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Cancer Metastasis

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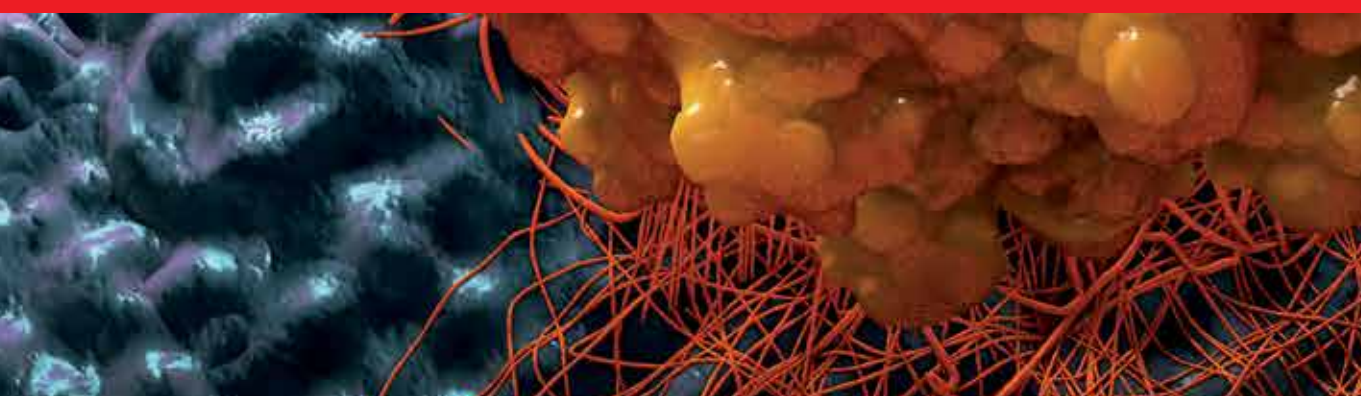

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Meet the editors

Dr. Yasemin Basbinar is a professor at the Department of Basic Oncology, Dokuz Eylul University, Institute of Oncology in Turkey. She graduated from the Faculty of Medicine, Ege University, Turkey in 1988. She completed her PhD degrees in Biochemistry and Basic Oncology at Dokuz Eylul University, Turkey, in 1995 and 2006, respectively. She worked in the Danish Centre for Human Genome Research as a visiting researcher. Dr. Basbinar is now the head of the Personalized Medicine and Pharmacogenomics/Genomics Center and Translational Oncology postgraduate training program in Dokuz Eylul University. Her primary research is focused on cancer proteomics and pharmacogenomics in terms of predictive medicine. She has been awarded by the Scientific and Technological Research Council of Turkey (TUBITAK), Turkish Association for Cancer Research and Control, for her achievements. She has more than 60 publications in prestigious journals, and is on the review boards of several journals and granting committees.

Dr. Gizem Calıbası-Kocal graduated from Ege University, Turkey, holding a Bachelor's degree in Biochemistry. She then received her MSc and PhD degrees in Basic Oncology from Dokuz Eylul University, Turkey. From 2013 to 2015, she worked in the USA in the field of microfluidic technologies related to cancer metastasis at Harvard University and Stanford University as a pre-doctoral research fellow. Her research interest is focused on metastatic processes of colorectal cancer in terms of tumor microenvironment and cancer metabolism, as well as pharmacogenetic applications in cancer management. She is an advisory board member of the Personalized Medicine and Pharmacogenomics/Genomics Center in Dokuz Eylul University. She also serves on the editorial boards of two journals and regularly reviews prestigious journals. Her achievements were recognized with several awards and honors from the Scientific and Technological Research Council of Turkey (TUBITAK), Turkish Association for Cancer Research and Control, Organization of European Cancer Institutes (OECI), and European Federation of Clinical Chemistry and Laboratory Medicine (EFLM).

Contents

Preface

Metastasis is the most common cause of cancer-related mortalities. Metastasis begins with the invasion of tumor cells into the neighboring stroma by epithelial mesenchymal transitions, then tumor cells intravasate into the vasculature and extravasate from the vessel to reach a secondary tumor location, later colonizing and forming micro- and macrometastasis at this new home. Although sequential steps have been described, the basic nature of metastasis is still unclear.

The most important concept is the understanding of the biological differences and similarities between primary tumors and their metastases, especially in terms of heterogeneity for genotype and phenotype, plasticity and resistance. Over the past decade, metastasis research has entered a new stage with its impressive progress. The accumulation of information on the comparison of primary tumor growth with metastatic dissemination and also molecular pathways that orchestrate the sequential steps of metastasis facilitates the understanding of metastatic behavior and rapid translation of these basic research findings to the clinical applications with the development of new approaches and therapies.

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Chapter 1

Introductory Chapter: Cancer Metastasis

Gizem Calibasi-Kocal and Yasemin Basbinar

Additional information is available at the end of the chapter

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1. Introduction

In malignant evaluation of cancer cells, metastasis is a commonly used terminology in which cancer cells gain the invasion ability to neighboring tissues and distant secondary organs and finally colonize in these organs. Metastasis is estimated as the main reason of 90% of cancer-related mortality due to its incurability by surgical resection and resistance of tumor cells to chemotherapeutic agents. Cells that have metastatic capability disseminate by several ways as hematogenous spread, lymphatic spread, or seeding into body cavities. Although lymphatic spread of cancer cells is commonly observed in metastasis and represents as a prognostic factor, hematogenous spread represents the major way in human tumors. Seeding into body cavities is routinely observed in colorectal and ovarian cancers [1].

The process from the spreading of cancer cells to distant parts of the body, termed as the invasion-metastasis cascade, involves sequential and interrelated steps: (1) invasion of local tissue, (2) intravasation into stroma and blood vessels, (3) survival in vasculature circulation, (4) extravasation into the parenchyma of distant tissues, and (5) survival in a new microenvironment and colonization to form micro- and macro-metastasis (**Figure 1**). The steps of invasion-metastasis cascade are explained below [1, 2].

2. Dissemination and local invasion

Dissemination process includes the initial step of invasion-metastasis cascade. During dissemination, cancer cells acquire ability to leave the primary tumor location to invade nearby tissues and travel to secondary tumor locations. Invasion ability of tumor cells is used to distinguish malignant tumors from benign tumors. Invasive growth and associated signaling pathways have role in tumor progression as well as metastasis. It basically includes the entering of tumor cells

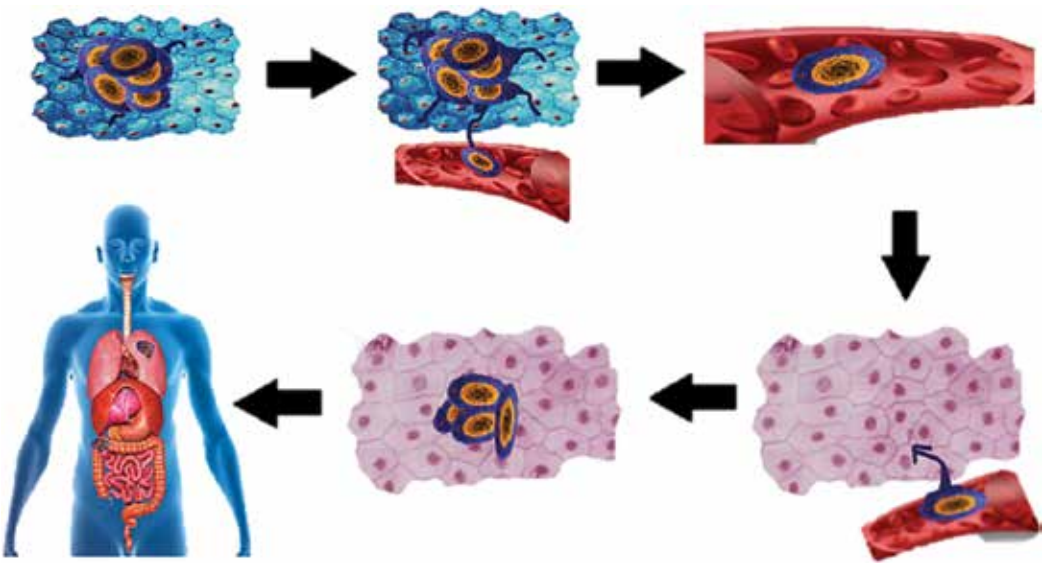

Figure 1. Steps of metastasis. During metastatic process, cells invade to local tissue (1), intravasate into the stroma and blood vessels (2), survive in the vascular circulation (3), extravasate into the distant tissues (4), survive and colonize to form micrometastasis (5), and finally form a clinically detectable macro-metastasis (6).

into the surrounding tumor stroma and adjacent normal tissue parenchyma. To gain invasion capability, cancer cells have to lose their adhesion ability to the adjacent cells and leave the primary tumor with gaining migratory feature. Therefore metastasis starts with the migration of tumor cells [3]. At this point, epithelial-mesenchymal transition (EMT), a process during the detachment of cancer cells from the epithelial stratum by losing of their epithelial markers and gaining motility, has a key role on the cancer progression. Gaining of EMT character and dissemination occur in early stage of metastasis. This complex process, EMT, is orchestrated by several EMT-inducing transcription factors such as Snail, Slug, Twist, Zeb1, Zeb2, Foxc2, Prrx1, etc. [4].

With the effect of EMT-related transcription factors, cancer cells present various alterations in gene expression. They lose their apical-basal polarity due to the loss of adhesion molecules (such as E-cadherin and integrins) and intercellular junctions; the remodeling of intracellular cytoskeleton molecules begins to form cellular protrusions; invasive growth and penetration into the surrounding stromal matrix start with the degradation of basal membrane and lacking intercellular contacts. Tumor cells, which have migratory and invasive feature, are more resistant to chemo- or radiotherapy than other cancer cells [5, 6].

3. Intravasation

Invasive and motile cancer cells invade into the vessels to travel through blood flow and access the secondary metastatic sites, where they may form micro- and macro-metastasis. During

intravasation, tumor cells pass through tissue and reach the endothelial vessel [7]. Intravasation can be facilitated by specific transcription factors, signaling molecules, enzymes (proteases), cells in tumoral microenvironment, and biophysical conditions of microenvironment and vasculature. The route of intravasation is led by the structural differences between blood vessels and lymphatics. Blood vessels have tighter junctions than lymphatics; therefore invasion through blood vessels and their connective tissue may be limited [7].

4. Survival in the circulation

When the cancer cells have achieved to intravasate into the blood vessels, they travel in the venous and arterial circulation, known as circulating tumor cells (CTCs). Survival in the bloodstream is crucial step for metastasis. Millions of tumor cells move from the tumor bulk and enter the circulation. CTCs travel as a single cell or CTC clusters, and they undergo molecular alterations to change their phenotype. However the relationship between immune system and tumor cells cannot be excluded; natural killer cells, monocytes/macrophages, and neutrophils mediate a clearance of CTC from the blood circulation. Therefore the success rate of metastasis is low due to the rare amount of CTCs [8]. Over time, cancer cells have developed various strategies to escape from the immune system. These strategies include the loss of immunostimulatory molecules and gain of immunoinhibitory molecules and increased expression of apoptosis-related molecules [9]. Also platelets, tiny blood cells that function against bleeding, facilitate the survival of CTCs by reacting to main threats in blood as shear stress and natural killer cells. Molecules related with coagulation such as tissue factor and thrombin lead activation of platelets, and this activation forms the platelet-cancer cell aggregates [10]. The popularity of CTCs is increasing in recent years due to their potential use in cancer diagnosis as well as prognosis. Up-to-date technological advances pave the way for detection of circulating tumor cells.

5. Extravasation

After the survival in the harsh blood stream, tumor cells become arrested at a secondary location and extravasate into parenchyma of distant tissues. Extravasation, which requires a tumor cell transendothelial migration, involves adhesion of tumor cells to endothelial cells and the transmigration through the endothelial wall. Endothelial cells can either allow or block the adhesion of tumor cells, as well as possible transmigration. Therefore endothelial cells are essential due to their role in the determination of secondary tumor location and regulation of metastatic formation. But still their all function in the metastatic cascade is still unclear [11]. Permeabilization of vascular structure is provided by the ATP production by active platelets and angiopoietin-like 4 (ANGPTL4) production. Increased transendothelial migration ability enhances the metastatic outgrowth. Also other molecules as VEGF, MMPs, ADAM12, and CCL2 disrupt the vascular integrity and increase both intravasation and extravasation. However, requirements can be various for different locations to achieve a successful extravasation process [4].

6. Micrometastasis and colonization

Extravasated cancer cells have to survive to form micrometastasis in the secondary locations. The microenvironment of secondary location is different from the microenvironment of primary tumor location due to the different types of stromal cells, extracellular matrix components, cytokines, chemokines, and growth factors, and metastatic cancer cells have to adapt to this secondary microenvironment of their new homes.

In 1889, Stephen Paget proposed "seed and soil" hypothesis for metastatic dissemination, which depends on numerous interactions between certain types of cancer cells and organ-specific homeostatic mechanisms of microenvironment. According to him, although tumor cells can disseminate in many locations, selected metastatic cancer cells (seeds) tend to form metastasis in one or more particular distant organ locations (soils) for survival and proliferation [12, 13].

Before the arrival of metastatic cancer cells to the secondary locations, an establishment process of a pre-metastatic niche was proposed for the survival and adaptation of tumor cells. Primary tumor cells induce the formation of their own pre-metastatic niches by releasing systemic signals that activate organ-specific orientation of resident tissue fibroblasts. Induced pre-metastatic niche is an essential parameter for metastatic propensity and tissue tropism [1].

7. Detectable macro-metastasis

Even if tumor cells can survive in the secondary locations, the proliferation of tumor cells and formation of macro-metastasis are not certain. They can be in long-term dormancy state and stay as microcolonies and also face with attrition problem due to the failure on the triggering neoangiogenesis. Because of this poorly understood attrition, high apoptotic rate balances continuously the proliferation rate [14].

Angiogenic switch is an essential need for the transformation of micrometastatic tumors (or dormant tumors) to macro-metastatic tumors. Reformed vasculature originate from the existing blood vessels or develop by endothelial progenitor cells [15]. With the existence of advantageous pre-metastatic niche and angiogenic signals, clinically detectable metastases are the final result of highly complicated invasion-metastasis cascade.

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Chapter 2

Epithelial-Mesenchymal Transition in Tumor Microenvironment Induced by Hypoxia

Görkem Eskiizmir and Erdoğan Özgür

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Abstract

A tumor microenvironment contains various noncancerous cells including adipocytes, fibroblasts, immune and inflammatory cells, neuroendocrine cells, pericytes, vascular and lymphatic endothelial cells, and the extracellular matrix that surrounds cancerous cells. In the tumor microenvironment, cancer cells interact and cross talk with noncancerous cells and orchestrate different mechanisms of cancer such as tumorigenesis, angiogenesis, and metastasis. Moreover, the expansive nature of cancer cells and chaotic angiogenesis affect microcirculation as well as alter the oxygen concentration progressively. Hypoxia, a key player in the multistep process of cancer metastasis, is important in different regions of the tumor microenvironment. Hypoxia may transform cancer cells to become more aggressive and invasive by triggering overexpression of several hypoxia-related factors that activate epithelial-mesenchymal transition (EMT). Herein, the current knowledge of how hypoxia-driven EMT is presented in the tumor microenvironment of solid cancers is discussed.

Keywords: cancer, cancer metastasis, epithelial-mesenchymal transition, hypoxia, tumor microenvironment

1. Tumor microenvironment: current perspective

The tumor microenvironment contains a multinetwork of cells, soluble factors, extracellular matrix (ECM) components, and signaling molecules that surround and neighbor cancer cells. It mediates aberrant tissue function and modulates subsequent progression in solid cancers [1]. In this microenvironment, the main structures are the parenchyma, stroma, growth factors, lymphokines and cytokines, and inflammatory and matrix metalloproteinase enzymes

(**Figure 1**). While cancer cells are located in the parenchyma, noncancerous cells and ECM constitute the stroma. Currently, it is known that noncancerous cells have key roles in several mechanisms of carcinogenesis, tumor progression, and metastatic cascade [2]. Noncancerous cells behave differently in the tumor microenvironment than healthy tissue. However, it is still unclear how noncancerous cells and noncellular components of the tumor niche collaborate and assist cancer cells to acquire invasive and metastatic features.

It is known that chronic inflammation is an important factor in shaping the tumor microenvironment. The major inflammatory cells located in the tumor microenvironment are T lymphocytes, natural killer cells, and tumor-associated macrophages (TAMS). TAMs are important in ECM destruction/restructuring of the tumor microenvironment, tumor cell motility, and triggering angiogenesis. These cells have both tumor-progressive and tumor-suppressive effects.

In the tumor microenvironment, fibroblasts have various roles under inflammatory conditions. However, they attain new characters and called as "*carcinoma-associated fibroblasts*" after the beginning of the neoplastic process. They constitute 50–70% of the volume of many solid epithelial tumors, such as pancreas, stomach, and breast cancers [3]. In addition, they are particularly effective in carcinogenesis, tumor progression, and metastasis [4, 5]. Studies on carcinoma-associated fibroblasts demonstrated that during the chronic inflammation and wound healing, only activated fibroblasts promote tumor growth. There are hypotheses about the production of cancer-associated fibroblasts. Genetic changes in normal fibroblast or exposure to EMT may directly arise from mesenchymal stem cells [6].

Cancer stem cells (CSCs) are also tumor microenvironment-specific cells. CSCs have been intensively researched recently. Today, we know many cancer types consist CSCs in their microenvironment, which is associated with aggressive tumor biology and treatment resistance. Moreover, CSCs are responsible for immune modulation during the carcinogenesis [7].

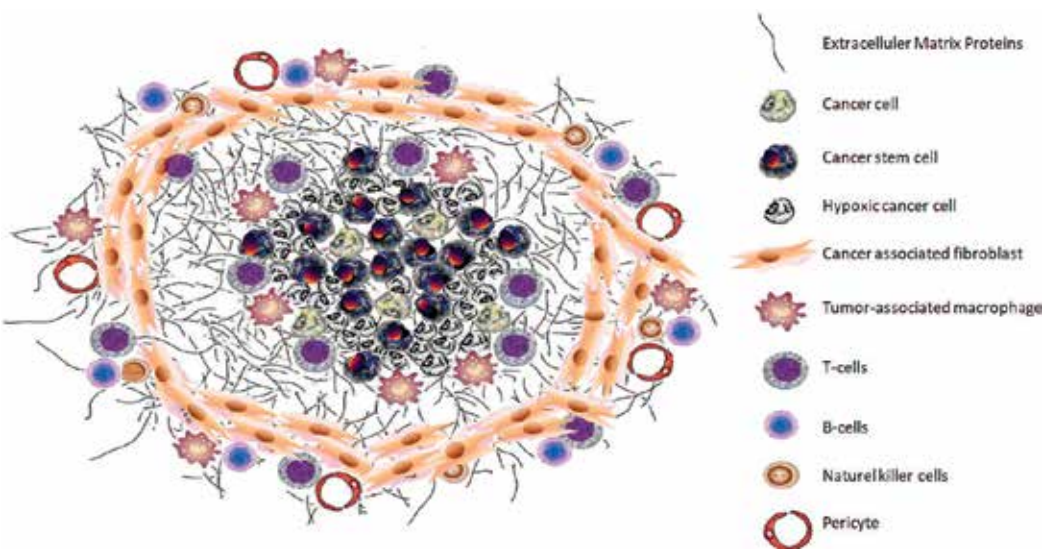

Figure 1. A schematic view of the tumor microenvironment.

In the tumor microenvironment, a unique network has been shown to be created by mainly carcinoma-associated fibroblasts and CSCs with the participant of other noncancerous cells. This network modulates and regulates different mechanisms of the neoplastic processes, such as carcinogenesis, tumor progression, angiogenesis, and metastasis.

Similar to healthy tissue, tumor tissue supplies oxygen and substances from blood and lymphatic vessels. Therefore, angiogenesis is a crucial step for tumor growth [8]. However, due to the rapid growth of tumor tissue, new blood vessel production is usually insufficient. This situation results with decrease of tissue oxygen levels termed as *hypoxia*. This new condition forces cells to acquire new and devastating behaviors such as resistance to environmental changes, invasiveness, and also metastatic phenotypes via different mechanisms. In this review, we aim to focus on the role of hypoxia and hypoxia-driven EMT in tumor microenvironment from the current perspective.

2. Hypoxia in tumor microenvironment

Hypoxia is a hallmark of tumor microenvironment. It emerges due to an inadequate blood source, which keeps proliferation cells viable. The cellular machinery uses several mechanisms in response to hypoxia. When a decrease in the level of oxygen develops, changes in numerous transcriptional regulators are altered.

2.1. Hypoxia: definition

Basically, hypoxia refers to the imbalance between the level of oxygen that the tissues require and that can be supplied. It is noteworthy that *normoxia* describes the "atmospheric" oxygen level which is approximately 20–21% (160 mmHg). However, every healthy tissue has lower and distinct oxygen levels; therefore, *physoxia* is a better terminology that defines the normal range of oxygen levels in different tissues [9]. The oxygen level of different tissues and cancers is presented in **Table 1** [10]. Therefore, hypoxic conditions often occur when the oxygen

Tissue/organ	Physoxia (% O_2)	Cancer	Hypoxia (% O_2)
Brain	4.6	Brain tumor	1.7
Breast	8.5	Breast cancer	1.5
Cervix	5.5	Cervix cancer	1.2
Kidney (cortex)	9.5	Renal cancer	1.3
Liver	4.0–7.3	Liver cancer	0.8
Lung	5.6	Lung cancer (nonsmall cancer)	2.2
Pancreas	7.5	Pancreas tumor	0.3
Rectal mucosa	3.9	Rectal cancer	1.8

Table 1. Physoxia and hypoxia of several tissues/organs and cancers.

tension (pO_2) decreases lower than 2.5 mmHg, even though tumor oxygen levels are dictated by the initial tissue and tumor microenvironment [11, 12]. Moreover, hypoxic regions are heterogeneously distributed particularly in the locally advanced tumors [13].

Cancer cells respond to hypoxia in two ways through apoptosis or resistance and survival, which is driven by the exposure time. If cancer cells are able to survive, they acquire new and unique features. Hypoxic conditions affect the gene transcription, which affords the ability of the cancer to survive through invasiveness, genetic instability, and metastasis. Furthermore, treatment (radiotherapy and/or chemotherapy) resistance may emerge [14]. A hypoxic response is mediated by hypoxia-inducible factors (HIFs), which control many facets of cancer cell viability [15].

2.2. Hypoxia-inducible factors

The HIFs orchestrate the responses to hypoxia in normal and cancer cells. Recently, three subtypes of HIFs have been introduced: HIF-1, -2, and -3. They are heterodimeric complexes and mainly act to mediate cellular processes including angiogenesis, cell proliferation, and tissue remodeling in response to hypoxia. HIFs are composed of basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) proteins including an O_2-labile alpha subunit (HIF-1α, -2α, and -3α) and a stable beta subunit (HIF-β). They interact with hypoxia-responsive elements that contain a conserved RCGTG core sequence [16]. HIF-1α was the first introduced prototypic member of HIF family, and has been shown to regulate O_2-dependent transcriptional responses [17]. After a while from the discovery of HIF-1α, a new HIF protein, HIF-2α, was introduced by independent research groups [18–21]. Currently, it is known that HIF-1α is the first biomolecule that responds to acute hypoxia, and HIF-2α is the major regulator under chronic hypoxic conditions. This phenomenon has been referred as the "*hypoxic shift*" [22]. Holmquist-Mengelbier et al. demonstrated that HIF-1α is active for a short duration particularly under hypoxia or anoxia (O_2 level <0.1%). However, HIF-2α is active for a long duration under less severe hypoxia (O_2 level <5.0%) [23]. Furthermore, Pietras et al. reported that the activation of HIF-2α may cause aggressive and infiltrative histopathological features under normal oxygen levels, which is termed as "*pseudohypoxic phenotype*" [24–26]. Tian et al. reported a correlation between HIF-2α and vascular endothelial growth factor mRNA expression levels in the endothelium [21]. Therefore, HIF-2α overexpression may lead to an increase in chaotic vascularization in the tumor microenvironment. In 2002, HIF-3 was introduced by Makino et al. [27]. Although the functions of HIF-3 are not clear yet, Heikkila et al. indicated that HIF-3 might regulate the activity of other HIF complexes [28].

Hypoxic conditions occur heterogenically in almost all types of solid cancers, which lead to HIF protein overexpression. Under physiologic conditions, HIF-1α is constitutively expressed; however, it is degraded rapidly upon its hydroxylation by prolyl hydroxylases (PHDs) [29]. In contrast, the O_2-dependent PHD inhibition develops under hypoxia and HIF-1α protein expression is increased [30]. Under physiological conditions, HIF-1α regulates the expression of important genes that regulate numerous biological processes. In

the tumor microenvironment, elevated HIF-α protein expression, which was induced by hypoxia or other oncogenic signals, promotes tumor growth, angiogenesis, and proliferation through the regulation of critical genes (**Figure 2**). Recent evidence has shown that HIF-1α/-2α can impact tumor development through critical oncoproteins and tumor-suppressor genes such as MYC, p53, and mTOR signaling pathway [31–35]. HIFs may also promote the immune-suppressive mechanisms that promote apoptotic resistance in the tumor microenvironment [36]. Therefore, HIF-1α overexpression due to pathological hypoxia is generally related to poor prognosis and tumor progression in solid cancer [37, 38]. Moreover, HIFs promote the progression of cancer through EMT induction. During the EMT, carcinoma cells undergo migration and invasion, leading to cancer progression and metastasis [39].

