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Squamous Cell Carcinoma

Hallmark and Treatment Modalities

Edited by Hamid Elia Daaboul

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Published in London, United Kingdom







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http://dx.doi.org/10.5772/intechopen.78167
Edited by Hamid Elia Daaboul

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First published in London, United Kingdom, 2020 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, 7th floor, 10 Lower Thames Street, London, EC3R 6AF, United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Squamous Cell Carcinoma – Hallmark and Treatment Modalities
Edited by Hamid Elia Daaboul
p. cm.
Print ISBN 978-1-78984-323-1
Online ISBN 978-1-78984-324-8
eBook (PDF) ISBN 978-1-78985-592-0

Meet the editor

Hamid Elia Daaboul, MD, PhD is an ASCO active member and researcher in the mode of action of new potential anticancer molecules in the Lebanese American University, Lebanon. He is a practicing Oncologist and also a former Head of Department of Oncology in Haroun Hospital, Lebanon. He earned his PhD in Oncology from Surrey University, London. He studied the mode of action of β-2-himachalen-6-ol, a new anticancer molecule, and succeeded in identifying its apoptotic effect via the inhibition of the mitogen-activated protein kinases (MAPK/ERK) and the phosphatidylinositol 3-kinase (PI3K/AKT) cellular signalling pathways. He is also engaged in clinical trials of other potentially active anticancer molecules.

Contents

Preface

Squamous cell carcinoma (SCC) is one of the most globally encountered kinds of malignancies. It is an aggressive metastasizing disease that can affect any human organ and maintains a high mortality rate despite all the research development. Currently, multiple new therapeutic methodologies founded on genetic identification have succeeded in confirming efficacy in extending disease-free survival, and were basically adopted in various scientific foundations. Targeted and immunotherapies are two different strategies of treatment that have remarkably succeeded in adding a fingerprint to the conventional chemo- and radiotherapies in prolonging the overall survival of several cancer types, keeping in mind the considerable side effects that may arise from their administration.

This book, therefore, was planned to cover every single data that might correlate with SCC, comprising kinds, classifications, investigative approaches, staging and treatment. It also aims to emphasize the latest approved therapeutical approaches with the ongoing promising clinical trials that may add benefit to the existing therapies. It also opens the opportunity for researchers and treating physicians to display and thoroughly present their newly discovered data and updated scientific results in their various SCC research, thus, paving the road for further investigations in the realm of cancer therapy.

Finally, I would like to express my sincere acknowledgments to the book authors and IntechOpen team that have contributed to the successful publication of this book.

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Section 1

Introduction to Squamous Cell Carcinoma

Chapter 1

Introductory Chapter: Squamous Cell Carcinoma (SCC)

Hamid Elia Daaboul

1. Introduction

Squamous cell carcinoma (SCC) is one of the most encountered types of cancers worldwide. It is an aggressive disease that affects the majority of the human body organs including the lungs, head and neck, esophagus, skin, genitourinary tract, thyroid, and other parts. SCC is a highly metastasizing disease with a relatively low overall survival rate. In addition to the traditional chemotherapy and radiotherapy, a combination of treatment modalities endeavors to ameliorate the survival rate of its various subtypes [1, 2]. Immunotherapy also tries to take part in SCC therapy by demonstrating durable improvements by hindering the immune system inhibitory interaction between the programmed cell death protein 1 (PD-1) and its ligand PD-L1 in the cells [3]. Moreover, the targeted inhibition of the cell signaling pathways as the PI3K and the MAPK has proven a novel promising therapeutic domain [4, 5]. Cyclin-dependent kinase (CDK) 4/6 inhibitors are another new group of small molecules targeting the cyclin D1-CDK4/6-Rb pathway involved in the cell cycle control [6, 7].

Oral squamous cell carcinoma is considered among the six most common cancers in the world. It is a subgroup of the upper aerodigestive tract and mostly affects the anterior tongue with the cheek, the floor of the mouth, the retromolar space, the gingiva, or any other part of the oral cavity [8]. The etiology and pathogenesis of all head and neck squamous cell carcinomas are majorly influenced by environmental and lifestyle risk factors, including tobacco use, excessive alcohol consumption, papilloma virus infection (predominantly HPV 16), and exposure to toxic substances, in addition to other dietary factors as salt-preserved food [9]. The esophageal squamous cell carcinoma is similarly affected by environmental and lifestyle-related factors such as tobacco use; alcohol overconsumption; salt-pickled or salt-cured and moldy foods; carcinogens as nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, various aldehydes, and phenols; vitamin (A, C, E, B) and mineral (zinc, selenium) deficiencies; extremely hot beverages; and fungal and HPV (16, 18) infections [10]. Human papilloma virus infection has also been implicated in the etiology of anal squamous cell carcinoma and especially in HIV-infected individuals, smokers, sexually perverted intercourses, and multi-sexual partners [11]. HPV infection is not known to be associated with the development of cutaneous squamous cell carcinoma; some sporadic cases, however, have suggested that cutaneous infection with HPV in immunocompetent hosts is prevalent in SCC development [12]. Other known risk factors implicated in SCC manifestation are exposure to solar ultraviolet radiation (UVR) and tanning bed usage, especially in the fair skin population [12–14]. Older age, male gender, cigarette smoking, chronic skin ulcers, and burn scars are also documented risk factors [12, 15]. Immunocompromised patients with organ transplantations are also at high risk of

developing SCC [12, 13]. Moreover, some genetic disorders as the recessive dystrophic epidermolysis bullosa, which is caused by loss-of-function mutations in the collagen type VII (C7), can lead to the appearance of aggressive form of cutaneous squamous cell carcinoma [16, 17]. Actinic keratoses, a form of premalignant lesions directly related to skin photodamage, are highly associated to SCC development as well [18].

In the following chapters, deepest information with each of these SCC subgroups will be widely discussed in order to decipher the basic data behind their mechanism of pathogenesis and possible therapeutic modalities. Nowadays, a variety of new therapeutic approaches based on genetic identification has proven efficacy in prolonging disease-free survival and was adopted in different scientific establishments. Targeted and immunotherapies have succeeded to add a fingerprint to the traditional chemo- and radiotherapies in prolonging the overall survival of many cancer types, even though a considerable amount of side effects has to be taken into consideration. This book, therefore, was designed to cover information related to SCC, including types, classifications, diagnostic methods, staging, and treatment, and to highlight the newest approved therapeutical methodologies, with the ongoing promising clinical trials that may add value to the existing treatments.

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Section 2

Oral Squamous Cell Carcinoma

Chapter 2

Molecular Pathogenesis of Oral Squamous Cell Carcinoma

Anshi Jain

Abstract

Oral carcinogenesis is a molecular and histological multistage process featuring genetic and phenotypic molecular markers which involves enhanced function of several protooncogenes, oncogenes and/or the deactivation of tumor suppressor genes, resulting in the over activity of growth factors and its cell surface receptors, which could enhance messenger signaling intracellularly, and/or leads to the increased production of transcription factors. Alone oncogenes are not responsible for carcinogenesis, genes having tumor suppressor activity, leads to a phenotypic change in cell which is responsible for increased cell proliferation, loss of cellular cohesion, and the ability to infiltrate local tissue and spread to distant sites. Understanding the molecular interplay of both onco and tumor genes will allow more accurate diagnosis and assessment of prognosis, which might lead the way for novel approaches to treatment.

Keywords: carcinogenesis, protooncogene, oncogene, tumor suppressor gene, intercellular signaling, cell surface receptors, growth factors

1. Introduction

According to the literature and current scenario it's a well-known fact that environmental and genetic factors modulate the multistep process of carcinogenesis. Genetic events lead to the disruption of normal regulatory mechanism that control basic cellular function of the body including cell division, differentiation and cell death [1]. Boyd and Reade (1988) described the mechanisms involved in carcinogenesis of the oral mucosa and distinguished between three major groups: chemical mechanisms, physical mechanisms, and viral mechanisms. Later Hanahan and Weinberg (2000) described six hallmarks of cancer (hallmarks I): acquisition of growth signaling autonomy (oncogenes), growth-inhibitory signals (tumor suppressor genes), evasion of apoptosis, cellular immortalization, angiogenesis, and finally, invasion and metastasis [2]. A decade later, an updating review (henceforth termed hallmarks II) added two emerging hallmarks: reprogramming energy metabolism and evading immune response, and two enabling traits: genome instability and mutation, and tumor-promoting inflammation [3].

Oral squamous carcinogenesis is the sixth most common cancer worldwide and commonest cancer in India, accounting for 50–70% of total cancer mortality rate. It predominantly affects anterior tongue, cheek, floor of mouth, retro molar area, gingiva or the buccal mucosa [4]. In carcinogenesis multiple genetic events alter the normal functions of both oncogenes and tumor suppressor genes. However, the importance of both the known gene alterations is unidentified and is still not fully understood. The histologic progression of oral carcinogenesis from hyperplasia to

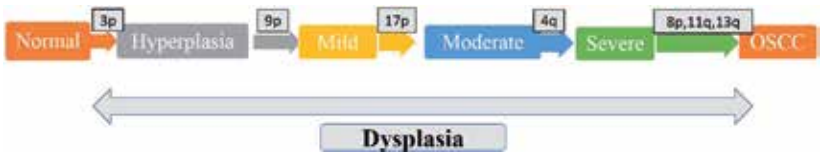

Figure 1.
Molecular model of oral carcinogenesis. The diagram shows the genetic progression from dysplasia to oral squamous cell carcinoma (OSCC), through changes in the p or q arm of chromosomes 3, 4, 8, 9, 11, 13, and 17 [2].

dysplasia, followed by severe dysplasia and eventual invasion and metastases, are believed to reflect the accumulation of these changes [5, 6] (**Figure 1**). Genetic alterations occurring during the carcinogenesis may present in the form of point mutations, amplifications, rearrangements, and deletions [5].

2. The genetic theory of cancer

2.1 Alteration of regulatory pathways during cancer development

Oral carcinogenesis is a complex, multistep process in which genetic events within signal transduction pathways governing normal cellular physiology are quantitatively or qualitatively altered.

Under normal conditions, cell biology of oral epithelia is tightly controlled by excitatory and inhibitory pathways which include cell division, differentiation, and senescence [1]. Cellular pathways of the oral keratinocyte may be diverse and contain the same fundamental elements. Binding of an extracellular ligand to a cell surface receptor forms a receptor-ligand complex that generates excitatory or inhibitory signals which are transferred intracellularly and further nuclear messengers can either directly alter cell function or can stimulate the transcription of genes which can affect protein synthesis [1] (**Figure 2**).

On contrary, oral cancer is the result of an accumulation of changes in these excitatory and inhibitory cellular signals that can occur at any level of a given pathway. Oral epithelial cells collect these alterations or mutations from cellular signals and become functionally independent from the surrounding oral epithelium made up of normal oral keratinocyte neighbors. These tumor cell divide more rapidly, sequester blood vessels to feed that growth, delete or amplify signals to produce abnormal structural or functional changes, and start invading normal tissue at local or distant sites [6].

Oncogenes and tumor suppressor genes constitute the cellular growth-regulatory genes which are widely expressed in normal cells and their protein products are required for cell to work normally. Any alteration or inappropriate expression of these genes can induce neoplasia [7].

The genetic damage of these genes found in cancer cells is of two sorts:

1. Dominant type: proto-oncogenes and oncogenes.

2. Recessive type: tumor suppressor genes, growth suppressor genes, recessive oncogenes, or anti-oncogenes.

The Former typically results in a gain of function, whereas latter causes loss of function [8].

The hallmark of cancer is rapid and uncontrolled growth. Cell cycle regulatory molecules (cyclin-CDK complex and retinoblastoma protein RB) play a key role

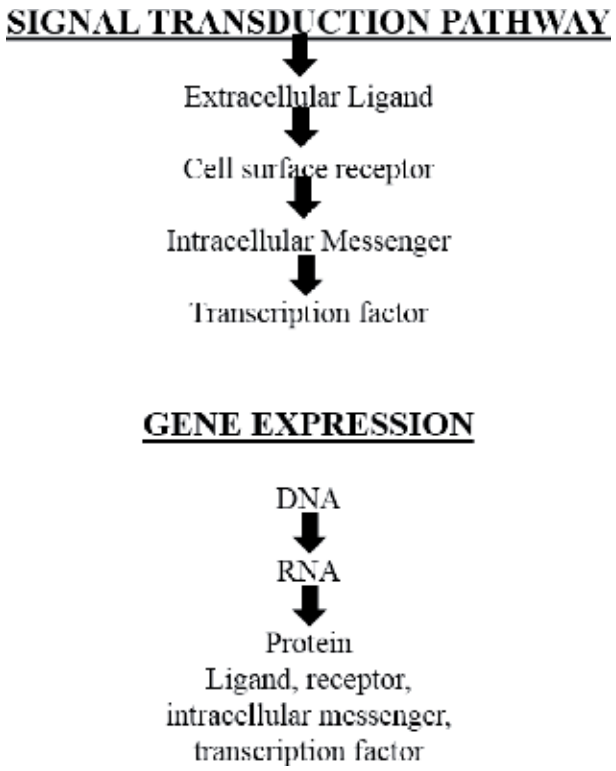

Figure 2.
Signal—transduction pathway.

in pathogenesis of head and neck cancers. Phosphorylation of RB by the cyclin/CDK there is a release of E2F, which transcribe the necessary components of the cell to continue through the G1/S transition. Specifically, RB function is mediated by cyclin E/CDK2 activity. In contrast, CDK4 and CDK6 act upstream of RB and inhibit RB function by phosphorylation [5]. In head and Neck cancers, both up and down regulation of RB function has been observed conferring a greater degree of malignancy and aggressiveness, dependent upon cellular context. Downregulation of RB function—cell cycle to remain unchecked and leads to continual cell division and cell proliferation; up-regulation of RB leading to a decrease in pro-apoptotic signals that are triggered during the cell cycle. In either case, changes in the RB pathway alter cell-cycle transition and allow for greater cancer cell survival [1].

3. Oncogenes and oncoprotein

Oncogenes can be classified according to the roles of their normal counterparts (protooncogenes) in the biochemical pathways that regulate growth and differentiation. These include the following

1. Growth factors (TGF, FGF, PDGF)

2. Cell surface receptors (EGFR, FGFR)

3. Intracellular signal transduction pathways (RAS)

4. DNA binding nuclear proteins transcription factors (MYC, FOS, JUN)

5. Cell cycle proteins (cyclins and cyclin dependent protein kinases)

6. Inhibitors of apoptosis (bcl-2)

Oncogenes are defined as "altered growth-promoting regulatory genes, or proto-oncogenes that govern the cell's signal transduction pathways" [5]. These genes were initially discovered in retroviruses which cause cancers in birds and cats by virtue of a highly tumorigenic 'molecular hitchhiker', a mutated gene (oncogene) not native to the virus but picked up from a homologue in the eukaryotic genome. Alteration or mutation of these proto-oncogenes results in either an overproduction or a "gain-of-function" alteration in these excitatory proteins. Although oncogenes alone are not sufficient to transform a normal oral keratinocyte to a malignant one, they are initiators of the process [6].

Aberrant expression of several oncogenes play a crucial role in development of oral carcinogenesis which includes proto-oncogene epidermal growth factor receptor (EGFR/c-erb 1), members of the ras gene family, c-myc, int-2, hst-1, PRAD-1, and bcl-1 (**Figure 3**) [6].

The potential of proto-oncogenes to participate in tumorigenesis arises from the fact that their protein products are relays in the elaborate biochemical circuitry that governs the phenotype of vertebrate cells polypeptide hormones that act on the surface of the cell, receptors for these hormones, proteins convey signals from the receptors to the deeper cell machinery, and nuclear functions that orchestrate the genetic response to afferent commands [5].

Three biochemical mechanisms which proto-oncogenes act are [8]:

1. The first mechanism is phosphorylation of proteins, with serine, threonine, and tyrosine as substrates.

2. The second mechanism by which the genes act is transmission of signals by GTPases. The role of these signaling devices in tumorigenesis was first

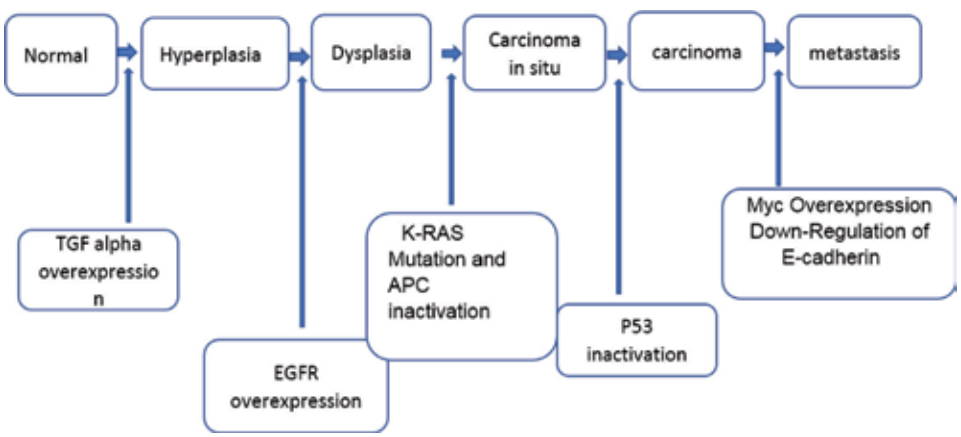

Figure 3.
Oral cancer progression model. The histopathologic progression of normal oral mucosa from hyperplasia to malignancy and metastasis appears driven by interplay of activation of oncogenes in early cellular transformation and inactivation of tumor suppressor genes closer to the initiation of malignancy and metastasis.

appreciated through the discovery of RAS oncogenes, which encode a previously unknown variety of GTPase.

3. The third mechanism consists of control of transcription from DNA. A still growing variety of transcription factors (FOS and MYC) are encoded by proto-oncogenes which may also participate directly in the replication of DNA.

3.1 Growth factor receptors and mechanisms

Activation of growth factor receptors in human tumors include mutations, gene rearrangements, and overexpression. Signaling pathways involved in the development of both cancer and stem cells are: the JAK/STAT pathway, NOTCH signaling pathway, the MAP-Kinase/ERK pathway, the PI3K/AKT pathway, the NFkB pathway, the Wnt pathway and the TGFβ pathways.

In the normal forms of growth factor receptors, the kinase is transiently activated by binding of the growth factors ligand to receptor, leads to rapid receptor dimerization and tyrosine phosphorylation of several substrates that are a part of the signaling cascade. The oncogenic growth factor receptors cause dimerization and activation without binding to the specific growth factor ligand. Hence, the mutant receptors deliver continuous mitogenic signals to the cell [1].

In oral carcinogenesis deregulation of growth factors receptors occurs through increased production and autocrine stimulation. Aberrant expression of transforming growth factor alpha (TGF-α) and beta (TGF-β) occur in carcinogenesis. TGF-α work in association with EGFR and TGF-β follows a pathway along with SMAD2 and 3.

TGF-α is reported to occur early in oral carcinogenesis, following the histological progression of hyperplastic epithelium first, and later in the invasive carcinoma within the inflammatory cell infiltrate, especially the eosinophils, surrounding the infiltrating epithelium. TGF-α stimulates cell proliferation by binding to EGFR and stimulates angiogenesis and has been reported to be found in "normal" oral mucosa in patients who subsequently develop a second primary carcinoma.

Microscopically "normal" oral mucosa of head and neck cancer patients who later develop second primary carcinomas overexpresses TGF-α suggesting a 'premalignant" lesion having rapid proliferation and genetic instability of the epithelium. Prognostically patients with oral tumors overexpressing TGF-α along with EGFR have been shown to have a significantly shorter survival than patients overexpressing EGFR alone [6].

TGFβ1 signals through the TGFβ receptors and these transduce the signal by phosphorylating SMAD2 and SMAD3, which, together with SMAD4, regulate the transcription of target genes.

Recently, a connection of TGFβ signalling pathway and nuclear factor-κB (NF-κB)99 has been studied, it's a transcription factor that provides an important survival signal to cells. Cohen et al. showed that abrogation of the TGF-β pathway was associated with activation of NF-κB, and this intriguing finding suggests that decreased TGFβ signalling is linked to NF-κB activation [9].

3.2 Cell surface receptors

Binding of cell surface receptor with ligands translates signals which are present extracellularly through the cell membrane by activating a cascade of biochemical reactions. Mutations or amplifications of genes encoding growth factor receptors can result in an increased number of receptors or production of continuous ligand-independent mitogenic signals.

EGFR, a 170,000-Da phosphoglycoprotein, is believed to be an important oncoprotein in oral cancer. Currently, three mechanisms have been postulated to activate the EGFR gene in carcinogenesis:

1. Deletion or mutations in the N-terminal ligand-binding domain.

2. Overexpression of the EGFR gene concurrent with the continuous presence of EGF or TGF-α.

3. Deletion in the C-terminus of the receptor that prevents downregulation of the receptor after ligand binding.

In human oral carcinogenesis EGFR is overexpressed as this gene is amplified. Therefore, it has been identified that in comparison to the normal counterpart, malignant oral keratinocytes possess 5–50 times more EGF receptor. Moreover, in oral carcinogenesis the mechanism of signal transduction is either because of overexpression of normal receptors due to mutated gene or because of the formation of many new receptors is not understood yet. Henceforth, oral tumors, having EGFR overexpression, have been shown to exhibit a higher response to chemotherapy than EGFR-negative tumors, presumably because of higher intrinsic proliferative activity leading to higher sensitivity to cytotoxic drugs [6].

3.3 Intracellular signal transduction pathways (RAS)

Like growth factor receptors, intracellular messengers can be intrinsically activated, thereby delivering a continuous rather than a ligand-regulated signal [6]. An oncogene can be activated either by gene amplification and/or mutation. In OSCC, the ras is one of the most frequently genetically altered oncogene. The mutations of three isoforms of ras gene such as Hras, Kras and Nras produce the same phenotype in the in vitro transformation assays. Mutations of the Hras appear to be highly prevalent in OSCC when compared to the Kras and Nras have been reported approximately from 0 to 55%.

3.3.1 Mechanism of ras activation

These genes encode closely related proteins that are located on the cytoplasmic side of the cell membrane and transmit messages from the cell surface receptors to intracellular regulatory enzymes [6].

RAS present on the cytoplasmic side of cell membrane get activated by growth factors through enhanced exchange of guanine nucleotide by forming Grb2 SOS complex. The molecular mechanism underlying in the functions activation of ras depends on the whole super family of small G-proteins because there exist a switch between GTP bound active and GDP-bound inactive state [10].

In normal human cell, an equilibrium is strictly maintained by the activity of GAPs (GTPase activating proteins) and GEFs (Guanine nucleotide exchange factors) between the active and inactive state because ras proteins have a minimal and a measurable activity on their own. The GAPs accelerate the GTP hydrolysis of ras and the antagonist GEFs such as ras-GRFs and ras-GRPs catalyze and weakens the GDP replacing with GTP. In a cell where ras is mutated, the equilibrium between the GTP and GDP-bound state is impaired. The ras is mutated predominantly at codon G12, G13 and Q61. In K-RAS and H-RAS because of point mutations GAP catalyzed hydrolysis of GTP to GDP, thereby generate constantly active ras and is responsible for the activation of downstream effectors whereby cell undergoes aberrant malfunctioning leading to malignancy (**Figure 4**) [10].

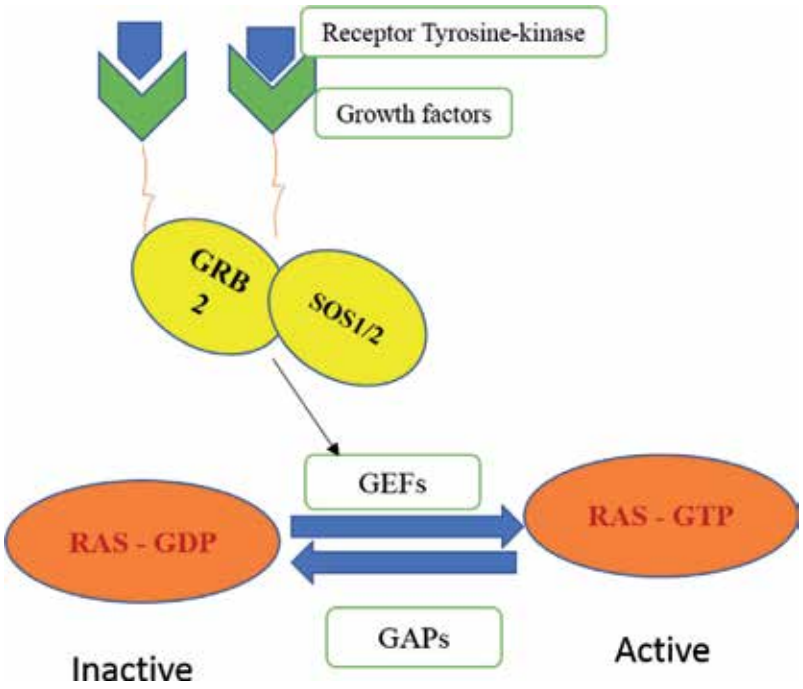

Figure 4.
Mechanisms of the ras activation.

3.3.2 Ras and its major signaling pathways

The ras oncogenes are associated with proteins that are involved in the transduction of extracellular growth, differentiation and survival signals. Ras activate receptor tyrosine kinases (RTKs), which further activate two key signal transduction components:

1. Small GTPase

2. Lipid kinase PI(3)K.

The activated ras stimulates mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K)/Akt pathways. The key downstream steps involve phosphorylation by RAF1 kinase on two distinct serine residues MEK1/2. The MEK1/2 further phosphorylates specific threonine and tyrosine residues in the activation loops of ERK1/2 and leads to growth and differentiation. On the other hand ras transduces PI3K/Akt signaling pathway which lead to cell cycle proliferation and survival [10].

