanced stage (Berek and Hacker 2011)

High risk sexual behavior is a risk factor due to the increase in exposure to sexually transmitted infections (STI). The more sexual partners one has the higher the chance that they will become infected with an STI. This is partly due to the fact that some STI's cause disruption of the epithelial cell layer that can facilitate the transport of infectious material. When the cervix is exposed to infection it undergoes reparative metaplastic changes that can also increase susceptibility of and STI. (Berek and Hacker, 2010)

Cigarette smokers are known to be at increased risk of cancer of the lungs and other body organs including the cervix and vulva. Cigarette smoke is an independent risk factor for cervical cancer. (Nishino, K. et al, 2008) The pathogenesis is due to the elevated levels of genotoxic breakdown products that are in the cigarettes, including nicotine, cotinine, hydrocarbons, and tars, that have mutagenic properties that are present in cervical mucus and cells. (Berek and Hacker, 2010) Coker et al (2009) found that smokers were 21% more likely to succumb to cervical cancer compared to those who did not smoke at all, suggesting that it expedites the disease process due to the inhibited epithelial immune response.

Although there has been a lack of sound evidence that vitamins and minerals has a clinically significant affect on the progression and prevention of cancer (World Cancer Research Fund, 2007), there have been several studies that have shown a benefit of certain nutrients and vitamins. Research continues to find a correlation between essential vitamin and nutrient intake and the progression/prevention of breast, cervical, prostate, and colon cancer. Vitamins A, C, D, and E have been depicted to have protective properties. Vitamin A has some role in regulating differentiation, growth, and apoptosis of normal as well as malignant cells. (Cui et al, 2008) The role of Vitamin C has been in the foraging of free radicals. Vitamin E works in conjunction with vitamins A and C to regulate cell differentiation and proliferation, to scavenge free radicals and oxidants, and may reduce the persistence of HPV as well as inhibit cervical carcinogenesis by augmenting immunological function and modulating the inflammatory response to infection. (Kim et al, 2010) Toner and Milner (2010) found that even though Vitamin D has been shown to be beneficial in cancer prevention the problem lies in finding a standard and most beneficial dosage and also showed that there were risk with overexposure.

There are several disease states that can result in immunosuppression. These include HIV, transplant patients, cancer patients undergoing chemotherapy, congenital immunodeficiency disorders, and immunosuppressive drugs, among others. The main physiological function of the immune cells is to monitor tissue homeostasis, to protect against invading pathogens, and to eliminate transformed or damaged cells. (Bremmes et al, 2011) When the physiologic function of immune cells are interrupted in any way, the body has an impeded response to the recognition and response to cancer cells. This delayed response and recognition aides in the

more rapid progression of cancer. Studies on immunotherapy in cancer patients have been ongoing and there have been great advances, giving validity that the immune response is an important mediator in preventing and fighting off cancer.

5. Pathogenesis

There have been many hypotheses about the pathogenesis of SSCC, including the basis of its aggressiveness. The theory that is longstanding and the most accepted is that there is a transformation from the squamous cell carcinoma component into a spindle cell cancer. This is mainly due to parallel immunohistochemical, molecular, and ultrastructural characteristics. In 1960, Hay-Roe et al found that the epithelial portion of SSCC of the esophagus had an apparent tendency to become spindled in tissue culture. This was also reported by Sherwin et al (1963) which also stated there is a probable loss of unity of the epithelial cells in the basal layer and this was the major feature causing the spindle cell transformation. Raptis et. al (1993) showed a relative decrease in desmosomes and tonofilaments within the spindled component therefore lacking the structural foundation of ordinary squamous cell carcinoma and might be more susceptible to the compressing effect of surrounding stroma. The fact that the spindle cells have desmosomes and tonofilaments confirms the squamous cell origin. Lastly, there has been speculation that the spindle cell component and the squamous cell component arise concurrently from distinct stem cell lines and thus SSCC has been termed a “collision” tumor and not a single tumor with conversion to a spindled cell type. (Otay et al. 2011)

6. Presentation

The disease process of Sarcomatoid Squamous Cell Carcinoma is very aggressive, patients typically present with extensive local disease or with metastasis on imaging and during surgery. The clinical signs and symptoms do not correlate to the severity of disease. Early nonspecific symptoms can include fatigue, anemia, pelvic pain, pelvic pressure, constipation, bloating, weight loss, and loss of appetite.

The most common presenting symptom of cervical SSCC was abnormal vaginal bleeding. There have also been cases of patients complaining of a foul smelling discharge (Brown et al 2003), postcoital spotting (Kong et al, 2010). In all of the cases of cervical SSCC there was a visible cervical lesion ranging from 1.6cm to 10cm. Vaginal lesions can present in a similar fashion with vaginal bleeding and a yellowish-white vaginal discharge. On physical exam a mass was usually palpable. Vulvar lesions usually present with the patient reporting a lesion that bleeds, is expanding, and/or is worrisome. In a small percentage of patients, there were no signs or symptoms of a genital mass. SSCC lesions of the cervix, vagina, and vulva, were all described similarly in the text as being ulceroproliferative, friable, of polypoid configuration, and necrotic in areas.

7. Diagnosis

The hallmark of diagnosing this disease is biopsy of the lesion. It is important to get an adequate specimen to increase the probability of accurately diagnosing the cancer. There is no consensus as to the diameter and depth of specimen that gives the highest yield for proper pathological evaluation.

An excisional biopsy is one in which the entire mass is removed with a margin of normal tissue. This is done in the OR for the most part under sedation or if the lesion is small enough in the office with local anesthesia. Since the lesion is friable, supplies for bleeding should be readily available. Excisional biopsy is preferred for smaller lesions in which margins are available and do not interfere with surrounding structures. An incisional biopsy, in which the surgeon removes a portion of the mass, may be done in the office or in the operating room. This is utilized in the event the mass is too large to obtain normal margin of surrounding tissue.

7.1 Histology

In general, the histopathological diagnosis of SSCC rests upon demonstration of a malignancy with regions of classic squamous cell carcinoma morphology, merging with those exhibiting a prominent spindle cell component. In cases where such a transition occurs, and/or where a squamous cell carcinoma in situ interface is evident, the diagnosis can be confidently rendered on morphologic grounds alone.

However, in situations where the spindled cell component predominates, without areas of classic squamous morphology or an interface with an in situ carcinoma component, the diagnosis is particularly challenging, as key differential diagnostic considerations would include a sarcoma. Such was the case with the current vaginal lesion, which was a tumor demonstrating exclusive spindled cell morphology; as immunohistochemistry for muscle markers (smooth muscle actin) was positive, the lesion was initially thought to reflect a leiomyosarcoma. In review of the cases, some report round to polygonal cells, scanty eosinophilic cytoplasm, irregular nuclei, prominent nucleoli, numerous mitosis, and areas of necrosis with interlacing bundles of spindle cells on microscopic exam.

7.2 Immunohistochemical methodologies including HPV detection

There are many tools in modern immunohistochemistry that may aid in facilitating the diagnosis of SSCC, but may also pose significant nuances. In our case of SSCC of the vagina, tumor cells which are exclusively spindled in morphology, were positive for markers associated with both, mesenchymal and epithelial differentiation, including cytokeratin, vimentin, desmin and smooth muscle actin. Indeed, while one report indicates that neoplastic cells of SSCC do not show reactivity for smooth muscle actin (C-P, Lin et al. 2006), another reports that this marker may be positive in tumor cells. (Brown et al., 2003). In our particular case of SSCC from the vagina, the tumor initially thought to reflect a leiomyosarcoma, was ultimately demonstrated to harbor high-risk HPV subtypes by in situ hybridization, as well as expression of p16, a tumor suppressor protein implicated in the HPV tumorigenesis pathway. These latter findings, despite the morphologic appearance of the tumor and the ambiguous immunophenotype, permitted a definitive final diagnosis of SSCC.

8. Staging

Due to the rarity of this cancer, the FIGO staging system that is used for squamous cell carcinoma is also used to stage SSCC of the vulva, vagina, and cervix.

Stage I	Tumor confined to the vulva
• IA	Lesions ≤ 2 cm in size, confined to the vulva or perineum and with stromal invasion ≤ 1.0 mm*, no nodal metastasis
• IB	Lesions > 2 cm in size or with stromal invasion > 1.0 mm*, confined to the vulva or perineum, with negative nodes
Stage II	Tumor of any size with extension to adjacent perineal structures (1/3 lower urethra, 1/3 lower vagina, anus)
Stage III	Tumor of any size with or without extension to adjacent perineal structures (1/3 lower urethra, 1/3 lower vagina, anus) with positive inguino-femoral lymph nodes
• IIIA	i. With 1 lymph node metastasis (≥ 5 mm), or ii. 1-2 lymph node metastasis(es) (< 5 mm)
• IIIB	i. With 2 or more lymph node metastases (≥ 5 mm), or ii. 3 or more lymph node metastases (< 5 mm)
• IIIC	With positive nodes with extracapsular spread
Stage IV	Tumor invades other regional (2/3 upper urethra, 2/3 upper vagina), or distant structures
• IVA	Tumor invades any of the following: i. upper urethral and/or vaginal mucosa, bladder mucosa, rectal mucosa, or fixed to pelvic bone, or ii. fixed or ulcerated inguino-femoral lymph nodes
• IVB	Any distant metastasis including pelvic lymph nodes

*The depth of invasion is defined as the measurement of the tumor from the epithelial-stromal junction of the adjacent most superficial dermal papilla to the deepest point of invasion. (FIGO Committee on Gynecologic Oncology. 2009)

Table 4. Carcinoma of the Vulva FIGO Staging. (2008)

Stage I	The carcinoma is limited to the vaginal wall
Stage II	The carcinoma has involved the subvaginal tissue but has not extended to the pelvic wall
Stage III	The carcinoma has extended to the pelvic wall
Stage IV	The carcinoma has extended beyond the true pelvis or has involved the mucosa of the bladder or rectum; bullous edema as such does not permit a case to be allotted to stage IV
• IVA	Tumor invades bladder and/or rectal mucosa and/or direct extension beyond the true pelvis
• IVB	Spread to distant organs

(FIGO Annual Report. 2006)

Table 5. Carcinoma of the Vagina FIGO Nomenclature

Cervical cancer is still staged clinically, which is not always very accurate in portraying the extent of the disease. Clinical staging can include palpation, inspection, colposcopy, endocervical curettage, hysteroscopy, cystoscopy, proctoscopy, intravenous urography, and e-ray examination of the lungs and skeletal system. (Berek and Hacker, 2010) CT and MRI are also utilized often as this can give a better idea of extent of disease, margins, lymphadenopathy, and other organ involvement. Positron emission tomography (PET) is mainly utilized for nodal status.

Stage I	The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded)
• IA	Invasive carcinoma which can be diagnosed only by microscopy, with deepest invasion ≤ 5 mm and largest extension ≤ 7 mm
IA1	Measured stromal invasion of ≤ 3.0 mm in depth and extension of ≤ 7.0 mm
IA2	Measured stromal invasion of >3.0 mm and not >5.0 mm with an extension of not >7 mm
• IB	Clinically visible lesions limited to the cervix uteri or pre-clinical cancers greater than stage IA*
IB1	Clinically visible lesion ≤ 4.0 cm in greatest dimension
IB2	Clinically visible lesion >4.0 cm in greatest dimension
Stage II	Cervical carcinoma invades beyond the uterus, but not to the pelvic wall or to the lower third of the vagina
• IIA	Without parametrial invasion
IIA1	Clinically visible lesion ≤ 4.0 cm in greatest dimension
IIA2	Clinically visible lesion >4.0 cm in greatest dimension
• IIB	With obvious parametrial invasion
Stage III	The tumor extends to the pelvic wall and/or involves lower third of the vagina and/or causes hydronephrosis or non-functioning kidney**
• IIIA	Tumor involves lower third of vagina, with no extension to the pelvic sidewall
• IIIB	Extension to the pelvic wall and/or hydronephrosis or non-functioning kidney
Stage IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to Stage IV
• IVA	Spread of the growth to adjacent organs
• IVB	Spread to distant organs

*All macroscopically visible lesions-even with superficial invasion-are allotted to stage IB carcinomas. Invasion is limited to a measured stromal invasion with a maximal depth of 5.00 mm and a horizontal extension of not >7.00 mm. Depth of invasion should not be >5.00 mm taken from the base of the epithelium of the original tissue-squamous or glandular. The depth of invasion should always be reported in mm, even in those cases with "early (minimal) stromal invasion" (~ 1 mm). the involvement of vascular/lymphatic spaces should not change the stage allotment

**On rectal examination, there is no cancer-free space between the tumor and the pelvic wall. All cases with hydronephrosis or non-functioning kidney are included, unless they are known to be due to another cause.

(FIGO Committee on Gynecologic Oncology. 2009)

Table 6. Carcinoma of the Cervix Uteri (2008)

9. Therapy

The rarity of these malignancies makes recommendations for standard treatments a formidable endeavor. In general, the stage of the cancer will dictate therapy.

In early stage disease of the vulva, vagina and cervix the role for surgery is more clearly defined. These lesions are usually treated with radical surgery followed by radiation therapy and/or chemotherapy in certain cases. Size, margin status, and local tumor biology might dictate the need for radiotherapy. Brown et al (2003) treated all Stage I and Stage II women with radiation therapy alone and this was successfully able to eradicate the tumor. This proves radiation to be an effective treatment option, even though some believe it to be a

source of the transition of this cancer from SCC to the intermingling of the spindle shaped cells. In 2010, Kong et al reported a case of IB1 SSCC of the cervix in a young patient, being treated by laparoscopic radical hysterectomy, bilateral pelvic lymph node biopsy, peritoneal washing cytology and transposition of both ovaries without adjuvant therapy. Despite the initial treatment for low stage cancers, recurrent cancer did not respond to second line therapy (Brown et al, 2003) which leads some to take a more aggressive approach with surgery and adjuvant therapy, especially since time from recurrence to death is less than a year as reported in the literature.

In more advanced stages, patients present with such extensive disease that surgery is usually done on a palliative basis if indicated. These tumors are usually treated with concurrent chemotherapy and radiation, extrapolating from the pure squamous counterparts. In contradiction to these lesions where there exist more robust standard treatment recommendations the response to similar treatment is largely unknown and the risk for recurrence is very significant with a very short disease free interval.

10. Prognosis

Prognosis of SSCC is very poor. It is a very aggressive cancer, is usually diagnosed at a later stage, and most recur within one year despite aggressive combined therapy. As with the majority of solid tumors, the survival of patients that were detected at early stages is very reassuring. Patients who are diagnosed at Stage I have a higher survival rate at 5 years, approaching 90%. In contrast, those who present at Stage IV, according to the studies, have a survival rate of less than 5% at five years, according to the review of the case reports. Prognostic factors have not been uniformed in any of the studies. Lane believed the extent of the grossly carcinomatous element was the best predictor of survival. Friedel et al found that not only was the degree of differentiation of the carcinomatous component an important prognostic factor but also the extent of invasiveness. Some suggest size and location are the sole factors impacting prognosis. (Randall et al.) Brown et al found that the younger patients tended to present at an earlier stage. Of these women who presented at stage 1, all were free of disease with the longest interval reported at 42 months. They also found that all patients less than 40 presented at Stage I and not a more advanced stage III or IV. One explanation for the fact that younger patients present at a lesser stage is that they are more prone to go to the doctors for acute visits and are more likely to voice their concerns over abnormal changes in their bodies. This is different to the belief that women >40 are more apt to cope with the symptoms and only present when the condition is debilitating or they are urged by family.

11. Conclusion

SSCC is a very rare cancer that has an aggressive and rapidly fatal course. Due to its rarity, there is no distinct staging or guidelines to direct therapy and care. As more cases become available and follow up is documented on patients with early FIGO stages, treatments, and surveillance decisions there will be a better chance of developing a set of guidelines based on the evidence. Since this cancer is most accepted as being a variant of SCC, the FIGO staging and treatment guidelines for squamous cell carcinoma are also used for SSCC.

Human papillomavirus has been implicated in SCC and is also found in the spindle cell component of SSCC. Even with the use of the widely available HPV vaccine and better

screening modalities, it is difficult to discern which squamous cell cancers will transition to SSSC so no risk prevention can be done. One would assume that with the reduction in squamous cell carcinoma cases of the female genital tract that there will be a parallel reduction of SSSC.

Diagnosis has traditionally been difficult due to the large ratio of sarcomatous to squamous cell component. Immunohistochemistry and ancillary testing, such as in situ hybridization for high-risk HPV subtypes are very important for the precise diagnosis which can guide treatment and counseling of the patient. One should perform immunohistochemistry for squamous epithelial markers as well as in situ hybridization for HPV when encountering a sarcomatoid neoplasm to rule out SSSC.

As with any cancer, the lower the FIGO stage at diagnosis the better the prognosis. Unfortunately even when adequately treated according to unmet standards, recurrence is very prevalent with disease free interval to death being very rapid. Once recurrence occurs there is very little recourse as the cancer does not respond to second line therapy and has usually extended out of the pelvis to distant organs. There have been few cases of a FIGO stage greater than II in which there has been long term survival but not enough to effectively alter the inevitable outcome.

12. Acknowledgements

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Basaloid Squamous Cell Carcinoma

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1. Introduction

Squamous cell carcinoma is the second most common cancer of the skin. This tumor arises predominantly in sun exposed actinically damaged areas. Implicated as predisposing factors, in addition to sunlight, these are industrial carcinogens, chronic ulcers, and ionizing radiation [1]. They are common cancer in immunocompromised and renal transplant patients [2]. Squamous cell carcinomas are known to be the most prevalent malignant tumor of the head and neck region [3]. They are also reported in many organs including cervix, lung, bladder, uterus, ovary, esophagus and teratomas [4;5;6,7].

Squamous cell carcinoma is characterized by squamous cells with large nuclei and abundant eosinophilic cytoplasm. The cells exhibit prominent intracellular bridges and variable keratin formation, depending on the degree of differentiation. Poorly differentiated tumors lack keratinization and usually form solid sheets of cells with marked pleomorphism to the extent that require special studies to establish the nature of the tumor.

2. Histologic variants of squamous cell carcinoma

Several histologic variant of squamous cell carcinoma are identified. These variants are based on certain morphological features accordingly, which may or may not have prognostic implications. The following are the most reported variant in the literature and include basaloid, warty verrucous, papillary, spindle cell, adenosquamous, clear cell, acantholytic and lymphoepithelioma-like type.

Spindle cell carcinoma is rather rare and is composed of atypical spindle cells with whorled arrangement (Fig 1), which mostly come from immunosuppressed renal transplant patients. The tumor needs to be differentiated from desmoplastic melanoma, atypical fibroxanthoma or metastatic carcinoma with spindle cell features. Immunohistochemistry is of value in differentiating these entities [8].

Clear squamous cell carcinoma is another variant first described as squamous cell carcinoma with extensive hydropic changes. The cells appear glassy looking, due to accumulation of fluid, and can be easily mistaken for sebaceous cell carcinoma. The differential also includes other clear cell tumors such as clear cell acanthoma, clear cell hidradenoma, metastatic renal cell carcinoma, balloon cell nevus and melanoma [9].

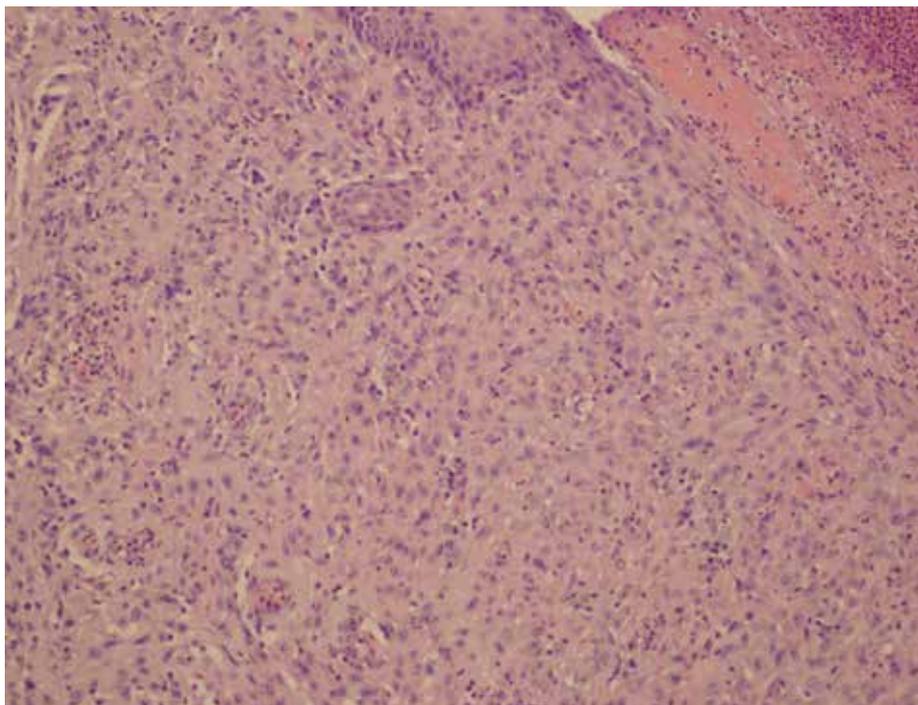


Fig. 1. Spindle cell variant of squamous cell carcinoma

Verrucous squamous cell carcinoma presents with rather non dysplastic epithelium with hyperkeratosis and elongation of the rete pegs. This is in contrast to the **papillary variant** of squamous cell carcinoma, which is characterized by malignant looking epithelium with papillary or exophytic architecture [10]. **Adenosquamous** cell carcinoma is very rare subtype, which is composed of admixed adenocarcinoma with squamous cell carcinoma. Mucin stain usually highlights the adenocarcinoma component.

Basaloid basal cell carcinoma is a rare variant of squamous cell carcinoma with more cases, which have been published since its first description in 1986 by Wain et al (11). These tumors affect both sexes but with predominance of male patients. They are frequently seen in the aerodigestive tract with most of the cases to be found in the tongue, floor of the mouth, the pyriform sinus, tonsil, and larynx [12]. These tumors have also been described in a variety of sites including nasopharynx, trachea, skin, cervix, bladder, thymus, anus, conjunctiva, and lung [13,14,15,16]. Clinically, patients have similar presentation to conventional squamous cell carcinoma depending on the site of the lesion.

Etiology and pathogenesis of basaloid cell carcinoma is similar to conventional squamous carcinoma. Most patients have a long history of smoking and alcohol drinking. In some cases there was a history of previous radiation to the head and neck region [17]. Both represent independent risk factors for the development of squamous cell carcinoma. Smokeless tobacco and other exogenous carcinogens such as occupational, environmental and nutritional factors may also play role in the pathogenesis of this cancer. EBV was detected in few cases using in situ hybridization technique from nasopharyngeal sites.

Recent studies detected a higher frequency of HPV and HSV in basaloid tumors than in conventional squamous cell carcinomas of the head and neck [18]. Basaloid squamous cell carcinoma in non smoker young patients revealed infection with HPV, high risk genotype 16. The expression is so significant, to the extent that it led some authors to consider the expression may be important for the diagnosis of this type of squamous cell carcinoma. The prognosis of HPV induced carcinoma appeared to have better outcome than the HPV negative cases [19]. It is not practical to perform in situ hybridization and sequencing techniques on every single case of basaloid squamous carcinoma as this is technically demanding and can be performed mostly in special centers [20]. The cell of origin of these tumors has been suggested to be a multipotential cell, which is able to differentiate into multiple cell type. However, the most acceptable origin for these cells is that they are from the surface epithelium since there is dysplastic or carcinoma in situ changes with direct continuity within the invasive component.

The tumor is considered by many authors as high grade with more aggressive behavior [11,16]. These lesions are capable of distant metastases, deep invasion, local recurrence and lymph node involvement. The most common sites for distant metastasis are the lung and liver. Multifocal disease includes other sites in the head and neck, which were also documented [17] However, some controversy is still present regarding the prognosis and conflicting results, which have appeared in recent literature. Some published papers claimed they have similar prognosis to traditional squamous cell carcinoma [21]. The majority of these cases are found at an advanced stage, which could explain the poor clinical outcome and prognosis. No general guidelines are present regarding the management of this disease; however, most published reports recommend a combination of surgery and postoperative radiotherapy, in order to prevent local recurrence and distant metastases [12,14].

3. Pathology of basal squamous cell carcinoma

Macroscopic appearances of these tumors show flat or slightly raised or polypoid exophytic lesions with or without a central ulceration in most cases reported in the literature [3] Microscopic examination of these lesions show characteristic invasive growth appearance, shared by most lesions. Generally they are composed of ribbons and or cords of basaloid cells with peripheral palisading and closely resemble traditional basal cell carcinoma figure 2. This lesion comes from rare urinary bladder flat lesion from a 66-year-old man seen on cystoscopy. In addition, the cellular arrangements of these lesions can closely mimic adenoid cystic carcinoma, due to the glandular or cribriform pattern, and have a tendency to have intracellular deposition of eosinophilic hyaline material figure 3. One of the major features of this tumor is that the cells exhibit high nucleocytoplasmic ratio and often have dense hyperchromatic nuclei and comedonecrosis may be seen in these tumor, figure 4, which was seen in a patient who presented with nasal sinus mass. This appearance represents common features of these lesions. Mitotic figures may be high and may include atypical forms. Careful search will reveal focal squamous differentiation with intercellular bridges or keratin formation, which is important for the accurate pathological assessment of these tumors. Another important feature of these lesions is dysplasia of the surface epithelium in cutaneous neoplasm. Sometimes the tumor show true neural type rosette

formation and other tumors may exhibit spindle shaped pleomorphic cells with elongated nuclei. Vascular or lymphatic invasion may also be present. In recent publication of cutaneous basaloid squamous cell carcinoma, this tumor may also have rather large pleomorphic cells with big nuclei widely scattered throughout the lesion. These pleomorphic cells present no significant biological behavior. The immunoprofile of these tumors show consistent positive staining to high molecular weight cytokeratin antibody 34 β E12, KL1, and MNF116, and focal staining for vimentin, EMA, CAM5.2, CK7, CEA, S100 and GFAP, and negative immunostaining for CK20, chromogranin, synaptophysin, BCL2, and Ber-EP4. Actin staining was positive in the basaloid cells and some cases were positive for CD99. More recent studies confirm strong and diffuse staining for P63 immunomarker in this tumor figure 5. Lastly, electron microscopic examination of samples may show tonofilaments and desmosome and do not demonstrate any characteristic findings, as the malignant cells show mainly undifferentiated cellular features, and the organelles are rather poorly developed(22).

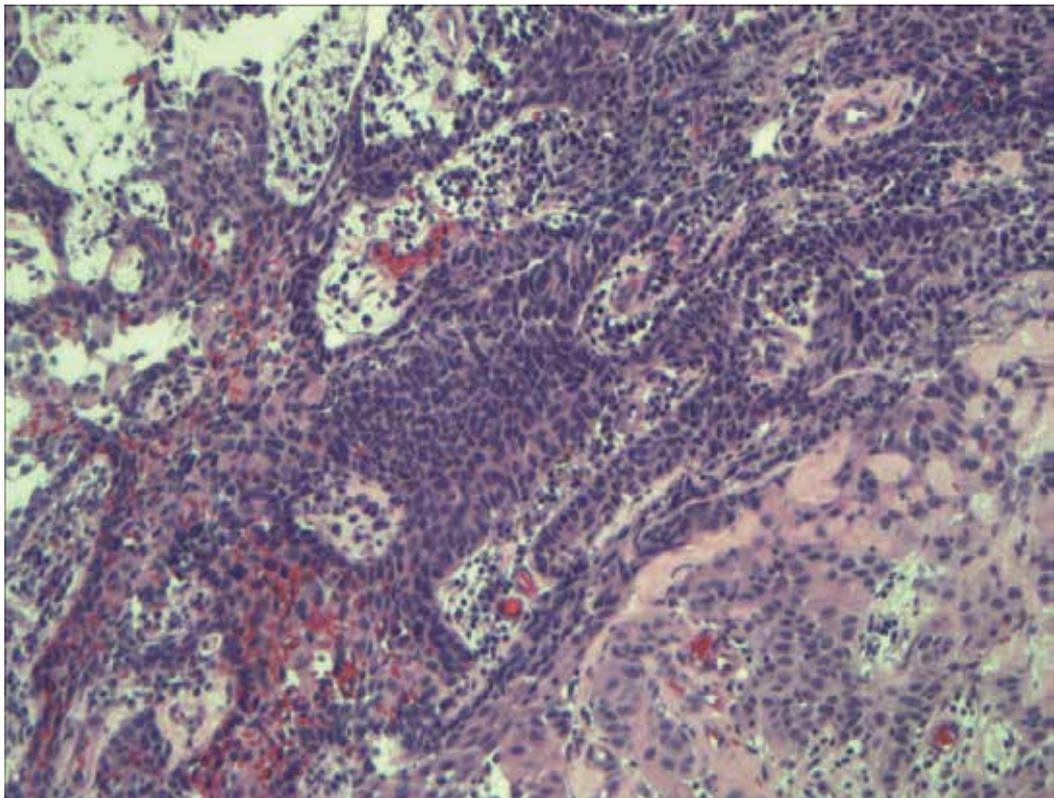


Fig. 2. Basaloid squamous cell carcinoma with peripheral palisading

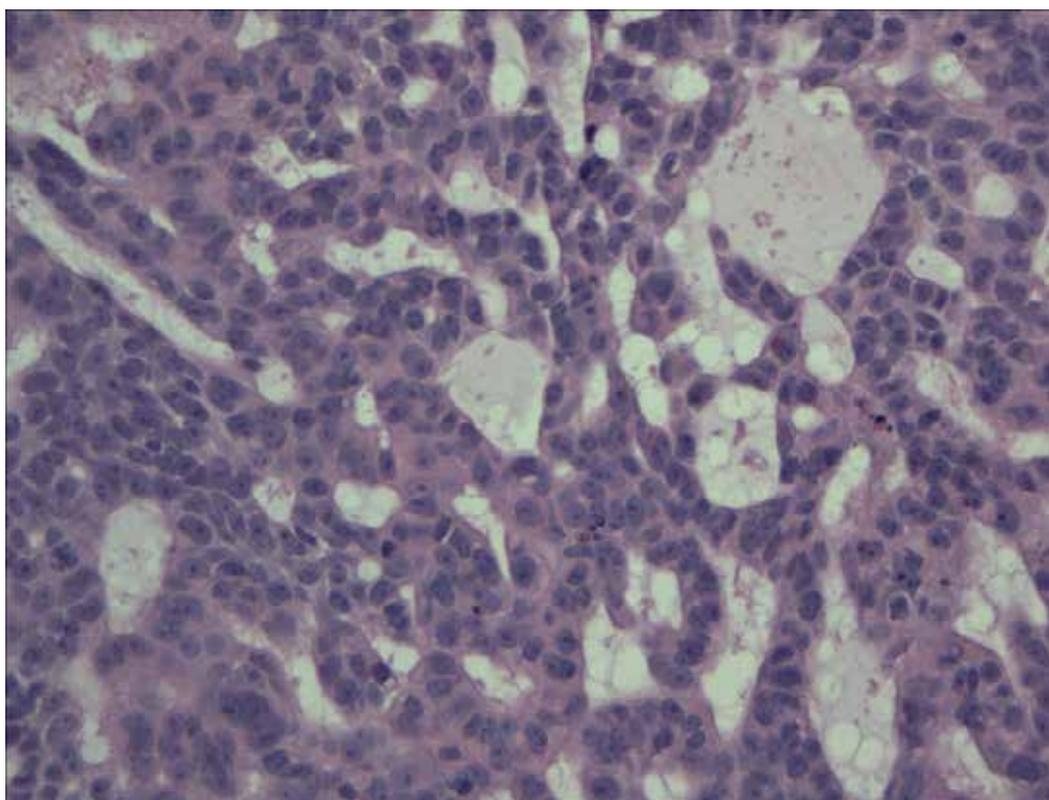


Fig. 3. Basaloid squamous carcinoma with glandular pattern

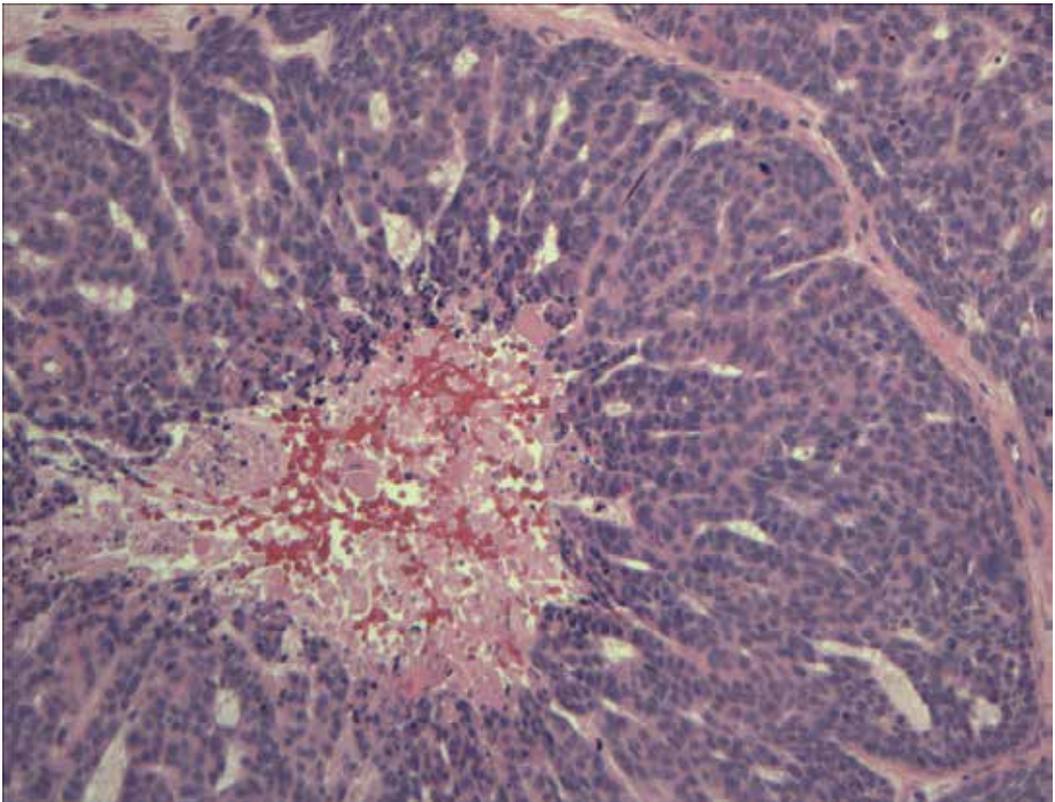


Fig. 4. The lesion shows prominent central necrosis

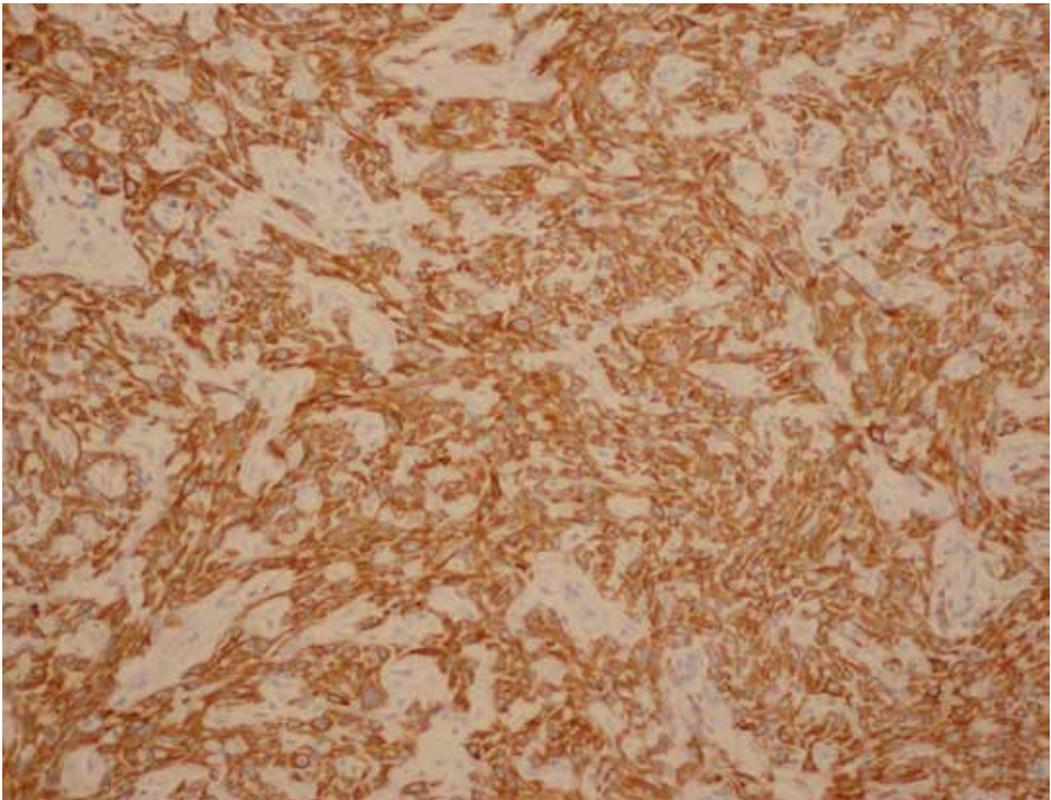


Fig. 5. Immunostaining with 34βE12

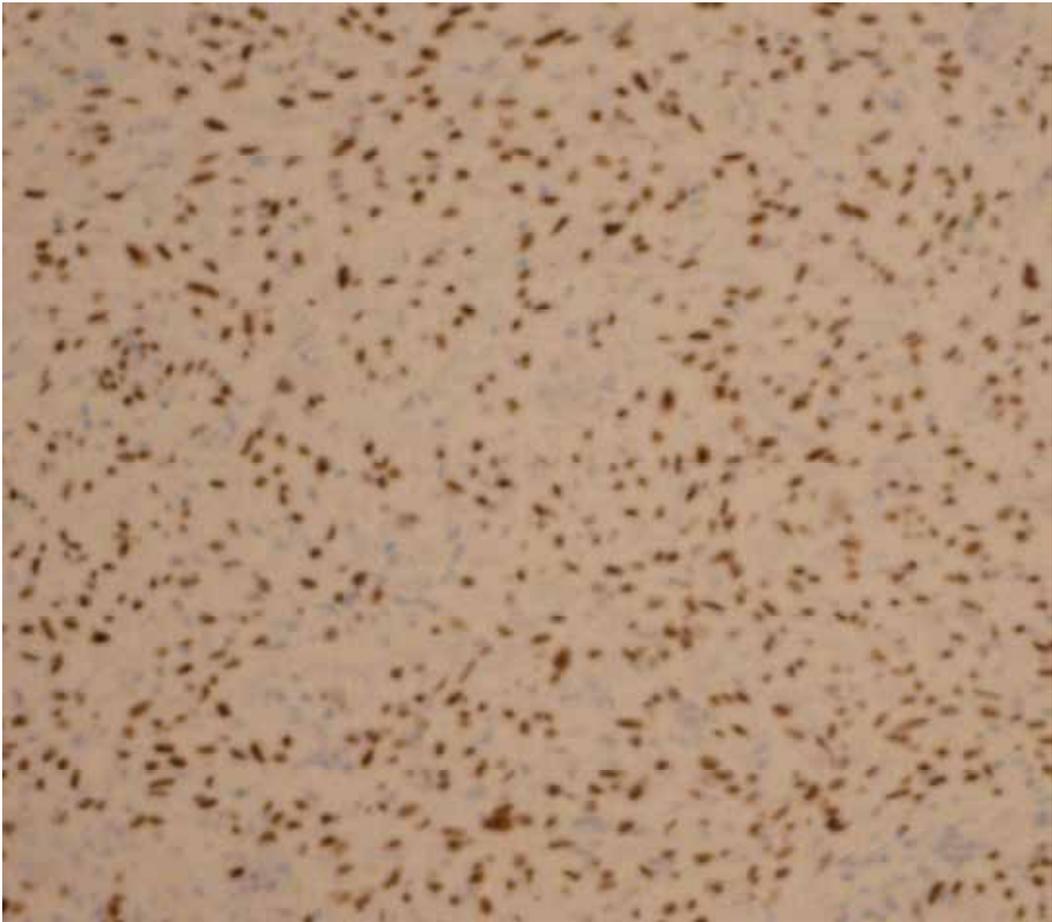


Fig. 6. Showing diffuse p63 positive malignant cell

The differential diagnosis of these tumors includes adenoid cystic carcinoma, small cell neuroendocrine carcinoma and other carcinomas depending on the anatomical sites.

Adenoid cystic carcinoma is characterized by basaloid-looking cells with predominant myoepithelial cells forming cribriform, solid or tubular structure. The tumor is slowly growing, less aggressive and with infrequent lymph node metastasis. Perineural invasion is a common feature of this lesion [23]. Immunohistochemistry show positive staining of the myoepithelial cells for S100, actin and calponin. The epithelial ductal cells of the tumor stain for cytokeratin, CEA and EMA. The stromal hyaline material can be highlighted with collagen IV and laminin. Small cell carcinoma is a more aggressive lesion with a different treatment approach. The tumor cells are positive for chromogranin, synaptophysin and dot-like staining for cytokeratin. These tumors are negative for 34 β E12 marker, which is normally present in basaloid squamous cell carcinoma [17]. Skin basal cell carcinoma share histologic features with basaloid squamous cell carcinoma and need to be differentiate; however it lacks surface epithelial dysplasia, pleomorphism and the comedonecrosis seen in basaloid squamous cell carcinoma [17]. Adenosquamous carcinoma, which comprised of

squalors and glandular differentiation, can have surface epithelial dysplasia. These lesions contain mucin and lack basaloid cells and peripheral palisading [24].

In conclusion, this variant of squamous cell carcinoma is reported in many sites and organs and present unique pathological and clinical features. This neoplasm is currently under more investigation to determine the nature and clinical behavior. The pathology of this entity is characterized by closely packed basaloid-looking cells with scanty cytoplasm. The cells are arranged in ribbons or with trabecular pattern. Occasional foci of squamous-looking cells are identified. The immunoprofile of these tumors are helpful to distinguish them from basal cell carcinoma, adenoid cystic carcinoma and small cell neuroendocrine carcinoma. The tumor cells are positive for epithelial marker 34B E12, EMA and P63.

Management of basaloid squamous cell carcinoma which is considered by many authors as more aggressive tumor requires radical excision followed by locoregional radiation and chemotherapy. For advance cases combination of radiotherapy and chemotherapy is a logical approach to control the disease. The overall disease free survival rate statically is slightly lower than in the conventional type of squamous cell carcinoma. Meanwhile some studies concluded that the prognosis is comparable to conventional type but the number of cases is too small to draw a definite conclusion. Metastatic disease is recorded in many patients with basaloid squamous cell carcinoma and it is advisable to perform metastatic work up. In conclusion the disease appears in most reported cases in the literature as more aggressive and capable of distant metastasis. [17, 25].

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Part 2

Adjuvant Therapeutic Strategies for Squamous Cell Carcinoma

Neoadjuvant Chemotherapy Using Platinum-Based Regimens for Stage Ib2-II Squamous Cell Carcinoma and Non-Squamous Cell Carcinoma of the Cervix

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1. Introduction

The methods used for treating stage Ib2-IIb cervical cancers, with a bulky mass, differ between Japan and Western countries. In Western countries, concurrent chemoradiation (CCRT) has been recommended as a standard therapy for such tumors based on the results of multiple large-scale randomized trials and meta-analyses (Morris et al., 1999; Rose et al., 1999; Whitney et al., 1999; Pearcey et al., 2002; Eifel et al. 2004; Green et al. 2001; Lukka et al., 2002). In Japan, Korea, Italy and some other countries, the neoadjuvant chemotherapy (NAC) approach has been extensively introduced to clinical practice (Sugiyama et al., 1999). NAC is considered to be clinically significant in 2 respects: it is expected to improve the radicality and safety of surgery by reducing tumor size; and it is expected to exert systemic effects, i.e., effects on lymph node occult micrometastases, etc. A disadvantage of NAC is delayed initiation of the primary treatment, suggesting the necessity of completing NAC as an auxiliary therapy within a short period of time. Therefore, we may find that NAC is valuable if it can exert efficacy rapidly with high platinum dose intensity (DI), assuring that subsequent primary surgical therapy can be performed as soon as possible. At our facility, a platinum-based regimen has been used for NAC in patients with cervical cancer. Herein, we review the efficacy and safety data on NAC for squamous cell carcinoma of the uterine cervix. We previously reported our interim data and now present the results of an ongoing pilot study on the efficacy and safety of NAC for non-squamous cell carcinoma of the uterine cervix.

2. Subjects and methods

2.1 Subjects

We studied 43 patients with locally advanced cancer of the uterine cervix (clinical stage Ib2 to IIb) who gave informed consent to participate in this study between January 2002 and

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September 2010. All 43 were scheduled to undergo a radical hysterectomy, including 23 with squamous cell carcinoma and 20 with non-squamous cell carcinoma.

2.2 Inclusion criteria

The following set of inclusion criteria was employed for selection of study subjects. (1) Histologically verified squamous cell carcinoma or non-squamous cell carcinoma of the uterine cervix; (2) locally advanced stage Ib2 to IIb; (3) age: 20 years upward and less than 70 years; (4) Eastern Cooperative Oncology Group (ECOG) performance status (PS): 0-2; (5) initially treated case; (6) the presence of an MRI-measurable bulky mass in the uterine cervix; (7) hematologic and blood biochemical findings meeting the following criteria [WBC count $\geq 4,000/\text{mm}^3$; neutrophil count $\geq 2,000/\text{mm}^3$; platelet count $\geq 100,000/\text{mm}^3$; hemoglobin ≥ 10.0 g/dl; AST and ALT levels ≤ 2 times the upper limit of normal reference range at study site; serum total bilirubin level ≤ 1.5 mg/dl; serum creatinine ≤ 1.5 mg/dl; and creatinine clearance ≥ 60 ml/min]; (8) life expectancy ≥ 6 months; and (9) written informed consent personally given by the subject.

2.3 Exclusion criteria

Exclusion criteria were prescribed as follows. (1) Patients with overt infection; (2) patients with a serious complication(s) (e.g., cardiac disease, poorly controlled diabetes mellitus, malignant hypertension, bleeding tendency); (3) patients with active multiple cancer; (4) patients with interstitial pneumonia or pulmonary fibrosis; (5) patients with effusions; (6) patients with a history of unstable angina or myocardial infarction within 6 months after registration, or with a concurrent serious arrhythmia requiring treatment; (7) patients in whom treatment with cisplatin (CDDP), irinotecan (CPT-11), paclitaxel (PTX), docetaxel (DTX) and carboplatin (CBDCA) is contraindicated; (8) patients with (watery) diarrhea; (9) patients with intestinal paralysis or ileus; (10) pregnant women, nursing mothers or women wishing to become pregnant; (11) patients with a history of serious drug hypersensitivity or drug allergy; and (12) patients who were inadequate for safe conduct of this study as judged by the attending physician.

2.4 Administration method and criteria for modification

2.4.1 NAC for squamous cell carcinoma

One course of NAC consisted of 21 days, with a CDDP dose of 70 mg/m² on Day 1 and intravenous CPT-11 doses of 70 mg/m² on Days 1 and 8. As a rule, 2 courses of NAC were administered to each patient (Fig.1).

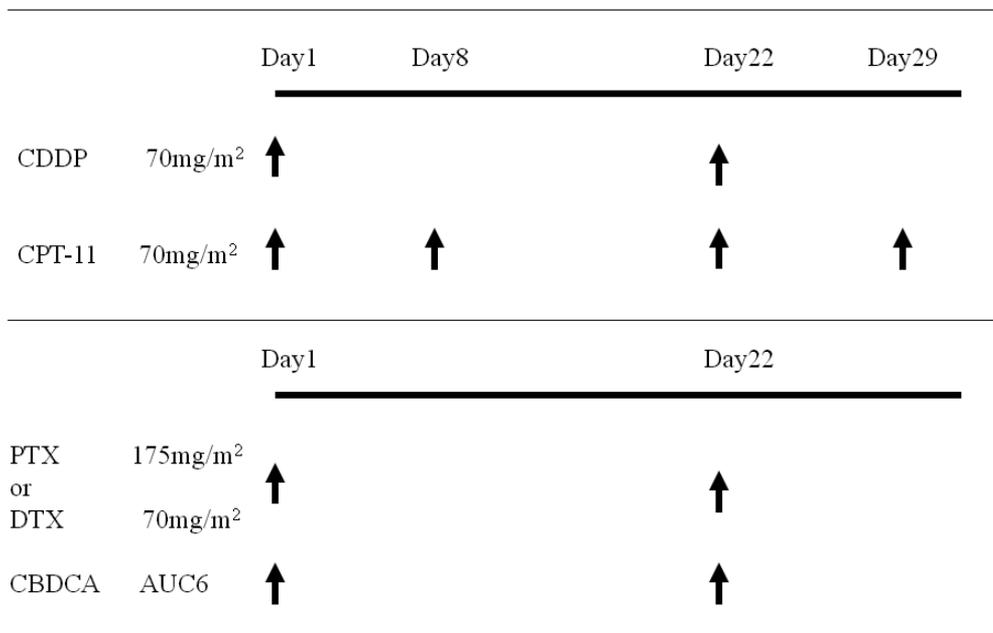
2.4.1.1 Criteria for skipping CPT-11

In cases in which hematological data within 2 days before Day 8 did not satisfy the following criteria, CPT-11 was skipped on Day 8: 1) neutrophil count $\geq 1,000/\text{mm}^3$, 2) platelet count $\geq 75,000/\text{mm}^3$.

2.4.1.2 Criteria for starting the next course of NAC

In cases in which hematological data within 2 days before the planned start of the next course of treatment did not satisfy the following criteria, starting the second course was

postponed by 2 weeks at a maximum: 1) neutrophil count $\geq 1,500/\text{mm}^3$, 2) platelet count $\geq 75,000/\text{mm}^3$, 3) serum creatinine $\leq 1.5 \text{ mg/dl}$.



CDDP; cisplatin, CPT-11; irinotecan, PTX; paclitaxel, DTX; docetaxel, CBDCA; carboplatin

Fig. 1. Treatment protocol of NAC for cervical cancer

2.4.1.3 Dose reduction criteria

In cases exhibiting the following signs of toxicity during the first course of treatment, the CPT-11 and CDDP doses for the second course were reduced from 70 mg/m² to 60 mg/m²: Grade 4 neutropenia lasting 7 days or more; febrile neutropenia lasting 4 days or more; Grade 4 thrombocytopenia; Grade 3 thrombocytopenia accompanied by bleeding; and Grade 3 or more severe non-hematological signs of toxicity other than nausea and vomiting.

2.4.2 NAC for non-squamous cell carcinoma

One course of treatment was 21 days, with a PTX dose of 175 mg/m² or DTX dose of 70 mg/m² on Day 1 and intravenous CBDCA AUC 6 on Day 1. As a rule, 2 courses of treatment were administered to each patient (Fig.1).

2.4.2.1 Criteria for starting the next course of treatment

In cases in which hematological data within 2 days before the planned start of the second course of treatment did not satisfy the following criteria, starting the second course was postponed by 2 weeks at a maximum: 1) neutrophil count $\geq 1,000/\text{mm}^3$, 2) platelet count $\geq 75,000/\text{mm}^3$.

2.4.2.2 CBDCA dose reduction criteria

In cases exhibiting the following signs of toxicity during the first course of treatment, the CBDCA dose for the second course was reduced from AUC 6 to 5. If signs of toxicity remained after this dose reduction, that for the third course of treatment was reduced from AUC 5 to 4: Grade 4 thrombocytopenia; and Grade 3 thrombocytopenia accompanied by bleeding.

2.4.2.3 PTX dose reduction criteria

In cases exhibiting signs of Grade 2 or more severe peripheral nerve toxicity during the first course, the PTX dose for the second course was reduced from 175 mg/m² to 135 mg/m². If Grade 2 or more severe peripheral nerve toxicity remained after dose reduction, the PTX dose for the third course was reduced from 135 mg/m² to 110 mg/m².

2.4.2.4 DTX dose reduction criteria

In cases exhibiting the following signs of toxicity during the first course, the DTX dose for the second course was reduced from 70 mg/m² to 60 mg/m². If signs of toxicity remained after this dose reduction, the DTX dose for the third course was reduced from 60 mg/m² to 50 mg/m²: Grade 4 neutropenia lasting 7 days or more; and febrile neutropenia lasting 4 days or more.

2.5 Supportive therapy

A granulocyte-colony stimulating factor (G-CSF) preparation was administered in patients developing Grade 4 neutropenia during the first course of NAC. Administration of the G-CSF preparation was permitted for prophylactic purposes during the second and subsequent courses of NAC in cases exhibiting Grade 4 neutropenia during the first course. Anti-emetics were additionally used for prophylactic purposes.

2.6 Observations and tests

The primary endpoint was anti-tumor response. Secondary endpoints were adverse events, surgery completion rate, progression-free survival period, and overall survival period. Hematological tests and urinalysis were carried out before the start of treatment and once weekly, as a rule, after starting treatment. Electrocardiograms and chest X-rays were obtained before the start and at the end of treatment.

2.6.1 Evaluation of anti-tumor response

Anti-tumor response was evaluated using Response Evaluation Criteria in Solid Tumors (RECIST) by comparing the baseline findings (before the start of treatment) on magnetic resonance imaging (MRI) with the MRI findings at the end of treatment courses. Efficacy evaluation adopted the best rating, without incorporating the response period.

2.6.2 Evaluation of adverse events

Adverse events were evaluated employing the National Cancer Institute Common Toxicity Criteria (NCI-CTCAE) version 3.0.

2.7 Primary treatment

Patients with stage Ib2-IIb carcinoma underwent a radical hysterectomy unless the response of the tumor to preoperative treatment was progressive disease (PD) and the tumor was up-staged. In cases in which surgery was not possible, concurrent CCRT was adopted.

2.8 Postoperative therapy

Postoperative radiotherapy or chemotherapy was undertaken additionally in patients with positive vaginal stump, positive lymphadenopathy, positive invasion of the cardinal ligament, or evident invasion of the vasculature.

3. Results

3.1. Results of NAC for squamous cell carcinoma

3.1.1 Background variables

The median age of the 23 patients was 40 (range: 25-63) years. PS was 0 in 20 cases (87.0%) and 1 in 3 (13.0%). The clinical stage of the tumor was Ib5 in 5 cases (21.7%), IIa in 2 (8.7%), and IIb in 16 (69.6%). All patients received 2 courses of NAC (Table 1).

		SCC (N=23)		Non-SCC (N=20)
Age years [Median, Range]		40 [25-63]		52 [32-63]
Performance status at entry	0	19 (82.6%)		15 (75.0%)
	1	4 (17.4%)		5 (25.0%)
	2	0 (0%)		0 (0%)
FIGO Stage at initial diagnosis	Ib	5 (21.7%)		5 (25.0%)
	IIa	2 (8.7%)		0 (0%)
	IIb	16 (69.6%)		15 (75.0%)
Cell type	SCC	23 (100.0%)	Mucinous	9 (45.0%)
			Endometrioid	3 (15.0%)
			Clear cell	1 (5.0%)
			Adenosquamous	7 (35.0%)
Number of Cycles	1	0 (0%)		1 (5.0%)
	2	23 (100%)		16 (80.0%)
	3	0 (0%)		3 (15.0%)

SCC; Squamous cell carcinoma

Table 1. Patient characteristics

3.1.2 Anti-tumor response

The response of the tumor to treatment was assessed in all cases. Five (21.7%) showed a complete response (CR), 15 (65.2%) a partial response (PR), 2 (8.7%) stable disease (SD), and 1 (4.3%) PD. Thus, the response rate was 87.0% (Table 2). Among the cases rated as showing

CR or PR, none showed tumor growth between the end of the first course and the end of the second course of treatment.

	CR	PR	SD	PD	Overall Response
SCC	5 (21.7%)	15 (65.2%)	2 (8.7%)	1 (4.3%)	20 (87.0%)
Non-SCC	4 (20.0%)	11 (55.0%)	5 (25.0%)	0 (0%)	15 (75.0%)

	Surgery completion rate	Median PFS (range)	Median OS (range)
SCC	100%	30 (8-93)	34 (8-93)
Non-SCC	75%	10.5 (3-70)	20 (6-70)

CR.; complete response; PR; partial response; SD; stable disease; PD; progressive disease
PFS; Progression-free survival, OS; Overall survival

Table 2. Response and clinical outcome

3.1.3 Adverse events

Grade 3 or more severe leukopenia and neutropenia were seen in 6 cases (26.1%) and 14 cases (60.9%), respectively. Grade 3 febrile neutropenia was seen in 1 case (4.3%). The G-CSF preparation was used in 11 (55.0%) of the 23 cases; during 17 (42.5%) of the 46 treatment cycles in total. The mean duration of G-CSF treatment during each course was 3.1 days. Grade 3 or more severe anemia was noted in 3 cases (15.0%), including one patient with Grade 1 anemia requiring blood transfusion. None of the patients developed Grade 3 or more severe thrombocytopenia. Signs of Grade 3 or more severe non-hematological toxicity included nausea in 2 cases (8.7%) and vomiting in 1 (4.3%) (Table 3). No treatment-associated deaths occurred. Chemotherapy was completed as scheduled in 21 (91.3%) of the 23 cases. In the remaining 2 cases, the CPT-11 dose on Day 2 of the second course was skipped. In these 2 cases, the dose was skipped at the discretion of the attending physician because of persistent Grade 3 nausea. There were 2 cases (8.7%) in which the start of the second course was postponed because the neutrophil count criterion was not satisfied. In both cases, the second course was started within 7 days. In one case (4.3%) showing febrile neutropenia lasting at least 4 days, the CDDP and CPT-11 doses for the second course were reduced from 70 mg/m² to 60 mg/m².