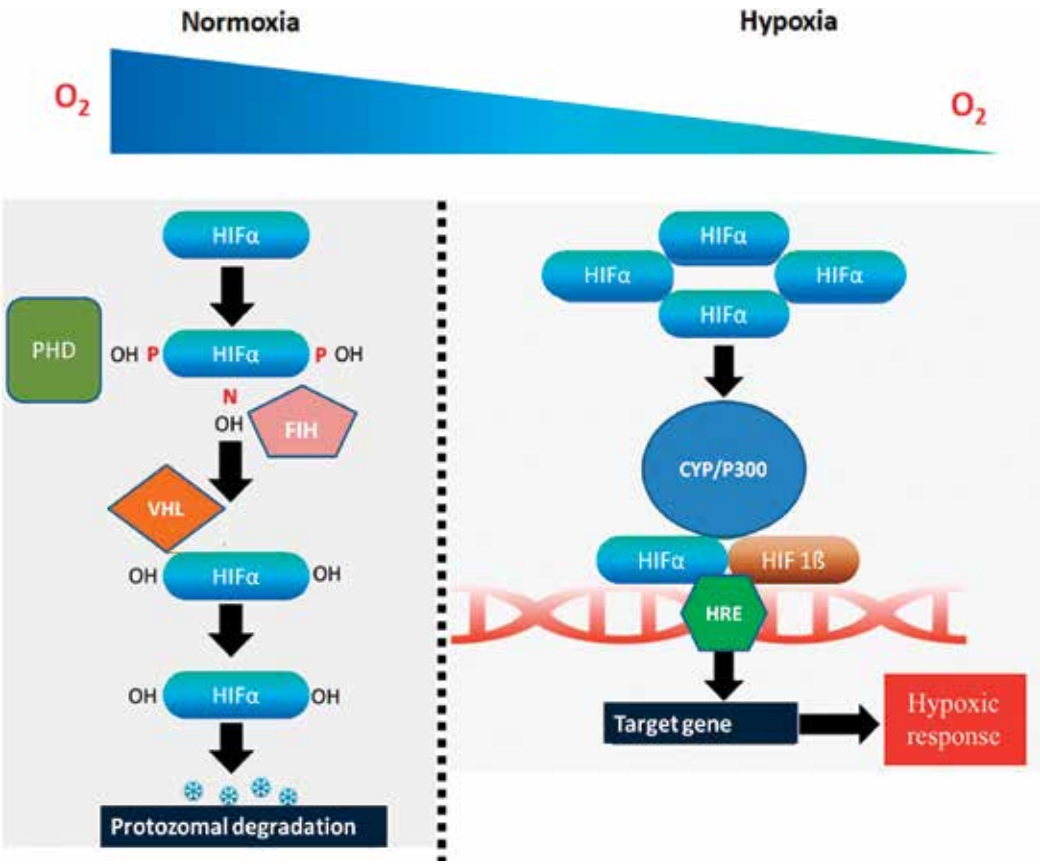

Figure 2. Regulation of HIF in normoxia and hypoxia. During normoxia, PHD enzymes and FIH take role in hydroxylation of HIF-α. Hydroxylation of HIF-α by PHDs creates a binding site for the Von Hippel-Lindau (VHL), HIF-α-VHL interaction leads to proteasomal degradation. Under hypoxic conditions, PHDs and FIH are inhibited due to lack of oxygen. Inhibition of PHDs and FIH lead to HIF-α stabilization and dimerization with its transcriptional partner HIF-1β. HIF-α-HIF-1β interaction leads to translocation to the nucleus and binding to consensus hypoxia-responsive elements (HRE) within the promoters or enhancers of HIF target genes.

3. EMT in cancer: an overview

3.1. Epithelial-mesenchymal transition: definition

Epithelial and mesenchymal cells have various functional characteristics. The epithelium is a thin layer which consists of the collection of cells with similar features that have been associated with one to another by cell-to-cell junctions such as tight junctions, adherens junctions, desmosomes, and gap junctions. Epithelium layer is polarized because apical side and the basal side have different properties that are referred as *apicobasal polarity*. Of note, cell-to-cell junctions consist of cadherins; however, cell-to-basal lamina or ECM junctions consist *laminin*. Moreover, actin is another cell-to-cell adhesion complex which has strong apicobasal polarity. All of these junctions provide immobility to the epithelium. On the other hand, mesenchymal cells do not have these features and only have focal points that adhere to their neighbor mesenchymal cells. Similar to epithelial cells, adhesions between mesenchymal cells can involve cadherin for cell-to-cell junctions and integrins for adhesion to ECM. However, they do not have junctions for basal lamina. In addition, interstitial collagen and fibronectin are important for the ECM adhesion of the mesenchymal cell. They do not have the same ECM molecules associated with the apical-basolateral surface (**Figure 3**).

In 1953, Abercrombie and Heaysman observed that the migration of epithelial cells slows down and they realign when contact each other by forming adhesive junctions [40, 41]. Conversely, mesenchymal cells, particularly fibroblasts, reorient their direction and move away by generating lamellipodia. This process is termed contact inhibition of locomotion. Thereafter, they demonstrated that any defect in contact inhibition of locomotion contributes to the development of invasive and aggressive characters of cancer cells [42–44]. Today, it is known that contact inhibition of locomotion is important in EMT.

EMT is a complex course where epithelial cells are transformed into mesenchymal cells. It is coordinated by several different influential factors that lead to behavioral changes in epithelial cells. Concisely, epithelial cells lose core properties including the apicobasal polarity, cell adhesion, and increase mesenchymal cell properties during the transition [45, 46].

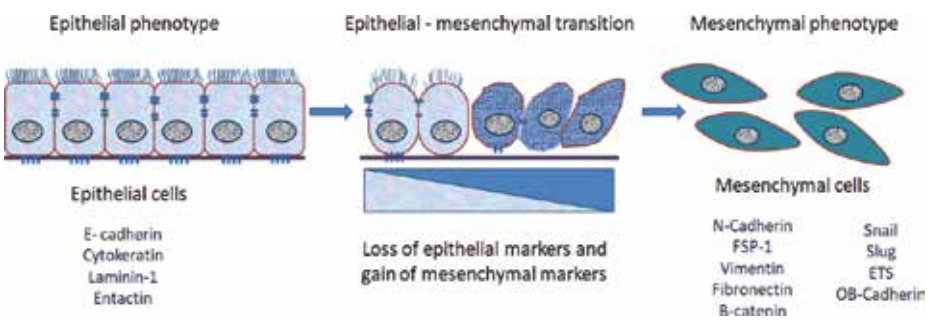

Figure 3. A schema for epithelial-mesenchymal transition. Loss of epithelial markers and gain of mesenchymal markers during the transition from epithelial phenotype to mesenchymal phenotype.

EMT is a naturally occurring transdifferentiation process and is critical during embryonic development and organogenesis. This phenomenon also occurs during wound healing, tissue regeneration, organ fibrosis, and carcinogenesis. In addition, a post-EMT behavior of a part of cells may include reverse transition, which is referred as the *mesenchymal-epithelial transition* [47].

The majority of tumors originate from epithelial tissues of lung, colon, breast, pancreas, prostate, bladder, ovary kidney, liver, and head and neck. Currently, EMT is important in cancer progression and metastasis. Epithelial cells may acquire several abilities such as motility, invasion, and malignant features via EMT [48]. Moreover, it is known that inflammation is the key inducer of EMT in cancer progression. Inflammation may trigger a number of signaling pathways involved in carcinogenesis. However, the specific signals that are induced during the pathologic EMT in epithelial cancers remain unclear [49].

3.2. EMT in physiology and diseases

The mechanisms under the induction and progression of EMT vary dramatically, even though motile cells with mesenchymal phenotype develop consequently. EMT is classified into three different subtypes: type-1, -2, and -3. Type-1 EMT (physiologic EMT) is related to implantation, embryogenesis, and organ development. It is impacted by remodeling and diversification of tissue during morphogenesis. Type-1 EMT is not related with inflammation, fibrosis, and systemic dissemination and generally occurs transiently. Type-2 EMT impacts tissue regeneration and fibrosis, and the process depends on continued inflammation in adults. It continues until the underlying injuries or infections are resolved/repaired. Type-2 EMT may produce mesenchymal cells that are activated. Most notably are the myofibroblasts that produce extreme levels of collagen-rich ECM. Type-3 EMT happens in the context of tumor growth/cancer progression and the tumors transform to a mesenchymal phenotype. The type-3 EMT induction is assisted by genomic changes by cancer cells. It may produce cells that have aggressive properties, which promote movement into the bloodstream in order to spread to other organs.

3.2.1. Type-1 EMT

Type-1 EMT is the exchange from epithelial cells to mesenchymal cells in the embryonic phase events such as implantation, embryogenesis, and organ development. After early embryogenic stages, fertilized egg implantation to the endometrium is associated with an EMT [50, 51]. This is the first step of type-1 EMT that is accompanied by embryonic morphogenesis. At the gastrulation stage, EMT continues with the generation of three germ layers and a primitive streak is made in the epiblast layer [52]. The formation of the primitive streak is the most important part of gastrulation. Primitive streak leads to three germ layers. Thereafter, all tissues are generated during organogenesis by cell migration and differentiation. The EMT coordinates almost every stage of this process [53].

At gastrulation level, the EMT is mainly orchestrated by Wnt signaling [54]. Of note, the TGF-β superfamily, including Nodal and Vg1, and FGF receptors are in close relation to Wnt signaling. Moreover, different signaling modalities through BMPs, c-Myb, and msh homeobox 1 (Msx-1) play roles in the regulation of type-1 EMT [55].

3.2.2. Type-2 EMT

Type-2 EMT is the transition of epithelial cells to mesenchymal cells, which occurs during wound healing and fibrosis due to inflammation. It is orchestrated by fibroblasts and inflammatory cells, which release multiple inflammatory molecules, signals, and ECM components such as collagens, laminins, elastin, tenascin, and other matrix molecules. A variety of studies demonstrated an association between EMT and progressive organ fibrosis such as kidney and lung disease [56, 57].

Inflammatory cells and fibroblasts produce proteins such as FSP1, S100 cytoskeletal proteins, α-SMA, and collagen I that develop during the development of organ fibrosis [57]. These proteins have been used as a biomarker for fibrosis of organs, which are undergoing an EMT associated with chronic inflammation. However, epithelial markers, including cytokeratin and E-cadherin, continue to be expressed until they gain a complete fibroblastic phenotype [58]. Rastaldi et al. evaluated the EMT in human renal biopsies of 133 patients with kidney fibrosis. The EMT was detected in the fibrotic kidney based on the staining for cytokeratin, vimentin, α-SMA, and zona occludens 1 (ZO-1) [59]. Kidney fibrosis has been associated with multiple inflammatory cells that induce EMT with various growth factors such as TGF-β, EGF, and FGF-2 [60]. As the role of TGF-β has been determined in kidney fibrosis, several researchers focused on the inhibition of TGF-β using BMP-7 [61]. Morrissey et al. demonstrated that BMP-7 provided the reversal of EMT and repaired tubular structural damage and repopulation of healthy tubular epithelial cells of mice with kidney fibrosis [62].

3.2.3. Type-3 EMT

Type-3 EMT is the transmission of epithelial cells to mesenchymal cells in cancer progression, also known as the "*oncogenic epithelial-mesenchymal transition*." Due to its complexity, oncogenic EMT is more complex than physiologic EMT. The role of type-3 EMT has been demonstrated in different cancer cells. For example, breast and prostate cancer cells can be classified as epithelial predominated or mesenchymal predominated [63, 64]. Zajchowski et al. studied different molecules to predict invasiveness of breast cancer by using gene array method and showed that epithelial proteins are related to noninvasiveness, whereas mesenchymal proteins are related to invasiveness [65]. Currently, several *in vitro/in vivo* studies demonstrated that mesenchymal status leads to an invasive phenotype, motility, and metastasis in cancers.

In solid tumors, loss of E-cadherin [66], cadherin transformation [67], adhesion loss, changes in apicobasal polarity, and tissue architecture modifications have been demonstrated in EMT. In addition, vimentin, N-cadherin, fibronectin, that are the mesenchymal markers, are highly expressed during the EMT [48]. In carcinogenesis and tumor progression, the loss of E-cadherin and increase in the N-cadherin, which is referred as "*cadherin switch*," are the most significant indicators of the EMT. Currently, it is known that cadherin switch breaks down cell-to-cell junctions and controls the contact inhibition of locomotion. Moreover, it may modulate signal transduction in metastatic cascade [68]. The association between tumor progression and cadherin switch has been demonstrated in prostate cancer, urothelial bladder carcinoma, and malignant melanoma [67, 69, 70].

In literature, there are evidences that support the idea of "*high levels of mesenchymal markers are often related to aggressive tumor behavior and poor prognosis*." In cervix cancers, the correlation between lymph node metastasis and vimentin positivity was also determined [71]. Nevertheless, this correlation has been reported in a small number of cancer types. Therefore, it is hard to mention that vimentin is a definitive predictor of aggressiveness for all cancer types. Ahmad et al. suggested another biomarker for metastatic breast cancer: *stromelysin-3*. Stromelysin-3 is a matrix metalloproteinase and marker for mesenchymal cells. Breast carcinoma cells that undergo EMT are able to express stromelysin-3, which may partly explain the increased metastatic propensity detected in these tumors [72].

Recently, the genetic and biochemical properties that underlie acquirement of cancer cell invasiveness and metastasis are the major areas of intensive research. Xue and colleagues demonstrated that cancer cells departing HER-2/neu expressed a GFP transgene that was facilitated by FSP-1. Moreover, the low rate of metastasis was detected in FSP-1 null mice [73]. This research provided important evidences for the mechanism of metastasis related to EMT. In addition, Yang et al. reported that tumor cells were able to behave like mesenchymal cells and express mesenchymal markers [74]. Besides the evidences about EMT in the metastatic process, some studies have also shown data on reverse EMT. They suggest that the reversibility of EMT is observed during embryonic development and also during the tumor growth at metastatic side. Tumor cells try to undergo not only growth but also cell differentiation to resemble the originating epithelium. Brabletz et al. demonstrated the similarity of epithelial nature between primary tumor side and metastatic tissue for colorectal cancers [75]. It indicates that the induction of an EMT is likely to be central and crucial for the metastatic cascade and implicates EMT during the colonization process.

3.3. Molecular mechanisms and pathways of EMT

In pathologic or physiologic events, the EMT is triggered and controlled by different signaling pathways. Several transcription factors have been described for the regulation of EMT. Tumor growth factor-β signaling appears to be one of the most important pathways. It generally acts as an epithelial cell proliferation suppressor. However, it may also positively affect the tumor progression and metastasis [76, 77]. TGF-β can induce the EMT via two signaling pathways. The first pathway involves Smad proteins that regulate the action of tumor growth factor-β by affecting ALK-5 receptors. Smad proteins mediate signaling pathway effects on motility of cells [61, 78]. Inhibitory Smad can induce autocrine production of TGF-β, thereby, reinforcing epithelial-mesenchymal transition [79]. Recently, β-catenin and LEF found to be relevant with Smad in PDGF-induced EMT [80]. Currently, it is known that TGF-β/Smad/LEF/PDGF axis has important effects on EMT during cancer progression. The second mechanism for TGF-β-induced EMT is MAPK-dependent pathway [81].

Several studies have demonstrated the association between reduced cancer cell E-cadherin levels and activation of EMT [82, 83]. Eger et al. showed that the cFos oncogene induction in mouse mammary epithelial cells induced the EMT by decreasing E-cadherin [84]. The movement of β-catenin from the cytoplasm to nucleus causes acquisition of mesenchymal phenotype by affecting E-cadherin expression. Nuclear buildup of β-catenin has been shown

to reduce E-cadherin expression and acquisition of invasive phenotype [85]. Scarpa et al. described the E-cadherin loss as an activation and contact-dependent cell polarity process via Rac signaling [86]. Currently, it is known that reduced E-cadherin levels are highly correlated with poor prognosis and decrease in survival in various cancers such as hepatocellular carcinoma, nonsmall cell lung, oral, esophageal, gastric, cervix and breast cancer, and bone and soft tissue sarcoma [87–95].

3.4. EMT in cancer metastasis: guilty or innocent?

Cancer metastasis is a complex multistep process with sequential molecular and cellular events that promote the transformation of cells, intravasation, survival and ultimately extravasation, implantation, growth, and colonization in a new and foreign tissue environment. As mentioned above, several evidences support that EMT has a major role in cancer metastasis. The EMT signifies the first step of the metastatic cascade. During EMT, cancer cells are able to invade adjacent cell layers following the loss of cell-to-cell adhesion and acquiring motility. Principally, the result of cellular motility is similar to the extensive cell migration and tissue reorganization that occurs during the embryogenesis and organogenesis; however, subsequent steps have different and complex events.

After a journey in the bloodstream, cancer cells that can escape from the immune system, extravasate from the circulation in order to implant and proliferate at the target organ, "*seed and soil theory*." Thereby, a colony of the primary tumor can regrow by inducing angiogenesis in a foreign and apparently "hostile" background. This process is induced by not only genetic/epigenetic factors but also by the nonneoplastic stromal cells [96]. *In vivo* studies demonstrated that this development is generally supplemented with partial or complete EMT. Therefore, the induction of EMT results in the acquisition of metastatic properties in different carcinoma cell lines. Main indicators for the acquisition of mesenchymal properties are the high level of mesenchyme-specific proteins [46]. In contrast to many studies, Tarin et al. reported that the acquirement of mesenchymal markers during tumor progression reflects genomic instability. Therefore, they advocated that EMT does not occur in carcinogenesis [97]. However, synchronized and complex gene-expression patterns are required to provide tumor cells with the mesenchymal properties. Moreover, genomic instability may have more important role in the regulation of EMT. For instance, SNAI1 regulates expression of EMT-associated genes in colorectal carcinoma [98].

A significant evidence for EMT during the metastatic process was presented by Yang et al. They reported that cancer cells were able to behave like mesenchymal cells and express α-SMA, FSP1, desmin, and vimentin [74]. Studies that include functional manipulations on EMT process also provide evidences. For instance, depletion of FSP1/S100A4-positive cells in tumors suppresses metastasis [73].

In adenomatous polyposis coli (APC) and β-catenin mutation-positive colorectal cancers, β-catenin levels are predominantly observed in tumor cells localized at invasion. Moreover, tumor cells with nuclear β-catenin seem to have undergone EMT [99]. Regardless of numerous studies, the major problem for the demonstration of the role of EMT in the metastatic cascade is the detection of cancer cells that have undergone EMT in primary human tumors.

The markers of EMT indicate epithelial phenotype or mesenchymal phenotype not the EMT in cancer metastasis. Therefore, *in vivo* studies with more sensitive indicators are required for understanding the role of EMT in cancer metastasis.

4. A new insight into the mechanisms of hypoxia-induced EMT

Hypoxia is a common situation in tumor microenvironment affecting cancer cell behavior, including progression and metastasis. Currently, it is clearly known that exposure to hypoxic conditions results in HIF-1α overexpression. As mentioned previously, overexpression of HIF-1a is related with promoting EMT for cancer cells. Additionally, it has been demonstrated that hypoxia-induced EMT includes the loss of cell adhesion and cell polarity. It has been observed that hypoxic conditions decrease the E-cadherin expression, but increase N-cadherin expression, a mesenchymal marker [100].

Azab et al. previously demonstrated that multiple myeloma cancer cells cultured in hypoxic conditions and injected into mice were able to spread to the new bone marrow faster than the cells cultured under normoxic conditions [101]. The hypoxia-induced EMT is mainly driven by stabilization and activation of HIF1α. It is controlled by epigenetic changes that result in a loss of tumor-suppressor functions and gain of oncogene functions (Ras, Raf, Src, mTOR, and Myc). Besides hypoxic conditions, the HIF pathway is also regulated by hypoxia-independent manner [102, 103]. Hypoxia-independent HIF-α stabilization and activation happens in response to cytokines, lipopolysaccharide (LPS), and growth factors in EMT mediated by PI3K/AKT/mTOR,29,30 MAPK,41 and NFκB pathways [104–106].

HIF-1α regulates hundreds of genes, and not only controls malignant and metastatic cancer cells but is also resistant to treatments. Thus, inhibition of hypoxia-induced EMT or HIF-1α may be promising as an anticancer therapy. Currently, there are many researches ongoing in this field. Besides targeting HIF-1α, another strategy is to block metastasis and target genes downstream of HIF-1α. Kaneko et al. have researched the hypoxia-induced EMT in oral cavity squamous cell carcinoma and showed that hypoxia-induced EMT in oral cavity cancer was improved by GSK3-β phosphorylation via PI3 K/Akt signaling [107]. Jiao and Nan showed that hypoxia-induced EMT and chemoresistance were supplemented with HIF-1α expression and Akt activation. Moreover, they demonstrated that PI3K/Akt and HIF-1α inhibition improved the therapeutic efficacy of hypoxic chemotherapy [108]. Lo Dico et al. reported that miR-675-5p promotes glioma growth through HIF-1α stabilization. Subsequently, they examined miR-675-5p specifically in colon cancer metastasis and demonstrated overexpression contributes to tumor progression through HIF-1α-induced EMT [109, 110].

5. Conclusion

Hypoxia is a hallmark of cells in the tumor microenvironment and has a major role in the carcinogenesis and metastasis processes. Hypoxia controls many crucial events such as tumor neovascularization, metabolism, cell survival, and cell death. Furthermore, hypoxia causes

EMT and CSC-like properties including resistance to treatment. Each step of the cancer adaptive process is regulated by HIF, NFκB, PI3K, and MAPK pathways. Understanding the impact of hypoxia and clarifying the hypoxia-induced responses and signaling modalities may pave the way to achieve important steps against cancer via hypoxia/HIF-targeted treatments.

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Chapter 3

Tumour Microenvironment and Metastasis

Ke Wang

Additional information is available at the end of the chapter

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Abstract

In recent years, cancer is more and more severe harm to the health of people in the world. Although tumour diagnosis and therapy have made some progresses, there is little improvement in overall. One of the main reasons is that the pathogenesis of cancer metastasis is still enigmatic. Cancer development and metastasis are a complicated process that depends on the antigenic properties of cancer cells and a favoured environment in organs. Cancer cells metastasis causes more than 90% cancer death in the lungs, liver, brain, and bone, and a primary tumour causes less than 10% death. Therefore, understanding the process of cancer metastasis is essential, and it is convenient to deal with the problem of cancer metastasis and reduce cancer-related thrombosis. It has shown that tumour microenvironment plays a significant role in cancer progression. A variety of carcinoma-associated fibroblasts, and tumour-related macrophages play expanding and critical functions in sustaining cell proliferation, evading growth suppressors, promoting survival, activating invasion and metastasis, and reprogramming energy metabolism, but the purpose of each constituent remains unknown. This chapter will focus on discussing the role of the microenvironment on tumour invasion and metastasis to improve molecular diagnostics and therapeutics.

Keywords: cancer, metastasis, tumorigenesis, migration, invasion, tumour microenvironment, exosomes, autophagy, BMPs

1. Introduction

Cancer cells can be distant metastasis at the late stage, and they can cause damage and injury to the body of the patients. Breast cancer is a common clinical malignancy; it can metastasis to liver, bone, brain, lung and pleural metastasis, as follows. 1. Metastasis to the liver: Experts point out that the rate of breast cancer liver metastasis is 10%, the metastatic pathway has

directly reached the liver through blood and lymphatic channel. There was no damage to the liver in the early stage. The liver function was normal. Liver volume could be enlarged. The condition of the patients deteriorated rapidly and died within a few months in the latter period. 2. Metastasis to bone: Bone metastases account for secondary blood metastasis of breast cancer. Common metastases places are vertebrae, ribs and pelvis. Bone metastases mainly encroach on the red bone marrow; the X-ray examination shows that it leads to irregular bone destruction, similar to the alteration of the insect specimen; some show double changes in osteoclast and osteogenesis. 3. Metastasis to brain: Brain metastases are rare, accounting for 5% of the metastatic cases of breast cancer, which can be divided into two types: meningeal metastasis and brain parenchymal metastasis. Brain metastases can cause brain oedema or brain swelling, the symptoms of increased intracranial pressure appear such as a headache, vomiting, visual impairment, convulsions and even coma. 4. Metastasis to lung and pleural: Lung and pleura are the most common metastatic sites of breast cancer. Lung metastases are often nodular and tend to be distributed in the peripheral lung field. The principal factors affecting the metastasis of breast cancer are the following factors.

2. Carcinoma-associated fibroblasts (CAFs) in the tumour microenvironment

Breast cancer mammary matrix fibroblasts can be regulated by the heat shock protein 1 [heat shock factor 1 (HSF1)] and promote the malignancy of a tumour, it indicates that CAFs may be activated before epithelial transformation [1]. CAFs are linked to the size of primary breast cancer. CAFs are triggered by the paracrine effect of various growth factors, cytokines and hormones, which can promote the proliferation of cancer cells. A large number of studies have reported that growth factors and their downstream signalling pathways produced by CAFs have a role in tumour cell survival, proliferation and cell cycle progression. At the same time, tumour cells can induce CAFs to synthesise growth factors and cytokines, and then form a positive feedback pathway to encourage tumour development. Studies have confirmed that CAFs secreted CXCL12 is linked to breast cancer cell surface homologous receptor CXCR4, promoting breast cancer cell growth [2]. HGF derived from CAFs can be bind to the C Met receptor and activate downstream signal proteins, such as tyrosine kinase, RAS/RAF/ERK, PI3K/AKT, and promote the proliferation of breast cancer cells [3]. In the co-culture of bone marrow stomatal cells and breast cancer cells, CAFs phenotype can be obtained, and the growth and aggregation of tumour cells are promoted by increasing the ratio of RANKL/OPG in breast cancer cells [4]. Besides, CAFs may be a significant source of local oestrogen. Cancer-associated aromatase can be written in CAFs, resulting in increased oestrogen production and tumour cell proliferation [5]. Also, distant metastasis is a significant cause of breast cancer death. Metastasis is an ongoing multistage process, including the invasion and growth of tumour cells, ECM degradation, tumour cells infiltrating into the blood and lymphatic systems, and the formation of metastatic clones in distant organs. CAFs activation can affect the invasiveness of breast cancer cells. Studies have demonstrated that CAFs can promote the invasive phenotype of breast ductal [6].

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3. Tumour-related macrophages (TAMs) in the tumour microenvironment

TAMs promote tumour cell proliferation and survival with expression and secretion of a large number of factors, such as epidermal growth factor, platelet source growth factor, transform growth factor-β1, liver cell growth factor, etc. In vitro, macrophages and tumour cells were co-cultured, the former can significantly promote the growth of the latter by high secretion of the above factors. The tumour cell growth and development were slowed down or even stopped mice knock out their macrophages, which confirmed the role of macrophages in promoting tumour growth. The results of genetic studies in mice showed that the low rate of tumour growth and low metastasis was strictly related to the smaller number of TAMs. The researchers established the CSF-1 spontaneous breast cancer mouse model to find tumour proliferation. Growth rate and lung metastasis rate were lower than those of the wild-type CSF-1 [7].

Three steps can summarise the role of TAMs in tumour invasion and metastasis: The tumour cells adhere to the extracellular matrix components to release protein hydrolase to degrade extracellular matrix and induce invasion migration with chemokines. The cancer cells are free of charge in the primary lesion after proliferation and adhere to the basement membrane. The enormous number of matrix proteases released by them will have a destructive effect on the extracellular matrix and basement membrane, and then invade the lymphatic vessels or blood vessels to cause damage to the tissue and form a metastatic focus. TAMs secrete a large number of proteolytic enzymes for tumour invasion and metastasis. For example, matrix metalloproteinases, include gelatinase-2, collagen enzyme-1, matrix degradation enzyme-3 and more than 20 kinds of proteins; these TMMPs enzymes can degrade the extracellular matrix and degrade the fibrous collagen. The researchers to establish a breast cancer mouse model and find that lung metastasis decreased the loss of systemic macrophages, indicating that tumour metastasis was affected by TAMs and that macrophages existed in the early stages of lesions, invasion and rupture of the basement membrane. And its proteolytic enzyme expression is increased (such as Cathepsin B), indicating that TAMs are also engaged when tumour cells are in normal tissues around them [8]. Studies have demonstrated that together with tumour cells and macrophages, the latter enhances the dynamic properties of the former by using the form of MMP and TNF-α [9]. The tissue structure and basement membrane can be hit by MMP expression, thus promoting tumour cell growth, diffusion and metastasis. MMP in invasive tumours is generally provided by TAMs [10]. TAMs are synergistic with stomatal tumour cells and epithelial cells to promote tumour metastasis [11]. The destruction of the basement membrane is TAMs, and the proteolytic activity of protease B in the local tissue is increased, indicating that TAMs affect the tumour cells to invade the normal tissues around the tissue [12, 13]. The study showed that TAMs in different tumour tissues synthesised a series of urokinase (urokinase, uPA) involved in tumour invasion, invasion, extracellular matrix degradation and tumour angiogenesis [14]. By separating TAMs from breast cancer cells, it was found that TGF–beta 1 could stimulate the transcription of uPA in TAMs and enhance the stability of uPA Mrna. In addition, TAMs secrete cathepsin to promote tumour development by the expression of cathepsin B, cathepsin D and cathepsin L in breast cancer [15].