3.4 DNA binding nuclear proteins transcription factors (MYC, FOS, JUN)

Transcription factors, or proteins that stimulate other genes to be activated, are also altered in oral cancer. A growing number of the known proto-oncogenes encode nuclear proteins. These nuclear proteins are further regulated by receptor activated second messenger pathways. Neutralization of these encoded genes result in cell cycle arrest which prevents mitogenic and differentiation responses to growth factors. C-myc is a gene which helps regulate cell proliferation and apoptosis and is frequently overexpressed in oral cancers as a result of gene amplification.

C-myc is often overexpressed in poorly differentiated tumors, although more recently c-myc has been shown to be overexpressed in moderate and well differentiated oral carcinomas, in which cell proliferation far outweighed the number of apoptotic cells present. For apoptosis, c-myc requires p53 for regulating cell proliferation. c-Myc interacts with retinoblastoma tumor suppressor gene Rb-1 nuclear protein pR6, preventing its transcription, and thus inhibiting cell proliferation. However, on phosphorylation of pR6, c-Myc is increased and cell proliferation proceeds. Another transcription factor which is also amplified in head and neck cancers is PRADl (also CCNDl or cyclin Dl) which acts too as a cell cycle promoter [5–6, 8].

Particular order of oncogene activation has not been shown in oral cancers; instead the accumulation of activated oncogenes should be of primary importance. The importance of the currently identified oncoproteins to oral carcinogenesis is under investigation. Other oncogenes linked to oral cancer development are hst-1, k-2, bcl-1, sea, men-1, and eM1s-1.3.4. Oncogenes alone, however. Not sufficient to result in oral cancer but appear to be initiators of the process and should work along with the inactivation of tumor suppressor genes. The critical event in the transformation of a "premalignant" cell to a malignant cell is the inactivation of cellular negative regulators, tumor suppressor genes.

3.5 Cell cycle proteins (cyclins and cyclin dependent protein kinases)

The cell cycle is a mammalian cells proliferation regulation process and has 4 functional phases:

a. S phase (DNA replication)

b. G2 phase (cells prepare for mitosis)

c. M phase (DNA and cellular components division into two daughter cells)

d. G1 phase (cells commit and prepare for another round of replication).

S and M phases are the major and common process in all cell cycles for replication of cells. It requires interplay of expression of cyclins and cyclin dependent kinases in response to growth factors.

3.5.1 Cdks, the cell cycle

Cdk2 and cdk1, together, direct S and G2 phase transit, while cdk1 governs the G2/M transition and mitotic progression. Cdks can be divided into two groups:

a. 'Cell cycle' cdks, which orchestrate cell cycle progression.

b. 'Transcriptional' cdks, which contribute to mRNA synthesis and processing.

The first group encompasses cyclin D-cdk4 and 6, as well as cyclin E-cdk2 complexes, which sequentially phosphorylate the retinoblastoma protein (RB), to facilitate the G1/S transition. Cyclin A-cdk 2 and 1 are required for orderly S phase progression, whereas cyclin B-cdk1 complexes control the G2/M transition and participate in mitotic progression [11].

The second group includes cyclin H-cdk7 and cyclin T-cdk9 (pTEFb). It phosphorylates the carboxy-terminal domain of RNA polymerase II to promote elongation of mRNA transcription. Cyclin T-cdk9 also regulates mRNA processing [12].

3.5.2 Cdk's and cancer

CDK's and cyclins are the biochemicals play a pivotal role in cell cycle progression and transcription. Errors and dysregulation like amplification, mutation, deletion and hypermethylation of cyclins and its cdk partners activity results in loss of cell cycle check points and apoptotic activity which is a major cause for proliferative disorders such as cancer and which has been directly linked to the molecular pathology of cancer [11].

Cell cycle progression through the G1 phase is regulated by the action of cyclin D-cdk4, cyclin D-cdk6, and cyclin E-cdk2. This transition is mediated through the RB, which is regulated through sequential phosphorylations by CDK. Various genetic and epigenetic alterations in human cancer including mutations and amplification of Cdk and positive regulatory Cyclin subunits, lead to a hyperactivation of Cdk regulatory pathways. Henceforth, alteration in cell cycle checkpoints causes abnormal cell proliferation and results in tumor progression. Although mutations of cdk genes in tumor cells are rather infrequent with the exception of Cdk4 and Cdk6 amplification, overexpression or hyperactivation of basic cell cycle regulators is a general feature of human tumors like leukemia or carcinomas and were associated with poor prognosis [11].

3.6 Inhibitors of apoptosis (Bcl-2)

Apoptosis "programmed cell death'—is a physiologic process of cell to undergo death following sequence of events once the function is over. Any alterations in the mechanism of cell undergoing apoptosis not only contribute to abnormal proliferation of cell but also enhance resistance to anticancer therapies, such as radiation and cytotoxic agents. One of the suggested mechanisms for developing resistance to cytotoxic antineoplastic drugs is the alteration in expression of B-cell lymphoma-2 (Bcl-2) family members.

A balance between newly forming cells and old dying cells is maintained by Bcl-2 family of proteins which consists of 25 pro- and anti-apoptotic members. When there is alteration or disbalance in ratio of distribution of pro and anti-apoptotic proteins resulting in the overexpression of anti-apoptotic Bcl-2 family members, apoptotic cell death can be prevented. Targeting the anti-apoptotic Bcl-2 family of proteins can improve apoptosis and thus overcome drug resistance to cancer chemotherapy [6].

Two major pathways of apoptosis are the intrinsic and extrinsic cell-death pathways.

The intrinsic cell death pathway/mitochondrial apoptotic pathway: mainly triggers apoptosis in response to internal stimuli and is activated by a wide range of signals, including radiation, cytotoxic drugs, cellular stress, DNA damage and growth factor withdrawal. This mechanism involves the release of proteins cytochrome *c* from the mitochondrial membrane space which in turn activates pro caspase-9 and induces apoptosis.

The extrinsic cell-death pathway: pathway functions independently of mitochondria and executes cascade activation of caspases. Activation of cell-surface death receptors, such as Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, directly activate the caspase cascade via an "initiator" caspase (caspase-8) the role of which is to cleave other pro-caspases into active "executioner" caspases which induces degradation of cytoskeleton and nucleus [13].

3.6.1 Role of Bcl-2 in oral carcinogenesis

Bcl-2 family members can be divided into three subfamilies based on structural and functional features [13].

1. **Bcl-2 homology**—the anti-apoptotic subfamily contains the Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl1/A-1, and Bcl-B proteins, which suppress apoptosis and contain all four Bcl-2 homology domains.

2. **Multidomain proteins**—some pro-apoptotic proteins, such as Bax, Bak, and Bok, contain Bcl-2 homology domains.

3. **BH3-only proteins**—pro-apoptotic proteins, such as Bim, Bad, and Bid, contain only the BH3 domain.

Recent studies have shown that Bcl-2 expression is upregulated in oral SCC. Bcl-2 inhibits cell death via inhibiting apoptosis. Hence, Bcl-2-mediated inhibition of apoptosis may be an important factor in the pathogenesis of oral SCC. Bax forms heterodimers with Bcl-2 and when present in excess, Bax overrides the anti-apoptotic activity of Bcl-2.

P53 tumor-suppressor protein is a direct transcriptional activator of the human Bax gene suggesting that p53 may, in some instances, induce apoptosis via Bax-mediated suppression of Bcl-2 activity. In mutagenesis experiments, single amino acid substitutions in Bcl-2 homology domains disrupted Bcl-2-Bax heterodimers. The Bcl-2 mutants that failed to complex with Bax could no longer inhibit apoptosis. According to the study done by Oltvai et al. (1993) it was suggested that anti-apoptotic activity of Bcl-2 was inhibited by Bax, whereas the findings of Yin et al. (1994) is converse to that of the previous findings, i.e. that the function of Bcl-2 is to inhibit the apoptotic activity of Bax. But it was further hypothesized that the possible mechanism was the formation of Bcl-2-Bax heterodimers which inhibits both apoptotic and anti-apoptotic activity and is only seen when there is a functional excess of Bax or Bcl-2, respectively.

Bcl-x and Bcl-2 form heterodimers with Bad. This dimerization displaces Bax from Bcl-x, and Bcl-2 thereby enhances apoptosis. Therefore, the Bcl-2 family of related proteins (as with the Myc family) functions in part through protein-protein interactions.

In conclusion, Bcl-2-mediated inhibition of apoptosis may be an important factor in the pathogenesis of oral SCC. Furthermore, by blocking apoptosis, Bcl-2 can increase tumor cell resistance to anti-neoplastic drugs.

4. Tumor suppressor genes

Genes that encode the proteins for negative signal transduction pathways and modulate excitatory pathways and negate their effect in a "checks and balances" have been called as growth regulatory genes, recessive oncogenes or anti-oncogenes, but they are most often referred to as tumor suppressor genes. Negative regulatory pathways allow the cell to perform its function in the face of changing internal and external stresses [1, 14].

As been mentioned earlier in the chapter "Oncogenes alone are not sufficient to cause oral cancer and appear to be initiators of the process".

The transformation of a premalignant cell to a malignant cell is due to the inactivation of tumor suppressor gene which is a major event leading to the development of malignancy.

This mechanism of inactivation is may be due to point mutations, deletions, hypermethylation and rearrangements in gene copies. It was identified that many tumor suppressor genes were initially identified in pediatric tumors that formed early in life because one mutated tumor suppressor gene was inherited [1].

This mechanism led the evolution of "Knudson two hit hypothesis" This theory suggested a genetic model for retinoblastoma development. According to this RB gene mutation is inherited is described as the first hit and the tumor-restricted mutation as the second hit. This model further includes genetic aberrations, such as inactivation of a tumor suppressor and activation of an oncogene, as hits. Currently an extensive research on "chromosomal walking" is highlighted in pediatric tumors were the first tumor suppressor genes isolated with large chromosomal alterations. Therefore, although the identification of these "cancer genes" is one of the primary focuses of molecular biologists today, still far less is known about tumor suppressor genes [1].

4.1 Function of p53 as a tumor suppressor gene

The many roles of p53 as a tumor suppressor include the ability to induce cell cycle arrest, DNA repair, senescence, and apoptosis. Due to many genotoxic or chemical insults when genomic DNA damage is being identified, p53 gene activated and stop cell to divide further at the G1-S boundary and it repairs rather than replicates the error in the genetic code. If the chromosomal damage is too great, p53 gene activate apoptotic pathways [15].

4.2 Mutant form of p53

Mutation of p53 allows tumors to pass through the G1-S boundary and propagate the genetic alterations that may lead to other activated oncogenes or inactivated tumor suppressor genes. In addition to the loss of function that a mutation in TP53 may cause, many p53 mutants are able to actively promote tumor development by other means like:

1. Dominant negative manner

2. Gain of function

4.2.1 Dominant negative manner

In a heterozygous situation, where both wildtype (WT) and mutant alleles exist, mutant p53 can antagonize the activity of WT p53 tumor suppressor functions in a dominant negative (DN) manner. The transcriptional activity of WT p53 depends on forming tetramer where mutant p53 interfered in DNA binding activity of WT p53. However, such a heterozygous state is often transient, as TP53 mutations are frequently followed by loss of heterozygosity (LOH) during cancer progression as WT p53 allele is either deleted or mutated [14].

4.2.2 Gain of function

This term refers to the acquisition of oncogenic properties by the mutant form of p53 protein, compared with the mere inactivation of the protein. During tumorigenesis both the dominant negative and GOF effects may play a significant role in missense mutations of TP53 protein [15].

4.3 Mechanistic views of how mutant p53 exerts its function

Various mechanisms by which mutant p53 works in tumor progression:

1. GOF properties acquired by mutant p53 drive cells toward migration, invasion, and metastasis. Recent work demonstrates that mutant p53 can augment

cell migration and invasion. It was studied that "oncogenic" Ras and "Tumor Suppressor" mutant p53 activities occurs in early neoplasms to promote growth and survival, they play an equally important role at late stages of tumor progression in empowering TGFβ-induced metastasis.

2. EMT—metastasis follow the properties of epithelial to-mesenchymal transition (EMT), including loss of cell-cell adhesion and an increase in cell motility., Mutant p53 was found to promote EMT by facilitating the function of the key transcriptional regulators of this process, TWIST1 and SLUG whereas WT p53 was shown to inhibit EMT mechanism.

3. Tp63—an additional mechanism through which mutant p53 was shown to augment cell invasion is via the inhibition of transcriptional activity of TAp63α, but is unable to inhibit this function of TAp63γ indicating a protooncogenic activity of TP 53 [14].

It appears that in certain cancers, p53 is mutated late in the tumorigenesis process or plays a significant role in those advanced stages, whereas other studies indicates its expression in early stages of tumor progression. Therefore, it was hypothesized that TP53 mutations at early stages of tumorigenesis results in uncontrolled proliferation, a feature of both benign and malignant tumors, whereas mutations at later stages synergize with additional oncogenic events to drive invasion and metastasis, the hallmark of malignant tumors. p53 inactivation as a single event results in the high proliferation rate. Inactivation of p53 in conjunction with oncogenic H-Ras expression activates the expression of a large set of chemokines and interleukins reported to promote angiogenesis, invasion, and metastasis.

In general, tumor suppressor genes are thought to act recessively so that both copies of the gene must be inactivated for malignancy to occur. LOH and p53 mutations have been reported in several tumors. There is also controversy about the relation between mutated p53 and detection of its expression by immunohistochemistry. Some authors have commented on high correlation between p53 expression and point missense mutation, whereas others have reported discrepancy in oral cancer and lack of expression of p53 as immunocytochemistry have been attributed to insensitive methods of detecting p53 mutation. In Li-Fraumeni syndrome, mutant p53 is unstable, like the wild-type p53 protein, which suggests that some other event may be necessary for stability, and that stability of p53 is not intrinsic to the mutant p53 structure but might vary in different cell backgrounds. This mechanism can be highlighted by p53 and mdm2 relation because when normal p53 is bound to mdm2 it is targeted for destruction by the ubiquitin dependent pathway. However, it appears that mutant p53 fails to stimulate transcription of mdm2 and therefore mutant p53 is not degraded. Another mechanism tells that if E6 protein forms complexes with wild-type p53 and promotes p53 degradation this could account for the lack of concordance between p53 mutation frequency and LOH [16].

Other tumor suppressor genes include doc-1, the retinoblastoma gene, and APC.

5. Role of HPV in pathogenesis of OSCC

The role of HPV in pathogenesis of human malignancies has become convincingly established. HPV is a strictly epitheliotropic, circular double-stranded DNA virus that is known to be the primary cause of cervical cancer and currently establishing important role in oral carcinogenesis. There are more than 100 subtypes of HPV, some of which are involved in oral carcinogenesis and have been designated as

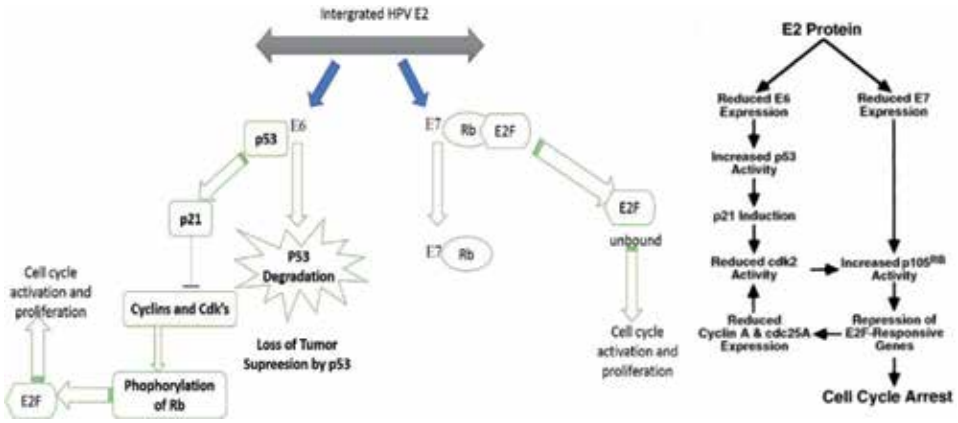

Figure 5.
Cell cycle deregulation by human papilloma virus activated by E6 and E7.

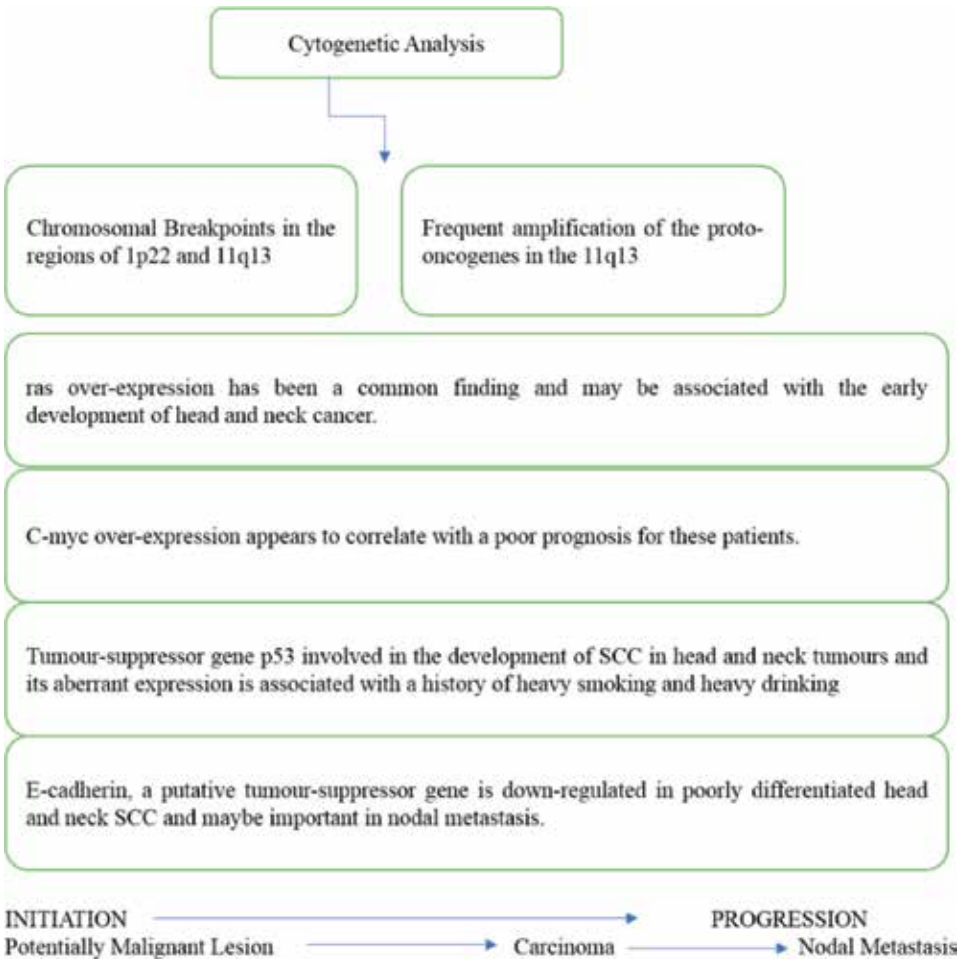

Figure 6.
Proposed molecular model for the genetic events in squamous cell carcinoma of the head and neck [19, 20].

high-risk HPVs. Approximately 85% of squamous cell carcinoma patients. The viral DNA gets incorporated into the host genome and is responsible for malignant transformation. The virus contains two oncogenes, E6 and E7, E1 and E2 open reading

frames will be interrupted and can lead to overexpression of E6 and E7 proteins. This E7 protein binds to underphosphorylated form of retinoblastoma results in the enhanced phosphorylation and degradation. Degraded form of pRb displaces E2F form of transcription factor and subsequent activation of gene promoting cell proliferation. E6 protein degrades p53 protein causing perturbation of cell cycle regulation in the infected cells which is considered to be the onset of HPV-mediated carcinogenesis. The virus is not easily cultured, therefore determining the role of virus in pathogenesis of OSCC is usually determined by detection of the viral DNA genome or expression of the viral genes using PCR methods. E6 and E7 have a crucial role in cervical cancer were also involved in HPV mediated carcinogenesis of the upper aerodigestive tract (**Figures 5** and **6**) [9, 17, 18].

6. Conclusions

Cellular signaling pathways are not isolated from each other but are interconnected to form complex signaling networks. Any change or diversification in this cellular signaling network such as increased production of growth factor or cell surface receptors, increase transcription or translation or intracellular messenger levels will give rise to abnormal proliferation of cell and is one of the reason for multifactorial oral carcinogenesis These changes can, in turn, cause a activation of protooncogene or loss of tumor suppressor activity which give rise to a phenotype capable of increasing cellular proliferation, weakening cell cohesion, and causing local infiltration and metastasis.

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Section 3

Esophageal Squamous Cell Carcinoma

Chapter 3

Squamous Cell Carcinoma: Esophagus

K.V. Veerendra Kumar, Ramesh Sagar and Joseph Mathew

Abstract

Esophageal cancer, according to GLOBOCAN 2018 data, ranks seventh in terms of incidence and sixth in mortality among all cancers worldwide. In India, it is considered the fourth most common cause of cancer-related deaths. Influenced by lifestyle, socioeconomic and environmental factors, striking geographic variations in incidence exist. With regard to histopathology, esophageal cancers are unique among malignancies of the gastrointestinal tract in that they principally comprise two variants: squamous cell carcinoma (SCC) and adenocarcinoma, with the former accounting for up to 80% of cases. Etiological factors for SCC show marked variations worldwide, with tobacco consumption, alcohol, hot beverages, and poor nutrition constituting the predominant predisposing factors. Although present day therapeutic interventions have begun to positively influence disease prognosis, with significant improvements in survival noted over the last 3 decades, cancer of the esophagus remains a highly lethal disease with a case fatality rate approaching 90%. Management of this disease includes all three primary modalities of treatment; surgery, chemotherapy and radiotherapy. Surgical resection, the only curative modality of treatment, remains a challenge even with advances like minimal access surgery and is feasible only in early stage disease. Early diagnosis and accurate staging are paramount for optimizing treatment and hence, prognosis.

Keywords: esophageal cancer, squamous cell carcinoma, adenocarcinoma

1. Introduction

Despite a better understanding of the biology of disease and a number of advances in diagnostic and therapeutic interventions, cancer of the esophagus remains a highly aggressive and lethal malignancy.

According to GLOBOCAN 2018 data, this disease ranks seventh in terms of cancer incidence (572,000 new cases) and sixth in overall cancer-related mortality (509,000 deaths), signifying that esophageal cancer was accountable for an estimated 1 in every 20 cancer-related deaths in 2018 [1]. In India, esophageal cancer is the fourth most common cause of cancer-related deaths.

Although advances in therapeutic interventions have begun to have a positive impact on survival evident over the past 3 decades, esophageal cancer remains a formidable disease with a case fatality rate of 90% [2].

Histopathologically, cancers of the esophagus are primarily of two types, squamous cell carcinoma (SCC) and adenocarcinoma. Marked geographic variation in incidence and cancer type has been known to occur and is influenced by life style, socioeconomic and environmental factors [3].

Alcohol consumption and smoking and the synergistic effects thereof are major risk factors for the development of SCC in the West. The incidence of esophageal adenocarcinoma in the West has seen a steep rise in the past 20 years, surpassing SCC as the most common type of esophageal cancer [4].

In India, squamous cell carcinoma accounts for up to 80% of all cases of esophageal cancers. Data from Kidwai Cancer Institute and Tata Memorial Hospital show that SCC of the esophagus is the second most common cancer among men and the fourth leading cause of cancer mortality. Although etiological factors implicated in SCCs show marked regional variation in different parts of India, tobacco consumption in various forms, alcohol, hot beverages, and poor nutrition remain the predominant predisposing factors in the subcontinent.

Despite the two pathological subtypes having different etiologic factors, biology and prognostic profiles, they have often been managed as a single entity. Today, management of esophageal cancer includes all three modalities of treatment, i.e., surgery, chemotherapy and radiotherapy. Considering that the esophagus spans three anatomic compartments: the neck, the thorax and the abdomen and its proximity to vital structures, surgery of this organ remains a challenge even with present -day therapeutic advances such as minimal access surgery. Surgery is the only curative therapeutic modality. However, its applicability is restricted to the early stages of the disease [5–7].

Most patients with esophageal cancer, on account of late onset of symptoms and a consequential delay in the final diagnosis, present with advanced disease which precludes definitive surgical intervention. Hence, the prognosis in general remains poor [5, 8]. Early diagnosis and accurate staging are considered vital for the optimal management of esophageal cancer.

2. Anatomy

The esophagus is a muscular tube beginning from the cricopharyngeal sphincter at the cricoid cartilage at the level of the sixth cervical vertebra and terminating at the gastroesophageal junction at the level of the 11th thoracic vertebra. It travels through the neck, chest and upper abdomen, and is anatomically divided into the cervical, the thoracic, and the abdominal segments [9] (**Table 1**). From its origin at the cricoid cartilage to the gastroesophageal junction, the length of the adult esophagus varies from 22 to 28 cm with the distal 3 cm lying intra-abdominally [10].

The cervical esophagus lies just left of the midline and is closely related to the trachea anteriorly and the prevertebral fascia posteriorly. Only a minimal amount of loose areolar tissue separates the trachea from the esophagus and malignancies are known to spread from the esophagus to the trachea and vice versa [11]. The upper

Part of esophagus	Extent	Distance from upper incisor (cm)
Cervical esophagus	Pharyngoesophageal junction to thoracic inlet	18
Upper thoracic	Thoracic inlet to the lower border of T6 vertebra	26
Mid thoracic	Lower border of T6 to lower border of T8 vertebra	31
Lower esophagus	Lower border of T8 to cardiac orifice	40

Table 1.
Parts of the esophagus according to UICC (1978).

portion of the thoracic esophagus curves slightly to the right and passes behind the tracheal bifurcation and the left main-stem bronchus. On either side of the thoracic esophagus are the lungs with their pleural linings. Additionally, the azygos vein, arching over the right main bronchus to drain into the superior vena cava, and the subclavian artery are important relations on the right. On the left are the aortic arch and the aorta which assumes a posterior course in relation to the esophagus. The lower portion of thoracic esophagus runs behind the pericardium and the left atrium, where it bends to the left to enter the abdomen through the esophageal hiatus. The left lobe of the liver bears an anterior relation to the abdominal esophagus [12].