3.1.4 Surgery completion rate and survival period

The completion rate of radical hysterectomy after NAC was 100%. The median follow-up period was 35 months (range: 8-93 months). The median progression-free survival period was 30 months (8-93 months). The median overall survival period was 34 months (8-93 months) (Table 2).

	Grade				
	1	2	3	4	≥3 (%)
Leukopenia	2	15	5	1	6 (26.1)
Neutropenia	1	8	7	7	14(60.9)
Thrombocytopenia	5	2	0	0	0
Anemia	9	12	1	1	2 (8.7)
Nausea	15	6	2	0	2 (8.7)
Vomiting	12	7	1	0	1 (4.3)
Diarrhea	1	0	0	0	0
Neurotoxicity	0	0	0	0	0
Renal toxicity	0	0	0	0	0
Fibrile neutropenia	0	0	1	0	1 (4.3)

CDDP; cisplatin, CPT-11; irinotecan

Table 3. Toxicity of CPT-11+CDDP therapy (n=23)

3.2 Results of NAC for non-squamous cell carcinoma

3.2.1 Background variables

The median age of the 20 patients was 51 (range: 30-63) years. PS was 0 in 15 cases (75.0%) and 1 in 5 (15.0%). The clinical stage was Ib2 in 5 cases (25.0%) and IIb in 15 (75.0%). The histological type was mucinous adenocarcinoma in 9 cases (45.0%), endometrioid adenocarcinoma in 3 (15.0%), clear cell adenocarcinoma in 1 (5.0%), and adenosquamous carcinoma in 7 (35.0%). One course of NAC was administered to 1 case (5.0%), 2 courses to 16 (80.0%), and 3 courses to 3 (15.0%) (Table 1).

3.2.2 Anti-tumor response

The response was rated as CR in 4 cases (20.0%), PR in 11 (55.0%), SD in 5 (10.0%), and PD in 1 (4.3%), with the response rate being 75.0% (Table 2).

3.2.3 Adverse events

Grade 3 or more severe leukopenia and neutropenia were seen in 10 (50.0%) and 19 (95.0%) cases, respectively. Grade 3 febrile neutropenia was noted in 2 cases (10.0%). The G-CSF preparation was used for 13 (65.0%) of the 20 cases; it was administered during 19 (45.2%) of the 42 cycles in total. The mean duration of G-CSF preparation treatment during each course was 3.0 days. None of the cases showed Grade 3 or more severe anemia or thrombocytopenia. The only sign of Grade 3 or more severe non-hematological toxicity was nausea, seen in one case (5.0%). None of the cases had signs of Grade 2 or more severe neurotoxicity (Table 4).

In 3 cases (15.0%), the start of the second course of treatment was postponed because the neutrophil count criterion was not satisfied. In all 3 of these cases, the second course was started within 7 days. Both cases (10.0%) with Grade 3 febrile neutropenia for 4 days or more had received DTX/CBDCA therapy prior to the development of neutropenia. In these 2 cases, DTX (from 70 mg/m² to 60 mg/m²) and CBDCA (from AUC 6 to 5) doses were reduced for the second course of treatment.

3.2.4 Surgery completion rate and survival period

A radical hysterectomy after NAC was completed in 15 of the 20 cases, i.e., the surgery completion rate was 75.0%. The median follow-up period was 20 months (6-70 months). The median progression-free survival period was 10.5 months (3-70 months) and the median overall survival period was 20 months (6-70 months) (Table2).

	Grade				
	1	2	3	4	≥3 (%)
Leukopenia	2	8	9	1	10(50.0)
Neutropenia	1	0	6	13	19(95.0)
Thrombocytopenia	10	0	0	0	0
Anemia	10	10	0	0	0
Nausea	9	2	1	0	1 (5.0)
Vomiting	5	2	0	0	0
Diarrhea	2	0	0	0	0
Neurotoxicity	18	0	0	0	0
Renal toxicity	0	0	0	0	0
Dyspnea	2	0	0	0	0
Fibrile neutropenia	0	0	2	0	2 (10.0)

TC; Paclitaxel+Carboplatin, DC; Docetaxel+Carboplatin

Table 4. Toxicity of TC or DC therapy (n=20)

4. Discussion

A meta-analysis of the results of NAC for squamous cell carcinoma of the uterine cervix ruled out the effectiveness of radiotherapy applied as the primary treatment but suggested the effectiveness of surgery employed as primary therapy. This analysis suggested the effectiveness of NAC, if: one cycle of treatment lasted no more than 14 days; and the DI of CDDP exceeded 25 mg/m²/week (Neoadjuvant Chemotherapy for Cervical Cancer Meta-analysis Collaboration., 2003). Sugiyama et al reported a CDDP/CPT-11 therapy schedule involving CPT-11 doses on Days 1, 8, and 15 (one course = 28 days) (Sugiyama et al., 1999). We evaluated the efficacy and safety of CDDP/CPT-11 therapy, reportedly an effective NAC regimen, using modified doses and administration schedules. In our study, a single

dose was set at 70 mg/m² for both CDDP and CPT-11, and the therapy was administered for 2 cycles at an interval of 3 weeks, with CDDP administered on Day 1 and CPT-11 on Days 1 and 8. In this way, the DI of CDDP was raised to 23.3 mg/m²/week, and this schedule was expected to reduce the need to skip treatments. Thus, it seems valuable to be able to reduce the time interval from NAC to surgery.

In an analysis of adverse events, Grade 3 or more severe neutropenia developed in 14 (60.9%) of the 23 cases, but subsided in response to short-term treatment with a G-CSF preparation. Severe diarrhea, specific to CPT-11, was not seen in any case when this agent was administered at a dose of 70 mg/m², suggesting that the quality of life (QOL) of patients was maintained during this therapy. The first course of treatment was administered as scheduled in all cases. The start of the second course was delayed, by no more than 7 days, in 3 cases. Furthermore, the CPT-11 dose on Day 8 was skipped in 2 cases. Dose reduction during the second course was necessary in only 2 cases, suggesting that this regimen does not increase the toxicity of these drugs as compared to the dosing regimen with 4-week intervals. Furthermore, the response rate (87.6%) and the surgery completion rate (100%) were satisfactory. Regarding the outcomes of patients treated with this regimen, further follow-up is needed.

Non-squamous cell carcinoma of the uterine cervix has been steadily rising in Japan, currently accounting for approximately 10% to 15% of all cervical cancer cases. Lymph node metastasis is more frequent in cases with invasive non-squamous cell carcinoma than in those with invasive squamous cell carcinoma (Aoki et al., 2002) and sensitivities to radiotherapy and chemotherapy are considered to be lower with non-squamous cell carcinoma (Landoni et al., 1997). Thus, squamous and non-squamous cell carcinomas must be analyzed separately. It is advisable to try new therapeutic strategies for non-squamous cell carcinoma, but the number of published studies involving cases with this type of cervical cancer is small, and the number of cases analyzed is also small. Thus, no high-level evidence has been obtained for this type of cervical cancer. The response rates of adenocarcinoma are reportedly 20% (Thigpen et al., 1986), 15% (Sutton et al., 1993), 14% (Look et al., 1997), and 12% (Rose et al., 2003) to uncombined therapies with CDDP, ifosmide, 5-FU, and oral etoposide, respectively, indicating that the response rates of adenocarcinoma to these therapies tend to be lower than those of squamous cell carcinoma. However, according to the report by Curtin et al, the response rate of adenocarcinoma was as high as 31% even when PTX was used independently (Curtin et al., 2001). DTX has also been attracting considerable interest. Nagao et al evaluated the efficacy of combined chemotherapy using DTX + CBDCA (DTX 60 mg/m² on Day 1, CBDCA AUC 6 on day 1 and then every 21 days) in 17 patients with advanced/recurrent cervical cancer, including 6 with adenocarcinoma and 1 with adenosquamous carcinoma, reporting that a PR was obtained in 6 of the 7 cases with adenocarcinoma (including the one with adenosquamous carcinoma) and that the response rate was thus 86% (Nagao et al., 2005). Following these findings, we conducted a pilot study involving standard regimens of PTX/CBDCA and DTX/CBDCA conventionally used for the treatment of ovarian cancer.

In the analysis of adverse events, Grade 3 or more severe neutropenia developed in 19 (95.0%) of the 20 cases, but subsided in response to short-term treatment with a G-CSF preparation (mean dosing period: 3.0 days/course). During the first course of DTX/CBDCA

therapy, Grade 3 febrile neutropenia developed in 2 cases. In these 2 cases, the dose was reduced during the next course of treatment (DTX, from 70 mg/m² to 60 mg/m²; CBDCA, from AUC 6 to 5). All signs of peripheral neuropathy specific to taxanes, observed during this study, were Grade 1 or less severe, allowing continuation of treatment while preserving the QOL of individual patients. No serious adverse events occurred, and the response rate was 75%, but the completion rate of surgery (radical hysterectomy) was 75%. Thus, the outcomes of treatment in this study were not satisfactory. Possible reasons are: rapid progression of non-squamous cell carcinoma, frequent invasion of tissues/organs surrounding the uterus, and frequent lymph node metastasis.

Numerous reports on phase II studies of NAC for cervical cancer have been published, demonstrating effectiveness in 70%-80% of all cases. Table 5 shows the results of the present study in comparison to those of previous reports (Sugiyama et al., 1999; Hwang et al., 2001; Dueñas-Gonzalez et al., 2001; D'Agostino et al., 2002; Di Vagno et al., 2003; Dueñas-Gonzalez et al., 2003; Umesaki et al., 2004; Shoji et al., 2010; Shoji et al., 2010) and this study.

Author	Year	N.P.	Stage	Histological type	Regimens	R.R.(%)
Sugiyama T, et al	1999	23	Ib2, IIb, IIIb	SCC	CDDP + CPT 11	78
Hwang YY, et al	2001	80	Ib2-IIb	SCC, Non-SCC	CDDP + VLB + BLM	94
Gonzalez DA, et al	2001	41	Ib2-IIIb	SCC, Non-SCC	CDDP + GEM	95
Agostino G, et al	2002	42	Ib2-IVa	SCC, Non-SCC	CDDP + EPI + PTX	79
Vagno G, et al	2003	58	Ib2-IIIb	SCC, Non-SCC	CDDP + VNR	85
Gonzalez DA, et al	2003	43	Ib2-IIIb	SCC, Non-SCC	CBDCA + PTX	95
Umesaki N, et al	2004	25	Ib2, IIb, IIIb	SCC	CPT 11 + MMC	76
Shoji T, et al	2010	15	Ib2-IIb	SCC	CDDP + CPT 11	87
Shoji T, et al	2010	66	Ib2-IIb	SCC	NDP + CPT 11	76
<i>Shoji T, et al</i>	·	23	<i>Ib2-IIb</i>	<i>SCC</i>	<i>CDDP + CPT 11</i>	<i>87</i>
<i>Shoji T, et al</i>	·	20	<i>Ib2-IIb</i>	<i>Non-SCC</i>	<i>CBDCA + PTX or DTX</i>	<i>75</i>

N.P.: number of patients

R.R.: response rate

CDDP: cisplatin, CPT 11: irinotecan, VLB: vinblastine, BLM: bleomycin, GEM: gemcitabine,

PTX: paclitaxel, EPI: epirubicin, VNR: vinorelbine, CBDCA: carboplatin, MMC: mitomycinC,

NDP: nedaplatin, DTX: docetaxel

Table 5. Phase II study of NAC for cervical cancer

Most of the reports shown pertain to evaluation of both squamous cell carcinoma and non-squamous cell carcinoma. There is an urgent need to conduct clinical studies on each histological type of cervical cancer and to establish new methods of treatment specific to each type. Only a limited number of reports have demonstrated a high response rate to correlate with a better outcome. Thus, randomized controlled trials (RCT) designed to assess improvement of long-term outcomes are essential. As an RCT evaluating outcomes after NAC, Sardi et al reported a study involving comparisons among 4 groups (NAC + surgery + radiotherapy, surgery + radiotherapy, uncombined radiotherapy, NAC + radiotherapy). They found that the survival rate improved significantly with NAC + surgery +

radiotherapy (7-year survival rate: 41%) as compared to surgery + radiotherapy (41%) (Sardi et al., 1997). Serur et al retrospectively compared the outcomes of treating stage Ib cases between a NAC + surgery group and a surgery alone group, demonstrating a higher 5-year survival rate in the NAC + surgery group although the difference was not statistically significant (80% vs 69%) (Serur et al., 1997). Tierney et al reported the results of a meta-analysis, stating that there was no prognostic improvement (Neoadjuvant Chemotherapy for Cervical Cancer Metaanalysis Colloaboration, 2003). Thus, there is no consensus on this issue.

The JCOG0102 was a representative randomized study of NAC conducted in Japan, designed as an RCT comparing the outcomes of treatment for stage Ib2-IIb cases with bulky tumors between radical hysterectomy (+RT) and NAC + radical hysterectomy (+RT). The JCOG0102 used bleomycin/vincristine/mitomycinC/cisplatin (BOMP) as the NAC regimen. In that study, the response rate to BOMP therapy was low as 61%, and the interim results did not endorse the usefulness of this therapy, forcing the study to be discontinued prematurely (Katsumata et al., 2006). The JGOG1065 was a phase II clinical study on NAC + radical hysterectomy, using nedaplatin and CPT-11 for NAC, carried out in 66 patients with stage Ib2-IIb cervical cancer with a bulky tumor. In that study, the response rate was 75.8% and the 2-year recurrence-free survival period was 73.8% (Shoji et al., 2010). This therapy is expected to reduce nephrotoxicity and adverse events such as nausea and vomiting and appears to be a useful regimen for patients with renal dysfunction and elderly patients from the viewpoint of QOL. However, the response rate to this therapy has not exceeded that to CDDP + CPT-11. At present, there is no plan to launch a phase III clinical study on NAC (NDP/CPT-11) + radical hysterectomy vs. CCRT. There is no evidence supporting the view that NAC improves the outcomes of patients with cervical cancer, and NAC has not been recommended in any set of guidelines. Further studies on the indications for and efficacy of NAC are clearly needed.

5. Conclusions

Irinotecan/cisplatin therapy for squamous carcinoma of the uterine cervix and PTX/CBDCA and DTX/CBDCA therapies for non-squamous cell carcinoma of the uterine cervix showed high anti-tumor efficacy, and the adverse reactions to these therapies could be dealt with satisfactorily, thus allowing safe treatment. In cases with squamous cell carcinoma, outcomes are expected to be improved by NAC, but further evaluation of the outcomes of patients with non-squamous cell carcinoma is awaited.

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Combined Therapy For Squamous Carcinoma Cells: Application of Porphyrin-Alkaloid Modified Gold Nanoparticles

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1. Introduction

Photodynamic therapy (PDT) is an established and useful modality for the clinical non-invasive treatment of cancer. This therapy requires a photosensitizing agent (photosensitizer) selectively taken up by tumor cells, visible light, and molecular oxygen to generate highly reactive oxygen species (ROS), which ultimately cause tumor destruction. The specificity achieved from drug uptake selectivity combined with light targeting makes PDT an appealing approach.

PDT consists of three phases: excitation of photosensitizers (PS) by light, production of ROS, and induction of cell death (Triesscheijn et al., 2006). In the first phase, irradiated light of a suitable wavelength, typically visible or near-infrared, excites the PS molecules. The light is generally selected to correspond with the maximum absorption wavelength of the PS. The PS molecules then absorb light energy and change to an excited singlet state. These excited molecules can fall back to their native state with emission of fluorescence. Thus, all PS molecules are also examples of fluorescent molecules. On the other hand, the molecules also have the ability to undergo an electron spin conversion to their triplet state followed by the transfer of this energy to oxygen molecules or to other substrate molecules in the surroundings which then react with oxygen.

1.1 History of PDT

The fact that sunlight can be used to treat a variety of diseases such as rickets, psoriasis, and skin cancer is known from ancient civilizations, i.e. Egyptian, Chinese and Indian (Ackroyd et al., 2001; Daniell & Hill, 1991; Fitzpatrick & Pathak, 1959). At the beginning of the 20th century the term “photodynamic action” was used by Tappeiner et al. to explain the oxygen-consuming chemical reactions induced by photosensitization (Moan & Peng, 2003; Szeimies et al., 2001). Tappeiner, in cooperation with Jesionek, successfully treated patients

suffering from stage II syphilis, lupus vulgaris, and superficial skin cancer with topical eosin red solution (Szeimies et al., 2001). In 1942, Auler and Banzer observed specific uptake and retention of hematoporphyrin in tumors followed by higher fluorescence in cancer cells as compared with the surrounding tissue, and induction of necrosis after irradiation (Szeimies et al., 2001). Afterwards, PDT had not been used until Dougherty initiated revitalization by treating a group of patients suffering from cutaneous and subcutaneous tumors with the injection of photosensitizer dihematoporphyrin and red light produced by laser. The majority of the treated tumors showed either complete or partial remission (Dougherty et al., 1975; Dougherty et al., 1978; Szeimies et al., 2001).

Particularly, PDT has grown in reputation in dermatology, mostly due to the simple accessibility of light exposure for the skin and the simplicity of topical use of photosensitizers. In the late 1970s, Thomas Dougherty initiated human clinical trials of PDT with hematoporphyrin derivative (HpD) for the treatment of cutaneous cancer metastases (Blume & Oseroff, 2007; Dougherty, 1996; Zeitouni, 2003). PDT has been revived and has become more applicable to common dermatology since 1990, when Kennedy et al. introduced 5-aminolevulinic acid (ALA) (Fig. 1), a topical porphyrin precursor causing local accumulation of the endogenous photosensitizer protoporphyrin IX (PpIX) (Fig. 2) with no significant prolonged phototoxicity (Kennedy, 1990). Nowadays, PDT is used to treat diseases in a variety of fields, including respiratory medicine (Ost, 2001; Sutedia & Postmus, 1996), urology (Jichlinski, 2006; Juarranz et al., 2008; Pinthus et al., 2006), ophthalmology (Mittra, 2002), and gastroenterology (Barr et al., 2001; Wiedmann & Caca, 2004), as well as dermatology. Mostly porphyrins or phthalocyanines have been studied (Marmur et al., 2004). On the other hand, for dermatological purposes, only hematoporphyrin derivatives such as porfimer sodium, or PpIX-inducing precursors such as ALA or methyl aminolevulinate (MAL) are of useful concern. As systemic photosensitizing drugs caused extended phototoxicity (Marmur et al., 2004), topical photosensitizers are preferred for the use in dermatology. Several drugs containing ALA or MAL are used for treating epithelial cancers and there is an increasing importance in the use of PDT (Braathen, 2001; Dragieva et al., 2004a; Dragieva et al., 2004b).

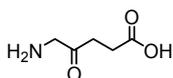


Fig. 1. Structure of 5-aminolevulinic acid

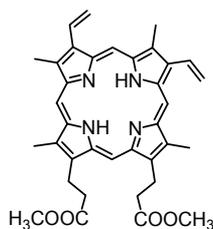


Fig. 2. Structure of protoporphyrin IX

1.2 Mechanism of PDT

PDT requires an interaction of three key elements: light, a photosensitizer, and oxygen. After exposure to particular wavelengths of light, the photosensitizer is excited from a ground state (S_0) to an excited singlet state (S_1) (Fig. 3) followed by intersystem crossing to a longer-living excited triplet state (T_1).

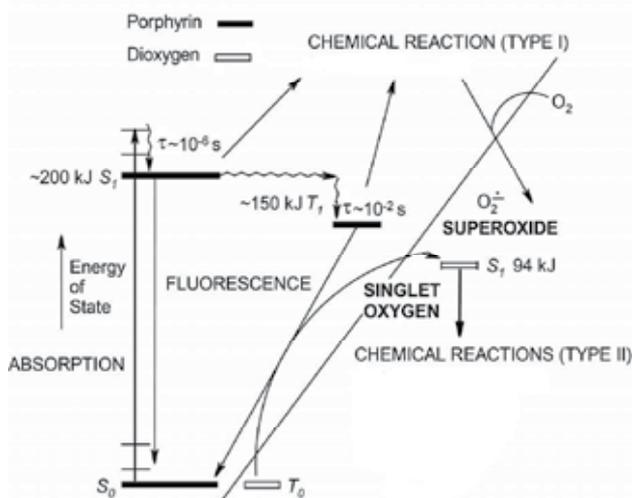


Fig. 3. Mechanism of PDT; State energies are represented by thick lines: **■** porphyrin sensitizer, **▬** dioxygen; reactive dioxygen intermediates are in bold

After that, the photosensitizer at T_1 state is able to go through two types of reaction with nearby molecules: either a type I reaction through hydrogen or electron transfer generating free radicals, or a type II reaction through energy transfer to oxygen, creating molecular singlet oxygen (1O_2). The type I reaction results in generation of reactive free radicals or radical ions, which then react with ground-state molecular oxygen to produce superoxide anion radicals, hydrogen peroxides and hydroxyl radicals (Foote, 1991). The type II reaction produces singlet oxygen which has an important role in the molecular processes initiated by PDT (Foote, 1991; Niedre et al., 2002). The singlet oxygen has a lifetime approx. 3 μ s and can diffuse no more than 0.07 μ m in cells (Moan, 1990; Hatz et al., 2007). Therefore, the initial damage is limited to the site of the PS molecule. This is usually the mitochondria, Golgi apparatus, plasma membrane, endosomes, lysosomes, and endoplasmic reticulum (Buytaert et al., 2007). Damage to the subcellular organelles and plasma membrane eventually leads to apoptotic, autophagic and/or necrotic cell death. Generally, PS molecules localized to the mitochondria or the endoplasmic reticulum cause apoptosis, while localization either in the plasma membrane or lysosomes is found to delay or block the apoptotic pathway. On the other hand, if the apoptotic route is blocked, damaged cells still die using the autophagic or necrotic pathways (Buytaert et al., 2007; Oleinick et al., 2002). Latest studies support apoptosis as probably the preferred path to cell death (Buytaert et al., 2007). Even though it is considered that 1O_2 is the main cytotoxic species and starts the pathway responsible for the damaging effects of PDT, free radicals formed by type I reactions significantly contribute to cell death as well (Foote, 1991).

1.3 Photosensitizers in PDT

The first generation of PS molecules was represented by HpD or its purified version porfimer sodium (Photofrin) (Fig. 4). Primarily, they were used as general PS and tested for cutaneous malignancies. On the other hand, general intravenous administration and the consequential prolonged phototoxicity, which can last 6–10 weeks, restricted their use (Dragieva et al., 2004; Fritsch et al., 1998).

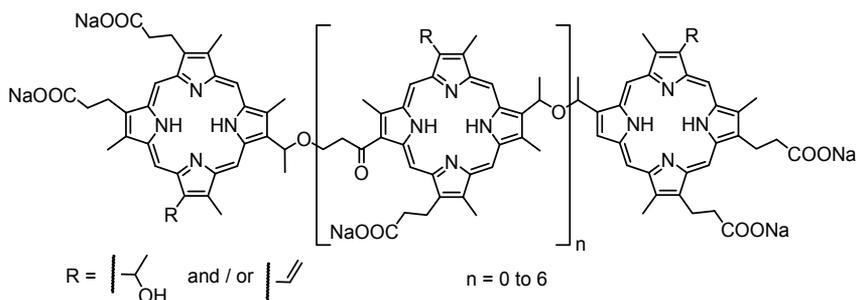


Fig. 4. Structure of Photofrin

Second generation PS molecules such as *m*-tetrahydroxyphenyl-chlorin, tin ethyl etiopurpurin, phthalocyanines, and chlorins (Fig. 5) are pure compounds that can be activated by light wavelengths in the range of 660–690 nm. Most significantly, they all have a lower tendency to cause prolonged photosensitivity compared with the first generation of photosensitizers (Moan & Berg, 1992).

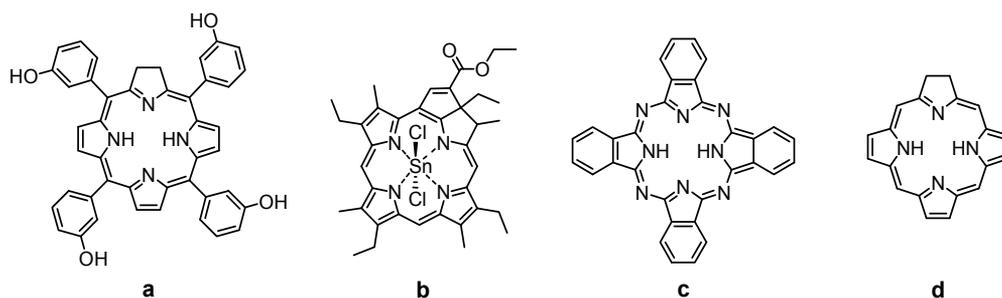


Fig. 5. Structure of *m*-tetrahydroxyphenyl-chlorin (a), tin ethyl etiopurpurin (b), phthalocyanines (c), and chlorins (d)

Third generation PS molecules (not yet approved) consist of antibody-conjugated PS (Josefsen & Boyle, 2008) and lutetium texaphyrin (Fig. 6) (Woodburn et al., 1998; Young et al., 1996). These drugs supporting deeper penetration into tissue with absorptions of 700–800 nm accumulate in tumor tissues with high selectivity.

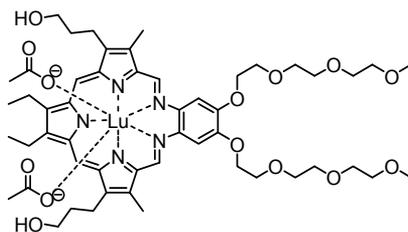


Fig. 6. Structure of lutetium texaphyrin

To avoid the prolonged photosensitivity caused by systemic administration, topically applied photosensitizers have been developed for the treatment of skin cancers. The most successful commercially accessible topical drugs are ALA and its methyl ester MAL. Levulan® using ALA and the Blu-U light source was accepted by the U. S. Food and Drug Administration for the treatment of nonhyperkeratotic actinic keratoses of the face and scalp in 1999 (Babilas et al., 2005; Kormeili et al., 2004). MAL was accepted in Europe for topical PDT of actinic keratosis (AK) and basal cell carcinoma (BCC) in 2001 (Morton, 2003; Morton et al., 2002) and for the treatment of AK in the USA in 2004 (Zeitouni et al., 2003; Garcia-Zuazaga et al., 2005). The endogenous photosensitizer PpIX generated from ALA or MAL can be fully metabolized to photodynamically inactive heme over 24–48 h (Blume & Oseroff, 2007; Morton, 2004), which radically decreases the unpleasant side effect of prolonged cutaneous phototoxicity.

1.4 Nanoparticles in PDT

In 2002 Konan et al. divided methods of PS molecules delivery into passive and active based on the presence or absence of a targeting molecule on the surface (Konan et al., 2002). The methods employed to bring the PS explicitly into diseased tissues using the target tissue receptors or antigens were designated active, whilst others that enable parenteral administration and passive targeting, such as PS conjugates of oil-dispersions, polymeric particles, liposomes, and hydrophilic polymers, were named passive. Active nanoparticles can be subclassified by their mechanism of activation and passive nanoparticles can be subclassified by material composition into (a) non-polymer-based nanoparticles, e.g. ceramic and metallic nanoparticles, and (b) biodegradable polymer-based nanoparticles.

1.4.1 Active nanoparticles

- Photosensitizer nanoparticles

Quantum dots (QDs) have great photostability, intensive fluorescent emission (high quantum yields) and possible use in specific pathological fields. They can be water soluble, and transfer energy to surrounding oxygen with resulting cellular toxicity. Many studies have been devoted to this field (Bakalova et al., 2004). The first report deals with cadmium selenide (CdSe) QDs and was published by Samia et al. in 2002. The authors presented the possibility to use semiconductor QDs alone to generate $^1\text{O}_2$ due to the intercalation of dissolved oxygen at the QD surface (Samia et al., 2003). They predicted a comparable interaction in water-soluble phospholipid-capped QDs. Moreover, they assumed that since the lowest excited state of CdSe QDs is a triplet state, the energy transfer was responsible for

the generation of singlet oxygen ($^1\text{O}_2$) from triplet oxygen ($^3\text{O}_2$). On the other hand, the efficiency of generation of $^1\text{O}_2$ was about 5% (with 65% emission quantum yield of QDs) as compared to 43% for the PS only. It may be due to carrier trapping and nonradiative carrier recombinations occurring on the early picosecond time scale and the very small fraction of QD - $^3\text{O}_2$ pairs created at any moment (Samia et al., 2003). To avoid the ineffectiveness of QDs alone to produce singlet oxygen, several experiments have been carried out to covalently conjugate PSs to CdSe/ZnS via organic bridges (Hsieh et al., 2006; Samia et al., 2003). These experiments have a frequent problem with lower water solubility.

- Self-lighting nanoparticles

Scintillation or persistent luminescence nanoparticles with attached PS molecules such as porphyrins were applied as *in vivo* agents for PDT (Auzel, 2004). After exposure to ionizing radiation such as X-rays, scintillation luminescence is produced from the nanoparticles and stimulates the photosensitizers, followed by production of singlet oxygen that increases the destruction of cancer cells by ionizing radiation. Employment of common radiation therapy with PDT allows application of lower doses of radiation. Using BaFBr:Eu⁺,Mn⁺ nanoparticles displaying luminescence, short X-ray exposures could be applied followed by extended PS excitation. The period of phosphorescent decay is increased *in vivo* due to higher local temperatures (Chen et al., 2006).

- Upconversion nanoparticles

Upconversion and simultaneous two-photon absorption occurs in luminescent materials with triplet excitation states (Auzel, 2004). Upconverting nanoparticles are modified nanometer-sized composites that generate higher energy light from lower energy radiation typically near or middle infrared (anti-Stokes emission) using transition metal ions doped into a solid-state host (Boyer et al., 2006; Pires et al., 2006). For biological use, the desired nanocrystalline core should have morphological and optical features that are appropriate for conjugation with biological molecules and exhibit high intensity emission as well (Pires et al., 2006). Preparation of high-quality nanocrystals is needed, and the surface properties and growth dynamics must be precisely controlled (Wang et al., 2006). Upconversion nanoparticles can be prepared via numerous different ionic materials – typically rare earth ions such as lanthanides and actinides doped in a suitable crystalline matrix (Zijlmans et al., 1999). Micrometer-sized Er³⁺/Yb³⁺ or Tm³⁺/Yb³⁺ co-doped hexagonal NaYF₄ are examples of nanoparticles that exhibit the highest upconversion efficiencies (Heer et al., 2004) and are precursors of upconverting nanoparticles with biological applications (Zhang et al., 2006). In 2006, the NaYF₄ nanocrystals doped with Er and Yb and coated with organic polymers were prepared and strong emission upon activation with 980 nm NIR laser was shown (Feng et al., 2006). One year later, Zhang et al. used upconverting nanoparticles (nanoparticles of NaYF₄:Yb³⁺,Er³⁺ coated with a porous thin layer of silica doped with merocyanine and functionalized with a tumor-targeting antibody) in PDT, but these nanoparticles were not activated in depth in animal tissue and the efficiency in killing cancer cells was very low (Zhang et al., 2007).

Another class of employed upconversion nanoparticles consists of zinc phthalocyanine (ZnPC) (Fig. 7) physically adsorbed to the surface of the nanoparticles with the encapsulation efficiency of 98 % (Ricci-Junior & Marchetti, 2006b). The fluorescence excitation spectrum of ZnPC exhibits an excitation maximum at 670 nm and greatly overlaps

the red emission peak for the upconversion nanoparticles. Creation of $^1\text{O}_2$ by irradiation of the ZnPC-nanoparticle complex with 980 nm light was confirmed through the photobleaching of disodium 9,10-anthracenedipropionic acid (Wieder et al., 2006).

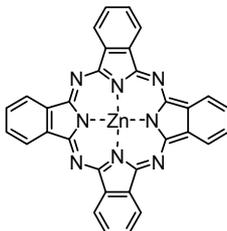


Fig. 7. Structure of zinc phthalocyanine

1.4.2 Passive nanoparticles

- Non-biodegradable nanoparticle carriers

In 2003 Roy et al. first reported ceramic-based nanoparticles used as a new drug-carrier system for PDT. It utilizes 30-nm silica-based spherical particles doped with the anticancer drug 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide (Fig. 8) (Roy et al., 2003).

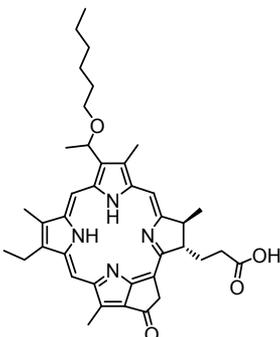


Fig. 8. Structure of 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide

Irradiation of the nanoparticles with light of appropriate wavelength led to efficient creation of singlet oxygen. On the other hand, noncovalent adsorption of PS into porous silica nanoparticles led to drug leakage. Covalent bonding of the PS into organically modified silica nanoparticles produced more stable material (Ohulchanskyy et al., 2007). Organically modified silica nanoparticles were also used for two-photon dye encapsulation (Kim et al., 2007). Cinteza et al. described a combination of magnetism and PDT using micellar polymeric diacylphospholipid-poly(ethylene glycol) capsules for encapsulation of the 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide PS and magnetic Fe_3O_4 nanoparticles (Cinteza et al., 2006). In contrast to the previous report (Kim et al., 2007), the magnetic nanoparticles were used for targeted delivery of PS to tumor cells and increased imaging (Cinteza et al., 2006). Wieder et al. described a delivery system consisting of gold nanoparticles modified with phthalocyanine (Wieder et al., 2006). Phthalocyanine derivative-modified gold nanoparticles have 2-4 nm in diameter and have a maximum absorption peak at 695 nm. They generated $^1\text{O}_2$ catalytically with high efficiency. Upon irradiation of these nanoparticles, significant improvement in PDT

efficiency was observed, probably thanks to 50% increase of $^1\text{O}_2$ quantum yields as compared to the free PS. In the same year El-Sayed reported efficient conversion of strongly absorbed light by plasmonic gold nanoparticles to heat energy. Easy bioconjugation of nanoparticles used suggests their application as selective photothermal agents in molecular cancer cell targeting (El-Sayed et al., 2006).

Two-photon dyes have received attention lately because of their ability to convert absorbed low-energy radiation to higher energy emissions. Dyes that can direct transfer of the higher energy to molecular oxygen for generation of $^1\text{O}_2$ can be very useful in PDT because they can be activated in deep tissues. The first use of two-photon dyes that are able to convert absorbed low-energy radiation to higher-energy emissions was recently reported using microemulsion to incorporate the two-photon dye porphyrin tetra(*p*-toluenesulfonate) into polyacrylamide nanoparticles (Gao et al., 2006).

- Biodegradable nanoparticle carriers

Biodegradable polymeric nanoparticles allow high drug loading and controlled drug release. They exist in a large variety of materials (Konan et al., 2002). Modifying the surface of nanoparticles with polymers such as poly(ethylene glycol) and poly(ethylene oxide) increases circulation times (McCarthy et al., 2005). Brasseur et al. described hematoporphyrin adsorbed in polyalkylcyanoacrylate nanoparticles (Brasseur et al., 1991), but the resulting materials showed poor carrier capacity and rapid drug release. Encapsulation of tetrasulfonated zinc phthalocyanine or aluminium naphthalocyanine into poly(isobutylcyanoacrylate) or poly(ethylbutylcyanoacrylate) nanocapsules or nanosphere was published in the same year (Labib et al., 1991). Then, second generation phthalocyanine derivatives were used in PEG-poly(lactic acid) nanoparticles (Allemann et al., 1995). The results showed that immobilization in the biodegradable nanoparticle improved PDT response of the tumor in contrast to conventional Cremophor EL emulsion by providing prolonged tumor sensitivity towards PDT (Allemann et al., 1995). After a few years, Konan et al. developed polyester poly(D,L-lactide-coglycolide) and poly(D,L-lactide) doped with PS with much higher loading than ever published before (Konan et al., 2003a; Konan et al., 2003b). In order to further investigate these nanoparticles, the efficacy of the encapsulated drug was assessed on the chick embryo chorioallantoic membrane model (Vargas et al., 2004). In another work the *in vitro* and *in vivo* photodynamic activities of verteporfin-loaded poly(D,L-lactide-coglycolide) nanoparticles were studied. The results showed improved photodynamic activity of PS (Konan-Kouakou et al., 2005).

The problem with side photosensitivity due to non-specific localization of the PS into healthy tissue or skin was studied by McCarthy et al., who developed a new nano-agent that has several desirable properties for use as photodynamic drug including no toxicity in extracellular spaces and time-dependent intracellular release of PS (McCarthy et al., 2005). They demonstrated in cell culture that the phototoxicity caused by non-internalized nanoparticles is minimal (9% cell death) in contrast to the effect of internalized nanoparticles (95% cell death under identical testing conditions) (Dougherty et al., 1978). In another study Ricci-Junior et al. reported the preparation, characterization, and results of the phototoxicity assay of poly(D,L-lactide-coglycolide) nanoparticles containing ZnPC for PDT use (Ricci-Junior & Marchetti, 2006a). Other photosensitizers that have been studied consist of Indocyanine green (Saxena et al., 2006) and Hypericin (Zeisser-Labouebe et al., 2006). These compounds have the potential to be used for both diagnostic and therapeutic purposes.

1.5 Combined therapy

Even if PDT has been used effectively for treating various tumors, it still has several restrictive factors for a target-specific response, such as an observed angiogenic effect and pronounced inflammatory reaction after PDT treatment (Pervaiz & Olivo, 2006). PDT in combination with other types of therapy is an attractive approach to suppress these problematic side effects.

PDT-induced hypoxia has been associated with an increase in the expression of many angiogenic growth factors, such as hypoxia-inducible factor 1 (HIF-1), fibroblast growth factor receptor-1 (FGFR-1), cyclooxygenase-2 (COX-2), and vascular endothelial growth factor (VEGF). Combination therapy using antiangiogenic agents (e.g., COX-2 or VEGF inhibitors) with PDT led to a significant decrease of PDT-induced expression of prostaglandin E2 and VEGF, as well as a marked improvement in tumoricidal response (Akita et al., 2004; Ferrario et al., 2002; Zhou et al., 2005).

In contrast to radiotherapy, surgery or chemotherapy, PDT can lead to a strong acute inflammatory response, generally as tumor-localized edema. This PDT-induced immune activation makes it possible to positively reverse the tumor-host relationship from one that is tumor dominated to one that is oriented against the tumor. The combination with immunotherapy can reinforce the immune response triggered by PDT and thus significantly improve the anti-tumor immune response (Pervaiz & Olivo, 2006). Numerous recent clinical trials conclude that enhanced clinical outcomes can be achieved by a combination of ALA-PDT and immunomodulation therapy for the treatment of premalignant skin diseases, such as Bowen's disease (BD), BCC and AK (Wang et al., 2007; Wang et al., 2008).

In several cases, combination therapy can be done by linking the photosensitizer directly to an anticancer drug or to a specific antibody to target highly tumor-expressed receptors (Palumbo, 2007). It would also be easily accomplished by combining them using nanotechnology.

1.6 Light sources in PDT

A variety of light sources that are used in PDT consist of light-emitting diodes (LEDs), filtered xenon arc and metal halide lamps, fluorescent lamps, and lasers. Lasers and filtered broadband sources provide comparable efficacy in topical PDT (Clark et al., 2003). Non-laser light sources are also important in topical PDT, because in contrast to lasers they are stable, cheap, and offer broad-area illumination fields. Recently, LEDs showed significant progress in design, creating these low-cost sources suitable for broad-area irradiation, and were accepted for patient use. These LEDs are focused on the 630-to-635-nm activation peak of PpIX while excluding the inappropriate wavelengths present in broadband sources, thus allowing shorter irradiation times. Biophysical calculations show that LEDs with peak emission of 631 ± 2 nm can have a deeper PDT action in tissue than filtered halogen lamps with 560–740 nm emission, and hence LEDs may be more successful in treating the deeper parts of tumors (Juzeniene et al., 2004).

PpIX has its main absorption peak in the blue region at 410 nm (Soret band) with smaller absorption peaks at 505, 540, 580 and 630 nm. Most light sources for PDT seek to utilize the 630-nm absorption peak, in order to improve tissue penetration. On the other hand, a blue fluorescent lamp (peak emission 417 nm) is usually used. Nowadays, there are several reports

that blue, green, and red light itself can be efficient in topical PDT of AK; however, the more deeply penetrating red light is better when treating BD and BCC (Morton et al., 2002).

The concept of ambulatory PDT to decrease hospital attendance for PDT was described by Moseley et al. (Moseley et al., 2006). In a study of five patients with BD, PDT was carried out with ALA and a portable LEDs device. Current studies have suggested that pulsed light therapy may be helpful for treatment in topical PDT of acne, AK and photorejuvenation. On the other hand, a recent controlled investigative study carried out in healthy human skin *in vivo* demonstrated that two pulsed light sources formerly reported in PDT brought evidence of minimal activation of photosensitizer, with a significantly smaller photodynamic reaction than observed with a conventional continuous wave broadband source (Strasswimmer & Grande, 2006). These sources deliver intense light in short periods (< 20 ms), which might suppress oxygen consumption (Kawauchi et al., 2004). Unplanned ambient light exposure may have considerably contributed to the clinical effect. However, three studies have recently addressed the possibility of using ambient light for ALA-PDT of AK (Batchelor et al., 2007; Marcus et al., 2007; Strasswimmer & Grande, 2006). Two of them report on therapeutic advantage. Nevertheless, the randomized ambient light-controlled study using ALA demonstrated no significant effect on lesion ablation. A randomized right/left intra-patient evaluation of conventional MAL-PDT combined with LEDs device *versus* daylight (for 2.5 h) for the treatment of AK of face and scalp demonstrated corresponding reduction in AK and significantly less pain with daylight (Wiegell et al., 2008).

Total effective light dosage is proposed as a concept for optimizing the accuracy of light dosimetry in PDT considering incident spectral irradiance and optical transmission through tissue and absorption by PS (Moseley, 1996). Actually, light dosimetry is explained as the irradiance rate (mW cm^{-2}) at the skin surface and the total dosage (J cm^{-2}) distributed to the surface, the second being a product of irradiance and time of exposure.

It has been suggested that lower fluence rates and fractionation of light exposure can improve lesional reaction by promotion of the photodynamic reaction (Henderson et al., 2004). A study of superficial BCC illuminated with 45 J cm^{-2} at 4 h and repeated at 6 h with 633-nm laser light at 50 mW cm^{-2} showed a total response of 84 % after a mean of 59 months (Star et al., 2006). Newer studies support advantages of the fractionation approach in BCC, although not in BD (Haas et al., 2006; Haas et al., 2007).

1.7 Synthetic meso-tetraphenylporphyrins in PDT

Extensive information about the application of various porphyrins and their derivatives in PDT has been published (Král et al., 2006). Accordingly, our laboratory synthesized porphyrin conjugates with glycol (Králová et al., 2008a), bile acid (Králová et al., 2008b), and cyclodextrins (Králová et al., 2006) and their *in vitro* and *in vivo* PDT activity has been tested. It was shown that these porphyrin conjugates are taken up preferentially by tumor cells and have the potential to be used for PDT to selectively ablate tumors (Králová et al., 2006; Králová et al., 2008a; Králová et al., 2008b).

Our contemporary strategy is to combine favorable features of gold nanoparticles mediating the photothermal effect with a photosensitizing compound mediating the photodynamic effect into one combined modality and thus introduce a therapeutic protocol efficient against SCC.

The key steps in our strategy are: i) generation of a synthetic ligand with photosensitizing properties, ii) ligand immobilization on the surface of modified gold nanoparticles to enable

combination of PDT and thermo-effect, and iii) verification of the biological activity by *in vitro* and *in vivo* studies.

2. Experimental

2.1 Preparation of modified gold nanoparticles

Porphyrin-brucine conjugates **1** and **2** (Fig. 9) were prepared according to the procedure described previously (Král et al., 2005). Gold nanoparticles (14.7 nm) were prepared by citrate reduction of potassium tetrachloroaurate(III) (**Au-citr**). After modification with 3- mercaptopropanoic acid, derivatives **1** and **2** were immobilized as described elsewhere (Řezanka et al., 2008). Here, a solution of **1** or **2** (5 mg) in methanol was added to 50 ml of **Au-citr**. Modified nanoparticles (**Au-1** and **Au-2**, respectively) were isolated by centrifugation after three days of incubation. Using redispersion in methanol, methanol-water, water and dimethylsulfoxide, unbound porphyrin derivatives were removed and **Au-1** and **Au-2** molecules were concentrated to a volume of 1 ml. According to the spectral analysis of supernatants, 0.8 mg of **1** or **2** was present in the final 1 ml solution of **Au-1** and **Au-2** nanoparticles. The core of modified nanoparticles was characterized by transmission electron microscopy and photon cross-correlation spectroscopy (Nanophox). The chemical modification, ligand, was analyzed by absorption and fluorescence spectrometry. Fluorescence spectra were recorded using a Fluoromax spectrometer (Jobin-Yvon, Japan). A volume of 1 ml of sample was placed into 1 cm plastic cuvettes. The excitation wavelength was 520 nm.

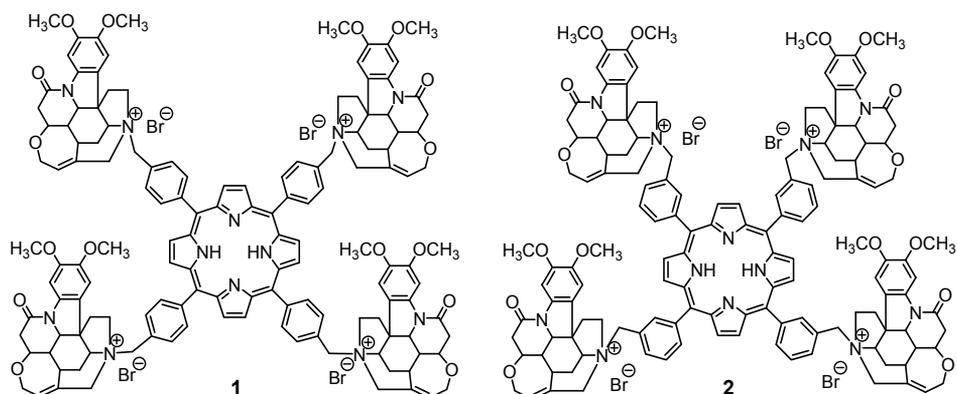


Fig. 9. The structure of **1** and **2**

2.2 Cell culture and *in vitro* experiments

4T1 (mouse mammary carcinoma) and A431 (epidermal squamous carcinoma) cells were purchased from ATCC and PE/CA-PJ34 (human basaloid squamous cell carcinoma) cells were purchased from ETCC. As described before (Králová et al., 2006), all cells were grown exponentially in RPMI 1640 medium with 10% fetal calf serum. For photodynamic experiments, $1-1.5 \times 10^5$ cells were seeded into 1.8 cm² wells and incubated overnight with the porphyrin-brucine conjugates or their counterparts immobilized on gold nanoparticles (1 and 2.5 μ M). After incubation, cells were rinsed with PBS, cultured for 1 h in fresh medium without phenol red and illuminated with a 75 W halogen lamp with a band-pass filter (Andover, Salem, NH) that emitted light at wavelengths between 500-520 nm. The

fluence rate at the level of the cell monolayer was 1 mW cm^{-2} , and the total light dose was 7.2 J cm^{-2} . Twenty-four hours post irradiation, the viability of PDT-treated cultures was determined by the Trypan blue exclusion method. In parallel, control “dark” experiments (without illumination) were performed.

2.3 Microscopic studies

Cells grown on coverslips in 35 mm Petri dishes were incubated with $2.5 \mu\text{M}$ porphyrin-brucine conjugates in culture medium for 16 h. After washing, porphyrin fluorescence was observed with a DM IRB Leica microscope equipped with a DFC 480 camera using a x63 oil immersion objective and Leica filter cube N2.1 (excitation filter BP 515–560 nm and long pass filter LP 590 nm for emission). To label lysosomes, 500 nM LysoTracker Green (Molecular Probes) was added to the culture media for 30 min. Cells were washed and examined by fluorescence microscopy using the Leica filter cube I3 (excitation filter BP 450–490 nm and long pass filter LP 515 nm for emission).

2.4 *In vivo* experiments

For *in vivo* experiments, the immuno-compromised nude mice with subcutaneously implanted human SCC tumors were used. When the tumor mass reached a volume of 100 mm^3 (10–14 days after injection), mice were intravenously injected with porphyrin-brucine conjugates (5 mg kg^{-1}) resuspended in a volume of 0.1 ml per 20 g mice and six hours later the tumor area (2 cm^2) was irradiated with a 500–700 nm xenon lamp ONL051 (maximum at 635 nm, Preciosa Crytur, Turnov, Czech Republic) with a total impact energy of 100 J cm^{-2} and fluence rate of 200 mW cm^{-2} . Each experimental group consisted of five or eight mice. The tumor size was measured repeatedly and the tumor volume was determined (Králová et al., 2006). All aspects of animal experimentation and husbandry were carried out in compliance with national and European regulations and were approved by the institutional committee.

3. Results and discussion

3.1 Modification by gold nanoparticles

Gold nanoparticles (14.7 nm) prepared by citrate reduction of potassium tetrachloroaurate(III) (**Au-citr**) were modified with 3-mercaptopropionic acid, and the derivatives **1** and **2** were immobilized. Gold nanoparticles modified with **1** and **2** are designated **Au-1** and **Au-2**, respectively.

3.2 Fluorescence spectra

The fluorescence intensity of **1** and **2** was strongly dependent on the solvent used. The influence of additional compounds on the intensity of emitted fluorescence wavelengths was tested by measuring the emission spectra (excitation of the first Q-band of porphyrins at 520 nm) of **1** and **2** in water, an inorganic salt solution (corresponding to the cell culture media) supplemented with a 50 mg ml^{-1} solution of human serum albumin (HSA) (Fig. 10A). In comparison with water, the emission bands of **1** and **2** measured in the media were red-shifted (for **1**, from 638 and 700 nm to 644 and 709 nm, and for **2**, from 643 and 707 nm to 647 and 710 nm) and the fluorescence intensity of **1** increased slightly whilst that of **2** decreased. After immobilizing the porphyrin conjugates on nanoparticles, the intensity of fluorescence emission

spectra significantly decreased (Fig. 10B) despite the concentration of porphyrins remained the same. The weak quantum yield may be attributed to that: (1) both porphyrins and nanoparticles absorb light at approximately 520 nm, (2) fluorescence quenching by porphyrin-to-metal energy transfer, (3) partial aggregation of the modified nanoparticles. In the case of **Au-1**, aggregation seems to be the cause (Fig. 10B, compare traces “**Au-1/water**” and “**Au-1/medium**”), as the intensity of emitted fluorescence was several times higher in cell culture medium compared to water only. These results demonstrate that both para- (**1**) and meta- (**2**) derivatives aggregate in a solution-dependent manner that is not affected by the presence of PS or immobilization on gold nanoparticles. Importantly, the presence of model plasma proteins present in the cell medium dramatically reduced the aggregation of modified nanoparticles. This observation led us to further test these compounds for *in vivo* PDT efficacy.

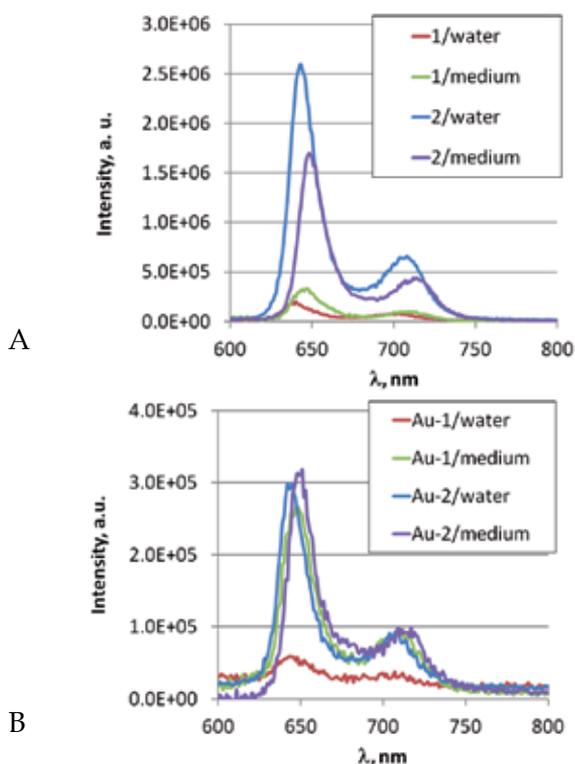


Fig. 10. The fluorescence emission spectra of porphyrins **1** and **2** (left) and porphyrin-modified nanoparticles **Au-1** and **Au-2** (right) in water and cell culture media. Excitation was performed at 520 nm. Porphyrin-brucine conjugates were used at a concentration of 3.5 μM . The concentration of human serum albumin used in growth medium was 50 mg ml^{-1} .

3.3 Intracellular localization

The porphyrin-brucine conjugates (**1** and **2**) were next analyzed for tumor cell uptake and intracellular distribution. The mammary carcinoma cell line, 4T1 was cultivated in the presence of the conjugates for 16 h, during which time the cells were well-dispersed and growing mostly as planar sheets, enabling focused images of fluorescence to be recorded. These cells exhibited punctate red fluorescence (Fig. 11).

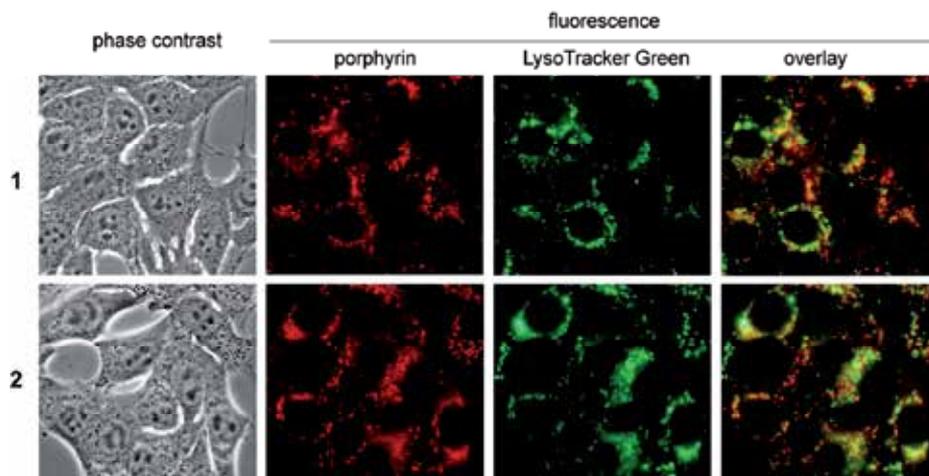


Fig. 11. The intracellular localization of porphyrin-brucine conjugates in 4T1 cells. The middle panels show the red fluorescence of **1** and **2** and co-staining with the lysosomal specific probe (LysoTracker Green); right panels represent an overlay of the green and red images and demonstrate co-localization (shown in orange/yellow). Porphyrin-brucine conjugates were used at a concentration of 2.5 μ M.

To identify the intracellular compartment where **1** and **2** accumulate, co-staining with the LysoTracker Green fluorescence probe was performed. The merged images revealed that **1** and **2** colocalized to a subset of LysoTracker-stained structures that represent lysosomes. Similar localization was also observed in PE/CA-PJ34 basaloid squamous cell carcinoma cells and A431 epidermal squamous carcinoma, cell lines that were predominantly used in our study (data not shown). Upon addition of gold nanoparticle-conjugated **1** and **2** to cell culture media, aggregates formed, which were visible as a reddish precipitate that covered parts of the cell. These were particularly abundant in the case of **Au-1** (Fig. 12).

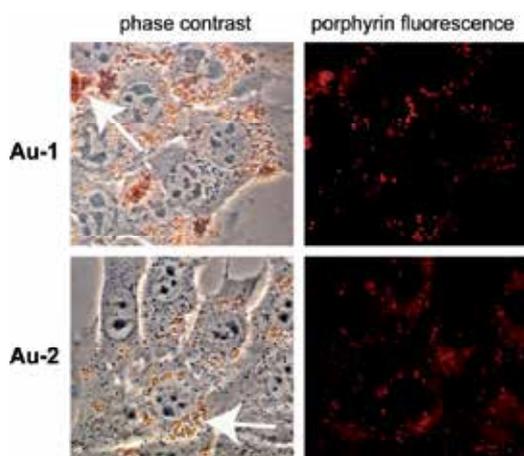


Fig. 12. Difference in aggregation behavior of porphyrin-brucine conjugates immobilized on gold nanoparticles (left panels). 4T1 cells were incubated with **Au-1** and **Au-2** at a concentration of 2.5 μ M for 4 h before pictures were taken. Aggregates are highlighted by arrows.

3.4 *In vitro* phototoxicity

To investigate the photodynamic potential of the free porphyrin-brucine conjugates or those immobilized on gold nanoparticles, we incubated PE/CA-PJ34 cells in the presence of the conjugates for 16 h and subjected them to PDT. In parallel, cells were incubated with porphyrins without illumination to serve as dark controls. Twenty-four hours following the illumination of cells with filtered light, the mortality of post-PDT cultures was determined (Fig. 13).