4. Exosomes in the tumour microenvironment

The 'seed' cells are situated in the primary site of a tumour. They regulate the 'soil' microenvironment of the target organs so that the scattered 'seed' cells can adapt to the new 'soil' and still survive. In the process of the formation of pre-metastatic niche, exosomes play an essential role in raising the chemokine receptor, changing the expression of cell adhesion molecules and creating an immunosuppressive microenvironment. CHEN and others analyse the proteomics in the serum of patients with colon cancer and normal human serum, they found that the expression of 36 exosomes proteins in colon cancer patients was up-regulated [16]. WANG and others found that a tumour could transfer to the liver by the animal model of subcutaneous colon cancer cells HT-29 in nude mice. The exosomes were secreted by HT-29 cells can collect and express chemokine C-X-C primitives on target organs by CXCR4 [chemokine (C-X-C motif) receptor 4, CXCR4] matrix cells to the tumour cells in this process. The organisation's transfer provides favourable conditions [17]. ZHOU and others found that miR-105 was carried in the exosomes secreted by breast cancer cells, which can specifically lead to the down-regulation of the tight connexin ZO-1 expression in endothelial cells and destroy the vascular endothelial barrier, which plays an important role in the early stage of microenvironment formation [18].

Exosomes promote the development of inflammatory response and create a pure metastatic microenvironment conducive to tumour metastasis. COSTA-SILVA and others found that the exosomes derived from pancreatic cancer cells expressed high expression of macrophage migration inhibitory factor [macrophage migration inhibitory factor (MIF)], and after absorption of these exosomes, the liver macrophages (Kupffer cells) secreted a significant amount of TGF beta, and TGF beta promoted the formation of immunosuppressive microenvironment and thus promoted EMT and blood. The TGF beta secreted by liver macrophages activates the hepatic stellate cells, up-regulated the expression of fibronectin, and then raises bone marrow-derived macrophages in the liver and prepares the microenvironment for the arrival of the tumour cells. The exosomes derived from chronic lymphocytic leukaemia, carrying protein molecules and miRNA can promote the transformation of matrix cells into a tumour-related fibroblasts, release inflammatory response factors, and form a microenvironment to tumour growth [19]. The miRNA (miR-21 and miR-29a) carried by the exosomes can combine the Toll-like receptor [Toll-like receptors (TLRs)] of the immune cells, resulting in the nuclear factor-kappa B [nuclear factor kappa B (NF-kappa)]. The release of tumour necrosis factor–alpha (TNF–a) and IL-6, the activation of the inflammatory response factor to encourage the proliferation and metastasis [20, 21]. Exosomes not only provides a suitable growth environment for migrating tumour cells but also mediates tumour-specific organ metastasis. Hoshino et al. research a variety of exosomes secreted by separate metastatic tumour organs [22]. It is found that these exosomes priorities are combined with their respective present points of receptor cells. The exosomes mediate the tumour's organ-specific transfer through the exosomes related and activate the Src phosphorylation pathway of the receptor cells to up-regulation the gene expression of S100 in order to promote the growth of tumour cells [23].

5. Autophagy associated with cancer metastasis

Autophagy inhibits metastasis of tumour cells by inducing anti-inflammatory effects and lysates can cause inflammation in the surrounding tissues, Degenhardt et al. have proved that the microenvironment of an inflammatory tumour may lead to invasion and metastasis of tumour cells [24]. At the initial stage of primary tumour metastasis, signal stimulation is needed to promote migration and invasion. Tumour cells get into the systemic circulation through vascular infiltration [25]. Hypoxia and oxidative stress usually affect solid tumours, which can lead to cell necrosis and inflammatory reaction and inflammatory cells infiltrate. Although some inflammatory cells, such as cytotoxic T cells and natural killer cells can antitumour immune responses and influence metastasis of tumour. Importantly, inflammatory mediators such as macrophages infiltration are often associated with poor clinical prognosis [25–27]. The PyMT (polyoma middle T) transgenic model of breast cancer metastasis has proved that macrophage infiltration in primary tumours is required for invasion and metastasis [28]. Degenhardt and others find that autophagy can indirectly inhibit the inflammatory response on the metastatic promoter site, by raising the survival rate of tumour cells under hypoxia and metabolic stress [29]. Also, autophagy can also regulate the inflammatory response directly by controlling the release of immunoregulatory factors such as the release of high mobility group protein 1 [high mobility group box protein 1 (HMGB1)]. Once HMGB1 is released, it will activate dendritic cells through the Toll-like receptor–4 (Toll-like) of these cells to play a role in inducing cells to produce potent antitumour immune responses that kill tumour cells and prevent their metastasis [30]. More interestingly, high levels of autophagy could be induced during cell death in malignant glioma cells treated with mycin, resulting in a substantial release of HMBG1 from the dead cells [30, 31].

6. Hypoxia-regulated genes implicated in cancer metastasis

The effect of hypoxia on tumour immunity is another essential influence factor. Under the condition of hypoxia, tumour cells can secrete a variety of immunosuppressive factors, transforming growth factor beta (transforming growth factor-beta) is one of the most critical factors. TGF-beta can make tumour cells acquire immune escape function through the following ways: (1) Inhibit the proliferation of cytotoxic T cells and the expression of cytotoxin genes, which makes T cells unable to play an antitumour effect and induces the production of CD4 + CD25+ regulatory T cells with immunosuppressive function. (2) Inhibit the expression of antigen-presenting molecules, conciliatory factors and chemokine receptors to prevent the dendritic cells from functioning normally and make dendritic cells unable to deliver tumour antigens to T cells [32–35]. (3) Inhibit the activation of natural killer (NK) cells and reduces the expression of multiple surface receptors in NK cells so that they cannot identify and dissolve tumour cells [36]. In addition, hypoxia can also directly activate myeloid-derived suppressor cells (MDSCs), dendritic cells, and programmed cell death receptor ligand 1 [programmed cell death-ligand 1 (PD-L1)] through HIF-1 alpha, which can reduce the expression of MDSCs secreting interleukin -6 and interleukin -10 p to promote activation in T cells [37–39].

7. BMPs effect on tumour microenvironment, migration and invasion

BMPs belong to the transforming growth factor-β (TGF-β) superfamily and were initially identified as obstetrician cytokines that can promote bone and cartilage formation in vivo. Recently, BMPs have turned out to be involved in the regulation of tumorigenesis, development and bone metastases, they have been shown to be involved in the regulation of tumour development and bone metastasis. Clement et al. find BMP2 can enhance the migration and invasion of breast cancer cells, those cells with high expression of BMP2 showed more cell migration than GFP and blank control group. Katsuno et al. find that BMP2 promotes the invasion and migration of MDA-MB-231 through BMPs/SMAD pathway, and the two BMPs receptors play an equally important role. Lack of anyone receptor affects the signalling process [40]. BMP2 can promote oestrogen receptor positive MCF7 to invade migration in vivo and in vitro [41]. Scherberich et al. research show that BMP2 enhances the tumour invasion by regulating the expression of skeleton protein M in the tumour microenvironment. BMP2 induces the expression of skeleton protein M by p38 MAPK and JNK signalling pathway. BMP2 can also promote the invasion and migration of breast cancer by up-regulation the ID1 expression [42]. BMP-4 increases the invasion and migration of breast cancer cell by CCN6, which has been shown directly antagonise the BMP-4 mediated invasiveness and metastases in vitro and in vivo to. Fibroblasts stimulated with BMP-4 enhanced the MCF-7 cell invasion, and these effects were inhibited by DMH1. BMP-4 increased the expression of MMP-3 and IL-6 in conditioned medium from treated mammary fibroblasts, suggesting BMP-4 can impact the tumour microenvironment to promote breast cancer invasion [43]. The latest research has found that BMP6 can inhibit the growth and migration of breast cancer cells. Takahashi finds that BMP6 and estradiol co-work can inhibit the proliferation of MCF-7 cells through p38 MAPK cell signalling, however, it will not play a role only BMP6 exists [24]. Yang et al. also find that BMP6 down-regulates the expression of miR-192 to inhibit the transcription of ZEB1, and the decrease of miR-21 expression to impede the migration ability of MDA-MB-231 and BMP6 could also reduce the proliferation ability of MDA-MB-231 cells [25]. Zeisberg et al. find that TGF-beta can reduce the expression of E-adhering to renal epithelial cells, but BMP7 can increase the expression of E-adhering. Buijs et al. Point out that BMP7 can induce the activity of E-adhering to breast cancer cells and reduce invasiveness. Therefore, BMP7 can induce the expression of E-adhering in normal epithelial cells and maintain the stability of epithelial cells, loss of BMP7 gene will decrease of the expression of E- adhering to lead the epithelial cells to the stomatal cells in the evolution of the tumour [44]. Alamo finds that BMP7 stimulates the growth of two breast cancer cell lines and inhibits the proliferation of four breast cancer cell lines. Exogenous BMP7 can significantly enhance the migration of MDA-MB-231 in vitro. The causes of these two differences are not yet clear, Ye Lin has found that the expression of BMP10 in breast cancer is reduced. It can inhibit the invasion and migration of MDA-MB-231 through the BMPs/SMAD pathway and suggests that BMP10 can serve as a target for molecular therapy of breast cancer [45].

8. BMPs and bone metastasis

There are comparatively few studies on the role of BMPs in bone metastasis of breast cancer. Recently, it has been noted that BMPs is involved in the process of breast cancer bone

metastasis. BMP has been involved in the development of bone metastases in up-regulating or down-regulating the corresponding regulatory factors in breast cancer cells. BMPs can increase the expression of Osteoblast bone saliva protein BSP. BSP is related to the formation of new bone, so it can connect to the process of breast cancer with bone metastasis. Runx2 similar to BSP is that the target gene of BMP, which is closely linked to the osteolytic metastasis of breast cancer [46].

The overexpression of BMP7 or exogenous BMP7 can significantly reduce the formation of bone metastasis by reducing the expression of romantic, increasing the expression of E-cadherin and reversing the EMT in the animal model of mouse breast cancer bone metastases. In contrast, additional studies found BMPs could increase the invasion of a tumour and the ability of bone metastases and the active SMAD1/5/8 was detected in primary and metastatic tumours [47].

In conclusion, BMPs involves the growth and invasion of breast cancer. Different types of BMPs have different roles in the same breast cancer cell line, even if the same kind of BMPs has different effects on various breast cancer cell lines. It is believed that this mechanism will be clarified with the research. BMP9 can inhibit the growth of breast cancer cells in vitro and in vivo, BMP-9 is also involved in the inhibition of tumour growth in bone by down-regulation of connective tissue growth factor(CTGF).

9. Conclusions

It has shown that tumour microenvironment plays an essential role in cancer progression. The more recent studies have demonstrated hypoxic and autophagy in both primary tumours and metastases, contributing to angiogenesis, invasion, BMPs can inhibit or promote the growth of breast cancer by different signalling pathway. It detects a promising therapeutic value for BMPs in the management of metastases by influencing the propensity to disseminate to and survive in the bone microenvironment.

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Chapter 4

The Landscape of Histone Modification in Cancer Metastasis

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Additional information is available at the end of the chapter

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Abstract

Metastasis represents one of the most devastating aspects of cancer. Epithelial to mesenchymal transition (EMT) has been shown to play a critical role in tumorigenic metastasis. During metastatic progression, both genetic and epigenetic modifications endow cancer cells with properties that modulate the capacity for metastatic success. Histone modification is profoundly altered in cancer cells and contributes to cancer metastasis by controlling different metastatic phenotypes. Here, we first review histone modifications and discuss their roles in EMT and metastasis, with a particular focus on histone methylation and acetylation. Second, we review the major histone modification enzymes that control chromatin in cancer metastasis. Third, we discuss the transcriptional regulation concerted by these enzymes with EMT transcription factors at different molecular layers. Finally, we discuss pharmacologic manipulation of histone modification enzymes for metastasis treatment. A comprehensive understanding of histone modification in metastasis will not only provide new insights into our knowledge of cancer progression and metastasis, but also offer a novel approach for the development of innovative therapeutic strategies.

Keywords: EMT, epigenetic, histone modification, metastasis, inhibitor

1. Introduction

Approximately 90% of cancer deaths are caused by metastasis [1]. Cancer metastasis is an exceedingly complex process involving tumor cell motility, intravasation, and circulation in the blood or lymph system, extravasation, and growth in new tissues and organs [2, 3]. During invasion, tumor cells lose cell–cell adhesion, gain mobility and leave the site of the primary tumor to invade adjacent tissues. In intravasation, tumor cells penetrate through the endothelial barrier and enter the systemic circulation through blood and lymphatic vessels. In

extravasation, cells that survive the anchorage-independent growth conditions in the bloodstream attach to vessels at distant sites and leave the bloodstream. Finally, in metastatic colonization, tumor cells form macrometastases in the new host environment [2, 3]. All of these steps, from initial breakdown of tissue structure, through increased invasiveness, and ultimately distribution and colonization throughout the body, are developmental characteristics of the processes, epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET). EMT is a distinctive morphogenic process that occurs during embryonic development, chronic degeneration and fibrosis of organs, and tumor invasion and metastasis [4–6]. The similarity of genetic controls and biochemical mechanisms that underlie the acquisition of an invasive phenotype and the subsequent systemic spread of cancer cells highlights the concept that tumor cells usurp this developmental pathway for metastatic dissemination. In total, EMT provides tumor cells with the proclivity for early metastasis, renders them resistance to therapeutics and endows cells with cancer stem cell (CSC)-like traits [6].

The hallmark of EMT is the loss of E-cadherin expression, an important caretaker of the epithelial phenotype. Loss of E-cadherin expression is often correlated with the tumor grade and stage because it results in the disruption of the cell–cell adhesion and an increase in the nuclear β-catenin. Several transcription factors have been implicated in the regulation of EMT, including the zinc finger proteins of the SNAIL family (SNAIL1/2/3), the basic helix–loop–helix (HLH) factor TWIST (TWIST1/2, E12/E47), and two double zinc finger and homeodomain ZEB family (ZEB1/ZEB2). These factors act as a molecular switch for the EMT program by repressing a subset of common genes that encode cadherins, claudins, integrins, mucins, plakophilin, occludin and ZO1, and thereby induce EMT.

EMT is a dynamic process that preserves plasticity [6]. In this instance, the reprogramming of gene expression provides a rapid and dynamic regulatory mechanism to switch between the epithelial and mesenchymal conditions during cancer progression. Consistent with this, these EMT-activating transcriptional factors (EMT-TFs) are liable proteins that turn over rapidly and do not have long residence times at their binding sites. Interestingly, disseminating cells orchestrate a metastatic cascade without a concomitant need for genomic mutations, which indicates that this dissemination is epigenetically templated. Both EMT and epigenetic modification (DNA methylation and histone modifications) are dynamic and efficient processes during development, differentiation and carcinogenesis. These studies indicate that the epigenetic mechanism plays an important role in modulating the induction of EMT and tumor metastasis.

2. Epigenetics and histone modification

2.1. Epigenetic and chromatin structure

The term “epigenetics” was first coined by Conrad H. Waddington in his Principles of Embryology textbook in 1942 to designate a process in which gene regulation modulated development. The final definition of epigenetics was confirmed in the Epigenetic Meeting held by the Banbury Conference Center and Cold Spring Harbor Laboratory in 2008 as “a stably heritable phenotype resulting from changes in a chromosome without alterations in

the DNA sequence." In general, epigenetic regulation includes changes that impact histone modification, DNA methylation, histone variants, chromatin looping, noncoding RNAs and nucleosomal occupancy and remodeling.

Genomic DNA is tightly packaged in chromatin by both histone and nonhistone proteins in the nucleus of eukaryotic cells. The basic chromatin subunits, nucleosomes, are formed by wrapping 146 base pairs (bp) of DNA around an octamer of four core histones: H2A, H2B, H3, and H4. Whereas the nucleosomal core is compact, eight flexible lysine-rich histone tails protrude from the nucleosome that modulate internucleosomal contacts and provide binding sites for nonhistone proteins. From the perspective of gene transcription, chromatin structure can be divided into two distinct categories: euchromatin and heterochromatin. "Euchromatin" is an open chromatin structure that affords accessibility of transcription factors to DNA, resulting in gene activation. In contrast, "heterochromatin" is a closed chromatin structure with a low interaction between transcription factors and the genome, leading to gene repression.

2.2. Histone modifications and histone code hypothesis

The histone code hypothesis was first proposed by Strahl and Allis in 2000. They suggested that "multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions" [7]. The histone "language," based on this "histone code," is encoded in these modifications and read by chromatin-associated proteins. So far, several histone post-translational modifications (PTMs) have been identified, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, proline isomerization, biotinylation, citrullination and their various combinations [8]. These modifications constitute a unique "code" to regulate histone interactions with other proteins and thereby allow modification (either overcoming or solidifying) of the intrinsic histone barrier to transcription. Accordingly, with these modifications, the various proteins that add, recognize and remove these PTMs, termed writers, readers and erasers, respectively, have been identified and structurally characterized. While "writer" and "eraser" enzymes modify histones by catalyzing the addition and removal of histone PTMs, respectively; "reader" proteins recognize these modified histones and 'translate' the PTMs by executing distinct cellular programs. In addition, numerous core histone chaperones also facilitate core histone deposition or removal from chromatin. Histone modifications control dynamic transitions between transcriptionally active or silent chromatin states, and regulate the transcription of genetic information encoded in DNA (the "genetic code") [9]. Analyses of genome-wide profiles of histone modifications and gene expression identified three distinct types of configurations: repressed, active and bivalent. First, the closed chromatin configuration is linked with suppression of gene transcription, the repressed state. Second, an open chromatin configuration is associated with active gene transcription, the active state. Third, bivalent chromatin consists of domains that have both repressive and active histone markers, predominately on developmental genes, which allows phenotypic plasticity before committing to a specific cell fate.

During EMT, histone modifications provide a regulatory platform to orchestrate the repression or activation between epithelial and mesenchymal genes. Here, we only focus on the well-studied histone acetylation and methylation, and discuss their diverse regulation and role in transcriptional reprogramming of tumor metastasis (**Table 1**).

Modification				Writer	Eraser
Acetylation	H3K9, H3K27			P300, PCAF, MOF, Tip60	HDAC
Methylation	Lysine	Activating	H3K4	MLL1–4, Set 1a, Set 1b, Ash1L, Set7/9, and SMYD	KDM1, KDM2, KDM5, JARID2 and NO66
			H3K36	KMT3, NSD2, NSD3 and SMYD	KDM2, KDM4/JMJD and NO66
			H3K79	KMT4	PHF8
		Repressing	H3K9	KMT1	KDM1, KDM3, KDM4, PHF8, and JHDM1D
			H3K20	KMT5, KMT7 and SET8	Unknown
			H3K27	PRC2	KDM6, UTY and JHDM1D
	Arginine	H4R3me2a, H3R8, H3R2me2a,H4R3me2s		PRMT1, PRMT2, PRMT3, CARM1/PRMT4, PRMT5, PRMT6, PRMT7, PRMT8, and PRMT9	Unknown

Table 1. Histone modifying enzymes involved in metastasis.

3. Histone acetylation

Evidence has established that histone acetylation is associated with gene activation. A genome-wide study demonstrated that all forms of histone acetylation are positively correlated with gene expression [10]. Histones contain amino acids with basic side chains that are positively charged and attracted to the negatively charged genomic DNA. Ultimately, histone acetylation reduces the positive charge on histones and decreases the interaction between nucleosomes and DNA. Generally, histone acetylation is greater in the promoters of active genes and influences both the initiation and elongation of gene transcription. Histone acetylation also stabilizes the binding of chromatin remodeling factors at promoter regions and induces nucleosomes unfolding as well as reduces nucleosome occupancy. The acetylation state of a chromatin leads to the structural modification of the nucleosome. Acetylated (or hyperacetylated) chromatin is in a relaxed confirmation and associated with active transcription. In contrast, deacetylated (or hypoacetylated) chromatin is condensed and supercoiled, and is associated with transcriptional silencing (and, in the context of cancer, the inhibition of tumor suppressor genes).

Histone acetylation is a rapid and reversible process controlled by histone acetyltransferases (HATs) and histone deacetylases (HDAC)s. The HATs transfer acetyl groups from acetyl-coenzyme A (CoA) to the ε-amino groups of lysine residues in histone tails, which results in gene activation. HATs contain a bromodomain that recognizes and binds to acetylated histones, categorized into three major families, GNAT (GCN5 and PCAF), MYST (Tip60 and MOF), and CBP/p300. The HDACs remove acetyl groups from lysine residues, leading to gene silencing. Sequence homology, subcellular location, and the features of the catalytic site have been used to classify the 18 members of the human HDAC family into 4 groups: class

I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9, and 10), class III (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7), and class IV (HDAC11) [11]. Class I HDACs have sequence homology to class II HDACs and class IV HDACs but not class III HDACs. Class I, II, and IV HDACs are zinc-dependent, whereas class III HDACs are nicotinamide adenine dinucleotide $(NAD)^+$-dependent. Genome-wide mapping of the binding of HATs and HDACs to the human genome demonstrate that these enzymes regulate the activation and repression of transcription, respectively. The dysfunctional balance between acetylation and deacetylation is clearly associated with human disease and tumorigenesis.

p300 cooperates in an epigenetic manner with a DOT1L-c-Myc complex to induce EMT in breast metastasis [12]. The elevated level of p300-DOT1L-c-Myc is associated with the acquisition of CSC-like properties during breast carcinogenesis, which implies that p300 functions as a potential oncogene to influence the clinical outcome of breast cancer. In addition, transforming growth factor-beta (TGF-β) and WNT co-operated to mediate EMT. TGF-β induces the translocation of β-catenin to the nucleus where it binds to T- cell Factor (TCF); this complex recruits p300/CBP to assemble a transcriptional complex on target gene promoters that promotes EMT signaling. Intriguingly, over-expression of SNAIL/SLUG up-regulates TGF-β-receptor 2 (TGFBR2) expression with an increase of H3K9 acetylation on TGFBR2 promoter to increase TGF-β signaling [13]. In contrast, however, p300 was reportedly recruited by the hepatocyte nuclear factor (HNF) 3 to the E-cadherin promoter, increasing expression, and thus reducing the metastatic potential of breast cancer cells [14]. Similarly, the p300-CBP-associated factor (PCAF) has functions that can differ among cancer types. PCAF is an anti-oncogene and its expression is down-regulated and negatively correlated with tumor metastasis in hepatocellular carcinoma (HCC) [15]. This complex plays an important role in suppressing EMT and HCC metastasis and by targeting Gli1 [16]. However, it was also reported that PCAF acetylates the enhancer of zeste homolog 2 (EZH2) at K348 to augment EZH2 stability, and thus promotes lung cancer cell migration and invasion [17]. These reports indicate that the role of PCAF is context-dependent. In several breast cancer cell lines, hMOF catalyzes promoter H4K16 acetylation, which is critical to maintain expression of EMT-related tumor suppressor genes [18]. Consistent with this, MOF also acetylates the histone demethylase lysine-specific histone demethylase 1 (LSD1), to suppress EMT, indicating that MOF is a critical suppressor of EMT and tumor progression [19]. Recently, we found that Tip60 appears to be an important regulator of TWIST activity by acetylating at H3K73 and H3K76 of the GK-X-GK motif, resulting in an interaction between BRD4 and TWIST, hence promoting the aggressiveness of basal-like breast cancer (BLBC) [20].

Dysfunctional class I HDAC expression and activity is associated with cancer metastasis. HDAC1 regulates invasiveness by increasing matrix metalloproteinase (MMP) expression. Furthermore, HIF-2α is a transcriptional regulator of the *HDAC1* gene, and hypoxia increase HIF-2α and HDAC1 expression [21]. TGF-β-driven E-cadherin silencing and EMT in human pancreatic cancer cells also depend on HDAC activity [22]. HDAC1 and HDAC2 form a transcriptional repressor complex with SNAIL to downregulate the E-cadherin expression of pancreatic cancer cells during metastasis. Intriguingly, SNAIL also recruits the HDAC1/2-containing SIN3A complex to deacetylate histones on the E-cadherin promoter for gene

silencing [23]. In a similar manner, SLUG recruits the HDAC1-containing CtBP complex to silence genes by binding to the E-box on the BRCA2 promoter [24]. ZEB1/2 also recruits the CtBP/HDAC1 complex to the E-cadherin promoter, while ZEB1 recruits SIRT1, a class III HDAC, to silence E-cadherin and promote EMT and metastasis in prostate cancer cells [25, 26]. Moreover, ZEB1-induced EMT is accomplished by repression of other epithelial genes, including EPCAM, ESRP1 and RAB25, and is accomplished by a reduced acetylation of H3K9 and H3K27 at their promoters. In fact, evidence suggests that H3K27 deacetylation is a key epigenetic event in ZEB1-induced transcriptional reprogramming [27]. In addition to the EMT transcriptional factors, HDAC1 co-purifies with TCF12 and promotes migration and invasion; elevated expression of TCF12 and HDAC1 correlate with a poor prognosis in gallbladder cancer. These findings suggest that this HLH transcription factor TCF12 could also target HDACs for epithelial gene silencing during EMT [28]. The expression of the HDAC1 and HDAC3 correlates with nuclear receptor (NR) (i.e., ER and PR) status. HDAC assembles a complex with ERα that binds to the SLUG promoter to repress expression [29]. Interestingly, HDAC6 and SIRT1 counteract the p300-catalyzed acetylation on Cortactin, which enhances its F-actin binding ability to facilitate EMT and tumor progression [30]. Conversely, HDAC6 and ERα are co-localized in the cytoplasm of renal cell carcinoma (RCC) cells and HDAC6 enhances cell motility by decreasing acetylated α-tubulin expression [21]. Loss of α-Tubulin acetylation by HDAC6 is also associated with TGF-β-induced EMT [31].