There are three areas of physiological/normal narrowing of the esophageal lumen: at the cricoid cartilage, at the point where it crosses the left main bronchus and the aortic arch and at the diaphragmatic hiatus.

2.1 Supports of the esophagus.

The outer longitudinal muscular layer of the esophagus inserts into the posterior ridge of the cricoid cartilage via the cricoesophageal tendon. The inner circular muscle layer is in continuity with the inferior laryngeal constrictor which inserts on the sphenoid. Bronchoesophageal and pleuroesophageal strands are fibromuscular bands which connect the esophagus with the trachea and bronchi and pleura, respectively. Inferiorly, the posterior gastric ligaments and the lesser omentum are the main anchors of the distal esophagus, the phrenoesophageal membrane serving as a weaker support [12].

The peritoneal reflections associated with the esophagus are the hepatogastric ligament and the gastrosplenic ligament. The former encloses the left gastric vessels, the hepatic division of the left vagal trunk and lymph nodes. The hepatogastric ligament continues to the left of the abdominal esophagus as the gastrosplenic ligament. The lesser sac lies posterior to these ligaments [10, 12].

2.2 Blood supply of esophagus

The esophagus has a segmental blood supply. The cervical esophagus is predominantly supplied by the inferior thyroid artery, the upper and mid thoracic esophagus by branches from the bronchial arteries and the descending thoracic aorta and the lower thoracic and intra-abdominal esophagus by branches from the left gastric and inferior phrenic arteries [13].

An extensive submucosal venous plexus communicates with the longitudinally oriented periesophageal veins through the muscularis. In the cervical esophagus, these veins drain principally into the inferior thyroid veins, in the thoracic esophagus into the azygos vein and in the abdominal esophagus, into the azygos and left gastric veins. Hence, in the distal esophagus, the caval and portal venous system are connected through the submucosal plexus. A rise in portal venous pressure can transform these submucosal veins into varices as is seen in portal hypertension [14].

Lymphatics form a dense submucosal plexus which runs along the long axis of the esophagus. Lymph flows primarily along the long axis of the esophagus the direction of which is cephalad in the proximal two thirds of the esophagus and caudad in the distal third. Nevertheless, a definitive watershed line for the demarcation of lymphatic drainage is not evident: lymph can course freely along the entire length of the esophagus via the esophageal plexus before draining into the regional nodes. This lymphatic network serves as a means for the spread of cancer intramurally. Consequently, cancers of the upper esophagus can metastasize to the superior gastric nodes or cancers of the lower esophagus to the superior mediastinal nodes. The submucosal plexus gives off branches which communicate with the peri-oesophageal

lymph plexus which then drains into the posterior mediastinal nodes. Again, these nodes can drain into both the supraclavicular and the left gastric nodes [12].

In general, lymph from the upper esophagus drains mostly into the cervical and paratracheal nodes and that from the lower thoracic and abdominal esophagus into the retrocardiac and celiac nodes.

3. Histology

The mucosal lining of the esophagus comprises a thick layer of stratified squamous non-keratinizing epithelium. Proximally, it is continuous with the mucosa of the oropharynx. Histologically, the gastroesophageal junction is delineated by an irregular line (the "Z line") between the stratified squamous epithelium proximally and the simple columnar epithelium distally. However, patches of gastric epithelium can be found proximal to the squamocolumnar junction. Deep to the mucosal lining are the lamina propria and the muscularis mucosa. The submucosa is a layer of connective tissue layer that lies deep to the mucosa. It contains small vessels, lymphatics, nerves and mucous glands. The submucosa is widely considered the strongest layer of the esophageal wall. Meissner's nerve plexus is found in the submucosa [10, 15].

The tunica muscularis comprises two layers; the external or longitudinal muscle layer and the inner circular muscle layer both beginning at the level of the cricoid cartilage. Auerbach's plexus lies in the connective tissue between the circular and longitudinal muscular layers.

The musculature of the pharynx and proximal esophagus is striated and is gradually replaced by involuntary smooth muscle in the distal esophagus reflecting the embryonic development of the esophagus. The lower esophageal sphincter although not an anatomically defined sphincter is a high pressure zone which serves to prevent acid reflux into the esophagus. The tunica adventitia is the outermost thin layer of loose areolar tissue. It contains small vessels, lymphatics and nerves. The esophagus lacks a serosal lining; anastomotic dehiscence following esophageal resection and anastomosis has been attributed to this absence of this outermost layer [15].

4. Embryology

The esophagus develops from the foregut of the primitive endodermal tube which is embryological precursor of the gastrointestinal tract. The foregut starts to divide into the laryngotracheal and the oesophageal tubes in the fourth week of gestation. Failure of division may result in congenital anomalies ranging from esophageal atresia to tracheo-oesophageal fistulae. Distal to the oesophageal tube, the foregut dilates to form the stomach [10].

Cephalad to the aortic arch, the esophageal musculature is derived from the branchial arches whereas caudal to the aortic arch, the embryonic esophagus is suspended in a mesentery, similar to the rest of the foregut [10]. Hence, the tunica muscularis of the upper third of the esophagus comprises skeletal muscle whereas that of the middle and lower third is predominantly smooth muscle.

5. Physiology

The esophagus primarily serves as a conduit to convey food through the thoracic cage.

Swallowing may be divided into three phases. The oral phase is voluntary and results in the food bolus entering the pharynx. The pharyngeal phase is involuntary and initiates a peristaltic wave propelling food through the upper oesophageal sphincter.

The esophageal phase is a continuation of the peristaltic wave initiated by the superior constrictor in the pharynx into the esophagus allowing the bolus to reach the stomach. Failure to do so results in esophageal distension which triggers secondary peristalses.

The lower esophageal sphincter is primarily a physiologic sphincter. The high pressure (15–25 mmHg) in this region serves to prevent the reflux of gastric juices into the esophagus. Other factors contributing to the functionality of the LES are the diaphragmatic crura, the gastric sling fibers, the valvular effect of the gastroesophageal angle and the positive intra-abdominal pressure. Gastroesophageal reflux disease is considered a predisposing factor for the development of adenocarcinoma of the esophagus [16–18].

6. Biology of esophageal cancer

Esophageal cancer shows marked variations in incidence, histopathological type of malignancy according to gender, ethnicity and geographic location. Environmental and socioeconomic factors also play a key role in carcinogenesis [3].

The two main histopathologic types of esophageal cancer are squamous cell carcinoma and adenocarcinoma. Other uncommon variants include squamous cell carcinoma with sarcomatous features, mesenchymal tumors, adenoid cystic carcinoma, mucoepidermoid carcinoma, neuroendocrine cancer and benign tumors.

The incidence of esophageal adenocarcinoma in Europe and in the United States has seen a steep rise in the past 2 decades, surpassing SCC as the most common type of esophageal cancer [4]. The rate of SCC of the esophagus has remained relatively stable or has seen a declining trend in Western countries [19–21]. Predisposing factors include gastroesophageal reflux disease and the ensuing Barrett's esophagus, obesity and smoking [20].

Nevertheless, squamous cell carcinoma remains the most common variety of esophageal cancer worldwide, arising as a result of long standing irritation of esophageal lining most commonly due to smoking and alcohol abuse, and occupational exposure. Tobacco and alcohol are strong, synergistic risk factors for the development of SCC [22]. Other notable predisposing factors are caustic injury, Plummer Vinson syndrome and achalasia cardia [20]. Squamous cell carcinomas of the esophagus are most likely to arise in the upper and middle thirds of the esophagus whereas esophageal adenocarcinomas are most common in the distal aspect of the esophagus.

7. Etiology of SCC

7.1 Environmental promoters of carcinogenesis

7.1.1 Alcohol

Alcohol abuse is a known risk factor for the development of esophageal. SCC more so when ingestion exceeds 170 g/week. This risk increases in a linear fashion with increasing consumption [23]. Key mechanisms in carcinogenesis include metabolic activation and decreased detoxification of potential

carcinogens, and increased cellular exposure to oxidants, a critical determinant of DNA damage. Also, production of acetaldehyde is increased, leading to diminished methyl transferase activity. The risk is compounded by synchronous exposure of tobacco [24].

7.1.2 Tobacco

Tobacco smoke contains polycyclic aromatic hydrocarbons, N-nitroso compounds, epoxides, lactones and tar, all of which are known carcinogens. They are irritant to the squamous epithelial lining of the esophagus and can give rise to metaplasia, a precursor of malignancy, on chronic exposure. Smoking is considered a risk factor for the development of both esophageal adenocarcinoma and SCC. Smoking was shown to contribute to a 12-fold greater incidence of atypical nuclei and a two-fold increase in incidence of in situ carcinoma within the basal layer of esophagus. Smokers have a nine-fold higher risk of developing SCC when compared to nonsmokers (hazard ratio 9.3; 95% CI: 4.0–21.3) [25, 26].

7.1.3 Nitrosamines

The human body is constantly exposed to N-nitrosamines at levels of 20–200 mcg/day. Nitrates and nitrites are precursors to N-nitroso compounds. These compounds are transformed in vivo into alkylating electrophiles that form adducts with DNA, by alkylating the N7 and O6 positions of guanine in the DNA helix.

7.1.4 Vitamin and mineral deficiency

Deficiencies of vitamins A, C, E and the B complex vitamins such as cyanocobalamine, riboflavin and folic acid may predispose to the development of squamous cell carcinoma of the esophagus. Among the micronutrients, zinc deficiency can induce carcinogenesis by the formation of O6-methylguanine DNA adducts by microsomal activation of N-nitrosomethyl-benzylamine. The trace element molybdenum is considered protective against the development of esophageal cancer by inhibiting the formation of nitrate reductases.

Selenium as an antioxidant plays a role in the inhibition of cell membrane lipid peroxidation. Deficiencies in these micronutrients have been linked to an increased risk of developing SCC of the esophagus [27–29].

7.1.5 Food and water contaminants

Fungi such as Fusarium, Alternaria, Geotrichium, Aspergillus, Cladosporium, and Penicillium have been associated with the development of esophageal cancer either by a direct mutagenic effect or through the formation of nitrosamines.

Other rarer causes of esophageal SCC are Helicobacter pylori infection, Plummer Vinson syndrome, caustic injury, achalasia cardia and human papillomavirus infection. HPV infection may account for as much as a third of all cases of esophageal cancer in high incidence areas as seen in Asia and South Africa [30, 31].

Barrett's esophagus, longstanding GERD and obesity are considered exclusive risk factors for the development of esophageal adenocarcinoma apart from other factors such as smoking, socioeconomic status, deficiency in dietary micronutrients which are also associated with SCC of the esophagus. Chronic gastroesophageal reflux leads to columnar metaplasia of the distal esophagus (Barrett's esophagus) which is associated with a 30- to 40-fold increased risk of progressing to esophageal adenocarcinoma [25].

7.2 Molecular oncogenesis

Epidermal growth factor (EGF) is an autocrine growth factor whose DNA is amplified in esophageal SCC. Overexpression of mRNA and the protein product appears to decrease survival. EGF receptor gene is the homolog of erb-B oncogene. The overexpression of the epidermal growth factor correlates with an increased frequency of lymph node metastasis [32].

Transforming growth factor-alpha is another autocrine growth factor that is co expressed with EGF and EGF receptor gene. They code for proteins that are homologous to EGF. The co-amplification correlates with advancing clinical stage and a worse prognosis in esophageal SCC [32, 33].

Ras encodes a protein product, p21, and has homology to G-proteins; a critical aspect of the signal transduction cascade. Over expression of p21 has been observed in esophageal SCC.

Tumor suppressor genes inhibit uncontrolled growth. They are necessary for repair to take place before damaged DNA is replicated. Gene inactivation in chromosome 17p is detected in at least half of esophageal cancers. PCR amplification and direct sequencing may detect p53 mutation in one third of specimens.

Human papilloma virus has been associated with the development of esophageal cancer. Low risk HPV genotypes are HPV 6 and 11.high risk genotypes are HPV 16, 18, and 33 [31].

Geographic distribution of esophageal squamous cell carcinoma SCC of the esophagus is the most common histologic type of esophageal cancer outside Western countries, where adenocarcinoma predominates. Incidence rates in China and some parts of Africa are estimated to be as high as 140 per 100,000 population [34]. Men and women are affected equally in these high-incidence areas [25]. However, in the United States and Europe, the incidence is much lower, estimated to be around 3 cases per 100,000 population with a declining trend [34].

8. Screening and early detection

Despite several potential preventive measures, none have been proven to decrease the risk of esophageal carcinoma in prospective well-designed trials [23]. The relatively low incidence of disease, absence of symptoms in the early stage, and the rarity of hereditary forms make population-based screening untenable except in certain high-risk areas of the world [35].

9. Diagnosis

The management of esophageal cancer remains a challenge even today because of the late stage at presentation of the majority and the overall poor prognosis of disease. It is estimated that only one in eight esophageal cancers are identified at an early stage (T1). These include cancers diagnosed incidentally during a gastroscopy performed for other reasons or during the course of surveillance programs. However, most esophageal cancers are diagnosed after symptoms develop. Typical symptoms which prompt patients to seek medical attention include dysphagia (which signifies a 50% reduction in the esophageal lumen) [36], vomiting, loss of body weight, and gastrointestinal bleeding. In general, these are manifested in tumors that are locally advanced and hence, inoperable. Moreover, unlike esophageal adenocarcinoma, which evolves from premalignant conditions such as Barrett's

esophagus in the background of gastroesophageal reflux disease, SCC lacks a premalignant stage. Hence, they tend to present at an advanced stage.

Gastroscopy remains the investigation of choice for the diagnosis of esophageal cancer as it permits the visualization of mucosal abnormalities and enables retrieval of tissue for histopathological examination. If erosions, ulcers, or strictures are found, the endoscopist decides whether these changes are neoplastic or not and whether they necessitate a biopsy. Dysplastic signs are discolorations, fine granulated surfaces (orange peel effect), as well as small elevations and troughs.

The sensitivity of endoscopy in detecting early-stage carcinoma may be improved by adjunctive techniques such as chromoendoscopy (using 1.5–3% acetic acid for adenocarcinoma and 0.5–1% Lugol's solution for SCC) or virtual chromoendoscopy which serves to highlight foci that are suspicious for malignancy.

A 'targeted' biopsy may be obtained from these areas for confirmation [37]. The current recommendation with respect to diagnostic tissue sampling in esophageal cancer is that a minimum of eight samples be taken from the lesion specifically from the margins and center; sensitivity for biopsies in detecting esophageal cancer has been shown to be 96% when multiple samples are taken from the lesion in question [37]. Alternatively, a diagnostic endoscopic mucosal resection may be performed [38].

Preoperative assessment and staging of esophageal SCC As in any malignancy, accurate staging is crucial for optimizing treatment in esophageal cancer. The depth of the tumor determines the feasibility of endoscopic management.

Several imaging techniques have been employed in the preoperative staging of these patients [39–45]. These include endoscopic ultrasonography (EUS), computed tomography (CT) and magnetic resonance imaging (MRI) among others. However, all these modalities have their limitations.

Numerous studies have demonstrated the superiority of EUS in both local tumor (T) and nodal (N) staging over CT [46]. EUS is the ideal modality for assessing the depth of invasion of the primary tumour with accuracy for T staging approaching 90% in superficial and partially obstructing esophageal cancers [47]. However, accuracy declines in cases of completely obstructing tumors wherein the luminal compromise associated with disease cannot be negotiated by the echo-endoscope [47–50]. This is considered the major limitation of this technique and precludes accurate staging in 16–50% of esophageal cancer patients [49–50]. Also, its ability to discriminate between subtle differences in T1 disease, that is, T1a versus T1b, is less exact [51]. For assessing regional lymph node metastases, EUS is reported to be more sensitive but less specific than CT and hence carries a risk of over-staging [48, 49]. Endosonographic characteristics of a malignant lymph node include size >10 mm, round and smooth features, proximity to the primary tumor, and hypoechogenicity. The accuracy of EUS for nodal staging based solely on these criteria approaches 80% [52, 53]. Accuracy of nodal staging can be increased to 92–98% when FNA of the lymph node is performed concurrently with EUS [53, 54]. However, false positive results, as a result of contamination by exfoliated cancer cells from the primary tumor site, are a possibility when EUS guided FNAC of suspicious nodes is performed [55].

EUS is not indicated for the evaluation of distant lymph node metastases, where CT is preferred [49]. Other limitations associated with EUS are its limited availability and operator dependence for accurate staging. With respect to its ability to provide accurate staging information after neoadjuvant therapy, EUS is not considered reliable due to the presence of post-treatment adherence and fibrosis [56].

To date, MRI has not gained widespread acceptance for the locoregional staging of cancers of the esophagus. Despite initial data [57] suggesting inferiority of MRI when compared to CT with respect to accuracy in staging esophageal malignancies,

subsequent literature [58] reported that the two modalities were comparable when assessing resectability of carcinomas of the esophagus. Nevertheless, CT remains the most widely used imaging modality on account of its utility in detecting metastatic disease and greater availability. Moreover, a CT scan also provides useful information regarding extension of the tumor especially with regard to involvement of the trachea or the aorta (T4b disease). Suspicion of direct invasion of the thoracic aorta or the tracheobronchial tree should be confirmed with MRI and bronchoscopy, respectively.

An abdominal ultrasound or preferably, a multi-slice CT scan of the thorax and abdomen are required for metastatic evaluation of the tumour before definitive therapy is initiated.

FDG-PET scan provides the most accurate information regarding potential metastatic disease, increasing the accuracy of detecting occult metastasis by as much as 20% over CT alone [59]. Also, FDG-PET is considered a reliable imaging modality for post-treatment reassessment and to assess the response to neoadjuvant therapy [60]. However, its specific indication and role in this scenario is yet to be defined [61].

AJCC 8th Edition [62]

TNM clinical classification—squamous cell carcinoma and adenocarcinoma

T—primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	High-grade dysplasia, defined as malignant cells confined to the epithelium by the basement membrane
T1	Tumour invades lamina propria, muscularis mucosae, or submucosa
T1a	Tumour invades lamina propria or muscularis mucosae
T1b	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4	Tumour invades adjacent structures
T4a	Tumour invades pleura, pericardium, azygos vein, diaphragm, or
T4b	peritoneum Tumour invades other adjacent structures such as aorta, vertebral body or trachea
N—regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1–2 regional lymph nodes
N2	Metastasis in 3–6 regional lymph nodes
N3	Metastasis in 7 or more regional lymph nodes
M—distant metastasis	
M0	No distant metastasis
M1	Distant metastasis
Definition of histologic grade (G)—squamous cell carcinoma and adenocarcinoma	
GX	Grade cannot be assessed
G 1	Well differentiated
G2	Moderately differentiated

G3	Poorly differentiated, undifferentiated		
Stage and prognostic group			
Clinical stage			
Stage 0	Tis	N0	M0
Stage I	T1	N0, N1	M0
Stage II	T2	N0, N1	M0
	T3	N0	M0
Stage III	T1,T2	N2	M0
	T3	N1, N2	M0
Stage IVA	T4a,T4b	N0, N1, N2	M0
Stage IVA	Any T	N3	M0
Stage IVB	Any T	Any N	M1

10. Treatment

10.1 Early stage cancer

Early esophageal cancer as an entity, according to the AJCC seventh edition, comprises all high grade dysplastic lesions and T1 malignancies [62]. Presence of intraepithelial malignant cells without a breach in the basement membrane is termed high grade dysplasia. T1 lesions include malignancies involving the mucosa (T1a) and submucosa (T1b) but not invading the muscularis propria.

In order to facilitate greater precision in staging and to further optimise stage-specific treatment in early esophageal cancer, T1a and T1b lesions have been further categorized into three subtypes (M1–M3 and SM1–SM3, respectively) according to the depth of invasion. Endoscopic mucosal resection is considered feasible in cancers involving the upper third of the submucosa (SM1 lesions) [63–66].

10.1.1 High grade dysplasia and T1a lesions

In cancers confined to the mucosal layer, the risk of lymph nodal disease correlates with the depth of tumour invasion and the histological type. For HGD or for intramucosal cancer, a systematic review of surgical literature, has reported that the rates of occult invasive cancer in patients undergoing esophagectomy for the treatment of HGD was 12.7% (pooled average in 441 patients from 23 studies) [67]. The rate of node positivity in high grade dysplasia and T1a cancers was estimated to be 0–2%. A retrospective review of 126 patients with T1 tumors of adenocarcinoma histology reported the rate of nodal involvement in T1a and T1b as 1.3–22%, respectively [64]. Data in early esophageal cancer has shown that M3 cancer (disease extending to the muscularis mucosa) has at least 6% risk of lymph metastases [63]. Additional characteristics which impact the risk of nodal involvement include vascular invasion, tumor size, and the degree of tumor differentiation. Given the low risk of node positivity in early stage esophageal cancer confined to the mucosa, there is general consensus that endoscopic management is adequate and reliable for the treatment of mucosal disease (T1a). Endoscopic resection is, therefore, curative in such lesions. Initially, options included argon beam coagulation, laser, and photodynamic therapy. More recently, endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), radio-frequency ablation (RFA), cryotherapy, and free-hand mucosal resection have

increasingly been applied [68]. However, data on these modalities of treatment are limited at present, and efficacy of one technique over another has not been established [69].

However, all visible lesions should ideally be removed by EMR for definitive histopathological staging and to ensure adequacy of resection margins. This recommendation is based on the poor accuracy of EUS to discriminate between T1a and T1b lesions. In this regard, EMR remains the sole technique able to stage the degree of invasion into the esophageal wall. For intramucosal cancer associated with Barrett's esophagus, eradication of the metaplastic mucosa is essential to prevent the development of potentially malignant lesions. For segments that measure ≤5 cm and harbor HGD or intramucosal cancer, an EMR approach is used. For patients with segments >5 cm, all focal lesions are resected with EMR or ESD and the residual base of the Barrett's lesion radiofrequency ablated which reduces the incidence of stricture formation [68].

10.1.2 T1B and T2 tumors

As mentioned above, lymphatic invasion and hence, nodal involvement in T1a lesions is uncommon. However, once the muscularis mucosa is breached, dissemination of cancer cells can occur via the submucosal lymphatic plexus. Thus, T1b and T2 cancers have a disproportionately higher incidence of node positivity when compared to T1a cancers [64]. The depth of invasion beyond which an endoscopic resection is considered inadequate treatment remains controversial. In one clinical series, it was demonstrated that EMR could be performed in low grade submucosal SM1 lesions (considered 'low risk' tumors) [70]. At a mean follow-up of 5 years, no tumor related deaths were reported. However, according to other series, rate of node positivity in SM1 tumors is in the range of 16.5–21% [64, 71–73]. For tumors invading beyond SM1, existing literature suggests that the incidence of nodal involvement in patients with T1b cancer ranges from 21 to 50% [59, 74].

Also, in a review of outcomes for T2 lesions, the current approach to clinical staging correlated with the pathological stage in just 13% of patients of those inaccurately staged, 63% were overstaged and the rest, understaged. Based on these results, the recommendation for treatment of T2 lesions is to proceed with definitive surgery as it is considered optimal in both patients who are overstaged and accurately staged.

With regard to T1b and T2 cancers, the general consensus is to proceed to surgical resection without neoadjuvant therapy [75]. Patients who are discovered to be understaged after esophagectomy can be considered for adjuvant therapy [76]. Indications for esophagectomy in early stage esophageal cancer include failures of endoscopic therapy and all incomplete endoscopic mucosal resections.

Invasion of tumour into the submucosa is now considered an indication for esophagectomy, although invasion into the superficial third of the submucosa does not carry the same risk of nodal metastasis as the deeper two thirds, and could be potentially treated endoscopically [77, 78]. Apart from tumor characteristics, the treatment modality chosen may also be tailored according to the patient preferences and characteristics and the surgical or endoscopic expertise available. A vagal sparing esophagectomy has also been proposed recently as an alternative to conventional esophagectomy. This procedure, which involves resection of the esophagus from the mediastinum using a stripping device leaving the vagi and the nodes intact, has been reported to offer several advantages in carefully selected patients including the preservation of meal size, gastric emptying and BMI [74, 79]. However, prospective data in support of this technique is not available.

10.1.3 Indication of neoadjuvant therapy in early stage cancer

Surgery alone in the form of an esophagectomy remains the standard treatment for early stage cancer. Data promoting the benefits of neoadjuvant treatment for localized esophageal cancer is scant. The Fédération Francophone de la Cancérologie Digestive (FFCD) 9901 assessed whether preoperative chemoradiotherapy (CRT) improved outcomes in patients with localized (stages I or II) esophageal cancer [75]. From 2000 to 2009, 195 patients were randomized and assigned either to the surgery only group (n = 98) or to the neoadjuvant CRT group (n = 97). Although postoperative morbidity rates were not statistically significant between the two groups, 30 day-mortality rates were 1.1% in the surgery alone group compared to 7.3% in the CRT group (p = 0.054). At a median follow-up of 5.7 years, the median survival was 43.8 months in the surgery group compared to 31.8 months in the CRT group (HR 0.92; 95% confidence interval 0.63–1.34; p = 0.66). The trial concluded that neoadjuvant CRT with cisplatin and fluorouracil does not improve overall survival but increases postoperative mortality in patients with stage I and II esophageal cancer compared with surgery alone.