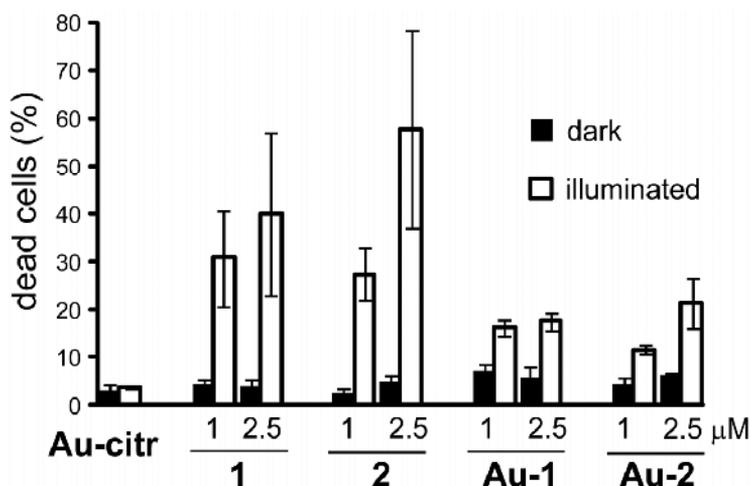


Fig. 13. The effect of free or immobilized porphyrin-brucine conjugates on the induction of cell death via PDT. PE/CA-PJ34 cells were incubated with either 1 or 2.5 μM of **1** and **2** or their modified Au-nanoparticles for 16 h. Cells were then illuminated with filtered light (500–520 nm, 7.2 J cm⁻²). The percentage of dead cells was established the following day by using the Trypan blue exclusion method. The average and standard deviation for three independent experiments is shown.

Satisfyingly, the induction of cell death was both light and drug-dose dependent. Control cells incubated with unconjugated gold nanoparticles (**Au-citr**) did not display any increase in cell death after illumination. Thus, under these *in vitro* conditions we can exclude the possibility that any case of cell death is due to the photothermal activity of the gold nanoparticles. Interestingly, the phototoxicities of unbound porphyrin-brucine conjugates **1** and **2** were higher than those immobilized on gold nanoparticles. This reduction of photodynamic efficacy is likely to be a consequence of **Au-1** and **Au-2** aggregation that occurs in the aqueous cell growth media (Fig. 12).

3.5 *In vivo* PDT efficacy

Using an *in vivo* mouse cancer model, the PDT effectiveness of the unbound porphyrin-brucine conjugates **1** and **2** was compared with those immobilized on gold nanoparticles (**Au-1**, **Au-2**). Nude mice (NuNu) bearing basaloid squamous cell carcinoma PE/ CA-PJ34 cells received by intravenous injection either unmodified porphyrins or their gold nanoparticle-modified counterparts. Six hours post injection, tumors were illuminated

with light at a dose of 100 J cm^{-2} . Mice not injected with unmodified porphyrins or nanoparticles served as controls. Tumor size was measured after PDT at regular intervals (Fig. 14).

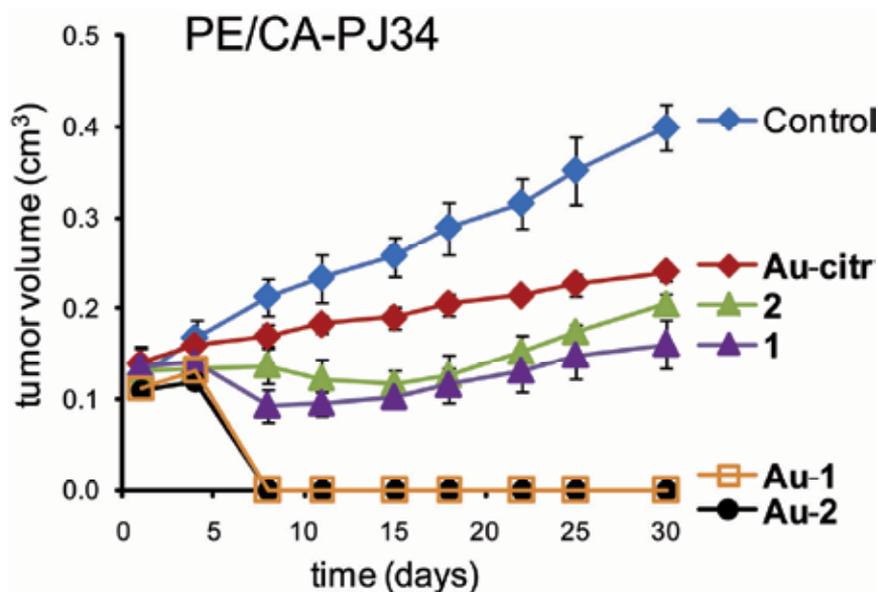


Fig. 14. The PDT effectiveness of **1** and **2** and their respective Au-immobilized nanoparticle counterparts to eradicate mouse tumors. Nude mice (NuNu) bearing subcutaneous PE/CA-PJ34 tumors ($n = 8$ per each group) received an intravenous dose of the drug (5 mg kg^{-1}). Tumors were illuminated with light (100 J cm^{-2}) six hours after injection. The tumor size was measured repeatedly and the tumor volume was determined. Control mice were exposed to illumination but did not receive the porphyrin drug. The **Au-citr** group represents mice injected with Au nanoparticles, **1** and **2** groups received porphyrin conjugates, **Au-1** and **Au-2** groups received porphyrin-modified Au nanoparticles.

We observed the greatest reduction in tumor growth in mice treated with **Au-1** and **Au-2**. All tumors were eliminated in animals that received these conjugated porphyrins and importantly, no detectable relapse of the primary tumor was observed. In contrast, animals treated with unbound **1** and **2** exhibited only a transient regression in tumor size that lasted until day 18, when the primary tumors began to gradually regrow. Presumably, this relapse in tumor growth comes from the small population of tumor cells that survived the PDT. Interestingly, mice treated with unconjugated gold nanoparticles exhibited slight tumor retardation in growth, which is most likely due to the photothermal effect described in other systems (Gamaleia et al., 2010; O'Neal et al., 2010; Řezanka et al., 2008).

These results clearly show that porphyrin alkaloid-modified gold nanoparticles are very effective against basaloid SCC *in vivo*. To verify more general applicability of porphyrin alkaloid-modified gold nanoparticles, the same approach was used against epidermal SCC tumors (Fig. 15). A431 cells formed fast progressing subcutaneous tumors, which were completely eradicated after **Au-2**-mediated PDT treatment in 60% mice or their growth was substantially reduced. These results demonstrate a high potential of porphyrin alkaloid-modified gold nanoparticles to fight SCC.

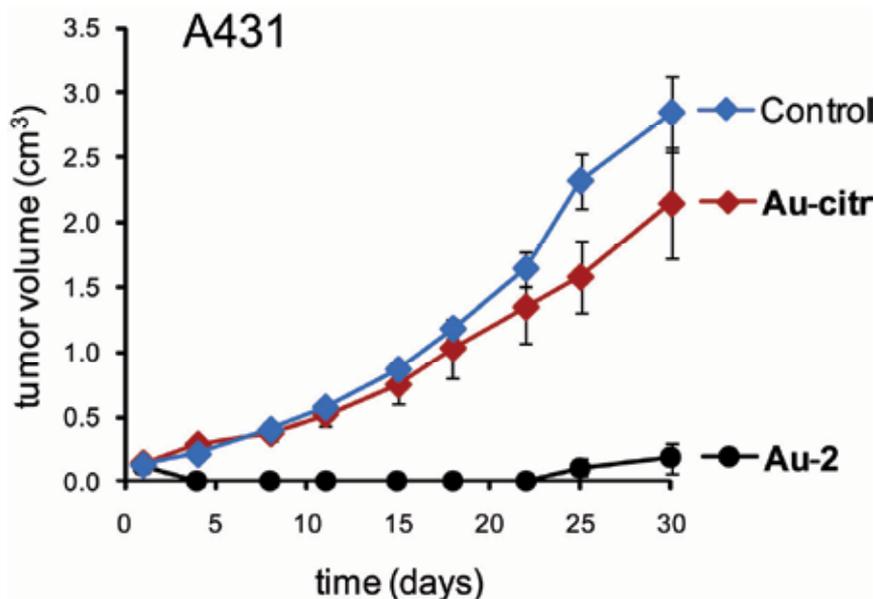


Fig. 15. The PDT effectiveness of Au-2 against fast progressing epidermal squamous carcinoma A431. Nude mice (NuNu) bearing subcutaneous A431 tumors ($n = 5$ per each group) received an intravenous dose of the drug (5 mg kg^{-1}). Tumors were illuminated with light (100 J cm^{-2}) six hours after injection. The tumor size was measured repeatedly and the tumor volume was determined. Control mice were exposed to illumination but did not receive the porphyrin drug. The **Au-citr** group represents mice injected with Au nanoparticles without porphyrin.

The apparent discrepancy in the *in vitro* and *in vivo* performance of unbound porphyrin-brocin conjugates **1** and **2** and those immobilized on gold nanoparticles (**Au-1** and **Au-2**) is likely to be due to the differing environmental conditions to which the porphyrin conjugates were exposed. The fluorescence data revealed that conjugates **1** and **2** were efficiently taken up by cells under the *in vitro* conditions tested. However, in culture media, **Au-1** and **Au-2** tended to aggregate, which resulted in their lower intracellular availability (Fig. 12) and lower PDT efficacy (Fig. 13). Under the *in vivo* conditions tested, the gold nanoparticle-immobilized conjugates were more effective than free conjugates alone. Both spectroscopic and ECD studies demonstrated that conjugated nanoparticles exhibited a strong interaction with plasma proteins (mainly HSA), which led to their self-assembly and to generation of supramolecular complexes. Subsequently, thanks to the enhanced permeability and retention (EPR) effect resulting in potent accumulation of **Au-1** and **Au-2** in the tumors, their PDT efficacy was increased. Moreover, the direct lethal effect of PDT on tumor cells combines well with the nanoscale size of gold-immobilized porphyrins that may limit the local blood supply (vascular impairment). This hypothesis of vascular damage after PDT with nanoparticles will be the subject of future work.

4. Conclusion

The spectroscopic studies demonstrated that fluorescence intensity of free and immobilized conjugates were strongly dependent on the solvent used. After immobilizing the porphyrin

conjugates 1 and 2 on nanoparticles, the intensity of fluorescence emission spectra significantly decreased. The weak quantum yield may be attributed to that: (1) both porphyrins and nanoparticles absorb light at approximately 520 nm, (2) fluorescence quenching by porphyrin-to-metal energy transfer, (3) partial aggregation of the modified nanoparticles. Importantly, the presence of model plasma proteins in the cell medium dramatically reduced the aggregation of modified nanoparticles and prompted their use *in vivo*.

The evaluation of the biological activity of porphyrin-brucine conjugates, either free or immobilized to gold nanoparticles, started with determination of their intracellular uptake. It was shown that both forms were effectively taken into the cell, although a lower level was observed for immobilized forms. To investigate the photodynamic potential of the conjugates, SCC were exposed *in vitro* to photodynamic treatment and cell mortality of post-PDT cultures was determined. The phototoxicities of unbound porphyrin-brucine conjugates were higher than those of conjugates immobilized on gold nanoparticles. This reduction of photodynamic efficacy is likely to be a consequence of nanoparticle aggregation that occurs in the aqueous cell growth media.

In contrast, when the PDT effectiveness was tested *in vivo*, the greatest reduction in tumor growth was observed in mice treated with porphyrin conjugates immobilized on gold nanoparticles. All tumors were eliminated and no detectable relapse of the primary tumor was observed. When animals were treated with unbound conjugates, they exhibited only a transient regression in tumor size that lasted until day 18, and then the primary tumors began to gradually re-grow. Importantly, mice treated with gold nanoparticles without porphyrin exhibited slight tumor retardation in growth that is most likely attributed to the photothermal effect described in other systems. Thus, under the *in vivo* conditions tested, the gold nanoparticle-immobilized conjugates were more effective than free conjugates alone. In addition, both spectroscopic and ECD studies demonstrated that conjugated nanoparticles exhibited a strong interaction with plasma proteins (mainly serum albumin), which led to their self-assembly and generation of supramolecular complexes, and thereby to the enhanced permeability and retention effect. It further contributed to potent accumulation of immobilized conjugates in tumors leading to increased PDT efficacy. Moreover, the direct lethal effect of PDT on tumor cells combines well with the nanoscale size of gold-immobilized porphyrins that may limit the local blood supply (vascular impairment).

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Part 3

Molecular Aspects of Tumor Invasion and Progression in Squamous Cell Carcinoma

Cadherin Expression and Progression of Squamous Cell Carcinomas of the Oral Cavity

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1. Introduction

Cell-cell adhesion plays fundamental and dynamic roles in the development and maintenance of multi-cellular organisms. Epithelial sheet is a typical structure and composed of cells that work together and separate from a lumen or space from underlying tissue. It lines most internal surfaces, including gastrointestinal tract and kidney tubes, and external layer of the epithelium as the epidermis of skin. The oral cavity is covered with stratified squamous cell epithelium in which keratinizing epithelial cells strongly connect with each other and differentiate from basal cells at the bottom to keratinized surface cells. Epithelial cells are connected together by junctional complexes that have distinct order with respect to their ultra-structures; zonula occludens (tight junctions), gap junctions, zonula adherens (adherence junctions) and macula adherens (desmosomes). Adherence junctions in epithelial sheet are belt like junctions and composed of cadherins that bind with proteins at the cytoplasmic domain. In other cell types, adherence junctions display different morphology; spotty and discontinuous in fibroblastic cells and punctate in the synaptic junctions. Desmosome is a spot-like junction associated with desmosomal cadherins (desmogleins and desmocollins) and tightly associated with adjacent cell membranes compared to adherence junctions. Stratified squamous epithelial cells express large amount of cadherins and well organize adherence junctions and desmosomes. Disruption of desmosomes by autoantibodies against desmoglein causes pemphigus that are multiple and bullous diseases in the skin and oral mucosa. Cadherins are most characterized cell-cell adhesion molecules and implicated in the development and progression of carcinomas of the epithelial origin. In this chapter, we overview the regulation and role of cadherins in the pathology of oral squamous cell carcinomas (OSCCs).

2. The cadherin superfamily

Cadherins are calcium-dependent transmembrane proteins that are evolutionary conserved and have two or more extracellular domains (EC domains). Yoshida and Takeichi (1992) cloned a transmembrane protein from the calcium-dependent junctions and termed cadherin. Since many related molecules were cloned, cadherins constitute a superfamily and the original cadherins are now called as "classic cadherins" (Fig. 1). Approximately twenty members of cadherins are included in the classic cadherin family depending on their

domain structures. In vertebrate, they have five repetitive EC domains that contains calcium-binding sequences and highly conserved cytoplasmic domain that directly interacts with catenins. The binding of calcium ions with the EC domains is prerequisite for the conformation and adhesive function of the extracellular region, and the extracellular region undergoes interactions with apposed cells. The classic cadherins are subdivided into type I and type II. Type I cadherin contains a His-Arg-Val sequence in the N-terminal EC domain, and other classic cadherins that do not contain the sequence are grouped into type II cadherin. The type I cadherin includes epithelial-cadherin (E-cadherin, CDH1), neural-cadherin (N-cadherin, CDH2), placental-cadherin (P-cadherin, CDH3) and others, and vascular endothelial-cadherin (VE-cadherin, CDH5), osteoblast-cadherin (OB-cadherin, CDH11) and others belong to the type II cadherins. Although it is still controversial, the classic cadherin basically binds with the same-type cadherin but not with other types. This nature of homophilic binding is implicated in the sorting of different cell types. Besides to the classic cadherins, a number of nonclassic cadherins that conserve EC domains but have divergent cytoplasmic sequences has been identified. Desmosomal cadherins are most closed to the classic cadherins and required for desmosome formation in the epithelium. Other nonclassic cadherins, including protocadherins, Fat and Flamingo, appear not to organize specialized junctions, nor to be the essential adherence junction components (Meng & Takeichi, 2009; Gumbiner, 2005).

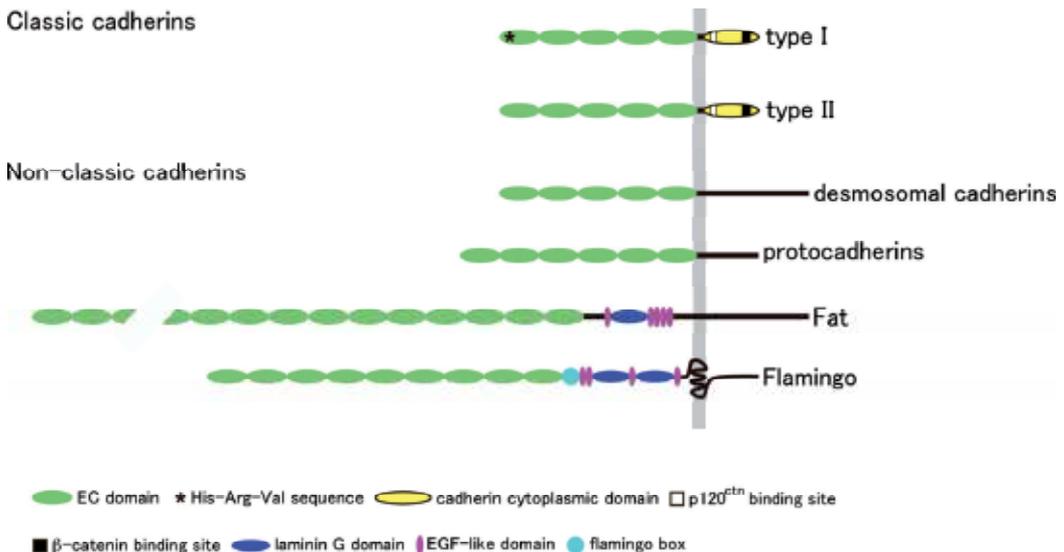


Fig. 1. Domain structures of cadherin superfamily. Molecules conserving EC domains in the extracellular region consist of the cadherin superfamily. The classic cadherins have five EC domains and the cytoplasmic domain possessing the binding sites for p120^{ctn} and β-catenin. They are subdivided into type I and type II groups according to the presence or absence of His-Arg-Val sequence in the N-terminal EC domain, respectively. Non-classic cadherins do not preserve the cytoplasmic domain and have unique cytoplasmic amino acid structures in each cadherin. Numbers of EC domains in each non-classic cadherin are different depending on members. For example, in desmosomal cadherins, desmoglein and desmocollin have four and five EC domains, respectively.

3. Cadherin functions at the adherence junction

The highly conserved cytoplasmic domains of the classic cadherins interact with catenins (Fig. 2). The juxtamembrane region of the cytoplasmic domain binds with p120-catenin

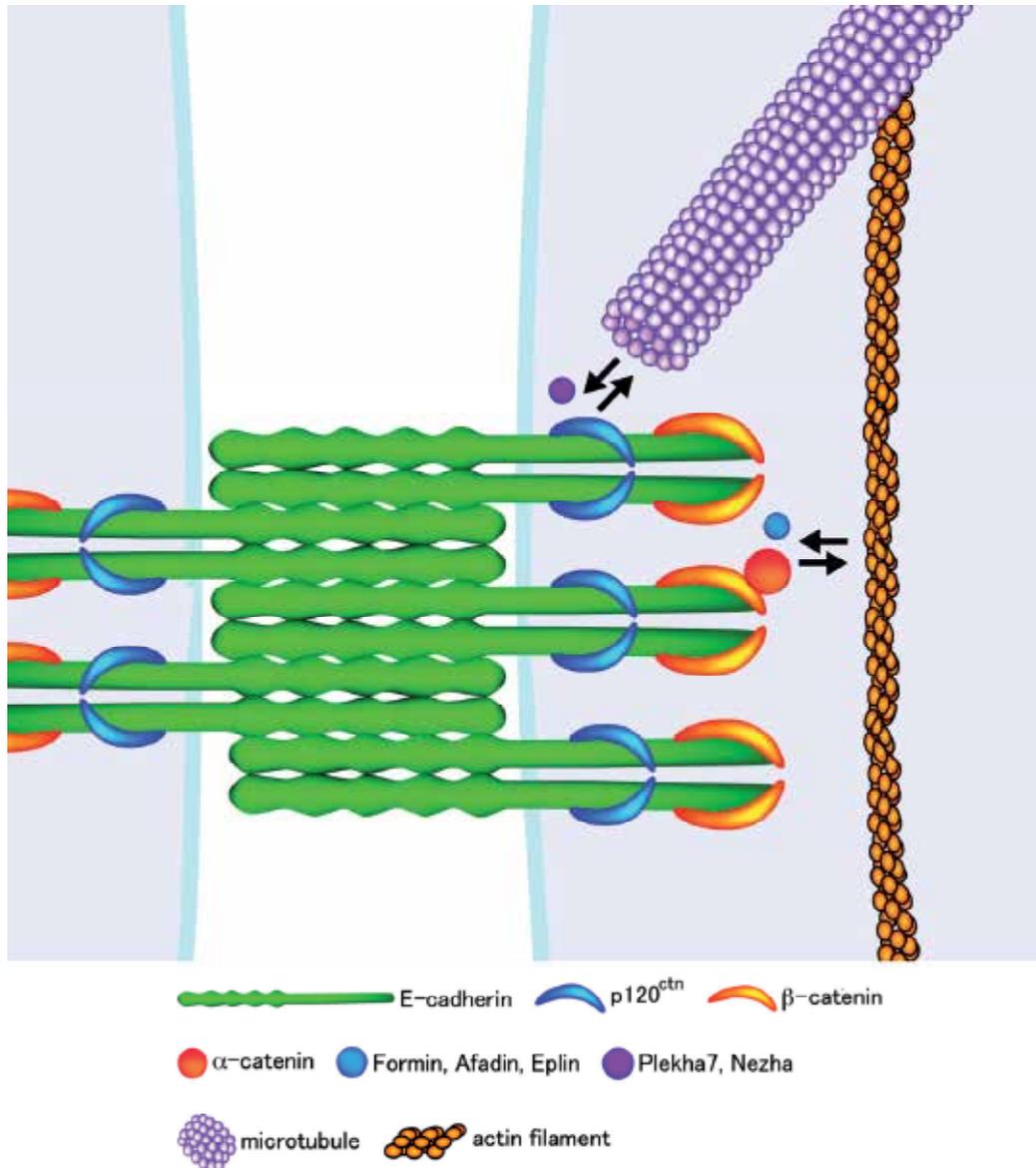


Fig. 2. Molecular structural organization of adherence junction containing E-cadherin. E-cadherins homotypically bind with adjacent cells by the EC domain interaction, and p120^{ctn} and β-catenin interact with the cytoplasmic domain of E-cadherin. p120^{ctn} directly associate with microtubules or indirectly mediated by Plekha 7 and Nezha. The β-catenin-α-catenin complex associates with actin filaments with or without linker proteins (Formin, Afadin and Eplin).

(p120^{ctn}), and the carboxy-terminal half with β -catenin. The cytoplasmic domain indirectly binds with α -catenin through β -catenin, resulting in formation of the cadherin- β -catenin- α -catenin complex. The complex ligates with actin filaments that are essential for assembly and integrity of adherence junctions. Although early studies suggested that α -catenin acts as a linker protein which in turn interact with the complex and actin filament, recent studies showed that free α -catenin can bind the filament and promote the bundling of actin filaments, but not α -catenin in the complex (Drees et al., 2005; Yamada et al., 2005). Several proteins, including Formin (Kobielad et al., 2004), Afadin (Mandai, 1997) and Eplin (Abe & Takeichi, 2008), are suggested to work as a linker between α -catenin and actin filament. p120^{ctn} protein also regulates actin reorganization and contractility by regulating RhoA activity (Anastasiadis et al., 2007). However, since the roles on the formation of adherence junction and the linkage with actin filament are appeared different depending on cell types (Meng & Takeichi, 2009), further studies are required to define the molecular mechanism for actin filament-binding to the adherence junction complex. Another cytoskeleton connected with the complex is microtubules. Microtubules extend to adherence junctions, and blocking the microtubules extension reduces accumulation of E-cadherin to the junctions (Stehbens et al., 2006; Harris & Tepass, 2010). Furthermore, depolymerization of microtubules disrupt the integrity of the junctions and inhibit disassembly of cell junctions (Waterman-Storer et al., 2000; Ivanov et al., 2006). Recent studies showed that microtubules interact with adherence junctions via p120^{ctn}, Plekha7 and Nezha (Meng & Takeichi, 2009). The adherence junction is a static structure but cadherin proteins are recycled in epithelial cells. E-cadherin is endocytosed and transported to recycling endosomes followed by trafficking in late endosomes to the cell surface (Meng & Takeichi, 2009). The surface-located cadherins are stabilized by their homophilic interactions at adherence junctions. p120^{ctn} also has a pivotal role in the microtubule assembly at adherence junctions. The p120^{ctn} protein consist of the N-terminal region, armadillo repeat domain and C-terminal tail region. The N-terminal region and the armadillo repeat domain are responsible for binding with microtubules and the juxtamembrane domain of E-cadherin, respectively (Ichii & Takeichi, 2007; Ishiyama et al., 2010). The binding of p120^{ctn} to E-cadherin masks a dileucine motif on the juxtamembrane domain, which is sensitive to endocytosis and ubiquitin-mediated degradation of E-cadherin (Ishiyama et al., 2010). It is suggested that p120^{ctn} stabilizes the microtubule polymerization independent of a mechanistic trait of E-cadherin-mediated cell-cell adhesion (Ichii & Takeichi, 2007). Thus, the assembly and the function of adherence junctions are regulated by multi-dimensional factors, including E-cadherin *per se*, catenins, the related molecules and association with actin filaments and microtubules.

4. Roles of E-cadherin in the epithelium

E-cadherin-null mutation is lethal and the conditional knockout mice in skin epithelium show hyperproliferation of basal cells with defects in terminal differentiation (Ohsugi et al., 1997; Tinkle et al., 2004). An animal model of pancreatic carcinomas demonstrated a direct role of E-cadherin in adenoma-to-carcinoma conversion (Perl et al., 1998). These studies indicate that E-cadherin plays a critical role in developmental and pathological events *in vivo*. Forced expression of E-cadherin in the intestinal epithelium represses migration of epithelial cells along with the crypt-villus axis and stimulates the apoptotic rate of epithelial cells (Hermiston et al., 1996). Recent studies implicate that cadherins regulate interaction of growth factor receptors with ligands and modulate their signaling. Cadherins bind to growth factor receptors, including transforming growth factor- β receptor (TGFB β),

fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR), vascular endothelial cell growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), and the cytoplasmic domain can suppress growth-promoting cell signaling, such as Src, phosphatidylinositol-3-kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK) pathways (Reddy et al., 2005; Suyama et al., 2002; Georgopoulos et al., 2010; Cavallaro & Dejana, 2011). Sensitivity to the EGFR inhibitor, cetuximab, requires intact E-cadherin expression and silencing of E-cadherin reduces responsiveness to the inhibitor (Black et al., 2008). Cadherins regulate the growth factor signaling by recruiting the receptors at the cell surface, stimulating the receptor dimerization, and modulating their activities (Cavallaro & Dejana, 2011). β -catenin is a leading player in WNT signaling, which has a predominant role in developmental and pathophysiological conditions. Binding of WNTs to the receptors protected β -catenin from the degradation by the ubiquitin-proteasome pathway and increases the cytoplasmic free pool (Maher et al., 2009). The cytoplasmic free- β -catenin translocates into the nucleus and modulates gene transcription by interacting with lymphoid enhancer factor (LEF) and T cell factor (TCF). Since the cadherin- β -catenin interaction is constitutive, cadherins interfere with the transcriptional activity of β -catenin. Therefore, unveiling the regulatory mechanisms of E-cadherin expression is a pivotal theme to understand the initiation and progression of carcinomas and develop a novel strategy for the treatment of carcinoma patients.

5. Regulation of E-cadherin expression

Loss or reduction of E-cadherin expression results from somatic mutations, chromosomal deletions, proteolytic cleavage, promoter hypermethylation and transcriptional repression. Germ-line mutation of the E-cadherin gene causing inactivation of one allele has been reported in several families from New Zealand and Europe, and is associated with hereditary diffuse-type high-grade gastric carcinomas (Guiford et al., 1998; Gayther et al., 1998). Although single nucleotide polymorphisms have been described to associate with the reduction of transcription efficiency of E-cadherin gene (Li et al., 2000), the mechanism of reduction awaits for future studies to establish. However, genomic deletion and germ-line mutation with loss of heterozygosity, referred to as Knudson's two-hit theory, are rare events in sporadic carcinomas (Brown, 1997). Loss of E-cadherin expression stimulates carcinoma progression and carcinoma cells in the metastatic loci frequently re-express E-cadherin (Cheng et al., 2001), indicating the genetic mutation and polymorphism are not a major cause in sporadic carcinomas. In mammalian genome, methylation emerges at a cytosine located 5' to a guanosine in a CpG dinucleotide. CpG islands are found in promoter region of approximately half of the genes in human genome. In the development of cancers, epigenetic silencing of tumor-suppressive genes as a result of cytosine methylation in CpG islands has been documented as one of most important alterations. Increasing evidence highlight the fact that there are target genes for CpG island hypermethylation in many types of cancers, especially carcinomas of the epithelial origin (Fazzari & Grealley, 2004; Jones & Baylin, 2002; Maeda et al., 2007a; Maeda et al., 2007b; Chiba et al., 2009). The hypermethylation at CpG islands determines the transcriptional status of a gene by blocking the access of certain transcription factors that are sensitive to cytosine methylation in their binding motifs, and by packaging chromatin into compacted nucleosomes with deacetylated histones and recruiting a methyl-cytosine-binding protein complex that represses transcription (Fazzari & Grealley, 2004; Jones & Baylin, 2002). The transcription repressors, including Snail, Slug, Twist, zinc finger E-box binding homeobox

(ZEB)1, ZEB2, and E47, bind to the E-box (5'-CANNTG-3') in the promoter and repress E-cadherin expression. After identification of Snail as a transcription repressor of E-cadherin in 2000 (Cano et al, 2000; Batlle et al, 2000), several other repressors were implicated in tumor progression and the epithelial-mesenchymal transition (EMT) induction. The EMT stimulates migration, earns the drug-resistance and the stem cell-like features of carcinoma cells and energizes carcinomas to an aggressive subset, and the loss of E-cadherin expression is the most prominent event (Hanahan & Weinberg, 2011). Expression of E-cadherin repressors is regulated by multiple pathways activated by growth factors. Among them, TGF β signaling is frequently activated in aggressive carcinomas and induces the EMT of carcinoma cells at the invasive front. High mobility group protein A-2 (HMGA2), which is specifically expressed in undifferentiated mesenchymal cells, are strongly misexpressed in oral carcinoma cells at the invasive front in patients with poor prognosis (Fig. 3; Miyazawa

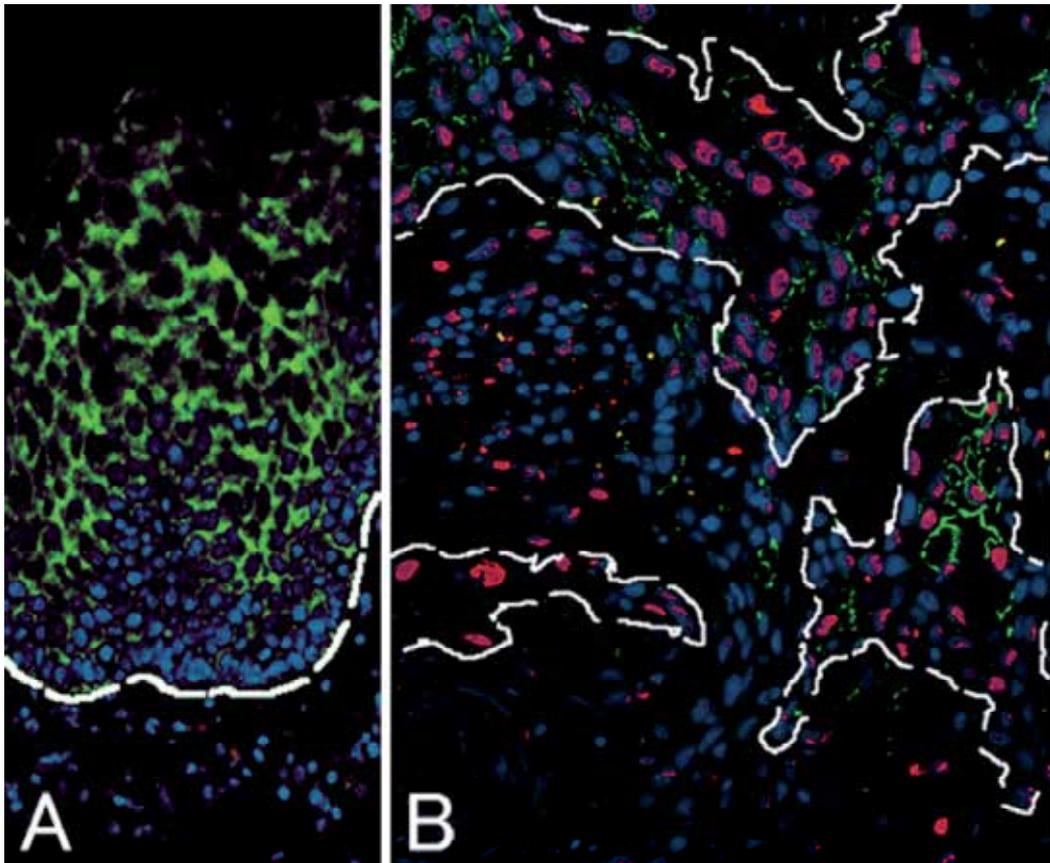


Fig. 3. Expression of E-cadherin and HMGA2 in oral epithelium and carcinomas. E-cadherin (green) expression is detected by immunofluorescent microscopy at the cell-cell boundaries of normal oral epithelium (A) while in carcinoma cells at the invasive dramatically lose the immunoreactivities (B). In contrast, the mesenchyme-specific HMGA2 expression (red) is localized in carcinoma cells that are negative for E-cadherin, and not detected in normal oral epithelial cells. The data in a panel B is reproduced from Miyazawa et al., [*Cancer Research*, Vol. 64, No.(6) pp.2024-2029, ISSN 1078-0432].

et al., 2004), and integrates the TGF- β -mediated EMT in carcinoma cell in combination with the induction of Snail, Slug and Twist (Thuault et al., 2006). In addition, inhibition of WNT signaling promotes degradation of Snail by the ubiquitin-proteasome pathway (Shou et al., 2004). Expression of E-cadherin is also post-transcriptionally regulated by the microenvironment of carcinoma cells. Furthermore, series of immunohistochemical studies suggest the differential roles of repressors; Snail in the induction of initial migratory phenotype of carcinoma cells followed by the maintenance of phenotype by Slug, Twist and ZEB1/2 (Peinado et al., 2007). The transcription repressors attenuate E-cadherin expression while they are negatively regulated by microRNAs (miRNAs). Expression of miR-200, which binds to ZEB1 and ZEB2 mRNAs and abrogates their translation into proteins, is inhibited by TGF- β signaling but stimulated a tumor suppressor gene p53 (Kim et al., 2011; Gregory et al., 2011). The miR-92, which directly targets E-cadherin mRNA, downregulates p53 expression (Neveu et al., 2010; Chen et al., 2011). TGF- β also upregulates expression of matrix metalloproteinases (MMPs), which liberate TGF- β from surrounding tissues to cells after degradation of extracellular matrix proteins (Imai et al., 1997). Since MMPs shed the extracellular region of E-cadherin (Zheng et al., 2009; Imai & Okada, 2009), the TGF β -MMP loop enhances disruption of E-cadherin-mediated adherence junctions of carcinoma cells. Therefore, the state of E-cadherin comprehensively regulated by the intrinsic and extrinsic factors of carcinoma cells (Fig. 4).

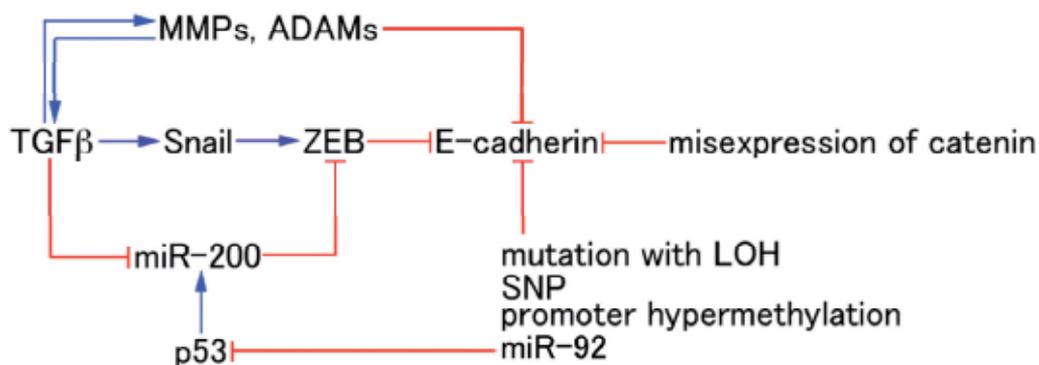


Fig. 4. A schematic representation for the E-cadherin expression and repression machineries. In carcinoma cells of epithelial origin, expression of E-cadherin is regulated by several pathways that directly or indirectly control the expression. Blue lines indicate stimulation and red lines indicate suppression.

6. Loss of E-cadherin expression in oral squamous cell carcinomas

As mentioned above, multi-factors may regulate the E-cadherin repression in oral carcinoma cells. Although germ-line mutation with the loss of heterozygosity is rare (Saito et al., 1998), epigenetic aberrations, including the promoter hypermethylation and expression of transcription repressors, are commonly observed in an aggressive subset of OSCCs. The hypermethylation is detected in 35-85% of OSCCs (Viswanathan et al., 2003; Yeh et al., 2002) and prompts carcinoma cells to develop invasive tumors (Nakayama et al., 2001). Kudo et al. (2004) reported that the hypermethylation was observed in oral carcinoma cells at the invasive front but not in non-invasive areas. Although increasing number of investigations

revealed the presence of E-cadherin transcription repressors, their expression is largely dependent on cell and tissue types (Peinado et al., 2004). Snail is a most studied molecule that is responsible for repression of E-cadherin gene expression in many types of carcinomas including OSCCs (Yokoyama et al., 2001).

Snail expression is observed at the invasive front oral carcinoma cells of patients with poor prognosis (Yu et al., 2011). Although we could not find a reverse correlation between expression of Snail and E-cadherin in oral carcinoma cells, ZEB2 expression was upregulated in the E-cadherin-low cells and detected in carcinoma cells at the invasive front of OSCC patients with poor prognosis (Maeda et al., 2005). Upregulation of Twist in OSCCs is reported while its significance to E-cadherin expression is uncertain (Vered et al., 2010; Liang et al., 2011). In addition to the loss of E-cadherin expression, the involvement of catenins is also documented. The role of p120^{ctn} in E-cadherin expression and carcinoma progression has been attracting a lot of attention. Loss of p120^{ctn} expression in the oral epithelium in mice spontaneously develops invasive OSCCs, induces the EMT of carcinoma cells, and recruits chronic inflammatory reactions within carcinoma tissues (Stairs et al., 2011). Cell membrane-associated E-cadherin become endocytosis upon the loss of p120^{ctn}, leading to the reduction of cell-cell adhesion (Liu et al., 2007). The loss or mislocalization of p120^{ctn} correlates with poor patient prognoses of carcinomas of the colon, bladder, stomach, breast, prostate, lung and pancreas (Thoreson & Reynolds, 2002). Cytoplasmic mislocation of p120^{ctn} in oral carcinoma cell lines, while it is localized at the cell membrane of normal oral keratinocytes, was reported previously (Lo Muzio et al., 2002). However, the loss of expression in the epidermis does not have an obvious effect on cell-cell adhesion but reduces expression level of E-cadherin. The mice show epidermal inflammation due to activation of nuclear factor-kappa B (NF- κ B) signaling (Perez-Moreno et al., 2006). Chronic inflammation increases production of inflammatory cytokines and reactive oxygen species and DNA damage, and results in development and progression of carcinomas (Meira et al., 2008). Oral carcinoma cells upregulate the E-cadherin-targeting miR-92 (Scapoli et al., 2010). Although expression of miR-200 in OSCCs is not known at present, nasopharyngeal carcinomas downregulate it which destabilizes ZEB1/2 mRNA (Chen et al., 2009). Regardless of the cause, loss of E-cadherin results in the liberation of β -catenin from adherence junctions and the increase of the cytoplasmic free-pool, which synergistically acts with the canonical WNT signaling. In fact, carcinoma cells at the invasive front, where loss of E-cadherin and expression of WNTs are observed, exhibit the cytoplasmic and/or nuclear staining of β -catenin (Uraguchi et al., 2004; Miyazawa et al., 2004). Silencing of β -catenin by RNA interference reduces proliferation of oral carcinoma cells (Duan & Fan, 2011). A recent study suggests that the loss of E-cadherin-mediated cell-cell adhesion and sequestering the β -catenin from E-cadherin have a differential role establishing metastatic properties of carcinoma cells (Onder et al., 2008). Although a precise mechanism is under debate, loss of E-cadherin expression and the gain of WNT expression may synergistically increase the cytoplasmic β -catenin and preserve it from degradation, allowing the nuclear translocation and transcriptional control of target genes toward the tumor progression. The WNT signaling represses transcription of E-cadherin gene but stimulates WNT protein expressions *per se*. The WNTs also upregulate E-cadherin suppressors, including Snail and Twist, and downregulate miR-200 (Saydam et al., 2009). E-cadherin suppresses activation of NF- κ B, which strongly enhances aggressive behaviors of oral carcinoma cells and is upregulated in patients with poor outcome (Solanas et al., 2008). A mouse EMT model

demonstrated the essential contribution of NF- κ B to the induction of EMT, maintenance of the mesenchymal phenotype, and metastasis (Huber et al., 2004). NF- κ B suppresses E-cadherin expression through ZEB1 and ZEB2 induction (Chua et al., 2007). Therefore, reduction and loss of E-cadherin expression in OSCCs is under the control of multiple factors and pathways including the gene transcription, catenins and growth factor signaling.

7. Cadherin switch and oral carcinoma progression

E-cadherin and N-cadherin are the most prominent members of the classic cadherins, and a numbers of studies have been reported about their roles in carcinoma progression. During the progression of many human gastrointestinal tumors, gradual loss of E-cadherin expression at the invasive front accompanies a *de novo* N-cadherin expression (Wheelock et al., 2008). Replacing the member of cadherins, usually E-cadherin-to-N-cadherin in carcinoma cells, is referred as the cadherin switch. Followed by the cadherin switch, carcinoma cells acquire motile, invasive and metastatic abilities. Although functional implications are unknown at present, expression pattern of N-cadherin in a belt-like structure in low-grade prostate carcinomas becomes a dotted pattern at the interface of interaction with stromal fibroblasts in parallel with loss of E-cadherin expression (Tomita et al., 2000). Inhibition of N-cadherin expression or function blocks motility and invasion of carcinoma cells (De Wever et al., 2004). The cadherin switch is initiated by the internal and microenvironmental programs of carcinoma cells. Carcinoma cell adhesion on extracellular matrix proteins, including laminin (Kim et al., 2011), type I collagen (Shintani et al., 2008) and fibronectin (Lefort et al., 2011), induces N-cadherin expression. Another key regulator of N-cadherin is TGF- β , which can act to carcinoma cells after the extracellular matrix degradation and promotes invasion of oral carcinomas (Imai et al., 1997; Lu et al., 2004).

In OSCCs, TGF- β and N-cadherin is predominantly expressed at the invasive front and stimulates the motility of cells (Franz et al., 2007). The TGF- β signaling promotes oral carcinoma cells to express N-cadherin without affecting E-cadherin expression and regulate the motility (Diamond et al., 2008). Li et al. (2009) reported that N-cadherin was positively stained in 92.4% of tongue carcinomas while E-cadherin in 11.3%. A previous study reported that N-cadherin was upregulated in OSCCs with reduced expression of E-cadherin, and that N-cadherin expressing OSCCs had a tendency to be less histologically differentiated, more invasive and metastatic to lymph nodes (Pyo et al., 2007). However, from a stand of view that the carcinoma cell EMT is a representative event at the invasive front, there is no clear experimental study to investigate the role of the EMT in OSCC progression so far.

8. Conclusion

Investigators have reported numerous molecules related to the stimulation and the suppression of OSCC progression. Among the molecules, E-cadherin is one of most well-studied and powerful suppressor of carcinoma progression. It is frequently downregulated in aggressive OSCCs at the invasive front. Its expression is negatively regulated by many factors, including genetic and epigenetic factors, transcriptional repressors, miRNAs, growth factor signaling, shedding and catenin expression. In addition to loss of E-cadherin expression, carcinoma cells become to express N-cadherin referred as the cadherin switch.

The cadherin switch takes an important part in the EMT, which strongly stimulate aggressive behaviors of carcinoma cells. Since E-cadherin expression is negatively regulated by multi-dimension, re-activation of E-cadherin in carcinoma cells may not be a straightforward strategy to treat OSCC patients. However, unveiling the regulatory mechanism and roles of E-cadherin downregulation and cadherin switch will greatly improve our knowledge on the pathology of OSCCs and contribute to establish the future direction for the patient treatment.

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The Role of EphB4 and EphrinB2 in Head and Neck Squamous Cell Carcinoma

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common cancer arising in the upper aerodigestive tract. It is an epithelial tumor most commonly affecting the oral cavity, hypopharynx, and larynx. HNSCC is the fifth most common cancer worldwide with approximately 900,000 cases yearly worldwide. In the United States, there were approximately 36,000 cases in 2010 and 8,000 deaths. Men are at significantly greater risk with tobacco and alcohol consumption the most important etiologic risk factors.

The main treatment modality for HNSCC has traditionally been surgery and postoperative radiotherapy. However, over the past 30 years, no significant change has been made in treatment strategy with minimal improvement in survival. Overall survival at five years ranges from 70-85% for patients presenting with early-stage disease (stage I and II) to 30-40% for advanced-stage disease (stage III and IV).

2. Pathogenesis of head and neck cancer

Mutations in specific genes and alteration of their expression lead to neoplasia in the head and neck. The development of HNSCC is a multi-step process with sequential mutations in genes responsible for tumor surveillance. A microsatellite analysis of allelic alterations showed that with the accumulation of genetic mutations, one can follow the transformation of cells from simple squamous hyperplasia to severe dysplasia, and, ultimately, invasive squamous cell carcinoma. These changes include mutation of the p53 tumor suppressor, overexpression of epidermal growth factor receptor (EGFR), and inactivation of the cyclin D dependent kinase inhibitor p16. Other changes such as Rb mutation, ras activation, cyclin D amplification, and myc overexpression are less frequent in HNSCC. There is also an alteration in those genes which control DNA repair, proliferation, immortalization, apoptosis, invasion, and angiogenesis in HNSCC.

The p53 gene is believed to be the most frequently mutated tumor suppressor in human cancer. p53 has been implicated in the early pathogenesis of HNSCC, as it controls cell growth through regulation of the cell-cycle and apoptosis. The p53 null keratinocytes possessing an activated ras oncogene proliferate at a higher rate than those expressing the tumor suppressor.

In a study by Kashiwazaki et al in HNSCC, 79% of cancers and 36% of dysplastic lesions were shown to have p53 mutations. Hyperplastic lesions were negative for p53 mutations in this study. A higher incidence of p53 mutations have been detected in invasive carcinomas (75%) than in non-invasive cancers (35%). p53 mutations were not detected in normal mucosal cells. This study also detected sequential mutations of different exons which suggested accumulation of alterations during neoplastic transformation. The incidence of p53 mutations correlated with the degree of dysplasia with significantly higher numbers found in smokers. In agreement with these studies, dysplastic lesions in non-smokers infrequently contained p53 mutations. These results indicate that p53 mutation and inactivation is an early event in head and neck tumorigenesis.

In addition to p53, alterations in the retinoblastoma (Rb) gene are involved in the pathogenesis of HNSCC. The Rb protein is also a tumor suppressor pathway. p16^{INK4A}, a major target of the Rb pathway is inhibited through a variety of pathways including loss of heterozygosity (LOH) of chromosome 9p21, where it is located. LOH of 9p21 is seen in 80% of malignant lesions.

In addition to the p53 and Rb genes, sphingosine kinase (SphK) has been implicated in HNSCC. SphK regulates levels of ceramide, sphingosine, and sphingosine-1-phosphate, influencing cells to enter proliferative states. SphK1, a SphK isozyme is upregulated in HNSCC with overexpression in recurrent and advanced stage tumors. Use of small molecular inhibitors or siRNA's targeting SphK1 sensitizes cells both in in vitro and in vivo studies leads to radiation induced cell death. As a cell cycle regulator overexpressed in HNSCC patients, SphK1 plays a significant role in the pathogenesis of HNSCC.

The Epidermal Growth Factor Receptor (EGFR) is of the most studied biomarkers in HNSCC. EGFR is a receptor tyrosine kinase that effects cell growth, angiogenesis, and invasion. The epidermal growth factor receptor gene encodes a transmembrane receptor for EGF and transforming growth factor (TGF)- α . Ligand binding to the extracellular domain induces receptor dimerization and activation of the cytoplasmic tyrosine kinase. Many epithelial cancers including that of the head and neck overexpress EGFR, its ligands, or both. EGFR has been detected in the basal layer of normal oropharyngeal mucosa. All cells from dysplastic head and neck lesions stain for EGFR as do the majority of carcinomas. Almost all cells in poorly differentiated head and neck tumors were positive for the receptor. Amplification of the EGFR gene has been demonstrated in cultured cells and tissues.

EGFR overexpression may result in constitutive activity of the kinase domain and consequently increase downstream signaling such as that of the mitogen activated protein kinase pathway. The tyrosine kinase activity of the receptor results in autophosphorylation and recruitment of a variety of intracellular signaling proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. This recruitment provides a means of assembling the complexes required for receptor signaling. Proteins such as Grb2 and Shc, which contain SH2 and SH3 domains, mediate interactions with signal transduction proteins linking EGFR with the ras/mitogen activated protein kinase (MAPK) pathway. Ras also interacts with many proteins such as raf and phosphatidylinositol 3-kinase (PI3-K) to simulate downstream effectors such as MEK and ERK. These MAPKs are translocated to the

nucleus where they activate a number of transcription factors which control cellular proliferation, migration, and differentiation.

With devastating effects on communication, swallowing, and most importantly, survival, new biomarkers and targeted therapies are needed to improve detection, treatment, and survival. Potential targeted therapies may be found in factors that regulate angiogenesis. Angiogenesis plays an important role in both tumor growth and metastasis. Tumors are unlikely to grow beyond 3mm without the growth of new vessels. Receptor tyrosine kinases (RTKs) have emerged as important molecules in the regulation of angiogenesis.

Abnormal RTK expression is characteristic of most human cancers. There are three families of receptor tyrosine kinases and their ligands important in vascular development, including the vascular endothelial growth factor receptor (VEGF) family, the angiopoietin family, and the ephrins and the Eph receptors. Of the three receptor tyrosine kinase families above, VEGF is the most extensively studied. VEGF has been shown to be overexpressed in tumor compared to normal cells. VEGF overexpression is associated with a 1.88 fold increased risk of death and is also shown to be associated with lymph node metastasis. This chapter focuses on the expression of EphB4 and EphrinB2 in HNSCC and possible therapeutic applications to reduce tumor burden and improve survival.

3. The Eph receptors and their ligands the ephrins

The Eph receptors (erythropoietin-producing human hepatocellular carcinoma) form the largest family of RTKs. In this group of proteins, there are 15 members divided into EphA and EphB classes. The EphA subclass is tethered to the cell membrane by glycosyl phosphatidylinositol, and the EphB subclass has a transmembrane domain that is followed by a short cytoplasmic region.

Eph receptors have an extracellular domain composed of the ligand-binding globular domain, a cysteine rich region followed by a pair of fibronectin type III repeats. The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues, a protein tyrosine kinase domain, a sterile α -motif (SAM), and a PDZ-domain binding motif.

The ephrins (Eph family receptor interacting proteins) are the ligands for the Eph receptors, with 13 members, also divided into classes A and B. Class B Ephrins have a transmembrane domain and cytoplasmic region with five conserved tyrosine residues and a PDZ domain. EphrinB2 is the exclusive ligand for EphB4. EphB4 is normally expressed on venous endothelial cells while EphrinB2 on arterial endothelial cells. In contrast, the A class ligands have a glycosylphosphatidylinositol membrane anchor.

Eph receptors are activated by binding of clustered, membrane attached ephrins indicating that contact between cells expressing the receptors and cells expressing the ligands is required for Eph activation. A corollary of this is that soluble ligands would act as inhibitors of Eph activation. Ligand binding to the Eph receptor autophosphorylates the juxtamembrane tyrosine residues to acquire full activation. Specificity of the ligand to its receptor is mediated by the N-terminal domain of the receptor. The interactions between the Eph receptors and their ligands form a bi-directional signaling pathway with forward Eph receptor signaling and reverse ephrin signaling (Figure 1).

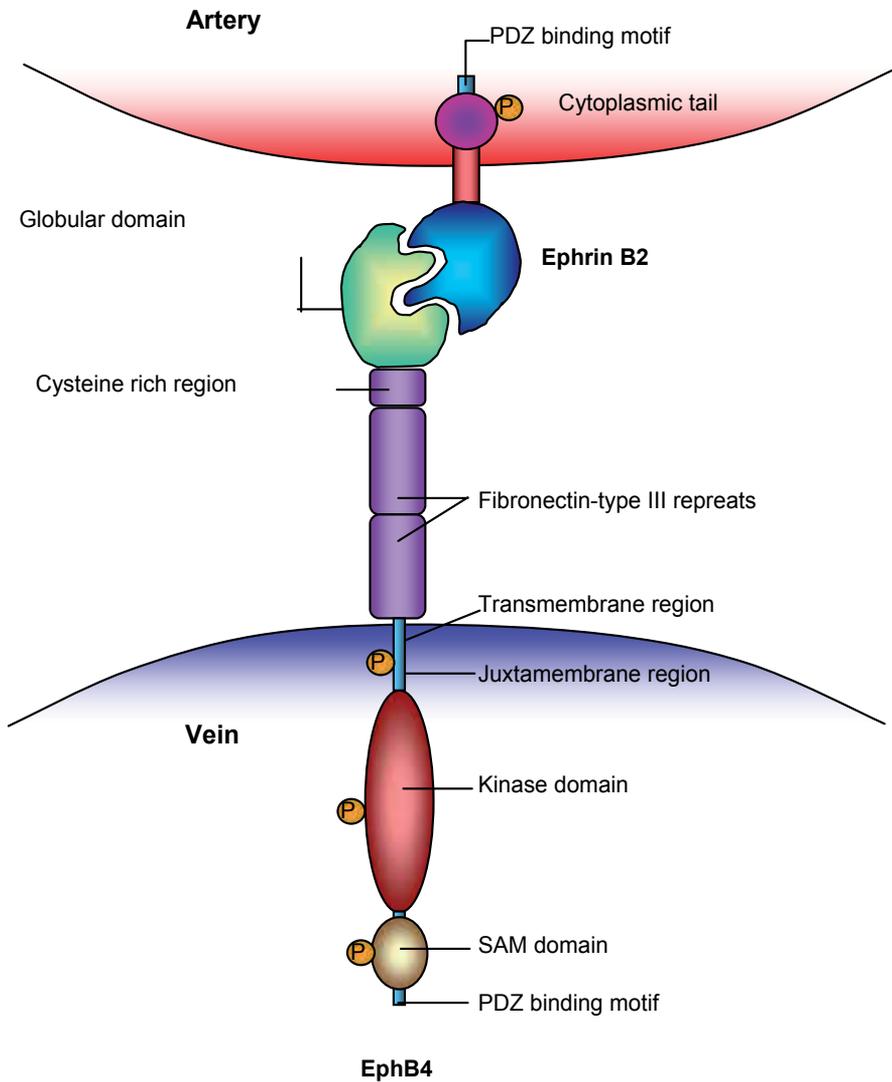


Fig. 1. Bidirectional signaling between EphB4 and EphrinB2.

When activated, EphB4 and EphrinB2 become phosphorylated, forming complexes with other proteins, and affect downstream signaling. Reverse signaling is initiated through recruitment of Src-family kinases followed by phosphorylation of ephrin B proteins. Evidence suggests that the Eph/ephrin interaction influences and is influenced in turn by other signaling pathways. The endothelial-specific receptor Tie-2 can directly phosphorylate ephrin cytoplasmic domains while EphrinB1 is phosphorylated by the PDGF receptor, and inhibits PDGF induced focus formation. Similarly, EphrinB2 inhibits VEGF signaling and the proliferation and migration of endothelial cells. The ephrins have also been shown to couple to GPCRs, such as the chemokine receptor CXCR4, via the PDZ linking proteins and a ternary complex involving the extracellular domains of EphrinB1, EphB2, and the 7-transmembrane GPCR subunit of the NMDA glutamate receptor has also been demonstrated.

The Eph receptor/ephrin system has been shown to play a role in several biologic processes. These processes include embryonic development, cell migration and aggregation, segmentation, pattern recognition, neural development, angiogenesis, vascular network development, and immune regulation. Recently, a role for these proteins has emerged in cancer.