Recently, the clinical relevance of HDACs and the therapeutic potential of HDAC inhibitors (HDACi) have been reported. HDACi can generally be classified into hydroximates, cyclic peptides, aliphatic acids, and benzamides [32], and grouped according to their specificity. Thus far three HDACi: vorinostat (SAHA), romidepsin (Istodax) and PTCL (Belinostat or Beleodaq) are approved by the FDA for some T-cell lymphomas [33]. However, these molecules have not produced favorable and expected outcomes in solid tumors. Currently, a number of small molecules HDACi were investigated in clinical trials with variety of solid neoplasms, including breast cancer, either alone or in combination with hormonal treatments. Entinostat (MS-275), a benzamide with high specificity for the class I HDACs, is currently in a phase II/III trial for advanced ER^+ breast cancer [34, 35]. Vorinostat exerts EMT reversal effects by restoring the expression of E-cadherin. An expanded screen on 41 HDACi further identified 28 HDACi compounds, such as the class I-specific inhibitors Mocetinosat, Entinostat and CI994, that restore E-cadherin and ErbB3 expressions in ovarian, pancreatic and bladder carcinoma cells [36]. Mocetinostat, but not other HDACi, specifically interferes with ZEB1 function, restores miR-203 expression, represses stemness properties, and induces sensitivity against chemotherapy by restoring histone acetylation on the E-cadherin promoter [37]. Given that persistent genes activation may require targeting of multiple epigenetic silencing machineries, a combination of HDACi with anticancer drugs and/or radiotherapy demonstrate synergistic or additive effects in clinical trials. For example, HDACi have been utilized in combination with 5 Aza-dC as a synergistic strategy [38]. However, recent reports also found that HDACi could promote EMT in prostate and nasopharyngeal cancer cells [39, 40], indicating the application of HDACi in anti-cancer therapy is cancer-context dependent and may limit application.

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4. Histone methylation

Histone methylation occurs at specific lysine or arginine residues on the histone tails. This modification is associated with either transcriptional activation or repression. Histone methylation does not change the electrostatic charge of histones or affect the chromatin structure. The functional effects of histone methylation are affected by both the position of the modified residues and number of methyl groups. Histone methyltransferases (HMTs) transfer methyl groups from S-adenosylmethionine (SAM) to either lysine or arginine residues, whereas histone demethylases (HDMs) remove methyl groups. The HMTs and HDMs specifically catalyze particular lysine or arginine residues.

4.1. Lysine methylation

Methylation of lysine residues on histones was first identified in the 1960s. Histone lysines can have four states of methylation at different lysine sites. Histones H2B lysine 5 (H2BK5), H3K4, H3K9, H4K20, H3K27, H3K36, and H3K79 are subject to unmethylated, mono-methylation (me1), di-methylation (me2), or tri-methylation (me3) on the ε-amino groups of lysine residues. These lysine methylations change the chromatin structure and regulate gene transcription. Histone lysine methylation is a reversible modification and is maintained by the balance lysine methyltransferases (KMTs) and lysine demethylases (KDMs). The KMTs recruit SAM as a cofactor and catalyze the addition of methyl groups to lysine residues through the SET domain. The KMTs are grouped into the SET domain-containing enzyme families (KMT1–3 and KMT5–7), the KMT4/DOT1 family, and others. The KDMs include the flavin adenine dinucleotide- (FAD-) dependent monoamine oxidase family (KDM1/LSD), the Jumonji C domain-containing demethylase (JMJD) families (KDM2–6), and others. Methylation of H3K4, H3K36, and H3K79 usually correlate with gene activation, whereas methylation of H3K9, H3K20, H3K27, and H3K56 are associated with transcriptional silencing.

4.1.1. Transcriptional activation and lysine methylation

H3K4: H3K4 methylation (H3K4me) is present in euchromatic regions and is usually associated with transcriptional activation. H3K4me3 occurs principally at the 5′ end of actively transcribed genes, near the transcription start site (TSS). H3K4me2 is located throughout genes, but frequently found towards the middle of the coding region of transcribed genes, and H3K4me1 is more abundant at the 3′ ends [41]. H3K4me2 marks can be present at both active and inactive euchromatic genes, whereas H3K4me3 is present exclusively at active genes. H3K4me favors transcriptional activation by facilitating H3 acetylation and the recruitment of RNA polymerase II, but it also antagonizes gene repression by preventing the binding of nucleosome remodeling and deacetylase co-repressor complexes, such as NuRD, and interfering with substrate recognition by the variegation 3–9 (SUV39H) methyltransferases [42]. The balance between KMTs and KDMs is an important dynamics for H3K4me and the regulation of gene transcription. More than ten H3K4 KMTs have been identified, including the mixed-lineage leukemia (MLL)1–4 proteins, along with Set 1a and Set 1b, Ash1L, Set7/9, and also the

SET and MYND domain-containing enzymes (SMYD) family members (SMYD1 and SMYD3). SMYD are involved in many cellular processes, including tumorigenesis and invasiveness. For example, SMYD3 is a novel histone H3K4-specific N-lysine di- and tri-methyltransferase, and highly characteristic active transcription. SMYD3 exerts its effects on initiation, invasion and metastasis of diverse tumors (e.g., esophageal squamous cell carcinoma (ESCC), gastric cancer, HCC, cholangiocarcinoma, breast cancer, prostate cancer, and leukemia). SMYD3 stimulates EZR and LOXL2 transcription to enhance proliferation, migration and invasion by directly binding to sequences of the promoter regions of these target genes [43]. SMYD3 is also capable of increasing cell migration through MMP-9 expression [44]. The MLL proteins (Trithorax homologs in Drosophila) are important for the regulation of developmental genes such as the Hox cluster, and deficiency of MLL1 or MLL2 causes embryonic lethality [45]. MLL1 coordinates with HIF1α and regulates hypoxia-induced HOTAIR expression to facilitate tumorigenesis [46]. In addition, MLL1 interacts with β-catenin to promote cervical carcinoma cell tumorigenesis and metastasis [47]. KMT2B/MLL2 is highly expressed in ESCC and promotes tumor progression by inducing EMT [48]. Another member of MLL family, MLL3 is reportedly mutated in multiple cancers. MLL3 regulates many migration-related genes and downregulation of MLL3 has a profound impact on the progression of ESCC [49]. Furthermore, Kim et al. [50] showed that KMT2D/MLL4 expression is associated with poor survival in breast cancer and regulates tumor proliferation and invasiveness.

Histone lysine methylation is a reversible process. H3K4 is demethylated by the KDM1 family (LSD1 and LSD2), the KDM2 family (FBXL10 and FBXL11), and the KDM5 family (JARID1A, JARID1B, JARID1C, and JARID1D) as well as JARID2 and NO66. The LSD subgroup of KDMs specifically targets the mono- and dimethylated lysines. This group demethylates substrates through a flavin adenine dinucleotide-dependent oxidative reaction, producing lysine and formaldehyde. KDM1A/LSD1 was the first H3K4 lysine-specific demethylase to be identified. We and others demonstrated that SNAIL recruits LSD1 to epithelial gene promoters with demethylation of H3K4me2 and subsequent silencing of target genes to enhance tumor metastasis [51]. SLUG also interacts with LSD1 to facilitate tumor metastasis [52]. In addition, both SNAIL and SLUG recruit LSD1 and bind to a series of E-boxes located within the BRCA1 promoter to repress BRCA1 expression. LSD1 overexpression promoted metastasis whereas knockdown of LSD1 inhibited tumor spread, suggesting that LSD1 is a key regulator of ESCC metastasis [53]. LSD1 and LSD2 act differently in the regulation of gene transcription and chromatin remodeling. However, both of KDM1A and KDM1B are overexpressed in invasive breast carcinoma, and depletion results in high levels of H3K4me1–2. The KDM5/JARID1 family is frequently found in the promoter region of transcriptionally active genes, and results in repressed expression of the target genes. KDM5A is highly expressed in ovarian cancer tissues and facilitates EMT and metastasis [54]. KDM5A promotes an increase in TNC expression, which augments breast cancer cell invasion and metastasis [55]. Reports indicate that, in gastric cancer cell, KDM5A is induced by TGF-β1 and recruited by p-SMAD3 to silence the *E-cadherin* promoter and promote tumor progression [56]. KDM5B plays a role in cell differentiation, stem cell self-renewal and other developmental progresses. Recent studies showed that KDM5B expression was increased in breast, bladder, lung, prostate and many other tumors and promote tumor initiation, invasion and metastasis. Mechanistically, KDM5B exerts its function through modulation of H3K4me3 at the PTEN gene promoter [57].

KDM5C/JARID1C is overexpressed in breast cancer, and its expression is significantly associated with metastasis. This demethylase modulates the status of H3K4 methylation in the breast cancer metastasis suppressor-1 (BRMS1) promoter, and thereby controls the expression of BRMS1 to inhibit tumor progression. Accordingly, the expression of KDM5C and BRMS1 are inversely correlated in human breast cancer [58].

H3K36: Because the level of H3K36me3 is high at the promoter site in active genes, H3K36me3 is involved in active transcription. In contrast, the H3K36me1 signal has a low association with active promoters. H3K36 is methylated by the KMT3 family (SETD2 and NSD1) as well as by NSD2, NSD3, SMYD1, SMYD2, SMYD3, SMYD4, and SMYD5. SETD2 plays a tumor suppressor role in tumor metastasis. Interestingly, SETD2 is frequently either deleted or mutated [59]. In contrast, H3K36 is demethylated by the KDM2 family (FBXL10 and FBXL11), the KDM4/JMJD family (JMJD2A, JMJD2B, and JMJD2C), and NO66. KDM2A expression is increased in breast cancer and associated with poor clinical outcomes [60]. KDM2A promotes lung tumorigenesis by epigenetically enhancing ERK1/2 signaling through demethylation of H3K36 [61]. In addition, KDM8/JMJD5 also demethylates H3K36me2, and overexpression of JMJD5 promotes cell invasion and is significantly correlated with clinical stage, histological grade and lymph node metastasis [62].

H3K79: H3K79me3 is associated with active transcription in yeast, whereas it is localized at both active and silent promoters in humans. H3K79me1 and H3K79me2 do not have any association with either active or silent promoters. H3K79 is methylated by the KMT4 family (DOT1L) and demethylated by PHF8 [63]. Methylation of H3K79 has been implicated in cell cycle regulation and the DNA damage response [63]. Disruption of this methylation can lead to cancers, making DOT1L a potential therapeutic target for cancers such as leukemia [64]. More recently, DOT1L has been implicated in the stimulation of proliferation, self-renewal, and metastatic potential of breast cancer cells [65]. DOTL1 cooperates with c-Myc-p300 complex to epigenetically activate EMT regulators in breast cancer progression. Clinically, DOTL1 expression is associated with poorer survival and aggressiveness of breast cancer [12]. PHF8 is highly expressed in metastatic prostate tissues and plays an important role in controlling invasion and metastasis [66]. PHF8 also interacts with β-catenin, and binds to the promoter region of vimentin, leading to the promotion of gastric cancer progression and metastasis [67].

4.1.2. Transcriptional repression and lysine methylation

H3K9: The methylation of H3K9 (H3K9me) was the first mechanism of gene repression to be linked to KMT. Studies in *Drosophila* showed that the gene *Su(var)39*, later shown to encode a H3K9 HMT, had an important role in the regulation of position-effect variegation [68] and similar enzymes were subsequently discovered in humans (SUV39H1/H2, G9a and Riz1 among others) [69]. H3K9 methylation is important for chromatin condensation and heterochromatin formation. H3K9me is recognized and bound by heterochromatin protein 1 (HP1), which recruits SUV39H, to reinforce the silencing process. H3K9 methylation plays a critical role in the formation of transcriptionally silent heterochromatin and the stable inheritance of the heterochromatin state. H3K9me1 and H3K9me2 are associated with euchromatic gene repression, whereas H3K9me3 is associated with stably silenced heterochromatin. H3K9me2 marks contribute to the maintenance of gene repression

in differentiated tissues in large genomic regions known as 'large organized chromatin K9–modifications (LOCKs)', and require the activity of the methyltransferase G9a [70]. H3K9me is methylated by the KMT1 family (SUV39H1, SUV39H2, G9a, GLP, SETDB1, and SETDB2). H3K9 is demethylated by the KDM1 family (LSD1), the KDM3 family (JMJD1A, JMJD1B and JMJD1C), and the KDM4 family (JMJD2A, JMJD2B, JMJD2C, and JMJD2D) as well as PHF8 and KDM7A/JHDM1D. SUV39H1 generates H3K9me3, and is involved in breast carcinogenesis. In addition, we found that SUV39H1 cooperates with SNAIL to repress the expression of E-cadherin. Knockdown of SUV39H1 blocked the formation of H3K9me3 and DNA methylation and inhibited cell migration, invasion and metastasis of BLBC [71]. Furthermore, we demonstrated that knocking down G9a resulted in suppression of H3K9 methylation and inhibition of tumor cell migration or invasion [72]. Mechanically, we found that G9a interacted with SNAIL and is critical for SNAIL-mediated E-cadherin repression in human breast cancer. Consistent with our research, Huang et al. [73] demonstrated that knocking down G9a or pharmacological inhibition of its activity suppressed tumor cell growth, colony formation, invasion and migration in non-small-cell lung cancer cells (NSCLC). G9a is also associated with an increased expression in lung cancer [74]. SETDB1 is the most significantly up-regulated epigenetic regulator in human HCCs and prostate cancer [75, 76]. Knockdown of SETDB1 decreases cell migration and invasion and reduces EMT and CSC properties [77]. SETDB1 indirectly up-regulates STAT3 expression and induces TWIST. KDM3A catalyzes the demethylation of H3K9 associated with transcriptional repression, resulting in the derepression and activation of genes involved with invasion and metastasis [78]. Global gene expression profiling demonstrated KDM3A regulates genes and pathways that augment cell migration and metastasis. KDM3A promotes both migration *in vitro* and metastasis *in vivo* by targeting melanoma cell adhesion molecule (MCAM) [79]. Surprisingly, increased expression of KDM3B correlates with improved clinical outcomes [80]. Accordingly, JMJD1B and JMJD2B are associated with PRL-3, a gene crucial to metastasis in colorectal cancer (CRC). However, JMJD1B seems to be a candidate tumor suppressor while JMJD2B seems to be a potential oncoprotein for CRC metastasis and progression [81]. With respect to the breast cancer, KDM4A is a regulator of cancer cell growth and metastasis, which correlates with breast cancer progression, and is associated with the attenuation of the tumor suppressor ARHI [82]. KDM4B is physically associated with β-catenin and binds to the promoter of the β-catenin target gene *vimentin* to increase its transcription by inducing H3K9 demethylation [83]. Inhibition of JMJD2B attenuates migration and invasion of gastric cancer cells *in vitro* and metastasis *in vivo*. KDM4C expression correlates significantly with genes driving metabolic alterations in breast cancer; the mechanism involves an interaction between KDM4C and HIF1α, which is recruited to a subset of genes involved in metabolic remodeling and metastasis [84].

H4K20: H4K20 methylation is also associated with repressed chromatin. A recent genome-wide analysis demonstrated that H4K20me3 was associated with heterochromatin and played a pivotal role in chromatin integrity. In addition, loss of histone H4K20me3 predicts poor prognosis in breast cancer and is associated with invasive activity. On the other hand, H4K20me1 is located in the promoters or coding regions of active genes and co-localizes with H3K9me1, which suggest that H4K20me1 is associated with transcriptional activity. H4K20 is

methylated by the KMT5 family (PR-Set7, SUV4-20H1, and SUV4-20H2) and the KMT7 family (SET7/9). KDMs that catalyze H4K20 demethylation have not been reported. Moreover, ectopic expression of SUV420H1 and SUV420H2 in breast cancer cells suppressed cell invasiveness, whereas knockdown of SUV420H2 activated invasion by normal mammary epithelial-cell *in vitro* [85]. Through its repressive H4K20me3 mark, SUV420H2 silences several key drivers of the epithelial state. Knockdown of SUV420H2 elicited MET on a molecular and functional level. An analysis of human pancreatic cancer biopsies suggests that high levels of SUV420H2 correlate with a loss of epithelial characteristics and progressively invasive cancer [86]. SET8 (also known as PR-Set7/9, SETD8, KMT5A), a member of the SET domain-containing methyltransferase family that specifically target H4K20 for monomethylation, physically interacts with TWIST to promote EMT and invasion by breast cancer cells [87]. Interestingly, SET8 acts as a dual epigenetic modifier on the promoters of E-cadherin and N-cadherin through its H4K20 monomethylation activity [88]. These bipolar roles of SET8 in EMT were also found in prostate cancer, which were mediated by ZEB1 [89]. A recent report indicates that the activation of the Shh pathway is required for EMT in NSCLCs [90]. SET7-mediated Gli3 methylations contribute to the tumor growth and metastasis in NSCLCs *in vitro* and *in vivo* [91].

H3K27: Another important repressive mark is H3K27 methylation which plays an essential role in embryogenesis, cell differentiation and organogenesis. H3K27me3 is associated with constitutive heterochromatin and maintenance of gene repression during early development. According to a genome-wide analysis, the levels of H3K27me2 and H3K27me3 are elevated in silent promoters and reduced in both active promoters and genic regions, whereas the level of H3K27me1 is high in promoters engaged in active transcription, especially downstream of the TSS [92, 93]. In embryonic stem cells (ESCs), H3K27 methylation usually overrides the effect of H3K4me3 in bivalent regions, maintaining them in a repressed state. Upon differentiation, these regions become exclusively marked by either of these modifications, leading to gene activation or repression [92]. H3K27 methylation is catalyzed by the polycomb repressive complex 2 (PRC2), which is composed mainly of suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED) and EZH2. H3K27 is demethylated by the KDM6 family (KDM6A/UTX and KDM6B/JMJD3), as well as UTY and JHDM1D [94, 95]. EZH2 is overexpressed in prostate and breast cancers and correlates with poor prognosis. Interestingly, EZH2 is essential for CSC self-renewal, and these CSCs provide the seeds for metastatic dispersal and differentiate into tumor-associated endothelial cells. Pre-clinical studies showed that EZH2 can silence several anti-metastatic genes (e.g., E-cadherin and tissue inhibitors of metalloproteinases), thereby favoring cell invasion and anchorage-independent growth. Accordingly, Tiwari and colleagues delineated an elegant pathway wherein TGF-β induces EZH2 expression to elicit EMT programs and metastasis of breast cancers by reprogramming the epigenome [96]. EZH2 represses TIMP2 transcription, which leads to increased activity of MMP-2 and MMP-9 and the invasive capacity of BLBC cells [97]. In pancreatic cancer cells, SNAIL recruits PRC to the E-cadherin promoter by binding to SUZ12 [98]. Increased KDM6A expression is associated with poor prognosis, along with derepression/activation of genetic programs that induce cell proliferation, luminal to basal-like transition, and metastasis. Furthermore, the function of KDM6A correlates with the activity of the MLL4, and increased expression of these epigenetic enzymes correlates with poor survival outcomes in breast cancer [50]. UTX interacts with the

MLL4 complex to activate several pro-metastatic genes including MMP9 and SIX1, leading to increased EMT and metastasis of breast cancer [50]. In colon cancer, KDM6A not only demethylates H3K27me3 at the E-cadherin promoter but also recruits CBP to the E-cadherin promoter, resulting in increased H3K27ac [99]. However, it was also reported that KDM6A inhibited EMT by epigenetic repression of EMT genes in cooperation with LSD1 and HDAC1 [100]. Therefore, the role of KDM6A an EMT suppressor or enhancer requires further investigation. KDM6B expression is also increased in invasive breast carcinomas and enforcing KDM6B overexpression induces EMT, invasive migration, stem cell-like traits, and metastatic properties. The mechanism involves demethylation associated with increased SNAIL or SLUG expression mediating the EMT [101]. Interestingly, KDM6B also modulates the tumor microenvironment and promotes melanoma progression and metastasis through upregulation several targets of NF-κB and BMP signaling, including stanniocalcin 1 (STC1) and chemokine (C-C motif) ligand 2 (CCL2) [102].

In summary, histone lysine methylation modulates chromatin accessibility, transcriptional status, and control of tumor suppressor and oncogene expression in aberrant cell metastasis. Dynamic regulation of the either permissive or repressive histone methylation at different genomic loci and through different molecular mechanisms facilitates the dynamic EMT process.

4.2. Arginine methylation

Histone arginine methylation also occurs in many arginine sites, histone H3 arginine 2 (H3R2), H3R8, H3R17, H3R26, and H4R3 undergo monomethylation (me1), symmetrical dimethylation (me2s), or asymmetrical dimethylation (me2a) on the guanidinyl groups of arginine residues. The N-arginine methyltransferases (PRMTs) are a class of enzymes that transfer a methyl group from SAM to the guanidino nitrogen of arginine. PRMTs generate three arginine methylation forms: monomethylarginine (MMA), asymmetric dimethylarginine (aDMA), and symmetric dimethylarginine (sDMA). Human PRMTs are composed of nine members that are categorized into three groups based on the type of arginine methylation reaction each member catalyzes. Type I is comprised of PRMT1, PRMT2, PRMT3, CARM1/PRMT4, PRMT6, and PRMT8; these catalyze both mono-methyl and asymmetric dimethyl arginine reactions. The type II group is made up of two members, PRMT5 and PRMT9, which catalyze both mono-methyl arginine and symmetric dimethyl arginine. Finally, PRMT7 is, at this point, considered the only bona fide type III methyltransferase and can generate only mono-methyl arginines. Many studies demonstrated that PRMTs regulate a wide range of genetic programs and cellular processes including cell cycle, RNA splicing and differentiation. Although the consequence of lysine methylation is relatively well studied, the role of PRMT action in tumorgenesis is poorly understood. Here, we provide a description of these PRMTs regarding tumor metastasis.

PRMT1: PRMT1 has been extensively studied in many fields. Its activity is responsible for a substantial percentage of methylated arginine residues and modulates a wide range of cell types. Specifically, asymmetric dimethylation on H4R3 by PRMT1 is involved in transcriptional activation, thereby driving oncogenic pathways. PRMT1 is an important regulator of EMT, cancer cell migration, and invasion. PRMT1 can generate H4R3me2a on the promoter region of ZEB1 and TWIST, which play a critical role in EMT [103, 104]. Furthermore, PRMT1

is overexpressed in melanoma; silencing PRMT1 significantly suppresses tumor growth and metastatic ability by targeting activated leukocyte cell adhesion molecule (ALCAM) [105]. Similarly, downregulation of PRMT1 inhibits cell migration and invasion in HCC and oral squamous cell carcinoma (OSCC) [106, 107]. Because of complex alternative splicing in the 5′ region of its pre-mRNA, there are seven distinct PRMT1 isoforms [108]. Each of these isoforms, named PRMT1v1-v7, has distinct characteristics in terms of expression. PRMT1v1 is the most abundantly expressed isoform and likely represents the isoform that is described as PRMT1 in most reports. The expression of alternatively spliced PRMT1 (PRMT1v2) isoform, which is generated through inclusion of alternative exon 2, is significantly altered in breast cancer and promotes invasiveness. The RNA binding protein RALY regulates the PRMT1v2 isoform and promotes metastatic potential [109].

PRMT2: PRMT2 is also reported to be overexpressed in breast cancer [110]. PRMT2 interacts with many NRs, including ERα and ERβ *in vitro* [111]. Interestingly, the activation of these receptors within cells has both distinct and in some cases opposing effects, which suggests that the functional role(s) PRMT2 are quite diverse. Recently, four alternatively spliced PRMT2 isoforms (PRMT2L2, PRMT2α, β, and γ) in addition to the original PRMT2 isoform were identified [112]. Several splice variants (*i.e.*, PRMT2-α, $-\beta$, $-\gamma$) were identified as induced in breast cancer, particularly in ER, PR-positive breast cancer [110]. PRMT2 directly binds and enhances estrogen-mediated transactivation of ERα, and enhances the promoter activity of the downstream target gene SNAIL. These findings suggest that the increased PRMT2 expression is associated with breast aggressiveness and metastasis [110].

PRMT4: PRMT4, more commonly known as coactivator-associated arginine methyltransferase 1 (CARM1), is involved in the regulation of a number of cellular processes including transcription, pre-mRNA splicing and cell cycle progression. The expression of CARM1 is dysregulated in colorectal, prostate and breast cancer. CARM1 methylates the chromatin-remodeling SWI/SNF core subunit, BAF155 in breast cancer [113]. The methylation of arginine 1064 residue of BAF155 is associated with breast cancer recurrence and metastasis, indicating that CARM1 plays an important role in tumorigenic activity through BAF155. Accordingly, CARM1-induced tumorigenic effects and its expression is increased in invasive breast cancer, and correlates with a high tumor grade [114]. Interestingly, the *CARM1* gene also transcribes four isoforms: the primary isoform CARM1 (CARM1v1) and three alternative isoforms, v2, v3 and v4 [115]. Whether these isoform are responsible for the methylation of distinct substrates and their individual functions requires further study.

PRMT5: PRMT5 is a type II enzyme that generates symmetric dimethylarginine (sDMA). The PRMT5 symmetrically methylates H3R8 site and functions in gene silencing. H3R8me2s strongly associates with H4R3me2s, because both modifications are catalyzed by PRMT5. However, acetylation of H3K9 and H3K14 prevents H3R8 methylation. PRMT5 also acts as a novel cofactor of SHARPIN (Shank-associated RH domain interacting protein), which plays a central role in controlling lung cancer cell metastasis. SHARPIN-PRMT5 is essential for the monomethylation of histones at key metastasis-related genes [116]. PRMT5 has another distinct function; PRMT5 coordinates with multiple Mediator complex subunits to dimethylate H4R3 at the promoter regions of immune response genes and C/EBPβ target

genes [117]. Conversely, PRMT5 methylation of histone H3R2 recruits WDR5 and the MLL complex, stimulating H3K4 methylation and euchromatin maintenance [118]. In the context of cancer metastasis, PRMT5 is involved in TGF-β-WDR77 signaling, which induces cancer cell invasion [119]; this report indicates PRMT5 interacts with the WDR77 complex to catalyze arginine methylation. With respect to the acquisition of EMT via TGF-β signaling, epigenetic PRMT5-WDR77 activity is necessary for tumor invasion and metastasis. Furthermore, PRMT5 appears to be recruited by AJUBA to SNAIL and functions as a co-repressor. PRMT5, AJUBA and SNAIL form ternary complex to repress E-cadherin, concomitant with increase arginine methylation at the locus [120]. PRMT5 also modulates metastasis by methylating KLF4. Methylation blocks ubiquitylation of KLF4 by the von Hippel–Lindau tumor suppressor, and as a result, arginine methylation of KLF4 via PRMT5 increases the level of KLF4 protein and increases the probability of breast carcinogenesis [121].

PRMT6: PRMT6 primarily catalyzes asymmetric dimethylation of H3R2. H3R2me2a counter-correlates with the methylation of H3K4, which suggests that H3R2me2a is a repressive marker. However, PRMT6 also methylates H3K4 since both H3R2me2a and H3K4me3 markers are likely to coexist. Furthermore, genome-wide analyses indicate that both H3R2me1 and H3R2me2a are associated with active genes [93]. Thus, the data on the H3R2me2a marker are contradictory, and further studies are required to resolve this issue. There is also emerging evidence of an oncogenic role of PRMT6 in cancer. Overexpression of PRMT6 is associated with several cancer types, including breast, cervix, prostate, and lung cancers, indicating that PRMT6 might play an important role for the onset, incidence, and metastasis of cancer [122]. Furthermore, Dowhan et al. [123] demonstrated a PRMT6-dependent signature that influences long-term survival in patients with breast cancer.