10.2 Locally advanced esophageal cancer

The vast majority of esophageal cancers are found to be locally advanced at presentation. Traditionally, both locally advanced esophageal SCC and adenocarcinoma have been managed with surgical resection. In this regard, esophagectomy with radical lymphadenectomy was considered to be the ideal treatment in terms of achieving local control. However, many patients developed locoregional recurrence or metastatic disease after surgery and survival was poor. Analyses of disease recurrence patterns and the dismal outcomes following surgery alone in this subset of patients of prompted the introduction of adjuvant treatment as a means of achieving locoregional control. However, esophagectomy being a major procedure with high morbidity, adjuvant therapy may not always be feasible and hence, management strategies have now adopted neoadjuvant therapy. In some cases of carcinoma esophagus and more so in esophageal SCC, definitive CRT has been advocated as the first line treatment, taking into consideration the excellent response achievable by this modality. In these cases, surgery is reserved as a second line therapeutic option for patients in whom definitive CRT has failed (termed a "salvage" esophagectomy).

10.2.1 Neoadjuvant chemotherapy or chemoradiotherapy

Both neoadjuvant chemotherapy and radiotherapy are known to improve overall and disease free survival. They improve locoregional disease control by downstaging the cancer and improving resectability rates. Moreover, chemotherapy eradicates systemic micrometatstic disease by impeding the dissemination of cancer cells. Ameta-analysis by Gebski et al. evaluated outcomes associated with neoadjuvant chemotherapy and chemoradiotherapy followed by surgery compared to surgery alone in patients with locally resectable esophageal cancer regardless of the histological type [80]. The analysis included pooled data from 10 randomized controlled trials comparing surgery alone with neoadjuvant CRT and 8 randomized controlled trials comparing neoadjuvant chemotherapy with surgery. In the neoadjuvant chemotherapy group, the hazard ratio (HR) for all-cause mortality was 0.90 (95% CI, 0.81–1.00; p = 0.05), indicating a 2-year absolute survival benefit of 7%. Survival benefit associated with neoadjuvant chemotherapy differed with the cancer histology: patients with SCC did not experience a survival benefit with neoadjuvant chemotherapy [HR for mortality 0.88 (0.75–1.03); p = 0.12] whereas

in the adenocarcinoma group, survival benefit was significant [HR for mortality 0.78 (0.64–0.95); $p = 0.014$]. In the neoadjuvant chemoradiotherapy group, the HR for all-cause mortality was 0.81 (95% CI, 0.70–0.93; $p = 0.002$), corresponding to a 13% absolute difference in survival at 2 years when compared to surgery alone. With respect to tumour histology, neoadjuvant CRT was associated with a significant benefit over surgery in both esophageal adenocarcinoma and squamous cell carcinoma [HR of 0.84 (0.71–0.99; $p = 0.04$) for SCC and 0.75 (0.59–0.95; $p = 0.02$) for adenocarcinoma].

The updated meta-analysis published by Sjoquist et al in 2011 included 4,188 patients from the 17 studies evaluated in the previous meta-analysis with an additional seven more recent studies [81]. The inter-group analysis demonstrated strong arguments for CRT compared to CT in patients with SCC or adenocarcinoma. The HR for all-cause mortality for neoadjuvant chemotherapy was 0.87 (0.79–0.96; $p = 0.005$). When comparing the histological subtypes, the HR for SCC only was 0.92 (0.81–1.04; $p = 0.18$) whereas that for adenocarcinoma was 0.83 (0.71–0.95; $p = 0.01$). The HR for all-cause mortality for neoadjuvant CRT was 0.78 (95% CI, 0.70–0.88; $p < 0.0001$); that for SCC only was 0.80 (0.68–0.93; $p = 0.004$) and for adenocarcinoma, 0.75 (0.59–0.95; $p = 0.02$). When comparing all-cause mortality for neoadjuvant CRT versus neoadjuvant chemotherapy, the HR for the overall indirect comparison was 0.88 (0.76–1.01; $p = 0.07$).

However, the above meta-analysis did not include data from the recent phase III 'CROSS' trial which compared the outcomes associated with concurrent CRT (involving carboplatine and plaxitaxel with 41 Gy) followed by surgery and surgery alone [82]. A pathological complete response was noted in 47 of 161 patients (29%) who received neoadjuvant CRT followed by surgery. Despite the rate of postoperative complications and in-hospital mortality being similar in both groups, the overall survival was significantly better in the CRT group [HR 0.657 (0.495–0.871; $p = 0.003$)]. Median OS was 49.4 months in the CRT followed by surgery group as against 24 months in the surgery alone group.

In conclusion, neoadjuvant CRT is strongly recommended and may be considered the standard of care in patients with locally advanced esophageal cancer compared to neoadjuvant chemotherapy alone. However, the optimal neoadjuvant treatment regimen has not been established yet, as the various trials conducted have employed different drugs, doses, and schedules of chemotherapy and radiotherapy.

10.2.2 CRT: sequential or concomitant?

Gebski et al, in their meta-analysis, concluded that there was no survival benefit of sequential CRT for patients with SCC [HR for mortality 0.9 (0.72–1.03); $p = 0.18$]; the results obtained in the sequential CRT group were similar to that of the neoadjuvant chemotherapy group [80]. Concomitant CRT in patients with SCC had a significant benefit [HR for mortality 0.76 (0.59–0.98); $p = 0.04$]. On this basis, concomitant CRT has been recommended in patients with locally advanced cancer of the esophagus planned for neoadjuvant therapy.

10.2.3 Neoadjuvant or adjuvant treatment?

This issue was addressed by the Japan Clinical Oncology Group which conducted two randomized controlled trials to assess potential benefits of adding adjuvant therapy to surgery in patients with SCC. The JCOG 9204 sought to identify the benefit associated with adjuvant cisplatin plus 5-FU when compared to surgery alone in patients with resectable stage I and II esophageal cancer [83]. Although overall survival was not significantly different between the two groups (5-year

survival rate 52 vs. 61%; $p = 0.13$), disease-free survival was significantly better in those receiving postoperative CT, especially in node positive disease. In the JCOG 9907 study, neoadjuvant cisplatin and 5-FU was compared with adjuvant cisplatin plus 5-FU in patients with clinical stage II or III esophageal cancer [84]. In terms of overall survival, neoadjuvant CT was found to be superior with a 5-year survival rate of 60% compared to 38% in the adjuvant group ($p = 0.013$). Based on the results of these studies, neoadjuvant chemotherapy followed by radical surgery is currently recommended as the standard in locally advanced SCC.

10.2.4 Neoadjuvant CRT followed by surgery or definitive CRT?

Definitive CRT as a treatment modality in the management of esophageal cancer was introduced following the Radiation Therapy Oncology Group (RTOG) 8,501 study [85]. This trial, which included both esophageal SCC and adenocarcinoma, compared outcomes after RT alone (64 Gy) with concurrent CRT (cisplatin, 5- FU, and radiotherapy 50 Gy). This study demonstrated the strong sensitivity of SCC to concomitant CRT which resulted in better overall survival and decreased local failure rates when compared to RT alone. Subsequently, a Japanese phase II trial analyzed the efficacy of definitive CRT (cisplatin and 5-FU with classic portal radiation 60 Gy) in squamous cell carcinoma of the esophagus [86]. Although a complete response was obtained in 68% with a 3-year survival rate of 46%, these results were not superior to those obtained with conventional surgical resection with or without chemotherapy. Among the trials conducted comparing definitive CRT with neoadjuvant CRT in esophageal SCC, the study performed by the German Esophageal Cancer Study Group reported that the 2-year overall survival was similar in the neoadjuvant CRT followed by surgery group (39.9%) and the definitive CRT treatment group (35.4%) [87]. The neoadjuvant therapy group was complicated by a higher rate of early postoperative mortality, while definitive CRT was associated with a higher incidence of local relapses. These results were reproduced in another large randomized study, the FFCD-9102, where surgery was proposed in responders to CRT. Although surgery was reported to improve local control, it did not translate to an improvement in survival as neoadjuvant therapy was associated with increased early mortality [88].

On the basis on these results, both definitive CRT and neoadjuvant CRT followed by surgery seem to have similar long-term results. Despite flaws in these studies, surgery appears to provide better local control of the tumor but without any impact on long-term survival outcomes. Cost of major surgery and the risk of postoperative mortality are important factors that should be considered in patients being planned for neoadjuvant therapy followed by esophagectomy.

10.3 Salvage esophagectomy

In Japan and in Western countries, medical and radiation oncologists have reported satisfactory outcomes with definitive CRT blurring the boundaries of traditional treatment strategies. Definitive CRT is now considered a treatment option even in potentially resectable patients. Another factor favoring definitive CRT is that a complete response has been noted in the resected specimen in 15–30% of patients undergoing neoadjuvant therapy followed by surgery [81]. However, persistent disease and risk of local failure after definitive CRT remains a concern. It should be noted that locoregional morbidity need not always be related to the neoplastic process; local toxicity secondary to CRT or mechanical complications such as stricture formation may also be associated. Locoregional recurrence is defined as tumor detected more than 3 months after CRT whereas persistent

disease is the detection of malignancy within 3 months of CRT at the same site [89]. Unfortunately, locoregional control is often quite poor with definitive CRT, and up to 40–60% of the patients have persistent or relapsed tumor at the primary site within 1 year [88]. Moreover, due to radiotherapy associated fibrosis, histological confirmation of the malignancy is achievable in less than 60% of cases [90]. The prognosis is dismal in 11–26% of patients who do not exhibit any morphologic tumor response following definitive CRT (median survival of 9 months) [91]. Salvage esophagectomy is considered the only curative option for a subset of carefully selected patient who have received up to 50 Gy of radiation and who are physiologically fit for surgery. A number of studies have demonstrated the utility of salvage esophagectomy as a therapeutic option in recurrent or persistent disease following definitive CRT [90–98] with a subset of patients being cured after salvage esophagectomy with acceptable long-term outcomes. However, the decision to proceed with salvage esophagectomy is seldom straightforward considering the high postoperative morbidity and mortality associated with this procedure; each case must be evaluated individually. Initial studies examining the utilization of 'salvage esophagectomy' indicated that the procedure was associated with a significantly higher incidence of post-operative mortality, anastomotic leak, pulmonary complications and an increased length of ICU and in-hospital stay [89–99]. Much of this concern originated from the historical impression that surgical resection 4–8 weeks following radiotherapy or CRT was technically more challenging and associated with increased postoperative morbidity and mortality. This opinion has recently been challenged [100] with several publications demonstrating that the selected utilization of salvage esophagectomy in patients who have failed definitive CRT for esophageal SCC resulted in acceptable morbidity and mortality rates [89, 90, 94]. Special attention should be paid to the dose of radiation given: salvage surgery is considered highly morbid when the volume dose of radiation exceeds 55 Gy [90]. A randomized clinical trial assessing long-term outcomes indicated that definitive CRT could potentially cause progressive deterioration in pulmonary function when compared to surgery alone [100].

10.4 Minimally invasive esophagectomy (MIE)

MIE includes total thoraco-laparoscopic esophagectomy, robot-assisted minimally invasive esophagectomy (RAMIE) and hybrid procedures. Over the last decades, MIE has expanded worldwide and is estimated that they account for 15–30% of all esophagectomies performed at present [101, 102]. It seems likely that importance of MIE will exceed that of hybrid techniques. There are now centers that are publishing consecutive series of over 1000 minimally invasive procedures [103]. The approach to esophagectomy varies from center to center, and any decision regarding the surgical approach should be tailored according to individual physiologic and tumor-related issues in each patient [104].

11. Conclusions

Management of esophageal cancer has been refined since the last decades. Surgery continues to play a pivotal role in the treatment of the disease, either alone or in combination with multimodal approach. Progress in anesthesia and in surgery has led to a significant decrease in the mortality rate. Mortality rates average 5% and are under 2% in some experienced and high volume centers. The progress made in the field of minimal access surgery has led surgeons to consider these techniques to reduce the morbidity and mortality that have traditionally been associated with

surgery of the esophagus. Qualified surgeons with a high-level of expertise in high-volume centers are essential in this context to ensure optimal outcomes.

12. Future perspective

Multimodality treatment involving the surgeon, gastroenterologist, oncologist (medical and radiotherapy), radiologist, pathologist, and palliative care physicians is fundamental in the management of esophageal cancer. This serves to individualize treatment, optimize outcomes and ensure the best possible quality of life for the patients. Minimally invasive techniques have been proven to be noninferior to open surgery in terms of oncological safety and will benefit the patient in terms of post-operative recovery. In future, advances in cancer genomics and gene testing can be expected identify key genetic and epigenetic alterations in cancers of the esophagus which initiate the growth and progression of disease. Identification of these genetic alterations may also result in the introduction of targeted therapies which may be individualized based on the molecular profile of the cancer.

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Section 4

Anal Squamous Cell Carcinoma

Chapter 4

Evolving Concepts toward Individualized Treatment of Squamous Cell Carcinoma of the Anus

Luc Dewit, Annemieke Cats and Geerard Beets

"If you do not change direction, you may end up where you are heading",
Lao Tsu, Chinese philosopher.

Abstract

Treatment of squamous cell carcinoma of the anus has evolved over the last 5 decades from radical surgery to combined chemoradiation therapy. Radiation treatment techniques have dramatically improved with the development of more powerful computers, algorithms and treatment machines. The clinical impact of the modern radiation treatment techniques, such as intensity-modulated radiotherapy and volumetric modulated arc therapy, is discussed. The standard-of-care regimen still is concurrent Mitomycin C, 5-fluorouracil and high-dose radiation, as was conceived 45 years ago. Variants of this schedule are discussed in this chapter. International guidelines have been generated and implemented. Whereas concurrent chemoradiation therapy is the treatment of choice for locally advanced tumors, early tumors are probably adequately controlled with either reduced dose chemoradiation therapy or radiation therapy alone. Prognostic factors, such as high-risk human papillomavirus, epidermal growth factor receptor and immune response, will be highlighted. The role of surgery in primary care is limited to local excision of T1N0 tumors ≤ 1 cm of the anal margin. Salvage radical surgery is limited to locoregional recurrent, non-metastasized and resectable tumors after chemoradiation therapy. In addition, new treatment modalities, such as targeted therapy and immunotherapy, will be discussed. Current research aims at refining prognostic subgroups to further individualize treatment strategy, implementing quality assurance protocols in international trials and investigating the molecular profile of squamous cell carcinoma of the anus, in order to identify new treatment avenues. This will hopefully change the landscape of anal cancer treatment in the future.

Keywords: anal carcinoma, radiotherapy, chemoradiation therapy, prognostic factors, surgery, biological agents

1. Introduction

Squamous cell carcinoma of the anus (SCCA) is a rare tumor with an increasing incidence over the last decades [1]. It originates from the basal cells of the epithelial

layer of the anal canal, which extends from the anorectal junction to the anal orifice, or anal margin, which extends from the anal orifice to a radius of 5 cm laterally [2]. Tumors arising from the anal margin have a different biological behavior, and this will be briefly discussed later in this chapter. Most, but not all, SCCA are causally related with high-risk human papillomavirus (HPV-HR), mainly subtypes 16 and 18 [3, 4]. These tumors develop from high grade anal intraepithelial neoplasia (AIN3) through a number of consecutive oncogenic steps, which are only partially understood [5]. Radical surgery, which usually implies an abdominoperineal resection with a permanent end colostomy, has been shown to yield 5-year survival rates of only 20–70%, depending on stage and resection margins [6]. Radiation therapy has demonstrated superior survival rates with a high probability of organ preservation. The seminal papers of Nigro and colleagues have shown that the combination of radiation and chemotherapy resulted in even better survival rates, at least for locally advanced cases [7, 8]. This has been confirmed in two landmark randomized phase III trials [9, 10]. Hence, chemoradiation therapy (CRT) has largely replaced radical surgery in the treatment of SCCA.

The focus of this chapter is to highlight the evolving concepts toward individualized treatment of patients with SCCA, based upon prognostic parameters. Emphasis will be given to improved radiation treatment techniques, concurrent and (neo) adjuvant chemotherapy regimens, the role of HPV status, molecular markers and immune response. In addition, the role of surgery will be addressed.

2. Improved treatment of SCCA

2.1 Technical improvement of radiation treatment of SCCA

2.1.1 Radiation dose and target volume

The efficacy of (chemo)radiation treatment for SCCA has been known for several decades. The acute and late toxicity, however, was considerable with the large, non-conformal treatment fields, which often resulted in moderate functional outcome and quality of life [11]. With the development of more powerful computers, algorithms and treatment machines, more sophisticated treatment techniques became available. This has resulted in a shift from standard opposed anterior-posterior fields (AP-PA) or a four-field technique in the fifties through eighties of the previous century to 3D-conformal radiotherapy (3D-CRT) in the nineties and intensity-modulated radiotherapy (IMRT) in the early years of this century and volumetric modulated arc therapy (VMAT) in the last decade.

The difference in toxicity between 3D-CRT and IMRT or VMAT has never been compared in a prospective randomized trial, but several retrospective studies and one recent prospective study have reported an improved toxicity profile with the newer techniques [12–17]. A recent national audit in the UK comparing these techniques confirmed the reduced toxicity with IMRT (**Table 1**) [18]. A few studies also claim a better disease-free survival (DFS) and locoregional control (LRC) with IMRT [12, 14, 19].

Toxicity is largely related to the radiation dose and the volume of normal tissues exposed to radiation, which in turn is related to the gross tumor volume (GTV) and clinical and planning target volume (CTV and PTV). The GTV is determined by the macroscopic local tumor extent and documented macroscopically involved regional lymph nodes, whereas the CTV is dependent on the site of regional lymph nodes that are considered to be at risk for microscopic metastatic disease. In addition, the PTV is determined by the set-up error of patient positioning. With the advent of magnetic resonance imaging (MRI) and fluor-18-deoxyglucose positron

Comparison of grade 3+4 acute toxicity during chemoradiotherapy (CRT) seen in the ACT2 publication, all UK audit patients, UK audit patients undergoing ACT2 regimen and UK audit patient treated in keeping with UK intensity-modulated radiotherapy (IMRT) guidance

	ACT2 trial*	All UK audit patients	Two-phase conformal CRT in UK audit	IMRT as per guidance in UK audit
	(n = 472†)	(n = 199 non-haematological; n = 192 haematological*‡)	(n = 45*‡)	(n = 127 non-haematological; n = 120 haematological*‡)
Non-haematological§	294 (62%)	87 (44%)	22 (49%)	51 (40%)
Gastrointestinal	75 (16%)	26 (13%)	5 (11%)	17 (13%)
Nausea	10 (2%)	6 (3%)	2 (4%)	4 (3%)
Vomiting	9 (2%)	4 (2%)	1 (2%)	3 (2%)
Diarrhoea	44 (9%)	18 (9%)	2 (4%)	13 (10%)
Stomatitis	14 (3%)	5 (3%)	1 (2%)	3 (2%)
Other gastrointestinal	16 (3%)	1 (1%)	0	1 (1%)
Skin	228 (48%)	60 (30%)	18 (40%)	32 (25%)
Pain	122 (26%)	28 (14%)	6 (13%)	16 (13%)
Cardiac	7 (1%)	3 (1%)	0	3 (2%)
Other non-haematological	34 (7%)	8 (4%)	2 (4%)	6 (5%)
Haematological§	124 (26%)	31 (16%)	6 (13%)	21 (18%)
Neutrophils	112 (24%)	25 (13%)	5 (11%)	15 (13%)
Platelets	21 (4%)	13 (7%)	3 (7%)	9 (8%)
Haemoglobin	2 (<1%)	2 (1%)	0	2 (2%)
Febrile Neutropenia	15 (3%)	2 (1%)	0	1 (1%)
Any toxic effect§	334 (71%)	104 (52%)	25 (54%)	62 (48%)

* Only the highest grade is counted and patients with more than one toxic effect of a particular grade were counted only once.
† Patients in the mitomycin/5-fluorouracil arm only were used for toxicity comparison.
‡ Numbers and percentages based on patients with submitted toxicity.
§ Patients with more than one toxic effect counted only once.

Table 1.
UK National Audit of anal cancer radiotherapy 2015 [18]. Reproduced with permission of Elsevier.

emission tomography (^{18}F FDG-PET), much improvement is made over the years in visualizing the primary tumor and involved regional lymph nodes and, hence, delineating GTV. In contrast, the estimation of microscopic metastatic disease remains poor and is largely based upon a few studies with documented locoregional recurrence in relation to tumor size and irradiated volumes [20–22]. The CTV for SCCA is notoriously complex, given the potential involvement of inguinal, iliac, mesorectal and presacral lymph nodes. Consensus contouring guidelines have been developed to assist radiation oncologists in setting up a treatment plan [23, 24]. With respect to the radiation dose, a two or three dose level for microscopic and macroscopic disease has emerged form clinical trials. For instance, in the Radiation Therapy Oncology Group (RTOG) 87-11 trial, a radiation dose of 30.6 Gy was given to the common iliac lymph nodes whereas a dose of 45 Gy was delivered to the lower iliac lymph nodes and 50.4 Gy to the primary tumor [25]. In contrast, in the United Kingdom Coordinating Committee on Cancer Research Anal Cancer Trial (UKCCCR-ACT) I and the European Organization For Research and Treatment of Cancer Radiotherapy (EORTC) 22861 trial the common iliac lymph nodes were not included in the elective radiation field, whereas a dose of 45 Gy was given to the lower iliac and inguinal lymph nodes with a boost to 60–65 Gy to the primary tumor [9, 10]. In the subsequent UKCCCR-ACT II the dose to the iliac and inguinal lymph nodes was limited to 30.6 Gy and the boost to the primary tumor to 50.4 Gy [26]. Despite these differences in radiation dose and volume, no striking difference in LRC was observed between these trials [9, 10, 25]. A number of retrospective studies have reported a better LRC with a higher radiation dose, at least in the locally advanced tumors [27–30]. This was confirmed in a systematic literature review [31] and a recent retrospective study from a large Scandinavian database [32]. However, in the French prospective randomized ACCORD-03 trial, which included only locally advanced cases, a marginal, non-significant increase in colostomy-free survival (CFS), a surrogate endpoint for LRC, was observed after 70 Gy, as compared with 60 Gy [33]. Consequently, in the absence of definitive evidence, current clinical guidelines do not advocate a higher radiation dose for larger tumors [34, 35].

2.1.2 The treatment gap

In the initial trials, a treatment gap of 6 weeks was included at an intermediate radiation dose [9, 10, 25]. This was done to allow for recovery from acute radiation toxicity, but also to give the tumor time to regress and to assess whether a radiation boost should be given with external beam irradiation or with brachytherapy. As results matured and further insight in tumor radiobiology was gained, this long treatment gap was considered to be potentially hazardous, due to the likelihood of tumor repopulation during the treatment gap. In the subsequent studies, the treatment gap was shortened to 2 weeks, which not only seemed to be feasible, but also resulted in better LRC in some studies [36–40] but not in others [41, 42]. With the advent of IMRT and VMAT, the entire radiation course could be administered without a treatment break. Today, most modern radiotherapy centers have implemented IMRT or VMAT for SSCA.

2.2 Chemotherapy and radiation for SCCA

2.2.1 Landmark studies

In June 1973, Dr. Nigro presented 3 cases with SCCA at a meeting of the American Proctologic Society in Detroit, that were treated with radiation therapy (RT) and concurrent Mitomycin C (MMC) and 5-fluorouracyl (5-FU) in a preoperative setting [7]. The rationale for this approach was to improve the LRC and overall survival (OS) of SSCA, since the results with radical surgery alone were modest, at best. Dr. Nigro realized that, in contrast with rectal cancer, SCCA originates from an organ which has an abundant lymphatic vessel supply, that allows rapid lymphatic tumor spread. In addition, there is limited space in the lower pelvis for radical surgery. The radiation dose was 30 Gy in 3–5 weeks via two large anterior-posterior opposed fields, and 30 mg of MMC was given on day 1 in a single bolus infusion and 1500 mg per day of 5-FU on days 2–6 in a continuous infusion. Six to 8 weeks later, two of them underwent an abdominoperineal resection, as planned. No tumor was found on microscopic examination of the operation specimen in these two cases. The third patient refused surgery and remained free of disease 1 year later [7]. This treatment regimen was expanded in a larger series, which confirmed the excellent results [43]. This pioneering work formed the basis for definitive CRT with higher, therapeutic radiation doses.

The superiority of this regimen compared with RT alone was established in two randomized phase III trials, the UKCCCR-ACT I and the EORTC 22861 [9, 10]. These trials were executed almost parallel in time and their design was strikingly similar, except for the eligibility criteria: in the EORTC trial only locally advanced patients were eligible, whereas in the ACT I all stages were accepted for inclusion. Despite this imbalance in patient selection, no major difference in the treatment outcome was observed between these two trials. Both studies showed a significant improvement in LRC control with CRT as compared with RT alone [9, 10]. In the ACT I, 3-year LRC increased from 47% after RT alone to 70% after CRT with concurrent 5-FU and MMC [9]. The corresponding figures in the EORTC 22861 trial were 55 and 68%, respectively [10]. The difference in LRC and progression-free survival (PFS) in the ACT I remained up to 12 years after treatment [44]. However, no difference in OS was found in either of these trials [10, 44].