Several studies have demonstrated that the Eph receptor/ephrin system plays a role in tumorigenesis. Dodolet et al and Wimmer-Kleikamp et al have shown involvement of the Eph receptor/ephrin system in angiogenesis, invasion, and tumor metastasis. There is also evidence that elevated expression of the Eph/Ephrin system correlates with increased invasiveness in tumors including malignant melanoma, ovarian carcinoma, breast cancer, kidney carcinoma, neuroblastoma, and prostate cancer. More specifically, elevated EphB4 expression has been shown in hematologic, breast, endometrial, prostate, bladder, ovarian, and colon cancers as well as malignant mesothelioma.

EphB4 activation has been shown to increase proliferation and survival of microvascular endothelial cells through increased phosphatidylinositol 3-kinase activity and phosphorylation of mitogen-activated protein kinase (MAPK) and protein kinase B (Akt). EphB4's involvement in cell migration and invasion is associated with EphB4 induction of MMP2 and MMP9, thus demonstrating a role for EphB4 in tumor metastasis.

4. Expression of EphB4 and EphrinB2 in HNSCC

As demonstrated in many other tumors, EphB4 is overexpressed in HNSCC. Through *in situ* hybridization, western blot analysis, and immunofluorescence of HNSCC tumor samples, EphB4 expression was found to be elevated in tumor tissue compared to normal adjacent tissue. In addition, EphB4 was overexpressed in metastatic lymph nodes (Figure 2). Furthermore, EphB4 overexpression correlated with advanced tumor stage (stage III or IV) and lymph node metastasis with stage III and IV tumors having 2.8 and 5.5-fold overexpression respectively compared to adjacent normal tissue.

Lymph nodes positive with tumor had 7.8-fold higher expression compared to normal adjacent tissue. In contrast, in patients with early-stage disease (stage I or II), EphB4 overexpression was 2.1-fold greater in tumor compared to adjacent normal tissue. Using

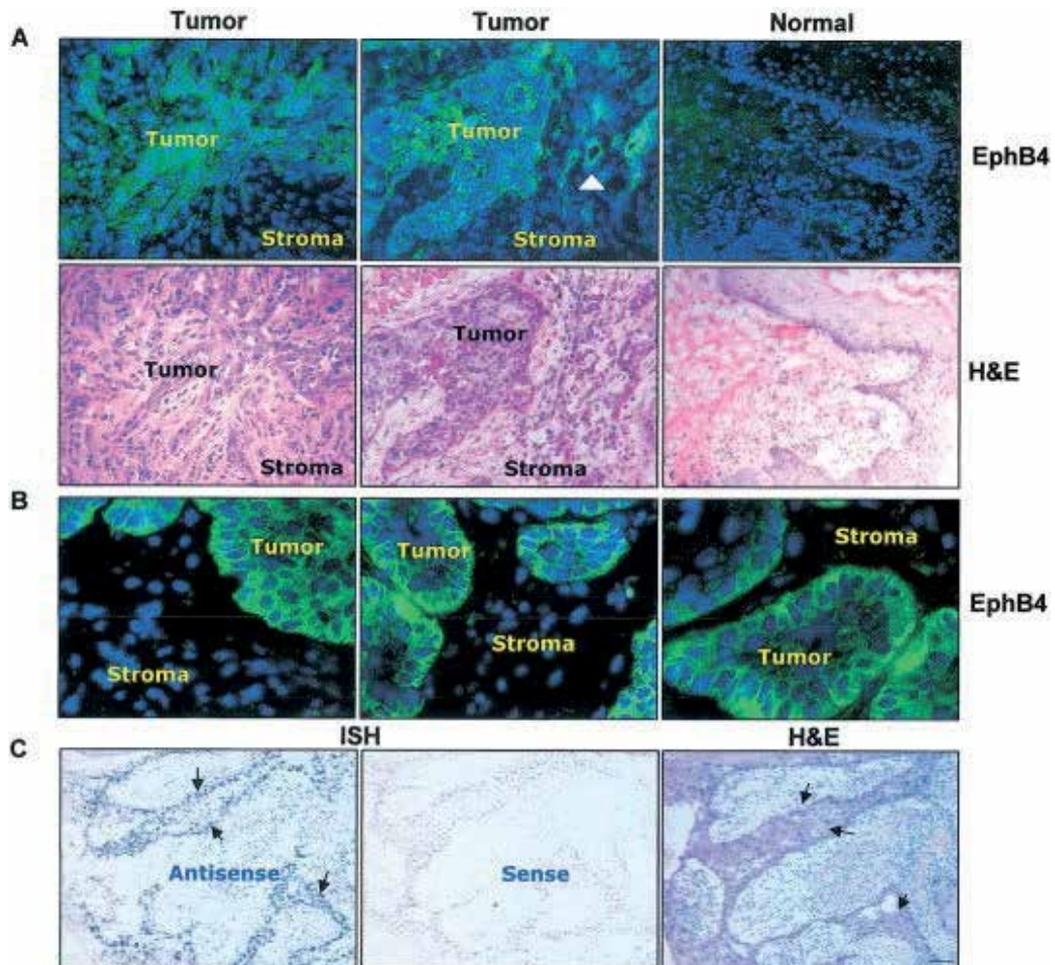


Fig. 2. EphB4 is expressed in HNSCC primary tissues and metastases. (a) Top panel: Immunofluorescence of representative fresh frozen sections of tumors (left and middle panels) or adjacent normal tissue (right panel) stained with EphB4-specific monoclonal antibody and visualized with FITC (green color). Sections were counter-stained with DAPI to identify cell nuclei. Bottom panel: Hematoxylin and Eosin (H&E) staining of the next serial section. Arrowhead in middle panel shows a vessel staining positive for EphB4. (b) Representative high power photomicrographs of tumor sections stained for EphB4 to document tumor cell membrane-specific expression. (c) *In situ* hybridization (ISH) of representative tumor sections with EphB4-specific antisense or sense probe. Arrows show positive signal for mRNA. H&E stain of the next section is shown in the right panel. Arrows indicate regions of the tumor.

quantitative PCR at the EphB4 gene locus, 30% of patients were found to have gene amplification of EphB4 with at least four copies of the gene locus. In a study of 42 patients with HNSCC, EphrinB2 expression was also analyzed with western blot analysis. EphrinB2 was found to be overexpressed in HNSCC tumor samples with an average overexpression of 2.2-fold greater when compared to normal adjacent tissue. Therefore, both EphB4 and EphrinB2 have been shown to be overexpressed in HNSCC.

5. HNSCC risk factors and EphB4 expression

The two main risk factors for HNSCC are alcohol and smoking. Studies have shown that tobacco use can lead to a 20-fold increased risk of HNSCC. Tobacco related substances can alter the genes and growth factors associated with HNSCC and can affect the genomic stability and extracellular environment in HNSCC. The expression of EphB4 in the oral mucosa of smokers without HNSCC was analyzed and results showed no expression of EphB4. However, in patients with HNSCC, EphB4 expression in tumor specimens in nonsmokers was compared to that of patients with a smoking history. There was a significantly increased expression of EphB4 in tumor samples from patients with a smoking history compared to nonsmokers, with a 3.8-fold overexpression of EphB4 in smokers compared to a 2.1 fold overexpression in nonsmokers. Therefore, tobacco-related substances may induce signaling changes that increase and activate EphB4 leading to changes in angiogenesis and tumor growth.

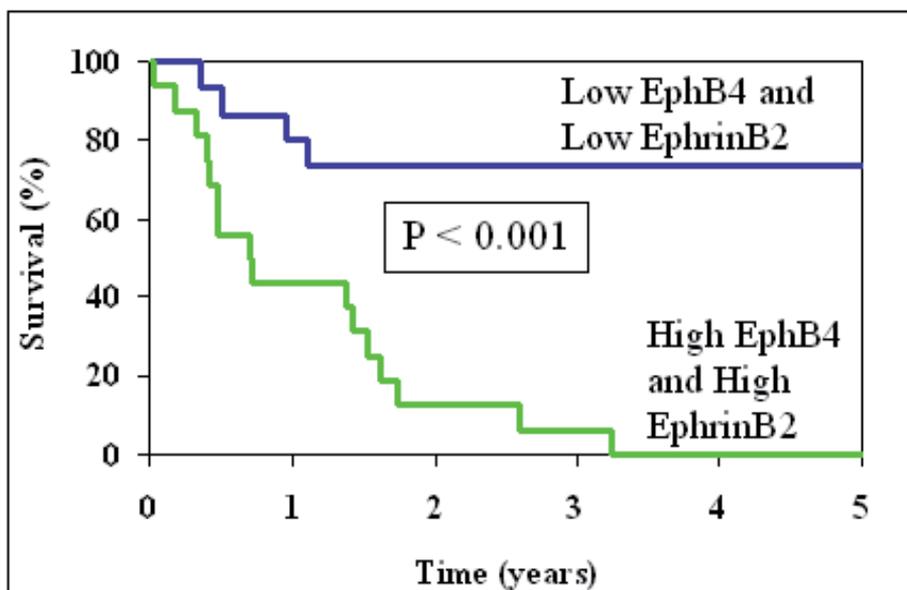


Fig. 3. Kaplan-Meier Curve for Overall Survival in Patients with Elevated Expression of EphB4 and EphrinB2.

In addition to smoking status, the effect of alcohol intake on EphB4 expression was also assessed in patients with HNSCC. Unlike with smoking status, EphB4 expression was not altered by a history of alcohol consumption. This is likely related to differing mechanisms of toxin induced carcinogenesis between alcohol and smoking.

6. EphB4 and EphrinB2 expression and survival

As increasing EphB4/EphrinB2 system expression is associated with advanced tumor stage and lymph node metastasis, the effect of EphB4 and EphrinB2 overexpression on survival was also studied. Patients who had high expression of EphB4 and EphrinB2 were compared to patients with low expression of EphB4 and EphrinB2. Those with high expression of EphB4 had a 5 year survival of 15% compared to 64% in patients with low EphB4 expression. Patients with elevated EphrinB2 expression had a 5 year survival of 9% compared to 79% in patients with low EphrinB2 expression.

In patients with elevated EphB4 and EphrinB2 expression, 5 year survival was 0% compared to 73% in patients with low EphB4 and low EphrinB2 expression (Figure 3). Therefore, elevated EphB4 and EphrinB2 expression is a significant predictor of poorer overall survival, even after adjusting for confounders including age, sex, race, stage, site of tumor, and mode of treatment. As all patients with high EphB4 and EphrinB2 expression died; this suggests a synergistic role between EphB4 and EphrinB2 in HNSCC.

7. Inhibition of EphB4 and tumor cell survival

EphB4 overexpression is associated with a worse overall survival in HNSCC; therefore, its inhibition in tumor cells is an important step to understanding possible therapeutic opportunities. Using small interfering RNA (siRNA) against the EphB4 sequence, which ablates EphB4 expression, results in a significant decrease in HNSCC tumor cells. In the presence of epidermal growth factor (EGF), which has been shown to induce EphB4 expression, inhibition with siRNA against EphB4 also led to a decrease in tumor cells (Figure 4). The population of cells exposed to the siRNA against EphB4 was found to accumulate in the sub-G0 phase, suggestive of apoptosis. EphB4 was shown to provide a survival advantage to cells by inhibition of apoptotic pathways. Inhibition of EphB4 in a murine HNSCC model showed a reduction in tumor growth. Its knockdown leads to an activation of capase-8 and subsequent cell death by apoptosis. Therefore, EphB4 expression in HNSCC provides a survival advantage to tumors cells and is an important potential biomarker whose inhibition may improve survival.

8. Therapeutic applications

Tumor biomarkers provide an opportunity with which one can improve early detection of tumor, monitoring, and treatment, and ultimately improve survival. Recently, several new biomarkers have emerged and are currently being studied for their effectiveness in HNSCC detection, prognosis, and treatment. One such molecule is cetuximab, a monoclonal antibody against the epidermal growth factor receptor. It is one of the most successful targeted therapies in HNSCC with a phase III clinical trial showing cetuximab in

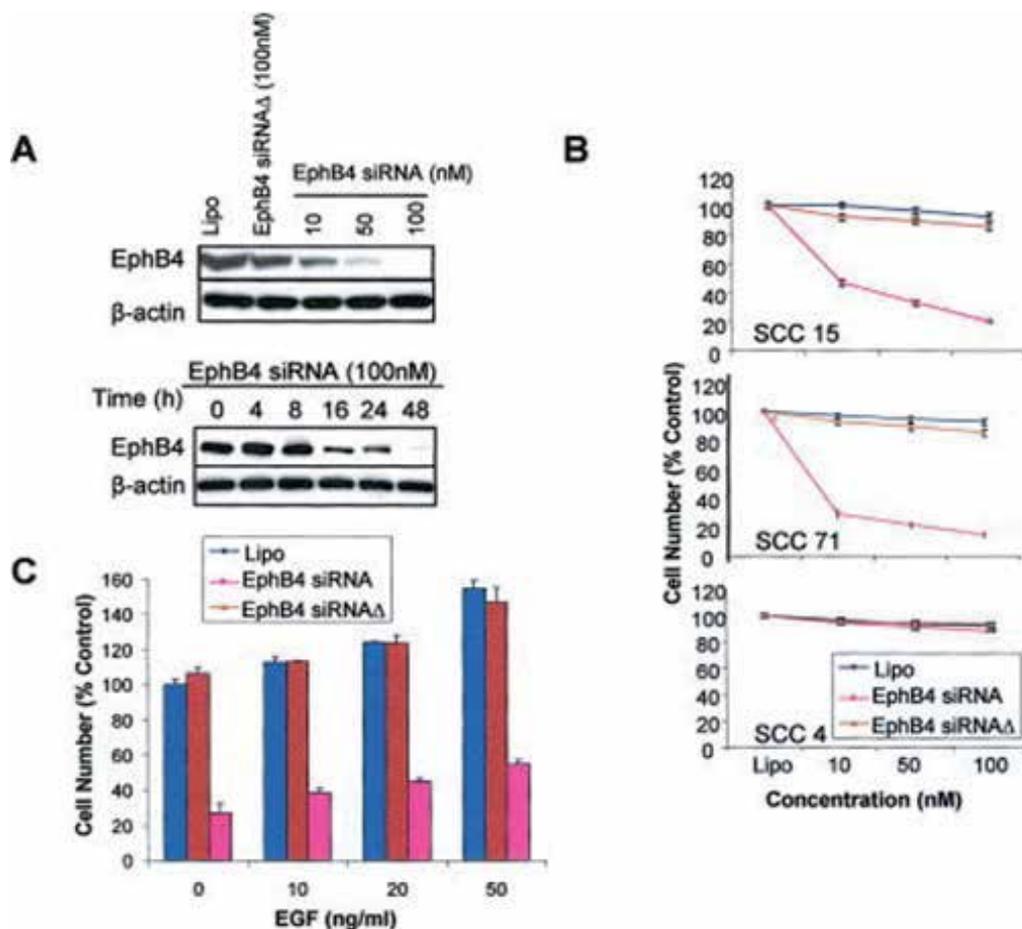


Fig. 4. Ablation of EphB4 in HNSCC cell lines results in reduction in cell numbers and inhibition of tumor cell migration/invasion. (a) Potent EphB4-specific siRNA chosen from their ability to block EphB4 was transfected at various concentrations into SCC-15 cells. A mutant siRNA (EphB4 siRNA Δ) with three base substitutions was used as negative control. Extracts of treated cells were analyzed by Western blotting to detect EphB4 and β -actin (upper panel). SCC-15 cells were transfected with 100 nM EphB4 siRNA and EphB4 expression analyzed at various time points (lower panel). (b) MTT cell number assays of EphB4-positive SCC cell lines (SCC-15 and -71) and an EphB4-negative cell line (SCC-4). Cell number was tested 48 hr following treatment with lipofectamine alone (Lipo), EphB4-specific siRNA (EphB4 siRNA) or mutant siRNA (EphB4 siRNA Δ). Data shown is mean \pm SEM of triplicate samples. (c) MTT cell number assays of SCC-15 cells following treatment with increasing doses of EGF and lipofectamine alone (Lipo), EphB4-specific siRNA (EphB4 siRNA) or mutant siRNA (EphB4 siRNA Δ). Data shown is mean \pm SEM of triplicate samples.

combination with radiotherapy provided an overall survival benefit of an additional 20 months compared to radiation alone. Downstream EGFR signaling activates the MAPK pathway as well as the PI3-K/Akt pathway. Signaling through the PI3-K/Akt pathway ultimately leads to inhibition of the tumor suppressor gene p53. EGFR has been shown to regulate EphB4 expression (Figure 5). EGFR signaling through the Akt pathway induces EphB4. Inhibition of EGFR through antibodies such as cetuximab may also downregulate EphB4 through Akt. Potentially, some of the survival benefit of cetuximab is achieved through EphB4 inhibition.

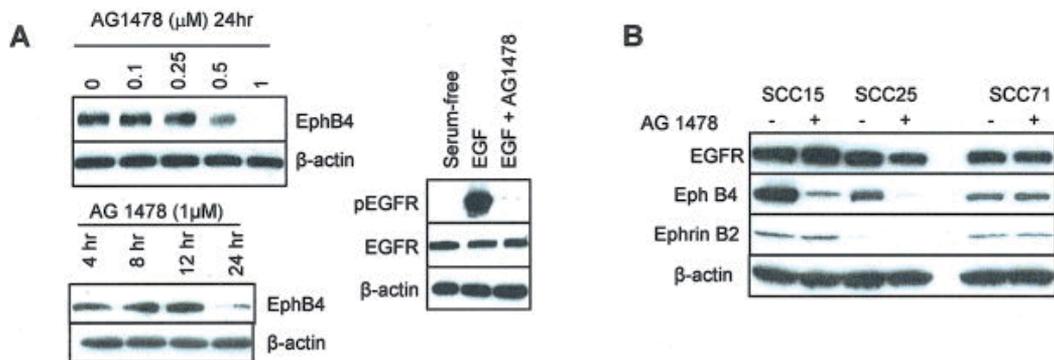


Fig. 5. Regulation of EphB4 expression by EGFR signaling pathway. (a) EGFR kinase inhibitor AG1478 was tested in SCC-15 for optimal dose (left upper panel) and time (left lower panel) for inhibition of EphB4 expression by Western blot of whole cell lysates. Equal loading of protein in each lane is shown by β -actin levels. Inhibition of EGFR activation by EGF in the presence of AG1478 (1 μ M) is shown in right panel. (b) Western blot analysis of SCC-15, -25, and -71 cell lines for regulation of EphB4, EGFR and EphrinB2 in response to AG1478. Serial stripping and probing for various proteins was performed from the same blot.

Monoclonal antibodies to EphB4 have not been applied clinically as of yet, however, their development is crucial to improve survival in HNSCC. Xu et al have developed a humanized version of a mouse monoclonal antibody to EphB4 that binds the human EphB4 receptor. Krasnoperov et al have also developed two anti-ephB4 monoclonal antibodies targeting different EphB4 domains. These antibodies have yet to be tested in humans. Bardelle et al have demonstrated a non-benzodioxole inhibitor of EphB4 that may have applications in vivo but is also yet to be studied further.

In addition to the direct inhibition of the EphB4, several receptor tyrosine kinase inhibitors are being studied. These may also inhibit EphB4 function. Sunitinib, sorafenib, vandetanib, semaxanib, and foretinib are small molecule tyrosine kinase inhibitors currently being studied in phase II clinical trials. Machiels et al reviewed Sunitinib in a phase II clinical trial of 38 HNSCC patients in which it was given as a palliative treatment achieving a disease control rate of 50%. Due to several complications that occurred including bleeding, skin ulceration, and fistulas, they recommended further study of the drug to assess which patients would benefit. In recurrent/metastatic HNSCC and nasopharyngeal carcinoma,

Sorafenib's effect was studied and a response rate of 3.7% was achieved. As a multikinase inhibitor, its effect cannot be attributed only to its anti-angiogenic activity. As a single agent, Semaxanib was also studied in HNSCC, but was discontinued due to several adverse effects and its difficulty with administration. Given the potential improvement in survival with EphB4 inhibition in HNSCC, new therapies targeting EphB4 are essential and further investigation is necessary.

9. Conclusion

Head and neck squamous cell carcinoma is the most common cancer of the head and neck with devastating effects on communication, swallowing, quality of life, and, most importantly, survival. The Eph receptor family and its ligands, the ephrins, specifically EphB4 and EphrinB2, have an important role in many physiologic processes including cell aggregation and migration, angiogenesis, and vascular network development.

EphB4 and its sole ligand EphrinB2 are overexpressed in all HNSCC patients, with EphB4 overexpression correlating with advanced stage disease and lymph node metastasis. In vivo, EphB4 has also been demonstrated to provide a survival advantage to tumor cells, and, its inhibition has been shown to decrease the survival of the HNSCC tumor cells. Furthermore, EphB4/EphrinB2 overexpression is associated with a significantly poorer overall survival. Given that EphB4 and EphrinB2 are overexpressed in HNSCC and that this is associated with worse overall survival, EphB4 and EphrinB2 are potentially useful biomarkers that may provide another target for HNSCC treatment. While there are several investigators examining the therapeutic role of EphB4 inhibition in cancer, there is still a great deal of progress to be made to apply EphB4 and EphrinB2 inhibition in head and neck squamous cell carcinoma treatment.

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Involvement of Squamous Cell Carcinoma Antigen in Invasion and Metastasis of Squamous Cell Carcinoma of Uterine Cervix

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1. Introduction

A tumor-related protein, squamous cell carcinoma antigen (SCCA) was first discovered in uterine cervical squamous cell carcinoma [1], and subsequently has been used as a useful tumor marker for squamous cell carcinoma of various organs [2-4]. Cloning and characterization of SCCA cDNA has revealed that SCCA belongs to serine proteinase inhibitor (serpin) family [5]. Since SCCA is present not only in squamous cell carcinomas but also in normal squamous epithelium, the biological function of SCCA is of great interest. The present paper reviews the current understanding of SCCA, focusing on its biological function in uterine cervical squamous cell carcinoma.

2. Characteristics of SCCA

SCCA consists of more than 10 protein fractions with different isoelectric points, ranging from 5.9 to 6.6, which are roughly divided into two groups: the acidic SCCAs with pIs of less than 6.25 and the neutral SCCAs with pIs of 6.25 or higher [6]. The neutral SCCAs are generally present inside the cell, whereas the acidic SCCAs are often increased in squamous cell carcinomas and is easily secreted by the cell [6]. In 1991, our laboratory reported the cloning of SCCA cDNA, which consist of 1,170 nucleotides coding for 390 amino acids [5]. Schneider et al. also found two SCCA genes (*SCCA1* and *SCCA2*) and these two genes were tandemly arrayed at the human chromosome 18q21.3 locus [7, 8]. The predicted amino acid sequences of *SCCA1* and *SCCA2* are 92% identical and have identical predicted secondary structures, which suggests that *SCCA1* gene encodes the neutral SCCA, while *SCCA2* gene encodes the acidic SCCA [7]. *SCCA1* inhibits the activities of serine proteinases, e.g. chymotrypsin and cysteine proteinases, e.g. cathepsin K, L, S and papain, whereas *SCCA2* inhibits serine proteinases such as cathepsin G and chymase *in vitro* [9-12] (Table 1). For these reasons, *SCCA1* and *SCCA2* are thought to have different biological functions. It is thus of interest to better understand the biological behaviors of SCCAs in normal squamous epithelium and squamous cell carcinomas.

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group of proteinases	proteinases	inhibitors	
		SCCA1	SCCA2
Serine proteinase	chymotrypsin	+	-
	chymase	-	+
	cathepsin G	-	+
	plasmin	-	-
	plasminogen activator	-	-
	thrombin	-	-
	trypsin	-	-
Cysteine proteinase	cathepsin B	-	-
	cathepsin H	-	-
	cathepsin K	+	-
	cathepsin L	+	-
	cathepsin S	+	-
	papain	+	-

Table 1. Inhibitory effects of SCCAs on proteinases.

3. Evaluation of SCCA in clinical practice

Serum SCCA levels have been used as an indicator of a variety of squamous cell carcinomas, including skin cancers, head and neck cancers, esophageal cancers, lung cancers, bladder cancers, epidermoid cancers of the anal canal, and malignant transformation of mature cystic ovarian teratoma [13]. Serum SCCA levels are especially useful for monitoring treatment efficacy, disease progression and recurrence. In general, increased serum SCCA levels reflect disease progression and poor prognosis in squamous cell carcinomas [13]. In advanced cancers, pretreatment serum SCCA levels are associated with clinical stages, tumor sizes, and lymph node involvement. Furthermore, over 6 ng/ml of serum SCCA level shows a significant independent effect on survival and disease-free survival [14]. Even in the early stage of uterine squamous cell carcinomas, elevated serum SCCA levels predict pelvic lymph node involvement and are associated with a poor prognosis [15]. Recently, patients with elevated SCCA2/SCCA1 mRNA ratios in uterine squamous cell carcinoma tissues were found to be at higher risk for recurrence in early stage uterine cervical cancers, suggesting SCCA2 is increased during cervical carcinogenesis [16]. In addition to malignant diseases, several benign and chronic inflammatory skin diseases, such as psoriasis, pemphigus, or eczema are often characterized by elevated SCCA levels [13]. SCCA will be a useful marker for monitoring the status of these diseases not only for malignant diseases but also for non-malignant diseases.

4. Role of SCCA in normal squamous epithelial cells

Human squamous epithelium is composed of four compartments; *stratum germinativum*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. Immunohistochemical staining

shows that SCCA is present in the spinous and granular compartments, but not in the basal and parabasal cells [17] (Fig. 1). SCCA is not present in the epithelial region adjacent to the squamo-columnar junction of the uterine cervix. Interestingly, SCCA levels begin to increase at 18-20 weeks of pregnancy for the first time when the fetal epidermis begins to cornify during the development of human fetal skins [18]. SCCA genes has been found in most of the eutheria (placental mammals), but not in other vertebrates [19]. Furthermore, several eutherian species show heterogeneous patterns of SCCA nucleotides in Southern blot analyses [19]. This suggests that SCCA has had a role in the stratification and differentiation of integuments during evolutionary change.

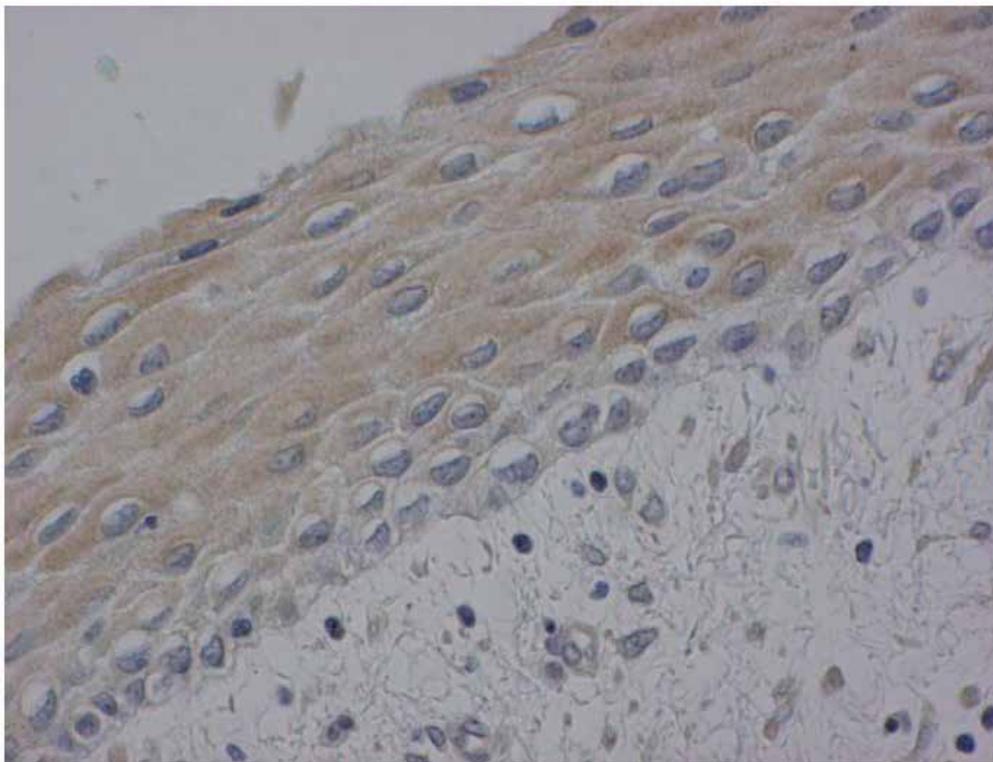


Fig. 1. Immunohistochemistry for SCCA expression in normal cervical squamous epithelium. SCCA is expressed in all epithelial layers except the basal layer (original magnification: X 100).

The stratification and cornification of normal squamous epithelial cells are influenced by extracellular calcium concentrations. Calcium concentrations are low in the parabasal layer but high in the granular layers. Keratinocytes begin to stratify and cornify in the presence of high concentrations of calcium [20]. High concentrations of calcium stimulate the production of neutral SCCA, whereas low concentration of calcium stimulate the production of acidic SCCA [21].

The final stage of differentiation of squamous epithelial cells is modulated by several cysteine proteinases, such as cathepsin L, calpain, and epidermal transglutaminase [20].

SCCA1 inhibits cathepsin L and some of the proteinases in the spinous and granular layers, suggesting that SCCA1 inhibits UV-induced apoptosis of squamous epithelial cells to maintain barrier functions in the squamous epithelium. On the other hand, SCCA2 may act outside of the cells to enhance the cell adhesion system in the parabasal layer [22, 23], suggesting that SCCA2 may play important roles to maintain the structure of the normal squamous epithelium, particularly structure of the thick stratum corneum in mammalian species.

5. Role of SCCA in squamous cell carcinoma of uterine cervix

Anti-tumor therapeutics inhibits the cancer cell proliferation and induce necrotic and apoptotic cell death. However, some cancer cells acquire the ability to resist anti-tumor therapeutics. Thus, proliferation, cell invasion and migration are the most crucial biological events in the progression of cancer.

Recently, much attention has been focused on the role of proteinases and their inhibitors in the malignant behavior of cancer cells. Proteinase inhibitors are thought to suppress the apoptotic process of cancer cells. Apoptosis involves complicated mechanisms with multistep pathways. Some serpins are involved in the apoptotic process. In squamous cell carcinoma tissues, the expression levels of SCCA2 are higher than those in normal squamous epithelial tissues, suggesting that SCCA2 plays a role in suppressing apoptotic cell death [24, 25]. Both SCCA1 and SCCA2 belong to the ov-serpin family, and some of the ov-serpins have been reported to inhibit apoptosis [5]. In fact, SCCA1 inhibits both serine proteinases and cysteine proteinases, and SCCA2 inhibits serine proteinases [9-12]. Although the target proteinases are different, both SCCA1 and SCCA2 inhibit apoptosis. SCCA1 suppresses apoptosis induced by activated natural killer cells, TNF- α , irradiation and anti-tumor agents, while SCCA2 suppresses apoptosis induced by irradiation and TNF- α [26-28]. Both SCCAs suppress the activity of caspase-3 and caspase-9 via down-regulation of p38 MAPK and/or MKK3/MKK6 [27]. These results suggest that SCCAs in tumor cells help to protect cancer cells from apoptotic cell death, both from therapeutic modalities and the immune systems. Proteinase inhibitors are also thought to suppress the invasion and metastasis of cancer cells by inhibiting proteinase activities that disrupt the cell-to-cell adhesion system. In the first step of cancer metastasis, loss of E-cadherin expression causes detachment of cancer cells from the primary tumor lesion. After the detachment from the primary tumor, cancer cells migrate, attach to vessels, and move to other organs through blood and lymph fluid flow. In fact, suppression of SCCA2 expression promoted cell invasion and cell migration with the decreased expression of E-cadherin [29, 30]. Blockage of E-cadherin action suppressed SCCA production in squamous cell carcinoma cell lines [31]. Our immunohistochemical study on cervical squamous cell carcinoma revealed that SCCA2 expression was significantly related with E-cadherin expression and that mixed pattern with loss and positive stained of SCCA2 and E-cadherin in primary lesions was strongly associated with high incidence of lymph node metastasis [32]. These facts strongly suggest that cancer cells with loss of SCCA2 expression, as well as loss of E-cadherin expression, metastasize to other organs including the lymph nodes. In contrast, increased expression of E-cadherin induces the increase of SCCA2 expression through a PI3K - Akt pathway in uterine squamous cell carcinoma cells [33]. These results suggest that the decrease in E-cadherin expression causes cancer cells to detach from the primary tumor, and acquire the

activated E-cadherin – SCCA system, which leads to their aggregation, survival, and growth into metastatic tumors (Fig. 2).

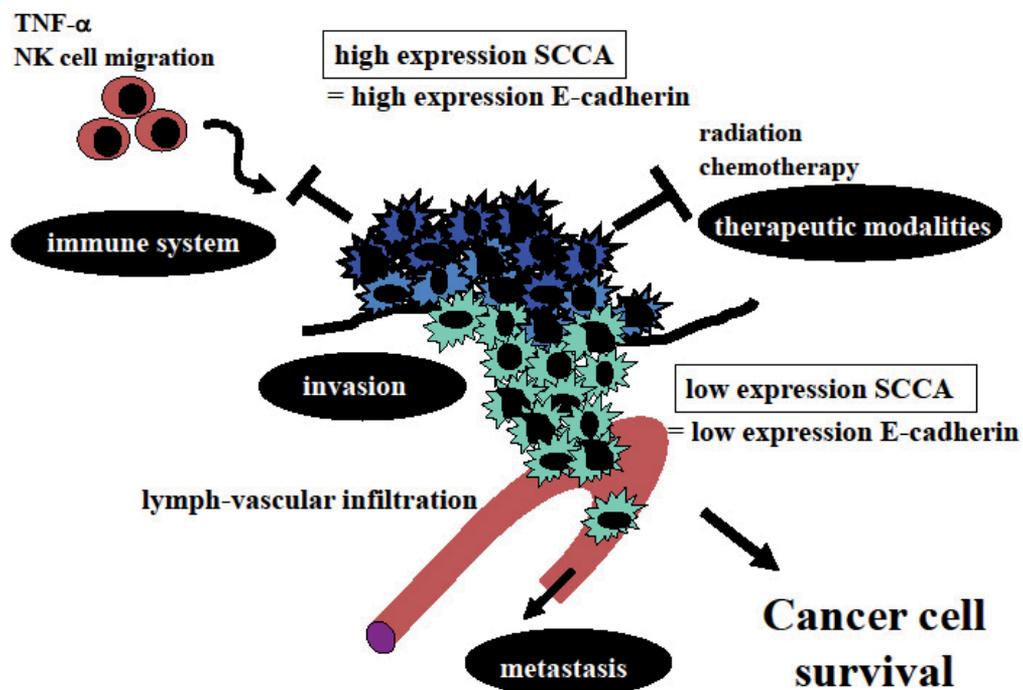


Fig. 2. Possible roles of SCCA in tumor cell survival and metastasis in uterine cervical squamous cell carcinoma. Cancer cells with abnormally high expression of SCCA are resistant to apoptosis induced by the immune system and therapeutic modalities. In contrast, cancer cells with abnormally low expression of SCCA show loss of E-cadherin expression, resulting in detachment from the primary tumor lesion. These cells migrate, attach to the vessels, and metastasize in other organs through blood and lymph fluid flow.

6. Conclusions

SCCAs have been regarded as a useful tumor marker for squamous cell carcinoma in clinical practice. Furthermore, they have some interesting biological functions. SCCAs are regarded as a useful tumor marker for squamous cell carcinoma in clinical practice. In normal squamous epithelium, SCCA may have roles in the stratification, cornification, barrier functions and structure of the epithelium. In squamous cell carcinomas, both SCCA1 and SCCA2 suppress apoptosis by inhibiting serine and cysteine proteinases concerned that function in the apoptotic pathway, resulting in the proliferation of cancer cells. Furthermore, suppression of SCCA2 promoted cancer cell invasion and migration with the decreased expression of E-cadherin, resulting in cancer cell metastases. Thus, SCCA appears to have roles not only in the normal squamous epithelium but also in the squamous cell carcinomas.

7. Conflict of interest

The authors declare no conflict of interest.

8. References

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Part 4

Role of Tumor Microenvironment in Head and Neck Squamous Cell Carcinoma

The Cellular Microenvironment of Head and Neck Squamous Cell Carcinoma

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) tumors function much like organs with support from multiple cell lineages. Tobacco and alcohol abuse are strongly correlated with the disease. Environmental carcinogen exposure introduces genetic alterations not only in the epithelial cells but also in the surrounding stroma contributing to tumor initiation and progression [1]. Factors and cells that do not support tumor growth are commonly downregulated or mitigated in the tumor microenvironment. Several classes of stromal cells that exist in close proximity with HNSCC tumors have been identified. These include fibroblasts, immune cells and cells involved in vascular growth. Each of these cell types are involved in molecular cross-talk with the tumor resulting in tumor progression (Figure 1). Here we highlight each of the major cell types present in the HNSCC tumor microenvironment. Well characterized molecular markers have been used to identify the specific stromal cellular components (Table 1). There continues to be a tremendous need for improved understanding of the role of each of these cell types in tumor growth, dissemination and resistance to therapies. Tumor-associated stroma can support tumor cell proliferation, angiogenesis and invasion making them potential therapeutic targets. Since de novo acquisition of genetic mutations is not common in stromal cells they may be less prone to developing resistance to therapy via genomic instability. The synergistic relationship between stroma and tumor cells suggests that stroma targeted intervention may have a synergistic role in primary cancer therapy. However, fibrosis that follows surgery, chemotherapy and radiotherapy may trigger the release of stromal factors that support recurrence and metastasis. Thus stroma targeted therapies may emerge as important in adjuvant setting.

2. Tumor associated fibroblasts

Fibroblasts are important components of the mesenchymal stroma. Though they appear morphologically similar, fibroblasts show large differences in their functions and patterns of gene expression depending on their anatomical site of origin. Under normal physiological conditions, fibroblasts help maintain the boundary between the epithelial cells and the

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underlying tissue by functioning as a physical barrier. Fibroblasts play a major role in regulating and maintaining extracellular homeostasis. Tissue injury triggers fibroblast activation [2]. Activated fibroblasts are responsible for wound contraction, fibrosis, scarring and regulation of inflammatory reactions. Upon activation, fibroblasts transdifferentiate into motile cells with abundant endoplasmic reticulum, Golgi and α -SMA stress fibers [3]. These α -SMA positive fibroblasts termed myofibroblasts synthesize extracellular matrix components, and several proteinases, growth factors and cytokines. Myofibroblasts have a morphology much like muscle cells with highly contractile microfilaments. Tumors are frequently regarded as wounds that do not heal. HNSCC tumors are frequently associated with desmoplastic stromal myofibroblasts also known as tumor-associated fibroblasts (TAFs) or cancer associated fibroblasts.

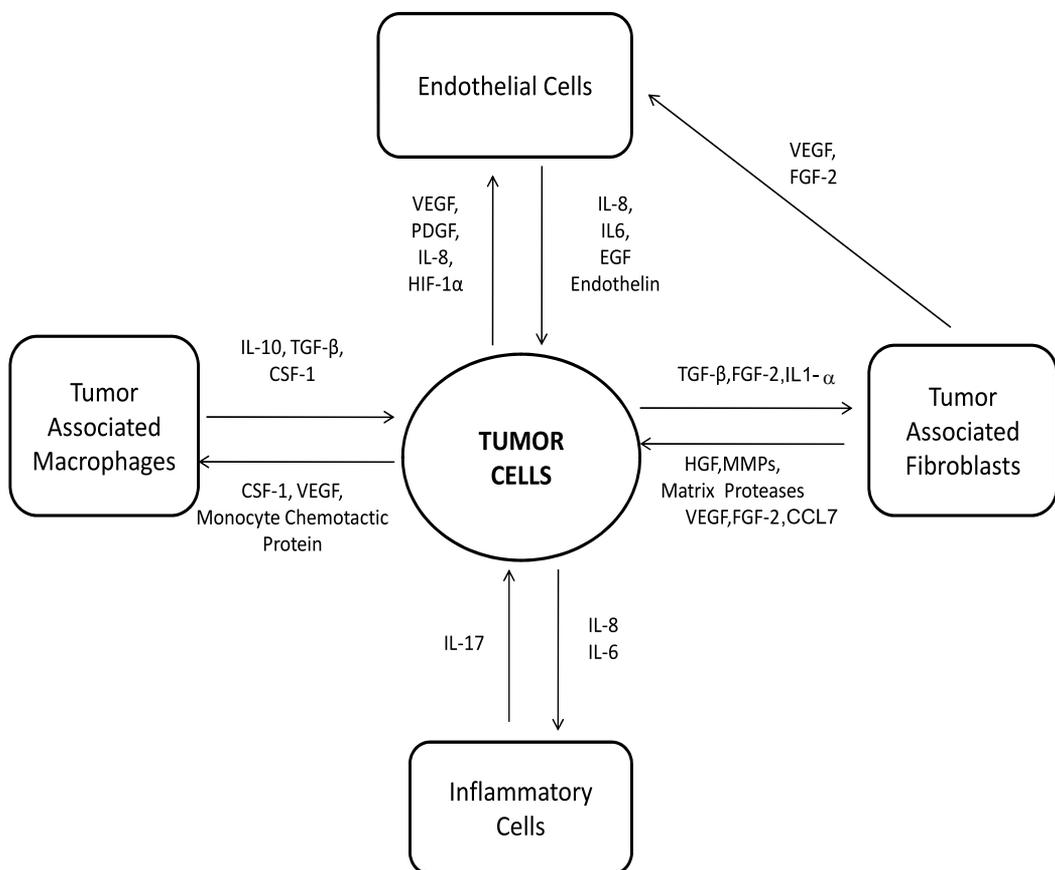


Fig. 1. Cross talk between HNSCC and stromal cellular components. Factors secreted by each cell type that influence target cells have been listed. Abbreviations include; VEGF- Vascular endothelial growth factor, PDGF-Platelet derived growth factor, IL-Interleukin, HIF-Hypoxia Inducible Factor, TGF-Transforming Growth Factor, CSF-Colony Stimulating Factor, EGF-Epithelial growth factor, HGF- Hepatocyte growth factor, MMP-Matrix metalloprotease, FGF- Fibroblast growth factor CCL7- Chemokine Ligand 7(C-C motif).

Cell Type	Molecular Marker
Tumor Associated Fibroblasts	α smooth muscle actin, Vimentin, Fibroblast activating protein
Tumor Associated Macrophages	CD-68, Macrophage inflammatory Protein-3 α
Tumor Infiltrating Lymphocytes	
T cells	CD-3 ⁺
NK cells	CD16/56 ⁺ CD3 ⁻
T helper cells	CD4 ⁺
Endothelial Cells	CD-31,CD34, VEGF-R1,VEGF-R2
Pericytes	α smooth muscle action
Mast Cell	Mast cell tryptase
Lymphatic Endothelial Cell	HIF-1 α , VEGF-C

Table 1. Molecular markers commonly used to identify cellular components of the stroma

TAFs constitute a major cellular component of the tumor associated stroma and are characterized by increased proliferation and aberrant expression of extracellular matrix components. They have been reported to change the phenotype of normal keratinocytes to that resembling squamous cell carcinoma [4]. In other tumor types including prostate, TAFs are reported to play a role in tumor initiation [5-7]. In addition, they play a role in tumor progression as evidenced by a correlation with tumor stage, metastasis and poor prognosis [8]. Although epithelial tumors undergo epithelial-to-mesenchymal transition to acquire a fibroblast-like morphology, they express epithelial cytokeratin markers that are otherwise not expressed on fibroblasts. Epithelial cells with mesenchymal characteristics are not included in these discussions. Several markers have been used to identify TAFs including α -smooth muscle actin, vimentin and fibroblast activating protein [3, 9]. However, these markers show only partially overlapping expression and no single marker consistently labels TAFs. TAFs in the tumor microenvironment are primed to facilitate HNSCC tumor invasion [8]. They are important modulators of tumor growth, invasion and metastasis producing extracellular matrix and angiogenic factors [10-12]. TAFs may be derived not only

from the fibroblasts in the locoregional vicinity of the tumor but also from circulating mesenchymal stem cells [13, 14]. TAFs are detected in both primary and metastatic HNSCC [15]. There are at least 4 possible explanations for the origin of the TAFs at metastatic sites; 1) they are derived from the stroma surrounding the metastatic site, 2) they co-metastasize along with the metastatic tumor cells from the primary tumor site or 3) they arrive at the metastatic site prior to the arrival of the tumor cells creating a metastatic niche permissible to the tumor growth or 4) they are derived from circulating mesenchymal stem cells. HNSCC stroma are either rich in TAFs dispersed throughout the tumor or have low levels of TAFs that are located at the periphery of HNSCC tumors or tumor islands [15]. TAFs are also commonly associated with the invasive margin of the tumor [10]. There is strong evidence to suggest that TAFs use protease and mechanical remodeling of the extracellular matrix to lay tracks along which HNSCC tumor cells invade [16]. They also influence the response of the tumors to conventional therapy [17]. Understanding the tumor microenvironment and the molecular mechanisms responsible for the highly invasive and metastatic nature of HNSCC tumors is vital in developing effective strategies to manage this disease.

TAFs differ in their phenotype, gene expression patterns and functionality from normal oral fibroblasts and normal-dermal fibroblasts derived from non-cancer patients [3, 4]. They are not contact inhibited and have a higher rate of proliferation than normal oral fibroblasts [3]. Somatic mutations such as in the *PTEN* and *TP53* tumor suppressor genes have been reported in TAFs derived from breast carcinoma [18]. There is extensive evidence to demonstrate that cross-talk between TAFs and HNSCC cells results in fibroblast activation and tumor promotion. Release of interleukin-1 α from HNSCC cell lines was reported to induce chemokine receptor ligand CCL7 from TAFs. CCL7 binds to its receptors on HNSCC cells promoting cancer cell migration [19]. Other cytokines released by HNSCC cells under the influence of fibroblasts include interleukin-1 β , -6, TNF- α and TGF- β [20, 21]. Several factors secreted by TAFs facilitate HNSCC invasion including MT1-matrix metalloprotease, [22]. Several aspects of the biology of TAFs suggest that targeting these cells may offer therapeutic benefits. Specific targeting of TAFs with CD8+ T-cells resulted in reduced growth and metastasis of colon and breast tumors [23]. Targeting galectin-1 expressed in TAFs reduced the secretion of monocyte chemoattractant protein-1 mitigating HNSCC migration and metastasis [24]. Several studies have demonstrated that TAFs express the hepatocyte growth factor which promotes the expression of angiogenic factors in HNSCC cells via the oncogenic c-Met receptor and its downstream effectors PI3 kinase and MEK [12, 25, 26].

3. Tumor associated macrophages

Monocytes are recruited by cytokine and chemokine gradients into tissues where further differentiation to macrophages is regulated by environmental signals. In neoplasms tumor associated macrophages (TAMs) represent a major component of the infiltrating leukocytes. The presence of TAMs can be beneficial for the growth of the tumor and sometimes they can cause the death of the tumor cells. For example it has been shown that the amount of TAMs in tumors can be associated with increased neoangiogenesis and worsened survival rates. TAMs also have potential for cytotoxicity towards tumor cells and some reports state an improvement in prognosis in relation to high number of TAMs in tumors. TAMs release various cytokines that cause further influx of monocytes in circulation into tumors. The cytokines released by the TAMs also play an important role in angiogenesis, lymphangiogenesis, invasion and metastasis. TAMs modulate the host immune response

against the tumor cell mass by releasing cytokines, chemokines, and enzymes that influence the function of antigen presenting cells and host lymphocytes.

In normal homeostasis, macrophages play an important role in immune surveillance and wound healing engulfing debris and dying cells. In addition they provide factors necessary for tissue matrix remodeling [27]. Depending on signals in the local microenvironment, macrophages mature into 3 distinct functional phenotypes namely classically, type I and type II activated. Macrophages induced by microbial products are classified as classically activated. Type 1 macrophages are antigen presenting cells capable of producing factors including cytokines, TNF α , reactive oxygen that trigger microbial and tumor cell kill [28]. In contrast, type II macrophages are anti-inflammatory, scavenge cell debris and promote angiogenesis, tissue remodeling and repair [29]. Macrophages develop into type 1 or type 2 phenotypes reversibly in response to changes in the microenvironment [30]. Tumor associated macrophages (TAMs) are typically type II cells reported to promote growth of various tumors including breast, prostate and lung [31]. CD68 stained TAMs are present at higher levels in HNSCC and modulate angiogenesis during tumor progression [32, 33]. Primary HNSCC tumor with high TAM infiltration is a strong predictor of lymph node metastasis, extracellular capsular spread and advanced HNSCC stage [34]. Further, expression of macrophage inflammatory protein-3 α was shown to promote oral SCC migration and invasion [35]. Thus, sufficient evidence exists to indicate that TAMs may be important therapeutic targets.

4. Tumor infiltrating lymphocytes

Pathologic examination of HNSCC demonstrates infiltration of cytotoxic T cell that are functionally inactive. Patients with stage 2 and stage 3 carcinoma of the glottis, tongue and hypo pharynx had significantly increased number of T lymphocytes compared to patients with stage 4 disease [36]. Further, increased T lymphocyte numbers at the margins of HNSCC tumors are associated with favorable prognosis. The T lymphocytes produce lymphokines and play an important role in the proliferation of cytotoxic effector cells, thereby play an important role in the local immune response in squamous cell carcinomas of head and neck.

T lymphocytes are the gatekeepers of autoimmune regulation. Failure of T lymphocytes to recognize and eradicate malignant cells contributes to tumor development [37, 38]. Tumors with a high infiltrate of lymphocytes are associated with improved prognosis [39-41]. HNSCC tumors are influenced by several classes of T lymphocytes including T helper cells, CD3, 4 or -8 positive T cells, natural killer cells, regulatory T cells and myeloid progenitor cells [42-45]. Depending up on the subtype of T cells infiltrating the tumor, the tumor experiences growth promotion or regression [46]. In Table 2 we list the tumor facilitating and tumor-promoting T cells. Myeloid-derived suppressor cells (MDSC) are reported to display antitumor effects or tumor promoting effects depending on the factors secreted in the tumor microenvironment [47]. In addition to modulating immune cells in its vicinity, HNSCC tumors actively recruit and trigger the production of tumor growth promoting interleukin-6 from CD34+ myeloid progenitor cells [48]. CD34+ progenitor cells differentiate into a variety of cell lineages including endothelial cells involved in angiogenesis [49]. Th17-T helper cells are characterized by the high levels of secreted pro-inflammatory cytokine interleukin-17. HNSCC tumor and draining lymph nodes are reported to be infiltrated with Th17 cells that are recruited by the tumor cells [45]. Interestingly, Th17 cells reduce HNSCC proliferation while increasing

angiogenesis. Natural killer cells on the other hand, are capable of profound antitumor effects. A deficiency in invariant CD1d-restricted natural killer cells was reported to predict a poor clinical outcome in HNSCC patients [42]. Dendritic cells and T regulatory (Treg) cells also play a role in HNSCC tumor suppression [43, 50]. Under normal physiological conditions these cells are responsible for antigen presenting and for discriminating between self and non-self-antigens, respectively. HNSCC use multiple mechanisms to evade immune surveillance including downmodulation of immunologic molecules, prevention of immune cell activation, inactivation or by triggering functional deficiencies in immune cells [51-54]. Immune evasion occurs not only in the primary HNSCC tumor but also during the process of metastasis allowing dissemination to regional lymph nodes and distant sites [55]. Reconstitution of immune cells with anti-tumor capabilities may be a feasible adjuvant immunotherapeutic strategy for HNSCC. Not all immune cells with anti-tumor activities are suppressed in HNSCC. Although the mechanisms remain unknown, in human-papillomavirus associated oropharyngeal carcinoma, large numbers of CD3 positive tumor-infiltrating lymphocytes correlate with higher overall survival and a decreased incidence of metastasis [44].

TILs Facilitating Tumor	TILs Antagonistic To Tumor
CD 34 +ve myeloid progenitor Cells	Cytotoxic T Cells
Th 17 cells (increasing angiogenesis)	Helper T Cells
	Natural Killer Cells
	Myeloid derived Suppressor Cells(MDSC)
	Dendritic Cells
	T regulatory Cells

Table 2. Tumor infiltrating lymphocytes that influence HNSCC tumors

5. Endothelial cells

Endothelial cells when stimulated by the growth factors form blood vessels that facilitate tumor growth and dissemination [56, 57]. HNSCC cells directly bind to endothelial cells through adhesion molecules including intercellular cell adhesion molecule-1, CD44, lymphocyte function-associated antigen-3, integrin chains $\alpha 6\beta 1$ and sialyl Lewis (x) [58]. Direct binding of HNSCC to endothelial cells is a prerequisite for penetration of and metastasis through the vasculature. In addition, direct interaction between HNSCC and endothelial cells trigger Notch-1 signaling in endothelial cells promoting capillary tubule formation [59]. Angiogenesis and neo-vascularization are complex processes involving cross-talk between multiple cell lineages in the vicinity [60]. HNSCC tumors and stromal cells secrete cytokines and growth factors including vascular endothelial growth factor

(VEGF), platelet-derived growth factor and interleukin-8 inducing angiogenesis [61]. VEGF plays an important role in endothelial cell survival [62, 63]. On binding to its receptor VEGFR2, VEGF induces expression of Bcl-2 and autocrine signaling through chemokines CXCL1 and CXCL8 facilitating proliferation of endothelial cells and sprouting of microvessels [64]. Global gene expression profiling revealed that HNSCC tumors induce angiogenesis by either expressing high levels of VEGF/fibroblast growth factor (FGF-2) and low levels of interleukin-8/CXCL8 or low levels of VEGF/FGF2 and high levels of interleukin-8/CXCL8 [65]. Tumor hypoxia also plays an important role in the release of angiogenic growth factors. Under hypoxic conditions stabilization of the hypoxia inducible factor 1 α (HIF-1 α) in tumor cells allows transcription of genes involved in angiogenesis and other critical aspects of tumor maintenance [66, 67]. Semaphorin 4D strongly induced by HIF-1 α , binds to plexin B1 on endothelial cells inducing migration [68]. In addition to the formation of new blood vessels, endothelial cells are also involved in a cross talk with squamous cell carcinoma cells resulting in a significant increase in tumor cell survival and migration [69]. Specifically, soluble factors secreted by endothelial cells including interleukin-8, interleukin-6, and epidermal growth factor induce phosphorylation of signal transducers and activators of transcription-3, extracellular-regulated kinase and Akt in HNSCC. Thus molecular targeting of endothelial cells may have tremendous therapeutic potential for HNSCC.

6. Lymphatic cells, pericytes, mast cells and other cells in the tumor microenvironment

In addition to blood vessels, HNSCC are typically infiltrated by lymphatic vessels a process known as lymphangiogenesis. Lymph vessels are typically distributed throughout the tumor as well as in the peritumoral regions [70-72]. Metastasis to regional lymph nodes commonly occurs in HNSCC and correlates with poor prognosis [73, 74]. Due to the paucity of lymphatic endothelial cell line models, most of the data generated pertaining to lymphangiogenesis are based on immunohistochemical analysis of xenograft or patient tissues. HNSCC tumors secrete VEGF-C, a member of the VEGF family, which plays an important role in tumor lymphangiogenesis [75]. Increased tumor lymphatic vessel density correlates with metastasis to lymph nodes in HNSCC [76, 77]. HNSCC tumors expressing high levels of HIF-1 α and VEGF-C had high lymphatic vessel density and increased metastasis [78].

Pericytes are contractile stromal cells closely associated with vascular endothelial cells that stabilize the capillary walls [79-81]. In the absence of pericytes, blood vessels are unstable and undergo regression. [82]. Pericytes influence the proliferation, migration and maturation of endothelial cells [83]. In tumors, pericytes are loosely associated with endothelial cells resulting in increased capillary leakiness [84]. Very few studies have focused on pericytes in HNSCC. Majority of reports use markers such as α -smooth muscle actin to stain pericytes associated with endothelial cells via immunohistochemical analyses [85, 86].

Mast cells are white blood cells that directly associate with endothelial cells stimulating vascular tube formation [87]. As HNSCC progresses, there is an increase in mast cell numbers that correlated with angiogenesis suggesting a role in angiogenesis [88].

The oral cavity and associated areas of the head and neck region are exposed to several microorganisms. Metaproteomic analyses of human salivary microbiota revealed a large number of oral bacteria that are metabolically active and actively engaged in protein synthesis [89]. The role of the human oral microbiome in tumor pathogenesis remains

largely unknown. It is well known that bacteria associated with periodontitis a condition caused by chronic inflammation of the gums, poses an independent risk factor for HNSCC [90]. Human papilloma virus (HPV) infection is a major risk factor for oropharyngeal squamous cell carcinoma [91, 92]. A recent study demonstrated that stromal cells expressing high levels of carbonic anhydrase IX (a sensitive marker for hypoxia) significantly correlated with reduced survival in HPV-negative HNSCC patients [93].

Tumor associated stroma are complex and influence tumor growth in a coordinated manner. Further studies on their contribution to tumor recurrence and new primaries are needed. The identification of promising targets for stroma-directed therapy will pave the way for enhanced anti-tumor effects and improved HNSCC patient survival.

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Role of Connective Tissue Growth Factor (CTGF/CCN2) in Oral Squamous Cell Carcinoma-Induced Bone Destruction

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1. Introduction

Oral squamous cell carcinoma cells in the gingiva frequently invade the maxillary or mandibular bone. The clinical consequences of oral squamous cell carcinoma-induced bone destruction include a worse prognosis, a high morbidity rate, hypercalcemia, and nerve paralysis (Brown, et al., 2002; Hicks, et al., 1997; Shaw, et al., 2004). Patients with oral squamous cell carcinoma and associated bone invasion require bone resection, which has a major influence on their functional outcome. However, the mechanism of bone destruction by oral squamous cell carcinoma remains unresolved.