PRMT7: The oncogenic role of PRMT7 has been emerging over the past few years. There are two isoforms, PRMT7α and β, which are active and have slightly different methylation profiles and locations. PRMT7α localizes to the cytoplasm and nucleus, whereas PRMT7β is exclusively cytoplasmic. R531 of PRMT7 is self-methylated and loss of PRMT7 automethylation leads to a reduced recruitment to the E-cadherin promoter by YY1, which consequently derepresses E-cadherin expression by decreasing the H4R3me2's level [124]. In terms of the functional role of PRMT7, this methytransferase is highly expressed in breast cancer and induces EMT by inhibiting E-cadherin. Baldwin et al. [125] also showed that PRMT7 promotes a well-known metastasis mediator, MMP9 and induces breast cancer cell invasion. Importantly, a gene expression analysis of independent data sets of more than 1200 breast tumors identified PRMT7 expression as significantly increased. In addition, this gene is located 16q22, where the chromosomal region was correlated with an increased metastatic potential of breast cancer [126].

PRMT9: PRMT9 and PRMT5 are the only known mammalian enzymes capable of forming sDMA residues as type II PRMTs. However, the specificity of these enzymes for their substrates is distinct and not redundant. Interestingly Yang et al. [127] showed that PRMT9 is also nonhistone methyltransferase. For example, it methylates the arginine 508 site of the alternative splicing factor SAP145. Given that alternative splicing is of paramount importance in RNA processing, PRMT9 might play a key role in many cellular programs including cancer biology. Recent reports demonstrate that overexpression of PRMT9 strongly promotes HCC invasion and metastasis through EMT by regulating SNAIL expression via activation of the PI3K/Akt/GSK-3β/SNAIL signaling pathway [128].

Many HMTs and HDMs inhibitors have been developed and evaluated in clinical trials, such as chaetocin, BIX-01294, BIX-01338, UNC0638 and DZNep. Chaetocin, a natural fungal substance, is the first inhibitor of an HMT, which targets SUV39H1 without high selectively [129]. Treatment with Chaetocin induces expression of E-cadherin while reducing H3K9me3 but does not produce a global H3K9 methylation on its promoter in multiple tumor cells [130]. By the contrast, BIX-01294 specifically reduces the dimethylation of H3K9me2 through an inhibition of the enzymatic activities of G9a and GLP [131]. Treatment of BIX-01294 activates E-cadherin expression and reverse EMT phenotypes in a variety of cancer cells, and is accompanied by reduced H3K9me2 and increased H3K9 acetylation on the E-cadherin promoter [132]. Another G9a/GLP inhibitor, UNC0638, was developed with higher potency and selectively [133]. UNC0638 treatment not only resulted in lower global H3K9me2 levels but also markedly reduced the abundance of H3K9me2 marks at promoters of known G9a-regulated endogenous genes. UNC0638 treatment activates E-cadherin expression and reverses EMT in PANC-1 pancreatic cancer cells and triple negative breast cancer (TNBC) and suppresses migration and invasion [134]. Because of the importance of H3K27 methylation in cancer, several highly specific EZH2 inhibitors have been developed, such as GSK2816126 and EPZ-6438, which are currently being evaluated in clinical trials for lymphoma and solid tumor/lymphoma respectively [135]. Another EZH2 inhibitor, 3-deazaneplanocin A (DZNep), selectively inhibits H3K27me3 and H4K20me3 [136]. DZNep dampens TGF-β-induced EMT signals and reduces tumor metastasis in pancreatic cancer and colon cancer [136, 137]. We found that Parnate, an LSD1 inhibitor, activates E-cadherin expression and suppresses motility and invasiveness in breast cancer cells [51]. Two highly specific LSD1 inhibitors, GSK2879552 and ORY-1001 are employed to clinical trials for the treatment of small cell lung cancer and acute leukemia [135]. Several inhibitors targeting HDMs also have been developed as well. For example, JIB-04, a specific inhibitor targeting the JMJC-domain, inhibits the activity of H3K4 and H4K9 and attenuates lung cancer cell proliferation [138]. The first reported small molecule PRMT inhibitors, including AMI-1 and AMI-5 were identified through virtual screening and high throughput screening [139]. AMI-1 was reported as type I PRMT and PRMT5 inhibitor [140]. AMI-1 inhibits proliferation and decreases cell migratory activity of CRC cells *in vitro* and in xenograft mouse models [141].

5. Histone modification readers

Sometimes, histone modifications can directly regulate the chromatin dynamic. However, in most cases, the modifications are recognized by proteins containing distinct recognition domains, which act as "readers" and bind to different histone modifications. For example, bromodomain acts as lysine acetylation "readers" of modified histones that mediate signaling transduction changes in gene regulatory networks. In the human genome, there are 61 bromodomains found within 46 proteins that can be divided into eight families based on structure/sequence similarity. Among them, bromodomain and the extra-terminal domain (BET) family recognize acetylated lysine residues in histones H3 and H4. BRD4 is a member of the BET family that carries two bromodomains. Recently, our studies revealed that the di-acetylated TWIST, mediated by Tip60, recruits BRD4 and related

transcriptional components to the super-enhancer of its targeted genes during tumor progression in BLBC [20]. In addition, pharmacologic inhibition of BRD4 with the BET-specific bromodomain inhibitors, JQ1 and MS417, effectively reduces WNT5A expression and suppresses invasion, CSC-like properties and tumorigenicity of breast cancer cells *in vitro* and *in vivo* [20]. Given the extensive cancer-related functions of BRD4 and the proof-of-concept demonstrated by disruption of the BRD4–acetyl-lysine interactions as a therapeutic target, significant efforts have thus been made to develop BRD4 inhibitors from both pharmaceutical and academic settings. BRD4 inhibitors have several chemical classifications including azepines, 3,5-dimethylisoxazoles, pyridones, triazolopyrazines, tetrahydroquinolines (THQs), 4-acyl pyrroles and 2-thiazolidinones [142]. BET inhibitor treatment results in AMIGO2 silencing and changes in PTK7 proteolytic processing, and thus inhibit melanoma metastasis [143].

Histone methylation provides docking sites and is recognized by specific reader proteins that contain a methyllysine binding protein, which has emerged as a focus of epigenetic research due to its critical role in gene regulation and oncogenesis. This reader harbors specific motifs, including Chromodomain (CD), MBT, WD40 repeat, PHD finger, PWWP, Tudor and Ankyrin repeat. Methyllysine binding proteins distinguish methylation marks on different residues as well as different methylation states on the same residue and in turn mediate distinct downstream functions [144]. CD-containing HP1 proteins were the first identified methyl-lysine binding proteins and recognize methylated-H3K9 (methyl-H3K9) [145]. HP1α was down-regulated in metastatic cells of colon cancer and thyroid carcinomas relative to non-metastatic cells, indicating HP1α may be directly involved in the silencing of genes that potentiate cancer cell invasive potential and metastasis. Recent evidence implicate HP1α in EMT. The association of HP1α to major satellite repeat sequences located in pericentric heterochromatin decreased during the initial steps of TGF-β-induced EMT in a SNAIL/LOXL2-dependent manner [146]. In addition, HP1α post-translational modifications could participate in the heterochromatin dynamics associated with EMT. In a different set of modifications, four MBT-repeats domain of SFMBT1 recognize H3K4me2/3 and form a stable complex with LSD1. SFMBT1 is essential for SNAIL-dependent recruitment of LSD1 to chromatin, demethylation of H3K4me2, transcriptional repression of epithelial markers, and induction of EMT by TGF-β [147]. H3K4me2/3 is also recognized by the WD40 repeat domain of WDR5, which is also important for the assembly and activity of the SET1 protein complex catalyzing H3K4me3 [148]. Under hypoxic conditions, WDR5 is induced, interacts with HDAC3 and further recruits SET1 complex to activate mesenchymal gene expression to promote EMT [149]. Furthermore, the PRC2 component, EED, also contains a WD40 repeat that recognizes H3K27me3. EED recruits PRC2 to chromatin with pre-existing H3K27me3 to spread the same methylation into adjacent regions [150]. Intriguingly, G9a and GLP itself contain a methyl-lysine binding module (the ankyrin repeat domains), which generates and reads the same epigenetic mark [151]. Several small molecule compounds targeting the lysine methylation reader domain have been developed, including UNC1215 and UNC3866 that block the methyl-lysine binding mediated by the MBT domain-containing protein L3MBTL3, and the CD-containing protein CBX4/7 respectively [152, 153]. However, whether these inhibitors reverse EMT and tumor progression remains unknown.

6. Coordinated histone modification regulation

Because different chromatin modifying enzymes coexist in the same protein complex, and because diverse catalyzed modifications have been implicated in regulating the same set of genes, it is likely that these processes act in concert to orchestrate transcriptional regulation during EMT. For example, HDAC1/2, G9a/GLP, LSD1, HP1 and ZEB1/2 were co-purified in the CtBP1 co-repressor complex [154, 155]. ZEB1/2 could first target the complex to E-cadherin promoter to initiate repression. Next, HADC1/2 would deacetylate histones while the primed H3K9 was methylated by G9a/GLP. Meanwhile, LSD1, which removes H3K4me1/2, whereby the un-methylated H3K4 could also prevent H3K9 from re-acetylation [156, 157]. An affinity purification of Flag-TWIST identified several components of the NuRD chromatin remodeling complex. Among them, TWIST directly interacts with Mi2β, MTA2 and RbAp46 and likely targets the NuRD complex for histone deacetylation and chromatin remodeling on E-cadherin promoter. Together, these epigenetic events lead to gene silencing and promote EMT and breast cancer metastasis [158]. In addition, TWIST was also co-purified with SET8, BRCA1-associated protein (BRAP), NF-kB subunit RelA, PPP2CA and HES6 in MCF7 breast cancer cells [88]. SET8 interacts with TWIST. However, SET8 and TWIST are functionally interdependent in promoting EMT. SET8 mediates E-cadherin repression and N-cadherin activation simultaneously via its H4K20 monomethylation to promote cell invasion and EMT. However, the molecular mechanism that underlies the same repressive protein complex that contributes to opposite functions on different genomic loci remains an open question. Our recent study found that TWIST is diacetylated by Tip60, which was further recognized by BRD4, thereby constructing an activated TWIST/BRD4/P-TEFβ/RNA-Pol II complex at the WNT5A promoter and enhancer to promote EMT and breast cancer cell metastasis [20]. In breast cancer cells, the UTX-MLL4 forms a complex with LSD1/HDAC1/DNMT1 on the promoter of several EMT-TFs and decreases H3K4mes and H3 acetylation. UTX facilitates epigenetic silencing of EMT-TFs by inducing competition between MLL4 and the H3K4 demethylase LSD1, which results in inhibition of EMT and CSC-like properties [100].

MPP8, another methy-H3K9 binding protein, bridges DNMT3A and G9a/GLP to assemble a repressive trimeric protein complex on chromatin by binding to different methyl-lysines. MPP8 also couples H3K9 methylation and DNA methylation to silence epithelial genes and EMT [159, 160]. Interestingly, MPP8 also cooperates with the SIRT1 in this process through a physical interaction [161]. SIRT1 and MPP8 reciprocally promote each other's function and coordinate epithelial gene silencing and EMT. SIRT1 antagonizes PCAF-catalyzed MPP8-K439 acetylation to protect MPP8 from ubiquitin-proteasome-mediated proteolysis. Conversely, MPP8 recruits SIRT1 for H4K16 deacetylation after binding to methyl-H3K9 on target promoters. Therefore, MPP8 not only promote DNA-methylation but also H4K16 deacetylation to fine-tune the transcriptional regulation of EMT.

7. Conclusions and perspectives

Increasing evidences show that aberrant profiles of histone modifications contribute to a dysregulation those results in the metastatic cascade. The biochemically reversible nature

of histone modifications provides a platform for rapid changes in a variety of epithelia and mesenchymal genes during EMT and MET. In concert with different ETM-TFs and oncogenic signaling, pleiotropic histone modifications form a sophisticated and regulated network to coordinate the plasticity and dynamic change required for EMT.

Recent research identifies the critical role of histone modifications in metastasis, but leaves many important, open questions. First, do tumor microenvironmental signals trigger the formation of histone modification enzyme complexes present on different EMT-TFs? Whether these extrinsic signals affect enzyme activity indirectly through intracellular signaling pathways or directly through the EMT-TFs remains to be determined. Second, how do these EMT-TFs form distinct complexes that coordinate the epigenetic regulation of gene expression programs during EMT? Third, EMT is usually activated only transiently and partially. Therefore, which and how do different histone modifying enzymes and the catalyzed modifications contribute to these dynamic changes? Finally, what consequences do epigenetic instabilities have on cancer cell fitness? Do these activities increase plasticity and/or lead to vulnerabilities that it could influence the metastasis?

We know that histone modification enzymes are highly correlated with tumor progression and a poor clinical outcome. Therefore, these enzymes can serve not only as effective biomarkers for earlier diagnosis, but also present multiple therapeutic opportunities. Over the last decade, considerable progress has been made in the discovery and development of potent and selective small molecule inhibitors targeting specific histone modifiers. Many of these molecules are currently under extensive preclinical testing or being evaluated in clinical trials. These inhibitors show great potential as clinically useful drugs. Additionally, inhibitors to specific histone modifying enzymes could serve as useful chemical probes to characterize the function of different epigenetic pathways in EMT *in vivo* as well as many other important pathological diseases.

In all, advances in our understanding of the landscape of histone modifications in metastasis will provide a better sense of the molecular mechanisms associated with metastasis and thus help speed the development of new therapeutic strategies and biomarkers for metastasis.

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Conflict of interest

The authors have declared that no conflict of interests exists.

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Chapter 5

Early Metastasis in Colorectal Cancer Poses an Option for New Diagnostic and Treatment Strategies

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Additional information is available at the end of the chapter

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Abstract

Metastasis is the spread of tumor cells from a primary site to a secondary site within the host's body. It is initiated by the detachment of the tumor cells from the primary tumor followed by invasion into the surrounding tissue. Thereafter the cells migrate across the endothelium and into the blood vessels (intravasation). During the intravasation the cells have to survive the sheer forces and the immune response. Upon arrival to the target organ, the cells leave the circulation and cross the endothelium to reach the host organ. Once there, the tumor cells are greeted with the organ's local immune cells and with a hostile or inappropriate environment, where they finally have to form proliferating colonies. Metastasis is therefore far from being a straight-forward or efficient process with less than 0.1% of disseminating tumor cells (around 1 × 109 cells per day for a 1 cm size tumor) succeeding in colonizing distal organs. The identification of the involved marker during the early metastasis process will be essential for establishment of new diagnostics tools, as well as development of novel treatment strategies.

Keywords: colorectal cancer, early metastasis, migration, invasion, homing

1. Introduction

Cancer metastasis is the major cause of cancer morbidity and mortality, and accounts for about 90% of cancer deaths [1]. Metastasis is a complex process requiring several processes, which involves the spread of cancer cells from the primary tumor to surrounding tissues and to distant organs. Cancer cells require the capacity to invade the surrounding tissues, then

migrate and survive in the circulation, colonize the foreign organ and eventually resume growth, to gain metastatic capability [2], Metastasis alone is an inefficient process because the tumor cells have to acquire some necessary abilities to regenerate a tumor at a distant site [3, 4]. Over the past few years, the main determinants of metastatic competence in CRC have begun to be characterized. In fact, the acquirement of a stem-like phenotype by cancer cells is very important for the tumor cells to regenerate in a foreign organ, in the absence of mutations associated with the metastatic process in CRC. These metastatic stem cells adopt multiple phenotypes and behaviors and critically depend on their interaction with the microenvironment to migrate, survive in the circulation and flourish in a foreign organ. The metastatic cascade consists of four essential steps. The first of which is the detachment of the tumor cells from the primary tumor. There after the separated cells undergo local invasion into the surrounding tissue, e.g. into the mesenchyma, followed by migration of the tumor cells across the endothelium and into the vessels in a process known as intravasation where disseminating tumor cells have to survive the circulatory system's sheer forces and swarming immune cells. Upon arrival to the target or appropriate organ, tumor cells have to leave the circulation and cross once again the endothelium to reach the host organ. Once there, the disseminated tumor cells are greeted with the organ's local immune cells as well as with a hostile or inappropriate environment where they finally have to form proliferating colonies. Metastasis is therefore far from being a straight-forward or efficient process with less than 0.1% of disseminating tumor cells (around 1×10^9 cells per day for a 1 cm size tumor) succeeding in colonizing distal organs. The cells have to face multiple ordeals, such as the immune system, at each step of the process thus making metastasis a process possible mostly out of the sheer number of disseminating cells entering the bloodstream [5]. Multi-biochemical events and other parameters affect the metastatic cascade such as extracellular matrix structure, growth factors, chemokines, matrix metalloproteinases. Hence, the biochemical markers along with the tumor microenvironment may serve as a crucial target for the inhibition and prevention of metastasis [5].

2. Metastasis in colorectal cancer

2.1. Cancer cell detachment

Cancer cell detachment is a process usually occurring from the extracellular matrix (ECM). Cell detachment involves both mechanical forces and protease-mediated cleavage, but also decreased expression of adhesion molecules and changes in glycosylation of cell membrane glycoproteins and proteoglycans. Mechanical forces are generated by actomyosin-driven contraction. The cytosolic dissociation of cell–substrate adhesions can also be performed by the calpain cysteine proteases, by phosphorylation/dephosphorylation of cytosolic adapter proteins and by posttranslational modification of integrins or adapter proteins.

Extracellular dissociation of cell-substrate adhesions can be achieved by proteolytic cleavage of matrix constituents that are mediated by matrix proteases. Moreover, the detachment

could occur by the shedding of matrix receptors such as integrins [6]. Anoikis is a form of programmed cell death that occurs when anchorage-dependent cells detach from their ECM [7]. When cells are detached from the ECM, there is a loss of normal cell-matrix interactions, and subsequently anoikis can occur through the down-regulation of Bcl-xL (an anti-apoptotic component of the mitochondrial pathway) and the up-regulation of Fas ligand (FasL) (an activator of the death receptor pathway) [8].

However, during the metastatic detachment, the tumor cells resist and escape the anoikis process. This escape includes the alteration of enzyme systems in the signaling pathways that regulate anoikis, such as small GTPases and effectors, receptor tyrosine kinases and other kinases such as NF-κB, and EMT factors [6, 9].

Furthermore, there are multiple anoikis-independent mechanisms by which normal epithelial cells would die once detached from the ECM. Metastatic cancer cells must overcome these anoikis-dependent and anoikis-independent barriers in order to survive once they lose the attachment to the ECM [6].

2.2. Detachment in mCRC

Metastatic colorectal cancer (mCRC) is a long and complex process involving several mechanisms, and molecular pathways. CRC is currently considered the third most common neoplasm in the world according to the World Cancer Research Fund International, and the second most frequent malignancy causing death [10] mCRC is a multi-step biological process (**Figure 1**). This process starts with a series of mutations in colonic epithelial cells, continues with their detachment from the large intestine, dissemination through the blood and/or lymphatic circulation, attachment to the hepatic sinusoids and interactions with the sinusoidal cells, such as sinusoidal endothelial cells, Kupffer cells, stellate cells, and pit cells. The metastatic sequence terminates with colorectal cancer cell invasion, adaptation and colonization of the hepatic parenchyma. All these events are termed the colorectal cancer invasion-metastasis cascade, which includes multiple molecular pathways. The cellular and molecular pathways of the metastatic process in CRC have been extensively analyzed over the last decades. The metastatic process in CRC involves a series of steps such as:

1. Lysis of the extracellular matrix

Enzymes produced by cancer cells alter the extracellular matrix and thus enable cancer cells to leave the original site of the primary tumor.

Matrix metalloproteinases (MMPs) are crucial components of cells that can degrade a range of extracellular matrix proteins allowing cancer cells to detach and migrate. MMPs are a family of zinc-dependent endoproteinases with an enzymatic activity directed against main Extra Cellular Matrix (ECM) components.

The mechanism by which MMPs aid cancer cells to escape and degrade the ECM consists of two main steps. First, proteinase acts by removing any physical barriers to invasion by the degradation of ECM macromolecules such as collagens, laminins, and proteoglycans. Second,

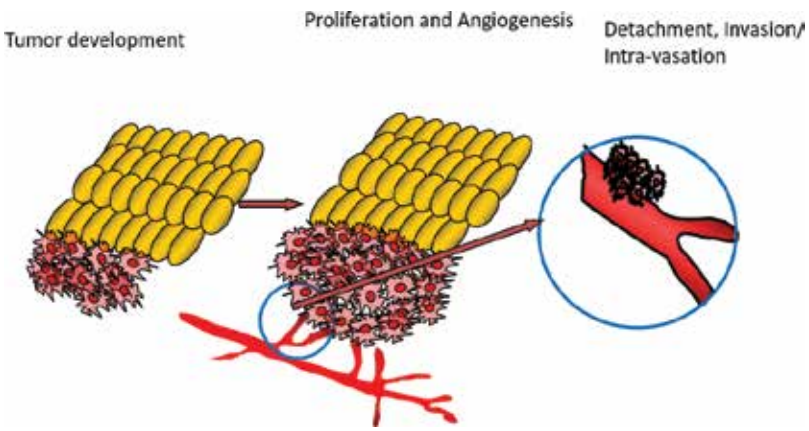

Figure 1. Illustration of the initial stages of the metastasis process. The tumor development is followed by tumor growth and angiogenesis. This stage is characterized by the down-regulation of cell attachment proteins such as the cadherins, as well as the tight junction proteins such as the claudins. In addition the expression of the matrix metalloproteinase is increased in this stage.

MMPs modulate cell adhesion. For cells to move through the ECM, they must be able to form new cell–matrix and cell–cell attachments and break existing ones. Overexpression of MMP-1, -2, -3, -7, -9, -13, and MT1-MMP has been demonstrated in human colorectal cancers (**Figure 1**). The degree of overexpression of some MMPs has been noted to be correlated with different stages of disease and/or prognosis [12].

2. Cellular adhesion

Cancer cells express adhesion molecules as cadherins, integrins, and carcinoembryonic antigen (CEA) that favor their adhesion to the extracellular matrix. These adhesion molecules have been under exclusive research regarding their roles concerning their regulation and expression in the process of early metastasis during detachment from the primary tumor.

A. Cadherins

Cell adhesion molecules (CAMs) regulate cell–cell and cell-matrix adhesion and are implicated in almost all stages of metastasis, therefore alterations in normal levels of CAMs such as E-cadherin will be significant in tumor progression. E-cadherin is the prototypical member of the type-1 classical cadherins and is found at adherens junctions (AJs), which are structures that mediate cell-cell interactions. E-cadherin is a single-pass transmembrane glycoprotein containing five extracellular repeats that mediate its Ca^{2+}-dependent homophilic interaction with opposing molecules on neighboring cells [11].

Studies exploring the expression of E-cadherin and α-catenin in tumor tissues have shown that loss of both molecules is linked to an increased invasiveness of tumor cells [12]. Evidence for this comes from in vitro and in vivo studies, which demonstrate that E-cadherin expression is inversely correlated with the motile and invasive behavior of tumor cells and also with metastasis in cancer patients [12]. Further studies have revealed that the relocalization of

β-catenin to the nucleus correlates with the acquisition of the mesenchymal phenotype [13], and is associated with the loss of E-cadherin.

Loss of the E-cadherin molecule is thought to enable metastasis by disrupting intercellular contacts. This can occur due to somatic mutations, chromosomal deletions, proteolytic cleavage, and silencing of the *CDH1* promoter. Silencing can also occur either by DNA hypermethylation or through the action of transcription factors such as Slug, Snail, and Twist [14].

B. Integrins

Integrins are heterodimeric cell-surface glycoproteins that serve to mediate cell–ECM interactions, thereby linking cues from the extracellular environment to the actin cytoskeleton [15]. These membrane-spanning proteins consist of 2 subunits, termed a and b, of which there are at least 18 a-subunits and 8 b-subunits. The resulting multitude of possible combinations gives rise to more than 20 different integrins, which act to differentially control a range of biological processes through selective binding to extracellular substrates.

The crosstalk between epithelial cell-cell adhesion and cell- matrix adhesion signaling, and the dynamic interplay between the two, contribute to the plasticity within tumor cells that allows them to respond to external cues, which in turn drives effective migration and invasion. Below, we will review data on the key signaling intermediates that regulate this crosstalk, as well as discuss recent work that is in support of a physical interaction between integrin- and E-cadherin-mediated adhesions that governs the adhesive strength of E-cadherin.

C. EMT

Epithelial-mesenchymal transition (EMT) is a reversible morphogenetic biological process that involves the transition from stationary polarized epithelial cells to motile, multipolar or spindle-shaped mesenchymal cells. The EMT is described in detail in another part of this chapter.

2.3. Hypoxia

Hypoxia in cancer cell metastasis is a tumor oxygen deficiency termed as the environmental stressor (**Figure 2**). This condition is known to induce genes involved in the regulation of cell proliferation, extracellular matrix production, cell adhesion, and other hallmarks of tumorigenesis [16].

The mechanism behind the hypoxia induced metastasis is influenced by transcription factors like the hypoxia-inducible factor (HIF) family. This family consists of three members, HIF-1, -2, and -3, that regulate vital cellular processes such as glucose metabolism, angiogenesis, cell proliferation, and tissue remodeling [17].

Hypoxia is linked to tumor early metastasis by several known molecular mechanisms. The first mechanism is the HIF-1α binding to hypoxia-response elements within the c-met promoter activating transcription of this gene. Overexpression of the Met protein on the cell surface of tumor cells leads them to be more susceptible to hepatocyte growth factor stimulation. This causes extracellular matrix degradation, cell dissociation, and escape from hypoxic areas

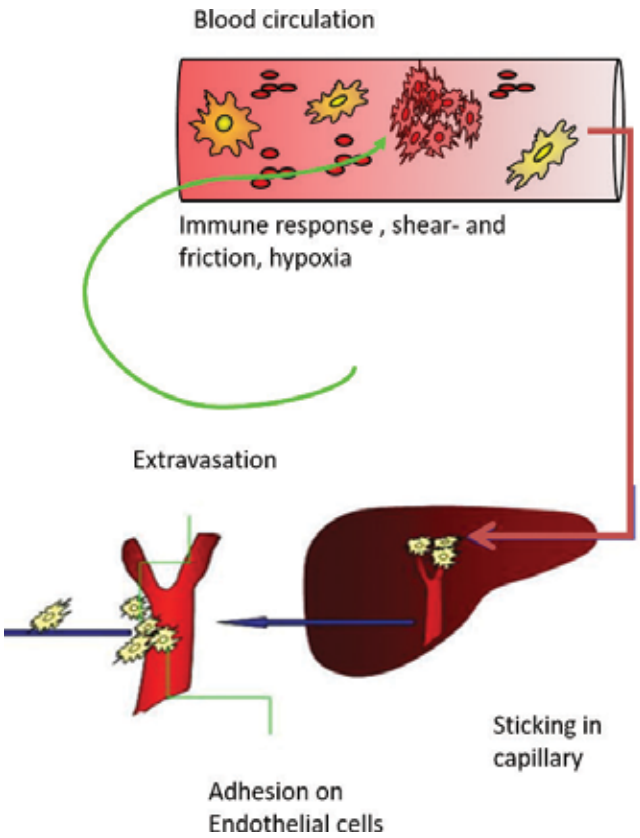

Figure 2. The post tumor cell intravasation stage poses the greatest obstacles for the tumor cells in the metastasis process. The immune response, shear and friction forces as well as hypoxia are responsible for considerable highest elimination of the tumor cells. The EMT is a special feature that defined the tumor cells in this time period.

to more oxygen-rich environments at a secondary [18]. Moreover, the HIF expression is necessary and sufficient to cause E-cadherin loss, a critical step in EMT [19].