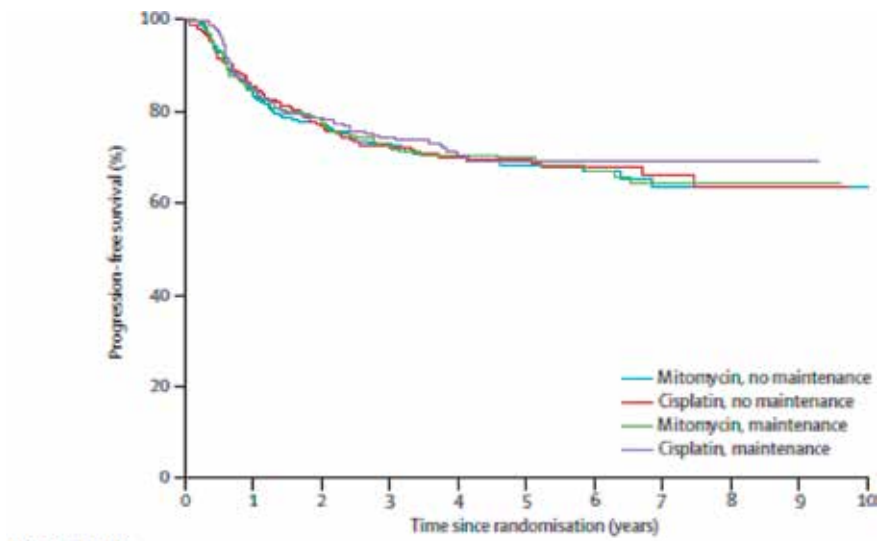
The value of MMC, in addition to 5-FU, was established in the phase III RTOG 87-04 study [25]. In this trial, however, MMC was given twice in the first and fifth week of the radiation treatment, as opposed to only once in the ACT I and EORTC 22861 trial. It resulted in considerably more grade 4-5 hematological toxicity than was seen in the European trials.

2.2.2 Subsequent pivotal studies

In the subsequent phase III RTOG 98-11 trial, the role of neo-adjuvant and concurrent cisplatin and 5-FU was addressed by comparing it with concurrent MMC and 5-FU [45]. While the combination of cisplatin and 5-FU was less toxic than MMC and 5-FU, the disease-free survival (DFS) and OS was significantly worse with the new regimen [46]. In the UKCCCR-ACT II, concurrent cisplatin, 5-FU and RT was compared with concurrent MMC, 5-FU and RT, with or without adjuvant cisplatin and 5-FU, in a 2 × 2 factorial design [26]. In this trial, which is the largest phase III trial carried out to date for anal cancer, no difference in PFS (**Figure 1**) and toxicity was observed between the four treatment arms [26]. The French phase III ACCORD 03 trial investigated the value of neo-adjuvant and concurrent cisplatin, 5-FU and RT, and radiation dose intensification, also in a 2 × 2 factorial design [33]. Whereas a marginal, non-significant increase in CFS was observed in the group that received the higher radiation dose, no difference in CFS was found between the patients with and without neo-adjuvant chemotherapy. Acute and late toxicity were similar between the four groups [33]. The EORTC 22011-40014 randomized phase II trial compared concurrent MMC, cisplatin and RT with MMC, 5-FU and RT [47]. The new combination proved to be highly effective, but more toxic, with a compliance of only 49% as opposed to 79% for the standard arm [47].

2.2.3 Variant schedules

In the UKCCCR-ACT I, EORTC 22861 and RTOG 87-04 trials, MMC was given once on day 1 [9, 10] or twice on day 1 and 29 of the radiation treatment [25],



Number at risk						
Mitomycin, no maintenance	223	266	117	48	15	0
Cisplatin, no maintenance	223	268	114	58	18	0
Mitomycin, maintenance	226	273	119	59	20	0
Cisplatin, maintenance	222	170	114	64	16	1

Figure 1.
MMC or cisplatin+5FU and radiation + or—adjuvant cisplatin/5-FU for SSCAC [26]. Reproduced with permission of Elsevier.

whereas 5-FU was administered in a continuous infusion day 1–4 or 5 and day 29–32 or 33. Variants of this treatment schedule have been explored with 5-FU given continuously in lower daily doses over the entire split-course radiation treatment [37], or by replacing 5-FU with capecitabine, an oral prodrug of 5-FU, given twice daily during the radiation treatment [48–51]. These schedules seemed feasible and equally effective as the standard schedule. In addition, capecitabine has the advantage of being able to be given on an outpatient basis.

Taken together, the original regimen of MMC and 5-FU remains the standard of care in CRT for SCCA, 45 years after its inception. There is a trend of using capecitabine instead of 5-FU because it is more patient friendly and equally effective. Arguably, MMC is more toxic than cisplatin in combination with 5-FU or capecitabine and RT [37], but this is dose dependent and seems to be equally effective in a single bolus of 10 mg/m^2 as 12 or 15 mg/m^2 or twice 10 mg/m^2 [9, 10, 25]. Furthermore, the combination of cisplatin and 5-FU is not more effective than MMC and 5-FU, but requires hospitalization for hydration procedures to prevent renal toxicity [26].

3. Prognostic factors in anal carcinoma

Well-known clinical prognostic factors in SCCA are age (>55 years better than ≤55 years), sex (female better than male), tobacco smoking (worse), primary tumor size and site (anal margin better than anal canal), T- and N-stage, tumor ulceration (worse if present) and histological differentiation grade [32, 52, 53]. Other prognostic factors include HPV-HR and certain genetic alterations.

3.1 Human papillomavirus

HPV-HR is causally related with the onset and progression of SCCA [5]. Once integrated into the host DNA, the main viral oncoproteins E6 and E7 interact with the tumor suppressor proteins p53 and retinoblastoma protein (pRb), respectively. P53 has a key role in maintaining DNA integrity, whereas pRb is a negative regulator of the cyclin-dependent kinase inhibitor p16. Upon persistent HPV-HR infection, p53 becomes permanently inactivated, disrupting DNA repair processes, and pRb inactivation induces upregulation of p16. As such, p16 is sometimes used as a surrogate marker of HPV-HR infection. These and other oncogenic processes lead to genomic instability, carcinogenesis and tumor progression. As a result, HPV-HR+ SCCA have a number of unique features, some of which have a prognostic or even a predictive value (**Figure 2**) [5].

Patients with HPV-HR+ SCCA have a significantly better outcome after CRT than HPV-HR-tumors [54–56]. Absolute difference in LRC/PFS varies from 32 to 67%, whereas the difference in OS varies from 22 to 52%. Interestingly, within the HPV-HR+ tumors, LRC and OS after CRT are significantly better in patients with tumors carrying a high HPV-HR DNA load than in those with a low HPV-HR DNA load [57]. Intratumoral p16 expression is also correlated with LRC and PFS after CRT for SCCA [58]. An even stronger discriminating effect on LRC and PFS is observed by combining p16 expression and HPV DNA tumor load [57].

P53 and p16 expression/HPV-HR+ are inversely correlated in SCCA [56, 58]. In addition, p53 expression and disruptive *TP53* mutations are associated with a significantly worse outcome after CRT [56, 58].

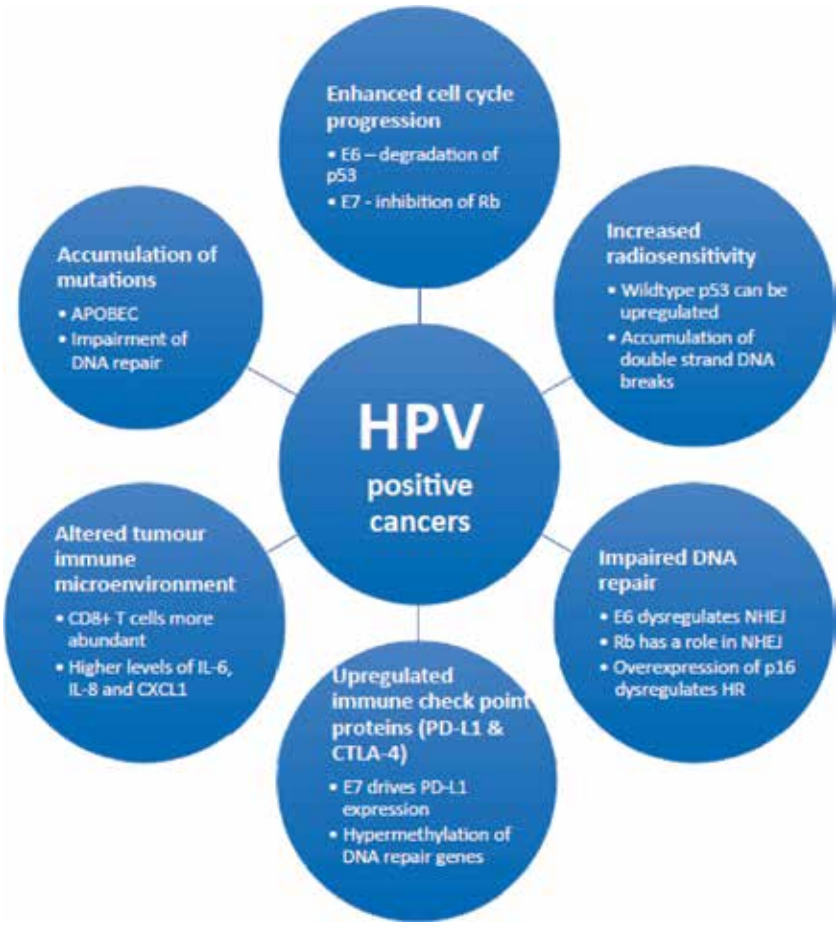


Figure 2.
Molecular features in HPV positive tumors [5]. Reproduced with permission of Elsevier.

3.2 Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is frequently overexpressed in SCCA and this may confer a growth and survival advantage. In a subgroup analysis of the RTOG 98-11 trial, overexpression of EGFR and a downstream proliferation marker Ki67 was associated with a significantly worse DFS and OS [59]. In a recent small series of recurrent SCCA, high levels of alterations in the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway, which is a growth and survival promoting pathway downstream of EGFR, were associated with poor OS [60].

3.3 Immune response

Persistent intratumoral HPV-HR infection can elicit a host immune response, which is mediated by immune checkpoint proteins such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1), expressed on activated T-cells and programmed cell death ligand 1 (PD-L1), expressed on tumors and various host cells [5, 61]. This can attract CD8+ T-lymphocytes into the tumor,

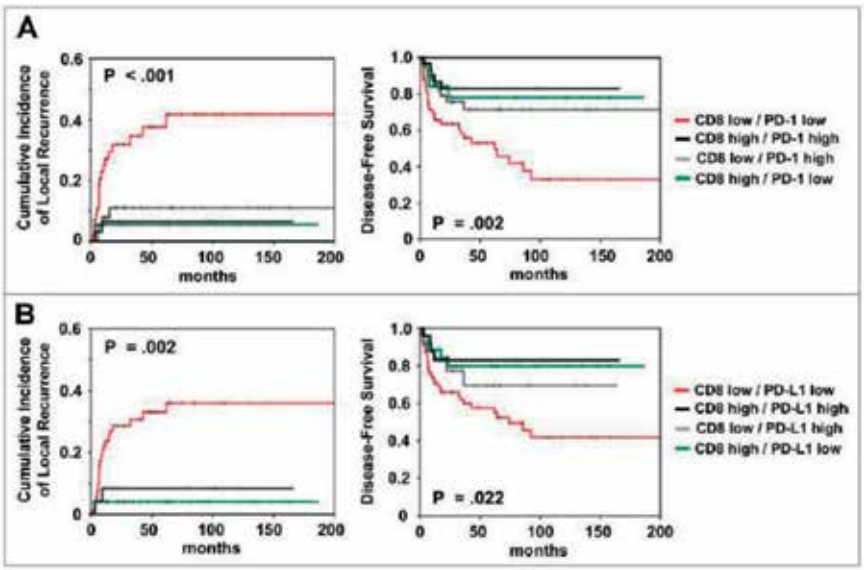


Figure 3.
Prognostic impact of CD8+/PD1 and DC8+/PD-L1 expression on LRC and DFS after CRT in SSCAC [62]. Reproduced with permission of Taylor and Francis.

so-called tumor-infiltrating lymphocytes (TILs). HPV-mediated intratumoral immune response has a significant influence on LRC and DFS, as illustrated by the amount of CD8+ TILs and PD-1 and PD-L1 expression levels after CRT in SCCA (**Figure 3**) [62].

4. Biological agents

Although the standard regimen of CRT with MMC and 5-FU is effective in SCCA, there is still room for improvement, in particular in the locally advanced cases and tumors that carry poor prognostic factors. Attempts have been made to investigate newer, promising agents. Here we focus on two avenues that have been explored.

Cetuximab is a chimeric IgG1 monoclonal antibody with a high affinity for EGFR. It has been tested in a few phase II trials in combination with concurrent CRT in SSCA, and turned out to be very toxic and probably also less effective than the standard regimen [63–67].

Two phase II trials have been published on the use of anti-PD-1 monoclonal antibodies in recurrent and/or metastatic SCCA, that is nivolumab [68] and pembrolizumab [69]. Objective responses were observed in 24 and 17%, respectively, and stable disease in 42% of the latter [68, 69]. Adverse events were acceptable.

5. The role of surgery in anal carcinoma

5.1 Salvage abdominoperineal resection

Radical surgery for SCCA is restricted to locoregional recurrent, non-metastasized and resectable tumors after CRT. The standard operation procedure is an abdominoperineal resection (APR), sometimes extended with resection of parts of the vagina or prostate, if involved, in order to obtain clear surgical margins [6]. This leaves a large pelvic floor defect, which preferably should be closed with a vertical

rectus abdominis myocutaneous flap (VRAM). Patients are left with a permanent colostomy. After APR, 5-year OS varies between 30 and 75%, depending upon whether or not clear resection margins have been obtained [6, 70]. Morbidity can be substantial, such as wound infections and poor healing of previously heavily irradiated organs and tissues. Wide resections into non-irradiated tissues and reconstructions with plastic flap techniques reduce these serious complications [6].

5.2 Curative local excision

A particular role for curative surgery in first line treatment of SCCA is reserved for small, T1N0 tumors of the anal margin, suitable for local excision (LE). This is not a trivial decision to make and these patients deserve to be seen by an experienced multidisciplinary team. Based on a recent pattern of care study in Australia, there is a wide variety in management of these small T1 tumors, depending upon the findings after a (non)excisional biopsy (**Figure 4**) [71]. In accordance with the guidelines and expert opinion, it is safe to say that T1N0 tumors < 1 cm, located in the anal margin, are good candidates for LE [34, 35]. This will probably account for only 4% of all anal cancers [72]. If pathological examination of the surgical specimen reveals that the resection is not radical, some form of additional treatment is warranted and should be discussed in a multidisciplinary team. If located in the anal canal, LE carries a risk of sphincter damage and is therefore relatively contraindicated. Nevertheless, a recent retrospective cohort study of

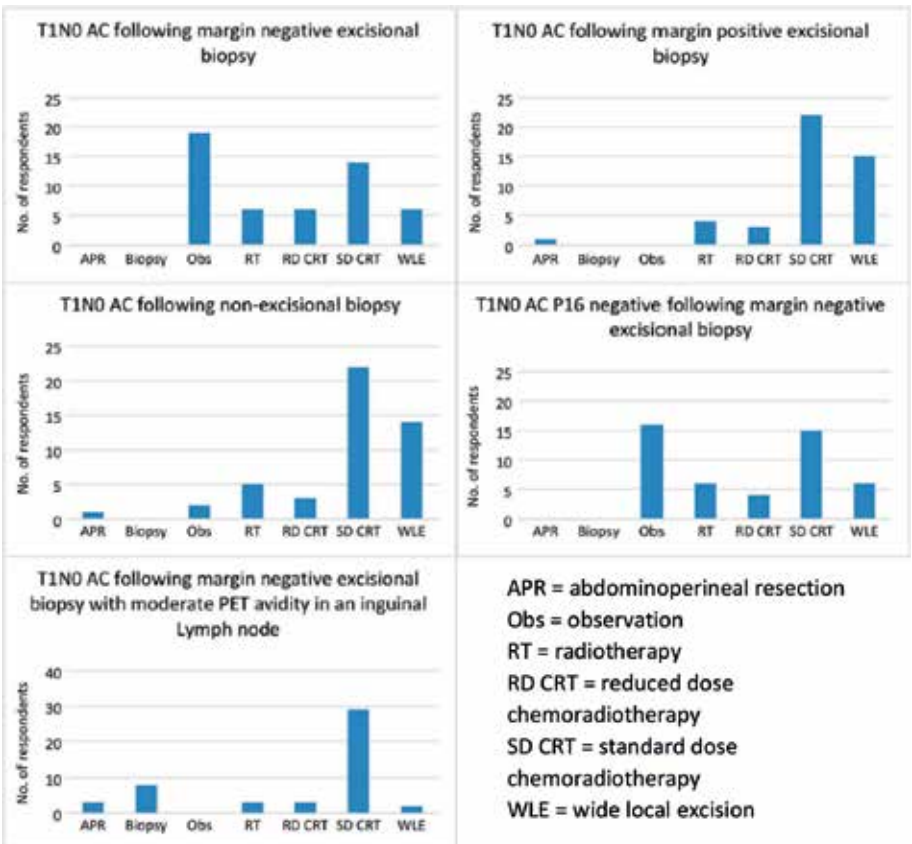


Figure 4.
Reported management of T1No anal cancer [71]. Reproduced with permission of Springer.

the US National Cancer Database on 2243 cases with T1 N0 SCCA has shown that over the period 2004–2012 LE was increasingly used in the more recent years, also for tumors of the anal canal [73]. Although criticized for its lack of information on the exact tumor location, LRC and DFS [74, 75], this study and the Australian survey [71] illustrate that clinicians are reluctant to treat these small tumors with standard CRT.

6. Treatment strategy

Today's clinical research on SCCA is focused on individualizing treatment as a function of estimated prognosis. A good example, for instance, is the UK trial "PersonaLising rAdioTherapy dOse in anal cancer" (PLATO), which offers a platform of 3 trials, ACT3, 4 and 5, for 3 different risk groups of SCCA [76].

ACT3 is a non-randomized trial for patients with low-risk T1N0 tumors of the anal margin, that undergo LE, followed by active surveillance if the resection margin is >1 mm. If the margin is ≤1 mm, postoperative reduced dose CRT is given locally (41.4 Gy in 23 fractions). In the Netherlands Cancer Institute, we use a somewhat different treatment policy for these tumors, taking a relatively new entity for SCCA into account, known as superficially invasive squamous cell carcinoma (SISCCA). SISCCA is defined as an invasive squamous cell carcinoma with an invasive depth of ≤3 mm and a horizontal spread of ≤7 mm that has been completely excised [77]. In the cervix, SISCCA is known to bear a minimal risk of microscopic lymph node metastasis and it is assumed to be similar for SISCCA of the anus, although the data supporting this are scarce [77]. We therefore have adopted a close surveillance policy for SISCCA of the anal margin. If the resection margin is too close or involved, a wider excision is performed, if possible. If not, postoperative reduced dose RT alone is given to the anus (45 Gy in 25 fractions). For T1N0 tumors that are microscopically >3 mm in invasive depth or >7 mm in horizontal spread, we also irradiate the inguinal lymph nodes to 45 Gy in 25 fractions. We do not advocate CRT in these cases, because the results with RT alone are excellent [35, 78, 79]. Furthermore, CRT is associated with an absolute increase of 9% of non-cancer related deaths compared with RT alone, mainly from cardiovascular cause and secondary tumors [44].

ACT4 is a randomized phase II trial for intermediate-risk tumors, T1–2 (≤4 cm) N0 or Nx, comparing LRC at 3 year after standard-dose CRT (50.4 Gy in 28 fractions) *versus* a reduced-dose CRT (41.4 Gy in 23 fractions). In the French guidelines, the advice for T1 and small T2 tumors is to treat them with RT alone [35]. In the Netherlands Cancer Institute, we follow the Dutch National guidelines, which advocate RT alone for T1N0 tumors and CRT for all other stages [80].

ACT5 is a randomized seamless pilot/phase II/phase III trial for high-risk SCCA, T1-2N1-3 or T3-4Nany, comparing 3-years' LRC after standard-dose CRT (53.2 Gy in 28 fractions) with that after 2 higher dose levels (58.8 and 61.2 Gy in 28 fractions) [76]. In the Netherlands Cancer Institute, we use CRT for these tumors with a relatively high radiation dose of 59.4 Gy in 30 fractions. We do not consider a lower radiation dose, because with VMAT the toxicity profile is acceptable [79].

7. Conclusions and future prospects

The treatment of SCCA has evolved over the last 5 decades from a mutilating radical surgical treatment with a modest survival probability to an individualized

radiation treatment with or without concurrent chemotherapy with good survival outcome and acceptable morbidity. Important improvements in radiation treatment techniques have been made, modern guidelines have been implemented and quality assurance is provided. However, there is still room for improvement. Quality of life analyses have infrequently been performed and are rarely taken into account in treatment decision making (e.g. [11, 81–83]). A good step forward in this respect is the development of a core outcome set of data, which should be the minimal information required in future clinical trials for anal cancer [84]. Radiation dose de-escalation and omitting concurrent chemotherapy for early tumors with good prognosis are important avenues to explore. On the other hand, new treatment modalities are needed for poor prognostic cases, such as HPV-HR negative SCCA. Immunotherapy seems to be a promising modality, either alone [68, 69] or in combination with chemotherapy [85]. Exploring the molecular profile of SCCA may reveal new potentially therapeutic targets and prognostic and predictive markers [60, 86, 87]. Circulating tumor DNA at baseline and in follow-up may become an important tool in treatment decision making [88]. These new insights and therapeutic avenues may eventually change the landscape of anal cancer treatment in the near future.

Conflict of interest

The authors have declared no potential conflict of interest.

Nomenclature

clinical target volume (CTV)	the microscopic tumor volume, based upon the estimated microscopic lymphatic tumor spread
CTLA-4	a member of the immunoglobulin superfamily, expressed on the cell surface of activated T-cells. It binds to B7-1 and B7-2 molecules of antigen presenting cells, which down-regulates the immune response, a process frequently occurring in cancer
3D-conformal radiotherapy (3D-CRT)	a 3-dimensional radiation treatment technique, which allows to shape the radiation dose distribution "conformal" to the shape of the planning target volume
epidermal growth factor receptor (EGFR)	a transmembrane protein, which is frequently overexpressed in a number of cancers. When activated, either by ligand binding (normal) or mutations (abnormal), it stimulates downstream signaling pathways, which promote DNA synthesis, cell growth and cell migration
gross tumor volume (GTV)	the macroscopic tumor volume as visualized with CT, MRI and/or PET
intensity-modulated radiotherapy (IMRT)	a refined version of 3D-conformal radiotherapy, where various segments within a radiation field allow to modulate the radiation fluency, in order to obtain conformity to irregularly shaped volumes

P16	a tumor suppressor protein, which slows down the cell cycle by inhibiting cyclin-dependent kinases
P53	a tumor suppressor protein, that plays an essential role in maintaining DNA integrity by various mechanisms. It can activate DNA repair proteins and induce cell cycle arrest to allow DNA repair, or, alternatively, initiate programmed cell death if DNA damage appears to be irreparable
PD-1	a member of the immunoglobulin superfamily, expressed on T-cells and pro-B-cells. It binds to PD-L1 on macro phages and dendritic cells, which down-regulates the immune system and promotes self-tolerance, a protective mechanism against auto-immune disease. PD-L1 is frequently overexpressed in many tumors, which promotes tumor tolerance
PI3K/AKT/mTOR pathway	an intracellular signaling pathway involved in cell cycle regulation. It is frequently overactive in many cancers, eliciting a growth and survival advantage
planning target volume (PTV)	the extension of CTV needed to account for systematic and random set up variation of the patient positioning
retinoblastoma protein (pRb)	a tumor suppressor protein, which prevents excessive cell growth by inhibiting DNA synthesis
volumetric modulated arc therapy (VMAT)	a refined version of IMRT, in which the radiation dose is delivered by rotating the gantry around the patient. The collimator head also rotates and contains moving leaves. The dose rate is also variable

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Section 5

Cutaneous Squamous Cell Carcinoma

Chapter 5

Mechanical Force and Actin Dynamics during Cutaneous Squamous Cell Carcinoma (cSCC) Progression: Opportunities for Novel Treatment Modalities

Sarah Boyle and Zlatko Kopecki

Abstract

Cutaneous squamous cell carcinoma (cSCC) accounts for 25% of cutaneous malignancies diagnosed in the Caucasian population. Surgical removal in combination with radio- and chemotherapy is an effective treatment; however, prognosis for patients suffering from aggressive cSCC is still relatively poor. Increasing prevalence coupled with high mortality and morbidity in aggressive metastatic forms of cSCC highlights the need for development of novel targeted therapeutics. Metastasis is a complex process requiring dramatic reorganization of the cell cytoskeleton. Recent studies have highlighted the importance of mechanical forces and actin dynamics in cancer cells' intrinsic ability to invade adjacent tissues, intravasate into vasculature, and ultimately metastasize. Tight regulation of the biochemical and mechanical properties of the actin cytoskeleton drives cellular processes involved in cSCC progression including polarity establishment, morphogenesis, and motility. Here we will provide a short introduction to disease pathogenesis, give an overview of the role of key regulatory proteins governing the mechanical forces and actin dynamics critical to cSCC progression, and describe the contribution of actin remodeling and actomyosin signaling to cSCC progression. We will also discuss how targeting protein regulating mechanical force and actin dynamics may have clinical utility in development of novel treatment modalities for patients suffering from aggressive cSCC.