Localization of tumor cells within the bone leads to the production of tumor-associated factors synthesized either directly by the tumor cell itself or as a result of tumor/stromal interactions. These tumor-associated factors converge on the pre-osteoblast or stromal cell to cause an increase in the level of receptor activator of nuclear factor kappa β ligand (RANKL) and/or a decrease in that of osteoprotegerin (OPG), which ultimately results in the activation and survival of osteoclasts, with osteolytic lesions being the result (Roodman GD & Dougall WC, 2008). Bone destruction then leads to the release of growth factors derived from bone, including transforming growth factor- β (TGF- β), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), and bone morphogenetic proteins (BMPs; (Kayamori, et al., 2010; Roodman GD, 2004; Roodman GD & Dougall WC, 2008; Shibahara, et al., 2005). These factors increase the production of tumor-associated factors or promote tumor growth directly. Thus, tumor cell proliferation and production of tumor-associated factors through the signaling of these pathways are promoted, and the vicious cycle continues.

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN family (Takigawa M, et al., 2003), which consists of 6 members: CCN1 (Cyr61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3; (Katsube K, et al., 2009; Kubota S & Takigawa M, 2007b; Perbal B, 2004), all of which possess an NH₂-terminal signal peptide

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indicative of their secreted-protein nature. CCN proteins share a common molecular structure consisting of an insulin-like growth factor (IGF)-binding protein-like module (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin type-1 repeat (TSP1), and C-terminal module (CT), except in the case of CCN5, which lacks the CT module. The N-terminal and C-terminal halves of the proteins are connected by a hinge region that is not conserved and is particularly sensitive to proteolysis (Dean, et al., 2007; Kireeva, et al., 1996). By means of these modules, the CCN2 protein interacts with a number of extracellular molecules. The IGFBP motif is responsible for binding IGF (Bork P, 1993), albeit studies with CCN2 have demonstrated that the interaction of CCN2 with IGF occurs with a much lower affinity than that of authentic IGFBPs (Yang DH, et al., 1998 Jul.). The VWC motif binds to integrin $\alpha\beta3$ (Perbal B & Takigawa M, 2005) and has been implicated as a binding site for BMP-4 and TGF- β family members, this binding modulating their activity (Abreu JG, et al., 2002). The TSP-1 motif is involved in binding to integrin $\alpha6\beta1$, $\alpha\beta3$ (Perbal B & Takigawa M, 2005), LRP1 and LRP6 (Gao & Brigstock, 2003; Segarini PR, et al., 2001), and VEGF (Inoki I, et al., 2002). Finally, the CT motif binds integrin $\alpha\beta3$ and cell-surface heparan sulfate proteoglycans (HSPGs; (Gao R & Brigstock DR, 2004). These different domains of CCN2 could be responsible for the differential signaling resulting in its various biological activities (Fig. 1).

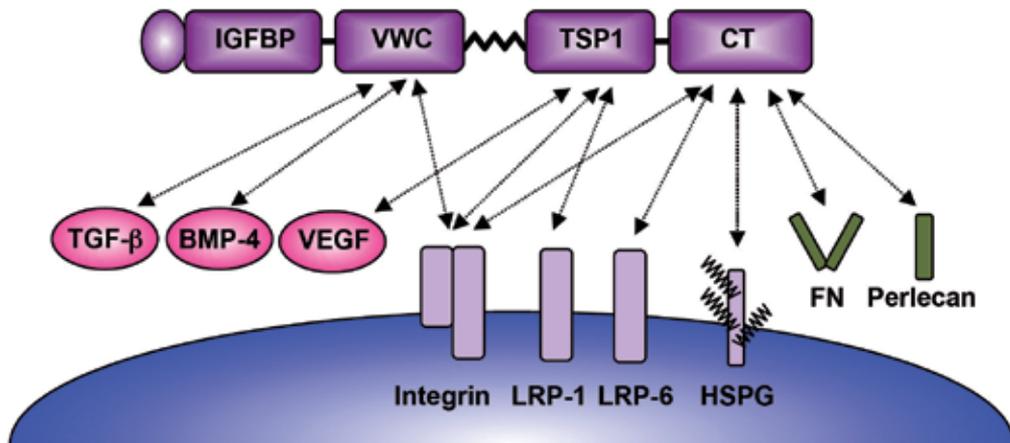


Fig. 1. CCN2-interacting proteins and receptors of CCN2. CCN2 protein interacts with a variety of cell-surface signal-transducing receptors and extracellular ligands, including various integrins, heparan sulfate proteoglycans (HSPG), and LRPs. Receptors and extracellular proteins that interact with 3 of the 4 conserved CCN2 domains are shown.

One of the most prominent functions of CCN2 is its role in cell adhesion. When immobilized on solid surfaces in cell cultures, CCN2 proteins can support the adhesion of most adherent-cell types through integrins and HSPGs and induce adhesive signaling. Adhesion of CCN2 to human skin fibroblasts occurs through $\alpha6\beta1$ -HSPGs and rapidly induces the formation of $\alpha6\beta1$ -containing focal adhesion complexes, activation of focal adhesion kinase (FAK), paxillin, and Rac, as well as reorganization of the actin cytoskeleton and formation of filopodia and lamellipodia (C. C. Chen, et al., 2001). CCN2 can serve as an adaptor for other extracellular matrix proteins to promote cell adhesion, as exemplified by the binding of CCN2 to fibronectin and perlecan (Y. Chen, et al., 2004; Nishida, et al., 2003). In addition to supporting cell adhesion, one of the ubiquitous activities of CCN proteins is the regulation of cell migration.

CCN2 proteins stimulate the migration of many mesenchymal cell types (Babic, et al., 1999; Grzeszkiewicz, et al., 2001; Lin, et al., 2003; Shimo T, et al., 1998; Shimo T, et al., 1999).

CCN2 was originally discovered in, and purified from, the conditioned medium of cultured vein endothelial cells (Bradham, et al., 1991). In 1998, Shimo et al. reported that knockdown of *ccn2* expression results in the suppression of the proliferation and migration of normal vascular endothelial cells (Shimo T, et al., 1998). Subsequently, CCN2 was shown to induce angiogenesis in corneal implants (Babic, et al., 1999) and chick chorioallatonic membranes (Shimo T, et al., 1999). CCN2 also induces chemotaxis (inducing directional cell migration) and chemokinesis (random cell movement) in endothelial cells (Babic, et al., 1999; Babic, et al., 1998; Lin, et al., 2003; Shimo T, et al., 1999). Through direct binding to integrin $\alpha\beta 3$, CCN2 can recapitulate angiogenic events *in vitro* by promoting endothelial cell adhesion, migration, proliferation, and tubule formation (Babic, et al., 1999; Leu, et al., 2002; Lin, et al., 2003; Shimo T, et al., 1999).

CCN2 knockout mice die just after birth due to respiratory failure (Ivkovic S, et al., 2003). This failure is attributed to hypoplasia of the thoracic skeleton and deformity of the oral cavity (palatal cleft and shortened mandible). CCN2 knockout mice also show skeletal dysmorphisms as a result of impaired chondrocyte proliferation and reduced extracellular matrix with altered composition within the hypertrophic chondrocytic zone in the growth plate. Histologically, angiogenesis and formation of tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells, as well as critical protease expression in the growth plate, are impaired and accompanied by defective replacement of cartilage by bone during endochondral ossification (Nakanishi T, et al., 2000; Nishida T, et al., 2000; Shimo T, et al., 2005). These results demonstrate that CCN2 is important for cell proliferation and matrix remodeling during chondrogenesis, and is a key regulator coupling extracellular matrix remodeling to angiogenesis at the growth plate. The biological activities of CCN2 also include the development of Meckel's cartilage (Shimo T, et al., 2004) and tooth germs (Shimo T, et al., 2002).

Next we will summarize research indicating the essential roles of CCN2 and related molecules in the bone destruction caused by cancer.

2. Cancers and CCN2

CCN2 proteins carry out their biological activity through binding and cell surface integrins (Lau LF & Lam SC, 1999), and elevated CCN2 expression has been observed in breast cancers (Xie D, et al., 2001), pancreatic cancers (Wenger C, et al., 1999), melanomas (Kubo M, et al., 1998), chondrosarcomas (Shakunaga T, et al., 2000), and squamous cell carcinomas (Shimo T, et al., 2008). Although CCN2 shows multiple roles in various cancer types, in breast tumor cells CCN2 over-expression has been linked to an increase in tumor size, lymph node metastasis (Chen PS, et al., 2007; Xie D, et al., 2001), and drug resistance through up-regulation of the survival pathway (Wang MY, et al., 2009). CCN2 is also regarded as a central mediator of tumor angiogenic factor in certain malignancies (Kondo S, et al., 2002; Shimo T, et al., 2001a; Shimo T, et al., 2001b). It should be noted that CCN2 is one of the contributors to bone metastasis, as it converts low-metastatic breast cancer cells to high-metastatic ones in collaboration with other factors (Kang Y, et al., 2003; Minn AJ, et al., 2005). Neutralizing antibodies against CCN2 significantly inhibit local tumor growth,

angiogenesis, and osteolysis caused by metastatic human breast cancer cells (Shimo T, et al., 2006). CCN2 and PTHrP are strongly expressed in cancer cells that have invaded the bone matrix, and CCN2 expression is regulated by PTHrP through PKA, PKC, and ERK1/2 MAPK pathways (Shimo T, et al., 2006). Furthermore, the CCN2 gene is significantly over-expressed in overt metastatic tumor cells as compared with its expression in disseminated tumor cells in the bone marrow of breast cancer patients by CT-guided bone metastasis biopsy and bone marrow biopsy (Cawthorn, et al., 2009). Fig. 2A illustrates a representative radiographic pattern of invasive bone destruction observed in a patient with oral squamous cell carcinoma in the mandibular region. In such cases, as shown in Fig. 2B, tumor cells fill the bone marrow space and destroy both the trabecular and cortical bone of the mandible.

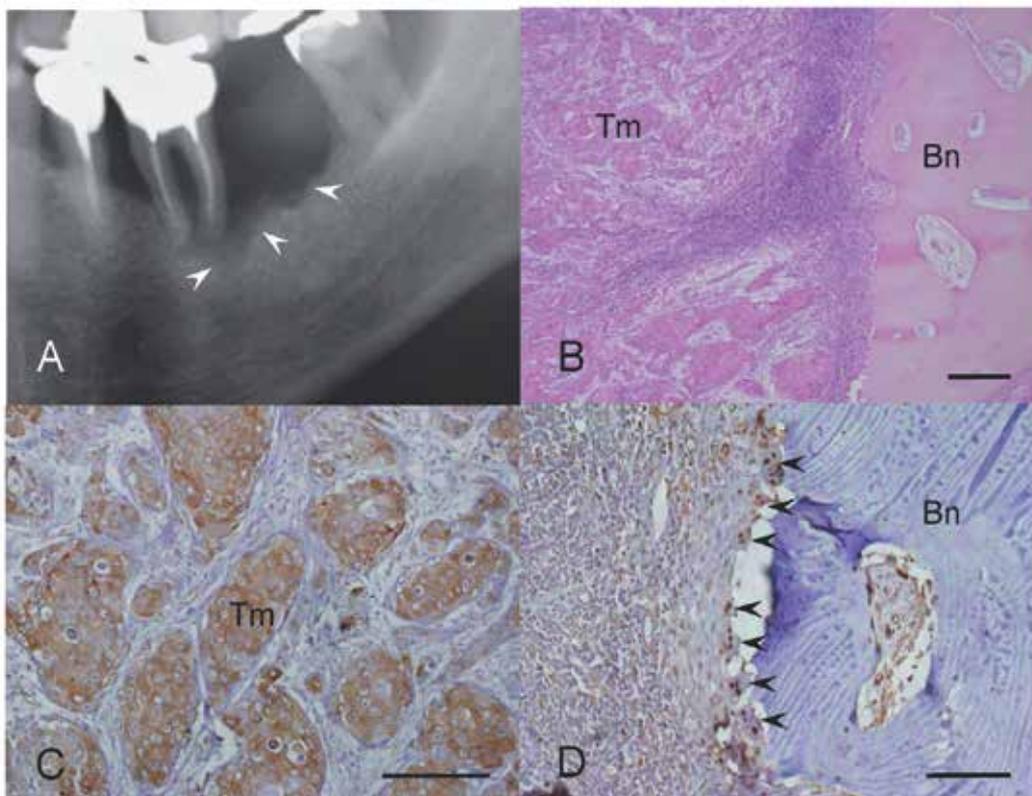


Fig. 2. Radiographic and immunohistochemical analysis of oral squamous cell carcinoma of the mandibular region. (A) Representative radiograph of an invasive oral squamous cell carcinoma in the mandibular region (arrowheads). (B) HE-stained sections of the resected mandible. Tumor tissue (Tm) has invaded into the marrow cavity and replaced the normal cellular elements. Significant loss of trabecular and cortical bone (Bn) has occurred. (C and D) Immunohistochemical staining of CCN2 in a section of invasive tumor (C) and of osteoclasts (D, arrowheads) of the resected mandible. Scale bar = 100 μ m. Bn: Bone, Tm: Tumor. The data were modified from Shimo et al. (Shimo, et al., 2008) (B and D).

CCN2 is abundantly produced by the tumor cells that have invaded the bone matrix (Fig. 2C); and, interestingly, CCN2 is also present in the osteoclasts at the destroyed bone/tumor cell interface (Fig. 2D, arrowheads). Of note, up-regulation of CCN2 in oral squamous cell carcinoma of the mandible is associated with increased bone destruction (Shimo T, et al., 2008). These data suggest that CCN2 can be considered both a diagnostic marker and target for treatment of oral osteolytic mandibular squamous cell carcinoma.

3. Relation between CCN2 and tumor associated factors and signaling

3.1 Insulin-like growth factor (IGF) and CCN2

The insulin-like growth factor (IGF) is the most abundant factor stored in the bone matrix (Hauschka, et al., 1986). The IGF system comprises hormone-like growth factors (IGF-I and II), cell-surface receptors (IGF-IR, IGF-IIR and insulin receptor), circulating binding proteins (IGFBPs), and IGFBP proteases. Activation of IGF-I/IGFR signaling plays an important role in cancer cells, leading to an increase in cell proliferation, invasion/migration, to a decrease in apoptosis, and to resistance to antineoplastic agents, suggesting that IGF/IGFR plays an important role in mammary tumorigenesis (Brady, et al., 2007; Kimura, et al., 2010; Saxena, et al., 2008). A larger family of secreted cysteine-rich proteins, made so by inclusion of the Twisted gastrulation (TSG), IGFBP, and CCN families, is termed TIC (Flint, et al., 2008; Pell, et al., 2005). Interestingly, members of the CCN protein family bind IGF with low affinity (Hwa, et al., 1999). However, there are only a few published reports on the association between IGF system and CCN proteins.

3.2 Parathyroid hormone-related protein (PTHrP) and CCN2

Parathyroid hormone-related protein (PTHrP) has important developmental roles in the embryonic skeleton and other tissues. Detection or increased plasma concentrations of PTHrP have been found in 80% of hypercalcemia patients with solid tumor (Burtis WJ, et al., 1990). When it is produced in excess by cancer cells, it can cause hypercalcemia; and its local production by breast cancer cells has been implicated in the pathogenesis of bone metastasis in that disease. Localized production of PTHrP by cancer cells in such lesions was shown to promote the survival and proliferation of cancer cells and osteolysis in a mouse model (Guise TA, et al., 1996). PTHrP induces both the production of RANKL and down-regulation of OPG production by osteoblasts, thereby stimulating osteoclastogenesis (Horwood NJ, et al., 1998; Lee SK & Lorenzo JA, 1999). Oral squamous cell carcinoma cells provide a suitable microenvironment for osteoclast formation by producing PTHrP (Kayamori, et al., 2010). Knock-down of PTHrP in oral squamous cell carcinoma cell caused decreased osteoclast formation *in vitro*, and suppressed tumor bone invasion *in vivo* (Y. Takayama, et al., 2010). Sections of resected mandibles from patients with invasive oral squamous cell carcinoma showed strong expression of PTHrP in tumor cells and great number of osteoclasts at bone invasion sites (Y. Takayama, et al., 2010). Type I PTH/PTHrP receptor (PTH1R) expression is specifically observed in cancer cells producing PTHrP and CCN2 that have invaded the bone marrow, and PTHrP strongly up-regulates CCN2 in MDA-MB-231 cells *in vitro* (Shimo T, et al., 2006). CCN2 is also critically involved in osteolytic metastasis and is induced by PKA- and PKC-dependent activation of ERK 1/2 signaling by PTHrP (Shimo T, et al., 2006).

3.3 Transforming growth factor- β (TGF- β) and CCN2

TGF- β is by far the second abundant cytokine in bone, and must be considered as a central player in bone turnover (Bonewald LF & Mundy GR, 1990) and potentially able to couple bone resorption with bone formation (Karsdal MA, et al., 2001; Takeshita S, et al., 2000). Restricted to the bone environment, target cells of TGF- β include cancer cells as well as osteoblasts, osteoclasts, their precursors in the bone marrow, and stromal cells (Bonewald LF & Mundy GR, 1990; Karsdal MA, et al., 2001). TGF- β is a pleiotropic cytokine that plays a central role in maintaining epithelial homeostasis. In early carcinogenesis, TGF- β acts as a tumor suppressor by inhibiting cell proliferation (Massague J, et al., 2000; Sun L, 2004). However, several studies showed that primary tumor cells in the late stage can reprogram their response to TGF- β by dysregulation or mutational inactivation of various components of the TGF- β signaling pathway and through cross-interaction with other oncogenic pathways (Nagaraj & Datta, 2010). TGF- β transduces its signal through 2 highly conserved single transmembrane serine/threonine kinase receptors, termed type I (T β RI) and type II (T β RII). T β RII activates T β RI upon formation of a ligand-receptor complex by hyperphosphorylating serine/threonine residues in the GS region of T β RI. Activated T β RI in turn phosphorylates Smad2 and Smad3, which interact with Smad4. Their complex is translocated to the nucleus, where it regulates the transcription of target genes. This signalling cascade initiates broad cellular and noncellular processes including proliferation and differentiation, migration and motility, and deposition of extracellular matrix, as well as induces the production of cytokines contributing to tumorigenesis, metastasis, and angiogenesis (Ge, et al., 2006; Petersen, et al., 2010). Due to its central role in TGF- β signalling, T β RI is emerging as a novel target for the blockade of the tumor-promoting and metastasis activities of the TGF- β pathway (Shinto, et al., 2010). Consequently, the TGF- β signal becomes a bone metastasis-promoting one (Kang Y, et al., 2005; Kominsky SL, et al., 2007; Yin JJ, et al., 1999). T β RI-positive signals are closely associated with destructive invasion of the mandible by oral squamous cell carcinoma cells, and a T β RI-inhibitor greatly reduces oral squamous cell carcinoma cell-induced bone destruction and osteoclast formation both *in vivo* and *in vitro* (Goda, et al., 2010).

TGF- β is one of the most potent inducers of CCN2, promoting CCN2 expression in bone metastatic cancer cells (Kang Y, et al., 2003); and the induction occurs through a complex network of transcriptional interactions requiring Smads, protein kinase C, and ras/MEK/ERK, as well as an Ets-1/transcription enhancer factor-binding element in the CCN2 promoter (Chen Y, et al., 2002; Leask A, et al., 2002; Van Beek JP, et al., 2006). TGF- β released from the bone causes a further increase in the expression of the TGF- β -responsive osteoclast-inducing genes, CCN2, RANKL, and TNF- α in oral squamous cell carcinoma cells, thus establishing a composite positive-feedback cycle of metastasis (Kang Y, et al., 2003; Shimo T, et al., 2006).

3.4 RANKL and CCN2

The RANK and its ligand RANKL signaling pathway play pivotal role in osteoclast-mediated bone resorption in both normal bone remodeling and in pathological conditions, including bone metastasis (Boyle WJ, et al., 2003; Lacey DL, et al., 1998; Simonet WS, et al., 1997). RANK is a transmembrane signaling receptor of the tumor necrosis factor (TNF)

receptor superfamily that is expressed on the surface of osteoclast precursors (Hsu H, et al., 1999; Nakagawa N, et al., 1998). Its cognate ligand, RANKL, is expressed almost exclusively within the bone marrow stromal cell compartment and is up-regulated by most hormones and factors that stimulate bone resorption (Boyle WJ, et al., 2003; Roodman GD & Dougall WC, 2008). The interaction between RANK and RANKL is necessary for osteoclast formation, function, and survival (Kong YY, et al., 1999; Lacey DL, et al., 1998). RANKL (50 ng/ml) stimulates osteoclastogenesis in mouse total bone marrow cells in the presence of 100 ng/ml CCN2 (Shimo T, et al., 2008). Stromal/osteoblastic cells are essential for *in vitro* osteoclastogenesis through cell-to-cell interactions (Kondo Y, et al., 2001). The expression of CCN2 is up-regulated in the cells of mouse macrophage cell line RAW264.7 after treatment with RANKL, and CCN2 synergistically promotes RANKL-induced osteoclast differentiation by interacting with dendritic cell-specific transmembrane protein (DC-STAMP) on the surface of osteoclast-like cells (Nishida, et al., 2011). Therefore, it has been hypothesized that CCN2 may facilitate cell-to-cell signaling by interacting with multiple molecules on the surface of these cells through integrin (Gao R & Brigstock DR, 2004; Hoshijima, et al., 2006), proteoglycans (Nishida, et al., 2003), and growth factors (Inoki I, et al., 2002).

3.5 Endothelin-1 (ET-1) and CCN2

Endothelin-1 (ET-1) is also a key mediator of osteoblastic bone metastasis, which is characteristic of breast and prostate cancers (Nelson JB, et al., 1995; Yin JJ, et al., 2003). Functional inhibition of ET-1 activity by blocking its receptor, ET_A, significantly decreases bone metastasis in an experimental bone metastasis model involving the osteoblastic breast cancer cell line ZR-75-1 (Guise, et al., 2003; Yin JJ, et al., 2003).

CCN2 is one of the secreted factors downstream of ET-1, as determined from microarray analysis of osteoblasts (Clines GA, et al., 2007). ET-1 activates the CCN2 promoter and induces CCN2 expression in cardiomyocyte cells (Recchia AG, et al., 2009). Furthermore, ET-1 induces CCN2 in an additive fashion with TGF- β through an element distinct from the TGF- β response element (Horstmeyer A, et al., 2005; Shi-Wen X, et al., 2008; Xu SW, et al., 2004).

3.6 Integrins and CCN2

Integrins have been shown to be critical in controlling how tumor cells interact with their microenvironment. The integrin $\alpha_v\beta_3$ is a receptor for osteopontin, fibronectin, and vitronectin, which are extracellular matrix proteins important in the bone matrix (Schneider, et al., 2011); and $\alpha_v\beta_3$ has been identified as one of the CCN2 receptors (C. C. Chen, et al., 2001). Bone metastatic cancer cells have a higher expression of $\alpha_v\beta_3$ than their primary tumor (Liapis, et al., 1996), promoting adherence to the bone matrix (S. Takayama, et al., 2005). The over-expression of $\alpha_v\beta_3$ in the tumor cells not only leads to increased tumor cell adhesion, migration, and invasion to bone, but also increases osteoclast recruitment at the tumor and bone interface (Pecheur, et al., 2002; Sloan, et al., 2006). Whereas, $\alpha_5\beta_1$, another signaling receptor mediating CCN2 action, plays a necessary role in the binding of prostate cancer tumor cells to the bone stroma (Van der

Velde-Zimmermann, et al., 1997), and in skeletal metastasis of breast cancer cells (Korah, et al., 2004).

Bone-invading destructive tumor cells enhance osteoclast function and recruitment. $\alpha_v\beta_3$ is the predominant integrin found on osteoclasts, and is responsible for mediating osteoclast-bone recognition (Crippes, et al., 1996; Liapis, et al., 1996; Ross, et al., 1993; Zamboni Zallone, et al., 1989) and subsequent attachment to the bone matrix (Chellaiah, 2006; Ross, et al., 1993). This signaling creates the characteristic resorptive ruffled membrane, as well as regulates OC spreading and the overall organization of the cytoskeleton (Faccio, et al., 2003; McHugh, et al., 2000).

3.7 Wnt signaling and CCN2

The Wnt signaling pathways are initiated by a combination of ligands and receptors formed from among 19 secreted Wnt ligands, 10 Frizzled receptors, with the involvement of the co-receptor LRP5/6, which is lipoprotein receptor-related protein 5/6. These ligand-receptor interactions then lead to the activation of multiple intermediate Wnt effectors including β -catenin, c-Jun-NH₂-kinase, and calcium-channel regulators. The accumulation of β -catenin in the cytoplasm and its translocation to the nucleus represent the hallmark of the activated canonical Wnt pathway. In the nucleus, β -catenin forms a complex with lymphocyte enhancer factor/T-cell factor family of transcription factors to activate many oncogenes, such as c-Myc, cyclin D1, metalloproteinases, c-Met, etc (Fuerer, et al., 2008; Rubin, et al., 2010).

The Wnt/ β -catenin signaling pathway is an important target for eliminating cancer stem cell in head and neck squamous cell carcinomas (Song, et al., 2010). The importance of paracrine Wnt signaling in bone metastasis was first revealed in multiple myeloma (Tian, et al., 2003), a plasma cell leukemia that causes severe osteolytic bone disease. The results revealed that one of the tumor-secreted factors responsible for the enhanced osteolysis is the Wnt-inhibitor DKK-1 (Tian, et al., 2003). In prostate cancer cells, high DKK-1 expression is correlated with osteolytic disease, consistent with the findings in multiple myeloma; whereas low DKK-1 expression is associated with osteosclerotic bone metastases (Schwaninger, et al., 2007). Functional inhibition of Wnt signaling by DKK-1 over-expression in prostate cancer cells favors the formation of osteolytic bone metastases (Hall, et al., 2005).

Si et al. (Si, et al., 2006) observed a significant up-regulation of CCN2 gene expression in mesenchymal stem cells that had been stimulated by Wnt3A. Osteoblasts and stromal cells of transgenic mice that over-express CCN2 display reduced Wnt- β -catenin signaling (Smerdel-Ramoya, et al., 2008). Over-expression of CCN2 in esophageal squamous carcinoma cells results in the accumulation and nuclear translocation of β -catenin leading to activation of TCF-LEF signaling and up-regulation of c-myc and cyclin D1 (Deng, et al., 2007).

4. Role of CCN2 in tumor/bone microenvironment

In the bone marrow microenvironment affected by a tumor, substantial bone marrow angiogenesis is present compared with that in healthy persons (Chavez-Macgregor, et al., 2005). CCN2, the best-characterized factor in its family is known to promote the proliferation

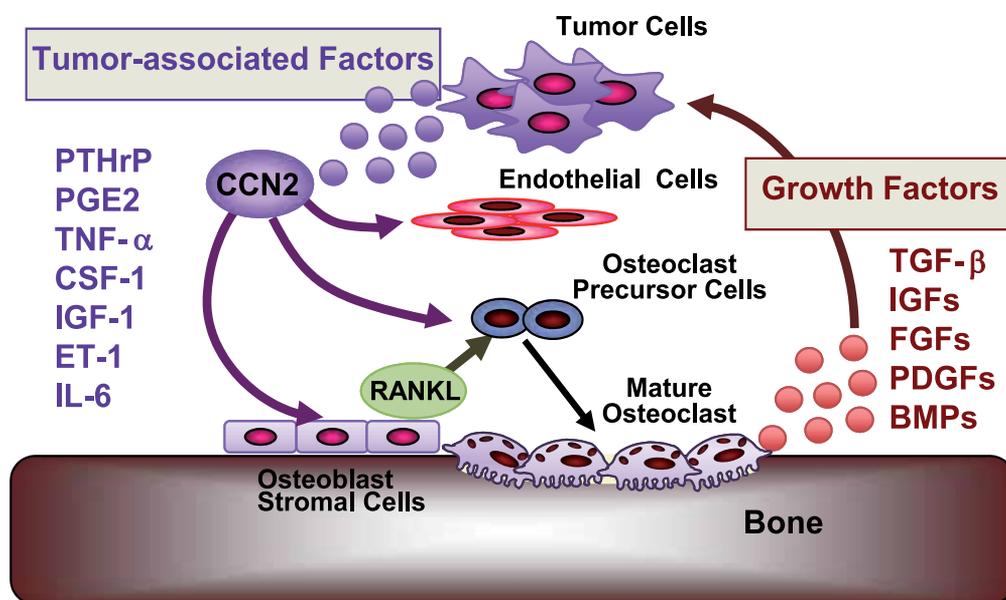


Fig. 3. Role of CCN2 in tumor-induced bone destruction. The cross-talk between tumor cells and osteoclasts is not direct, but involves molecular and cellular intermediates; e.g., tumor cells secrete parathyroid-hormone-related peptide (PTHrP), which is the primary stimulator of osteoblast production of RANKL (Roodman GD, 2004). PTHrP induces CCN2 in tumor cells (Shimo T, et al., 2008); on the other hand, PTHrP both up-regulates the production of RANKL and down-regulates OPG production by osteoblasts, thereby stimulating osteoclastogenesis (Horwood NJ, et al., 1998). Other factors produced and secreted by tumor cells (TNF- α , TGF- β , macrophage colony-stimulating factor (M-CSF), IL-6, and prostaglandin E2) also increase the expression of RANKL. The increased expression of RANKL in the tumor environment leads to increased formation, activation, and survival of osteoclasts, which cells cooperate with these tumor-induced growth factors (CCN2, TNF- α , TGF- β , and M-CSF), resulting in osteolytic lesions. Osteolysis then leads to the release of growth factors derived from bone, including transforming growth factor- β (TGF- β), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), and bone morphogenetic proteins (BMPs). These factors increase the production of PTHrP and CCN2 or promote tumor growth directly and cause neovascularization. Bone destruction increases local extracellular calcium (Ca^{2+}) concentrations, which have also been shown to promote tumor growth and the production of PTHrP (Roodman GD, 2004). Thus, tumor cell proliferation and production of tumor-associated factors through the signaling of positive-feedback pathways is promoted, thus giving rise to a “vicious cycle.” The Scheme was modified from (Shimo T, et al., 2011).

and differentiation of not only vascular endothelial cells but also fibroblasts and osteoblasts (C. C. Chen, et al., 2001; Nishida T, et al., 2000; Shimo T, et al., 1998; Shimo T, et al., 1999). CCN2 protein is able to interact with multiple molecules in the bone microenvironment, which interaction results in the modulation of the extracellular molecular network therein. The angiogenic effect of CCN2 is the result of the interaction of it with adhesion molecules (Gao R & Brigstock DR, 2004), cell-surface signal transducing receptors (Wahab, et al., 2005), proteoglycans (Nishida, et al., 2003), and growth factors (Inoki I, et al., 2002).

Bone-derived growth factors, such as TGF- β , FGFs, PDGFs, BMPs, and IGF-1, are activated and released into the bone microenvironment. Elevated TGF- β does not appear to affect tumor growth, but rather leads to the production of PTHrP (Guise TA, 2000) and CCN2 (Kang Y, et al., 2003; Shimo T, et al., 2006) in cancer cells, thus establishing a continuously destructive cycle termed the "vicious cycle" through up-regulation of RANKL and accelerated bone resorption. Of note, CCN2 is known to interact with these growth factors (Abreu JG, et al., 2002; Inoki I, et al., 2002) or regulate the gene expression of some of them (Shimo T, et al., 2001b). As a result, CCN2 may be anticipated to modulate the effects of these growth factors on the osteoblast-induced RANKL and OPG expression, osteoclast formation or osteoclast activation in regions affected by bone metastasis (Fig. 3). The other critical function of CCN2 is exerted by its interaction with extracellular matrix molecules and cell-adhesion molecules. By interacting with integrins, functions and other proteins and proteoglycans, CCN2 may promote adhesion and migration of osteoclast precursor cells and stimulate osteoclast formation and activation (Shimo T, et al., 2008). CCN2 not only promotes the expression of DC-STAMP, which plays an important role in cell-cell fusion, but also interacts with this molecule to promote osteoclast differentiation (Nishida, et al., 2011). CCN2 may thus be an integrator/modulator of extracellular information and appears to allow the establishment and progression of tumor angiogenesis and bone destruction within the skeleton (Kubota S & Takigawa M, 2007a; Sasaki A, et al., 1998; Sasaki A, et al., 2003).

5. Conclusions

The initiation of osteoclastogenesis and angiogenesis is the most fundamental step leading to tumor-induced bone destruction. From a clinical point of view, osteoclast formation and angiogenesis would be the major targets of therapeutic drugs for tumor bone metastasis. The major modulator of these processes, referring to osteoclast formation and angiogenesis has been shown to be the CCN2 molecule, which is thus now regarded as a potential target of anti-osteoclastogenic and angiogenic therapy (Aikawa, et al., 2006; Shimo T, et al., 2006). These findings strongly suggest that CCN2 may be a suitable molecular target for therapy of advanced oral squamous cell carcinoma.

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Role of Inflammation in Oral Squamous Cell Carcinoma

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1. Introduction

The most common malignant oral disease is oral squamous cell carcinoma (OSCC), and most of the time this term is used synonymously with oral cancer (1). Oral cancer is a serious and growing problem in many parts of the world. When grouped together with pharyngeal cancers, it is the sixth most common cancer globally (2). There is a wide geographic variation in the incidence of this cancer. This usually depends on the culture, life style factors and level of country development (1). In the South and Southeast Asia, parts of Western (e.g. France) and Eastern Europe, parts of Latin America and the Caribbean and in the Pacific regions, oral cancer rates are higher than the other parts of the world (3). The major risk factors of the disease are cigarette smoking (4), alcohol abuse (5), and viral infections such as HPV (6). These risk factors are primarily based on life style but do not adequately explain the increasing incidence of this cancer among the young population (7) and non-smoking females (8). In addition, genetic susceptibility may play an important role (9, 10, 11). Epidemiological studies have shown that chronic inflammation is associated with various types of cancer (12). It is estimated that 15-20% of all deaths from cancer worldwide are linked to infections and inflammatory responses (13). In the last two decades most chronic diseases, including cancer, have been associated with dysregulated inflammatory response. The identification of transcript factors such as NF- κ B, AP-1 and STAT3 and their gene products such as tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), chemokines, cyclooxygenase-2 (COX-2), 5 lipooxygenase, matrix metalloproteases (MMP) and vascular endothelial growth factor (VEGF), adhesion molecules and others has provided the molecular basis for the role of inflammation in cancer. These inflammatory pathways are activated by tobacco, stress, dietary agents, obesity, alcohol, infectious agents, irradiation, and environmental stimuli, which, combined, account for as much as 95% of all cancers (14).

2. Inflammation and cancer

2.1 A short overview of inflammation

Inflammation is a crucial, complex host defense against biologic, chemical, physical, and endogenous irritants. The contribution of inflammation to physiological and pathological processes such as wound healing and infection needs to be understood for a better understanding of the role of inflammation in cancer formation. When tissues are injured, a

multifactorial network of chemical signals initiates and maintains a host response designed to heal the afflicted tissue. The response includes activation and directed migration of leukocytes (neutrophils, monocytes and eosinophils) from the venous system to sites of damage. Neutrophils are thought to coordinate recruitment of these inflammatory cells to sites of tissue injury and to the provisional extracellular matrix (ECM). This is a four-step mechanism: first come selectins that include adhesion molecules (L- P-, and E-selectin) that facilitate rolling along the vascular endothelium; signals are then generated that activate and upregulate leukocyte integrins mediated by cytokines and leukocyte-activating molecules; neutrophils on the surface of the vascular endothelium are immobilized by means of tight adhesion through $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins binding to endothelial vascular cell-adhesion molecule-1 (VCAM-1) and MadCAM-1, respectively; this brings about transmigration through the endothelium to sites of injury and is presumably facilitated by extracellular proteases, such as matrix metalloproteinases (MMPs) (15).

Cellular components

Platelet activation and aggregation, in addition to accelerating coagulation, provide a bolus of secreted proteins and α -granule contents to the immediate area, all of which help initiate and accelerate the inflammatory response by the host. Examples of such secreted proteins include arachidonic acid metabolites, heparin, serotonin, thrombin, coagulation factors (factor V), adhesive proteins (fibrinogen and von Willebrand factor), plasma proteins (immunoglobulin- γ and albumin), cell growth factors (platelet-derived growth factor (PDGF), platelet-derived angiogenesis factor, transforming growth factor- α (TGF- α), TGF- β and basic fibroblast growth factor (bFGF)), enzymes (heparinase and factor XIII) and protease inhibitors (plasminogen activator inhibitor-1, $\alpha 2$ -macroglobulin and $\alpha 2$ -antiplasmin). Following platelet-induced hemostasis and release of TGF- $\beta 1$ and PDGF, formation of granulation tissue is facilitated by chemotaxis of neutrophils, monocytes, fibroblasts and myofibroblasts, as well as synthesis of new extracellular matrix (ECM) and neoangiogenesis.

Neutrophils produce cytokines/chemokines required for effector cell recruitment, activation and response (16). These phagocytic cells initiate wound healing by serving as a source of early-response pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) (17), and interleukin (IL)-1 α and IL-1 β (18). These cytokines mediate leukocyte adherence to the vascular endothelium, restricting leukocytes to areas of repair, and initiate repair by inducing expression of matrix metalloproteinases (MMPs) and keratinocyte growth factor (KGF/FGF-7) by fibroblasts (19).

Mononuclear phagocytes migrate from the venous system to the site of tissue injury, in response to tissue injury. Chemotactic factors, including PF-4, TGF- β , PDGF, chemokines (monocyte chemoattractant protein-1, -2 and -3 (MCP-1/CCL2, MCP-2/CCL8 and MCP-3/CCL7), macrophage inflammatory protein-1 α and -1 β (MIP-1 α /CCL3 and MIP-1 β /CCL4), and the cytokines IL-1 β and TNF- α , guide them to the site. Deployment of monocytes/macrophages to the site of injury causes the number of neutrophils to decline as they are phagocyted by macrophages. Once present, however, they differentiate into mature macrophages or immature dendritic cells. After activation, macrophages are the main source of growth factors and cytokines (TGF- $\beta 1$, PDGF, bFGF, TGF- α , insulin-like growth factor (IGF)-I and -II, TNF- α and IL-1) that modulate tissue repair. Cells in their local microenvironment (e.g., endothelial, epithelial, mesenchymal or neuroendocrine cells) are

profoundly affected by macrophage products (20, 21). Following their activation, mast cells are full of stored and newly synthesized inflammatory mediators. This cell type synthesizes and stores histamine, cytokines and proteases complexed to highly sulphated proteoglycans within granules, as well as producing, lipid mediators and cytokines upon stimulation. Once activated by complement or by the binding of antigens to immunoglobulin E (IgE) bound to high-affinity IgE receptors (FcεRI), mast cells degranulate, releasing mediators including heparin, heparinase, histamine, MMPs and serine proteases, and various polypeptide growth factors, including bFGF and vascular endothelial growth factor. These function both in the early initiation phase of inflammation (e.g. vascular reaction and exudation), and in the late phase where leukocyte accumulation and wound healing takes place (15).

Chemotactic cytokines

Chemokines represent the largest family of cytokines (~41 human members), forming a complex network for the chemotactic activation of all leukocytes. Chemokine receptors, members of the seven-transmembrane-spanning G-protein-coupled receptors, vary by cell type and degree of cell activation (22). There is considerable redundancy in chemokine-receptor interaction, as many ligands bind to different receptors.

The composition of chemokines produced at sites of tissue wounding not only recruits downstream effector cells, but also dictates the natural evolution of immune reactivity. For example, MCP-1/CCL2, a potent chemotactic protein for monocytes and lymphocytes, simultaneously induces expression of lymphocyte-derived IL-4 in response to antigen challenge while decreasing expression of IL-12 (23). The net effect of this alteration facilitates a switch from a TH1-type to a TH2-type inflammatory response (15).

Tissue repair

In response to wounding, fibroblasts migrate into the wound bed and initially secrete collagen type III, which is later replaced by collagen type I. Synthesis and deposition of these collagens by fibroblasts is stimulated by factors including TGF-β1, -β2 and -β3, PDGF, IL-1α, -1β and -4, and mast cell tryptase. Once sufficient collagen has been generated, its synthesis stops; thus, during wound repair, production as well as degradation of collagens is under precise spatial and temporal control.

The final phase of the healing process is re-epithelialization and migration of epithelial cells across this amalgam. This is a process that requires both dissolution of the fibrin clot and degradation of the underlying dermal collagen. Epithelial cells at the leading edge of the wound express the uPA receptor, which is important for focal activation of uPA and the collagenolytic enzymes of the MMP family. In the absence of the fibrinolytic enzyme plasmin, derived from plasminogen after activation by uPA and tissue-PA, re-epithelialization is dramatically delayed (24).

The profile of cytokine/chemokines persisting at an inflammatory site is important in the development of chronic disease. The pro-inflammatory cytokine TNF-α (tumor necrosis factor-α) controls inflammatory cell populations and also mediates many of the other aspects of the inflammatory process. In addition, TGF-β1 is important, because it influences the processes of inflammation and repair in both a positive and negative manner. The key

idea is that normal inflammation – i.e., inflammation associated with wound healing – is usually self-limiting; however, dysregulation of any of the converging factors can lead to abnormalities and ultimately, pathogenesis. This seems to be the case during neoplastic progression (15).

3. OSCC and inflammation

Pathologists have known for more than 100 years that almost all tumors are accompanied by inflammatory cells. At present, there is almost unanimous agreement about the causes. The functional association dates back to Virchow, who in 1863 hypothesized that cancer arises in sites of inflammation (12). Today it is accepted that chronic inflammation resulting from low grade, persistent chemical, bacterial, viral agents predisposes the formation of the preneoplastic foci and promotes tumor development (25).

Infectious agents such as *Helicobacter pylori*, with its strong association to gastric cancer, or the relationship of non-infectious chronic inflammation like chronic pancreatitis to pancreatic cancer (12, 15) are examples of infection and inflammation leading to tumor growth. Chronic inflammation caused by infections and chronic irritations are being deeply researched in order to locate the exact mechanism that triggers the cancer.

3.1 Infections of oral cavity and OSCC

OSCC is a multifactorial disease where no single clearly recognizable causative factor has been identified. Inflammation or infection-related carcinogenesis of the oral cavity is currently under investigation. Considering the oral cavity which comprises a variety of different surfaces with a huge diversity of microorganisms, including more than 750 distinct taxa of bacteria, it is not surprising that one or more of these microbes would take part in the carcinogenesis of their habitat (26). Table 1 summarizes the infectious agents and related carcinogenic mechanisms in OSCC development.

The first species of bacteria that has been classified as a definitive cause of cancer in humans is *Helicobacter pylori*, which is associated with gastric adenocarcinoma (27). After this discovery many other possibilities were investigated. Gall bladder carcinoma was associated with *Salmonella typhi*, cervical carcinoma with *Chlamydia trachomatis*, lung cancer with *Chlamydia pneumonia* and intestinal cancer with *Streptococcus bovis* (28). No such direct link was established in OSCC. As mentioned before, the oral cavity is home to a rich microflora which changes composition and quantity from person to person and throughout the lifetime of an individual as a response to a variety of factors (26). In the studies with OSCC, it is essential to identify the organisms in the tumor specimens. Specific bacteria detected in the tumor specimen were *Exiguobacterium oxidotolerans*, *Prevotella melaninogenica*, *Staphylococcus aureus* and *Veillonella parvula* (29). In another study using saliva samples, out of 40 samples three bacteria were found to be elevated in OSCC, namely *Capnocytophaga gingivalis*, *Prevotella meningica* and *Streptococcus mitis* (30).

It has been suggested that specific oral bacteria play a part in carcinogenesis, either through induction of chronic inflammation or by interference, either directly or indirectly, with eukaryotic cell cycle and signaling pathways, or by metabolism of potentially carcinogenic substances like acetaldehyde causing mutagenesis (28).

There are also a number of yeasts sharing the same environment with the bacteria. The most common yeast found in the human oral mucosa and generally regarded as commensals is a species of *Candida* (26). When host defense mechanisms are compromised or when changes occur in the local oral microenvironment *Candida spp.* act as 'opportunistic pathogens' leading to a wide range of oral mucosal infections (31). Besides being opportunistic, it has been shown that leukoplakia with candidal infection (formerly known as candidal leukoplakia) has a higher rate of malignant transformation than non-infected leukoplakia, and the estimated rate is up to 10% (32). Moreover, it has been observed that oral carriage of the most common type of candida, *Candida Albicans*, is higher in patients presenting with leukoplakia or OSCC than in patients without oral pathology (33). *C. albicans* may have a direct or indirect role in oral carcinogenesis. *Candida* might induce OSCC by directly producing carcinogenic compounds (e.g. nitrosamines) (26). The tubular hyphal structure of *C. albicans* is an important factor as it allows access of precursors from saliva and the release of nitrosamine product to keratinocytes, potentially initiating OSCC (34). In a recent study in a mouse model of oral carcinogenesis Dwivedi et al. (35), found that infection with *C. albicans* alone was not capable of inducing dysplasia or OSCC, but it was suggested that *Candida* creates an environment favorable to cell proliferation that may lead to clonal expansion of genetically altered cells. Alcohol consumption is a well-known risk factor in OSCC development. Although ethanol itself is not carcinogenic, its metabolites comprise highly toxic compounds such as acetaldehyde, hydroxyethyl radicals, ethoxy radicals, and hydroxyl radicals (31). The metabolism of alcohol starts in the oral cavity with the conversion of ethanol with enzymes catalyzed by alcohol dehydrogenase (ADH) from the epithelium and also from the oral microorganisms. Acetaldehyde in the mouth can also be derived from tobacco smoke, which contains a number of toxic aldehydes and other substances. Therefore tobacco and alcohol use has a synergistic effect on the risk of developing OSCC (5, 26, 31). From the molecular perspective, mucosal bacterial infections may influence carcinogenesis by inducing chronic inflammation in the adjacent connective tissue leading to upregulation of cytokines and growth factors. Similarly, *C. albicans* has been found to induce IL-8 secretion of endothelial cells by stimulating the cells to produce TNF- α (31, 36). The transcript factor NF- κ B, a key coordinator of innate immunity and inflammation, is also an important tumor promoter (14, 15). Candidal infection may activate particular toll-like receptors (TLRs), which are known to be activated after tissue damage and microbial infection. They can also communicate with the tumor promoter NF- κ B. NF- κ B is involved in carcinogenesis, especially where cancer-related inflammation is evident. The association between *C. albicans*, TLR and NF- κ B, and the production of cytokines and enzymes in the prostoglandin synthesis pathway, such as COX-2, is another potential mechanism that shows how *C. albicans* might influence the development of OSCC (31). Hooper *et al.* (26) suggested that 'Whether or not there is a causal relation between microbes and cancer, there is also a possibility that changes commensal microflora occur in conjunction with cancer development, which could have been used as a diagnostic indicator'. Meurman (37) proposed that it would be fascinating to control oral cancer by controlling oral microbes. The idea is truly fascinating and may not be as far-fetched as thought.

Apart from bacteria and yeasts there is also evidence that viruses take part in oral carcinogenesis. The role of the human papilloma viruses (HPV) and herpes simplex viruses (HSV) has been investigated in a number of studies (38, 39, 40). More than 100 types of HPV are identified, but only 12 types of HPV isolated from the oral cavity were associated with

malignant lesions, including HPV-2,-3,-6, -11,-13,-16,-18,-31,-33,-35,-52 and -57 (41). Studies indicated that HPV-16 and -18 were the most common types detected in individuals with OSCC (42). HPV-16 DNA, in particular, was detected predominantly in oropharyngeal SCCs located in the lingual and palatine tonsillar regions (39). Although the role of HPV in OSCC is smaller than in oropharyngeal cancers, it is important to distinguish HPV (+) OSCC since they are regarded as different entities (43, 44). HPV (+) OSCC are clinically found at young ages and generally in subjects without tobacco or chronic alcohol consumption. The histologically well differentiated and faster growing cancers which respond to chemo-radiotherapy have a clinical outcome – in term of overall survival – better than HPV (-) OSCC patients (44).

Recent studies revealed a synergistic effect between alcohol and HPV, but surprisingly tobacco use did not affect their relation (45). The mechanism of oral carcinogenesis by HPV is related with the E6 and E7 genes. Its genome is made up of early genes (E) with a primary function of episomal replication and late genes (L), which encode viral capsid proteins (41). There are 7 early genes identified and two of these, E6 and E7, have the capacity to immortalize the keratinocytes through inactivation of tumor growth suppression genes p53 and Retinoblastoma (Rb) respectively (39, 41). Generally, there is no clinic lesion or sign of inflammation in HPV (+) OSCC patients, but there is a relation proposed by Tezal et al. (40) that chronic inflammation in periodontal pockets may give an opportunity to initiate HPV infection and its persistency. In this study the base of tongue in squamous cell carcinoma patients were found to be 70% positive for HPV-16 and HPV (+) tumors, and had significantly higher rates of alveolar bone loss, which is indicative of chronic periodontitis.

Infections in the oral cavity are likely to play a role in oral carcinogenesis. Since there are numerous factors that cannot yet be distinguished, further studies with larger sample sizes are warranted.

Risk factor	Potential carcinogenic mechanism	Reference
Oral biofilm (Dental plaque)	Induction of cellular proliferation, inhibition of apoptosis, interference with cellular signalling mechanisms	46
	Mutagenic interaction with saliva	47
	Microbial action on oncogenic inflammatory reactions and proto-oncogenes	48
Periodontal disease	Providing opportunity to initiate HPV infection and serve resevoir for latent virus	40
	Interference with cellular signalling mechanism	49
Viridans streptococci		50
	Converting ethanol to acetaldehyde	
Candida albicans	Dysplastic changes in oral leukoplakia	26
	Converting ethanol to acetaldehyde	51
Human papilloma virus	Epithelial cell immortalization	52
Herpes simplex virus	Activation of proto-oncogenes inactivation of p53 tumor supressor gene	52

Modified from ref 37.

Table 1. Infectious agents and attributed carcinogenic mechanisms in oral carcinogenesis

3.2 Non-Infectious chronic inflammation and OSCC

Chronic inflammatory diseases such as ulcerative colitis, atrophic gastritis and Barrett's esophagus (53) have been causally associated with cancer development. Within the oral cavity, the best example of chronic inflammation are periodontal disease (as mentioned before) and oral lichen planus (OLP), which is regarded as having a malignant potential in a wide range of 0-12,5% (48, 53, 54). OLP was proposed as a unique disease model for studying non-infectious chronic inflammation and its relation to cancer in a recent publication. In the tissue microenvironment of OLP it is expected to find cytokines/chemokines directly associated with oral carcinogenesis, and suggested that OLP-related OSCC is very likely to develop from another pathway than non-OLP OSCC (53). Chronic traumas in the oral cavity were also associated with oral carcinogenesis in some recent studies and case reports (55, 56, 57). Recently, we conducted a study on the etiological factors of tongue carcinoma. Patient and control groups each consisted of 30 male and 17 female subjects with mean ages 53,17 (\pm 12,565) and 52,55 (\pm 11,542) respectively. Smoking and alcohol abuse proportions were significantly higher in the patient group as expected ($p=0.0001$, $p<0.0001$ respectively). Chronic traumas were observed in 44,7% of the patients and 17% of the control group ($p=0.004$). On regression analysis chronic traumas, such as alcohol abuse or a family history of cancer and smoking ($p=0.0001$) (58) appeared as significant etiologic factors.

We believe that field cancerization is evident in oral and orofarengeal mucosa (in the existence of epigenetic factors) with multiple steps of molecular changes starting from the first sign of dysplasia. In our opinion, the nuance is that, the site of chronic trauma reaches the point of cancer before any other competitive sites of oral mucosa. This finding might be supported by studies that associate inflammation with OSCC.

4. Role of chemokines in cancer

Chemokines are low molecular weight proteins (approximately 8-17kDa) and were originally defined as potent attractants for leukocytes in all inflammatory settings—as well as being regarded as mediators of acute and chronic inflammation (59, 60). More than 45 non-allelic chemokine genes and more than 20 chemokine receptors, which interact combinatorial, have been identified in human genome (59). Chemokines are classified on the basis of the presence of variations on their cysteine group. The first group, the CC subfamily, is composed of 28 members, whereas the CXC subfamily comprises 17 members. The other two smaller subfamilies are the CX3C and XC families, and each is presented with one member. The CXC chemokines are further classified into ELR+ and ELR- subgroups based on presence or absence of their 'glu-leu-arg' motif. ELR+ CXC chemokines are angiogenic, whereas ELR- members (except CXCL12) function as angiostatic to inhibit the formation of blood vessels (60). These are shown in Table 2 with their subgroups, receptors and tumoral impacts.

Chemokines carry a great significance in many biological events, both in physiological such as embryogenesis, lymphoid organ development, in pathology as wound healing angiogenesis, Th1/Th2 development, leukocyte homeostasis and inflammatory diseases (25). Chemokines attract leukocytes to the site of inflammation. Chemokines affect both the pro- and anti-tumor effect in the tumor microenvironment by regulating immune cell infiltration (12).

Systematic name	Chemokine reseptor	P/M/A
<i>CXC chemokine</i>		
ELR+ chemokines		
CXCL1	CXCR2>CXCR1	P
CXCL2	CXCR2	P
CXCL3	CXCR2	P
CXCL4	Unknown	A
CXCL5	CXCR2	P
CXCL6	CXCR1,CXCR2	P
CXCL7	CXCR2	P
CXCL8	CXCR1,CXCR2	P
ELR- chemokines		
CXCL9	CXCR3	A
CXCR10	CXCR3	A
CXCR11	CXCR3	A
CXCR12	CXCR4,CXCR7	M,P
CXCR13	CXCR5	
CXCR14	Unknown	P
CXCR16	CXCR6	
<i>CC chemokine</i>		
CCL1	CCR3	P
CCL2	CCR2	P
CCL3	CCR1,5	P
CCL3L1	CCR1,5	
CCL4	CCR5	P
CCL5	CCR1,3,5	P
CCL6	Unknown	
CCL7	CCR1,2,3	P
CCL8	CCR3,5	P
CCL9/10	CCR1	
CCL11	CCR3	P
CCL12	CCR3	
CCL13	CCR2,3	
CCL14	CCR1,5	
CCL15	CCR1,3	P
CCL16	CCR1,2	P
CCL17	CCR4	
CCL18	Unknown	P
CCL19	CCR7	P
CCL20	CCR6	P
CCL21	CCR7	P,lymph node metastasis
CCL22	CCR4	
CCL23	CCR1	P/M
CCL24	CCR3	
CCL25	CCR9	
CCL26	CCR3	
CCL27	CCR10	
CCL28	CCR3,10	
<i>C chemokine</i>		
XCL1	XCL1	
<i>CX₃CL1 chemokine</i>		
CX ₃ CL1	CX ₃ CL1	P/M

P-tumor progression; M-metastasis; A-Angiostatic (Modified from ref 25)

Table 2. Chemokine superfamily and their receptors

Leukocytes infiltrate the tumor in response to chemokines secreted by the tumor itself. This immune cell recruitment may promote anti-tumor activities such as elimination of tumor cells by macrophages and recruitment of innate and adaptive immune cells (25). As the tumor progresses, the attraction of immune cells by chemokines being secreted from the tumor tissue itself results in an accumulation of leukocytes in order to increase tumor growth and angiogenic mediators for tumor vasculature. Mostly, receptors of these particular chemokines are up-regulated in tumor cells which allow them to take advantage of the persistent chemokines in their microenvironment. Tumors act as immune cells which have the ability to secrete chemokines for progression. The best example is macrophages present in the tumor lesions which secrete chemokines involved in tumor cell proliferation and survival as well as angiogenesis and metastasis (12, 13). In studies based on solid tumors such as breast and prostate cancers, cancer cells were found to express higher levels of chemokine receptors CXCR4, CCR7, CCR9 and CCR10 (61, 62). This might explain the metastatic tropism of each type of cancer, depending on the receptor present on cancer cells and chemokines produced at the site of metastasis. The ligand of CXCR4, CXCL12, is best expressed in the lung, liver and lymph nodes, which are frequently involved in tumor metastasis. Moreover, CCL21, the ligand of CCR7, is produced by lymph nodes, and CCL27, the ligand of CCR10, is secreted by skin (63). The step of tumor progression includes growth of the primary tumor, angiogenesis and metastasis. The chemokines and their receptors described in these steps are as follows: CXCR4/CXCL12 is the most efficient chemokine/chemokine receptor pair in enhancing cell growth (60), CXCR2 ligands, CXCL1, CXCL2 and CXCL8 in promoting angiogenesis (64), CXCR4/CXCL12 (in bone metastasis), CCL19-CCL21/CCR7 (in lymph node metastasis) and CCL27/CCR10 (in skin metastasis) pairs in metastasis (63). Recently, chemokines and their receptors have been identified as molecular targets of cancer therapy. CXCR4 is the most targeted receptor in these studies since it was the first chemokine receptor found to be related with metastasis. CXCR4 antagonists significantly reduced the size of primary tumors in mouse models of melanoma, osteosarcoma, breast and prostate tumors (65). Another promising target is the angiogenic chemokine receptor, CXCR2, and antagonists for this receptor are under consideration for melanoma therapy. Some others, such as CCR5 antagonist, have been approved by the FDA for the treatment of HIV-infected patients. Clinical trials involving a CCR9 antagonist are also in progression for Crohn's disease (60).