While hypoxia has been strongly linked to tumor metastasis and poor clinical outcome of patients, it seems to actually have a dual role: insufficient oxygen limits tumor cell division while at the same time selecting for more malignant cells and inducing cell adaptations allowing for more invasive behavior. This is likely because low oxygen tension is able to increase cell invasiveness, cause cells to switch to anaerobic metabolism, increase genetic instability, and promote angiogenesis [20].

2.4. Invasion and endothelial transmigration

2.4.1. Mechanisms of invasion in CRC

The colonic epithelium in particular is composed of polarized cells with a characteristic apical membrane, forming a barrier with the components of the colon lumen, a basal membrane attached to the basement membrane and lateral membranes attaching to adjacent cells. In order

to invade other tissues, tumor cells may move individually, as clusters or as collective sheets by changing their phenotype and morphological features either by epithelial to mesenchymal transition (EMT), collective to amoeboid transition (CAT) or mesenchymal to amoeboid transition (MAT). EMT is the process of transition of the tumor cells from an epithelial phenotype into a mesenchymal phenotype by losing E-cadherin and upregulating vimentin [21]. This mechanism of invasion has been observed in colorectal cancer (CRC), breast cancer, hepatocellular carcinoma, pancreatic cancer, prostate carcinoma and lung cancer cells. CAT involves individual tumor cells detaching from cell clusters and developing amoeboid migration such as in melanomas. MAT describes the transition of mesenchymal tumor cells to amoeboid cells as observed in fibrosarcomas, melanomas and breast cancer. Amoeboid cells decrease their interactions with the extra-cellular matrix (ECM), which allows them to move easily through intact ECM gaps without resorting to proteolysis or ECM degradation and thus independent of protease activity [22]. EMT is the mode of invasion most observed and described in CRC.

2.4.2. EMT

EMT was first described as a process in developmental biology in embryogenesis. It was observed that a similar mechanism was employed by invading tumor cells, which undergo several phenotypical changes to resemble mesenchymal cells. This process requires the loss of cell–cell interactions such as the loss of epithelial cadherin (E-cadherin), α-catenin, claudins [23], occludin and ZO-1, with transcription factors' activation increasing the expression of mesenchymal proteins such as neuronal cadherin (N-cadherin), fibronectin and vimentin, reorganization of the cytoskeleton and production of proteases and ECM degrading enzymes [24]. The entirety of these processes is linked together, starting with the loss of E-cadherin. It binds extracellularly with its adjacent cell's E-cadherin while it binds intracellularly to α- and β-catenin and p-120 catenin, which is responsible for signal transduction and connecting the junctions to the cytoskeleton. Factors that have been known to induce EMT include transforming growth factor β (TGF-β), which plays an essential role in the transition and progression by activating Smad, integrins, platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). They induce the expression of EMT-related transcription repressors such as Snail, Slug, Twist and zinc finger E-box binding homeobox 1 (ZEB1). The downregulation of E-cadherin is generally accompanied by the upregulation of mesenchymal proteins, notably N-cadherin. This transition from E- to N-cadherin is called "cadherin shift" and is essential in EMT [25]. Upon loss of E-cadherin, the membrane-bound β-catenin is translocated to the nucleus where it regulates several genes' transcription, notably cyclin D1 and c-myc thus contributing to the progression of malignancy. In turn, N-cadherin expression mediated the formation of Rho-induced stress fibers forming lamellipodia (major actin projection leading the cell forward) by Ras-related C3 botulinum toxin substrate 1 (Rac1) protein activation and filopodia (thin actin projections from the leading edge of the cell) by cell division control protein 42 homolog (Cdc42) activation. Therefore, invading tumor cells undergoing EMT are able to detach themselves from clusters of epithelial cells; they lose their epithelial phenotype and are capable of moving individually similar to a mesenchymal cell.

Furthermore, EMT is known to preserve stem cell properties, evade apoptosis and the immune response as well as confer resistance to radiotherapy and chemotherapy, which was described in CRC. As of late, various microRNAs (miRs) have been shown to regulate EMT such as miR-9, which interacts with E-cadherin thus facilitating cell detachment and motility and increases VEGF levels leading to neoangiogenesis. Interestingly, epithelial cell differentiation may be driven by the miR-200 family including miR-200a, miR-200b, miR-200c, miR-429 and miR-141. Zinc Finger E-Box Binding Homeobox 1 and 2 (ZEB1 and ZEB2) transcription factors repress the miR-200 family at transcription, while this miR family itself inhibits ZEB1 and ZEB2 (EMT inducers) at a post-transcriptional level. Furthermore, ZEB induces EMT and stem cell characteristics by upregulating Sox2, Klf4 and Bmi1, which are ordinarily inhibited by the miR-200 family. This process has been conjectured to occur in CRC [26].

2.4.3. Collective cell invasion

Epithelial cancers such as breast and colorectal cancers witness the occurrence of collective cell or bulk invasion. The cells maintain their intercellular junctions, adherence and desmosomes, and thus stay attached together during movement [27]. However, there is a front to back polarity where tip or leader cells at the front of migration are phenotypically different than following cells. The asymmetry in actomyosin filaments affecting the tip cells are mediated by Rho GTPases and myosin II. Tip cells therefore become more similar to a mesenchymal cell as opposed to the following cells which maintain an epithelial phenotype with intact intercellular contacts. Cell movement occurs due to coordination in the polar CRC cells' cytoskeleton generating traction [28]. In order for movement to be possible, ECM and BM remodeling is required [29]. Stromal-cell derived factor (SDF1/CXCL12), FGF and TGF-β family are factors known to provoke collective cell migration. A two-dimensional invasion by a monolayer of cells, three-dimensional invasion or detachment of a cell cluster from the primary tumor are, among others, all different possible types of collective cell invasion (**Figure 2**).

In order to pull cells at the front and push those at the back, it is crucial to generate traction force. This is done by integrins present in the tip cells such as β1 and β3 integrins expressed therein, which attach to constituents of the ECM such as fibronectin by focal adhesion complexes [27]. Leading cells also express $\alpha 2\beta 1$ integrins, which bind to collagen and $\alpha v\beta 3$ integrins, which attach to fibrin. Following cells also form lamellipodia underneath the cells at the front whose $\alpha 6\beta 1$ integrins attach to the BM formed by the invading leading cells paving the way. Integrin binding to the ECM causes cytoskeletal changes such as activation of contractin, talin, paxilin and vinculin, which are cytoskeletal adaptor proteins. This induces actin reorganization, which is important for the formation of filopodia. Pseudopodia are regulated by Rac while filopodia are regulated by Cdc42. On the other hand, Rho is mainly involved in individual rather than collective cell invasion [30].

ECM degradation and remodeling is crucial for paving the way for migrating cells and is highly dependent on ECM density, gap size, orientation and dimensions as well as on the migrating cell [22]. Leading cells produce membrane type 1 matrix metalloproteinase; MMP14 (MT1-MMP), which degrades the initial outlet for movement through the ECM, which is then widened by the following cells. The latter continue ECM degradation followed by the production and deposition of laminin, perlecan, nidogen 1 and collagen type IV [31].

2.4.4. Mesenchymal cell invasion after EMT

In carcinomas, mesenchymal cells mainly originate from clusters of epithelial cells, which have undergone EMT. They generally invade as single cells [27]. Mesenchymal cell migration is a five-step process starting with pseudopodia formation at the front of the cell, which initiates focal contact with the ECM. Then focal proteolysis is followed by the contraction of the actomyosin filaments to pull the cell forward, which is finally followed by detachment of the trailing end from the ECM in order for the cell to be pulled forward [32]. The mesenchymal cell is partially polarized due to reorganization of the cytoskeletal F-actin resulting in a front able to bind tightly to the ECM and a trailing end or a tail that contracts refraction fibers in order to move [33].

TGF-β and nuclear accumulation of Smad2 were found to be the primary culprit in the detachment and de-differentiation of single tumor cells from moving epithelial clusters. On the other hand, it was found that Smad2 was retained in the cytoplasm in collectively invading cells and non-moving cells. Interfering with TGF-β type II receptor hindered intravasation or hematogenous metastasis for individual mesenchymal cells but tumor cell clusters moving by collective invasion were still observed in the lymphatic system [30]. Therefore, this study showed that TGF-β is crucial for individual cell movement via activation of the EGF receptor, fibrinogen/angiopoietin-related protein (FARP), E3 ubiquitin-protein ligase (Nedd4), Myosin phosphatase Rho-interacting protein (M-RIP), Smad4 and RhoC. However, it is necessary that TGF-β is downregulated thereafter to allow for tumor cell adhesion and subsequent colony formation at distal sites [12].

2.4.5. Role of TME

EMT is promoted by TGF-β, HGF, FGF, endothelial growth factor (EGF) and insulin-like growth factor (IGF). Along with interleukin (IL)-1α, these factors act on the tip cells and activate collective cell invasion. TGF-β in particular plays a major role in invasion by inducing EMT, and myofibroblast formation, as well as by producing autocrine mitogens and targeting CD8+ T cells to evade the immune response [34].

MMPs are upregulated in most cancers and are related to enhanced tumor growth, angiogenesis, invasiveness and metastasis. They are secreted from tumor cells, leading cells from collective invading cells, myofibroblasts and immune cells. They are central in the degradation of ECM proteins, the cleavage of cellular adhesion molecules like E-cadherin and the activation of cytokines and growth factors [35].

Immune cells from the TME were found to play tumor-promoting functions as demonstrated the observation that chronic inflammation often leads to the development of cancer, as for chronic Hepatitis C infection which causes hepatocellular carcinoma by its sustained and persistent inflammation. NF-κB is secreted by tumor cells and tumor-associated immune cells. It induces expression of the inflammatory cytokines IL-1, IL-6, TNF and RANKL, which stimulate invasion and metastasis. Tumor cells can actively regulate the tumor microenvironment (TME) to shield themselves from the immune system as part of the cancer immunoediting process. They do so by secreting and expressing a variety of immunosuppressive molecules that downregulate or inhibit the immune system, such as TGF-β, thus converting

macrophages from an antitumor state to a pro-tumor state. Macrophages in the protumorigenic state help the growth and propagation of tumor cells by inducing ECM cleavage, tissue remodeling, angiogenesis, chemoresistance, and tumor-associated macrophage recruitment. Mutations in cancer cells may also result in the exposure of different epitopes on the surface of the cells, thus equally modulating the immune system [36].

The most relevant tumor-related cells in the TME are tumor-associated macrophages (TAMs) and cancer associated fibroblasts (CAFs) or myofibroblasts, both of which aid in tumor progression. TAMs stimulate metastasis while myofibroblasts re-organize the ECM to facilitate metastasis and epithelial tumor cell migration.

2.4.6. TAMS

TAMs or macrophages infiltrating the tumor bed are found to stimulate angiogenesis as well as tumor development and progression. Macrophages represent the largest percentage of the immune cell population present in the TME. Ideally as a part of the immune system, the macrophages should eradicate tumor cells and halt progression when activated correctly. However, macrophages were found to be mostly pro-tumoral in the TME. Their presence in the TME was significantly related to poor prognosis in several types of cancer such as breast, ovarian cancers, and lymphomas [37]. These two opposing states, pro and anti-tumoral, can be classified as M1 and M2. M1 or classically activated macrophages are generally anti-tumorigenic, expressing pro-inflammatory cytokines such as TNF-α, IL-6, IL-12, and inducible NO synthase (iNOS). However, M2 or alternatively activated macrophages have increased expression of anti-inflammatory cytokines such as IL-10 and IL-1 decoy receptor, which inhibits T effector cells. This classification is however simplified. It has been shown that within each polarization, macrophage activation states are heterogeneous calling forth further subdivisions, each initiated by different regulators and having different functions. In the liver, additional phenotypes for macrophages have been identified such as the one associated with hepatocarcinoma. These TAMs exhibit mainly the M2 phenotype and promote angiogenesis by increased expression of VEGF, they also facilitate matrix remodeling to accommodate angiogenesis by producing matrix metalloproteases [38].

Tumor cells secrete colony stimulating factor 1 (CSF1), also known as macrophage colony-stimulating factor (M-CSF), which recruits CSF1 receptor-expressing macrophages to the TME [39]. TAMs secrete enzymes that help degrade the ECM as well as various growth factors such as EGF, which promotes EMT and invasiveness. They also express Chemokine (C-C motif) ligand 18 (CCL18), which stimulates the clustering of integrin in tumor cells thus providing an anchor to the ECM and facilitating invasion and tumor cell motility. Furthermore, TAMs secrete MMP, produce cysteine cathepsins and serine proteases, which contribute to the re-organization of the ECM to facilitate metastasis. For instance, collective cell invasion is promoted by MMP2 and MMP9 secretion from immature myeloid cells at the leading edge of the invading cluster in CRC.

2.4.7. CAFs

CAFs play an important role in the TME by secreting various cytokines such as IL-8, or growth factors such as VEGF, or MMPs or chemokines such as chemokine (C-X-C motif) ligand 12

(CXCL12) which drive tumor growth, neoangiogenesis and local invasion [34]. They were found to communicate with tumor cells via the CXCL12/CXCR4 axis, which promotes tumor migration. More importantly, CAFs rearrange the ECM forming channel-like structures through which cancer cells may grow and invade the surrounding tissue without having to undergo EMT, thus maintaining their characteristic epithelial features. In a study, CAFs were incubated with supernatants from CRC cells, which led to marked activation of the TGF-β pathway [40]. As seen previously, TGF-β is a known pro-tumoral factor inducing metastasis, which is also secreted from CAFs upon interaction with the TME. The secreted TGF-β in turn stimulates CAFs for further TGF-β secretion in an autocrine loop.

CAFs are also the primary source of ECM and connective tissue formation, such as collagens I, III, IV, V, and XII, and proteoglycans [41]. CAFs treated with TGF-β in vitro showed an overexpression of collagen type I, fibronectin, urokinase type plasminogen activator (u-PA), MMPs such as MMP-2 and MMP-9, and tissue inhibitors of metalloproteinases (TIMPs) [40]. On the genetic level, tenascin-C and laminin-B1 were also upregulated when compared with normal colon fibroblasts [41]. CAFs produce more MMPs when compared to CRC cells, thus making it the major player in ECM remodeling. Interestingly, collagen type IV, which is the main constituent of the basement membrane, is degraded by MMP-2 and MMP-9, which thus facilitate mesenchymal invasion [40].

Co-culturing of spheroid CRC cells and CAFs in a collagen invasion experiment showed that the spheroidal CRC cells in contact with CAFs had irregular edges with both individual cell and collective cell invasion of the matrix as opposed to the smooth-edged control CRC spheroids [42]. In another collagen invasion experiment, CRC cells treated with CAFs supernatants were found to have a five-fold increase in matrix invasion as opposed to control CRC cells [43]. Treated cells were also found to have a more elongated shape compared to the control CRC cells. CAFs were indeed shown to over-produce FGF-1, which activates FGFR-3, a receptor tyrosine kinase, leading to local invasion and cellular migration [44]. The pro-invasion morphological changes induced in CRC cells upon contact with CAFs supernatant confirm the multi-functional role they play in metastasis [45]. A recent study showed that CAFs were able to induce metastasis at a very early stage in tumor development, even when the tumor was of microscopic size, in a small number of cancer cells which remained associated with CAFs upon entering the circulation, potentially forming micrometastatic niches in distal organs which are only visible upon progression [46]. These mechanisms that induce tumor cell invasion and migration may be an interesting target for novel therapies in order to inhibit metastasis.

2.4.8. Role of the ECM

The ECM in particular may be defined as the collection of molecules surrounding the cells in a certain tissue, generally structural proteins such as collagens and elastins, enzymes such as metalloproteinases, polysaccharides, glycoproteins, water, signaling molecules and ECM bound growth factors. The ECM is vital for maintaining tissue homeostasis, proper scaffolding and structure. It is characteristic and unique for each type of tissue. It is a dynamic pool of molecules constantly edited by the surrounding cells in order to cater for their needs. Furthermore, ECM is an important motor for tissue growth, healing and cellular differentiation as demonstrated by studies in the developmental biology field. ECM is not only altered

by the type of cells present in the tissue but also by their state such as inflammation, injury, etc. Tumor beds in general share characteristic abnormalities in their ECM such as disorganized and disrupted ECM with extensive and uncontrolled neoangiogenesis. These changes in the ECM may drive the progression and invasiveness of tumors.

The colonic epithelium in particular is composed of polarized cells with a characteristic apical membrane, forming a barrier with the components of the colon lumen, a basal membrane attached to the basement membrane and lateral membranes attaching to adjacent cells. The basement membrane (BM) is a distinct structure of the ECM composed mainly of collagen type IV rich in disulfide bridges conferring the BM its rigidity, and of laminin, fibronectin and proteoglycans. Collagen type I replaces collagen type IV from the BM in the stromal ECM, which does not form disulfide bonds, making it less stiff than the BM [47].

Generally, tumors share common features with the ECM of unhealed wounds such as increased stiffness and epithelial contractility. They are characterized by dense growth in connective and fibrous tissues known as *desmoplasia* following injury or BM degradation [48]. Degradation of the BM is currently considered a marker for CRC and carcinoma progression in general. It has been associated with higher metastasis rates and worse prognosis as well as reduced patient survival [49]. Local tumor invasion is driven by two main consecutive mechanisms: enzymatic breakdown of the BM and migration of the malignant epithelial cells through the cleared ECM. A decrease in lateral cell–cell adhesion molecules like E-cadherin [50] is expected to allow for malignant cell detachment and migration. However, it has been shown that CRC may migrate as collective sheets [51]. Along with the enzymatic cleavage of the BM by MMPs, metastatic clones undergo cytoskeletal changes and form cell protrusions such as pseudopodes in order to migrate across the BM towards the mesenchyma, thus initiating tissue invasion [52]. BM degradation actively contributes to the progression of CRC since the ECM initially binds and presents growth factors and other modulators to surrounding cells. Disruption of the BM then releases these signaling molecules lodged within it such as angiogenic factors, growth factors and chemokines [53] thus advancing tumor growth, metastasis, neoangiogenesis and modulation of the immune system to a pro-tumoral state.

Laminin, a glycoprotein abundant in the BM, binds to integrin in epithelial cells, controls cell adhesion to the ECM, interacts with cell-surface receptors and adheres to other laminins thus giving the BM its strength [54]. Laminin-332 is ubiquitous and unique to the epithelium and was thus hypothesized to play a role in carcinoma development. Indeed laminin-332 cleavage products were found to activate the endothelial growth factor receptor (EGFR) pathway known to drive tumor proliferation, loss of cellular adhesion to the ECM and boosting migration [57]. It is a heterotrimeric structure composed of α3, a β3 and γ2 chains. The α3 chain, specifically its large globular domain 3 (LG3 domain) interacts with $\alpha3\beta1$ integrin thus enhancing cellular migration, adhesion and spreading on the ECM. Laminin-332 also interacts and binds to $\alpha6\beta4$ and $\alpha6\beta1$ integrins regulating actin-cytoskeletal protrusion, cellular migration and tissue invasion [55]. Degradation of the α3 chain resulting in cleaved LG3 and 4 domains were shown to be over-expressed in carcinomas which activate phosphoinositide 3-kinase (PI3K) and matrix metalloproteinases precipitating tumor growth and invasiveness. This was reversed in vivo with the use of antibodies against this domain of the α3 chain [56]. In colon and breast cancer, γ2 chain cleavage by membrane type-1 MMP was found

to stimulate cellular migration [57]. The migration-inducing effects of laminin-332 cleavage products contrast with that of intact laminin-332 itself, which promote epithelial cell adherence to the BM. These opposing effects are hypothesized to alternate under the effects of MMPs cleaving the laminin, thus maintaining tissue homeostasis. In CRC, MMPs are secreted by the tumor cells and surrounding inflammatory cells causing a shift in laminin activity to its cleaved form's migration and invasion inducing effects. All of these cleavage products expose a repeat of an EGF-like domain in the short arm of laminin-332 and become ligands to cell-surface EGFR, thus activating its proliferative and anti-apoptotic pathway [58].

In addition the non-collagenous proteins bone sialoprotein II and osteopontin have been found to be involved in the regulation of the ECM proteins MMP-7 and 9. The inverse regulation of Hoxc8, Runx2 implicates that these genes may be regulated in a feed-back loop manner [59, 60].

The formed dense collagen reorganized by the overexpressed LOX and the various metalloproteinases has a different orientation in CRC compared to normal healthy tissue. The collagen fibers become radially disposed at the interface between the epithelium and the stroma at an angle of 50°C as opposed to 10°C in the healthy colon. This structural change helps tumor cells migrate along the steeply aligned collagen fibers, thus aiding in local invasion of adenocarcinoma cells beyond the epithelium to the mesenchyma transformation [61]. High grade dysplasia was found to hoard changes in the ECM, harboring a denser and ordered collagenous fibers deposition, which reinforces the importance of ECM changes in malignancy, tumor progression and local invasion [62].

2.5. Intravasation

2.5.1. Mechanism

Intravasation is the process of invasion of the tumor cells into the blood or lymphatic vessels. In order for tumor cells to make contact with endothelial cells, complex interactions are needed with proteins lipids and carbohydrates. Carcinoma cells release various mediators and growth factors such as VEGF or vascular endothelial growth factor to promote the process of forming new blood vessels, also known as angiogenesis. The newly formed blood vessels have a leakier endothelial membrane than normal blood vessels, which may aid the process of intravasation and metastasis [63]. The lymphatic vessels are known to be formed of a mono-layer of endothelial cells without intercellular tight junctions. They also lack a basement membrane and smooth muscle cells to cover the endothelial cell layer unlike blood vessels making them an easy target for invasion [64]. Tumor cells are known to release VEGF-A, which is a potent activator of vascular endothelial receptor 1 (VEGF-R1) and R2, known to induce angiogenesis as well as the release of VEGF-C and –D, which activate VEGF-R3 known to induce lymphangiogenesis oriented towards the tumor cells. In turn, lymphatic vessels are thought to secrete chemokines such as CCL21, which might attract invading tumor cells [64].

It remains a topic for debate as to whether tumor cells migrate actively towards the blood and lymph vessels or whether it is a passive migratory process. The fact that the newly formed tumor blood vessels are immature, lacking organization and intercellular junctions may support the claim that tumor cells simply grow through the fragile endothelium, forming

clumps in the lumen due to intravascular proliferation [65]. However, strong evidence exists to support active migration, such as the change in tumor cell expression of growth factors and their receptors influenced by TME components such as TAMs and CAFs. Transient TGF-β activation of TGF-β type 2 receptor/Smad4 induced EMT and stimulated their invasion of blood vessels whereas prolonged TGF-β activation hindered the process [30]. In the lymphatic vessels, on the other hand, invading cells were found to be organized solely in non-EMT clusters and independent of TGF beta signaling [66]. It is therefore safe to assume that both active and passive mechanisms occur in the intravasation process of tumor cells, requiring both EMT and non-EMT cells for hematogenous intravasation (**Figure 2**).

2.5.2. Survival of tumor cell against circulating immune cells and sheering forces

In order for metastasis to be successful, disseminated tumor cells have to survive the immune system as well as the shearing forces of the circulation. It was shown that disseminating tumor cells are shielded by adherent platelets which protect the cancer cells from shearing forces and natural killer cells, as well as aid in extravasation [67]. Disseminating tumor cells were found to express membrane-bound tissue factor, which is activated by coagulation factors such as VIIa and X. Proteinase activated receptor 2 (PAR2) is activated by the Tf-VIIa complex causing immunomodulation, angiogenesis and evasion of apoptosis in the tumor cells [68]. Elevated platelet count and concentration was found to be correlated in a clinical setting with lower survival in colorectal, breast and lung cancers while treatment with anticoagulants lowered metastasis in cancer patients [69].

2.6. Extravasation

2.6.1. Mechanism

Once circulating tumor cells (CTCs) have found their way into the lumen of the blood vessels, they will extravasate and attempt to invade foreign tissues. Knowledge of CTC extravasation is modeled after leukocyte migration across the endothelium into target inflammatory tissue [35]. The extravasation process maybe both, active or passive. Organ specificity or tissue tropism of certain carcinomas may be due to a number of factors. The vascular structure may be more favorable in certain organs such as in the bone marrow, which has a single layer of endothelial cells thus facilitating the movement of red blood cells in and out of the bone marrow. This route constitutes an easy target for CTC extravasation, making the bone marrow a preferred destination for metastasis of various carcinomas such as breast, gastric and prostate cancers [70].

In the case of CRC, the organization and arrangement of the circulation is the major determinant of CRC metastasis. CRC is known to have a strong tropism for metastasis to the liver although the cells themselves may be poorly adapted to the liver environment. However, the portal circulation, draining directly from the mesentery into the liver, transports millions of tumor cells over from the colon to the liver microvasculature, making liver metastasis possible although otherwise it would have been highly unlikely. In these cases, extravasation and homing into a certain tissue is passive and dependent of the organization of the circulation

as well as its structure [5]. However, active processes are also at work where CTCs bind to specific features in the organ of interest such as TIMP1, which drives CRC metastasis to the liver by stimulating the HGF pathway [71]. We may thus conclude that extravasation at a certain site or homing of CTCs is dependent on mechanical and organizational features of the circulation as well as on specific interactions between the tumor cells and the endothelium in question (**Figure 3**).

2.6.2. Passive extravasation

Passive extravasation entails no active migration involving molecular interactions between CTCs and endothelial cells. The endothelium often sheds endothelial cells from its wall creating an opening through which CTCs enter into the parenchyma of the invaded tissue. The CTCs may also proliferate inside of the lumen which causes an increase in size and ends with the rupture of the vascular wall, giving full access to the target organ [72]. Furthermore CTCs are known to associate with platelets for protection within the circulation, therefore damage in the blood vessel wall, exposing fibrinogen on endothelial cells, attracts platelets and tumor cells alike. Fibrin clots can further damage the blood vessels, which attract even more platelets in CTC to the site of injury. Furthermore tumor cells may also migrate into other tissues following the migration pattern of white blood cells within the circulation.