Keywords: cutaneous squamous cell carcinoma, actin cytoskeleton remodeling, mechanical force, contraction, systemic therapy

1. Introduction

Cutaneous squamous cell carcinoma (cSCC) most commonly arises in actinically damaged skin and accounts for 25% of cutaneous malignancies diagnosed in the Caucasian population [1]. The incidence of cSCC continues to rise annually, with an estimated 50–200% increase in incidence in the last three decades in USA alone, and is predicted to increase in future years due to an aging global population [2]. Solar ultraviolet radiation is the primary environmental extrinsic cause of cSCC. Intrinsic

immunosuppression, the second most common cause, leads to the formation of aggressive cSCC in organ transplant patients, patients on immunomodulatory therapies, and those suffering from recessive dystrophic epidermolysis bullosa, a genetic skin blistering disease [3–5]. The incidence of cSCC is higher in individuals who are fair-skinned and have a sun-sensitive phenotype; however, the aggressive forms of cSCC are more common in men and the elderly [3]. Despite its prevalence, the relatively low fatality rate of cSCC means that its health and economic burden is often substantially underestimated [3], albeit latest data showing that in addition to significant morbidity cSCC accounts for up to 8000 deaths per year and costs approximately $4.8 billion annually in USA alone [6].

cSCC generally presents as a scaly, red or bleeding abnormal lesion on sun-exposed areas, and is associated with relatively benign outcomes and a low risk of metastasis. However, cSCC can demonstrate dramatic histopathological heterogeneity, resulting in a wide range of clinical outcomes [7]. Histopathologic subtypes of cSCC are broadly divided into low-grade SCC (**Figure 1A**) that are well-differentiated but have low metastatic potential (keratoacanthomas, SCC *in situ* and verrucous carcinoma), or high-grade SCC (**Figure 1B**) that are poorly differentiated, have high potential of metastasis and recurrence, and are associated with a poor prognosis for patients (desmoplastic cSCC, adenosquamous cSCC and cSCC associated with non-healing ulcers or scarring processes arising from chronic wounds) [2, 7]. Once developed, the natural history of untreated SCC is one of local invasion followed by metastasis via the lymphatic system, blood or perineural invasion, which can lead to death [7]. In most cases, cSCCs are detected early and can be successfully eradicated by surgical excision. However, if not detected and/or left untreated, disease progression to high-grade cSCC will often lead to mortality. Clinically, the most powerful predictor of disease pathogenesis is nodal metastasis and size, followed by invasion beyond fat, location, and lastly perineural invasion. These parameters are used in clinical staging systems for cSCC [3]. Management of cSCC is primarily surgical, with adjuvant chemoradiation approaches based on risk factors, patient and tumor features, as well as care features including access to treatment and associated costs [2].

With increasingly longer life expectancy, the health and economic burden of cSCC is likely to continue to increase significantly. Hence, a better understanding of factors contributing to cSCC progression and metastasis is necessary to aid development of novel therapies, aimed at combatting cSCC in the community. Despite

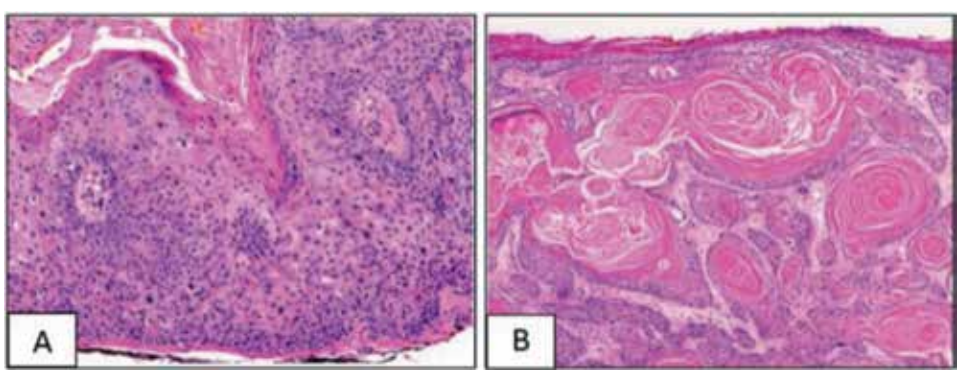

Figure 1.
Representative histopathological features of low-grade and high-grade cSCC. (A) Low-grade cSCC in situ with prominent dyskeratosis and aberrant mitosis at all levels of the epidermis, with marked parakeratosis and intact basement membrane. (B) High-grade poorly-differentiated cSCC lesions showing prominent keratinization and the formation of "pearl like" structures where dermal nests of keratinocytes attempt to mature. Adapted from Yanofsky et al. [7] and modified with approval.

recent advances in gene expression screening technologies that have begun to identify candidate genes commonly mutated in patients with cSCC (including TP53, CDKN2A, Ras and NOTCH1), which may be responsible for regulating motility and invasion in cSCC, a comprehensive understanding of the factors contributing to cSCC invasion including mechanical tension and actin dynamics is still emerging [8]. One thing that is clear is that patient outcome directly correlates with the degree of local and regional invasion, and coordinated regulation of the actin cytoskeleton is critical to cell motility, invasion and metastasis [9]. Consequently, the signaling pathways involved in mediating chemotactic cues from the extracellular environment that regulate the actin cytoskeleton and mechanical forces, guiding cancer cell invasion and metastasis, have been and continue to be an area of intense study.

Recent studies have revealed a number of proteins and molecules that are aberrantly expressed in cSCC. These proteins link cell migratory signals to the actin cytoskeleton, thereby playing an instrumental role in the ability of cancer cells to resist chemotherapy and/or metastasize [10]. In this chapter we will describe the actin dynamics and mechanical force governing tumor cell migration, invasion and metastasis. We will outline the main signaling pathways governing the formation of invasive protrusions by cancer cells with regard to the function of key regulatory proteins involved in actin cytoskeleton remodeling in cSCC.

The metastatic spread of aggressive cancers, including cSCC, is a highly selective process involving a series of sequential and orchestrated steps in the so-called "metastatic cascade": detachment from the primary tumor site, cell migration and invasion of the surrounding extracellular matrix (ECM), intravasation into vasculature, extravasation at a secondary site, and interaction with the extracellular environment to form metastatic tumors [11]. Each of these steps offers the potential for design of different therapeutic approaches to combat aggressive cSCC. Indeed, a number of recent studies have identified novel therapeutic approaches including both adjuvant and neoadjuvant treatments, with clinical trials utilizing epidermal growth factor receptor inhibitors and immune checkpoint blockers (nivolumab, pembrolizumab, and ipilimumab) showing promising early results as potential treatments of cSCC [12]. Recent trials using cytotoxic chemotherapy have, however, shown limited advances for the treatment of cSCC, and trials investigating combined immune checkpoint inhibitor and radiation therapies, which may have synergistic effects in treatment of cSCC, are still pending [13]. This highlights the need for increased research to close the gaps in our knowledge of cSCC biology, including better understanding of the factors that lead to aggressive cSCC, the role of microbiomes and HPV infection, the role that mechanical force and actin dynamics plays in this process, prediction of clinical response to therapies including immune checkpoint blockade, and how to tailor better prevention and treatment strategies to individual risk factors and needs [6]. Emerging evidence on the crosstalk between different components of the cytoskeleton in metastatic progression combined with clinical data illustrating strong relationships between cytoskeletal alterations and metastasis in various cancers pinpoints important opportunities for potential therapeutic targets [11]. Later in this chapter we will describe current research that has attempted to identify the steps of the metastatic cascade suitable and most amenable for therapeutic intervention, with a focus on harnessing our knowledge of actin cytoskeleton remodeling and mechanical forces to postulate therapeutic strategies targeting cytoskeletal and cytoskeletal-associated proteins critical in cSCC.

2. Cytoskeletal dynamics and regulation during cSCC progression

The skin is exposed to and responds to a wide range of mechanical signals throughout homeostasis and through to malignancy. Mechanical forces have been

shown to regulate these normal cellular processes including stem cell renewal, lineage differentiation and proliferation, wound healing, as well as transformation through changes in the actin cytoskeleton—the ability to protrude, adhere to the ECM, migrate through tissue and invade into the underlying basement membrane.

The types of mechanical forces exerted upon the skin can vary depending on the context. In homeostasis, tensile/stretch forces and compressive forces arise as a result of muscle and joint movements, and physical location—skin stretched over bone is under significantly more stress than when over fat or muscle [14]. Tensile forces cause cells to elongate and expand, and therefore are generated at sites of wounding as epithelial cells migrate in and contract to close the wound. This can generate scarring and fibrosis, which can lead to skin cancer including cSCC [15]. Compressive forces generate different biomechanics in skin cells compared to tensile force [16]. Compressive forces are able to activate Rho-ROCK signaling (described below) in the skin, which has been shown to play a role in tumor progression [17]. In melanoma, it was found that stress-bearing areas of the foot were more conducive to cancer development due to increased mechanical compressive stress [18]. Changes in substrate stiffness underlie these mechanical signals (**Figure 2**), and it has been shown that stiff stroma can lead to an activation of integrin signaling and subsequent cSCC development [19].

Cells have the ability to sense these changes in their environment (process referred to as "mechanosensing"). The mechanical signal is then converted to a biochemical signal in a process called mechanotransduction, and the biochemical signals initiate a cascade of changes within the cell at the transcriptional, translational and post-translational levels that result in a cell that can appropriately and reciprocally respond to the extracellular signals (process referred to as "mechanoreciprocity") [20]. In disease states including cSCC, the heightened and/or constitutive extracellular signals generate a detrimental loop of ever-increasing mechanoreciprocal signaling, hence leading to enhanced tumor progression, invasion and eventually metastasis [20].

Triggered by the changes in the cell microenvironment, the actin cytoskeleton undergoes a number of changes that allows a cell to become more motile and/or invasive. The ability of a cell to undergo directed migration is essential to its ability to metastasize, and is characterized by an ordered process (**Figure 3**) of membrane protrusion at the leading edge (filopodia and lamellipodia) and sides (invadopodia)

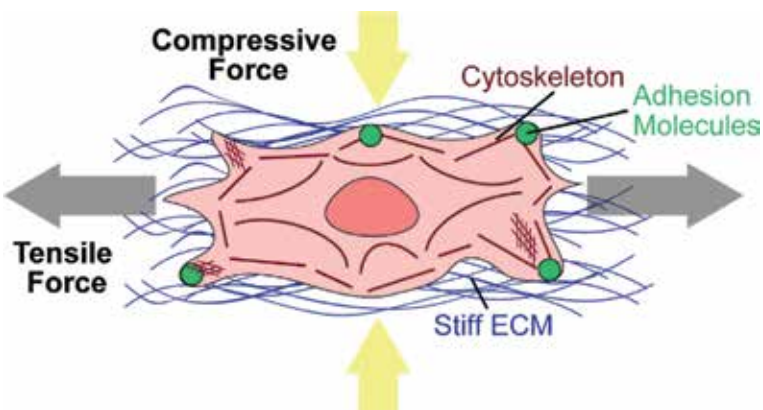

Figure 2.
Mechanical forces acting upon skin cells. The major types of mechanical forces experienced by skin cells are compressive (inward pushing) and tensile (stretching) forces, which in cSCC progression are generated by an increase in extracellular matrix stiffening. These are sensed by the cell, which then is able to respond accordingly.

of the cell, contact and adhesion between the protrusion and the matrix, movement of the main cell body, and retraction of the trailing edge [21, 22]. Lamellipodia are flat membrane protrusions containing dendritic arrays of actin filaments that branch out like a sheet from the leading edge of a cell. This particular form of protrusion is thought to have a major role in cell migration as their morphology allows the cell to make multiple contacts with the underlying substrate and pull the cell forward. Filopodia are narrow protrusions made up of bundled and cross-linked actin filaments that also stretch out from the leading edge. Invadopodia, unlike lamelli- and filopodia, are protrusions branching out from the sides of a cell, which have increased membrane remodeling and matrix degradation proteins [21]. These particular membrane protrusions are often seen in cancer cells including during cSCC progression [23].

Adhesions between the cancer cells and matrix are necessary for invasion and are largely mediated by integrins (discussed in detail below) [22]. During cSCC progression, there is an increase in cellular attachment to the ECM and, concurrently, a decrease in attachment to neighboring epithelial cells signified by a reduction in levels of E-cadherin expression. Following matrix adhesion, the trailing edge of the cell contracts, allowing the cell to move forward. Myosin II is required for this actin filament contraction, and is largely regulated by signaling through the small G-protein Rho. Once the cell contracts, its tail detaches as focal adhesion complex components are cleaved [22]. Actin remodeling proteins are essential in these processes, and often regulate cell-cell and cell-stroma attachment and turnover of focal adhesions, allowing cell traction and movement to take place. The coordination of actin polymerization and contraction allows the cSCC cell the ability to migrate through dense, stiff ECM and stroma to metastasize to lymph node or surrounding organs.

A number of different pathways are activated downstream of mechanical signals, causing changes in the actin cytoskeleton. Signaling pathways involved in cytoskeletal dynamics during cSCC progression are also activated as a result of constitutive or heightened growth factor signaling [24]. Together, the combination of mechanical and biochemical signals can trigger a multitude of intracellular signaling cascades that ultimately affect cell morphology. In this section, we will discuss the broad families of proteins regulating actin dynamics and mechanical forces in cSCC, an overview of which is illustrated in **Figure 4**. The overview provided in this chapter will not cover all the pathways or actin remodeling proteins involved but focus on those most relevant to cSCC progression, invasion and metastasis.

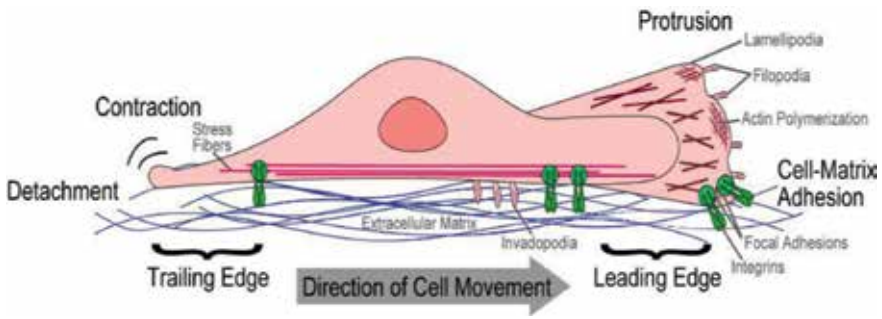

Figure 3.
Cell motility as controlled by the actin cytoskeleton. Upon sensing of extracellular cues, intracellular signaling cascades generate cytoskeletal protrusions (lamellipodia and filopodia) at the leading edge containing actin filaments, as well as invasive protrusions (invadopodia) at the sides of the cell, necessary for cSCC invasion. The cell adheres to the matrix, forming integrin-mediated focal adhesions. The nucleus and cell body are then pushed forward as the trailing edge contracts, via stress fibers. The rear of the cell then detaches, allowing the cell to migrate forward.

2.1 Mechanosensing: integrins

Integrins are well-characterized as the first point of contact for mechanical signal transduction. Inactive heterodimers on the cell surface, they are partially activated by intracellular proteins ("inside-out" signaling) before full activation upon binding to extracellular ligands ("outside-in" signaling) [25]. Binding to the extracellular ligand results in full activation of the integrin receptor and leads to the formation of either an intracellular focal adhesion complex to link the ECM to the actin cytoskeleton, or hemidesmosomes, linking the ECM to intermediate filaments. The integrin that is activated is context-dependent in regards to the particular focal adhesion complexes that are formed, broadly encompassing a range of adaptor proteins including: talin, vinculin, paxillin, Flightless I (Flii), focal

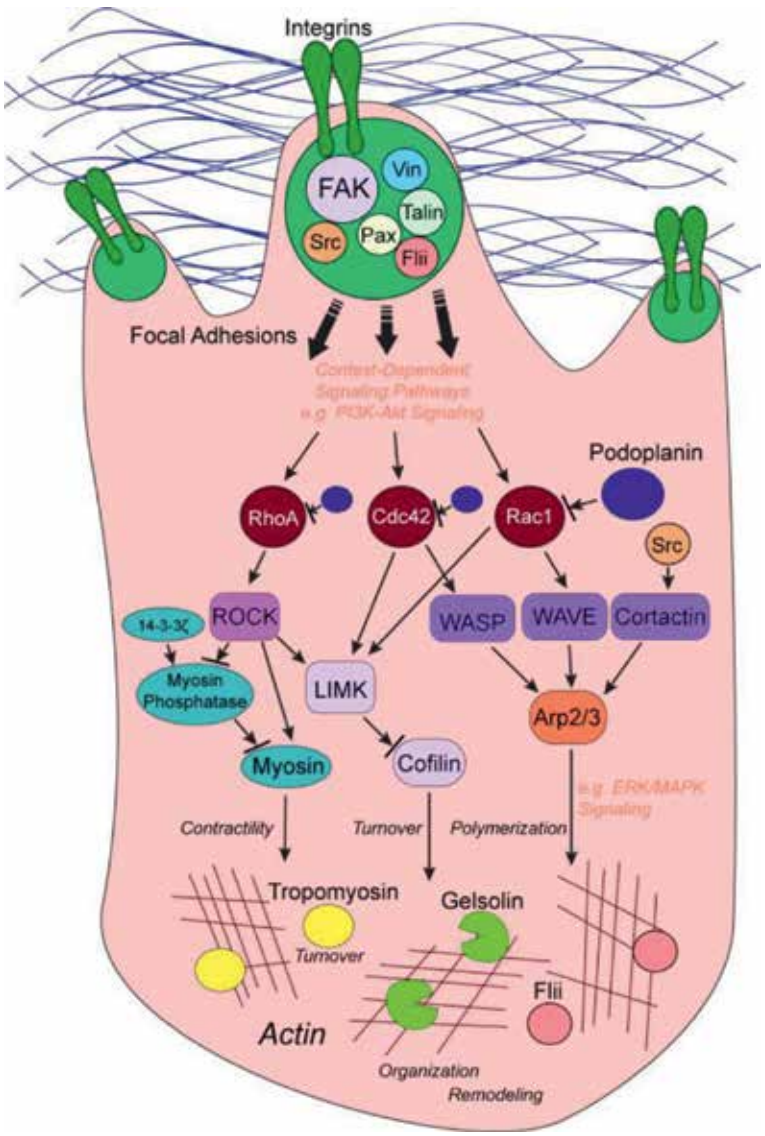

Figure 4.
Major pathways regulating cytoskeletal dynamics downstream of mechanical signals in cSCC. Graphical representation of signaling pathways which respond to mechanical force and govern downstream actin remodeling during cSCC progression.

adhesion kinase (FAK) and integrin-linked kinase (ILK). FAK is rapidly recruited to focal adhesions upon integrin activation and is auto-phosphorylated, driving downstream signaling. FAK phosphorylation, stimulated by integrin activation, allows binding of Src-family kinases that are then able to trans-phosphorylate FAK. This leads to activation of ERK/MAPK signaling, and this complex is therefore able to control cell shape and regulate focal adhesions [26]. In cSCC, a step-wise increase in activation of FAK from unaffected margin skin to hyperproliferative skin and invasive cSCC [27] results in elevated integrin-FAK-Src signaling that stimulates keratinocyte migration [28] and drives progression of benign papillomas to aggressive cSCC [29, 30]. In cSCC, it has been demonstrated that FAK function is required for cancer stem cell maintenance, regulating cSCC initiation, growth, regression, and progression [31]. Actin remodeling protein Flii, which will be discussed in detail in Section 2.4, has been shown not to directly bind integrin receptors but form focal adhesion complexes with adaptor proteins and regulate integrin activation, downstream Src/paxillin signaling and focal adhesion turnover in a Rac1 dependent manner [25, 32]. Additionally, latest research has shown that actin remodeling proteins Flii and gelsolin, which have always been thought to be intracellular, have also both been shown to be secreted where they function to sequester extracellular actin post tissue injury, modulate inflammation and affect collagen VII anchoring fibril formation. Flii is able to modulate inflammation via toll-like receptor 4 (TLR-4) signaling, as Flii leucine-rich repeat (LRR) domains have 50% similarity to LRR domains of TLR-4, by which the immune system is able to detect infection or injury. The binding of LRRs to PAMP and DAMP molecules activates intracellular TLR signaling and ultimately results in the release of proinflammatory cytokine secretion [33]. The extracellular roles of Flii and gelsolin in respect to mechanosensing and cSCC progression are still to be examined. Nevertheless, it is clear that the coordinated activation of integrin receptors, focal adhesion complex formation and downstream signaling stimulation is essential for cytoskeletal changes that are necessary for cell migratory and invasive capability during cSCC progression.

2.2 Mechanotransduction: Rho GTPases

Downstream of integrin activation are the Rho small GTPases, which are part of the Ras superfamily and key regulators of cell cytoskeletal dynamics through both actin polymerization and organization, hence driving cancer cell motility [34]. Of this subfamily, RhoA, Rac1 and Cdc42 are the best-characterized.

RhoA is involved in actomyosin contractility, formation of actin stress fibers and assembly of focal adhesion complexes. The main regulator of cytoskeletal dynamics leading to formation of stress fibers is myosin II, and its regulatory subunit myosin regulatory light chain-2 (MLC2) can be activated by RhoA signaling leading to contraction of actin fibers. Rho-associated kinases ROCK1 and ROCK2 are serine/threonine kinases that contain a Rho-binding domain and are activated by RhoA in its active GTP-bound form, directly activate MLC2 via phosphorylation. Due to its roles in cell contractility and movement, Rho-ROCK signaling has been implicated as a driver for invasiveness during cSCC progression but also plays a positive role in physiological normal wound healing processes [35]. In human cSCCs, ROCK is not only highly expressed but also activated in the hyperproliferative skin and invasive regions of the tumor, as shown by phosphorylation of the ROCK substrate myosin phosphatase (MYPT1) [27]. In the skin, this ROCK-mediated actomyosin contractility is required for proliferation of the epidermis, as ROCK activation stabilizes β-catenin through phosphoinositide 3-kinase (PI3K), Akt, and inhibition of its phosphorylating kinase GSK3β [36]. During cancer progression from normal skin through to hyperproliferative and invasive cSCC, nuclear localization of active

β-catenin and inactivation of GSK3β is increased, accompanied also by a progressive increase in FAK activation [27, 36]. It has further been demonstrated that a negative regulator of ROCK signaling, 14-3-3ζ, is significantly down-regulated in human cSCCs. As genetic deletion of 14-3-3ζ results in significantly larger papillomas in the two-stage chemical carcinogenesis (DMBA-TPA) mouse model of SCC, this suggests that uncontrolled ROCK signaling can drive cSCC tumor growth [35].

Cdc42 is involved in formation of F-actin microspikes and filopodia in both normal and cSCC cells, by via actin polymerization at the leading edge and at the sides of the cells, contributing to cSCC invasion. Traf6 has been demonstrated to regulate Cdc42 to induce these F-actin microspikes in SCC cells [37]. When Cdc42 is absent in keratinocytes, cells are no longer able to properly process and deposit ECM components or integrin receptors, hence halting cellular migration [38]. Taken together, these studies demonstrate roles for Cdc42 in both skin cell migration and invasion, necessary cellular processes for progression of cSCC. Cdc42 is necessary for proper cellular polarity in normal and migratory cells [34], and this in turn activates G-proteins of the Rac subfamily of Rho small GTPases [39].

Rac1 is hyperactivated in cSCC via integrins including α3β1, and is important for keratinocyte cell proliferation [40, 41]. Rac1 stimulates polymerization of actin via multiple kinase signaling cascades including that of MAP kinase (elevating the transcription factors AP-1, NFκB and CRE). This therefore allows the cell to form a branched actin network, necessary for leading-edge lamellipodia formation and membrane ruffling [42]. Indeed, actin remodeling protein, Flightless I (Flii), has been shown to regulate focal adhesion by inhibition of paxillin phosphorylation via a Rac1 dependent pathway [32]. Rac1 can be activated by Tiam1, and it was shown in the two-stage chemical carcinogenesis cSCC model that genetic deletion of Tiam1 significantly reduced tumor incidence, burden and growth. However, SCC tumors that did arise in Tiam-null mice were significantly more invasive and malignant, potentially due to a loss of cell-matrix adhesion [43]. This highlights the dual homeostatic and tumor-promoting roles that actomyosin regulatory pathways can play during cSCC progression.

Rho-associated kinase ROCK can also phosphorylate and activate LIM kinases 1 and 2, which are then able to phosphorylate and inactivate cofilin, an F-actin severing protein, resulting in F-actin filament stabilization [44]. Accordingly, it has been shown that LIMK1 levels are increased in cSCC tumor tissue compared to normal skin, and that LIMK1 silencing can suppress cell growth and invasion in cSCC cell lines [45]. In addition, it has been suggested that LIMK is required in the microenvironment in leading fibroblasts, to allow for efficient remodeling of the ECM and subsequent cSCC invasion [46]. It has been demonstrated that cofilin phosphorylation can be abolished by treating cSCC cells with LIMK inhibitors. This reduces β-catenin accumulation and epidermal proliferation via reversing actomyosin contractility [36], however clinical trials using these inhibitors are still pending. The involvement of Rho GTPases in cellular migration and invasion in cSCC due to cytoskeletal rearrangement implicates this family of proteins as drivers of cancer initiation, progression and metastasis. Hence, targeting Rho pathway signaling, in particular that of RhoA-ROCK signaling, is an attractive therapeutic option that will be explored later in this chapter.

2.3 Actin polymerization: WASP, cortactin, and Arp 2/3

Actin nucleation promoting molecules are activated downstream of Rho GTPases and growth factor receptors. Wiskott-Aldrich syndrome protein (WASP) family members, via Erk, paxillin, and Src signaling together with cortactin, act to stimulate the actin-related protein (Arp) 2/3 complex which in turn mediates actin polymerization [47].