4.1 Chemokines and OSCC

Ammar et al. (66) conducted one of the first studies in oral squamous cell carcinoma and chemokine expression and revealed the association of CXCR4 expression in primary site and lymph node metastasis, mode of invasion, tumor recurrence and prognosis of the patients. Parallel with this finding Ishikawa et al. (67) found a highly significant correlation ($p=0.0035$) between CXCR4 expression and lymph node metastasis of OSCC. Another study on chemokine expression and OSCC was reported in 2004, investigating the role of tumor-associated macrophages in oral cavity and oropharyngeal squamous cell carcinoma. CCL2 was found to be up-regulated significantly in tumors compared with normal mucosa (68). Later on Ferreira et al. (69) reported the role of CCL2 in lymph node metastasis of OSCC. Lymph node metastasis was also associated with other chemokine expressions. For example, CCR7 was found to be significantly associated with five clinical factors, including lymph node metastasis. Other factors were large tumors, progressive stages, local recurrences and cancer death (70).

The association of CCR7 expression and lymph node metastasis was confirmed by another study in 2009 that demonstrated CCL21 stimulation increased the ability of CCR7-positive cells, which in turn showed stronger adhesion to lymph nodes (71). Another axis related with lymph nodes was CCL3/CCR1. Silva et al. (72) reported that CCL3/CCR1 expression was significantly higher in OSCC patients than controls and they suggested that CCL3/CCR1 axis may have a role in the spread of tumoral cells to the lymph nodes.

CCL5/CCR5 axis is also studied in OSCC and found related with enhanced migration of oral cancer cells through the increase of matrix metalloproteinase (MMP)-9 production (73).

Beyond the expression profiles there is another important factor related with predisposition and progression of cancer. Single nucleotide polymorphisms (SNPs), in genes for susceptibility factors, may influence gene expression, protein function and disease predisposition in certain individuals (74). Recently, many studies revealed certain functional polymorphisms influencing expression of genes related with inflammation, and have been correlated with an increased risk for developing oral malignancies (75, 76, 77). Vairaktaris et al. (74) studied polymorphisms of a group of interleukins and tumor necrosis factors α and β 162 OSCC patients. Among studied cytokines, IL-6 and TNF- α polymorphisms were found to be related with OSCC occurrence. Gupta et al. (78) confirmed the results of the previously mentioned study in tobacco-related OSCC in Asian Indians. They studied SNPs in TNF- α and TNF receptor genes and TNF- α -308 G/A was found related with susceptibility to OSCC. In a Southern Thailand study on polymorphism of proinflammatory cytokines genes, susceptibility to OSCC appeared to be influenced by variants in inflammatory and immunomodulatory genes (79). Another study, again from the Greek group, was published in 2009, showing that PAI-1, MMP-9, TIMP-2 and ACE polymorphisms, which effect their expression, contributed significantly in OSCC prediction (80). Currently there are not many studies on OSCC and polymorphism of chemokines. In one study for SDF-1 (CXCL12) and CCR5 polymorphisms in head-neck cancers, only SDF-1 genotypes among studied polymorphisms were found to be significantly different from the control group distribution and this was correlated with susceptibility of SCC of the head and neck—but salivary gland tumors were excluded (81). In the other study on CCR5 and its receptor CCL5 polymorphism conducted in Taiwan, 253 OSCC patients were enrolled and SNPs in CCL5-28 and -403 genes revealed increased risk for OSCC, whereas the combined effect of CCL5-28 CG and -403 TT genes were found to increase the risk of OSCC but reduce the clinicopathological development of OSCC patients (82).

There is also a recently published study about chemokine polymorphism and OSCC of our group from Istanbul University (83). We studied the CCL2/CCR2 axis since CCL2 has been identified as a major chemokine inducing the recruitment of macrophages in human tumors, including those of the bladder, cervix, ovary, lung and breast (84, 85, 86, 87, 88). CCL2 expression was detected at the protein level in tumor cells, both in primary tumors and in the metastatic sites (89). Studies indicated that lower levels of CCL2 did form tumors but with substantial delay in onset and growth rate (90). It is shown that the polymorphism A-2518G in the regulatory region of the CCL2 gene influences CCL2 expression in response to inflammatory stimuli (91). The level of expression may vary due to polymorphism in CCL2 and its receptor CCR2 (89). In Istanbul University we therefore studied CCL2 and its receptor CCR2 polymorphisms in OSCC, and to the best of our knowledge it was the first time in the literature. In this study, we hypothesized that genetic polymorphisms in

chemokines and their receptors (CCL2 A-2518G and CCR2-V64I) are involved in leukocyte trafficking and may thus influence the risk of OSCC.

We found a statistically significant difference between the control and OSCC groups for CCL2 A2518G genotypes ($p=0.012$). The frequencies of CCL2 2518 GG genotype and G allele in the OSCC group were higher than those of the control group ($p=0.043$ and $p=0.006$, respectively). Individuals carrying the G allele (GG+AG genotypes) had a 1.89-fold increased risk for OSCC ($p=0.011$; $\chi^2=6.45$; OR=1.89; 95%CI= 1.15-3.09).

The CCR2 V64I genotype frequencies for controls and cases were not significantly different ($p=0.08$). CCR2 V64I wt/wt genotype frequency in the control group was higher than that of patient group ($p=0.027$; $\chi^2=4.88$) and individuals carrying the 64I allele and wt/64I genotype had an increased risk for OSCC individuals ($p=0.027$, $\chi^2=4.88$; $p=0.048$; $\chi^2=3.91$ respectively).

While CCL2 G allele, CCL2 GG genotype, CCR2 64I allele, gender, smoking and alcohol consumption were associated with OSCC in univariate analysis, only CCL2 G allele, CCR2 64I allele, gender and alcohol consumption were associated with this disease in multivariate logistic regression analysis.

Association of tumor progression and the possibility of CCL-2 A2518G and CCR2 V64I polymorphism playing a role in OSCC as a prognostic marker has been studied. No statistically significant differences were found between genotypes.

The genotype distributions of both CCL2 A-2518G and its receptors CCR2-V64I vary in many cancer studies (92, 93, 94). Our findings indicated a relation between CCL2 A- 2518 GG genotype and G allele and OSCC ($p=0.043$ and $p=0.006$ respectively). It seems that individuals carrying the G allele had increased risk for development of OSCC ($p=0.011$). To our best knowledge, six papers have reported an association of CCL2 2518 A/G polymorphism with various cancer types including breast (93), bladder (95, 96), nasopharynx (94), endometrial (97) and non-small cell lung cancer (98). Among these studies CCL2 2518G GG genotype was found to be a risk factor in endometrial cancer (6.7-fold increased risk) (97) and in bladder cancer (3-fold increased risk) (95). In a breast cancer study CCL2 2518 GG genotype and G allele frequency were also found to be significantly different ($p=0.020$ and 0.026 respectively) in patients with metastatic tumors (93). These studies indicated that GG genotype and mutant G allele stand on the tumor side as reported in our study. These results are also consistent with the report suggesting the association of G allele with higher levels of CCL2 expression (89). However, in a nasopharynx cancer study (94), CCL2 2518G AA and AG genotypes, which were suggested to have an association with relatively lower expression of CCL2, were found to be more prone to distant metastasis than those with GG genotype. GG genotype and G allele were also found significantly decreased in non-small cell lung cancer (98) and bladder cancer (96) patients.

The CCR2 V64I wt/wt genotype frequency in the control group was higher than the patient group ($p=0.027$; $\chi^2=4.88$), and individuals carrying the 64I allele and wt/64I genotype had increased risk to develop OSCC.

Although results presented in the current study have suggested that CCR2-V64I polymorphism leading to increased risk for OSCC is similar to bladder (95) and endometrial cancer (97) types in Turkish population, the other results related to hepatocellular carcinoma (99) and non-small cell lung cancers (98) remain controversial.

These conflicting results may be explained in many different ways. First, as these studies were mostly conducted in different countries; ethnic differences may play a part. Several CCL2 A-2518G and CCR2-V64I polymorphism studies were conducted around the world, and the distribution of control group data in these studies reveals genetic variations in distinct geographic areas. These results strengthen the idea that ethnic variations affect gene polymorphisms. Secondly, all mentioned studies were unique in cancer types and none was repeated either in the same or different nation. Thirdly, the sample sizes in the studies are relatively small when compared with the number of patients in their own nation—including our study.

The current study is the first report showing the influences of CCL2 and its receptor CCR2 gene variants on OSCC. Our results suggest that the genetic variants in the CCL2 and CCR2 genes may be associated with susceptibility of OSCC in the Turkish population. We can speculate that CCL2 polymorphism might increase the biological activity of the CCR2 receptor and the development of OSCC risk within this group.

5. Conclusion

Inflammation is a recently defined contributor of oral carcinogenesis. In this multi-step process, inflammation might have a role in initiation as well as progression. Important components of this association are cytokines and chemokines produced by activated innate immune cells, which stimulate tumor growth and progression. Moreover, genetic susceptibility and gene/environment interactions are becoming more important in the attempt to eliminate the burden of cancer. The evidence found so far is sending out signals that OSCC may cease to exist in the future, and the referral will only be for a group of diseases that manifests symptoms of a similar sort. Further studies with larger sample groups in premalignant diseases of oral mucosa as well as OSCC are required to confirm these findings.

6. Summary

Oral cancer accounts more than 2% of all body cancers worldwide and more than 95% of them were found to be squamous cell carcinoma. Despite its relative rareness, high mortality rates (survival is not more than 50% in 5 years), which have not improved over the past 3 decades, have drawn the attention of the investigators. The well-known risk factors of oral squamous cell carcinoma (OSCC) like smoking, alcohol abuse and HPV infection, which are mainly based on life-style factors, seem inadequate to explain the increasing incidence especially among young population. In addition, genetic susceptibility may play an important role but the underlying mechanism of the disease still remains obscure.

Many theories about pathogenesis of the disease have been produced as a result of the clinical observations. One of the best known is about inflammation. Clinicians have experienced that tumor mass is almost always accompanied by an inflammatory zone and pathologists have always observed inflammatory cells in and around the tumors. This is not a new finding and in fact so old, which dates back to famous hypothesis by Virchow in 19th Century and even ancient back to Celsius in the year 50 BC. So what is new? The new issues are the molecular developments which elucidate many unanswered questions about cancer. The relation between inflammation and cancer was one of them.

In clinical researches about the relationship between inflammation and cancer, oral squamous cell carcinoma has an important advantage which is being easily detected by naked eye. Our previous clinical studies about etiology of tongue squamous cell carcinomas revealed that chronic traumas and irritations which lead to chronic inflammation were associated with tongue SCC formation. This encouraged us to move another step into the molecular field to understand the mechanism.

It is suggested that the relationship between cancer and inflammation occurs through two pathways: an extrinsic pathway driven by inflammatory signals such as infections and an intrinsic pathway driven by genetic alterations that cause both inflammation and neoplasia. Main mediators at the intersection of these pathways include transcription factors and primary proinflammatory cytokines.

Chemokines are a family of cytokines which are important mediators of leukocyte trafficking. They involve in defense of microbial infection, angiogenesis and metastasis. Several important polymorphisms of chemokine and chemokine receptors which deregulate chemokine system have been found and it is suggested that they may interfere with inflammatory and other diseases.

This chapter will include a brief review of molecular mechanisms of inflammation that seem responsible for etiology, pathogenesis and prognosis of oral squamous cell carcinoma and the new findings of our study group on chemokine.

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Part 5

Cancer Stem Cells in Squamous Cell Carcinoma

Molecular Mechanisms Involving Therapeutic Resistance in Head and Neck Squamous Cell Carcinoma (HNSCC) – Roles of Hypoxic Microenvironment and Cancer Stem Cell

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1. Introduction

Locally advanced diseases accounting for most HNSCC have a poor prognosis. The main reason for this is that corresponding symptoms of HNSCC are not always obvious or ignored by patients at early stage, which is mostly reflected by the fact that more than 2/3 HNSCC patients present with stage III/IV disease (AL-Sarraf, 1987; Argiris, 2008). Patients characterized with advanced HNSCC are subjected to worse prognosis than those with confined disease, exhibiting 5-year survival of 10-40%, cure rate of 30% and median survival time of 6-10 months (Argiris, 2008; Vokes et al, 1993; Cohen et al, 2004). Our recent study in a large series (X. Li et al, 2009) demonstrated that overall survival rates of patients with distant metastases in clinic were 56.8% at 1 year, 9.1% at 3 years, and 6.8% at 5 years, respectively. In addition, traditional treatment related morbidities could negatively influence quality life of patients, which involves loss of speech, permanent tracheostomy or gastrostomy dependence, dysphagia and other systematic side effects. Therefore, it is necessary to seek novel strategies to cure advanced HNSCC aiming at organ preservation, prevention of metastases as well as second malignancies and improvement in quality of life.

2. Necessities for adjuvant therapies in HNSCC

Surgical ablation plays a major role in the management of locoregional diseases of HNSCC. At early stage of HNSCC, current novel surgical alternatives, such as laser surgery, can achieve curable effects for 5-year survival rate of 80% even without prominent functional detriments. However, many tumors in the advanced stage are inoperable either due to the invasion of some major structures by tumor or due to the unfavorable general conditions of patients. Moreover, even with very skillful surgeons, some tumors remain after surgical resection, leading to postoperative recurrence if additional complimentary treatment is not carried out. In tumors with regional lymph node metastasis, extracapsular nodal spread always implicates poor prognosis even after a comprehensive neck dissection. For these conditions, adjuvant therapies such as radiotherapy, chemotherapy and chemoradiation are

due to undertake to increase the chance of cure or to prolong duration of survival of advanced cases.

2.1 Traditional adjuvant therapies improve outcome of advanced HNSCC

The participation of radiotherapy improved effects of surgery alone. In 2008, *Cancer* journal (Lavaf et al, 2008) reported a large-scale analysis with regard to effects of combined surgery and radiotherapy on survival of patients with lymph node-positive HNSCC patients. In 8795 patients meeting the inclusion criteria, 54.9% of 3-year overall survival and 43.2% of 5-year overall survival for adjuvant therapy could be gotten compared with 44.4% and 33.4% for surgery alone. More recently, a new analysis with large series (Shrime, 2010) reported that postoperative radiotherapy improved 5-year overall survival rate in patients with T₁₋₂N₁ oral squamous carcinoma (41.4% for surgery alone vs. 54.2% for surgery plus radiotherapy). Although statistically significant, slight improvement in survival has to indicate the limitation of single radiotherapy addition, which appeals the need of chemotherapy in the management of advanced HNSCC. In 2009, the journal of *The Lancet Oncology* published a 10-year follow-up report of a trial for chemoradiotherapy for locally advanced head and neck cancer conducted by The UK Head and Neck (UKHAN) cancer group (Tobias et al, 2009). In this follow-up analysis, patients who did not undergo previous surgery benefited from scheduled simultaneous addition of chemicals to radiotherapy, exhibiting 4-7 years in the median overall survival. However, the median overall survival of patients undergoing surgery was still higher without substantial benefit from chemotherapy alone. Furthermore, sequent toxicity reactions, such as mucositis and xerostomia, are due to occur. All these findings suggest that the effects of traditional chemicals in treating the HNSCC are limited due to their unspecific hallmarks.

2.2 Limitations of traditional adjuvant chemoradiation therapies

As is known, HNSCC depend on many intrinsic or extrinsic factors to protect against traditional chemotherapeutic agents, such as cisplatin and 5- fluorouracil. As evidenced by clinical observations, HNSCC possesses a decreased sensitivity and increased resistance to chemo- and radiotherapy, giving rise to a poor tumor control efficacy of these treatment modalities. This situation is mostly reflected by the fact that many HNSCCs (including primary and recurrent carcinomas) have less or no response to the adopted treatment regimens in the course of chemotherapy and/or radiotherapy. For this reason, some tumors regenerate or relapse following a short- or long-term paracmasis during which time the tumor bulk contracts or even disappears visually in response to therapy. Therefore, chemo- and radiotherapeutic resistance and post-treatment relapse has been always a puzzling problem that needs to be solved urgently.

3. Role of hypoxia in therapeutic resistance in HNSCC

The mechanisms underlying resistance to chemo- and/or radiotherapy by HNSCC are very complicated. Among various factors that are associated with therapeutic resistance in HNSCC, hypoxic microenvironment resulting from hypoxia in local cancer lesions is thought to be important one. It has been demonstrated that, most solid tumors have a lower pressure of oxygen (PO₂) compared with normal tissues from which they originate. Hypoxia occurs due to rapid proliferation of cells and/or insufficient supplies of blood. The latter

attributes to poor drugs delivery, leading to a common cause of chemoresistance. In addition, activated intrinsic pathways within tumor cells contribute to comprehensive radio- and chemo-resistance under hypoxic condition. In this section, we will focus on these intrinsic responses underlying resistance under hypoxia.

3.1 The general responses to hypoxia in tumor cells

General responses of tumor cells under hypoxia include translation inhibition, paradoxical translation and genetic instability. ATP defect caused by hypoxia invokes global translation inhibition for maintaining energy homeostasis. However, paradoxically, tumor cells activate some factors which are always unexpressed under normal conditions for adaptation to hypoxic stress. These proteins act as mediators of PH and metabolism, as well as function to propagate therapeutic resistance. In the long run, hypoxia-induced reactive oxygen species (ROS) and/or defective DNA repair induce mutagenesis of tumor cells to confer selection of heterogeneous population with hypoxia tolerance (see Fig. 1).

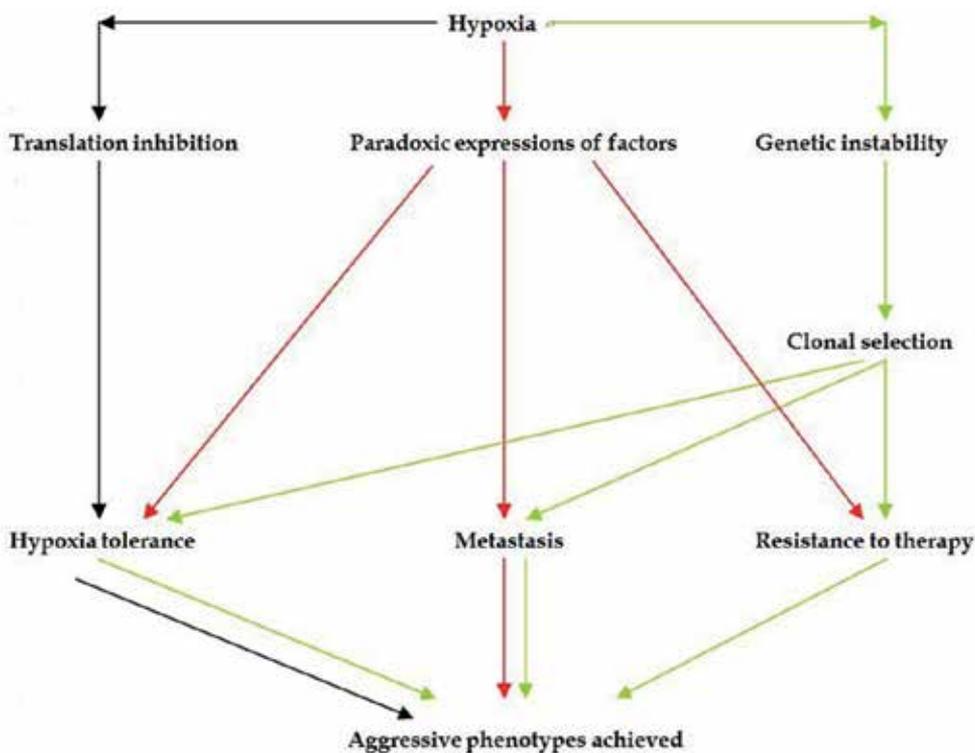


Fig. 1. General responses to hypoxia of tumor. Hypoxic tumor cells inhibit translation via mTOR pathways as well as UPR for energy homeostasis. Meanwhile paradoxically, they express some factors, such as HIF-1 α and GRP78, to degrade nonfunctioning protein, regulate PH and counter apoptosis. These factors also act as resistance to therapy and metastasis. Hypoxia induced mutagenesis select clonal subset characterized with aggressive phenotypes, which confer more malignant biological behaviors including therapeutic resistance.

3.2 Hypoxia-related translation inhibition

3.2.1 The mammalian target of rapamycin (mTOR) pathways

Protein synthesis is processed as a result of energy-consumption. To date, emerging data have evidenced that inhibition of translation is an important category of cellular hypoxic tolerance, and the process occurs during the initial step of translation. The initiation of translation involves two components, the eukaryotic initiation factor 4F (eIF4F) as well as 43S preinitiation complex. The former consists of the cap binding protein eIF4E, a scaffolding protein eIF4G and an ATP-dependent helicase eIF4A (RNA helicase activity), among which eIF4E and eIF4G mostly participate in the regulation of initiation of translation. Under nutrient and/or oxygen repletion, mTOR phosphorylates eukaryotic initiation factor 4E binding protein 1 (4EBP1) that has a high affinity with eIF4E to low the affinity. Together with eIF4G, released eIF4E contributes to assembly of eIF4F complex that binds to the 5' m⁷GpppN cap structure of mRNA to facilitate the recruitment of 43S preinitiation complex that includes the 40S ribosomal subunit and the ternary complex (eIF2-GTP and the methionine-loaded initiator tRNA) for the start of translation initiation. The ribosomal constituents of preinitiation complex scan through the 5' untranslated region (5'UTR) until the AUG initiation codon is recognized. Subsequently, the 60S ribosomal subunit joins to form 80S ribosome for the elongation of translation. Lots of recent reports have implicated that hypoxia can disturb the process above via hypoxic activation of the tuberous sclerosis protein 1 (TSC1)-TSC2 complex-mediated downregulation of mTOR. Thereby, corresponding expressions of translation initiation-related proteins, such as phosphorylated 4EBP1 and eIF4F, other mTOR-mediated targets and S6 protein kinases (S6K 1 and 2), all of which also function in translation, would be inactivated accordingly. In addition, hypoxia also interferes with formation of preinitiation complex to inhibit translation via PERK-mediated cascade of unfolded protein responses.

3.2.2 Unfolded protein responses (UPR)

UPR is an evolutionary conserved protective response to microenvironment stress. Because of abnormal vascular structure and aggressive cellular growth, hypoxia and glucose starvation always occur in tumor microenvironment, resulting in defective usage of energy in response to accumulation of many unfolded protein in endocytosplasmic reticulum (ER). Tumor cells must adapt to this stress though inhibiting mRNA and protein synthesis as well as degrading excessive useless protein, which is executed through activation of UPR. Glucose-regulated protein 78 (GRP78/BiP) is the core regulator in ER-stress and overexpressed in most tumor as a predictor of poor prognosis. Routinely, GRP78 binds to ER-stress sensors, IRE1 α (inositol-requiring 1 alpha), PERK (double-strand RNA-activated protein kinase-like ER kinase), and ATF6 (activating transcription factor 6), to inactivate their downstream targets. Once ER-stress occurs, the role of GRP78 is shifted towards that of a chaperone. After dissociation, GRP78 handles unfolded protein to facilitate degradation and binds to Ca²⁺ to inhibit apoptosis. Importantly, the dissociation activates these integral ER membrane sensors PERK, IRE-1 and ATF6. Activated PERK phosphorylates eukaryotic initiation factor 2 subunit α (eIF2 α), leading to either inhibition of global protein translational attenuation or paradoxical expression of the transcription factor 4 (ATF4) that immediately blocks eIF2 α phosphorylation and subsequently encodes genes upregulating stress-adaptive factors, such as GRP78 and hypoxia inducible factors (HIFs). UPR also

includes IRE-1 and ATF6 arms. Activated IRE-1 serves as an endoribonuclease to remove a 26 nucleotide intron from X-box binding protein 1 (XBP1) pre-mRNA. The resulting XBP1 protein by spliced XBP1 mRNA can activate lots of ER chaperones and enzymes to remove mis/unfolded protein and help ER-localized protein maturation as well as ER-associated degradation. Similar with XBP1, ATF6 needs cleavage for its activation. Briefly, upon UPR activation, ATF6 is transmitted from ER to Golgi apparatus where ATF6 completes its splicing process. Subsequently, cleaved ATF6 also activates target genes functioning in protein degradation and upregulation of molecular chaperones. As a matter of fact, their functions and target genes overlap one another. Overall, these sensors play a critical role in inhibition of mRNA and protein synthesis and upregulation of stress-adaptive factors. As a final step, unfolded protein is transported to cytoplasm and degraded via ubiquitin-proteasome pathway (UPP) or autophagy. To our understanding, UPR is a double-edged sword. On the one hand, PERK--eIF2a-ATF4 pathway is a dominant UPR arm offering survival advantage under hypoxia. On the other hand, once severe stress persists, UPR will induce ER-stress-related cell death (apoptosis, autophagy associated programmed death or necrosis). To date, GADD153/CHOP induced by ATF4, has been identified as a pro-apoptosis factor that can activate cascades of caspases, mediating type I programmed cell death (known as apoptosis). It remains to be determined by what mechanisms the UPR induces autophagic death and necrosis.

3.3 Factors expressed paradoxically under hypoxia

3.3.1 HIF-1 α

HIFs are core factors regulating oxygen and energy supplies of the tumor bulk, by which tumor cells adapt to hypoxia through inducing expressions of related genes to overcome such an unfavorable low-oxygen condition. HIFs are members of bHLH-PAS protein family including HIF- α and HIF- β subunit, the former of which also includes HIF-1 α , HIF-2 α and HIF-3 α . Functionally, HIF- α (HIF-1 α , HIF-2 α) can be stably sustained in the hypoxic niche. In the event of cell signaling, HIF- α (HIF-1 α , HIF-2 α) and HIF- β can form a heterodimer that binds to promoters or enhancers of target genes. Hypoxia not only induces the expression of HIF-1 α , but also activates many specific biological effects of HIF-1 α gene protein, which functions either to acquire the tolerance to hypoxia or to commit the capability of invasion, metastasis and therapeutic resistance: 1) inducing expression of carbonate dehydrates (CAH) to maintain a stable cytoplasmic PH to promote the survival ability of cancer cells in response to apoptosis-inducing factors; 2) upregulating the expression of MDR gene and its product, P-gp, resulting in resistance to multiple chemotherapeutic agents; 3) mediating the overexpression and activation of DNA kinase (DNA-PK), contributing essentially to the repair of DNA double-strand breaks (DSBs); 4) acting as an upstream regulator of genes encoding vascular endothelial growth factor (VEGF) as well as some key enzymes related to glycolysis, responsible for angiogenesis and glycometabolism within tumors; 5) promoting expressions of anti-apoptosis proteins such as Survivin and XIAP to inhibit the activation of pro-apoptosis proteins Bax and caspases, rendering the tumor cells the ability to escape from apoptosis.

3.3.2 Signal transducer and activator of transcription 3 (STAT3)

STAT3 is an important factor overlapped by many intracellular signal pathways. It can be activated though Janus kinases (JAKs) or tyrosine kinase receptors such as EGFR. Upon

phosphorylation at the Tyr705 residue, p-STAT3 translocates to nucleus to bind DNA for inducing the transcription of downstream targets. Emerging reports have demonstrated that STAT3 is associated with poor prognosis in many cancers including HNSCC. STAT3 induces resistance to therapy in tumors via activation of anti-apoptosis factors, such as Bcl-2, Bcl-xl as well as Survivin and downregulation of P53. Recently, a study demonstrated that STAT3 participates in inhibition of apoptosis caused by PIs in HNSCC (C. Li et al, 2009). Under hypoxia, STAT3 can be activated by ROS (Simon et al, 1998). Selvendiran et al (Selvendiran et al, 2009) found that STAT3 can be activated by production of ROS under 1% O₂ in ovarian cancer. In their study, overexpressed STAT3 contributed to similar rate of proliferation as that under normoxia but increased drug resistance under hypoxia. Through blockage of STAT3 using RNAi technique, ovarian tumor cells with defective expression of STAT3 exhibited affected proliferation as well as increased sensitivity to traditional chemotherapeutics under hypoxia. In HNSCCs, STAT3 was also found to be constitutively activated and associated with cervical lymph node metastasis in laryngeal cancer. Silencing STAT3 gene with specific siRNA enhances the sensitivity of Hep-2 human laryngeal carcinoma cells to ionizing radiation both in vitro and in xenotransplanted mice model. (X. Li, et al, 2010a, 2010b)

3.4 Hypoxic dynamic complication in solid tumor

3.4.1 Category of hypoxia

In solid tumor, hypoxia can be categorized as chronic continuing hypoxia and cyclic hypoxia (also called intermittent hypoxia or fluctuating hypoxia) depending on distances of tumor cells from the adjacent vessels. The former is incurred from the condition that tumor cells locating far from vessels result to diffusion-limited and relatively stable delivery of oxygen. On the other hand, the latter characterized by acute hypoxia/reoxygenation is caused by status of nearby vessels that suffer from dynamic changes in blood perfusion not least as a result of the abnormal vasculature and the mechanical instability of microvessel walls caused by proliferating tumor cells and/or circulating blood cells. With regard to insufficiency in blood or oxygen supply, hypoxia is classified as mild hypoxia (PO₂: 1-3%), moderate hypoxia (PO₂: 0.1-1%) and severe hypoxia (PO₂: 0-0.1%) ((Koumenis & Wouters, 2006). Additionally, in term of duration of persistent hypoxic condition, hypoxia is divided into acute hypoxia lasting several minutes to several hours as well as prolonged chronic hypoxia during which cells are exposed to hour-to-day intracellular low PO₂. The complex nature of hypoxia and different responses to distinct hypoxia by tumor cells may explain why it is so difficult to antagonize hypoxia-induced therapeutic resistance in HNSCCs.

3.4.2 Chronic versus cyclic

In most lab experiments, there is an important difference ignored by us. That is the parameters of hypoxic condition selected by most investigators are usually simple and fairly stable. However, reoxygenation may occur during manipulation of assorted cells. To date, cyclic hypoxia has been less studied than chronic hypoxia. The setting of cyclic hypoxic condition was not consistent among various studies on cyclic hypoxia. Here we introduce the difference between the two as follows with special emphasis on the importance of cyclic hypoxia. Firstly, cyclic hypoxia confers more potential therapeutic resistance than chronic hypoxia. It has been demonstrated by many studies that increased expression of HIF-1 α contributes to cyclic hypoxia-induced resistance. In addition, it has been confirmed that chronically hypoxic tumor cells are more susceptible to ionizing radiation (IR) or DNA-damaging drugs than acutely hypoxic tumor cells because of decreased homologous

recombination (HR) function, a main pathway to repair DNA double-strand breaks (DSBs) in the S and G2 phases of the cell cycle. Secondly, cyclic hypoxia induces an enhanced metastasis. It was found that mice bearing melanoma xenografts exposed to cyclic hypoxia suffered from increased incidence of pulmonary metastases (Rofstad et al, 2010). Furthermore, tumor cells treated by cyclic hypoxia up-regulates the expression of VEGF-A, the ligand of VEGFR-1 on bone marrow derived cells confirmed to form “premetastasis niche”. Therefore, induction of VEGF-A by hypoxia may be an important promoter of metastasis. Thirdly, cyclic hypoxia enhances metabolism of Tirapazimine (TPZ), an agent with specific hypoxic cytotoxicity, by intratumoral vessels adjacent to the populated tumor cells, which attenuates the effects of TPZ (Cárdenas-Navia et al, 2007). Finally, cyclic hypoxia is pervasive. As early as 1996, Kimura’s group (Kimura et al, 1996) measured microvessel red cell flux (RCF) and perivascular PO₂ in xenotransplant of R3230Ac mammary carcinomas using intratumoral dorsal flap window chambers. They found that the baseline RCF and PO₂ underwent a highly dynamic process, demonstrating that fluctuating hypoxia is a common phenomenon within a tumor. More recently, another group (Cárdenas-Navia et al, 2008) used phosphorescence lifetime imaging to detect fluctuation of vascular PO₂ in rat fibrosarcomas, 9L gliomas and R3230 mammary adenocarcinomas transplanted in dorsal skin-fold window chambers. By short interval periodic imaging, they found O₂ delivery to tumors is constantly instable. In addition, hypoxia, including acute and chronic hypoxia, causes genomic instability. Cyclic hypoxia mostly induces DNA double-strand breaks (DSBs) by generating reactive oxygen species (ROS). Chronic hypoxia causes genomic instability due to the defective HR ability. Thereby, both types of hypoxia facilitate mutagenesis leading to clonal selection with therapy-resistant, invasive and metastatic phenotype (see Fig. 2).

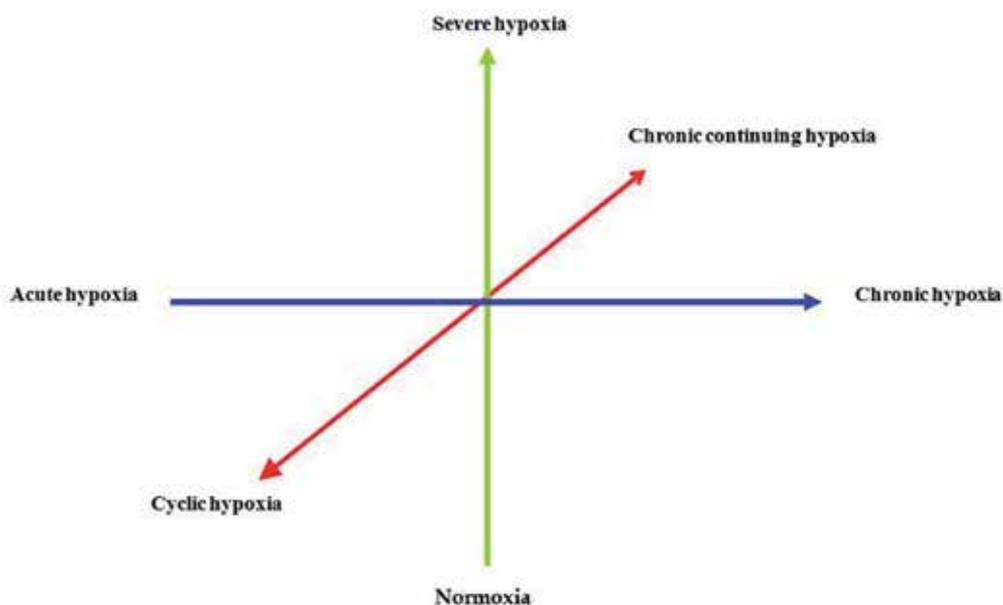


Fig. 2. Dynamic heterogeneity of hypoxia in solid tumor. Distinct hypoxia patterns exist in solid tumor. These patterns may overlap and co-exist in the same tumor bulk, which can be reflected through the 3D axis. Any point of this axis represents a combined type of hypoxia pattern in a tumor.

3.4.3 Hypoxic duration- and/or degree-related responses

The existing status of oxygen level in tumor bulk is very heterogeneous, which is reflected by detected PO₂ ranging from 0% to 100%, namely, from anoxia to normoxia. It is plausible that paradoxical activation of associated factors under hypoxia is also heterogeneous and dynamic in solid tumor, implying that focusing on one single target factor is insufficient to carry out an effective therapy. To date, although HIF-1 α has been comprehensively studied under different hypoxic conditions, it is not the case in the study of short period of severe hypoxia as well as chronic moderate hypoxia. This can be partially explained by the fact that regulation of HIF-1 α is actually a negative feedback loop via HIF-1 α -dependent induction of prolyl hydroxylase enzymes that promote the von Hippel-Lindau (VHL) tumour suppressor protein-mediated HIF-1 α degradation by ubiquitin-proteasome system under moderate hypoxia. On the contrary, the induction of prolyl hydroxylase enzymes is inhibited under severe hypoxia. Activation of eIF2 α has been indicated as a transient process during severe hypoxia, which is decreased following the prolonged duration of hypoxic status. Under moderate hypoxia, eIF2 α exhibits a gradually elevated activation along with elongation of hypoxic time.

4. Role of cancer stem cells (CSCs) in hypoxia-induced therapeutic resistance in HNSCC

4.1 Identification of CSCs in HNSCC

The theory of CSCs, as a milestone of cancer research, has a history of 150 years. The focus of this theory is that there exists a sub-group of tumor cells, like stem cells of normal tissues, with stem traits characterizing growth stasis and self-renewal with specific cell surface markers. This subset of cells within the tumor bulk is known as “cancer stem cells” (CSCs) or “tumor initiating cells (TICs)”. Other tumor cells that are considered as progeny of CSCs would face a final differentiation followed by programmed cell death. In term of CSC theory, tumor bulk only originates from CSCs; therapeutic failures in cancer management are a result of insufficient elimination of these heterogeneous subpopulation. In addition, the subset is believed to play important roles in invasion, metastasis and therapeutic resistance in various malignancies. To date, CSCs or CSC like cells have been identified in different cancer including HNSCC. In 2007, a subpopulation of cells with characterized stemness and CD44 marker in HNSCC was first isolated and identified (Prince et al, 2007). It was also demonstrated that CD133⁺ cells in Hep-2 human squamous laryngeal carcinoma cells have stem cell-like characters (Zhou et al., 2009). Subsequently, other investigators identified a CD133⁺ CSC-like subset with chemoresistance in oral squamous carcinoma (Zhang et al., 2009). More recently, CD44⁺ CSCs have also been isolated and identified from human laryngeal squamous carcinoma. All these data confirm the existence of CSCs in HNSCC, which shed light on a novel area to get further insight into the mechanisms of chemo- and radioresistance in HNSCC with respect to SCCs.

4.2 CSCs and microenvironment

4.2.1 “Seeds and soil” theory

The microenvironment of CSCs also called “niche”, consists of cellular and non-cellular components surrounding CSCs (Scadden, 2006), including direct cell contacts, cell-matrix

contacts, cytokines, blood vessels, mesenchymal cells and so on. It serves to protect CSCs from differentiation and apoptosis, and keeps the status of self-renewing (Iwasaki & Suda, 2009). As a matter of fact, the significance of the niche function is far beyond these, as it affects the physiology of CSCs to a far more great extent. As early as 1889, through analysis of 735 cases of breast cancers, British assistant doctor Stephen Paget (Paget, 1989) found that breast cancer cells preferred liver to settle in rather than spleen that has vascular supply as abundant as liver. Nearly a century later, after the quiescence of the “seeds and soils”, Hart and Fidler (Hart & Fidler, 1980) injected melanoma cells into mice implanted with ovary tissue, kidney tissue and lung tissue in muscle or under the skin, and these implanted tissues had previously established vascular supplies of their own. Finally, they demonstrated that melanoma cells just metastasized to grafted ovary and kidney, suggesting that the formation of tumor is not only influenced by the characters of tumor cells but also depends on the niche. Because CSCs are a kind of cells that can self-renew in malignancy, the niche of CSC must be critical for preserving the property of self-renewal.

Recently, many studies provided evidence for the “seeds and soil” even as they further disclosed the relationship between CSC and its niche. A group attenuated the adhesion between CSCs and some components of the niche, such as hyaluronan, through interfering with CD44, thereby, inhibited the neoplasia of myeloid leukemia (Jin et al, 2006; Krause et al, 2006). Calabrese et al (Calabrese et al, 2007) found that most medulloblastoma stem cells grew by adhering to endothelial cells selectively, and these CD133+ cells could give rise to new tumors only when co-transplanted with endothelial cells. Kaplan et al (Kaplan et al, 2005) introduced a concept of “premetastatic niche”, which meant the microenvironment of metastasized organs had been induced to transform into a condition better for the formation of secondary tumor. All of the findings above suggest that targeting the niche will be a very significant approach to eliminate CSCs.

4.2.2 Niche and heterogeneity

Species are selected by adaptation to environmental changes as proven by the earliest dinosaurs to today’s diverse biological species. Such is Darwinian evolution, a possible explanation for survival. The development of tumors might be a process of survival of the fittest by the pressure of microenvironment.

It is known to all that cells in various types of tumors are different from each other in many aspects, such as size, appearance, antigen expression, cell membrane composition and sensitivity to different treatment modalities (Ichim & Wells, 2006; Heppner, 1984; Axelson, 2005). There are two explanatory models to the potential heterogeneity of the tumor cells, the stochastic model and the hierarchical model. Firstly, the stochastic model attributes the tumor development to the “genetic instability”, through which the ones best adapting to the microenvironment are selected to obtain the advantage of proliferation (Nowell, 1976; Tysnes & Bjerkvig, 2007). This model shows that the tumor parenchyma contains many types of tumor cells with the ability to form tumors in the microenvironment (Bjerkvig, 2009). Secondly, the hierarchical model supports that the initial tumor and the metastatic tumor are both evolved from CSCs, which seems contradicting to the first model.

The two controversial models are currently interpreted by some recent investigations. Odoux et al (Odoux et al, 2008) found that a small number of CSCs subsets with the ability of self-renewal and differentiation exist in liver metastases in patients suffering from colon cancer, and this sub-group of cells are more invasive and expanding than the CSCs in the primary tumor. Surprisingly, as a considered decisive element in the evolution of tumor (Cahill et al, 2009), genetic instability was present in this subset. A recent genomics study found that aberrant stem cells significantly express the regulatory protein molecules which function to adapt to microenvironmental stimuli with respect to the gene expression profile of murine embryonic stem cell lines and its malignant counterpart, murine teratocarcinoma cell lines (Heffron, 2007). Campbell and Polyak (Campbell & Polyak, 2007) integrated the results of their research on breast cancer and a number of related reports, and ended up with that the heterogeneity of tumor cells may be due to the combination of some levels of the stochastic model and the hierarchical model. They found that the origin of breast cancer may initially be normal CD44-expression stem cells or progenitor cells, which undergo self-renewal, differentiation and mutation-driven clonal evolution motivated by the environmental changes and gene mutations, resulting in a bunch of different genotypes and diverse stages of development of tumor cells. This indicates that the hierarchical model in cancer stem cell theory is the extension of the stochastic model. Tumor tissues are evolved through genetic alterations, phenotypic changes and the impacts of micro-environment. So there may be more than one type of stem cell subsets with different characteristics in parenchyma, which exhibits different genetic or epigenetic phenotypes and ability to adapt to microenvironment, and the distinct characteristics of epithelial to mesenchymal transition (Werb & Evans, 2004). Therefore, not all CSCs have the ability to survive and metastasize, only those with the ability to adapt to the microenvironment are selected (Odoux, C et al, 2008; Lagasse, 2008) to do so.

4.2.3 Hypoxia and CSCs

As is known, CSCs are a subpopulation of tumor cells that co-exist with differentiated tumor cells in the same microenvironment, in which hypoxia serves as a necessary component for CSCs growth, self-renewal and differentiation (Keith & Simon, 2007). It has been demonstrated that HIF-1 α could induce the expression of some crucial genes related with CSCs' self-renewal and multipotency, including Oct4 and Notch1. Under hypoxia, stable expression of HIF-1 α in CSCs can stimulate expressions of Oct4 and Notch1, and activate the associated signaling of critical pathways, promoting specific properties of CSCs and related multipotency. In view of the mentioned above, we can introduce the concept of interaction between tumor hypoxic microenvironment and CSCs. Traditionally, the standard for evaluating the efficacy of a treatment regimen is the sizable contraction of the tumor bulk. However, most tumor relapse after a period of paracmasis, probably because traditional chemo- and radiotherapy only kill tumor cells in rapid proliferation and differentiation rather than CSCs, the latter of which with are in slow divisions and proliferation, conferring therapeutic resistance. The formation of the relapsed tumor is driven by CSCs under proper microenvironment at a certain time after the treatment regimens are completed.

4.2.4 CSC's resistance and related mechanisms

Lots of convincing data showed that CSCs subset displays powerful resistance to traditional chemo- and/or radio-therapy compared with non-CSCs of in same tumor or parent cells in

vitro. Currently, the mechanisms of CSCs-related therapeutic resistance have not been well understood. Perhaps, basic principles regarding CSC-caused therapeutic resistance can be categorized as follows.

4.2.4.1 Quiescence

Lots of cytotoxic drugs mostly kill tumor cells with rapid proliferation, which settle in cellular s-phase cycle. However, it is believed that CSCs, like normal stem cells, mostly reside in G₀/G₁ phase, which reduces efficacy of anti-cancer agents.

4.2.4.2 Overexpression of protective genes

Another cause of CSCs-related resistance is that this subset overexpresses some factors that protect CSCs from apoptosis and cytotoxicity. It has been well confirmed that CSCs express high levels of ABC drug transporters. Due to ATP hydrolysis, these proteins function to efflux drugs from tumor cells to protect against cytotoxicity. ABC superfamily includes 7 subfamilies from ABCA to ABCG (ABCB1 is P-gp). Among the superfamily, ABCG2 has been studied most extensively and is believed to be the most critical transporter of drugs. However, in clinic, targeting on ABCG2 alone has a minimal effect in the correction of chemoresistance by cancers, suggesting other ABC components also participate in chemoresistance or CSC's resistance is not determined only by ABC transporters. Liu et al (Liu et al, 2006) isolated CD133-positive tumor cells from glioblastoma and demonstrated that along with ABC transporters, these CSCs overexpressed anti-apoptotic factors, such as BCL-XL, Xiap, Survivin as well as cIAPs and DNA repair protein MGMT, which suggests that powerful repair ability combined with anti-apoptotic features may be partially responsible for CSC's resistance. Through studying CSCs from hepatocellular carcinoma (HCC), Ma et al (Ma et al, 2008) demonstrated that HCC CSCs confer chemoresistance via preferential induction of AKT/PKB and BCL-2 survival pathways. Using specific AKT1 inhibitors, survival of HCC CSCs can be abolished.

5. How to cope with the therapeutic resistance induced by hypoxia

5.1 Targeting genes related with hypoxia

Based on the mentioned above, it is evident that blockage of paradoxical activation of genes by transgenic techniques or improvement of hypoxic status in tumor microenvironment could overcome hypoxia-induced therapeutic resistance and relapse in HNSCC. Gene therapy mostly pointing to some critical target genes and associated gene products in HIF-1 α , UPR and mTOR pathways and some activators and regulators of HIF-1 α in alternative pathways such as EGFR and STAT3 pathways offers hope in this regard. However, transgenic techniques using either viral or non-viral vectors have limitations for application in human body. As previously described, dynamic hypoxic heterogeneity exists in solid tumors. It is difficult to determine the specificity and effectiveness of a single-gene targeted therapy to hypoxic cells in a huge tumor bulk. Therefore, there should be a long-term exploration before gene therapy can be used as an efficient method of modifying therapeutic resistance in HNSCC. It is likely that a strategy targeting multiple genes would be a potential solution to CSC-associated therapeutic resistance under the condition that hypoxic status in each individual tumor is evaluated objectively.

5.2 Targeting CSCs and hypoxic microenvironment

As stated, only CSCs can facilitate tumorigenesis and confer therapeutic resistance, which is the major cause of therapeutic failure. Therefore, successful targeting on CSCs is expected to provide a chance of cure for cancers. To date, targeting on CSCs has been faced with difficulties, because mechanisms underlying CSC-related therapeutic resistance have not been well understood. Although Notch, Oct-4, Wnt, Bmi and other stemness related factors were demonstrated to play a critical role in CSCs physiology, it is difficult to target them specifically in CSCs among the huge population of cancer cells. It is interesting to note that the clinical course of anti-ABCG-2 drugs in cancer treatment mirrors that of anti-bacterial agents in the control of infection. Based on this observation, some scholars believe that CSCs also experience evolutionary processes, and the driving force for these processes, selection stress by microenvironment, should be the target for cancer therapy. Although more CSCs markers have been identified in cancers, CSCs isolated by these markers are in minority, approximately 2-5%. However, increasing evidence has revealed that CSCs are not rare when isolated based on stem traits, which suggests that CSC markers are limited and not all CSCs express the same markers. Therefore, it is possible that CSCs are existing in separate subpopulations with distinct biological features, which are affected by their niche, and what is worse, these features are in constant change. Given that targeting CSCs is a putative approach, it would be much more important to concentrate on the manipulation of niche as the direct target in curing cancers. For example, we can resort to approaches to maintain the homeostasis of the niche by manipulating non-cellular components, especially fluctuating hypoxia. Consistent with this idea, traditional Chinese medicine (TCM) is to cure the disease by rectifying imbalance in body environment and re-establish the homeostasis of the human body, which may offer some hope in this regard. And intriguingly, lots of herbs have been identified as antioxidant compounds. Cai et al (Cai et al, 2004) have demonstrated that 112 traditional chinese medicinal plants used as anti-cancer herbs have a more powerful antioxidant activity compared with common vegetables and fruits which are considered as good natural sources of dietary antioxidants. Tang et al (Tang et al, 2004) also identified the antioxidant function of TCM extracts. These pieces of evidence implicate that TCM is a promising strategy capable of targeting on ROS-induced evolution of CSCs. Indeed, data from several reports have provided direct evidence that some herbs in TCM could target CSCs. Observations made by Jiang et al (Jiang et al, 1983) demonstrated that camptothecin and harringtonin could inhibit the clonal formation of human stem cells. Furthermore, anti-tumor and therapeutic resistance-reversing effects of some phytochemicals such as Curcumin have been confirmed and proven to be prospective, which exhibit the capability of targeting side population cells (Fong et al, 2010). More recently, high inhibitory effect on breast cancer cells was acquired by combining stealthy liposomes from vinorelbine and parthenolide (Liu et al, 2008). Taken together, chemotherapy combined with TCM may dominate anti-cancer treatment if the niche is properly manipulated to overcome the chemoresistance of CSCs resulting from genetic instability.

5.3 Inducing UPR pro-death arms

As is known, UPR is a double-edged sword. On the one side, it can help tumor cells relieve hypoxic stress ; On the other hand, UPR can induce apoptosis or autophagy-related cell

death under severe stress. Induction of UPR pro-death arms may be a promising modality to reverse hypoxia-induced resistance to traditional therapy. At this point, some agents, such as PIs, which can enhance ER overload, represent a promising perspective. PIs inhibit proteasome to reduce ERAD, which can intensify ER-stress caused by accumulation of unfolded protein. Recently, Fels et al (Fels et al, 2008) found that PIs can effectively enhance UPR responses of hypoxic tumor cells and ameliorating ER-stress can reverse PIs effects. They demonstrated that hypoxic tumor cells treated by PIs underwent apoptosis, autophagy and necrosis. Intriguingly, some groups reported that tumor cells can activate STAT3 to resist PIs therapy in HNSCC (C. Li et al, 2009). Therefore, PIs combined with STAT3 inhibition could achieve potential efficiency.

5.4 Chopping off hypoxia from the “root”

For strategies used to improve local hypoxia, some groups have tried using inhalation of Carbogen (95%O₂ and 5%CO₂) and hyperbaric oxygen chambers to improve local hypoxic condition within tumors, and thus therapeutic resistance, but the results are not as satisfactory. In this regard, it is necessary to modify traditional approaches and to explore a new way of oxygen delivery to rectify the intratumoral hypoxic condition. It is a common sense that vascular structure of tumor is very different from its counterpart of normal tissue, the former of which exhibits architectural distortion, higher permeability and irregular infuse, facilitating fluctuating hypoxia and providing specific target strategy. Vascular disrupting agents (VDAs) serve as a novel type of anti-cancer target agent. In contrast with angiogenesis inhibitors (AIs) that mostly prevent neoformation of vascular structure, VDAs directly block or damage existing blood vessels in tumor bulk to commit necrosis. To date, VDAs have been in phase of clinic trails, and small molecular VDAs have been mostly studied. The mechanisms of VDAs action include: 1) induce TNF- α secretion by tumor cells to cause apoptosis of endothelial cells constituting microvessels; 2) through binding to microtubule protein, VDAs facilitate disaggregation of microtubules to damage cell skeleton of vascular endothelium. VDAs have been believed to cause intratumoral necrosis, leaving the remaining periphery to be oxygenated. Therefore, combination of VDAs may cut both fluctuating and continuous hypoxia from the “boot”.

Although improvement of tumor hypoxia has been achieved, imaging results are not always consistent with changes of endogenous markers of hypoxia, indicating that the improvement of intratumor hypoxia as observed by imaging does not represent the thorough rectification of intracellular hypoxic metabolisms of the cancer cells. Therefore, it is highly likely that there exist a “time gap” between improvement of intratumor hypoxia and thorough rectification of intracellular hypoxic metabolisms. Currently, length of this window phase is unclear. It is of paramount importance for hypoxic cells to gain thorough recovery of the intracellular oxygenation using this compensation time and become more susceptible to chemoradiation.

6. Future directions

Up till now, the impact of hypoxia on CSCs in HNSCC and its relation between chemo- and radiotherapeutic resistance is largely unknown. To further elucidate the causes of chemo- and radiotherapeutic resistance and post-treatment relapse in HNSCC with respect to effects

of tumor microenvironment on tumor cells, the first step is to study how hypoxic microenvironment regulates CSCs in HNSCC. Through establishment of HIF-1 α knock-down cell lines (HIF-1 α ^{-/-}), the correlation between induction of HIF-1 α and related gene expressions associated with self-renewal as well as multipotency of CSCs is to be observed. Meanwhile, the differential expression of these genes between CD133⁺ and CD133⁻ cells must be documented. Furthermore, the proliferative activity of CD133⁺ CSCs should be measured by culturing HIF-1 α ^{+/+} and HIF-1 α ^{-/-} cells under normal or hypoxic conditions, thereby to understand whether hypoxic microenvironment modulates the differentiation and proliferation of CSCs by regulation of HIF-1 α in HNSCC.

The established concept of interaction between tumor hypoxic microenvironment and CSCs helps us to further understand the mechanisms behind the therapeutic resistance in HNSCC. If CSCs are taken as anti-cancer targets, the strategies by focusing on tumor microenvironment will be promising for purposely intervention of CSCs (Iwasaki & Suda, 2009). It can be inferred that CSCs are the critical element responsible for therapeutic resistance in HNSCC. Improving hypoxic conditions and regulating CSCs-related signaling pathways during chemo- and radiotherapy of HNSCC offers hope for reversion of therapeutic sensitivity in HNSCC and elimination of therapeutic resistance and relapse, aiming at improving outcomes of HNSCC.

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New Therapeutic Strategies in Small Cell Lung Cancer: The Stem Cell Target

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1. Introduction

In 1889, Sir S. Paget introduced the *soil and seed* hypothesis of metastasis to medicine and credited the idea to Fuchs. In Paget's study, he concluded that the distribution of metastases cannot be due to chance alone and that different tissues provide optimal conditions for the growth of specific cancers. In the *soil and seed* metaphor, the *soil* refers to the secondary site of tumour growth and development and perhaps the chemical signals produced in the microenvironment at the sites of metastasis. The *seed* is the ostensible stem cell or tumour-initiating cell from the primary tumour. These tumour-initiating cells are the tumorigenic force behind tumour initiation, growth, metastasis, drug resistance, and relapse. In a variation of this idea, called the *homing* hypothesis, a secondary signal secreted by cells at the future metastatic sites "calls" the tumour cells to the site and permits them to proliferate in the new environment. In this hypothesis, the *seed* produces cell surface receptors that are able to recognise the site demarcated by the *soil*. Although the mechanisms that define tissue specificity remain obscure, researchers have focused on small messenger molecules as attractants and larger cell surface receptors that guide the tumour-initiating cells. Based on the hypothesis introduced by Paget, other groups have focused on chemokines and their receptors as viable candidates for *soil and seed* signalling and have proposed a "spatial and temporal code" composed of specific combinations of such molecules, while other molecules are responsible for neovascularisation, metastasis, and immunosurveillance avoidance. Lung cancers result from complex genetic and epigenetic changes and are characterised by stepwise malignant progression of cancer cells with an associated accumulation of genetic alterations. This process, referred to as multistep carcinogenesis, develops through the clonal evolution of initiated lung cells. Initiation consists of the acquisition of defined genetic alterations in a small number of genes that confer a proliferative advantage and facilitate progression towards invasive neoplasia. Although many of these genetic changes occur independently of histological type, their frequency and timing of occurrence with respect to cancer progression differ between small cell lung carcinomas (SCLC), which may originate from epithelial cells with neuroendocrine features, and non-SCLCs, which

originate from bronchial, bronchiolar or alveolar epithelial cells. Furthermore, a number of genetic and epigenetic differences have been identified between squamous cell carcinoma (SCC), which arises from bronchial epithelial cells through a squamous metaplasia/dysplasia process, and adenocarcinoma (ADC), which is derived from alveolar or bronchiolar epithelial cells. Hence, lung tumours have been classified according to tumour morphology, but classification is complicated by the fact that a number of different histologic tumour characteristics frequently exist within the same neoplasm. In the 1990s, SCLC accounted for approximately one-quarter of all lung cancers, but a recent Surveillance Epidemiology and End Results (SEER) database analysis found that the incidence has since decreased to approximately 13%. SCLC now accounts for 15% of all newly diagnosed lung cancers and 60% to 70% of patients present with extensive stage (ES) tumours. For patients with limited-stage (LS)-SCLC, standard treatment has consisted of chemotherapy combined with radiotherapy (RT), while chemotherapy alone has been the standard for ES-SCLC patients. Despite a high initial rate of response to chemotherapy, most patients die from rapid recurrence. The median range of survival time after diagnosis for patients with ES-SCLC is 8 to 10 months, and only 5% to 10% of patients survive for as long as 2 years. Although chemotherapy is an essential component in the treatment of SCLC, improvements in survival in the past two decades have primarily been achieved through the appropriate application of radiotherapy. The standard treatment for patients outside of clinical trials is as follows: LS-SCLC patients receive combination chemotherapy, which generally consists of cisplatin and etoposide, with concurrent thoracic radiotherapy; and ES-SCLC patients receive combination chemotherapy (etoposide and cisplatin or carboplatin). The current standard treatment for most cancers involves some combination of chemotherapy, hormonal therapy, radiation treatment, and a growing list of molecularly targeted therapeutics, depending on the tumour characteristics and stage. Following treatment, tumour regression is normally used as an indicator of therapeutic success. To better treat cancer, the new ideas regarding CSCs must be integrated into our strategies for clinical intervention. One approach to inhibit cancer stem cells is to target the proteins that are essential for the growth and maintenance of stem cells, such as the growth regulatory pathways that function in embryonic cells. One pathway, controlled by the Hedgehog (Hh) signalling molecule, contains several genes that function as either tumour suppressor genes or oncogenes. Other pathways that are critical to embryonic development and are potentially important in cancer have also been described, including the Wnt and Notch pathways. These pathways are also subjects of drug development for the treatment of a number of conditions.