2.6.3. Active extravasation

Countless studies have shown that homing and extravasation might be more than simply a mechanical response to a faulty environment. TAMs were shown to secrete TNF-α, VEGF and TGF-β into the circulation to distal tissues where they activate tissue macrophage production of S100A8, which is a chemoattractant for CTCs [46]. CTC attachment to the endothelium is allowed by endothelial cell P- and E-selectins binding to tumor cells as well as tumor glycan patterns and adhesion molecule interactions such as integrin and CD44 interactions [73]. Overexpression of mucin carbohydrate is correlated with increased metastasis in CRC. Constituents of the ECM such as laminin and fibronectin may increase the arrest of

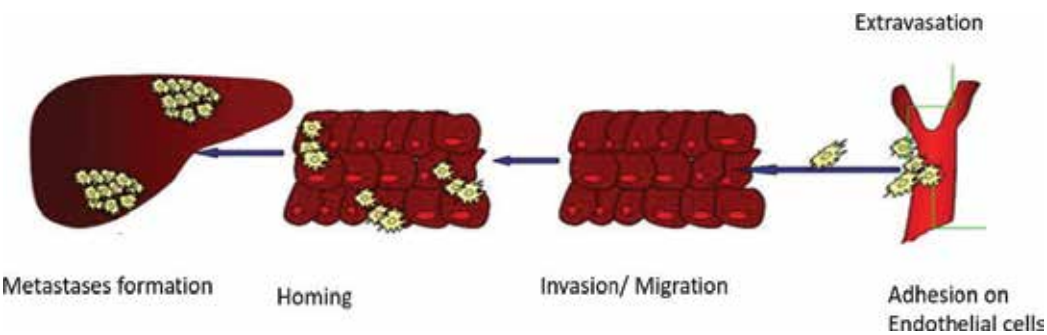

Figure 3. The tiny fraction of the disseminated tumor cells that were able to survive the previous steps must undergo extravasation, migration and homing into the distant organ with different histological nature. Adaption of the new environment demands further physiologic elasticity from the cells.

tumor cells at a certain site in the vasculature. However, when this interaction is counteracted by targeting peptides against fibronectin and laminin, it was found that it reduces the formation of metastasis. Circulating tumor cells may also increase vascular permeability and cause retraction of endothelial cells to expose the ECM for attachment by secreting VEGF which activates SRC (**Figure 3**).

2.6.4. Chemokines and colorectal cancer

Multiple interactions between tumor and stromal cells often contribute to the tumor progression. These interactions are mediated by a variety of growth factors, enzymes and chemokines. In this regard, chemokines play their role in three possible routes, which are enhanced proliferation by auto-or paracrine manner (a), modulating the immune response (b) or favoring the angiogenesis (c). Effects of chemokines in CRC are quite vital in terms of tumor growth and its metastasis. Many of the clinical studies have indicated the expressional changes of chemokines in CRC development and progression. CXC-chemokines are more important in this regard, as these chemokines have been shown to modulate the anti-tumor immune response, behavior of the epithelial cells and cross talk with the stroma. Chemokines are of vital importance in CRC metastasis, where chemotactic signals from different organs facilitate the directional migration of CRC cells, which lead to the metastatic state of the disease. In this context, several studies have highlighted the high expression of chemokine receptors (e.g. CXCR4, CXCR3, CCR6, CCR1 and CCRL2, CCR7 and CCR5) which favor the metastasis of CRC to lymph nodes and liver [47, 74, 75, 23].

2.7. Homing

For the immune response in host tissues, a focus is made on Kupffer cells in CRC liver metastasis. In the case of CRC metastasis to the liver, Kupffer cells (KC), which are the resident liver macrophages, have been reported to directly kill cancer cells through the secretion of cytotoxic molecules such as TNF-α, reactive oxygen species and enhancing the antitumor response of other immune cells such as T effector cells. They have also been reported to have a protumorigenic effect by producing signaling molecules such as cytokines and chemokines, which promote angiogenesis, ECM remodeling and cleavage and recruitment of TAMs. KC are the only characterized macrophages that exhibit dectin-2 receptor mediated cancer cell phagocytosis [76].

In a mouse model of colorectal cancer liver metastasis, KC were chemically depleted by gadolinium chloride before tumor induction and at a later stage of tumor growth, after liver colonization. Absence of KC at the early stage of liver metastasis and invasion led to an increase in tumor burden compared to mice with intact KC at the same stage of liver metastasis. Depletion of KC however in the later stages of liver colonization (18 days) decreased the tumor load with an increase in the number of activated cytotoxic T-cells (CD3+ T cells) and infiltrating cells expressing iNOS with a decrease in the number of VEGF-expressing infiltrating cells, as opposed to non-depleted animals. These results point towards a bimodal role of KC in liver metastasis and tumors, with an antitumor effect in early stages of metastasis, before tumor

establishment in the liver, and a protumorigenic effect at later stages of liver colonization and metastasis. Deeper understanding of the precise contribution of KC in CRC liver metastasis may be beneficial for timing immunomodulatory therapies [77]. It was also observed that the occurrence of CRC liver metastasis is rare in patients with cirrhotic livers. Upon closer investigation, it was found that a rat colon cancer cell line (RCN-9) pretreated with conditioned media of KC from cirrhotic rat livers and then inoculated into rat liver showed a reduced incidence of hepatic colonization. In vitro, RCN-9 cells were found to be sensitized to TIL-FasR-mediated killing after treatment with cirrhotic KC media by upregulation of FasR on RCN-9 cells [78]. This further confirms the versatility and importance of the role KC may play in tumor progression.

3. Antimetastatic treatment for CRC

3.1. Targeted therapies for metastatic detachment

To effectively eliminate metastatic cancer cells, it is suggested that both anoikis-dependent and anoikis-independent pathways should be targeted. Fortunately, many of the signaling pathways are already the targets of current FDA-approved therapeutic drugs such as bevacizumab (Avastin) against VEGF, ramucirumab (Cyramza) against VEGF receptor, cetuximab (Erbitux) and panitumumab (Vectibix) against EGF receptor [6].

However, there are significant molecular differences between tumors, which can affect both prognosis and response to treatment. Personalized medicine aims to tailor treatment according to the characteristics of the individual patient and is now a clinical reality as testing for *KRAS* mutations to guide treatment with the anti-EGFR monoclonal antibodies cetuximab and panitumumab is now part of routine clinical practice. However, not all patients who are *KRAS* wild type respond to anti-EGFR therapy and a validated biomarker for antiangiogenic therapy is still lacking. Therefore, other molecular biomarkers are needed to assist with predicting response to both existing drugs as well as to drugs currently under investigation [79].

3.1.1. Role of personalized medicine in metastatic detachment

Advances in the treatment of metastatic colorectal cancer have led to an improvement in survival from 12 months with fluorouracil monotherapy to approximately 2 years. However, there are significant molecular differences between tumors which can affect both prognosis and response to treatment. Personalized medicine aims to tailor treatment according to the characteristics of the individual patient, specifically for early metastatic prognosis and biomarker precision application to personalized treatments. In metastatic colorectal cancer an improved understanding of the underlying pathology and molecular biology has successfully merged with advances in diagnostic techniques and local/systemic therapies as well as improvements in the functioning of multidisciplinary teams, to enable tailored treatment regimens and optimized outcomes. Indeed, as a result of these advancements, median survival for patients with mCRC is now in the range of 20–24 months, having approximately tripled in the last 20 years.

3.1.2. Anti-epidermal growth factor receptor therapies in mCRC personalized treatment

The first true use of personalized medicine in mCRC was the clinical testing of *KRAS* mutations (which occur in approximately 45–50% of patients with CRC) [80]. Subsequently the anti-EGFR treatment is given only to patients who are *KRAS* wild type. However, not all patients who are *KRAS* wild type respond to anti-EGFR therapy and therefore there has been substantial research into other potential predictive biomarkers for future precision application [81].

3.1.3. BRAF mutation in personalized therapy of mCRC

After KRAS mutations, BRAF V600E mutations currently have the strongest evidence to support their use as a predictive biomarker for EGFR-targeted mAb activity. Overall, BRAFV600E activating mutations occur in approximately 10–15% of CRC tumors and are generally mutually exclusive to KRAS mutations [82]. Most but not all of the available evidence links BRAF V600E mutations with resistance to EGFR-targeted mAb therapy [83], however, the impact of tumor BRAF status on efficacy of these treatments has not yet definitively been addressed due to the relatively small number of patients with BRAF mutations.

3.1.4. The PI3K pathway in mCRC personalized therapy

The main alterations in the PI3K pathway in CRC are mutations in PIK3CA and loss of PTEN protein expression. These molecular alterations may coexist with KRAS and BRAF mutations and this makes it more challenging to ascertain their clinical significance [84]. However, PTEN loss correlates with advanced and metastatic tumors and has been associated with worse survival outcomes in CRC.

Several studies revealed that PIK3CA mutations or PTEN loss are associated with a lack of response to anti-EGFR therapies and these alterations therefore appear to have a negative predictive role [85].

Moreover, the personalized strategy has yet developed to be exon specific. For example, mutations in exon 20 of PIK3CA have been associated with a low response rate to anti-EGFR therapy, whereas mutations in exon 9 do not appear to have this effect, which leads to taking research on mutation correlation with the metastatic therapy more specific [86].

Huge advances have already been made, which can be exemplified by recent progress in the management of mCRC, particularly the discovery and implementation of *KRAS* as a predictive biomarker. Indeed, the implementation of new technologies is leading to the accumulation of huge amounts of genomic and proteomic data and the identification and validation of predictive biomarkers for existing and new targeted therapies and will likely improve patient outcomes in the future.

True personalized medicine in mCRC currently remains an aspiration for the future rather than a clinical reality. However, it is likely that a molecular screening approach to treatment will become increasingly used in the future to fully characterize tumors and identify patients who are most likely to benefit from targeted treatments. This holds great promise for the

improvement of patient outcomes but brings its own logistical and financial challenges as well as new complexities, such as how to overcome tumor heterogeneity, how to interpret a patient's molecular profile to select the most appropriate treatment and how to prevent rapid development of treatment resistance.

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Chapter 6

Prognostic Biomarkers for Breast Cancer Metastasis

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Additional information is available at the end of the chapter

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Abstract

Breast cancer treatment has improved rapidly through the years, starting from surgery, to hormonal therapy, to targeted therapy. Despite this, tumor metastasis remains the highest cause of breast cancer–related death. The current regime to deter metastasis is through adjuvant therapy, but such therapy frequently yields undesirable side effects. As such, prognostic markers for metastasis are important to stratify patients for adjuvant therapy so as to ameliorate the standard of living of patients with low metastatic potential. So far, only a few well-characterized prognostic biomarkers for metastasis are used in clinics. This chapter will cover both established and novel prognostic biomarkers for breast cancer metastasis and metastatic breast cancer prognosis. The potential of using these biomarkers as predictive biomarkers or new targeted therapy will also be discussed.

Keywords: metastasis, prognostic biomarker, metastatic breast cancer, relapse, recurrence, distant-free metastasis survival, overall survival

1. Introduction

Breast cancer remains the most frequently diagnosed cancer in women worldwide. In the United States (US), the American Cancer Society estimates that in 2018, the highest frequency of cancer diagnosed and the second highest cancer-related death in women will be breast cancer, at 30 and 14%, respectively [1]. Breast cancer survival statistics have improved tremendously over the years with a decrease of 39% mortality from 1989 to 2015 [1, 2]. This is mainly due to mammogram screening resulting in early detection and intervention [3, 4]. When diagnosed at a localized stage, the 5-year survival rate is 99% [5]. However, metastasis remains the major cause of mortality in breast cancer patients. Five-year survival rate decreases dramatically according to spread of cancer, with regional and distant metastasis

spreads at 85 and 27%, respectively [1]. Ten-year survival rate for stage IV metastatic breast cancer female patients is only approximately 13% [6]. These statistics indicate that metastasis is the major barrier against breast cancer eradication.

Breast cancer can be categorized largely into two types, *in situ* and invasive breast cancer [2]. *In situ* represents the subset in which the cancer is still confined within the transformed origin. Ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) are the two main types of frequently diagnosed *in situ* breast cancer at 83 and 13%, respectively [2]. DCIS, as its name suggests, refers to cancer originating from the epithelial cells of the breast ducts, whereas LCIS arises from the lobules of the breast. On the other hand, majority (80%) of breast cancer will become invasive, i.e., that they will outgrow into surrounding breast tissue [2].

The primary treatment for *in situ* breast cancer is surgery. This includes lumpectomy where only the tumor and surrounding tissues are removed or mastectomy in which the entire breast is removed [2]. Very often, radiation and adjuvant therapy are recommended after surgery to prevent recurrence and to eliminate breast tumor cells which might have spread [2]. Examples of adjuvant therapy are cytotoxic chemotherapy, hormone therapy, and targeted therapy [2, 3]. These will be discussed in more detail in the later sections.

Although adjuvant therapy has been shown to be beneficial in preventing metastatic recurrence, the patient's quality of life is severely affected in many cases. Side effects include fatigue, osteoporosis, increased thromboembolic events, premature menopause, weight gain, and mild memory loss [7, 8]. Although as many as 80% of patients receive adjuvant treatment, only 40% of them relapse and die from metastatic breast cancer indicating that majority of patients are over-treated and suffer unnecessary side effects [3]. In addition, only 15% of patients treated with tamoxifen after surgery will have distant recurrence, indicating that 85% of patients will be overtreated if chemotherapy is mandatory [9].

One solution to overcome unnecessary treatment is through identifying patients with high and low risk of metastasis using prognostic biomarkers. Current established metastasis biomarkers are available but have poor predictive power [10]. With new concepts such as gene expression profiling and circulating tumor cells, new prognostic markers with greater accuracy could be identified. The following sections will describe established markers as well as new upcoming markers for the prediction of survival, metastasis risk, and recurrence risk for metastatic breast cancer. Current and potential use of these markers in the clinic as predictive biomarkers for treatment and as potential targets is also discussed.

2. Established biomarkers

2.1. Tumor size and lymph node status

The tumor-node-metastasis (TNM) staging system is commonly used to stage breast cancer progression during initial diagnosis [11]. It constitutes a manner to measure the aggressiveness of the cancer. The abbreviations represent different characteristics of the cancer. "T" represents tumor size, "N" indicates the number of lymph nodes that the cancer has spread to,

and "M" conveys the presence of distant metastasis [11]. In the absence of distant metastasis ("M"), tumor size and lymph node status are established prognostic markers for likelihood of metastasis. Specifically, primary tumors that are less than 2 cm have low prognosis for developing into metastatic breast cancer. Tumor sizes of 2–5 cm and more than 5 cm have a high and very high likelihood of progressing into metastatic cancer respectively [12–15]. Likewise, breast cancer patients with no detectable lymph node metastases are at low risk of distant metastasis. Patients with presence of lymph node metastasis have high risk of metastasis, and more than four lymph node metastases represent a high probability progressing to distant metastasis [12–15].

2.2. Histological grade

Histological grade is the determination of how differentiated a tumor is. As the histological grade increases, the tumor appears more poorly differentiated [16]. The determination of histological grade is performed by a trained pathologist using certain characteristics of the cancer tissue section such as mitotic count, extent of tubule or gland formation, and nuclear pleomorphism [16]. Histological grade 1 tumors have low risk of metastasis, while grade 2 and 3 tumors have intermediate- and high-risk tumors [12, 14, 17]. Integrating histological grade with the aforementioned tumor size and lymph node status, several prognostic indices such as the Nottingham Prognostic Index, the Kalmar Prognostic Index, and the St. Gallen guidelines have been established and used in clinics to aid in adjuvant treatment decision [16].

2.3. Angioinvasion

Angioinvasion is the presence of blood vessel invasion by cancer cells. In lymph node negative patients, angioinvasion has some prognostic value in predicting metastasis [18, 19]. In particular, tumor emboli in more than three blood vessels suggest a high risk of metastasis [18, 19]. Although these tumor characteristics (tumor size, lymph node status, histological grade, and angioinvasion) represent a simple and cheap method to predict metastasis, statistics show that these prognostic factors only accurately predict metastatic outcome in 30% of patients [3, 16]. As such, better prognostic markers are required for the remaining 70%.

2.4. Molecular subtype

Invasive breast cancer can be divided into four main molecular subtypes based on the presence of hormone receptors (estrogen or progesterone) (HR) and human epidermal growth factor receptor 2 (HER2) [2]. The four subtypes are luminal A (HR+/HER2−), luminal B (HR+/HER2+), HER2 enriched (HR−/HER2+), and triple negative (HR−/HER2−) at frequencies of 71, 12, 5, and 12% of all invasive breast cancer, respectively [2].

In general, luminal A and B subtypes are associated with the most favorable prognosis and least aggressive, followed by HER2 enriched and triple negative sequentially [2, 20–23]. In a 6.9-year follow-up study on patients who were initially diagnosed with localized breast cancer, the frequency of distant metastasis increases progressively in the following order, luminal A (6.4%), luminal B (12.1%), HER2-enriched (19.2%), and triple negative (27.4%) [24]. Another

study involving metastatic breast cancer found that luminal A exhibited the longest survival rate (34.4 months), followed by luminal B (24.8 months), HER2 enriched (19.8 months), and triple negative (8.8 months) [25]. Similarly, follow-up on patients with early stage breast cancer found that survival with distant metastasis showed similar patterns [26]. Luminal A survived the longest duration (2.2 years), followed by luminal B (1.6 years), HER2 enriched (0.7 years), and triple negative (0.5 years) [26]. These findings strongly suggest that molecular subtype correlates with metastasis rate and survival and can be used as a prognostic marker for metastasis.

In fact, molecular subtypes are already routinely used in clinics as prognostic and predictive biomarkers for overall survival and stratification of patients to targeted therapy [2]. As a predictive biomarker, patients who are either HR+ or HER2+ benefit majorly due to targeted therapy options. The estrogen receptor (ER) in breast cancer plays an important tumor promoting role by activating downstream intracellular signals for proliferation and survival [27]. As such, patients with ER+ breast cancer are routinely treated with selective estrogen receptor modulators (SERMs) [8]. Tamoxifen is the first approved SERM to be used for the treatment of ER+ metastatic breast cancer [8]. Five-year use of tamoxifen as an adjuvant significantly reduced local and distant recurrences by 40–50% making ER status both a prognostic and predictive biomarker [28].

Apart from using SERMs, aromatase inhibitors (AIs) are an alternative targeted treatment for ER+ breast cancer patients. These inhibitors function to block estrogen production by inhibiting the aromatase enzyme [8]. Studies have found that there is no difference in efficacy and time to distant recurrence when compared to tamoxifen treatment [29]. In addition, both tamoxifen and AI treatment are associated with increased overall survival and distant metastasis-free survival [30]. Since ovaries are the main source of estrogen, ovarian surgery, ovarian irradiation, or ovarian suppression by drugs have been shown to improve therapeutic outcomes [2, 31]. Particularly, in a clinical study, 5-year disease free survival was as high as 91.1%, when ER+ premenopausal women were treated with adjuvant ovarian suppression combined with AI treatment [31]. Additionally, use of AI with ovarian suppression significantly decreased recurrence as compared to tamoxifen with ovarian suppression [31]. These evidences strongly illustrate the use of ER status for metastatic survival prognosis and for tamoxifen or AI adjuvant therapy decision.

Historically, HER2-enriched metastatic breast cancer is associated with high aggressiveness and has a poor prognosis [2, 32–34]. That is until the first anti-HER2 targeted therapy Trastuzumab clinical trial emerged, which showed improved clinical outcomes by including Trastuzumab into adjuvant treatment with chemotherapy for HER2-enriched metastatic breast cancer [35]. Trastuzumab is a monoclonal antibody which binds and targets the extracellular portion of the HER2 receptor protein [8]. Specifically, combining Trastuzumab with standard chemotherapy for HER2-enriched metastatic breast cancer resulted in increased time to progression, overall survival, and duration of response [35, 36].

In recent years, many new biologics targeting HER2 have been approved for advanced metastatic HER2-enriched breast cancer [37]. Pertuzumab, another monoclonal antibody which inhibits HER2 dimerization, is approved for use in combination with trastuzumab

in metastatic HER2+ breast cancer [38]. Treatment of HER2-enriched metastatic breast cancer patients using a combination of Pertuzumab, Trastuzumab, and Docetaxel resulted in a significant increase in median overall survival of 15.7 months as compared to just treating with Trastuzumab and Docetaxel [38]. Increase in progression free survival and duration of response by 6.3 months and 7.7 months, respectively, were also noted when Pertuzumab was used in combination [38].

Another recently approved biologic for HER2+ advanced breast cancer is the antibody-drug conjugate T-DM1 [39, 40]. It involves the ingenious exploitation of Trastuzumab's specificity to HER2+ breast cancer cells to deliver the linked cytotoxic microtubule-inhibitory drug DM1 directly to HER2+ cancer cells [39, 40]. It is particularly effective in slowing disease progression for HER2+ advanced breast cancer patients who were initially treated with first line Trastuzumab/Taxane combination [39]. Progression free survival when treated with T-DM1 was significantly longer (9.6 months) as compared to the standard second line treatment (6.4 months) [39]. Overall survival improved significantly from 25.1 to 30.9 months [39]. Additionally, patients treated with T-DM1 experienced less toxicity as compared to the standard second line treatment [39]. Taken together, it is recommended that the Docetaxel/Trastuzumab/Pertuzumab combination be used as a first line choice and T-DM1 as a second line therapy [37].

The importance of HER2-targeted therapy for metastatic breast cancer is further emphasized by the finding that as many as 16% of initially HER2 negative breast cancer exhibits HER2 expression upon metastasis [37]. This indicates that HER2-targeted therapies could be extended to treat metastasis in this select group of patients, and it is recommended in clinics that HER2 status in metastatic cells be tested by fluorescence in situ hybridization or immunohistochemistry staining to evaluate eligibility for HER2-targeted therapy regardless of initial subtype of the primary tumor [37]. Overall, these findings highlight the importance of using HER2 status as both a prognostic and predictive biomarker in clinics for metastatic breast cancer.

As for triple negative cancers which do not currently have their own targeted therapy, neoadjuvant anthracycline-based chemotherapy has been found to benefit this group [41]. Clinical response in triple negative was 85% as compared to luminal (47%) or HER2 positive (70%), and all subtypes had equally good prognosis after treatment [41]. However, this only applies to triple negative patients who exhibited pathologic complete response from treatment, which constitutes only 27% [41]. As such, for majority of triple negative patients who do not display complete pathologic response after chemotherapy, more studies need to be done to discover targets specific against triple negative breast cancer.

In addition to metastasis frequency and survival, molecular subtypes could potentially predict distant metastasis tumor sites and distant relapse sites. With the exception of triple negative basal subtype, bone metastasis is the most common metastasized site among all subtypes, with the luminal subtypes displaying the highest frequency [26, 42]. Correspondingly, bone is the most frequent metastatic relapse site, with luminal subtypes exhibiting the highest frequency [43, 44]. The subtypes with the highest brain and lung metastasis rates are HER2 enriched and triple negative [26]. Among all subtypes, metastatic lung and brain relapse are

highest for triple negative [43, 44]. HER2-enriched subtype has the highest liver metastasis rate, whereas triple negative has more distant lymph node metastasis as compared to other subtypes [26, 42]. The importance of determining site of metastasis is covered in the next section.

2.5. Site of distant metastasis

The significance of predicting site of metastasis for metastatic breast cancer patients is highlighted by the intrinsic correlation with overall survival and survival after distant recurrence. In the following order, breast cancer patients with single site brain, lung or liver, and bone metastasis have the worst to best prognosis [42, 45]. Median overall survival rates for patients with brain, lung, liver, and bone metastasis are 11 months, 30 months, 31 months, and 41 months, respectively [42]. In addition, the survival trend holds true when patients are stratified based on HR indicating that it is independent of HR status [45].

Postmetastasis distant recurrence is also associated with the site of recurrence. In particular, first visceral (including brain) site recurrence is associated with a poorer prognosis as compared to first bone recurrence with 3-year breast cancer specific survival (BCSS) rate at 13 (visceral) and 23% (bone), respectively [46]. When compared to recurrences closer to the primary tumor, first local and first lymph node recurrences 3-year BCSS are significantly higher at 83 and 33%, respectively, indicating that metastatic site proximity to primary tumor origin site is also strongly linked to prognosis [46].

Apart from the site of distant metastasis, the number of initial metastatic sites is also prognostic of survival. Patients with multiple metastatic sites have significantly poorer overall survival than patients with single metastatic site in both HR+ (9 months) and HR− (5 months) patients [45]. Multiple metastatic sites are also more prone to occur in HR− patients, which are in line with the poorer prognosis of HR− patients [45].

2.6. Age of diagnosis

Indubitably, as with many diseases, age is a major determinant of prognosis in metastatic breast cancer. Survival rate in stage IV invasive breast cancer patients significantly decreases with age [6]. Ten-year breast cancer specific survival rates for three groups of stage IV patients namely, below the age of 40 years, between 41 and 50 years, and between 51 and 70 years, drops from 15.7 to 14.9% to 11.7%, respectively [6]. Likewise, another study found similar trends in metastatic breast cancer patients, where overall survival decreases significantly with age, from 32 months (age < 50 years) to 25 months (50–69 years) to 16 months (>69 years) [47]. One plausible explanation is that younger patients are more physically fit to endure treatment than elder patients, and this is supported by a significantly higher rate of surgery and radiation therapy underwent by patients below 69 years [47].

Age is also a determinant in the prediction of distant metastasis site. In accordance with the age-related survival trend, frequency of the deadlier lung metastasis increases significantly with age from 5.9% (age < 50 years) to 7.6% (50–69 years) to 14.2% (> 69 years), respectively [47]. Correspondingly, a significantly lower rate of the less lethal distant lymphatic metastasis

is observed as age increases with rates at 7.3, 5.4, and 4.0% for patient age of less than 50 years, 50–69 years, and more than 69 years, respectively [47]. Metastasis to the brain, liver or bone is not dependent on age [47]. Interestingly, multiple metastatic sites, which are associated with poorer prognoses, occurred more frequently in younger patients (<69 years) than in older patients (>69 years) at approximately 34.9 (age < 50 years) and 36.2% (50–69 years), and 28.3% (>69 years), respectively [47]. This discrepancy could be explained by the higher rate of treatment in younger patients, suggesting that patients with multiple metastatic sites could benefit from surgery and radiation therapy [47]. Overall, age at diagnosis is a strong independent prognostic factor for metastatic breast cancer patient survival and for predicting the site of metastasis [47].

2.7. Urokinase-type plasminogen activator (uPA) and plasminogen activator type 1 inhibitor (PAI-1)

The urokinase-type plasminogen activator (uPA), which is a serine protease, and its inhibitor plasminogen activator type 1 inhibitor (PAI-1) are involved in the degradation of extracellular matrix, which is a crucial process in the initial stages of metastasis [48]. Although PAI-1 inhibits uPA activity, it has been found to promote tumor invasion and angiogenesis through other means [49]. As such, both uPA and PAI-1 could potentially be used as metastasis prognostic markers. Supporting this, high uPA and PAI-1 levels are correlated with lower metastasis-free survival and overall survival in breast cancer patients [50–54]. In addition to being a prognostic marker, both uPA and PAI-1 could be used as predictive biomarkers for adjuvant therapy. In a study, patients with high uPA and PAI-1 levels benefited significantly from adjuvant chemotherapy compared to patients with low uPA and PAI-1 levels [55]. This indicates that patients with high uPA and PAI-1 levels could be treated with chemotherapy after surgery.