The WASP family consists of WASP proteins and WASP-family verprolin-homologous (WAVE) proteins. WASP proteins interact with Rho GTPases in order to form cellular protrusions and allow the cell to migrate, for example, N-WASP is involved in formation of filopodia and invadopodia upon its activation by Cdc42. WASP proteins bind to G-actin and Arp2/3 resulting in their activation and hence triggering actin filament production. WASP and WAVE proteins also bind profilin, which transports actin monomers onto the growing ends of actin filaments, and is therefore also an important factor in cell motility [47]. As a loss of N-WASP in keratinocytes causes epidermal hyperplasia and a reduction in epithelial cell tight junctions [48], this highlights the need for proper control of these processes. The formation of cellular protrusions also relies on the Src protein cortactin, which binds to and activates the Arp2/3 complex independently of WASP, thereby regulating actin filament nucleation. Cortactin binds to F-actin, stabilizing actin filaments and allowing it to properly activate Arp2/3 [49, 50]. In head and neck SCC, overexpression of cortactin increases cancer cell proliferation and increases cell survival in anchorage-independent conditions [51], while in oral SCC, silencing of cortactin was shown to significantly impair invasiveness and downregulate the levels of epithelial markers, indicating an epithelial to mesenchymal transition (EMT) [52], a process by which epithelial cells lose their adhesion to one another and acquire a migratory and invasive mesenchymal phenotype (discussed in further detail below). Indeed, Arp2/3 complex proteins are required for cell proliferation and migration in other forms of SCC [53, 54], and based on the Arp2/3 role in actin filament polymerization, it is clear that Arp2/3 is also critical for actin cytoskeletal remodeling leading to cancer cell motility and invasion.

2.4 Actin remodeling: tropomyosin, Flightless I, and podoplanin

The actin cytoskeleton is composed of three distinct elements including microfilaments, microtubules and intermediate filaments. Tight regulation of cytoskeletal elements must be coordinated, and latest research has shown that interplay between actin and microtubules is bidirectional [55]. Actin-based motility is also dependent on the balanced activity of number of specific actin remodeling proteins. In this section we will highlight main actin remodeling proteins that have been shown to have specific functions in cSCC progression, including members of the tropomyosin family of actin-associated proteins, the gelsolin family of actin remodeling proteins, and Podoplanin, a simple glycoprotein with important roles in cSCC progression.

Members of the tropomyosin family of actin remodeling proteins display a tissue- and time-specific expression, while their association with actin filaments impairs isoform-specific regulation of actin filament dynamics [56]. Tropomyosin proteins assemble as polymers in the major groove of the polymerized actin filament and their association drives actin filament turnover, hence playing an important part in a number of cellular functions including motility and metastasis [57]. There are over 40 different isoforms of tropomyosin and few have been described as having an important role in cSCC progression. High expression of Tm5NM1, a specific cytoskeletal tropomyosin isoform, has been shown to inhibit cell migration and invasion as well as impair normal wound healing via its effects on Src activation, focal adhesion stabilization, increased actin filament tension, and paxillin phosphorylation [58]. Current research is examining the effect of Tm5NM1 inhibitor TR100 on cSCC progression (see Section 3). On the other hand, downregulation of tropomyosin-1 and complete loss of β-tropomyosin has been identified in human esophageal SCC, while α-tropomyosin has been shown to be preferentially

expressed in keratinocytes of the multistage model of murine cSCC, collectively suggesting isoform specific functions [59–61].

The dynamic remodeling of the actin cytoskeleton is also tightly regulated by the gelsolin family of actin remodeling proteins, which includes: gelsolin, villin, adseverin, capG, advillin, supervillin, and Flightless I (Flii) [62]. These actin binding proteins function in the cytoplasm of the cells where they control actin organization by severing pre-existing filaments, capping the fast growing filament ends and bundling filaments, enabling filament reassembly into new cytoskeletal structures that are required for cell motility, invasion and metastasis [63]. Studies have shown that downregulation of gelsolin proteins counteracts cancer cell invasion *in vitro* [64], however in cSCC, gelsolin and Flii have been the most studied to-date. Gelsolin over-expression has been shown to promote cell growth and motility in oral SCC [64, 65], while Flii, through its effects on apoptosis, has been linked to promotion of breast cancer progression and invasion and progression of cSCC [23, 66]. Flii is an important regulator of cell adhesion, migration and proliferation and a number of previous studies have described the role of Flii protein in wound healing and demonstrated the therapeutic effect of Flii neutralizing antibodies (FnAb) in acute and chronic wounds, skin blistering diseases and inflammatory skin conditions [25, 32, 67–72]. Flii modulates cell adhesion and paxillin signaling, and regulates actin polymerization, tight junction formation and ECM production during wound repair suggesting that similar roles may govern Flii activity in cSCC progression [23, 25, 32, 72]. Indeed, altering Flii levels both genetically and using Flii neutralizing antibodies significantly augments cSCC progression [23]. Therapeutic approaches targeting Flii in cSCC are described in Section 4.

The expression of Podoplanin, a small mucin-like protein, has also been linked to remodeling of the actin cytoskeleton in cSCC. Podoplanin is upregulated in the invasive front of a number of human carcinomas including cSCC and has been shown to induce collective cell migration by filopodia formation, via downregulating the function of Rho small GTPases [73, 74]. Podoplanin has also been linked to an increase in the migration of cancer-associated fibroblasts as well as endothelial network formation [75]. Collectively these findings suggest that Podoplanin is able to induce an alternative pathway of tumor cell invasion in the absence of traditional epithelial-mesenchymal transition.

Taken together, these studies highlight the important role of actin remodeling in cSCC progression and outline the importance of bidirectional stimulation of actin remodeling by both intrinsic factors and the microenvironment, critical to tumor invasion/metastasis. These findings provide a rationale for development of novel therapeutic strategies that target tumor invasion and metastasis.

3. Physiological effects of actin remodeling

Changes in the actin cytoskeletal structure result in changes to cell morphology, creating a cell more conducive to invasion. One of the commonly recognized requirements of metastasis is a cellular transition from epithelial to mesenchymal phenotype (EMT). This transition is characterized by upregulation of genes including vimentin, SNAI1 (snail), SNAI2 (slug) and Zeb1, and a downregulation of epithelial genes including cadherins, as well as concurrent loss of cell-cell junctions [76]. For example, in head and neck SCC an increase in matrix stiffness and hence increased mechanical signaling caused an increase in EMT markers in tumor-initiating cells [71]. Cells undergoing EMT develop an elongated spindle-like morphology, due to the enhanced membrane protrusion formation [77]. It has been shown in A431 cells, a human epidermal SCC cell line, that loss of T-cadherin induces elongation of cells and formation

of lamellipodia and multiple leading edges via changes in EGF-stimulated motility and invasion, as T-cadherin influences EGFR localization and responsiveness [78]. Of note, RhoA activation was also increased upon the loss of T-cadherin [79]. Likewise, Podoplanin is also capable of transforming cells to an invasive state without having to undergo EMT, due to rearrangements of the actin cytoskeleton [74].

The remodeling of the actin cytoskeleton also creates intracellular reciprocal forces that balance out the forces received by the cell from the extracellular microenvironment. Actin polymerization extends the filament network, and as filaments in the leading edge are compressed between transient associations with the cell membrane and the bulk of the actin cytoskeletal network behind them, intracellular force is generated. As protrusions are extended and retracted, actin filaments experience tension from transient bonds with the membrane, becoming bent or compressed [80]. Activation of the mechanotransduction pathways described above, downstream of ECM stiffness in cSCC, can also increase the propensity for augmented interactions with the stroma, and generate a tumor-promoting environment that enhances mechanoreciprocal signaling [20, 36].

4. Therapeutic approaches targeting actin cytoskeletal regulatory pathways

Metastasis is a complex process requiring significant reorganization of the actin cytoskeleton and coordinated involvement of number of key proteins. These proteins interact directly and indirectly with both actin and microtubule networks, hence significantly influencing migratory and metastatic cell phenotypes. Strong clinical relationships between actin cytoskeletal alterations and cutaneous cancer metastasis have been previously described [11], offering potential opportunities for therapeutic intervention. For example, up-regulation of cortactin, an actin-binding adaptor protein in melanoma, has been directly linked to increased distal metastasis and reduced disease-free survival, while up-regulation of Ras mRNA has been directly linked to Stage III and Stage IV disease in head and neck SCC [81, 82]. The complex nature of cellular migration and invasion presents challenges in developing therapeutic approaches, as compensatory pathways may overcome the effects of specific inhibitors. This highlights the need for development of combinational and adjuvant therapies targeting multiple pathways that are involved in actin dynamics to treat aggressive cSCC. Pharmacological inhibitors of actin have failed clinical development due to non-specific effects on normal actin function in tissue, resulting in high levels of cardiotoxicity. Hence, research efforts have centered on therapeutic approaches that can modify signaling pathways regulating the actomyosin cytoskeleton and/or target cytoskeletal and cytoskeletal-associated proteins [11].

Increasingly it has been recognized that microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are involved in regulating cytoskeletal dynamics through regulation of gene expression. lncRNAs have been shown to regulate lamellipodia formation by downregulating integrin expression in cSCC [83]. A number of miRNAs have also been shown to play a role in regulating cell cytoskeletal dynamics and interactions with stroma in cSCC, including: miR-340 [84], miR-20a [45], miR-31 [85] and miR-125b [86]. These miRNAs act via inhibiting RhoA, LIMK1, WAVE3 and matrix metalloproteinase (MMP)-13 respectively. The use of miRNAs in the clinic has clear potential, however clinical trials are yet to be undertaken.

One of the signaling pathways participating in regulation of cancer cell motility, invasion and metastasis is the ROCK signaling pathway, described in detail above. Hyperactivation of this pathway promotes cancer cell invasion in many solid tumors and studies have shown that Rho signaling through ROCK promotes the rounded

bleb-associated mode of amoeboid motility, thereby promoting tumor cell metastasis [87]. Earlier reports have shown that treatment with the selective ROCK inhibitor Y-27632 increases SCC cell adhesion, upregulates expression of E-cadherin, and decreases the phosphorylation of cofilin (thereby activating it), resulting in altered actin cytoskeleton rearrangement [88]. In the two-stage chemical carcinogenesis model, Y27632 treatment resulted in significantly smaller and fewer papillomas, with a reduced rate of cSCC conversion. This was associated with reduced collagen deposition in the ECM, which would indicate a decrease in mechanical signaling due to ECM stiffness [36]. This illustrates that inhibition of ROCK is a potential strategy for treatment of solid cancers including cSCC.

A potential upstream regulator of ROCK-mediated cell migration is gamma-actin. Modulation of gamma-actin changes the directional cell migration via effects on microtubule dynamics and cell polarity, hence highlighting the crosstalk between actin cytoskeleton and microtubule signaling as a potential modality for targeting specific components of the network [89]. More recent studies, using newer generations of ROCK inhibitors and pharmacological small molecule inhibitors of the downstream effectors of ROCK that have micro-tubule stabilizing effects, are also showing some promise in regulating tumor metastasis, however no compounds are yet clinically approved [90]. Another potential therapeutic strategy is harnessing the ability of 14-3-3ζ, a negative regulator of ROCK signaling, to moderate mechanoreciprocity in cSCC [35]. These approaches are particularly enticing due to the negative effects that clinical targeting of ROCK itself can potentially have [91].

Interestingly, studies investigating the interactions between SCC cells and cancer-associated fibroblasts have shown that ROCK activity is also an important requirement for adjacent stromal fibroblasts. ROCK activity positively influences the JAK1-STAT3 signaling pathway resulting in increased actomyosin contractility and proinflammatory cytokine secretion, favoring cSCC cancer cell invasion [92]. Consequently, these studies suggest that approaches aimed at inhibiting ROCK signaling have the potential to interrupt both intrinsic and microenvironment-derived signals during cSCC progression.

Actin remodeling proteins have long been implicated in cSCC, as a dysregulated actin cytoskeleton and an aberrant tumor microenvironment is a hallmark of aggressive cSCC [11, 93]. One particular actin remodeling protein, Flightless I (Flii), has been identified as a tumor promoter with transcriptional activity in colorectal, breast and hepatocellular carcinoma cell lines [66]. However, recent studies have also shown that Flii is significantly increased in human and mouse cSCC tissue samples, while secreted Flii is elevated in the sera of patients with cSCC and is increased in different cSCC cell lines established from human primary, recurring and metastatic cSCC as well as immortalized keratinocytes [23]. Human cSCC samples show positive staining for Flii in invading keratinocytes, surrounding tumor stroma and the outer hyperkeratotic layer of cSCC nodules present in the deep dermis [23]. Together, these data suggest that Flii is not only an important regulator of the actin cytoskeleton involved in cSCC progression but also a potential therapeutic target and diagnostic marker of cSCC severity. Indeed, overexpression of Flii resulted in severe cSCC development via evasion of apoptosis, while reducing Flii expression using intradermal injections of FnAb during cSCC initiation and progression significantly reduced Flii expression in both the tumor microenvironment and in the serum, and led to significantly smaller tumor size (**Figure 5**) and decreased cellular sphere formation and invasion *in vitro* [23].

Remodeling and polymerization of actin filaments is critical during cSCC invasion and formation of invadopodia. Increased Flii levels have shown to weaken cell-stroma and cell-cell adhesions via alteration of GTPase and Src/paxillin signaling pathway activity [32] and augmented integrin-facilitated cell migration [25]. This

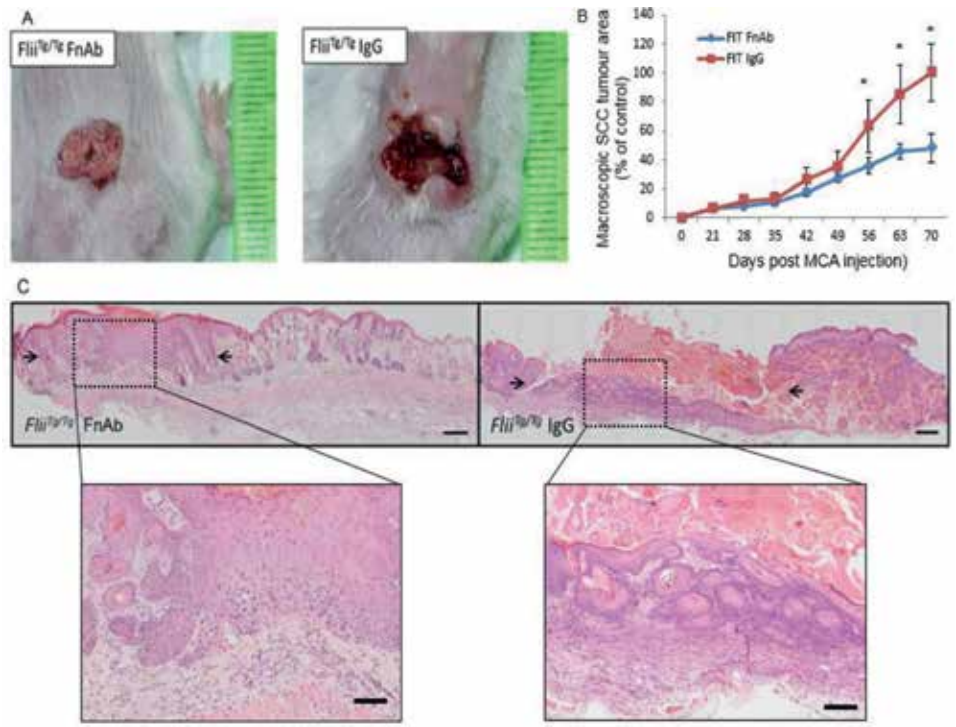

Figure 5.
Reducing Flii expression prior to initiation and development of cSCC using FnAb results in decreased cSCC progression. As Flii is increased in human SCC samples, the effect of preventative FnAb treatment prior to SCC induction was investigated in Flii$^{Tg/Tg}$ mice. Mice were treated with FnAb 2 weeks prior to cSCC induction and every second week throughout the trial and SCC development. (A and B) Reducing Flii levels in mice skin using preventative FnAb treatment prior to cSCC induction and during development resulted in decreased tumor progression and size relative to IgG control mice that have significantly larger and more developed necrotic and ulcerated tumors. (n = 12/treatment) Mean ± SD $^{*}p < 0.05$. (C) Representative images of H&E stained tumors treated with FnAb or IgG control show more severe ulcerated tumor pathology in the IgG control group (black arrows). Scale Bars = 500 μm and 100 μm.

promotes tumor progression and facilitates invadopodia formation and subsequent tumor invasion into surrounding tissue [23]. Indeed, Flii is significantly increased in invading cSCC and has been demonstrated to associate with cortactin at leading edges of invadopodia and to regulate the invasive properties of cSCC keratinocytes [23]. Systemic and topical therapeutic approaches using FnAb are currently in development with FnAb as a therapy for wound healing now entering the final pre-clinical validation stage [68, 94]. Flii has been shown to colocalize with structural (Claudin-1, -4 and -6) and adaptor (ZO-1 and -2) tight junction proteins and its overexpression in keratinocytes results in an altered F-actin/G-actin ratio, which can be restored using FnAb [72]. Therefore, taken together, these studies suggest that therapies targeting Flii may be a potential strategy for reducing the severity of cSCC in the community, however clinical trials using FnAb are still pending.

Pharmacological inhibition of actin-associated proteins aimed at compromising the survival and invasion of tumor cells may also have clinical benefit. One example of this strategy is harnessing TR100 inhibition of the tropomyosin isoform Tm5NM1. Tm5NM1 belongs to a family of actin-associated proteins that regulate the activity of several effectors of actin filament dynamics [95], as described above. The TR100 inhibitor has been shown to preferentially disrupt the actin cytoskeleton of tumor cells, impairing tumor cell motility and viability, and reducing melanoma growth both *in vitro* and *in vivo*. This therefore provides a pathway for development of a novel class of anti-actin compounds for the potential treatment of wide variety of cancers including cSCC [96].

Microtubule targeting agents of both synthetic and natural design, and microtubule stabilizing and destabilizing agents, have been the focus of anti-cancer therapy in the last decade and remain one of the most successful group of agents in the clinic [97, 98]. Their ability to regulate the tubulin-microtubule equilibrium disrupts the mitotic spindle, halting the cell cycle and resulting in cell death. They have been shown to be effective in combination with anti-angiogenic and anti-vascular properties and in some cases have demonstrated the ability to overcome multi-drug resistance, supporting their utilization as a chemotherapy [99]. Epothilones are a new class of anti-microtubule agents currently in clinical trials. Epothilones have shown activity in cSCC cell lines and in melanoma clinically, however clinical trials on cSCC patients are still pending [100, 101]. Other examples of microtubule-targeting agents, which have shown clinical promise in different subtypes of SCC including metastatic and recurrent disease, include semisynthetic compounds docetaxel and eribulin and a natural compound called rhizoxin [102–104]. While microtubule-targeting compounds are widely used as chemotherapeutic agents, they do have variability in different cancers, cancer cells frequently develop resistance to them, and they can be toxic to normal tissue, highlighting the need for better research and refinement of these compounds as well as a need to further understand their interactions with microtubule-associated proteins [105]. It is possible that microtubule-targeting agents also exert broader effects on tumor cell migration, invasion and metastasis and future studies should explore their effects on cSCC in combination with actin pathway inhibitors. Gaining a better understanding on the interplay of regulatory proteins governing the mechanotransduction and actin cytoskeletal remodeling involved in tumor cell migration, invasion and metastasis will lead to increased efforts to exploit therapeutic avenues targeting the actin cytoskeleton to treat aggressive cSCC.

5. Conclusions

The contribution of actin cytoskeletal remodeling and actomyosin signaling during SCC progression is significant and cannot be undervalued in the search for new treatment modalities. Recent research has identified a number of potential novel therapeutic targets within regulatory actin and microtubule signaling pathways that should be explored as potential therapeutic adjuvants to immunomodulatory therapies currently in clinical trials. A comprehensive understanding of the regulatory network of cutaneous mechanotransduction, mechanical forces and actin dynamics in cSCC, as discussed in this chapter, will facilitate the development of novel approaches to curb the incidence and progression of aggressive cSCC in the community, generating new inroads toward development of novel, individually personalized and efficient therapeutic approaches.

Acknowledgements

Dr. Zlatko Kopecki is supported by a Future Industries Institute Foundation Fellowship from University of South Australia. Dr. Sarah Boyle is supported by a Royal Adelaide Hospital Research Fund Early Career Fellowship.

Conflict of interest

The authors declare no conflict of interest.

Nomenclature

Akt	protein kinase B—serine/threonine-specific protein kinase
AP-1	activator protein 1—transcription factor that regulates various cellular processes downstream of stimuli
Arp2/3	actin-related proteins 2/3 complex—protein complex regulating the actin cytoskeleton
Cdc42	cell division control protein 42—protein involved in regulation of cell cycle
CDKN2A	cyclin-dependent kinase inhibitor 2A
CRE	cAMP-response element
cSCC	cutaneous squamous cell carcinoma
DAMPs	damage-associated molecular pattern molecules
DMBA	7,12-dimethylbenz[a]-anthracene—polycyclic aromatic hydrocarbon that acts as a carcinogen
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
Erk	extracellular signal-regulated kinase—kinase involved in regulation of cellular processes such as meiosis and mitosis
FAK	focal adhesion kinase—a focal adhesion-associated protein kinase involved in cellular adhesion
Flii	Flightless I
FnAb	Flii neutralizing antibody
GSK3β	glycogen synthase kinase 3-beta—serine/threonine protein kinase
GTPase	enzyme that binds and hydrolyzes guanosine-5′-triphosphate
H&E	hematoxylin and eosin
HPV	human papillomavirus
ILK	integrin-linked kinase
JAK	janus kinase
LIMK	LIM-domain kinase
lncRNA	long non-coding RNA
MAPK	mitogen-activated protein kinase
miRNA	microRNA
MLC2	myosin regulatory light chain-2—subunit of myosin, which regulates cell contractility
MMP	matrix metalloproteinase—endopeptidases that degrade various ECM proteins
MYPT1	myosin phosphatase target subunit 1—subunit of myosin phosphatase, which dephosphorylates MLC and thereby opposes contractility
NFκB	nuclear factor kappa-light-chain enhancer of activated B cells—protein complex that regulates transcription and other cellular processes
PAMPs	pathogen-associated molecular pattern molecules
PI3K	phosphoinositide 3-kinase—intracellular signal transducer enzymes ROCK: Rho-associated coiled-coil containing kinases
SNAI1	snail—transcription factor promoting repression of E-cadherin
SNAI2	slug—transcription factor promoting repression of E-cadherin
STAT	signal transducer and activator of transcription proteins—intracellular transcription factor

Tiam1	T-lymphoma invasion and metastasis-inducing protein 1—regulates Rho-like proteins and transduces extracellular signals
TP53	tumor protein p53
TPA	12-o-tetradecanoylphorbol-13-acetate—tumor promoting phorbol ester
TLR-4	toll-like receptor 4—transmembrane receptor that activates NFκB signaling
Tm5NM1	tropomyosin isoform 5NM1
Traf6	tumor necrosis factor (TNF) receptor associated factor 6
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous
Zeb1	zinc-finger E-box-binding homeobox 1—transcription factor that induces EMT by repression of E-cadherin and other genes

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Section 6

Molecular Aberrations in Squamous Cell Carcinoma

Chapter 6

Comprehensive Molecular Characterization of Squamous Cell Carcinomas

Corina Lorz, Carmen Segrelles, Ricardo Errazquin and Ramon Garcia-Escudero

Abstract

Over the last two decades, a number of high-throughput technologies (genome- and proteome-based) have been developed and applied on different cancer types such as squamous cell carcinomas (SCCs) arising from aerodigestive and genito-urinary tracts. These analyses, when comprehensively utilized, have clearly contributed to a better understanding of the molecular hallmarks, oncogenic pathways and immunological features of SCCs. This chapter aims to describe the SCCs most important molecular aberrations as well as their molecular classification, highlighting the commonalities and differences among them, independent of their body site origin. The most frequently altered oncogene is PIK3CA, involved in the PI3K/AKT/mTOR pathway and frequently activated in many human cancers. However, alterations in the cell-cycle control TP53 gene occur in the vast majority of SCCs. New possible molecular therapies, common to all SCCs, are discussed in light of a comprehensive, panSCC analysis.

Keywords: squamous cell carcinoma, human papillomavirus, genomics, Fanconi anemia, TCGA, mutation, copy number alteration, cancer treatment, biomarker

1. Introduction

Squamous cell carcinomas (SCCs) represent highly common solid cancers that arise from stratified and pseudo-stratified epithelia of the skin, and aerodigestive and genitourinary tracts. Although SCCs from different body sites share histological characteristics, they are molecularly and clinically heterogeneous, and a major cause of cancer mortality [1]. Reported risk factors for SCCs, depending on the body site, include alcohol intake (head and neck, and esophagus), cigarette smoking (bladder, lung, head and neck, and esophagus), UV light exposure (skin) and infection with human papillomavirus (HPV) (skin, head and neck, and cervix uteri). HPV infects epithelial cells and transforms them through the oncogene action of viral genes. E6 and E7 genes from some HPVs infecting head and neck and cervix uteri inhibit the function of the important tumor suppressors p53 and pRb, respectively [2, 3]. The initiation of SCCs is due to genomic perturbations, genetic mutations, and/or altered expression of key molecules mainly involved in cell-cycle control, signaling and cell adhesion pathways, squamous differentiation and chromatin regulation [1, 4]. A number of reports show that SCCs from different

anatomical locations have common features despite the fact that they are clinically treated as separate entities. These findings suggest an integrated view of the disease and possible new methods for prevention and treatment.

Here we review reports in which hundreds of SCCs have been comprehensively characterized at the molecular level using different high-throughput technologies. Such analyses highlighted commonalities and differences between SCCs, independent of body site origin, and allow their classification based on molecular aberrations. New possible molecular therapies, common to all SCCs, are discussed in light of the comprehensive, panSCC analysis.