2. Development of the airway

The respiratory system is an outgrowth of the ventral wall of the foregut, and the epithelium of the larynx, trachea, bronchi, and alveoli originates in the endoderm. The cartilaginous, muscular, and connective tissue components arise in the mesoderm. In the fourth week of development, the tracheo-oesophageal septum separates the trachea from the foregut, dividing the foregut into the lung bud anteriorly and the oesophagus posteriorly. Lungs are composed of two primary tissue layers, namely epithelium and mesenchyme. Previous investigations have demonstrated that mutual interactions between these two tissues are essential for the sequential events of organogenesis, determination, growth, morphogenesis,

and cytodifferentiation. This mutual interaction is defined as embryonic induction. The morphogenesis and cytodifferentiation of embryonic lung epithelial components are modulated by surrounding mesenchymal components. In embryonic organs that are formed by a process of progressive branching of the epithelium, such as the lung, the mesenchyme plays a determining role in the formation of the characteristic morphology of the organ. Increasing evidence has suggested that the formation of the tracheo-bronchial tree and alveoli results from heterogeneity of the epithelial-mesenchymal interactions along the developing respiratory tract. Genetic data have supported this idea and shown that this heterogeneity is likely the result of activation of distinct networks of signalling molecules along the proximal-distal axis. Among these signals, fibroblast growth factors, retinoids, Sonic hedgehog and transforming growth factors appear to play prominent roles. Variable levels of FGFs, Shh, TGF β , EGF, retinoid receptors, and other signals that play a role in lung morphogenesis have been reported in the adult lung. Increasing genetic evidence has suggested that the Gli genes play multiple roles during prenatal development, particularly in the lung. All three genes are widely expressed during embryonic development in distinct but sometimes overlapping domains. The extent to which these regulators are expressed during adult life to mediate cellular activities in processes such as post-injury repair and compensatory lung growth is currently unclear. Lung bud initiation has been well-established to be regulated by the Sonic hedgehog (Shh) signalling pathway, by fibroblast growth factor (FGF) receptor signalling, and likely by retinoid-related signalling. Branching morphogenesis is a dichotomous branching process that involves defining the proximal-distal structure of the conducting airway prior to the saccular stage and is dependent on the integrated effects of the conducting airway prior to the saccular stage. Several growth factors have been implicated in branching morphogenesis. Epidermal growth factor (EGF) and transforming growth factor (TGF α) are expressed in embryonic murine lung; both factors influence growth and branching morphogenesis. During early lung branching, the EGF protein is present in bronchial epithelial cells, whereas the EGF mRNA is localised to the mesenchyme; this discordance between the location of the protein and mRNA suggests that EGF is produced by the mesenchyme and acts on the epithelium. EGF receptors (EGFR) have been found in epithelial cells and in the mesenchyme surrounding the branching epithelium of the mouse lung. These data are compatible with the notion that EGF acts in an autocrine and paracrine fashion. Retinoic acid (RA) and glucocorticoid signalling pathways have long been appreciated as major contributors to prenatal and postnatal lung maturation, and some evidence exists for their coordination or antagonism during lung development. Retinoic acid also plays an important role in morphogenesis. RA stimulates lung epithelial branching activity via an epithelial-mesenchymal interaction that, in part, involves the up-regulation of the expression of EGFR, Insulin-like Growth Factors (IGF), basic Fibroblast Growth Factor (bFGF-2), and PDGF.

3. The airway stem cells

For several years, a consensus has been achieved that various types of stem cells exist, differing according to their position within the pulmonary tree, and that the stem cells often form pools that are ready to proliferate in response to injury and effect local repair. The classical subdivision of the airway tree into regions with individual stem cell harbours was

accepted many years ago. Thus, the local repopulating cells of the trachea (basal, mucous secretory), bronchus (basal, mucous secretory), bronchiole (Clara) and alveolus (type II pneumocytes) remain, for the most part, the first reserve of airway stem cells. Stem cell research in the lung has progressed rather slowly due to the anatomical and functional complexities associated with the numerous distinct cell types. This organ must be divided into various anatomical regions when considering multipotent progenitor or stem cells. Evidence has clearly suggested that multipotent progenitors of the conducting airway epithelium and gas-exchange alveolar regions are derived from different populations of stem cells that are anatomically separated in the lung. Stem cell niches in the conducting airways must also be uniquely divided between the proximal and distal regions. Bronchial airways harbour at least two distinct progenitor cell populations. Both basal and non-ciliated secretory cell types of the bronchial airways have been shown to exhibit proliferative capacity. The disparity between bronchial and bronchiolar airways is consistent with a mechanism in which the activity of distinct progenitor cell pools accounts for the regional differences both in lineage specifications during lung development and in the cellular composition of tracheo-bronchial and bronchiolar airways (Table 1).

Tissue	Epithelial stem cell niche	Daughter cells
Lung proximal	Tracheal basal cell	Mucous, ciliated, neuroendocrine
	Tracheal mucus-gland duct cell	Mucous, ciliated, neuroendocrine
	Tracheal secretory cell	Mucous, ciliated, neuroendocrine
	Bronchiolar Clara cell	Mucous, ciliated (Type I/II pneumocyte)
Distal	Alveolar type II pneumocyte	Type I and II pneumocytes (Clara cells),
	Neuroendocrine	PNEC (and Clara cells)

Table 1. Stem or progenitor cell characteristics in the airway

Epithelial cell composition and zone boundaries depend on both the species and the individual animal history. In normal mice, a renewing cell system encompassing a gland-containing, pseudostratified epithelium with Clara cells and few goblet cells is present in the upper trachea. In rats, a similar system, but with more goblet cells and no Clara cells, is present in the entire trachea, whereas this zone in humans penetrates many bronchial generations. Distally, the airway epithelium becomes glandless and cuboidal. This region is dominated by a Clara cell based lineage system before its transformation into a type II cell-based system in the alveoli. Stem cell niches in the airway have been characterised through experiments with rodent models. Stem cells in the proximal mouse trachea reside in the submucous gland duct, whereas those from the bronchi and bronchioles come from a subset of cells expressing a Clara-cell-specific protein located near neuroendocrine bodies and bronchoalveolar-duct junctions.

4. Stem cells and lung cancer

Stem cells give rise to a number of different cell types that can be classified into three groups: fully differentiated cells, transit-amplifying cells, and stem cells. The fully differentiated cells are mitotically inactive cells. These cells are at the end stages of cellular differentiation and will never re-enter the active cell cycle. The transit-amplifying (TA) cells are fast growing cells that are not fully differentiated. TA cells are able to proliferate for several generations, but they eventually terminally differentiate and must be replenished by

the SC. Pluripotency is the ability of a SC to differentiate into the heterogeneous population of cells that comprise a tissue or, in the case of cancer stem cells (CSCs), a tumour. There is growing evidence that some, if not all, tumours are derived from cells with the stem cell properties of self-renewal, multilineage potential, and proliferative capacity. Stem cells are candidates as the “cell of origin” for cancer because they have a pre-existing capacity for self-renewal and unlimited replication. In addition, stem cells are relatively long-lived compared to other cells within tissues. They therefore have a greater opportunity to accumulate the multiple additional mutations that may be required to increase the rate of cell proliferation and produce clinically significant cancers. Recent work has suggested that a subpopulation of cancer cells with stem-cell-like properties may be critical for triggering tumour development. Insights into the function and characteristics of CSCs offer a novel approach to understanding the progression of metastasis. Given that a single cancer cell can drive the formation of a metastatic tumour, CSCs are likely responsible for distant tumorigenesis and primary tumour formation. Thus, research focussed on the role of CSCs in primary lesions has led to discovery that CSCs can drive tumour formation in leukaemia and various solid tumours. While little work has been done to elucidate the role of CSCs in metastasis, properties of CSCs, such as self-renewal and differentiation, make them logical candidates as metastatic colonisers. To facilitate the discussion of CSCs with different metastatic ability, a distinction should be made when referring to two potential subtypes of CSCs: primary tumour cancer stem cells (pCSCs) and metastatic cancer stem cells (mCSCs). The first, pCSCs, constitute the original population of tumorigenic cells that initiate the formation of haematopoietic and solid tumours and are the centre of most CSC. The second group, mCSCs, represent a distinct population of cells with the intrinsic properties to disseminate from the primary site and generate the distant metastases. Although other cell subpopulations may break free of the primary tumour and invade the blood stream, mCSCs, like their pCSCs counterparts, are solely responsible for the initiation of tumours. mCSCs are related to pCSCs in the essential properties of self-renewal and differentiation that are needed for the propagation of the bulk of the tumour, but the two cell types differ in key ways. Unlike pCSCs, mCSCs disseminate from the tumour, colonise foreign tissue, and likely have additional alterations (whether mutational, epigenetic, or adaptive) that allow survival and propagation in secondary sites. The key to developing effective future therapies thus seems to be the identification and characterisation of these cancer stem cells and the development of drugs that specifically target these cells. Classically, the stem/progenitor cells of the pulmonary epithelium have been considered the basal cells in the proximal airways, Clara cells in the bronchioles and type II pneumocytes in the alveoli. There is evidence that the basal and parabasal cells are stem cells in the human lung. Clara cells have been shown to be the progenitors of themselves and of ciliated cells in the bronchioles. Recent research has established that a subset of Clara cells fulfils the criteria of adult, niche-specific stem cells. Pools of stem cells have been discovered that express Clara cell secretory protein (CCSP) but are not typical Clara cells. These variant CCSP-expressing (or vCE) cells show multipotent differentiation. The vCE cells are located in discrete pools in neuroepithelial bodies and at the broncho-alveolar duct junction. In the trachea and bronchi, the basal cells are widely believed to be stem cells. The basal cells and the parabasal cells that lie just above them certainly form a pluripotential reserve cell that, unlike the surrounding epithelium, usually survives injury. Procedures that involve denuding the trachea have demonstrated the capacity of basal cells to produce all of the major cell phenotypes found in the trachea, including basal, ciliated, goblet and granular secretory

cells. Controversially, pulmonary neuroendocrine cell (PNEC) populations have been suggested to be able to proliferate and serve as a reservoir of progenitor/stem cells that are capable of epithelial regeneration.

Stem/progenitor	Daughter	Lineage progression
Basal	Basal	
	Mucous	Ciliated
	Secretory	Ciliated
Tracheal Gland duct	PNEC	
	Basal	
	Mucous	
Clara	Ciliated	
	Clara	
	PNEC	
Type II	Type II?	
	Type II	
	Type I	
	PNEC	
PNEC	Clara	
	Clara	

Table 2. Possible lung cell lineages. Adapted from Otto WRJ. Pathol. 2002.

5. Small cell lung cancer

SCLC is the most common lung tumour in the spectrum of pulmonary neuroendocrine malignancies, which include typical carcinoid (TC), atypical carcinoid (AC), large-cell neuroendocrine carcinoma (LCNEC), and small-cell lung carcinoma (SCLC). The histological classification of SCLC has evolved substantially over the past several decades

	WHO (1967)	WHO (1981)	IASLC (1988)
Oat cell	Lymphocyte-like	Oat cell	Small-cell carcinoma
Polygonal	Polygonal	Intermediate	Small-cell carcinoma
	Fusiform		Mixed small-cell/large-cell carcinoma
	Other	Combined oat cell carcinoma	Combine small-cell carcinoma

WHO: World Health Organization

IASLC: International Association for the Study of Lung Cancer

Table 3. Classification of small-cell lung carcinoma

Interestingly, a large proportion of SCLC contains a component of NSCLC. Approximately 5% to 10% of patients diagnosed with SCLL will have mixed tumours, meaning that other pathologies, such as adenocarcinoma or squamous cell carcinoma, can be found within the pathologic specimen. The WHO classification of SCLC includes only one variant, combined small cell carcinoma, an SCLC with a mixed non-small-cell component (adenocarcinoma,

squamous cell carcinoma, large cell carcinoma, or spindle cell or giant cell carcinoma). Although various synonyms are in the current clinical terminology (anaplastic small-cell carcinoma, small-cell undifferentiated carcinoma, small-cell neuroendocrine carcinoma, oat cell carcinoma, and mixed small-cell/large-cell carcinoma), the use of these terms is discouraged to avoid confusion. Although the precise cell of origin is not known for SCLC, there is probably a pluripotent bronchial precursor cell that can differentiate into each of the major histologic types of lung cancer. However, within the spectrum of neuroendocrine tumours, a closer morphologic and genetic similarity exists between large cell neuroendocrine carcinoma and small cell carcinoma than either typical or atypical carcinoid. Although classified as a neuroendocrine (NE) tumour, the biological origins of this cancer have remained a matter of conjecture. Recently, SCLC has been shown to be dependent on the activation of Hedgehog signalling, an embryonic pathway implicated in the regulation of stem cell fates. This finding sheds new light on the potential histogenesis of SCLC. SCLC and carcinoid tumours both show high-level expression of neuroendocrine genes. Only a few markers are shared between SCLC and carcinoids, whereas a distinct group of genes defines carcinoid tumours, suggesting that carcinoids are highly divergent from malignant lung tumours, as has been reported. Recent studies have shown that the most useful neuroendocrine markers for SCLC in formalin-fixed, paraffin-embedded tissue sections are chromogranin A, synaptophysin, Leu-7, and certain neural cell adhesion molecules (NCAMs). Bombesin or gastrin-related peptide (GRP), keratin (AE1/AE3) and membrane antigen (EMA). DNA analysis of SCLC reveals a high percentage of aneuploidy in up to 85% of cases. Finally, the expression of proliferative markers, such as PCNA, thymidylate synthase, MCM2 and MCM6, is highest in SCLC, which is known to be the most rapidly dividing lung tumour.

6. Targeted agents that have been evaluated in SCLC

Various chemotherapy schemes have been evaluated for SCLC, but the combination of cisplatin and etoposide is widely considered the standard, with observed response rates of 80-85% and approximately 25% of patients obtaining a complete response. However, most patients experience disease relapse, and neither maintenance chemotherapy nor dose-intensive chemotherapy regimens have led to improved outcomes.

6.1 Topoisomerase I and II inhibitors

A topoisomerase I inhibitor, Topotecan, has shown response rates of 14% to 38% in chemosensitive patients, but the response rates in patients with chemorefractory disease are lower. Irinotecan, another topoisomerase I inhibitor, has demonstrated 10% partial response and 22% stable disease in refractory or relapsed SCLC. Etoposide-containing regimens currently remain the standard first line therapy in North America, while irinotecan-containing regimens are used in Japan. Thus, the combination of carboplatin and irinotecan may be a viable alternative to etoposide-containing regimens. Novel topoisomerase I and II inhibitors appear to continue to exhibit activity in patients with SCLC and warrant further investigation in this disease (particularly in non-Asian populations). However, whether these agents will be more active than etoposide remains to be determined.

6.2 Alkylating agents

The results are similar to those seen with other regimens.

6.3 Picoplatin

The role of picoplatin in SCLC is still not well defined and should be further explored in the future.

6.4 Antimetabolites

Pemetrexed has been shown to have minimal activity as a second-line agent in the treatment of patients with SCLC. Elevated thymidylate synthase expression in SCLC tumours has been proposed as one of the reasons for the observed lack of efficacy.

6.5 Antiangiogenic agents

Bevacizumab combined with standard first line therapy of cisplatin plus etoposide has shown a 64% response rate (RR), 4.7 months of progression-free survival (PFS), 30% of PFS at 6 months and 10.9 months of overall survival (OS). Upon employing bevacizumab to cisplatin plus irinotecan, the RR, PFS and OS were similar to those in the study conducted by ECOG. Another trial has reported an 84% overall RR, with PFS of 9.1 months and OS of 12.1 months. The importance of maintenance bevacizumab following combined modality treatment in patients with LD-SCLC is questionable; the response rate and OS are similar to what is seen with traditional chemotherapy with cisplatin, etoposide and radiation alone. Cediranib, a potent inhibitor of both VEGFR-1 and VEGFR-2, also has activity against c-kit, platelet derived growth factor beta (PDGFR- β), and FMS-like tyrosine kinase 4 (Flt-4). The response rate for Cediranib in recurrent SCLC that had progressed following platinum-based chemotherapy did not meet the predefined target. Vandetanib is an oral inhibitor of angiogenesis that targets VEGFR-2 and VEGFR-3 and inhibits tumour growth through activity against RET and EGFR/HER1. No difference in PFS or OS exists in vandetanib-treated patients compared with placebo-treated patients. Sorafenib, an oral multi-kinase inhibitor that targets both tumour proliferation via inhibition of Raf, stem cell factor receptor (KIT), and Flt-3 and angiogenesis by targeting VEGFR-2, VEGFR-3, and PDGFR- β , has been recommended for further evaluation in SCLC. Sunitinib is a novel, multi-targeted, small-molecule inhibitor of VEGFR-1, -2, and -3, PDGFR- α and - β , Flt-3, c-kit, the receptor encoded by the rearranged during transfection (*ret*) proto-oncogene, and Flt3. Thalidomide initially appeared to be a promising drug, but inclusion of this drug has ultimately failed to show any benefit in OS. Thalidomide in combination with chemotherapy in patients with SCLC shows, contrary to the results of the prior study, no significant difference between the thalidomide-treated patients and placebo-treated patients in OS. Based on the results of these trials, the role of anti-angiogenic therapy in the treatment of patients with SCLC remains to be determined. All agents studied to date appear to produce similar response rates and OS that are similar to the results achieved with chemotherapy alone (in most cases). Maintenance therapy with these agents does not appear to be beneficial in patients with SCLC.

6.6 MMP inhibitors

Many trials with MMPi in SCLC have been equally disappointing. Of the multiple MMPs elevated in SCLC, marimistat targets MMP-1, MMP-2, MMP-9 and MMP-12 at low concentrations, while BAY 12-9566 targets MMP-2 at low concentrations.

6.6.1 mTOR inhibitors

At this time, mTOR inhibitors do not appear to be beneficial in the treatment of patients with SCLC.

6.7 Kit inhibition

Imatinib appears not to be beneficial in SCLC, even in patients with known c-kit mutations.

6.8 B cell leukaemia/lymphoma-associated gene 2 (Bcl-2)

Despite these discouraging results, a new class of oral BCL-2 antagonists is currently being developed and evaluated in patients with SCLC.

7. Signalling pathways that drive cancer stem cells

In cancer tissues, homeostasis is tightly regulated to ensure the generation of mature cancer cells throughout life without a depletion of the cancer stem cell pools. Each tissue is composed of a cellular hierarchy including stem cells able to generate all progeny, committed progenitors, and terminally differentiated cells. The stem cells in each tissue are believed to communicate with their microenvironment or surrounding stroma to maintain their homeostasis. Thus, the pathways that control stem cell self-renewal and the microenvironment in which the cancer stem cells (CSCs) reside may both play roles in targeted therapies

7.1 Hedgehog (Hh)

The Hh gene family encodes several secreted glycoproteins, including Indian Hedgehog (Ihh), Desert Hedgehog (Dhh), and Sonic Hedgehog (Shh). These proteins mediate signalling in embryogenesis and development through activation of the Gli family transcription factors. The Hh pathway is somewhat unique in that the signals serve to relieve a series of repressive interactions. The receptor for Hh, the transmembrane protein Patched 1 (Ptch), normally binds and inhibits smoothed (Smoh), a G-protein-coupled receptor that is related to Frizzled (Frz). When secreted Hh binds both Ptch and Hedgehog-interacting protein (Hip), Smoh initiates a transcriptional response. Specifically, Smoh activates the serine/threonine kinase Fused (Fu) to release Gli from sequestration by Suppressor of Fused (SuFu). Subsequently Gli proteins are able to translocate to the nucleus and regulate transcription of cyclin D and E, c-myc, and other genes involved in cell proliferation and differentiation. Shh is one among several important factors derived from the lung endoderm and is required for proliferation, differentiation, and patterning of the mesenchyme. Shh regulates pattern formation of a variety of developing structures, including the formation of the primary lung buds. However, Shh is expressed in the ventral foregut endoderm. Shh is subsequently expressed in a gradient fashion (in the developing lung epithelium) with the highest levels in cells at the tips. In turn, most components of the Shh pathway, including Shh target genes and its receptor Ptch1, are found in the mesenchyme. Shh signalling is initiated upon binding to Ptch1 and results in activation of Shh target genes by Gli transcription factors. Ptch expression in the lung follows the proximal-distal gradient of Shh. Gli1, 2, and 3 are expressed in overlapping but

distinct domains in the lung mesenchyme. The proximal-distal gradient is evident in *Gli1*, which together with *Ptch*, is transcriptionally upregulated by *Shh* and is expressed in the subepithelial mesenchyme. All three *Gli* genes are expressed in the lung mesenchyme during the pseudoglandular stage of development, and mutations in the *Gli* genes give rise to various lung and foregut defects. *Shh* signalling has been implicated in the regulation of *Gli* genes, notably in *Gli1* and *Gli3* transcription in the lung. *Gli2* has also been implicated in the regulation of *Ptch1* and *Gli1* components of the *Shh* signalling cascade in the lung. Thus, *Shh* is part of an epithelial network of regulators that restricts fibroblast growth factor 10 (FGF-10) expression. *Shh*-FGF-10 interaction supports a model in which the growing epithelial bud, which expresses high levels of *Shh*, interacts with a chemotactic source (FGF-10) in the distal mesenchyme for its elimination. This model supports the idea that not only the presence of FGF-10, but also its correct spatial distribution, is necessary for patterning. If FGF-10 signals are diffuse rather than localised, direct clues are lost and branching is disrupted. Importantly, the data suggest that under normal conditions, *Shh* plays a role in controlling FGF-10 expression in the distal lung. Expression of *Shh* and *Ptch* does not seem to be influenced by FGF-10; however, both genes are down-regulated by FGF-7 in lung explant cultures.

7.2 *Gli* genes

The vertebrate *Gli* gene family currently consists of three members, *Gli1*, 2 and 3, which are orthologous to *Drosophila cubitus interruptus* and encode DNA-binding proteins with five zinc fingers.

7.3 BMP-4

Bone Morphogenetic Protein (BMP) belongs to the TGF β superfamily of growth factors, and at least three members (BMP-4, -5 and -7) are present in the developing lung. BMP-4 is an important regulator of epithelial proliferation and proximal-distal cell fate during lung morphogenesis. During branching morphogenesis, BMP-4 is dynamically expressed in the distal epithelium of branching airways. BMP-4 stimulates distal lung formation but might preferentially induce alveolar type I cell fate.

7.4 TGF β -1

TGF β -1 is a member of a sub-family of peptides having at least two other members, all expressed in the developing lung. TGF β signalling is mediated by serine-threonine kinase receptors (type I and II) and Smad transcription factors. TGF β -1 transcripts are uniformly expressed in the sub-epithelial mesenchyme. TGF β -1 protein accumulates later at sites of cleft formation and along proximal airways. TGF β -1 promotes the synthesis of the extracellular matrix, which, when deposited in the epithelial-mesenchymal interface, is thought to prevent local branching.

8. Perspectives and future directions in therapy for SCLC

The recurrence of tumours after initial tumour regression by conventional therapies is also frequent. One potential reason for this recurrence is the failure of current therapies to target CSCs. The design and development of new cancer treatments is therefore necessary to target

stem cell properties, i.e., self-renewal and differentiation. If the malignancy results from a blocked ontogeny, the treatment of cancer by inducing differentiation should be possible. These strategies have had variable success. In addition to inducing differentiation, a number of stem cell self-renewal pathways have been targeted for the treatment of various human tumours. If most solid tumours are composed of a minor population of self-renewing (stem) cells and a large fraction of non-renewing cells, cancer therapy failure following radiation and chemotherapy treatment is not the result of a rare cell evolving from within the tumour but the result of regrowth of the cancer stem cells. Of course, tumour stem cells could accumulate genetic changes that render them even more drug resistant, radiation resistant, or aneuploid. Because cures are achieved for many types of cancer, the cancer stem cells must be eliminated by a given therapeutic strategy. Regardless of which therapeutic paradigm turns out to be most effective, SCLC will clearly have to be treated with a "targeted medicine" approach if chemotherapy is to be widely successful in the clinic. This approach requires that each patient be segregated into a specific treatment group according to the constellation of molecular alterations that define his or her disease. The remarkable variation in genetic profiles across patients suggests that each tumour represents a distinct disease state that can only be effectively treated with precision therapy that targets the specific signalling pathway that is unique to each tumour. An important molecular mechanism that promotes cell differentiation is signal transduction. Signal transduction pathways ensure the reception of the concentration gradients of morphogens and their transformation into the differentiation of cells within tissues and organs. Hence, the key molecular rearrangements at the molecular level may be assumed to be related to changes in genes that participate in signal transduction pathways. In some contexts, these signals may be independently responsible for distinct aspects of tissue self-renewal, such as survival, proliferation and inhibition of differentiation. In other cases, the various signalling cascades may act in a hierarchy and regulate each other. Studies in which pathways are antagonised by treatment with pharmacological agent antagonists and/or agonists of Hh pathway signalling further demonstrate an ongoing requirement for pathway activity in the growth of additional cancer types. As a specific Smo antagonist, cyclopamine may be generally useful in the treatment of such cancers and represents a therapeutic strategy that may be further supported by the absence of observable toxicity in cyclopamine-treated animals. Cyclopamine inhibits Hh pathway activation by binding directly to Smo. This binding interaction is localised in the heptahelical bundle. Moreover, the binding influences the Smo protein conformation. Cyclopamine binding is also sensitive to Ptch function and provides biochemical evidence for an effect of Ptch on the structure of Smo. Cyclopamine appears to interfere with these signalling events by influencing Smo function; cyclopamine antagonises Hh pathway activity in a Ptch-independent manner and exhibits attenuated potency toward an oncogenic, constitutively active form of Smo. Pharmacologic inhibition of the Hh pathway has been necessary as a research tool to understand Hh pathway biology and is an attractive mechanism to evaluate antitumour activity. The first evidence that Smo could be antagonised came with the isolation of compounds called cyclopamine and jervine from corn lilies, which caused teratogenic effects (including cyclopia) in lambs. Significant new therapeutic strategies in SCLC will result from a deep understanding of the biology of response and resistance to targeted therapy. These approaches are in development to block embryonic pathways that play a role in cancer stem cells, including the Notch, Hh, and Wnt pathways.

9. Conclusions

The introduction of effective targeted agents for SCLC has lagged behind that for non-small-cell lung cancer. However, the number of agents now being tested has increased and includes agents that have shown some anti-tumour activity against other types of cancer, such as inhibitors of the Hh signalling pathway. This activity has prompted the development of agents that can inhibit Hh signalling. If the cancer stem cells that are responsible for driving the growth of cancer types associated with Hh pathway activation indeed come from stem cells trapped in a state of active renewal by pathway activities, then a logical therapeutic approach for these cancers would be to impose a state of pathway blockade. As we look towards the future, an important area of investigation will clearly involve analysing how the Hh pathway exerts its effect and whether shared molecular targets are involved in influencing self-renewal in the context of stem cells and cancer. Additionally, Hh probably integrates with other niche-derived signals, such as BMP (Bone Morphogenic Protein), Wnt and Notch. By understanding the molecular events governing CSCs, the development of therapeutics aimed at targeting these cells will become possible. The development of such therapeutics is of paramount importance because CSCs may mediate the resistance to current treatment and the relapse of the most aggressive tumours. This resistance may in part result in the reactivation of several signalling cascades, such as Hh, Wnt, Notch, and EGF, in the CSCs combined with an increase in DNA repair mechanisms and ABC transporter-mediated multi-drug resistance.

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Part 6

Genetic Manipulation and Its Possible Clinical Implications for Squamous Cell Carcinoma

MicroRNA Dysregulation in Squamous Cell Carcinoma of Head and Neck

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1. Introduction

Head and neck cancers refers to cancer arising in the head or neck regions including paranasal sinuses, nasal cavity, nasopharynx, oral cavity, salivary gland, oropharynx, pharynx, hypopharynx, larynx, and lymph node. Histologically, squamous cell carcinoma is the predominant form. The cancer progenitor cells are premalignant cells in the mucosa layer of head and neck. Cumulative genetic and epigenetic alterations lead to behavioural changes from hyperplasia to invasive carcinoma. Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. It is the 4th most common cancer among men in the European Union (Black et al., 1997). In United State, over 12,000 patients died from HNSCC every year (Altekruse et al., 2008). Globally, there are approximately 650,000 new cases of HNSCC and 350,000 patients dying from HNSCC annually (Parkin et al., 2005). Most patients will develop local-regional disease with cervical lymph node involvements. HNSCC is heterogeneous in nature. Early disease might not have any symptoms. Further, the inconspicuous locations of some HNSCC make it difficult to be identified at the early stages. Thus, patients arrive at the clinic by large present late and have poor prognosis. The overall survival rate of HNSCC patients is about 50% (Stell, 1989; Argiris et al, 2004). Development of local recurrence, distant metastasis and secondary primary tumor is also common in HNSCC. Despite the advances of cancer treatment in last several decades, the overall survival rate of HNSCC did not have much improvement (Stell, 1989; Argiris and Eng, 2003).

HNSCC is a multifactorial disease. Major risk factors are alcohol consumption and tobacco use (Jaber et al., 1999). HNSCC is particularly common in countries with high alcohol and tobacco consumption e.g. southern Africa, Australia, Brazil, France, India, The Netherlands, Papua-New Guinea and Switzerland (Parkin et al., 2005). Smoking and drinking habit is associated with early onset of HNSCC (Farshadpour et al., 2007). Patients with alcohol and tobacco use generally have poor prognosis and poor survival rate (Farshadpour et al., 2011). Other risk factors of HNSCC include age, environmental exposures (including UV exposure and viral infection such as Epstein-Barr Virus and Human Papilloma Virus), sex, hygiene, industrial inhalants, and gender (Argiris et al., 2003). Regional lymph node involvement is also an indicator of poor prognosis. About 20-50% N0 patients will develop nodal

metastasis. The overall survival rate reduced to 50% in case if lymph node metastasis is observed (von Buchwald et al., 2002).

Management of HNSCC is based primarily on the tumor locations and stages (Akervall, 2005). For early HNSCC (stage I and II) surgical resection together with radiotherapy is the primary treatment regime. For advanced disease (stage III and IV) multidisciplinary treatment including surgery, radiation and chemotherapy is adopted (Posner, 2010). For loco-regionally advanced HNSCC, concurrent chemo-radiotherapy is used in case where the tumor is unresectable or adverse functional loss will be resulted from the operation (Wong et al., 2011). For oral squamous cell carcinoma, surgical excision of the primary tumor and/or selective neck dissection is the major treatment (Bilde et al., 2006). For pharyngeal and laryngeal SCC, radiotherapy and/or concomitant chemotherapy are commonly used (Lajer et al., 2011). It has been shown that the use of concomitant chemo- and radio-therapy is more effective in advanced HNSCC (Robbins, 2005).

2. MicroRNA

MicroRNA are small non-protein-coding RNA, which regulate mRNA at post-transcriptional level. MicroRNA are small epigenetic regulators usually about 19–22 or 19–25 nucleotides long (Ambrose, 2004). They are highly conserved molecules among different species including nematode, drosophila, vertebrate, and human indicating its significance in cellular functions. MicroRNA was first discovered in 1993 in nematode *Ceanorhabditis elegans* (*C. elegans*) (Lee et al., 1993). Later, the tumor suppressing microRNA let-7 was identified in *C. elegans* and mammalian models. By then, it was proposed that microRNA had a trans-regulatory role through direct binding to the target mRNA. Computational prediction suggested that microRNA are regulating about 30% of human genes (Lewis et al., 2005). Up till now (1 July 2011), 16,772 microRNA are reported in the miRBase (see miRBase at <http://www.mirbase.org>, Release 17) at which 8.9% (1,492) are human microRNA. MicroRNA could bind to the target mRNA in a partial or complete complementary manner. They regulate gene expression by promoting target mRNA degradation and/or hindering mRNA translation (Bushati and Cohen, 2007).

MicroRNA are transcribed in genomic DNA. The genes encoding microRNA are located throughout the human genome in intron, exon, coding / non-coding genes (Lee et al., 2002). MicroRNA are first transcribed by RNA polymerase II into long precursor microRNA. This long RNA will be cleaved by Dorsha (RNase III-type nuclease in the nucleus) generating primary microRNA (60–70 nucleotides hairpin molecules). The primary microRNA are later exported into the cytoplasm by Exportin-5 (a Ras-GTP-dependent dsRNA-binding protein). Primary microRNA will be further processed by Dicer (RNase complex) and TRBP [TAR (transactivation-responsive RNA of HIV-1) RNA-binding protein] forming an asymmetric microRNA: microRNA* intermediate duplex (microRNA* is usually functionless and are degraded subsequently). This duplex molecule is then incorporated with Argonaute-containing RNA-induced silencing (RISC) complex forming a functional post-transcriptional regulator (Bartel, 2004). This functional complex usually binds to the 3' untranslated region of the target mRNA (Lim et al., 2005; Wightman et al., 1993). The complementary binding between microRNA and the target mRNA is not necessary perfect in order to carry out its function as negative regulator. The binding of seed sequence (2-7

nucleotides on the mature microRNA) of the microRNA with the target mRNA would suffice to induce mRNA destabilization and degradation (Filipowicz et al., 2008).

So far, microRNA were identified as negative regulator of specific mRNA (Lim et al., 2005). However, recent findings suggested that microRNA might also act as gene activator. Vasudevan *et al.* demonstrated that miR369-3 could activate translation (Vasudevan et al., 2007). Later, Place *et al.* observed that miR-373 could induce E-cadherin expression in prostate cancer cells (Place et al., 2008). MicroRNA Let-7 can induce upregulation of gene involved in cell cycle arrest (Vasudevan et al., 2007). Although such activating mechanisms are not yet clear, it revealed that many remain to be explored if we want to uncover the exact functions of microRNA in human cells.

3. MicroRNA and cancers

In comparison with the normal counterparts, cancer displays a differential microRNA expression patterns (Lu et al., 2005). Association between human cancers and microRNA dysregulation was first observed in leukemia. Downregulation of miR-15 and miR-16 was first discovered in peripheral blood of chronic lymphocytic leukemia (Calin et al., 2002). For HNSCC, study on individual microRNA was first performed by Jiang *et al.* in 2005 (Jiang et al., 2005). Later, Tran *et al.* performed microRNA expression profiling on head and neck cancer cell lines (Tran et al., 2007).

The microRNA profile of nasopharyngeal carcinoma, oral tongue carcinoma, and laryngeal carcinoma are emerging in the subsequent years (Li et al., 2010; Li et al., 2011; Liu et al., 2009; Rentoft et al., 2011; Scapoli et al., 2010). The underlying mechanism concerning microRNA dysregulation in head and neck cancers is not yet clear although it has been reported that the microRNA processing machinery is upregulated in head and neck cancers (Zhang et al., 2009). Zhang noticed that the microRNA processing enzymes Dicer and Drosha are overexpressed in salivary gland tumor (Zhang et al., 2009). Expression of Drosha (microRNA processor) will affect the phenotype of squamous epithelial cells (Muralidhar et al., 2011).

4. Mechanisms of MicroRNA dysregulation in head and neck cancers

4.1 Chromosomal rearrangement

Chromosomal abnormalities are associated with the development of head and neck cancers (Akervall, 2005; Gollin et al., 2001). Common chromosomal gains in HNSCC include 3q, 5p, 7p, 8q, 9q, 11q13, and 20q. In comparison, losses of chromosomal region were frequently detected on 3p, 9p, 5q, 8p, 13q, 18q, and 21q. Cromer *et al.* reported that genes related to tumorigenesis and metastasis of hypopharyngeal carcinoma were located on 3q27.3, 17q21.2-q21.31, 7q11.22-q22.1 and 11q13.1-q13.3. Chromosomal rearrangement (e.g. deletion or translocation) will result in dysregulation of the gene on the abbreviated loci (Akervall, 2005).

About 50% of the microRNA are located in minimal deleted regions, minimal amplified regions, and breakpoint regions involved in human cancers (Calin et al., 2004). Recently, Persson *et al.*, has shown that t(6;9)(q22-23;p23-24) will lead to fusion of MYB oncogene to the transcription factor gene NFIB in head and neck cancers (Persson et al., 2009). As the 3'-

UTR of MYB is targeted by miR-15a/16, the chromosomal translocation allows the cancer cells to escape control by miR-15a/16. Lee *et al.*, identified that miR-204 located in 9q21.1-22.3, a cancer genomic-associated region of head and neck cancers, is linked to progression of HNSCC (Lee *et al.*, 2010). Loss of heterozygosity (LOH) in these loci are common in HNSCC (Bauer *et al.*, 2008; Spafford *et al.*, 2001).

4.2 DNA hypermethylation

DNA hypermethylation is usually found in the CpG island of tumor suppressor genes. Methylation of the clustered CpG dinucleotides in the CpG island would result in transcriptional silencing of the genes. It is now known that the methylated CpG dinucleotide could also link to the regulation of microRNA expression. Promoter methylation will affect binding of the transcriptional machinery (Zhang *et al.*, 2011). Demethylation treatment of the nasopharyngeal carcinoma cells with demethylating agents would result in let-7 upregulation indicating the involvement of DNA methylation in regulating let-7 expression in nasopharyngeal carcinoma cells (Wong *et al.*, 2011). Kozaki *et al.* identified that DNA methylation is linked to the transcriptional silencing of miR-137 and miR-193a, both of which are tumor suppressing microRNA associated with oral SCC (Kozaki *et al.*, 2008). Apart from microRNA, the methylated microRNA promoter can also be used as a biomarker for HNSCC patients. Langevin *et al.* reported that methylated miR-137 promoter is associated with the clinical pathological characteristic of HNSCC patients (Langevin *et al.*, 2010)

4.3 Genetic polymorphism of microRNA-encoding region

Similar to mRNA, microRNA are also encoded by genomic DNA. Theoretically, any variation in genomic materials will affect the biogenesis and final sequence of the mature microRNA (the seed sequence especially) and affect the specificity of microRNA to their target mRNA. Thus, any genetic variation in the microRNA biogenesis pathway gene, primary microRNA, precursor microRNA or mature microRNA sequence will eventually affect the microRNA regulatory pathways (Slaby *et al.*, 2011)

4.3.1 Single nucleotide polymorphism (SNP) of microRNA-encoding genes

The association between microRNA and SNP has been demonstrated recently (Duan *et al.*, 2007). It has already been demonstrated that SNP will affect the processing of pre-microRNA (Yu *et al.*, 2007). In HNSCC, SNP of miRNA-146a (rs2910164; guanine to cytosine), miR-149 (rs2292832; guanine to thymine), miR-196a2 (rs11614913), and miR-499 (rs3746444; adenine to guanine) are associated with the risk of developing HNSCC (Liu *et al.* 2010). Christensen *et al.* confirmed the association of SNP in miR-196a2 (rs11614913, C/T) with HNSCC. They demonstrated that miR-196a2 polymorphism is associated with the risk of HNSCC (Christensen *et al.*, 2010).

4.3.2 Single nucleotide polymorphism (SNP) of microRNA-targeted genes

Apart from microRNA itself, SNP on microRNA target genes will also affect the binding efficacy of microRNA. Zhang *et al.* demonstrated that SNP associated with microRNA biogenesis pathway genes and microRNA-targeted genes are associated with the prognosis of HNSCC patients (Zhang *et al.*, 2010). They proposed that microRNA-related genetic

polymorphisms might be used as predicative markers for secondary primary and/or recurrence in early HNSCC patients (Zhang et al., 2010).

4.4 MicroRNA dysregulation by candidate oncogene

MicroRNA expression could be controlled by oncogene such as *myc*. He *et al.* reported that the miR-17-92 cluster is transactivated by *myc* (He et al., 2007). In addition, p53 is also shown to be involved in microRNA dysregulation through inducing miR-34 expression (Chang et al., 2008; He et al., 2005; Melo and Estella, 2011; Suzuki et al., 2009).

5. MicroRNA as molecular markers in circulation and body fluids in HNSCC

With the advance of molecular techniques and understanding in cancers, molecular markers are now considered as an effective auxiliary test in conjunction to histological examination in assisting clinical decision making (Hui et al., 2010). The existence of differential microRNA patterns between cancer and the normal counterparts opens up the possibility of using the differential expressed microRNA in monitoring cancers (Krutovskikh et al., 2010). In view of the myriad of functions of microRNA in cancers, de Planell-Saguer and Rodicio suggested that microRNA is potentially useful as biomarkers for cancer onset, prognosis, risk of diseases and cancer classification (de Planell-Saguer and Rodicio, 2011).

MicroRNA is suitable cancer marker as it is highly stable and is resistant to degradation (Li et al., 2007). Significant amount of extracellular microRNA had been detected in peripheral blood, urine, saliva and semen (Mitchell et al., 2008; Hanke et al., 2009; Park et al., 2009; Zubakov et al., 2010) making it a candidate biomarker for detection and surveillance of HNSCC in a non-invasive manner. In addition, it could be extracted in formalin-fixed paraffin-embedded tissues (Li et al., 2007). Circulating microRNA have been found to be significantly elevated in the peripheral blood of head and neck cancer patients. However, there is no direct evidence showing that primary tumor is the only source of the circulating microRNA. Reduction of the circulating microRNA after removal of the primary tumor suggested that primary tumor is one of the major sources of circulating microRNA (Iguchi et al., 2010; Kosaka et al., 2010).

Peripheral blood has high RNase activity, however, circulating microRNA could still exist in cell-free form and remain stable in the blood (Mitchell et al., 2008). Circulating microRNAs are existed in membrane-bound vesicles (about 50 nm to 1 μ m) and are released from the cancer cells through exocytosis (Février & Raposo, 2004; Heijnen et al., 1999; Hunter et al., 2008). Recently, Turchinovich *et al.* performed physical analysis on the characteristics of the circulating microRNA. They noticed that circulating microRNA could exist independently without the vesicle provided that they are bound to the Ago2 protein (Turchinovich et al., 2011).

6. Examples of microRNA dysregulation in HNSCC

Depending on the functions, the dysregulated microRNA could be classified into oncogenic microRNA (onco-miR) and tumor suppressing microRNA (Kozaki et al., 2008; Iorio et al., 2005). Identifying the dysregulated microRNA patterns in HNSCC is useful in selecting suitable microRNA biomarkers for use in HNSCC monitoring (Chang et al., 2008; Ferdin et

al., 2010). We here performed a review on the potential oncogenic microRNA and tumor suppressing microRNA identified in HNSCC.

6.1 Let-7 Family

Human let-7 has multiple isoforms. They are let-7a-1 [Mature sequence: 6 - ugagguaguagguuguauaguu - 27 (MI0000060)], let-7a-2 [Mature sequence: 5 - ugagguaguagguuguauaguu - 26 (MI0000061)], let-7a-3 [Mature sequence: 4 - ugagguaguagguuguauaguu - 25 (MI0000062)], let-7b [Mature sequence: 6 - ugagguaguagguuguguguu - 27 (MI0000063)], let-7c [Mature sequence: 11 - ugagguaguagguuguauaguu - 32 (MI0000064)], let-7d [Mature sequence: 8 - agagguaguagguugcauaguu - 29 (MI0000065)], let-7e (Mature sequence: 8 - ugagguaggagguuguauaguu - 29 (MI0000066)], let-7f-1 [Mature sequence: 7 - ugagguaguagauuguauaguu - 28 (MI0000067)], let-7f-2 [Mature sequence: 8 - ugagguaguagauuguauaguu - 29 (MI0000068)], let-7g (Mature sequence: 5 - ugagguaguaguuugucaguu - 26 (MI0000433)], and let-7i [Mature sequence: 6 - ugagguaguaguuugucaguu - 27 (MI0000434)].

In human cancers, expression of let-7 family is usually reduced suggesting its tumor-suppressing role. In comparison with the normal nasopharyngeal cells, let-7 levels were significantly decreased in the nasopharyngeal carcinoma. Reduced expression levels of let-7 (-a, -b, -d, -e, -g, and -i) were detected in nasopharyngeal carcinoma cells compared to normal nasopharyngeal cells. Ectopic expression of let-7 in nasopharyngeal carcinoma cells reduced cell proliferation (Wong et al., 2011). Moreover, c-Myc expression was inhibited in NPC cells transfected with precursor let-7 (Wong et al., 2011). Association of let-7 with cell proliferation was also observed in oral cancer cells (Jakymiw et al. 2010). Let-7a microRNA expression was inhibited in both laryngeal squamous cancer tissues and in laryngeal cancer cell lines (Hep-2 and BEAS-2B). Let-7a could inhibit proliferation and induce apoptosis in laryngeal carcinoma cells (Long et al., 2009). In Hep-2 cells, overexpression of let-7a could also suppress RAS and c-MYC protein expression (Long et al., 2009). It was demonstrated that up-regulated RAS and c-MYC protein levels had inverse correlation with the down-regulated let-7a levels in cancer tissues (Long et al., 2009). Further, let-7a could enhance the chemosensitivity of head and neck cancer cells and might link to the stemness gene expression pathway (Yu et al., 2011).

6.2 MiR-15a [Mature sequence: 14 - uagcagcacauaugguuugug - 35 (MI0000069)]

Regulation of miR-15a is altered in head and neck cancer cells (Persson et al. 2009). Expression of miR-15a was inversely correlated with protein kinase C which was usually overexpressed in primary HNSCC (Cohen et al., 2009). It has been shown that overexpression of miR-15a suppressed cyclin E protein expression and inhibition of miR-15a enhanced cyclin E protein expression in laryngeal cancer cell line. Precursor miR-15a could also affect DNA synthesis in laryngeal carcinoma cells but the related mechanisms are not yet identified (Cohen et al., 2009). These results indicated that miR-15a might function as a tumor suppressor through regulating the gene associated with the proliferation pathways of cancer cells (Cohen et al., 2009).

6.3 MiR-21 [Mature sequence: 8 - uagcuuaucaugacugauguuga - 29 (MI0000077)]

Overexpression of miR-21 was first reported in human glioblastoma and miR-21 is now recognized as a potent anti-apoptotic factor (Fu et al., 2011). Upregulation of miR-21 is observed in multiple human cancers including breast, cervical, colon, leukemia, liver, lung, ovarian, pancreas, prostate, stomach and thyroid as well as head & neck (Krichevsky et al., 2009; Volinia et al., 2006). Elevated expression of miR-21 is observed in tongue squamous cell carcinomas. Suppressing miR-21 in tongue SCC cell lines (SCC-15 and CAL27) reduced cell survival and induced apoptosis (Li et al., 2009). It has been found that the expression level of miR-21 was reversely correlated with TPM1. The observation suggested that miR-21 may inhibit cell apoptosis partly via silencing the expression of TPM1 (Li et al., 2009). Furthermore, it has been shown that miR-21 expression was an independent prognostic factor associated with survival rate (Li et al., 2009). Moreover, repeated injection of miR-21 antisense oligonucleotide could inhibit tumor formation in nude mice (Li et al., 2009). Laryngeal cancer cell line (JHU-O11) transfected with miR-21 displayed enhanced cell growth (Chang et al., 2008).

6.4 MiR-29 [Mature sequence: 54 - uagcaccuuugaaaucggguua – 75 (MI0000735)]

MiR-29c expression was suppressed in nasopharyngeal carcinomas in comparison with normal healthy nasopharyngeal epithelia (Sengupta et al., 2008). The function of miR-29c is not yet clear. In HeLa cells, transfection of miR-29c precursor could suppress expression of collagen 3A1, 4A1, 15A1, laminin, and thymine-DNA glycosylase (TDG) linking to tumor cell invasiveness and metastasis (Sengupta et al., 2008).

6.5 MiR-100 [Mature sequence: 13 - aaccgguagaucgaacuugug - 34 (MI0000102)] and miR-125b [Mature sequence: 15 - uccugagaccuaacuuguga - 36 (MI0000446)]

Suppression of both miR-100 and miR-125b were reported in HNSCC. Expression levels of miR-125b and miR-100 were decreased in oral squamous cell carcinoma cell lines and tumors of alveolar ridge, buccal mucosa, floor of mouth, retromolar trigone and tongue (Henson et al., 2009). Overexpression of miR-100 and miR-125b inhibited cell proliferation in buccal mucosa cell lines (Henson et al., 2009). Suppressed expression of miR-100 and miR-125b in oral cancer cells may lead to cancer progression and loss of sensitivity to ionizing radiation (Henson et al., 2009).

6.6 MiR-133 family

MiR-133 has 2 isoforms: miR-133a [Mature sequence: 53 - uuuggucccucaaccagcug – 74 (MI0000450)] and miR-133b [Mature sequence: 66 - uuuggucccucaaccagcua – 87 (MI0000822)]. Downregulation of miR-133 had been reported in HNSCC including tongue SCC (Child et al., 2009). Decreased expression of miR-133a and miR-133b was observed in tongue SCC cells. Tongue SCC cell lines (Cal27, HN21B and HN96) transfected with miR-133a and miR-133b precursors showed reduced proliferation rate and elevated apoptosis rate (Wong et al., 2008a). Overexpression of miR-133a and miR-133b reduced the expression of pyruvate kinase type M2 (PKM2) in tongue SCC cell lines (Wong et al., 2008a). The elevated expression of PKM2 in tongue SCC tissues was associated with the down-regulated expression of miR-133a and miR-133b (Wong et al., 2008a).

6.7 MiR-137 [Mature sequence: 59 - uuauugcuaagaauacgcuag – 81 (MI0000454)]

Expression of miR-137 was downregulated in tongue carcinoma cells. Ectopic expression of miR-137 could inhibit cell growth in tongue SCC cell line HSC-6 and HSC-7 (Kozaki et al., 2008). MiR-137 is essential to cell cycle control of HNSCC. MiR-137 mimics enhanced the accumulation of G0-G1 phase cells, suggesting that it was associated with cell cycle arrest at the G1-S checkpoint (Kozaki et al., 2008). Expression of CDK6, E2F6, and NCOA2/TIF2 was suppressed by miR-137 in tongue SCC cell lines (Kozaki et al., 2008). Apart from the microRNA itself, the methylation status of miR-137 promoter has potential clinical value. Methylated miR-137 is a potential prognostic marker in HNSCC and is associated with survival (Langevin et al., 2011).

6.8 MiR-138 [Mature sequence: 23 - agcugguguugugaacaggccg - 45 (MI0000476)]

MiR-138 is linked to cell invasion, cell cycle arrest and apoptosis of HNSCC (Liu et al., 2009). Reduced expression of miR-138 was reported in oral tongue cell lines UM1, UM2, Cal27, SCC1, SCC4, SCC9, SCC15, SCC25 (Liu et al., 2009). High level of miR-138 could reduce migration and invasion rate of tongue cancer cell UM1 and UM2 (Jiang et al., 2010). It has been demonstrated that overexpression of miR-138 could reduce expression of two key genes in the Rho GTPase signaling pathway, RhoC and ROCK2, leading to the reorganization of the stress fibers (Jiang et al., 2010). In contrast, inhibition the expression of miR-138 increased RhoC and ROCK2, contributing to an elongated cell morphology and enhanced cell migration and invasion (Jiang et al., 2010). The expression level of miR-138 was inhibited in hypopharyngeal carcinoma cell line (1386Tu) and oropharyngeal carcinoma cell line (686Tu) compared to non-tumorigenic cells (OKF4-E6/7 and NHOK) (Liu et al., 2009)

6.9 MiR-141 [Mature sequence: 59 - uaacacugucugguaaagaugg – 80 (MI0000457)]

Dysregulation of miR-141 was observed in head and neck cancer. However, its role in the pathogenesis remains unknown. Enhanced miR-141 expression was observed in NPC specimens in comparison with normal nasopharyngeal epithelium. Suppression of miR-141 affected cell cycle, apoptosis, cell growth, migration and invasion in NPC cells (Zhang et al., 2010). It has been shown that miR-141 directly targeted BRD3, UBAP1 and PTEN that are involved in NPC carcinogenesis (Zhang et al., 2010). Furthermore, inhibition of miR-141 affected the expression levels of some important molecules in the Rb/E2F, JNK2 and AKT pathways (Zhang et al., 2010). In contrast, Nurul-Syakima *et al.* demonstrated that miR-141 was downregulated in HNSCC and the results were different from those observed in NPC (Nurul-Syakima et al., 2011). Further studies are warranted to elucidate the role of miR-141 in head and neck cancers.

6.10 MiR-184 [Mature sequence: 53 - uggacggagaacugauagggg – 74 (MI0000481)]

MiR-184 was overexpressed in early oral SCC (Cervigne et al., 2009). Cervigne *et al.* demonstrated that miR-184 was upregulated during the progression of progressive dysplasia and oral SCC suggesting that miR-184 might potentially be used as a biomarker for malignant transformation. In tongue SCC, primary tumor has higher level of miR-184 in comparison with the paired normal epithelial cells. Inhibition of endogenous miR-184 in

tongue SCC cell lines (Cal27, HN21B, and HN96) resulted in reduced cell proliferation rate and enhanced apoptotic rate (Wong et al., 2008b). The observations that miR-184 levels were increased in the plasma before operation and decreased significantly after surgical treatment suggested that plasma miR-184 levels might serve as biomarker in oral tongue SCC patients (Wong et al., 2008b).

6.11 MiR-193a [Mature sequence 21 - ugggucuuugcgggagagauga – 42 (MI0000487)]

The expression of miR-193a was inhibited in buccal mucosa cell line HO-1-N-1 cell line. Furthermore, HO-1-N-1 cell line transfected with miR-193a mimics displayed suppressed cell growth and induced apoptosis (Kozaki et al., 2008). In addition, miR-193a mimics reduced the protein levels of E2F6 and PTK2/FAK (Kozaki et al., 2008).

6.12 MiR-204 [Mature sequence 33 - uucccuuugucauccaugccu – 54 (MI0000284)]

The expression of miR-204 was suppressed in tongue SCC cell lines (SCC58, SCC61, SCC151) and hard palate cell line SCC135 (Lee et al., 2010). Overexpression of miR-204 inhibited migration, adhesion and invasion of HNSCC cell (Lee et al., 2010). MiR-204 expression was reduced in NPC cell lines JSQ3 (Nasal cavity) and SQ38 (pyriform sinus) compared to samples of pooled normal buccal mucosa. NPC cell lines transfected with miR-204 mimics displayed suppressed cell-matrix interaction, motility and invasiveness (Lee et al., 2010).

6.13 MiR-205 [Mature sequence: 34 - uccucauuccaccggagucug – 55 (MI0000285)]

MiR-205 is associated with the epithelial-mesenchymal transition of head and neck carcinoma (Zidar et al., 2011). It was proposed that high expression levels of miR-205 can be used to detect HNSCC positive lymph nodes (Fletcher et al., 2008).

6.14 MiR-222 [Mature sequence: 69 - agcucaucuggcuacugggu – 89 (MI0000299)]

MiR-222 is associated with the aggressiveness of tongue cancer cell lines (Liu et al., 2009b). Overexpression of miR-222 in UM1 resulted in reduced cell invasion (Liu et al., 2009b). It has been shown that miR-222 directly targeted metalloproteinase 1 (MMP1) and manganese superoxide dismutase 2 (SOD2) and suppressed their expression in oral tongue SCC cell lines (Liu et al., 2009b). These results indicated that miR-222 may serve as a novel therapeutic target for oral tongue SCC patients (Liu et al., 2009b).

6.15 Others microRNA dysregulation

It was recently shown that the expression levels of miR-221 to miR-375 could be used to distinguish tumor from normal tissue with high specificity and sensitivity (Avissar et al., 2009). The expression levels of miR-196b, miR-138, miR-155, miR-142-3p, and miR-18a were elevated and expression levels of miR-204, miR-449a, miR-34c-3p, miR-143, and miR-145 were reduced in NPC samples in comparison with normal nasopharyngeal tissues (Chen et al., 2009). Several biological pathways including TGF-Wnt pathways, G1-S cell cycle progression, VEGF signaling pathways, apoptosis and survival pathways, and IP3 signaling pathways are targeted by these down-regulated microRNA (Chen et al., 2009).