2. Molecular features of SCCs

SCCs from different anatomical sites have been molecularly characterized using various genome-wide technologies (**Table 1**). Despite early reports describing most frequent mutations using next-generation sequencing (NGS) such as whole-exome sequencing (WES) in many cancer types [5], most of the comprehensive analyses have been done within the context of The Cancer Genome Atlas (TCGA) consortium. TCGA is an USA project which has generated comprehensive, multi-dimensional maps of the key genomic changes in the main types of cancer (http://cancergenome.nih.gov/). Microarray- and/or NGS-based technologies have been used in order to determine mutations in protein coding genes, expression levels of messenger RNA (mRNA) and micro RNA (miRNA) molecules, DNA-methylation and genome copy-number variation (CNA) (**Table 1**). Moreover, an important subset of cancers has been characterized at the protein level, using Reverse Phase Protein Array (RPPA) (**Table 1**). Recently, the TCGA launched a set of publications reporting pancancer analyses of more than 11,000 tumors from 33 types of cancers [4] (https://www.cell.com/pb-assets/consortium/pancanceratlas/pancani3/index.html), including SCCs from 5 individual body sites: lung (LUSC), head and neck (HNSC), esophageal (ESCA), cervical (CESC), and bladder (BLCA) cancers. Most of the molecular features described here are based on the TCGA panSCC analysis, in which around 1400 SCCs from those body sites were analyzed simultaneously [6]. Although skin SCC is the second most frequent cancer in Caucasians [7], no comprehensive, genome-wide analysis has been reported. Interestingly, most frequent mutations using NGS-based technologies in skin SCC showed many similarities with SCC from other body sites [8, 9].

Body site	Sample size (SCC size)	Genome-wide molecule*	Reference
Head and neck	279 (279)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[10]
Lung	178 (178)	DNA-meth, CNA, DNA-seq, mRNA, miRNA	[11]
Esophagus	164 (90)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[12]
Cervix uteri	228 (144)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[13]
Bladder	131 (19)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[14]
Bladder	412 (42)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[15]
Pancancer12	3,527 (546)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[16]
Pancancer33	~10,000 (~1,400)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[4]
PanSCC	~1,400 (~1,400)	DNA-meth, CNA, DNA-seq, mRNA, miRNA, proteome	[6]

** DNA-meth: DNA methylation; CNA: DNA copy number alteration; DNA-seq: whole exome sequencing; mRNA: messenger RNA; miRNA: micro RNA; proteome: reverse phase protein assay (RPPA).*

Table 1.
List of publications with genome-wide analysis of SCC.

2.1 Mutations in cancer genes

The most frequent mutated gene found in SCCs is *TP53* (64% in panSCCs) [4, 6, 16], (**Figure 1**) a tumor suppressor gene whose main function is to prevent genome mutations [17]. Missense "hot spots" mutations are very common, which result in dominant-negative and/or gain-of-function properties [18]. Although *Tp53* was found to be highly altered in many other cancer types [19], frequencies depends on the type, stage, body site, and other factors. Mutations in *TP53* are infrequent in HPV(+) SCC cancers, possibly because p53 functions are compromised as the protein is degraded by the activity of the viral E6 oncogene. Individually, frequent *TP53* mutations are found in SCCs within BLCA [15], ESCA [12], HNSC [10] and LUSC [11], and to a lesser extent in CESC whereby the majority of tumors are HPV(+) [13]. Other mutated genes involved in cell-cycle control include CDK inhibitor *CDKN2A* and the *RB1* gene, although less frequently. The incidence of *CDKN2A*/*RB1* mutations is much reduced in HPV(+) HNSC and CESC, as the E7 viral oncogene can bind and inactivate pRb protein, coded by *RB1*, thus rendering direct genetic mutation dispensable [10, 13]. Another important group of mutated genes include regulators of squamous differentiation, such as *NOTCH1*, *AJUBA* or *ZNF750* (**Figure 1**) [4, 6, 16].

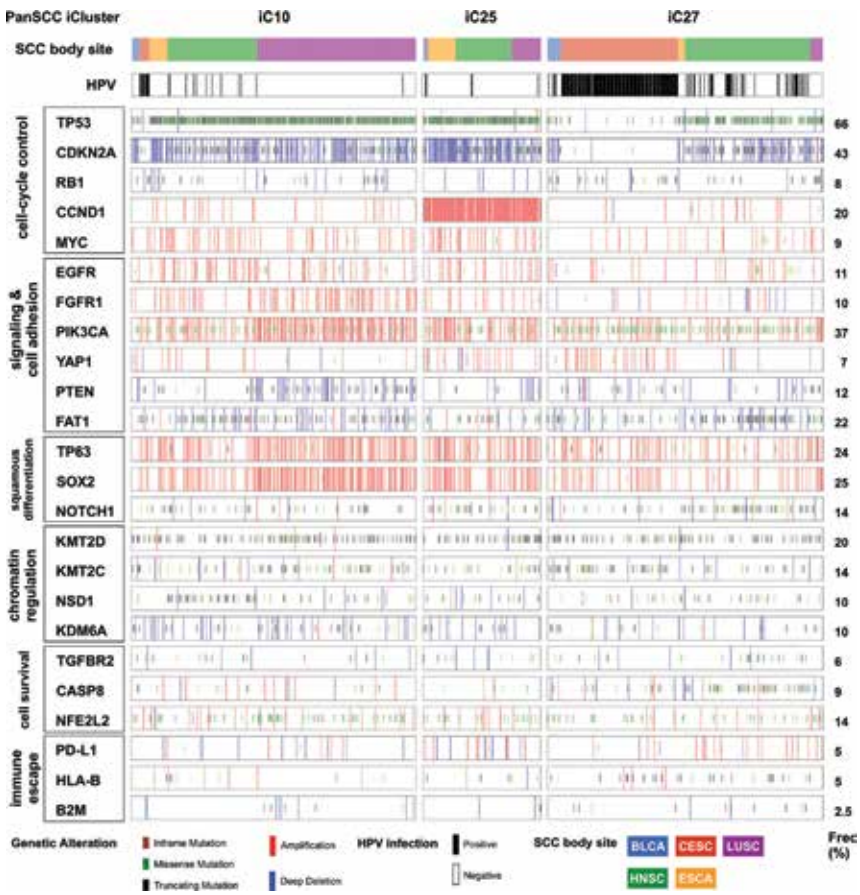

Figure 1.
Relevant mutations and CNA alterations in SCCs from BLCA, CESC, LUSC, HNSC and ESCA. Tumors are grouped into iC10, iC25 and iC27 clusters. Genes are grouped into functions. Frequency of alterations per gene is shown. HPV infected samples are indicated. Tumors having WES and CNA data, and belonging to iC10, iC25 and iC27 clusters are shown (n = 1098). Data are from the cBioportal for Cancer Genomics (http://www.cbioportal.org/) [20].

Other mutated genes include *KMT2D*, *NSD1*, *EP300*, or *KDM6A*, all of them involved in chromatin regulation through histone post-translational modifications. *PIK3CA*, *PTEN*, *FAT1*, *EPHA2* or *RASA1* genes, also mutated, are involved in important signaling and cell adhesion pathways of epithelial cells. There are also mutations in genes important in cell survival, like *TGFBR2* or *CASP8*. Mutations of *HLA-A* and *HLA-B* and deletions of *B2M*, implicated in immune escape, also exist (**Figure 1**) [4, 6, 16].

2.2 panSCC molecular clustering

High-throughput technologies have allowed the identification of tumor subgroups within specific cancer types, like the 'intrinsic subtypes' of breast cancer [21], occasionally having important clinical differences and outcomes [22]. Tumor subgroups based on genome-wide molecular analyses have been reported also for HNSC [10], LUSC [11], BLCA [14, 15], CESC [13] and ESCA [12]. Such classifications are based on molecular features like mutations, CNA, DNA-methylation, or expression of mRNAs, miRNAs, proteins [10–15] and long non-coding RNAs [15, 23, 24].

The existence of hundreds of primary tumors from different cancer types within TCGA having multiplatform molecular data have allowed the integrated identification of their differences and commonalities, regardless of body site [4, 6, 16]. One of such analyses, performed over 1400 SCCs from five different locations (LUSC, HNSC, CESC, ESCA and BLCA), discovered the existence of different SCC tumor clusters based on CNA (six clusters), DNA methylation (five clusters), mRNA expression (six clusters), miRNA expression (five clusters), and RPPA-based protein expression (eight clusters) [6]. These clusters highlight significant molecular features in SCC versus non-SCC, and between SCCs. Moreover, the iClustering method [25], which performs clustering from multi-type genomic data, showed the presence of 3 main iClusters: iC10, iC25 and iC27 [6] (**Figure 1**). Most HPV(−) tumors grouped in iC10 and iC25, associated with smoking history, organ site and molecular aberrations (**Figure 2A**), while most HPV+ CESC and HNSC samples mapped within iC27 having non-smoking individuals (**Figure 2A**). All tree SCC-clusters displayed significant chromosome 5q and 3p copy gains, concomitant with overexpression in 3q genes SOX2, TP63, and TP73, implicated in squamous differentiation and stemness (**Figure 1**) [6]. iC25 cluster bear 11q gains, and iC10/iC25 included 9p losses. Most iC10/25 HPV(−) SCC tumors displayed genome-wide hypomethylation with high DNA CNA, and associated augmented mRNA and miRNA levels. Some HPV(−) SCCs and most iC27 HPV(+) HNSCs and CESCs, showed wider hypermethylation and reduced CNAs, correlated with reduced mRNA and miRNA expression [6].

Kaplan-Meier curves demonstrated significant differences in overall survival and progression-free interval between the iClusters, even after adjusting for distinct body sites or disease stages (**Figure 2B**). Patients within iC25 display poorer prognosis, possibly associated to higher CNA aberrations and genome instability (**Figures 1** and **2B**). Therefore, panSCC analysis showed the existence of a prevalent SCC group, having a combination of recurrent CNA and other alterations, and other subtypes whereby HPV infection and other alterations have a greater role.

2.3 Cancer genes in CN alterations

Oncogenic transformation from normal tissues occurs upon the accumulation of small mutations and also larger alterations, giving rise to deletion (DEL) or amplification (AMP) of regions and altering the normal diploid state of the genome. Negative regulators of cell-cycle control like CDKN2A and RB1 are frequently deleted in SCCs (**Figure 1**). Contrarily, CCND1, MYC, and CCNE1 genes appear frequently amplified, and therefore, their function in cell proliferation. Important

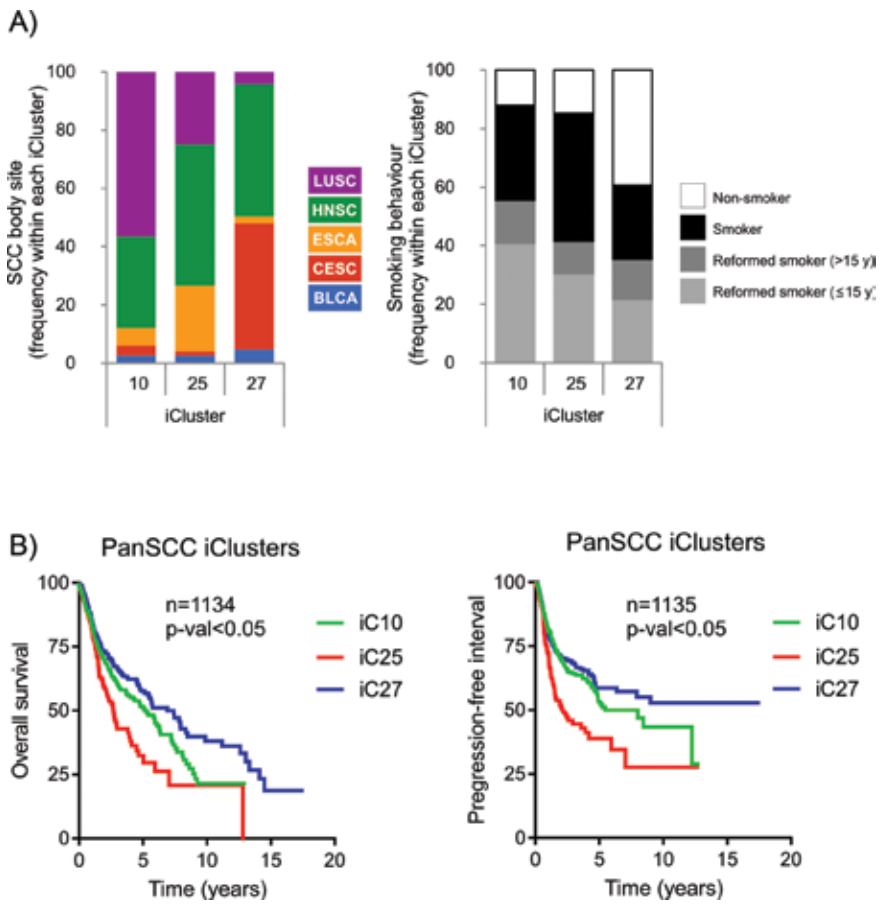

Figure 2.
Clinical features of main panSCC iClusters, including body site distribution and patient smoking history frequencies within each iC10, iC25 and iC27 (A), and survival curves with 2 endpoints: overall survival and progression-free interval (B). Clinical data obtained from Liu et al. [26]. P-values were calculated after multivariate Cox regression analysis, using iClusters and body sites or pathologic tumor stage.

positive mediators of signaling and cell adhesion pathways are frequently amplified (EGFR, ERBB2, FGFR1, PIK3CA, AKT1, AKT3, MAPK1, YAP1), and tumor suppressors like PTEN or FAT1 are deleted. Chromosome 3q genes TP63 and SOX2 are highly frequently co-amplified, and overexpression of their mRNAs is a common SCC feature as mentioned above (**Figure 1**) [6]. Squamous differentiation genes which are deleted also exist, like NOTCH1 and ZNF750. Frequent deletion of chromatin regulation genes occurs, like ARID1A, NSD1, KMT2C or KDM6A. There are also alterations in cell survival genes, like NFE2L2 (AMP), BCL2L1 (AMP), and BCL2L2 (DEL). Importantly, some main immune escape regulators are segregated in CNA regions, like PD-L1 (AMP) or B2M (DEL) (**Figure 1**).

3. SCC and Fanconi DNA repair pathway

Fanconi anemia (FA) is a rare autosomal recessive genetic disorder in which patients can develop a life-threatening bone marrow failure in the early years after birth [27], which frequently requires allogeneic hematopoietic stem cell transplant [28]. In addition to this blood disorder, FA patients can develop leukemias and solid tumors, mainly SCC in the head and neck, skin, and anogenital regions [29].

Incidence of HNSC in FA is >500 times higher than in the general population, and average age of appearance is significantly earlier. Mutations occur in genes involved in the 'FA pathway' which is activated as a result of DNA replication or DNA damage, especially the damage triggered from DNA crosslinking agents. Some of these FA genes include *BRCA1* and *BRCA2* genes, well known breast cancer-susceptibility genes. Hitherto, there is no explanation for the high incidence of FA-HNSC, but it has been suggested that FA pathway defects might accelerate oncogenic transformation through the accumulation of mutations in a DNA repair-defective context [30]. In this sense, a number of reports showed tumor suppressor functions by FA genes, both in the FA as well as in non-FA human cancer [31].

Campbell et al. reported an unexpectedly high frequency (around 12%) of molecular aberrations involving top 10 FA pathway genes in panSCC from TCGA [6]. An analysis using all 22 FA pathway genes reported so far, demonstrated that almost 30% of SCCs within iC10, iC25 and iC27 clusters from BLCA, CESC, ESCA, HNSC and LUSC (314 out of 1098) display either point mutations or deletions in any FA gene (**Figure 3**). Whether all of these FA gene alterations are associated with defects in DNA-repair is unknown, but clinical implications would be important

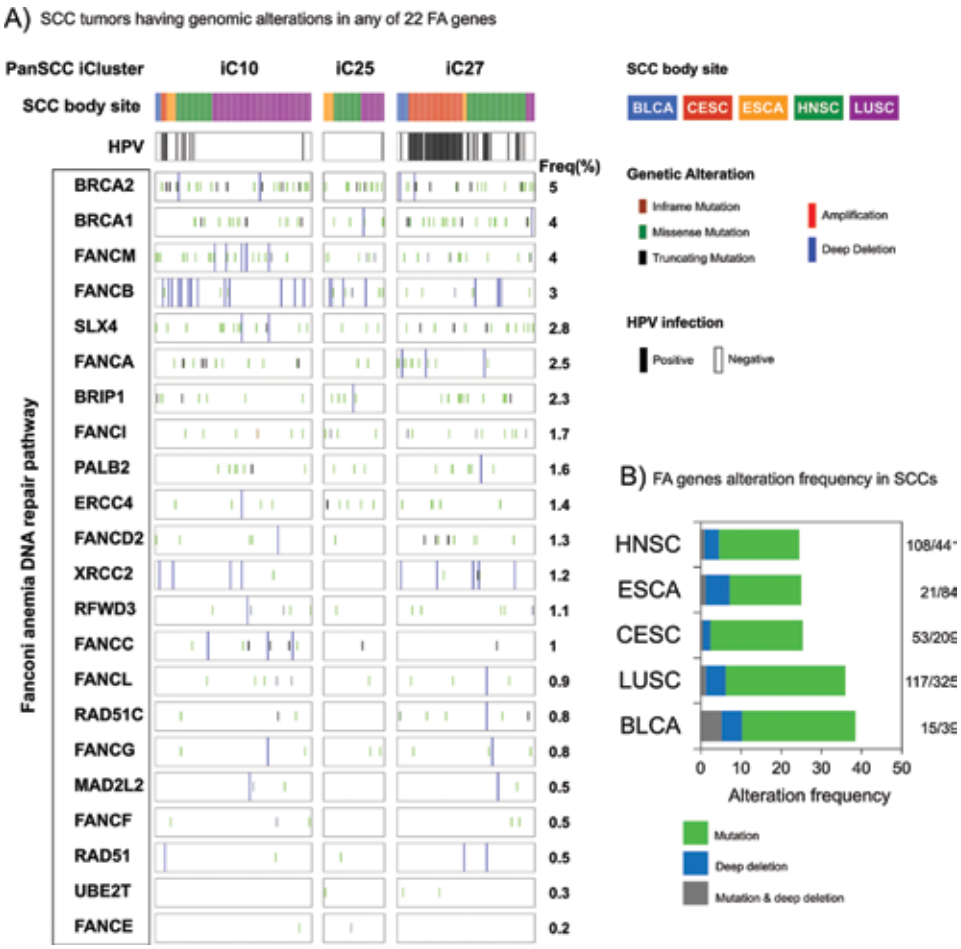

Figure 3.
Mutations and deep deletion in all 22 FA pathway genes in SCCs from BLCA, CESC, LUSC, HNSC and ESCA. (A) Tumors are grouped into iC10, iC25 and iC27 clusters. Frequency of alterations per gene is shown. HPV infected samples are indicated. (B) Alteration frequency in any FA gene is shown per body site. Tumors having WES and CNA data, belonging to iC10, iC25 and iC27 clusters, and having mutation/deep deletion are shown (n = 314). Data are from the cBioportal for Cancer Genomics (http://www.cbioportal.org/) [20].

as the FA pathway is a major predictor of cisplatin response in HNSC [32]. These findings suggest that acquired as well as germline alterations in this pathway may contribute to the development of a subset of SCC.

4. Molecular therapies against SCC

Patients suffering squamous cell carcinoma display poor overall survival, and the disease is difficult to treat. Independent of body site, the standard of care is based on surgery, radiotherapy and chemotherapy. Still, few molecular therapies are being used so far, and only in the latest stages of the disease, such as immunotherapies, cetuximab (antibody to EGFR) in HNSC or bevacizumab (antibody to VEGF) in cervical cancer. There is a clear need to develop new targeted therapies accompanied with accurate response biomarkers, so we can give more effective and less aggressive treatments to SCC patients. The profound knowledge about the molecular biology of SCCs that we have acquired over the last recent years, together with comparative efforts of tumors from different body sites, should help to design new clinical trials challenging current treatment modalities.

4.1 Immunotherapies in SCCs

As understanding of the underlying cancer biology and the complex interactions within the tumor microenvironment improves, there is gathering interest in and evidence for the role of immunomodulating agents in the management of cancer. Immune checkpoint inhibitors, which aim to hinder the inhibitory interaction between programmed cell death protein 1 (PD-1) and its ligand PD-L1, have demonstrated durable improvements in patient outcomes in many cancer types. Thus, pembrolizumab (anti-PD1) has been approved to treat HNSC, CESC, LUSC, and BLCA [33–35]. Clinical trials for pembrolizumab in ESCA are giving good responses [36]. Nivolumab has also reach FDA approval for HNSC, BLCA and LUSC [33–35]. Other existing immunotherapies include avelumab, atezolizumab and durvalumab for BLCA [34]. Although the use of immunomodulating agents in SCC treatment is giving good results, none of them are being used in first line so far and many patients do not respond. Therefore, future analyses and trials should focus on developing accurate response predictors to accelerate their use as first line in therapy.

4.2 Possible new therapies targeting SCCs biomarkers

Deep molecular analyses of SCCs, as explained above, suggest that certain targeted therapies, at different stages of clinical trial or approval, might be adequate for SCC treatment. These include targeting the following biomarkers:

i. PIK3CA, which encodes p110α, a catalytic subunit of phosphoinositide 3-kinase (PI3K). Activated PI3K can activate PDK1 and AKT, triggering downstream effects on transcription, protein synthesis, metabolism, proliferation and apoptosis. The gene is amplified or mutated in about 37% of SCCs (**Figure 1**), and constitutes the most frequently mutated oncogene in cancers like HNSC, CESC, ESCA and LUSC. A number of clinical trials with p110α inhibitors as possible antitumor therapies are currently running. We have recently identified that HPV(−), HNSC tumors that overexpress PIK3CA display poor outcome and activation of the YAP1-nuclear function, a transcriptional co-factor within the Hippo growth pathway [37]. Therapies targeting nuclear YAP1 might also be effective in a subgroup of SCC patients [38].

ii. CCND1, which encodes cyclin D1, is a cell-cycle protein that regulates transition from G1-to-S phase through the formation of complexes with cyclin dependent kinases (CDKs), such as CDK4 and CDK6. CCND1 is amplified in 20% of panSCCs, and in 93% within the iCluster iC25 (**Figure 1**). CCND1 amplification is associated with poor prognosis, cisplatin resistance and EGFR-inhibitor resistance in HNSC [39]. Although targeting of cyclin D1 is not currently feasible, there are inhibitors of its binding partners CDK4/CDK6, which might be useful in the CCND1 amplification setting [40].

iii. CDKN2A, which encodes $p16^{INK4A}$, is a CDK4/CDK6 inhibitor that regulates cell-cycle. CDKN2A is mutated or deleted in 43% of panSCCs, mostly in the iC25 cluster (50%) (**Figure 1**). Similar to CCND1 amplified tumors, CDKN2A mutated/deleted tumors might respond to CDK4/CDK6 inhibitors [40].

iv. EGFR, which encodes the epidermal growth factor receptor protein, is mutated or amplified in 11% of panSCCs, mainly in HPV(−) tumors. Although EGFR is an attractive target for therapy by either small-molecule inhibitors [41] or blocking antibodies [42], current EGFR-related therapies in SCC are limited to cetuximab antibody in HNSC. Good responses are observed to inhibitors in lung tumors with activating EGFR mutations, but they occur in adenocarcinomas not in lung SCCs. Further research should be done with EGFR-therapies in panSCCs, understanding mechanisms of action as well as probing response efficacy in preclinical models.

5. Conclusions

Squamous cell carcinomas arising from five different body sites (bladder, cervix uteri, lung, head and neck, and esophagus) share many molecular aberrations, so that the majority of them can be classified in 3 main molecular clusters (iC10, iC25 and iC27). Principal differences between clusters include HPV infection, genome-wide DNA-methylation and CNA, and mutations/CNA in subsets of cancer genes. Amplification in CCND1 is prevalent in iC25 samples, and TP53 and CDKN2A deleterious modifications in HPV(−) tumors. iC25 tumors are HPV(−), display frequent genome alterations and smoking patients, as well as poorer clinical outcome. Importantly, there exist common features between panSCC clusters, such as oncogene PIK3CA mutations/amplifications, amplification in TP63 and SOX2, or mutations in chromatin modifier regulators (like KMT2C and KMT2D). The comprehensive, panSCC molecular analyses suggest that current and future clinical trials targeting aberrations in signaling/cell adhesion pathways (PIK3CA and EGFR inhibitors) and cell-cycle control (CDK4/CDK6 inhibitors) might have a great impact on SCC treatment and independently of their body site. Future research efforts should focus on developing accurate biomarkers of immunotherapies. Finally, basic and clinical investigators should work together to discover SCC vulnerabilities and derive new treatments, as well as understanding basic mechanisms of oncogenesis, tumor progression and therapy resistance.

Acknowledgements

This work was supported by FEDER cofounded ISCIII [grant numbers PI18/00263, CB16/12/00228] and a grant from Fundación Anemia de Fanconi [grant number 2018/127].

Conflict of interest

No 'conflict of interest' to declare.

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Edited by Hamid Elia Daaboul

With the support of multinational specialists, each with different background and separate field of expertise in oncology, this book had the occasion to emerge and to offer physicians, researchers and academics efficient, well-organized and updated scientific information related to the characteristics and treatment modalities of squamous cell carcinoma. It provided in-depth information regarding the comprehension of the molecular interaction of signalling pathways and new phenotypes that might result and lead to further cell proliferation and metastases. It also emphasized on the management and individualization of treatment strategies in different types of SCC, applying molecular profiling and approved protocols in order to identify new treatment opportunities. This book also discussed the potential therapeutic modalities that might arise upon understanding and exploring the role of key regulatory proteins that govern the process of cutaneous SCC progression. And Lastly, it underlined the molecular aberrations present in SCC of different organs and the possibility of emerging new therapeutic drugs by targeting these abnormalities. It is really an innovative record that combined novel information from various medical sources and mixed it to become unified in its scientific profit.

Published in London, UK

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ISBN 978-1-78985-592-0