Tumor sites	Sub-sites	MicroRNA	Dysregulation	Related functions	References
oral cavity carcinoma	alveolar ridge	miR-100	down-regulated	proliferation	(Henson et al., 2009)
		miR-125b	down-regulated	proliferation	(Henson et al., 2009)
	buccal mucosa	miR-100	down-regulated	proliferation	(Henson et al., 2009)
		miR-125b	down-regulated	proliferation	(Henson et al., 2009)
		miR-193a	down-regulated	growth	(Kozaki et al., 2008)
	floor of mouth	miR-100	down-regulated	proliferation	(Henson et al., 2009)
		miR-125b	down-regulated	proliferation	(Henson et al., 2009)
		miR-138	down-regulated	migration, invasion	(Jiang et al., 2010; Liu et al., 2009a)
	hard palate	miR-204	down-regulated	migration, invasion	(Lee et al., 2010)
	retromolar trigone	miR-100	down-regulated	proliferation	(Henson et al., 2009)
miR-125b		down-regulated	proliferation	(Henson et al., 2009)	
tongue		miR-100	down-regulated	proliferation	(Henson et al., 2009)
		miR-125b	down-regulated	proliferation	(Henson et al., 2009)
		miR-138	down-regulated	migration, invasion	(Henson et al., 2009; Liu et al., 2009a)
		miR-184	up-regulated	invasion	(Liu et al., 2009a)
		miR-204	down-regulated	apoptosis, proliferation	(Wong et al., 2008b)
		miR-222	down-regulated	proliferation	(Lee et al., 2010)
		miR-21	up-regulated	migration, invasion	(Liu et al., 2009b)
		miR-133a	down-regulated	invasion	(Li et al., 2009)
		miR-133b	down-regulated	invasion	(Wong et al., 2008a)
miR-137	down-regulated	apoptosis, survival, proliferation, apoptosis, proliferation, apoptosis, growth	(Wong et al., 2008a) (Kozaki et al., 2008)		
naso pharyngeal carcinoma		miR-196b	up-regulated		(Chen et al., 2009)
		miR-138	up-regulated	proliferation	(Chen et al., 2009)
		miR-155	up-regulated	metastasis	(Chen et al., 2009)
		miR-142-3p	up-regulated	apoptosis, invasion	(Chen et al., 2009)
		miR-18a	up-regulated	invasion	(Chen et al., 2009)
		miR-204	down-regulated	migration, invasion	(Chen et al., 2009)
		miR-449a	down-regulated	invasion	(Chen et al., 2009)
		miR-34c-3p	down-regulated		(Chen et al., 2009)
		miR-143	down-regulated		(Chen et al., 2009)
		miR-145	down-regulated		(Chen et al., 2009)
		let-7 family	down-regulated		(Wong et al., 2011)
		miR-29c	down-regulated		(Sengupta et al., 2008)
		miR-141	up-regulated		(Zhang et al., 2010)
miR-204	down-regulated		(Lee et al., 2010)		

Tumor sites	Sub-sites	MicroRNA	Dysregulation	Related functions	References
pharyngeal carcinoma	oropharynx	miR-138	down-regulated		(Liu et al., 2009a)
	hypo pharynx	miR-138	down-regulated		(Liu et al., 2009a)
laryngeal carcinoma		miR-let-7a	down-regulated	Proliferation,	(Long et al., 2009)
		miR-204	down-regulated	apoptosis	(Lee et al., 2010)
		miR-21	up-regulated	migration,	(Chang et al., 2008)
		miR-15a	down-regulated	invasion growth proliferation	(Cohen et al., 2009)

Table 1. MicroRNA dysregulation in HNSCC

7. The role of viral-encoded microRNA in head and neck cancers

7.1 Epstein-Barr Virus (EBV)

EBV is a member of gamma-Herpes virus and is closely associated with the progression of undifferentiated nasopharyngeal carcinoma (Wei and Sham, 2005). EBV is the first identified oncogenic virus. Expression of EBV-encoded oncoproteins are linked to epithelial-mesenchymal transition of metastatic nasopharyngeal carcinoma (HoriKawa et al., 2011). EBV could alter somatic gene expression by controlling the microRNA biogenesis machinery of the host cells. Li *et al.* observed that LMP1 could induce expression of miR-10b and promote metastasis of nasopharyngeal carcinoma cells (Li et al., 2010). Du *et al.* reported that EBV oncoprotein LMP1 and LMP2A could activate miR-155 expression in nasopharyngeal carcinoma cells which is associated with the nodal status and metastasis of nasopharyngeal carcinoma patients (Du et al., 2011).

Apart from the viral oncoprotein, the microRNA encoded by EBV virus itself is also playing a part in pathogenesis of nasopharyngeal carcinoma cells. EBV-encoded microRNA was first discovered in 2004 (Pfeffer et al., 2004). At present, 25 precursors and 44 mature microRNA were identified (Sanger database Release 16). The identified EBV microRNA are encoded in 2 major clusters: BHRF1 cluster and BART cluster (Lung et al., 2009). Barth *et al.* demonstrated that EBV-BART2 could target EBV DNA polymerase BALF5 hindering the lytic replication of EBV (Barth et al., 2008). EBV-encoded microRNA could regulate the activity of EBV and enhance the survival of the host cells (Lo et al., 2007). For example, BART5-5p could target pro-apoptotic gene PUMA contributing to the resistance to apoptotic agents (Choy et al., 2008).

As mentioned above, expression of the EBV oncoprotein LMP1 (Key viral oncoprotein linked to the pathogenesis of nasopharyngeal carcinoma) is critical in the pathogenesis of nasopharyngeal carcinoma. LMP1 act as tumor necrosis factor receptor (TNFR). It is the activator in multiple cancer-related pathways and could enhance proliferation, migration, and cell cycle progression in nasopharyngeal carcinoma cells (Kung et al., 2011). It is now known that LMP1 expression is partly controlled by the EBV-encoded microRNA. Lo *et al.* demonstrated that BART1-5p, BART16-5p and BART17-5p are involved in the regulation of LMP1 in nasopharyngeal carcinoma cells (Lo et al., 2007). In addition, LMP1 can suppress

somatic gene expression by inducing somatic microRNA expression (Anastasiadou et al., 2011; Motsch et al., 2007).

7.2 Human Papilloma Virus (HPV)

HPV is a DNA virus and could infect squamous epithelial cells (Muno et al., 2003; Tran et al., 2007). HPV infection was closely associated with cervical cancer and account for 70% of the cervical cancers (No et al., 2011). Recent data suggested that it could also play a role in HNSCC. In general, HPV could be found in about 30% of the HNSCC. According to Heller and Münger, HPV is associated with 24% oral cavity cancer and 36% in oropharynx cancer (Hellner and Münger, 2011). HPV infection has also been reported in nasopharyngeal carcinoma (Lo et al., 2010). Increasing evidence suggested that HPV is closely associated with tonsillar cancer with prevalence ranged from 50-100% (Hammarstedt et al., 2006; Nasman et al., 2009; Syrjanen, 2004). Alcohol and tobacco consumption is linked to the risk of HPV infection (Chaturvedi et al., 2008; Tran, 2007). HPV status greatly influences the clinical features and prognosis of head and neck cancer patients (Lajer and Buchwald, 2010). The viral-encoded oncoprotein is a sensitive and specific marker for identifying tonsillar carcinoma patients (Hellner and Münger, 2011).

HPV-infected HNSCC cells had a different microRNA expression pattern in comparison with the HPV-negative counterpart (Wald et al., 2011; Wang et al., 2008). It is now clear that HPV could affect the host microRNA expression patterns resulting in the distinct clinical features (Lajer and Buchwald, 2010). Similar to EBV, HPV-encoded microRNA could modulate the microRNA expression machinery of the host (Wang et al., 2009). Lajer *et al.* reported that HPV infection is closely associated with the alteration of miR-127-3p and miR363 in oral and pharyngeal SCC (Lajer et al., 2011). By interfering the E6-p53 and E7-pRb pathways, HPV E6 and E7 oncoproteins could control expression of miR-15/16 cluster, miR-17-92 family, miR-21, miR-23b, miR-34a, and miR-106b/93/25 cluster in the host cells (Zheng and Wang, 2011).

By the time of writing, there is still no HPV-encoded microRNA reported and its role is largely unknown. In addition, the oncogenic role of HPV is affected by geographic factors (Lajer et al., 2010). The prevalence of HPV-associated HNSCC varies between different geographic regions. Low prevalence is reported in Asia, Central Europe, and Latin America (Kreimer et al., 2005; Ribeiro et al., 2011). HPV is nearly undetectable in tonsillar carcinoma of the Chinese patients (Li et al., 2003). The data suggested that HPV infection is a risk factor for a subset of HNSCC and the molecular pathways associated with HPV-negative HNSCC remain to be elucidated.

8. Methods used in microRNA detection

Similar to gene expression patterns, head and neck cancers had specific microRNA expression patterns. With microRNA profiling, Lu *et al.* could distinguish poorly differentiated carcinoma from the rest (Lu et al., 2005). Thus, there is a need to develop molecular techniques to (1) detect and quantify known microRNA; and (2) identify novel microRNA; and (3) perform global and high throughput microRNA profiling. Since identification of the first microRNA in *C. elegans*, the technologies employed to examine microRNA are fast evolving. The following session will briefly describe the common

methods used in microRNA research. Among all the method, northern blotting is nearly the first use to detect and quantify specific microRNA expression (Lau et al., 2001). To date, this technique is largely replaced by others in detecting and quantifying microRNA. *In situ* hybridization detection is used to monitor the cellular and subcellular distribution of microRNA (Wienholds et al., 2005). *In situ* hybridization could be used on both frozen section and on archival formalin-fixed paraffin-embedded (FFPE) allowing localization of microRNA in clinical specimens. Real-time quantitative PCR is now the most commonly used technique in detecting and quantifying microRNA of interest. With the growing number of microRNA sequence published in the miRBase, real-time quantitative PCR primers and probe set could be designed to amplify specific microRNAs. For high throughout microRNA profiling, different form of microRNA array are commercially available. The oligo-nucleotide arrays allow detection of the whole miRBase library in a single run and are very suitable to use in examining the expression patterns of samples (Liu et al., 2008). Recently, next generation sequencing (deep sequencing) is employed to identify novel microRNA. The technique allows sequencing of the whole genome within weeks. In addition, deep sequencing can be used to identify posttranscriptional modifications in mature microRNAs. Initial studies have suggested that these post-transcriptionally modified, so-called isomiRs, might be evidence of tissue-specific or even tumor-specific distribution (Lee et al., 2010; Kunchenbauer et al., 2008). Commonly used system for microRNA identification includes Solexa (Illumina), SOLiD (ABI), and 454 (Roche) which allows detection of microRNA in low abundance (Fridlander et al., 2008).

9. MicroRNA and epigenetic therapies

MicroRNA could target multiple gene transcripts making it a good choice for systemic therapy of cancers. The rationale of microRNA-based therapy is similar to siRNA-based therapy. Based on the gene sequence, the microRNA/siRNA of a target gene could be synthesized chemically and delivered to the cancer patients. Synthetic microRNA can be used to restore the levels of basal tumor suppressing microRNA in cancer cells. In addition, microRNA antagonist (partially or completely complementary to specific microRNA sequences) can be designed based on the mature microRNA sequence to inhibit the overexpressed oncogenic microRNA in cancer cells (Krutzfeldt et al., 2005). The therapeutic microRNA could be packed into microvesicles and delivered to the cancer sites directly or through the circulation system (Skog et al., 2008). Cancer cell could take up the microvesicles at high efficiency as the constituent of microvesicle are similar to the plasma membrane (They et al., 2002). Elmén *et al.* tested this idea using mouse models and non-human primate models [African green monkeys (*Chlorocebus aethiops*)] (Elmén et al., 2008). They synthesized the miR-122 antagonist and delivered it into the animal model. MiR-122 is related to the cholesterol mechanisms in liver cells. The miR-122 antagonist could be taken by the liver cells resulting in decreased plasma cholesterol levels without any toxicity. Similar to drug treatment, the major challenge of microRNA-based therapy is the efficiency to deliver the therapeutic microRNA to cancer tissues as microvesicle in circulation is actively cleaned up by macrophage and kidney. Further, large microvesicles are difficult to pass through the capillary endothelium and extracellular matrix of head and neck tissues (Bader et al., 2011). Advances in the microRNA delivery system are necessary in order to put microRNA-based therapy into clinical practice.

10. Concluding remarks

HNSCC is a complex disease caused by accumulating genetic, epigenetic and proteomic alterations. MicroRNA is a potent regulator controlling multiple biological processes including cell growth, differentiation, cell death, development and immune responses (Flynt et al., 2008; Stefani et al., 2008; Lodish et al., 2008). With emerging data supporting that microRNA plays a central role in gene dysregulation in human malignancies, unraveling the microRNA expression patterns in different HNSCC is essential and critical if we want to develop better diagnostic and prognostic system for our patients. On the other hand, gaining better insight into the regulatory mechanisms of microRNA would allow us to design therapeutic regime, which targets the disease with better outcome. We could anticipate that our knowledge to HNSCC will be changed with the increase in understanding of microRNA in the coming decades. Translating our knowledge into clinical management will be a beneficial to the treatment and prognosis of our patients.

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Structural Features, Biological Functions of the Alpha-1 Antitrypsin and Contribution to Esophageal Cancer

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1. Introduction

Alpha-1 antitrypsin (AAT) is a member of the serine protease inhibitors (serpin) family. Hepatocytes are the major source of synthesis and secretion of AAT into the blood stream, however macrophages of the lungs also take part in this process to a lower extent [1]. AAT is a proteolytic enzyme which plays major role in the normal physiological processes such as angiogenesis, intravascular fibrinolysis, and wound healing. However it may also participate in pathological conditions such as tumor invasion and metastasis which require degradation of the basement membrane, stimulation of angiogenesis, and migration [2, 3].

Following to synthesis AAT diffuses into tissues where it targets neutrophil elastase, a powerful protease capable of cleaving elastic fibers of alveolar walls and other structural proteins [4]. Apart from synthesis in the liver, AAT may also be synthesized and secreted by the epithelial cells of stomach, intestine, pancreas, and respiratory tract. Additionally, it can be produced by certain cancer cells, including cancers of gastric, colon, and lung. Tumor cells synthesize and release not only an intact native form of AAT, but also a variety of cleaved and/or degraded forms of alpha-1 antitrypsin. AAT has multiple effects on tumor cell viability and play diverse roles in tumorigenesis [2].

Being the most abundant human serum protease inhibitor, AAT is encoded by a single gene of 12.2 kb in length, which is located on the long arm of chromosome 14 (14q31-32.2). The protein is highly polymorphic and a number of alleles have so far been identified for it. These alleles are classified into the following four groups; group 1 or normal allele, whose product is AAT with normal function and serum level ranging from 150 up to 350 mg/dL -1. Group 2 or the deficient alleles is associated with serum AAT level less than 35% of normal subjects. In addition group 2 alleles may also not function normally. Group 3 includes the null allele as this group display no detectable serum AAT; and finally group 4 which includes dysfunctional alleles. The last group encodes AAT present at normal level; however, the AAT produced by this group is a non-functional AAT [5, 6]. Mutations in the AAT gene has shown to be associated with a number of diseases including Cirrhosis, COPD, pneumothorax, asthma,

wegener's granulomatosis, pancreatitis, gallstones, bronchiectasis, pelvic organ prolapse, primary sclerosing cholangitis, autoimmune hepatitis, emphysema (predominantly involving the lower lobes and causing bullae), renal, and arthritis. In addition in other malignancies such as Hepatocellular carcinoma, Bladder carcinoma, Gallbladder cancer, Lymphoma, and lung cancer defects and mutations of AAT have also been reported [7, 8].

2. Alpha-1 antitrypsin deficiency (AATD), conformational disease

Alpha-1 antitrypsin deficiency (AATD) is an autosomal recessive genetic disorder caused by defective production of AAT, which leads to the decreased AAT activity in blood and lungs, and deposition of excessive abnormal AAT protein in liver cells. Severe AAT deficiency causes panacinar emphysema or COPD in adults with complications, especially if they were exposed to cigarette smoke. It also include subjects with various liver diseases in a minority of children and adults [9].

Symptoms of AATD include short dyspnea, wheezing, rhonchi, and rales (Crackles). The patient's symptoms may resemble recurrent respiratory infections or asthma that doesn't respond to treatment. Individuals with AATD may develop emphysema during their thirties or forties even without a history of significant smoking, though smoking greatly increases the risk for emphysema. AATD also causes impaired liver function in some patients and may lead to cirrhosis and liver failure (15%). It is a leading cause of liver transplantation in newborns.

The conformational diseases [10], which include diverse disorders such as Alzheimer's and Parkinson's, amyloidoses, AAT deficiency and the prion encephalopathies, take place due to conformational rearrangements of a specific protein that endows a tendency to aggregate formation and deposition within tissues or cellular compartments [11]. AAT deficiency serves as an excellent model for conformational disease because it is one of the few members of this class for which detailed structural data are available on both the wild type and mutant proteins [11]. Indeed, familial conformational diseases occur when a mutation alters specific conformation of protein resulting in abnormal intermolecular interactions, protein aggregation, and consequent tissue damage. The molecular mechanisms of conformational disease are best understood for the serine protease inhibitor (serpin) superfamily of proteins. The serpinopathies include alpha-1 antitrypsin (SERPINA1) deficiency and the newly characterized familial encephalopathy with neuroserpin inclusion bodies (FENIB) resulting from mutations in the neuroserpin (SERPINI1) gene [12].

Robin Carrell and Lomas [11] have described structural rearrangements that take place when AAT meets and inactivates its target, the serine proteases. In the case of AAT, this inherent instability allows the proteins to undergo loop-sheet polymerization, creating an abnormal structure in which the loop from the active site of one AAT molecule inserts itself as another β -strand into a pre-existing β -sheet of an adjacent molecule [11], [13, 14]. In the figure 1, the mechanism of inhibition of proteases by serpins and mutations resulting in disease has been shown [14]. This intrinsic tendency of wild-type AAT to undergo structural transformation is markedly enhanced in mutant forms. As such forms are more prone to accommodate the extraneous strand from an adjacent molecule since mutations destabilize the sheet, allowing an increased mobility of its constituent strands. This loop-sheet insertion is an example of conversion of a loop to a beta- strand through interactions

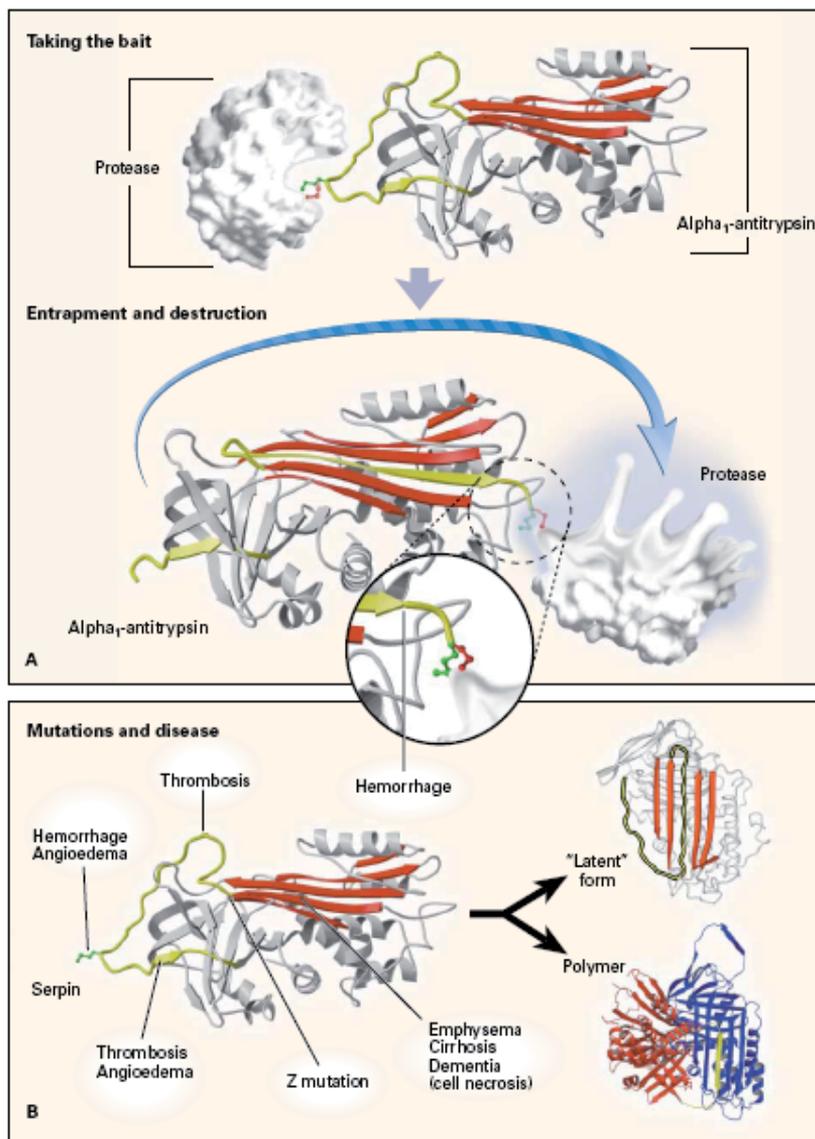


Fig. 1. Mechanism of inhibition of proteases by serpins and mutations resulting in disease.

The mechanism of inhibition of the serpins, represented in Panel A by AAT, is like that of a mousetrap, with a springlike shift from a metastable to a hyperstable state. The protease attacks the reactive center loop (yellow) of alpha1-antitrypsin, with the active serine of the protease (small red side chain) forming a link to the amino acid at the base of the reactive center (small green side chain) of alpha 1-antitrypsin. The resulting cleavage of the reactive loop allows it to snap back into the main *b* sheet (red ribbons with arrows) of the alpha 1 -antitrypsin. This spring-like movement flings the tethered protease to the opposite end of the alpha1 -antitrypsin molecule, distorting its active site (inset) and altering its structure so that it can be destroyed.

A sum 200 different mutations in serpins are known to result in disease (Panel B). In particular, mutations affecting antithrombin confer a predisposition to thrombosis, those affecting C1 inhibitor confer a predisposition to angioedema, and those affecting antiplasmin confer a predisposition to hemorrhage. Mutations at the reactive center result in a loss of function (e.g., causing familial angioedema) or more rarely result in a change in function (e.g., causing hemorrhagic disease). The insertion of an amino acid into the peptide loop containing the reactive center of another serpin, alpha2-antiplasmin, reduces the distortion of the catalytic site (inset) of plasmin, allowing its release, with consequent fibrinolysis and hemorrhage. The most common cause of loss of function of serpin molecules are mutations affecting the critical mobile hinges of the molecule. These lead to spontaneous changes in conformation that allow either the insertion of the intact reactive loop into the main *b* sheet, resulting in the formation of an inactive "latent" form, or the insertion of the loop of one molecule into the *b* sheet of the next, resulting in the formation of polymers. Polymerization occurs in AAT with the common Z variant and with mutations at the opening of the sheet, leading to emphysema and cirrhosis. Mutations at the same site in a neuron-specific serpin result in neurodegeneration and dementia (Carrell R.W. and Lomas D.A. 2002) with pre-existing β -sheet leading to pathological consequence. The tendency to undergo loop-sheet polymerization is not restricted to AAT as other serpins undergo the same transformation. In a rare form of familial encephalopathy where neuronal inclusion bodies (FENIB) form, it was found that inclusion body formation to be the result of a mutant neuroserpin which undergoes loop-sheet polymerization. Structural modeling of the neuroserpin mutants indicate that it may lead to the instability of β -sheet structure, increasing its propensity to gain an extraneous strand. Robin and Carrell [10] have suggested that such β -promiscuity may account not only for the pathologic properties of serpins, but also could explain the 'pathologic property of β -sheets in prion disorder that seems to be caused by the induced transition from α -helix to β -strand [11].

3. AAT, a response to malignancy and inflammation

AAT augments in the serum of gastrointestinal [15], prostate [16], brain [17] as well as biliary tract cancer [18] patients. Also reports indicate increased serum AAT in pancreatic adenocarcinoma [19], breast tumors [20], and esophageal cancer [21]. A significant correlation between serum AAT level and stage of cancer have also been proposed [22, 23]. Several means by which AAT plays role in malignancy and inflammations have been proposed so far as described in the following paragraphs:

- a. Equilibrium hypothesis; it is assumed that changes in the ratio of a particular protease to its cognate inhibitor account for the increased potential of tumor formation [23]. Neutrophil elastase and AAT constitute a pair including protease and protease inhibitor counterpart which are in equilibration. Perturbation of this equilibration causes tissue damage and provides a favorable environment for carcinogenesis and tumor progression. Laboratory and clinical findings have indicated that deficiency in AAT is associated with the increased risk of cancers such as liver, bladder, gall bladder, malignant lymphoma, and lung cancer. Conversely elevated concentration of neutrophil elastase may promote development; invasion and metastasis of many types of cancers as a result of tissue damage and air trap which foster longer exposure to the carcinogens and hence promotion of cancer by degradation of extracellular matrix. In this regard tumor-necrosis-factor signaling pathway plays a role [24].

- b. The other hypothesis suggests the roles that are played by a protease inhibitor *per se*. While imbalanced equilibration between protease to its cognate inhibitor would affect malignancy (as described above), however the inhibitor by itself seems to play more complicated function. The finding of a high serum concentrations of protease inhibitor even in the advanced stage of cancer at first glance was paradoxical, since inhibitors such as AAT are supposed to counteract the destructive activity of proteolytic enzymes (e.g. trypsin). However, it became clear that the role of protease inhibitors is rather complex and that, in most types of cancers, they play important role in modulating the dynamics of the proteolysis, in which proteases, inhibitors, regulators, cytokines and growth factors interact with each other through unknown mechanisms that have yet to be explored [25]. Tissue dependency of protease inhibitor activity is another phenomenon observed in malignancies. Cancers originated from several tissues often produce tumor associated trypsin inhibitor (TATI), however the strongest expression of which could be seen in mucinous ovarian tumors, both in benign and malignant type of tumors. Thus it appears that expression of TATI is regulated by different mechanisms in different tissues. In the other word expression of TATI is tissue dependent. TATI is a 6 kDa peptide, which is synthesized by several tumors and cell lines and produced by the mucosa of the gastrointestinal tract, where it is thought to protect the mucosal cells from proteolysis. Elevated serum and urine level of TATI occurs in connection with many types of cancer, especially mucinous ovarian cancer, pancreatitis, severe infections and tissue destruction. Thus TATI may behave as an acute phase reactant. While elevation of TATI in cancer and pancreatic disease is associated with expression of trypsin, but such a relationship has not been observed for the inflammatory disease. TATI inhibits trypsin-mediated degradation of extracellular matrix by tumor cells. Therefore it might control activation of tumor-associated trypsinogen [26].

Regarding malignant diseases; increased level of TATI has been observed both in serum and urine. In most cancers the increased secretion is caused by tumors, however in acute-phase reaction which is induced by tissue destruction and advanced disease TATI secretion is associated with cancer invasion. The concentration of TATI in serum and urine correlates strongly with tumor invasion. However there is more variation in urine concentrations of TATI; therefore the serum concentration of TATI is preferred if it is going to be used as an indicator of the degree of invasion [27].

3.1 AAT as an acute phase response

AAT is secreted into circulation and increased level of which is the result of at least three mechanisms: production by tumors, leakage from a diseased pancreas and as a reaction against tissue damage and by impaired renal function [28]. For supporting this proposal, Solakidi, *et al*, have shown that elevated serum tumor associated trypsin inhibitor (TATI) could be due to production of TATI by tumors [28]. They reached to this conclusion because none of patients had any signs or previous history of pancreatic disorders or impaired renal function. Moreover gastric and colorectal neoplasms of patients under study were positive for TATI immunoexpression which could explain the elevated TATI in serum as a result of tumor secretion. To find whether elevation of TATI could be explained in terms of acute-phase reaction, measurement of TATI and CRP (C-reactive protein), a prototype of acute-phase reactant proteins was done; the result of which indicated statistically significant correlation between serum TATI and acute-phase reactant protein level. This finding has

indicated regulation of TATI synthesis as an acute-phase reaction. In supporting this notion, Peracaula, *et al*, [29] have suggested that acute-phase proteins might play important role as sensor of diseases. Both level of acute-phase protein and glycosylation have reported to be altered in the inflammation and other diseases including cancer. Factors that promote acute-phase protein synthesis and enhance the expression of specific glycosyltransferases, such as sialyltransferases and fucosyltransferases, may be up-regulated in some tumors which could explain the changes in acute-phase proteins level and specific *N*-glycosylation modifications of some acute-phase proteins in cancer.

4. AAT as a tumor marker and its clinical applicability

Elevation of serum AAT, assessment and association of its phenotype and genotype with regard to specific type of cancer has been subject of many studies on different types of cancers such as gastrointestinal cancers, brain tumors, biliary tract cancer, pancreatic adenocarcinoma, cancers of the prostate, breast, lung and liver [22, 23, 30-32]. Regarding esophageal cancer, there are limited reports available from Japan and Korea as well as our recent report [21, 33]. These reports have suggested that serum AAT level could be considered as tumor marker. Our results show that the mean range of trypsin inhibitor capacity (TIC) and AAT level are significantly higher in patients than in healthy controls [34]. Hong and colleagues have observed significant increase in serum AAT in malignant esophageal cancer patients compared to benign tumors and healthy controls [35].

Recently Hsu and colleagues identified AAT as a potential biomarker of gastric cancer in gastric juice. They showed gastric juice AAT concentration is markedly higher in gastric cancer patients than in healthy subjects, gastric ulcer patients, and duodenal ulcer patients [2].

Investigating the histological pattern and tumor location of patients, Schena and colleagues [36] showed that AAT represents a diagnostic index of neoplastic diseases, highly sensitive but less specific. Saito and colleagues [37] have investigated severe septic complications as the major cause of post-surgery mortality in esophageal cancer patients. They assessed acute phase proteins in the infection related complications post-surgery in a large number of patients with esophageal cancer and have compared this group of patients with a group of gastric cancer patients and the healthy controls. Elevation of AAT, alpha-1 acidglycoprotein, haptoglobin, and ceruloplasmin was more prominent in patients with esophageal cancer. Stenman and colleagues [27] showed that the TATI level increased in serum of patients with pancreatic, gastric, hepatocellular, biliary tract, and colorectal cancer. They concluded that TATI is a sensitive marker. It increased in 75–95% of pancreatic patients, 40–65% of gastric patients, 60–80% of hepatocellular patients, 75–100% of those with biliary tract, and 34–74% of patients with colorectal cancers [27].

An outstanding study carried out by Varela, and López Sáez [38] indicates that plasma level of A1AP (alpha-1 antiprotease); a member of serpins superfamily increases in clinically active cancer compared to the normal controls and normal range values for clinically defined complete remission. The mean value of A1AP was lower in healthy individuals than individuals with chronic non-malignant diseases. Notably A1AP in both groups was lower than individuals with malignant tumors. They also defined a correlation between plasma A1AP level and the type of malignancy such that increased plasma A1AP follows the following scheme; breast, gastrointestinal, head and neck, and lung cancers. Also the mean

range of A1AP has shown to increase in the following clinical order: complete remission, local disease, local-regional disease and metastatic disease. Thus it was concluded that A1AP could be considered as a cancer marker that discriminates cancer from chronic non-tumoral diseases as well as complete clinical remission from relapses[38]. Furthermore Solakidi and colleagues [28] have assessed level of tumor associated trypsin inhibitor (TATI) as well as the carcinoembryonic antigen (CEA), C-reactive protein (CRP), and AAT in association with malignancy or inflammation to demonstrate the role of TATI in gastric and colorectal cancers. Their results showed elevated level of TATI in 50% of patients with gastric cancer and in 41.7% of colorectal cancer patients. Interestingly, elevated level of TATI was observed in only 8% of patients with benign gastrointestinal malignancies. Thus, TATI can be used as a complementary tumor marker in addition to CEA for gastrointestinal cancers. This finding supports our [34] and other reports that elevation of protease inhibitors was observed in the advanced tumor stages. Whether such elevated protease inhibitors, such as TATI and AAT, are functionally effective in the inhibition of proteases or not could be the subject of further investigations. Summarizing our [34] and other reports, it could be concluded that AAT plays role as a biomarker for malignancies including esophageal cancer.

5. AAT and esophageal cancer

Esophageal cancer ranks among the top 10 most frequent cancers, characterized by poor prognosis and 5-years survival rate less than 10%. Despite many efforts and investigations, the mechanism underlying development of esophageal cancer is not well understood [39]. Iran is located in the so-called Asian esophageal cancer belt where reports indicate the highest incidence rate of squamous cell carcinoma of esophagus (SCCE) of the world from certain parts of this country. Although recent reports [40-43] indicate attempts for identifying the molecular etiology of SCCE in addition to achievement of suitable tumor markers for this cancer, such efforts have so far been unconvincing and further efforts are therefore required [34]. Delayed diagnosis is a major problem associated with SCCE that most often results in diagnosis of the disease in the advanced stages of tumorigenesis. In addition, the high invasive phenotype of SCCE together with metastatic potential leads to low curative resection and high frequency of relapses. For developing effective approaches of diagnosis, treatment, and follow-up of SCCE availability of appropriate molecular markers is an asset. In this regard assessing proteases and their inhibitors such as AAT level could be helpful [21, 36, 37].

In a recent study, we investigated the level of AAT in serum of SCCE patients, its trypsin inhibitory capacity (TIC), and association of its phenotype with genotype [21, 36, 37]. AAT deficiency is an inherited disease as it is characterized by the reduced level of AAT in the serum. The two common genotypes of AAT deficiency are type Z (PiZ) and type S (PiS), which are associated with several malignancies. We assessed the AAT phenotype as well as genotypes Z and S in SCCE and their association with malignancy in Azeri patients. Azerbaijan is a region in the north west of Iran composed of at least three provinces where epidemiological studies have indicated a high rate of esophageal cancer from this region in addition to the north eastern region of the country where the highest incidence rate of esophageal cancer in the world has reported from there. AAT phenotype identification was done using isoelectric focusing (IEF) and its genotype was determined by restriction fragment length polymorphism (RFLP). Results indicate that the mean range of trypsin inhibitory capacity (TIC) and AAT nephelometry are significantly different in patients than that of healthy

controls. Measurement of AAT indicated higher level of AAT in patients' serum that was in accordance to what previously reported with regard to patho-physiological status and malignancies (as described in detail above). However, and as a significant finding we found that the augmented AAT is non-functional which accounts for further dysfunction as well as reduction of AAT proper protease inhibitor activity in SCCE patients. Moreover, 97.3% of SCCE patients were homozygote for MM (PiMM) (normal genotype), and only 2.7% were MS heterozygous. Neither of the PiZ and PiS genotypes were identified in the patients ($P < 0.05$). Thus AAT is among those tumor suppressors whose augmentation doesn't correlate with proper function, though it might be dysfunctional in tumors.

Finding a cogent relationship between stages of SCCE at the time of diagnosis and change in marker serum level is important since it affects survival rate following to surgery as well as helping in choosing proper method of treatment. Assessing the pathology records of patients, we found that most diagnosis were done in the late stages of tumorigenesis when tumors were fully grown and developed into highly invasive and metastatic phenotype. This was unfortunately a shortcoming in some studies [21, 36, 37]. Due to nature of SCCE, disease related complications appear late. As a result, diagnosis by clinical examinations becomes only possible in the advanced stages of tumor development. This has been true for 70.3% of cases in our study. Thus low rates of curative resection and high frequency of relapses was observed post-surgery (67.56% of mortality). This finding is in accordance with Yunping and colleagues[39] who found poor prognosis of esophageal cancer with an overall 5 years survival rate less than 10% [39]. Thus measurement of AAT in the late stages of SCCE raises further challenges for the applicability of which as a tumor marker in order to be applied for early stage diagnosis. We propose that further studies are required regarding to the change in the level of AAT as a marker along with analysis its defective functionality in a large sample size to achieve a definite correlation between serum AAT, tumorigenesis, and stages of tumors. Further study is also required to establish a rational relationship between increased level of ATT as a response to defect in its function or as a response to malignancy and inflammation as suggested by other researchers (above) at cellular and molecular level. It should also be kept in mind that most SCCE are diagnosed in the late stages of tumorigenesis due to late referral of patients to clinics. Thus determining augmented AAT level in the early stages remains to be investigated in future studies. One way for elucidation AAT level in early stages of malignancies would be establishing definite relationship between inflammatory diseases and cancers, though the level of AAT increases in inflammation. In addition most malignancies exhibit increased production of inflammatory cytokines. This is also true for SCCE in which increased cyclooxygenase has been well documented [2, 5, 15, 21, 22, 34, 36, 37, 44].

6. Conclusion

The poor prognosis of malignancies including SCCE in addition to late diagnosis in the advanced stages of tumorigenesis for most cancers demand further efforts for achieving specific and appropriate tumor markers for early stage cancer detection. While we did not have access to the patients at the early stage of SCCE, combining our results with other investigations indicate that AAT is a suitable prognostic rather than early stage diagnostic tumor marker as both we and others found its correlation with the advanced stages of tumors. As a tumor marker, AAT is highly sensitive, however, like most other tumor markers; it lacks tissue specificity which in fact is a drawback for its organ or tissue specific

applicability. Increasing the size of the population under study, establishing a rational correlation between malignancies and inflammatory diseases as well as combining AAT with other tumor markers might be helpful for achieving a better picture of AAT applicability for early stage SCCE and other tumors detection as well as specificity for prediction and evaluation of curative treatment.

7. References

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Part 7

Miscellaneous

Vitamin D3 and Its Role in the Treatment of Head and Neck Squamous Cell Carcinoma

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common type of malignancy worldwide, and represents over 6% of the global cancer burden ¹. Each year, HNSCC accounts for nearly 650,000 new cases of cancer, and over 35,000 deaths worldwide ^{1, 2}. Historically, HNSCC has been a challenging disease to manage, with locally advanced disease often requiring a multidisciplinary approach of surgery, chemotherapy and radiation. However, despite significant advances among the aforementioned fields, the current 5-year survival rate of approximately 50% has improved very little over the last 30 years ^{2, 3}. This poor improvement in prognosis is a reflection of the unique treatment challenges presented by HNSCC, which include advanced stages at diagnosis, high recurrence rate after surgical removal and second primary tumor development ⁴. Moreover, not only are current treatment options often non-curative, but they are associated with significant morbidity, including substantial physical deformity and functional deficits. Together, these challenges underscore the importance of developing novel anti-neoplastic treatment strategies which may improve survival and quality of life among HNSCC patients.

One of the more intriguing novel anti-neoplastic agents is 1 α ,25-dihydroxyvitamin D₃ [(1,25(OH)₂D₃), calcitriol], the hormone first identified as an effective treatment for rickets, which has recently garnered wide-spread support as a possible anti-neoplastic agent in a number of different cancer subtypes ⁵. Epidemiological observational studies, performed mainly in the United States, have long postulated a correlation between higher latitude residence and overall cancer incidence and mortality. The first such theory was put forth in 1937 by Peller and Stephenson, who together hypothesized that increased sunlight exposure lowered the risk of cancer ⁶. Four years later, observational studies defined the relationship further, demonstrating a significant association between geographic latitude and cancer mortality ⁷. Since that time, scientists have continued to hypothesize that inadequate serum vitamin D levels at higher latitudes increases one's risk for a number of different cancers including colon, breast, prostate and ovarian cancers ⁸⁻¹¹. Large cohort studies have

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confirmed similar findings among HNSCC patients as well. Such studies have not only demonstrated significantly reduced 25-hydroxyvitamin D [25(OH)D] serum levels among HNSCC patients, but also found disease-free survival time to be significantly associated with 25(OH)D serum levels¹².

However, a definitive causal relationship is perhaps not as clear as it seems. Though these reports have evoked widespread enthusiasm within the scientific community, such zeal has remained tempered. Most of the data regarding an association between 25(OH)D and cancer is derived from retrospective ecological observational studies. To date, no large-scale randomized control trials have ever been done examining serum 25(OH)D levels and a primary outcome of cancer. In fact, smaller randomized control studies are extremely limited and yield conflicting results as to the significance of this relationship¹³. There are several prospective studies examining the relationship, but such studies have offered no consensus findings regarding the association^{14, 15}.

The continued optimism regarding vitamin D's role as an anti-neoplastic agent is sustained, however, by a number of molecular studies which support the biologic feasibility of the theory. In particular, proponents cite the widely expressed vitamin D receptor (VDR), which to date has been found in over 30 different human tissues including lymphocytes, muscle, skin and cancer cells^{16, 17}. Furthermore, the terminal enzyme in the synthesis of active Vitamin D, 1- α -hydroxylase, also exhibits widespread expression allowing for synthesis of the hormonally active metabolite in a number of different cell types. Not only is vitamin D capable of ubiquitous synthesis and action, but *in vivo* studies within both human and animal models suggest that the active metabolite of vitamin D (calcitriol) is capable of eliciting a number of non-calcemic downstream effects. Many of these actions can be considered anti-neoplastic in nature, including anti-inflammatory, proapoptotic, and antiangiogenic properties, the promotion of cell differentiation, and the inhibition of cancer-cell proliferation.

In this chapter we describe the relevant molecular and biological mechanisms regarding Vitamin D metabolism. We also describe the basic and inherent anti-cancerous properties of vitamin D metabolites, with a specific emphasis on its role in the management of HNSCC.

2. Overview of vitamin D biological activity

In 1919, Sir Edward Mellanby conducted a series of elegantly designed experiments on rachitic canines. By placing strict restrictions on both diet and sunlight exposure, he was able to establish a causal relationship between the bone disease rickets and a deficiency of an unidentified trace dietary substance. Mellanby concluded, at that time, that the unknown entity was most likely a previously undescribed fat-soluble vitamin. It was not until 1932, however, that Vitamin D's chemical structure was formally characterized, revealing that Vitamin D was not actually a vitamin, but a steroid hormone. In the late 1960s it was discovered that vitamin D was actually a precursor of a new steroid hormone, 1,25(OH)₂D₃, produced intrarenally. As both the physical structure and physiologic actions of 1,25(OH)₂D₃ continued to be better characterized, it was soon found to be a critical component of both calcium homeostasis and overall bone health. As research progressed into the 21st century, scientists discovered that the hormone impacted a number of important non-calcemic cell regulatory functions.

Today, what is commonly referred to as “Vitamin D” is actually a combination of ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Both compounds are readily available in diet form, with vitamin D₂ being found in plant products and vitamin D₃ found predominately in fatty fish, fish liver oil, and eggs with smaller amounts found in cheese and meat products. In either case, the total amount of either vitamin found within food items is relatively small; thus, many developed countries, including the United States, have turned to fortifying numerous food items, such as milk and juices, with both vitamin D₂ and vitamin D₃. Despite food fortification, diet is not the body’s primary means for acquiring vitamin D₃ stores. Instead, the majority of the body’s cholecalciferol is synthesized *in vivo* through a photosynthetic process occurring in the epidermal layer of sun-exposed skin. Upon exposure to ultraviolet radiation, the epidermis is capable of transforming a cholesterol derivative (7-dehydrocholesterol) into vitamin D₃ (cholecalciferol). The catalysis, however, is not uniform among all populations and is subject to significant and intense variations based on skin pigmentation, sunscreen use, age, gender and even obesity¹⁸.

After synthesis, cholecalciferol enters the blood stream and is transported to the liver. There it undergoes intra-hepatic hydroxylation at the 25 position to create 25-hydroxyvitamin D₃ [25(OH)D₃ or calcidiol]. 25(OH)D₃ is the circulating form of the hormone within the plasma as well as the most accurate biomarker of overall Vitamin D₃ status¹⁹. 25(OH)D₃ is next catalyzed into one of two metabolites: the biologically inert 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] or the biologically active metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]. Exactly which metabolite is created depends entirely on what enzyme is expressed within the local tissue. If the tissue expresses 24-hydroxylase (also known as CYP24A1), then 25(OH)D₃ is modified to become 24,25(OH)₂D₃. This particular metabolite has poor affinity and avidity toward the VDR and is considered the first step in vitamin D decomposition. Alternatively, if the tissue expresses 1- α hydroxylase (also known as CYP27B1), the biologically active metabolite 1,25(OH)₂D₃ is formed. This particular metabolite is most well known for its regulation of calcium homeostasis and is the metabolite capable of exerting anti-neoplastic effects on cancer cells.

3. Vitamin D mechanism of action

The majority of Vitamin D’s hormonal action involves direct interaction with a single VDR. An intranuclear regulator of gene transcription, the VDR is a member of the class II steroid hormone receptors and is closely related to the retinoic acid and thyroid hormone receptors¹⁷. The human receptor is located on chromosome 12q12q14 and consists of nine exons. Fully transcribed, the receptor is a 427-amino acid peptide with relatively simple binding configuration consisting of a DNA-binding domain termed the C-domain, a ligand-binding domain called the E-domain and an activating domain called the F-domain. The receptor acts by interacting with portions of DNA called vitamin D-responsive elements (VDREs). Located in the promoter region of target genes, VDREs are commonly found within 1 kilobase from the gene start sequence. Such areas are composed of hexanucleotide repeats separated by 3 nonspecified nucleotide bases (for example CGACTA-NNN-CGACTA where N represents any nucleotide).

Once 1,25(OH)₂D₃ is transported into the cell, it enters the nucleus where it is capable of binding to VDR. After binding, the 1,25(OH)₂D₃-VDR complex synergistically

heterodimerizes with retinoid-X receptor (RXR). The $1,25(\text{OH})_2\text{D}_3$ -VDR-RXR complex then binds specifically to a VDRE sequence complex, with the VDR occupying the 3' region and the RXR positioned at the 5' end. Conformational changes which occur after $1,25(\text{OH})_2\text{D}_3$ binding results in the release of corepressors and the simultaneous exposure of binding sites for transcriptional coactivators. VDR coactivators are then capable of binding to the complex, which results in the acetylation and subsequent release of histones from the DNA strand. Without histones in place, the appropriate transcription factors are capable of binding to the naked DNA strand and transcription of the target gene is able to initiate.

Certain variables that construct this transcriptional machinery apparatus may have a direct role within the formation of cancer. Recent resequencing of *VDR* gene identified 194 single-nucleotide polymorphisms (SNPs). It has been hypothesized that *VDR* SNPs themselves may have a direct impact on formation and prognosis of certain types of cancer, including HNSCC. The two most widely investigated receptors regarding their impact and role within cancer include a *FokI* restriction fragment-length polymorphism (RFLP) in exon 2 and an adjacent *TaqI* RFLP in exon 9. These two polymorphisms were recently investigated with respect to their association with HNSCC incidence. Such studies yielded mixed results. Findings from one study demonstrated that the heterozygous genotype of *Taq I* (Tt) polymorphism may be associated with an increased susceptibility to HNSCC ²⁰. In a separate study, however, both homozygous variant genotypes, *TaqI* (tt) and *FokI* (ff), were associated with a reduced risk of HNSCC as compared to the more common *TaqI* (TT) and *FokI* (FF) genotypes. Thus, it remains uncertain as to whether or not specific *VDR* SPNs provide a protective or deleterious effect regarding HNSCC risk ²¹. However, demonstrations of both increased and decreased HNSCC risk warrant continued research into how *VDR* SNPs may not only impact cancer incidence, but also mortality and treatment response.

3.1 Biological plausibility within cancer

The general biologic characteristics and mechanisms of action detailed above presented two main molecular hurdles for scientists considering $1,25(\text{OH})_2\text{D}_3$ as a plausible anti-neoplastic agent. First, there was the seemingly restricted localization of $1-\alpha$ hydroxylase. The enzyme responsible for converting vitamin D into its active form is highly expressed in renal proximal tubules and was once thought to be solely expressed intra-renally. However, it is now known that many non-renal cells, including bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes, dendritic cells and several cancer cells all express the converting enzyme capable of producing the hormonally active metabolite $1,25(\text{OH})_2\text{D}_3$ ²². This particular formation of Vitamin D contains a high affinity for the VDR and is capable of eliciting an anti-neoplastic response. It is this formation with which we concern ourselves for the remainder of the discussion.

The second major impasse was the restrictive localization of VDR to skeletal tissues. However, since VDRs were first discovered their localization has been confirmed on a variety of tissues. With respects to SCC, it has been demonstrated that not only do human cancer cell lines (SCL-1, SCL-2) exhibit VDR expression, but in fact they express the receptor in higher amounts than normal human skin cell lines ²³. The discovery of extra-renally expressed $1-\alpha$ hydroxylase and extra-skeletally expressed VDR was paramount in vitamin D's acceptance as a possible anticancer agent. Such findings sustained the molecular

integrity of the theory, paving the way for the initiation of many phase I and phase II clinical trials.

4. Vitamin D and cancer

Thus far, 1,25(OH)₂D₃ has been presented as a secosteroid whose fundamental intra-nuclear action is critical for appropriate regulation of calcium homeostasis and proper bone mineralization. Vitamin D, however, has other non-calcemic regulatory functions, including many which make it an intriguing anti-neoplastic agent. Four separate cellular functions tend to be compromised in order for cancer to thrive within a normal host environment: 1) aberrant regulation of differentiation, 2) dysregulated cellular proliferation, 3) newly acquired ability permitting invasion and metastasis, 4) dysregulated cell destruction of altered self-cells. Numerous published reports have demonstrated that 1,25(OH)₂D₃ is capable of restoring or repairing each of the aforementioned hallmarks of carcinogenesis. It does so in a number of ways, including inhibition of cellular proliferation, disruption and inhibition of angiogenesis, promoting apoptosis, and improving tumor immunosurveillance.

4.1 Cellular proliferation and differentiation

The processes of cellular proliferation and differentiation are intimately intertwined, and the aberrant regulation of each is critically important to the prosperity of growing cancer cells. The normal cell grows along a continuum of cellular differentiation. As a cell proceeds toward terminal differentiation, its ability to divide and proliferate is decreased. This feature is exploited and utilized by many newer anti-neoplastic agents, including 1,25(OH)₂D₃. A promising feature of 1,25(OH)₂D₃ is its ability to manipulate the cell's state of differentiation. *In vitro* studies in human cell lines demonstrated that 1,25(OH)₂D₃ is capable of reducing cell proliferation by inducing cell cycle arrest in G₀/G₁ phase while simultaneously promoting cellular differentiation^{24, 25}.

These results were replicated within murine SCC cell lines which showed that administration of 1,25(OH)₂D₃ to cells induces G₀/G₁ cell-cycle arrest via the transcriptional activation of *CDKN1B* and subsequent dephosphorylation of pRB²⁶. The study demonstrated that in both *in vivo* and *in vitro* studies, the increases seen in *CDKN1B* were the result of downregulated p21 activity, a potent CDK inhibitor²⁶. Human SCC cell lines that positively express VDR showed a potent decrease in cellular proliferation when exposed to 1,25(OH)₂D₃ and its analogues²⁷. Vitamin D's ability to force cells into differentiation may be of critical importance, as such actions can aid in the inhibition of tumor cell proliferation²⁸.

4.2 Angiogenesis

Cancer cells will often acquire the ability to escape the confines of the original tumor nidus. The tumor cell often relies heavily on its angiogenic properties to spread. In theory, limiting the tumor's ability to aberrantly generate rogue blood supplies may limit its ability to thrive and metastasize, offering a potential avenue to achievement of loco-regional control. Several studies have demonstrated that 1,25(OH)₂D₃ contains potent antiangiogenic properties capable of reducing the invasiveness of cancer cells. *In vitro* studies demonstrated that

1,25(OH)₂D₃ and its analogs directly inhibit tumor-derived endothelial cell proliferation in addition to disrupting angiogenic signaling in cancer cells ²⁹.

Within HNSCC cell-lines, treatment with 1,25(OH)₂D₃ resulted in a significantly lower production of pro-angiogenic cytokines, VEGF and PDGF ²⁷. Meanwhile, the pro-inflammatory and proangiogenic cytokine, IL-8, exhibits no change after administration of 1,25(OH)₂D₃ ²⁷. The finding of decreasing concentrations of VEGF has prompted considerable interest, as it has not only been cited as a marker for tumor metastasis, but also used as a prognostic factor in HNSCC patients ^{30,31}.

4.3 Apoptosis

Apart from its actions on the cell cycle, 1,25(OH)₂D₃ can also suppress cancer growth via induction of apoptosis ³²⁻³⁴. Results carried out on human HNSCC cell lines demonstrated that exposure to 1,25(OH)₂D₃ results in the downregulation of several anti-apoptotic genes. In particular, 1,25(OH)₂D₃ represses the expression of the anti-apoptotic, pro-survival gene BCL-2 while simultaneously increasing pro-apoptotic gene products of BAX and BAK ³⁵. Studies completed on mouse SCC tumor cells with 1,25(OH)₂D₃ revealed a form of caspase-dependent apoptosis involving the growth-promoting/pro-survival signaling molecule, mitogen-activated protein kinase kinase (MEK) ³⁶. The study was able to demonstrate that exposure to 1,25(OH)₂D₃ resulted in increased VDR expression and concomitant cleavage of the pro-survival signaling molecule MEK via a caspase-dependent manner.

4.4 Immune escape

Key to the survival of cancer cells is the evasion of destruction, a critically fundamental step of which is the ability to evade the immune system. HNSCC, like all cancers, is capable of evading the host's immune system via a number of different mechanisms. Cancer can evade antigenic recognition via tissue sequestration, improper lymphocytic homing due to improper expression of adhesion molecules, and antigenic shedding and modulation. In certain instances, however, antigenic recognition remains intact, despite a poor immune response. In such cases, cancers continue to thrive by releasing immunosuppressive cytokine, downregulating MHC I complex, or instilling dysregulated or improper costimulation

Numerous studies performed on HNSCC patients have investigated the effects of 1,25(OH)₂D₃ on the immune profile of patients. Within the past 10 years, it has been shown that not only do monocytes, B-cells, and T-cells express VDR, but also contain the enzyme 1- α -hydroxylase capable of converting 25(OH)D₃ into 1,25(OH)₂D₃ ³⁷. Such findings confirmed long-standing speculation that vitamin D is critical for the appropriate management and integrity of the immune system. Numerous studies have since attempted to understand exactly how vitamin D modulates the host immune profile.

HNSCC itself is associated with profound immunosuppression ³⁸. This is in part due to tissue infiltration by immunosuppressive immature progenitor cells with surface marker CD34 ^{39, 40}. In one study, HNSCC patients treated with 1,25(OH)₂D₃ exhibited decreased levels of intratumoral CD34⁺ cell populations and diminished the preexisting profound

immunoinhibitory effect of the cancer. Such findings suggest that $1,25(\text{OH})_2\text{D}_3$ may be helpful in overcoming the profound immunosuppression associated with HNSCC ⁴¹.

Tissues of HNSCC patients who received treatment with $1,25(\text{OH})_2\text{D}_3$ contained increased levels of CD4^+ cells and, more significantly, CD8^+ T cells. Such findings indicate an increased infiltration of immune effector cells into the tumor microenvironment. Also prominent was an increase in cells expressing the lymphoid activation marker CD69, which represent mainly T cells and monocytes. The same study showed that HNSCC patients who received $1,25(\text{OH})_2\text{D}_3$ prior to treatment had a lengthier time to tumor recurrence compared with patients who were not treated before surgery. The treatment arm exhibited a median time to recurrence that was almost 3.5-fold longer than control patients who were untreated before surgery ⁴². These findings are complemented by separate studies suggesting that treatment with $1,25(\text{OH})_2\text{D}_3$ may be an appropriate method of restarting the immune system ⁴³.

5. Future clinical considerations

One of the most discussed aspects regarding the implementation of vitamin D as an anti-cancer medication is dosing regimen. The Institute of Medicine recently addressed vitamin D dosing regimens in its newly published Dietary Referenced Intakes for Vitamin D. Their review of the literature yielded the suggested dietary intake of 600 IU per day for persons 1 to 70 years of age and 800 IU per day for persons over 70. Such intakes corresponded to serum $25(\text{OH})\text{D}$ levels of at least 20 ng per milliliter (50 nmol per liter) with recommended daily ingestion not to exceed 4000 IU per day ⁴⁴. The same review concluded that the prevalence of vitamin D deficiency in North America, which previously published reports had suggested to be as high as 54% in some populations, has been drastically overestimated ⁴⁵. The authors concluded that the vast majority of North American individuals have serum $25(\text{OH})\text{D}$ concentrations above 20 ng per milliliter, which is adequate for bone health in at least 97.5% of the population

Another area of debate is the concept of vitamin D screening as a predictor for cancer incidence. Since its inception, many have taken disagreed with such practices. One of the main areas of contention rests in the number of confounding variables associated with low serum $25(\text{OH})\text{D}$ levels. These variables, which include obesity, lack of physical activity, and poor diet or supplementation practices, have the ability to impact cancer risk. Critics also cite reverse-causation bias, noting that in certain individuals poor health reduces one's ability to participate in outdoor activities, resulting in lower vitamin D levels.

6. Conclusion and future perspective

It is the opinion of the authors that the role of $1,25(\text{OH})_2\text{D}_3$ as an anti-cancer agent warrants continued clinical and laboratory exploration. Further investigation into the relationship calls for randomized control trials of vitamin D for cancer prevention. As noted by the recently published IRAC review, observational studies themselves are unlikely to "disentangle the complex relationships between vitamin D and known cancer risk factors ⁴⁶." Such reports, however, have paved the way for numerous ongoing phase I and II clinical trials which should begin to elucidate what role, if any, $1,25(\text{OH})_2\text{D}_3$ may play in the treatment of HNSCC.

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This book points to some new areas for investigation on squamous cell carcinoma (SCC). Firstly, the features and management of some specific SCC is discussed to give the readers the general principles in dealing with these uncommon and sophisticated conditions. Some new concepts in adjuvant therapy including neoadjuvant therapy and gold nanoparticle-based photo dynamic therapy are introduced. Secondly, a detailed discussion of molecular aspects of tumor invasion and progression in SCC is provided with the emphasis on the roles of some important factors. The role of tumor microenvironment in head and neck SCC is specifically discussed. Thirdly, the roles of cancer stem cells (CSC) in cancer therapy of SCC are described. Molecular mechanisms involving therapeutic resistance and new therapeutic strategies targeting CSC are discussed in detail. Finally, other aspects concerning SCC are included, which involve the assessment, genetic manipulation and its possible clinical implications for the treatment of SCC.

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