Furthermore, since uPA functions by binding to its receptor, urokinase plasminogen activator receptor (uPAR), the interaction could be exploited for metastasis targeted therapy. Indeed, one of the developments in this area is the use of an antibody to target uPAR [56, 57]. Remarkably, the antibody is shown to inhibit invasion of cancer cells and induce apoptosis, indicating its potential use for metastatic breast cancer [57].

2.8. Gene expression profiling

With the advent of gene expression profiling, treatment options are expected to shift toward a more personalized approach [58]. Although the idea of sequencing every cancer patient for individualized prognosis and treatment remains elusive, using multigene signatures to stratify patients into groups with different prognosis and therapeutic options has been very well established and routinely used in clinics. The first report of using high throughput methods for stratification of patients started in diffuse large B-cell lymphoma (DLBCL) patients [59, 60]. Based on their gene signatures from microarray, two subtypes of DLBCL, namely germinal center B-like DLBCL and activated B-like DLBCL, were characterized and were prognostic of overall survival [59]. In fact, molecular subtypes of breast cancer (mentioned in previous sections) were also identified using microarray-based gene expression profiling and are routinely used in clinical settings for prognosis [20–23].

Following the clinical success of using multigene signatures to identify and stratify patients with different clinical outcomes, two different gene expression profiling panels have emerged and are currently used in clinical settings. Each platform relies on different gene panel and is routinely used for predicting metastasis risk, local and distant metastasis recurrence, and for treatment decisions [61]. These are Oncotype DX and MammaPrint. Other gene expression profiling panels such as the PAM50 [62], two-gene expression ratio [63], and MapQuant DX [64] are not covered here [65].

Oncotype DX is the most widely used multigene panel tool for the prediction of distant recurrence risk in the United States [2]. It is a reverse-transcription-polymerase-chain-reaction (RT-PCR) based assay which measures the expression of 16 cancer-related genes and 5 reference genes in tumor tissue [9]. Based on the expression level of the 21 genes, an algorithm computes a recurrence score which quantifies the probability of distant recurrence [9]. Using the recurrence score, a patient with higher score is considered high risk and would likely benefit from chemotherapy as compared to a patient with lower score who could avoid chemotherapy altogether [2, 9].

Oncotype DX is currently utilized in clinics to predict distant recurrence for ER+, lymph node negative breast cancer patients who had prior tamoxifen treatment [9]. In the original paper, 51% of ER+, lymph node negative, tamoxifen-treated patients were classified under low-risk, and indeed, only 6.8% of this group had distant recurrence within 10 years [9]. This is in comparison with the high-risk group consisting of 27% of patients who had a 30.5% distant recurrence rate within 10 years [9]. In addition, recurrence score could also predict overall survival and relapse-free survival [9]. In support, a recent prospective validation study showed that Oncotype DX could potentially select low recurrence patients with high probability to forgo chemotherapy [66]. Five-year recurrence free rate from all sites and distant site, for patients with low recurrence score and only underwent tamoxifen treatment, were a high 98.7 and 99.3%, respectively [66]. Overall survival and invasive disease-free rates were up to 98 and 93.8%, respectively [66]. These findings show the clinical applicability of Oncotype DX to select for patients who could forgo chemotherapy and its unnecessary side effects.

The second most commonly used multigene panel for prognosis and treatment decision is MammaPrint [65, 67]. It utilizes an oligonucleotide microarray to measure the expression of 70 genes to identify gene signatures that stratify patients into a “good” or “poor” prognosis that predicts metastasis risk in lymph node negative early breast cancer patients [68–72]. Sensitivity and specificity of MammaPrint are 91 and 73%, respectively [3]. Genes involved in “poor” prognosis signature include angiogenesis, cell cycle, invasion, and metastasis [69].

In one of the earlier studies depicting the prognostic value of MammaPrint patient stratification, 10-year distant metastasis free probabilities were lower for “poor” prognosis group at 50.0% than in “good” prognosis group at 85.2% [68]. Overall survival rates were also significantly different between groups at 50.6 and 85.2% for “poor” and “good” prognosis groups correspondingly [68].

In a follow-up study with longer term survival statistics, 25-year distant metastasis free survival was significantly lower for “poor” prognosis group (41.6%) as compared to “good”

prognosis group (60.4%) [72]. Overall survival at 25 years also showed the same trend at 44.5 and 57.3% for "poor" and "good" prognosis groups, respectively [72]. These statistics show the relevance of using MammaPrint in clinical settings as a prognostic biomarker for metastasis risk and overall survival. It could also be applied as a predictive biomarker for selecting patients with "poor" prognosis for adjuvant treatment.

Overall, the importance of multigene expression profiling tools for prognosis and treatment decision in the clinic is apparent when compared to classical clinicopathological parameters which are determined by a trained pathologist. It is found that low agreement exists among pathologists in breast cancer grading as tumor grading includes a degree of subjectivity [9]. A study comparing the different gene expression profiling tools found that although different gene sets were used among different panels, 4 out of 5 (including Oncotype DX and MammaPrint) achieved high concordance in relation to predicting outcomes [73]. As such, tools like Oncotype DX and MammaPrint stand out in this aspect.

3. New biomarkers

3.1. Improved gene expression profiling

Although the first generation gene expression profiling tools, Oncotype DX, and MammaPrint have greatly advanced the prognosis of breast cancer patients for metastasis risk and distant recurrence, a major drawback is that they are unable to accurately predict late distant recurrence of more than 5 years [74]. Two newer gene expression profiling tools, EndoPredict and The Breast Cancer Index, have emerged successful in this aspect [74].

EndoPredict is a RT-PCR-based assay, which measures the expression of eight cancer genes and three housekeeping genes to stratify patients into high- and low-risk distant recurrence groups [75–77]. A newer version of it combines the 11 gene expression with tumor size and nodal status to calculate a risk score termed the EPclin [76]. Patients with an EPclin score of less than 3.3 are classified as low-distant recurrence risk, while more than or equals to 3.3 are classified as high-distant recurrence risk [76]. The EPclin score is the best predictor of late relapse (>5 years), when compared to the earlier version of EndoPredict or to nodal status and tumor size alone [76]. Metastasis free survival for short term (less than 5) and long term (5–12 years) are also significantly different between the EPclin-stratified high- and low-risk groups, validating its applicability to predict metastasis [76]. In addition, distant recurrence free rates at 10 years in EPclin low-risk group is higher than in EPclin high-risk group, at 98.20 and 87.69%, respectively, depicting its ability to predict distant recurrence for longer time frames [76].

The breast cancer index (BCI) is another RT-PCR-based assay, which combines two independent biomarkers namely a set of five cell cycle genes and the HOXB13 and IL17BR gene ratio to determine the recurrence probability of early stage ER+, lymph node negative breast cancer patients [63, 78, 79]. Independently, both the five gene panel and the two gene ratio are associated with distant metastasis free survival rates [78]. However, when combined, three

groups could be formed, low-, intermediate-, and high-risk groups, which are significantly predictive of metastasis occurrence [78]. Ten-year distant metastasis free survival for low-, intermediate-, and high-risk groups are 98, 87, and 60%, respectively [78]. Additionally, in a study comparing the prognostic ability of BCI with Oncotype DX and another gene panel, BCI emerged as the only test capable of significantly predicting both early and late distant metastasis recurrence, whereas the other two were only able to predict early recurrence [80].

In general, both EndoPredict and BCI seem to be superior as compared to the first-generation counterparts. This is particularly in terms of predicting longer term distant recurrence while also predictive of early recurrence [74].

3.2. Circulating tumor cells

An essential part of distant metastasis requires cells from the primary tumor to migrate into the bloodstream to spread throughout the body till it finds a secondary site to establish a secondary tumor [3]. As such, it is not surprising that circulating tumor cells (CTCs) in peripheral blood could be utilized as a prognostic indicator. The peripheral blood also represents an easily accessible region, which is an added advantage of using CTCs for prognosis [81].

The history of CTCs dates back as far as the nineteenth century when researchers have just begun to study the concept of tumor cells shedding from primary tumor [82, 83]. Today, there are multiple platforms for the isolation and detection of CTCs in the peripheral blood in clinical use [84]. Termed the "golden standard", the CellSearch system is the only FDA-approved platform for such purpose in breast, prostate, and colorectal cancer [84]. The problems associated with detection of CTCs are its rare amount in the peripheral blood and the absence of a universal surface marker for different cancer cell types [84]. The CellSearch system overcomes these sensitivity and specificity issues through the use of an antibody to capture CTCs and poststaining the captured CTCs for identification [84]. Specifically, an avidin-biotin anti-EpCAM antibody complex is used to bind CTCs followed by a magnetic capture to isolate CTCs [84–86]. Following which, the captured pool of cells is stained with DAPI and cytokeratins CK8, CK18, and CK19 to select for nucleated and epithelial cells, respectively [84–86]. Additionally, to differentiate from circulating white blood cells, anti-CD45 is used to further isolate CTCs [84–86]. Other systems using size [87–89], density [90], and microfluidic [91] characteristics of CTCs for isolation exist but are not covered in this chapter [81].

Enumerating CTCs in peripheral blood holds immense potential in clinics. The early paper using the CellSearch system to study progression of metastatic breast cancer provided many useful information [86]. The first thing noted was that CTCs were only present in metastatic breast cancer patients. Two or more CTCs per 7.5 mL of blood were present in metastatic breast cancer patients, while CTCs were rare (less than or equal to 1) in both healthy and benign breast cancer women [86]. Next, CTCs were independent prognostic marker of overall survival and progression-free survival [86]. After new treatment, patients with high level of CTCs (CTCs $\geq$5) had a significantly lower median progression-free survival and overall survival than patients with low level of CTCs (CTCs <5), at 2.1 months versus 7.0 months for progression-free survival and 8.2 months versus >18 months for overall survival, respectively [86]. Additionally, the prognostic value of CTCs for overall survival and progression-free

survival is also validated in two other studies, wherein one of it is a prospective study with metastatic breast cancer patients who were not treated previously [92, 93].

In terms of treatment, median progression-free and overall survival differed significantly between patients with CTCs ≥5 before treatment and CTCs <5 after treatment (1st group) and patients with decrease in CTCs after treatment but still ≥5 after treatment (2nd group) [86]. Median overall survival and progression free survival for 1st group are 7.6 months and 14.6 months, and for the 2nd group, 2.1 months and 9.2 months, respectively [86]. This suggests that CTCs could potentially be used to measure treatment efficiency, although the authors cautioned against this interpretation [86]. Following this, two other studies have also observed CTCs as a predictor of therapy efficiency for metastatic breast cancer [94, 95].

3.3. TIP60

Tat-interactive protein 60 kDa (TIP60) is a haploinsufficient tumor suppressor involved in both early and late stage breast cancer [96–99]. In particular for late stage progression, TIP60 is known to regulate epithelial to mesenchymal transition, an important pathway for cellular metastasis [96, 97]. Both *in vitro* and mouse models have shown that loss of TIP60 results in increased metastatic breast cancer cell migration and invasion, indicating that therapies to increase TIP60 in breast cancer could be a potential therapeutic approach for metastatic breast cancer [96, 97]. Additionally, the microRNA miR-22 inhibits the expression of TIP60, thereby making it a promoter of metastasis and a potential therapeutic target for metastatic breast cancer [97]. More importantly, both miR-22 and TIP60 are prognostic of overall survival and metastasis free survival [96, 97]. As such, both miR-22 and TIP60 expression levels would be invaluable tools in clinics to predict metastatic probability and prognosis of overall survival.

4. Future directions/conclusions

Using only clinicopathological characteristics for assessing metastasis risk, the St Gallen criteria and National Institutes of Health criteria each classified a low 15 and 7% of lymph node negative breast cancer as low metastasis risk, respectively [3, 68]. However, after 10 years, up to 25% of the low-risk group developed distant recurrence [3, 68]. In addition, only slightly less than half (45%) of high-risk patients developed metastasis, indicating that the remaining 55% of "high-risk" patients had adjuvant treatment and tolerated its unnecessary effects [3, 68]. In contrast, using MammaPrint in the same cohort, a high 60% of total patients were categorized as low risk, out of which only 13% of these low-risk patients developed metastasis after 10 years, showing that as many as 52.2% of overall patients were safely spared from adjuvant therapy, as compared to 5.25 to 11.25% of overall patients stratified using clinicopathological characteristics [3, 68]. These findings clearly delineate the superiority of gene expression panels for prognosis as compared to just clinicopathological characteristics.

A more beneficial solution would be to combine both clinicopathological markers and multigene expression profiling to have an additive or synergistic effect in prediction for the betterment of patient prognosis and prediction of treatment outcomes. An example is the multigene

expression EPclin risk score which combines its predecessor, the EndoPredict with tumor size, and nodal status to better predict distant metastatic recurrence as compared to if the markers were used individually [76].

However, a significant problem still exists in the field of prognosis for metastatic breast cancer. Many of the gene expression profiling tools, such as Oncotype DX and BCI, are suitable only for prediction of distant metastasis recurrence in ER+, lymph node negative metastatic breast cancers [74, 100]. As such, it represents a gap in the identification of prognostic markers for other subtypes. For this, newer markers like the detection of CTCs which does not discriminate between subtypes may be used. TIP60 which also does not discriminate between subtypes in risk-free survival rates [98] may be explored and could potential be used as a prognostic biomarker for breast cancer metastasis. Other upcoming biomarkers which are not discussed here such as blood-based biomarkers [101] and long noncoding RNA [102] also holds immense potential as prognostic markers in breast cancer metastasis.

Overall, the field of metastatic breast cancer prognosis has come a long way, beginning with clinicopathological markers to molecular subtypes to multigene expression profiling and eventually CTCs. Continuing on, it is likely that future direction for this field will entail combining existing biomarkers together or with newly identified biomarkers, leading to tremendous improvements in metastatic breast cancer prognosis and in predicting metastasis.

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Chapter 7

Ovarian Clear Cell Carcinoma: Metastatic Pathways

Chrisostomos Sofoudis

Additional information is available at the end of the chapter

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Abstract

Ovarian carcinoma reflects the biggest challenge among the field of gynecologic oncology. It represents the most common death cause of genital carcinomas throughout years. The major classification consists of epithelial and non-epithelial types. Due to the histologic origin, epithelial types of ovarian carcinoma are endometrioid, serous-mucinous, and clear cell types. Due to intense metastatic infiltration and rapid tumor spread, clear cell ovarian carcinoma constitutes type of lesion with the most poor prognosis, decreased overall survival, decreased free survival, and poor quality of life of the patient. The metastatic infiltration is strongly accompanied with all significant prognostic factors. All biochemical pathways at the time of the infiltration are correlated with tumor size, lymphatic spread, staging of the lesion, histologic type, and grade of differentiation of the lesion.

Keywords: clear cell, chemotherapy, debulking, metastasis, ovarian carcinoma

1. Introduction

According to current literature, ovarian cancer represents a high mortality neoplasm in gynecologic malignancy. The 2017 incidence estimates 22,400 new cases in the United States [1]. The increased mortality rate is strongly accompanied with staging of the lesion at the time of the diagnosis. Many predisposition factors influence the therapeutic mapping. Age of the patient, parity, staging, cluster of differentiation, surgical margins, and lymphatic infiltration consist the gold standard of therapeutic strategy (**Figure 1**).

The frequency of the lesion increases in ages between 55 and 65 years old. There are also studies implicating younger or older patients. The lesion is more frequent in developed countries of the Western World and less in Asian countries [2]. Ovarian neoplasms express

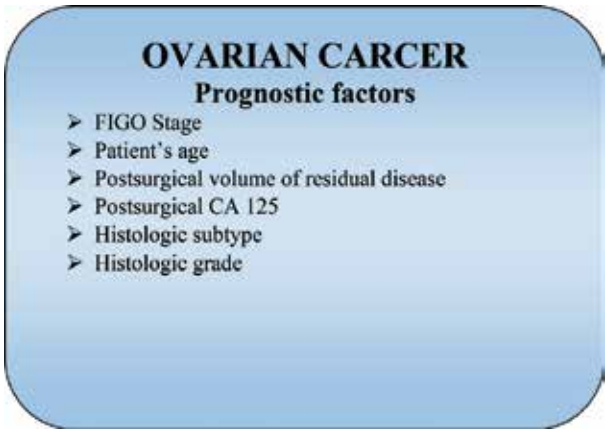

Figure 1. Prognostic factors in ovarian cancer. Ozols RF et al. Cancer Principles and Practice of Oncology. 5th ed. 1997;1510.

a wide variety. The most practical and useful classification depends on the histogenetic origin. Histological classification represents an autonomic entity with independent subtypes, disease-free survival, and quality of life of the patient (**Figure 2**).

On the other hand, depiction of histopathology, immunohistochemistry, and molecular genetic analysis reveal five basic types of ovarian carcinoma: high-grade serous carcinoma (HGSC 70%), endometrioid carcinoma (EC 10%), clear cell carcinoma (CCC 10%), mucinous carcinoma (MC 3%), and low-grade serous carcinoma (LGSC <5%) [3] (**Table 1**).

All recent conducted studies with classification parameter of the histogenetic origin express in 90% of cases the epithelial type as the most common type of ovarian carcinoma. Many useful tools, such as physical examination, transvaginal ultrasonography, Ca-125 levels, abdominal CT, or MRI, are mandatory in order to establish a more accurate clinical diagnosis [4]. Depending on clinical diagnosis, proper therapeutic mapping can be performed.

Among histologic subtypes of epithelial ovarian carcinoma, the most significant type with chemoresistance and poor prognosis consists clear cell ovarian carcinoma (CCC).

Clear cell carcinoma represents a distinct entity of epithelial ovarian carcinoma with an incidence less than 5% of all ovarian lesions [5]. Gold standard concerning therapeutic strategy of epithelial ovarian cancer and, respectively, of clear cell carcinoma is based on abdominal total hysterectomy, bilateral salpingo-oophorectomy, partial omentectomy with peritoneal sampling, and lymphadenectomy, adding cytoreductive surgery in advanced cases.

In many cases, surgical mapping for clear cell carcinoma remains a controversial issue. Many studies underline the decreased impact of adjuvant chemotherapy in patients with stage I clear cell carcinoma and the relation of the lesion with overall survival [6]. The ultimate scope of cytoreductive surgery in patients with clear cell carcinoma reflects the acknowledgment of high-risk patients correlated with recurrence of the lesion.

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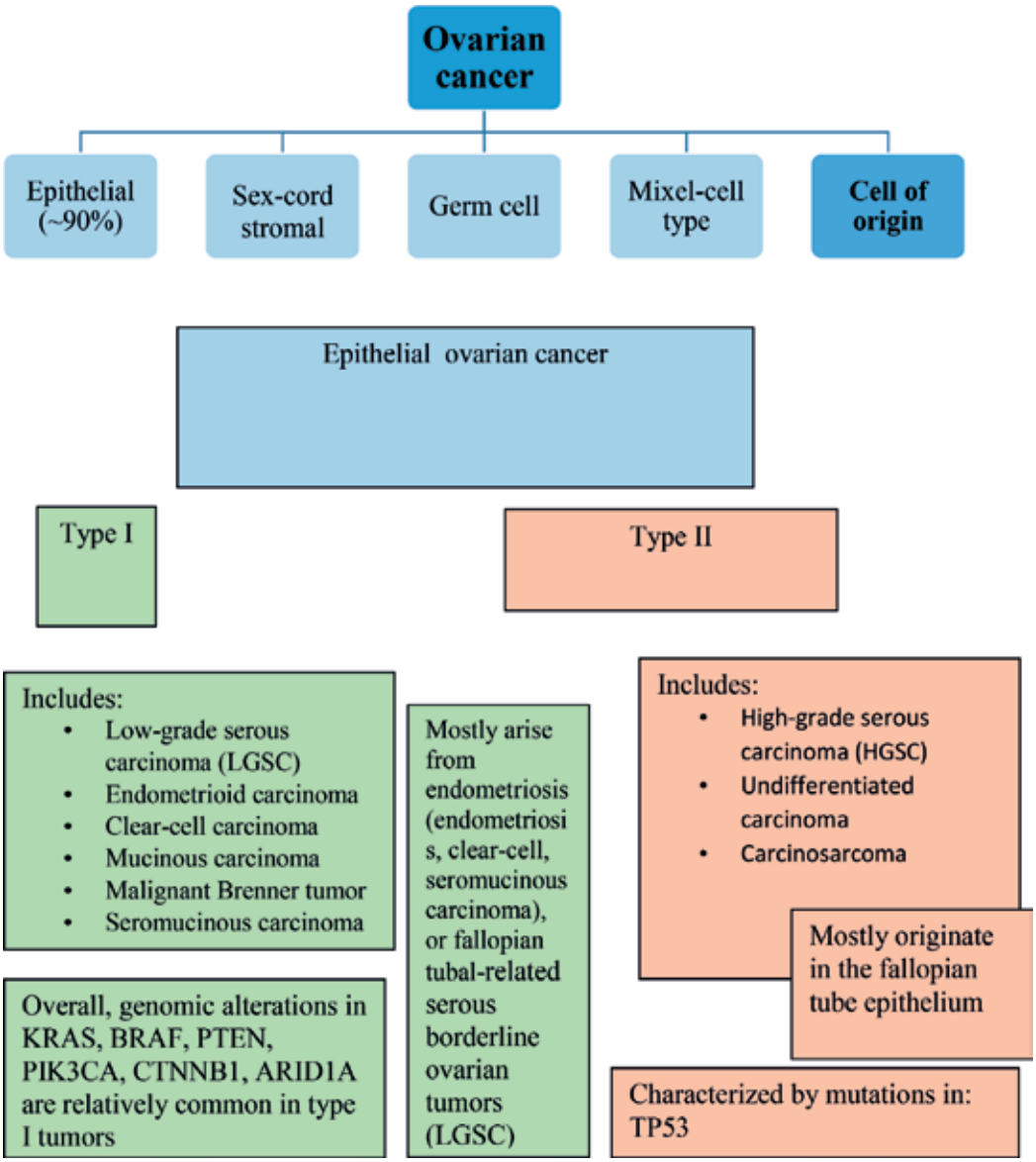

Figure 2. (A) Histological subtypes of ovarian cancer and (B) widely accepted epithelial ovarian cancer classification paradigm based on clinic, pathologic, and molecular evidence that type I and type II tumors develop through different pathways. *Indicates rare tumor. †Mucinous and malignant Brenner tumors are considered to be possible exceptions that may arise from transitional cells at or close to the junction of the fallopian tube and the peritoneum. Kurman RJ, Shih Ie M. The dualistic model of ovarian carcinogenesis: Revisited, revised, and expanded. Am. J. Pathol. 2016, 186, 733–747.

The significant role to metastatic pathways of clear cell carcinoma and the therapeutic mapping reflects the histologic configuration of the lesion. Histologic figures of clear cell carcinomas express clear cytoplasm with large nuclei and prominent nucleoli and a partial hobnail appearance (**Figure 3**).

	HGSC	LGSC	MC	EC	CCC
Risk factors	BRCA1/BRCA2	?	?	HNPCC[2]	?
Precursor lesions	Tubal intraepithelial carcinoma	Serous borderline tumor	Cystadenoma/ borderline tumor?	Atypical endometriosis	Atypical endometriosis
Pattern of spread	Very early transcoelomic spread	Transcoelomic spread	Usually confined to the ovary	Usually confined to the pelvis	Usually confined to the pelvis
Molecular abnormalities	BRCA, p53	BRAF, KRAS	KRAS, HER2	PTEN, ARIDIA	HNF1, ARIDIA
Chemosensitivity	High	Intermediate	Low	High	Low
Prognosis	Poor	Intermediate	Favorable	Favorable	Intermediate

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HGSC, high-grade serous carcinoma; LGSC, low-grade serous carcinoma; MC, mucinous carcinoma; EC, endometrioid carcinoma; CCC, clear cell carcinoma. [a]Hereditary nonpolyposis colorectal carcinoma.

Table 1. Ovarian carcinoma: clinical and molecular features of the five most common types.

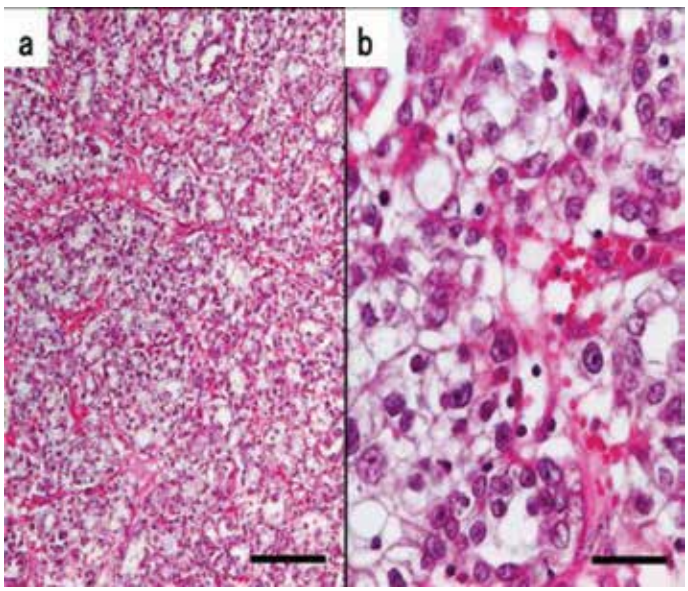

Figure 3. Histology of the original tumor. The left ovarian tumor is a clear cell carcinoma, with cells harboring clear cytoplasm and a partial hobnail appearance, as shown by hematoxylin and eosin (HE) staining ((a) bar = 200 μm; (b) bar = 50 μm). Yamada T et al. Characterization of a Novel Cell Line (HCH-3) Derived from a Human Ovarian Clear Cell Carcinoma. Yamada et al., J Carcinogene Mutagene 2017.

2. Discussion

Despite poor prognosis, overall survival, and quality of life of the patient, all conducted studies are focusing on the pathologic and metastatic pathways of the lesion. This issue remains controversial.

Szubert et al. described the correlation of endometriosis and clear cell ovarian carcinoma [7]. All the efforts lead to correlate the risk factors of endometriosis and clear cell carcinoma. We must never forget the role of endometriosis as trigger point and prominent risk factor of ovarian cancer. On the other point, many conducted studies depict the opposite statistic conclusion, gaining the impression of controversial issue. Zafrakas et al. correlated all the current data without an informative meta-analysis [8]. More conducted studies were mandatory in order to establish such a hypothesis.

Critical points of clear cell ovarian carcinoma remain the understanding of carcinogenesis, the genetic changes of the lesion, and most of all the mechanisms of target therapy.

Mabuchi et al. described and correlated all the critical genetic changes in clear cell carcinoma [9] (**Table 2**). Focusing on gene mutation, pathway bridge, and following tumor implications, we can explain the carcinogenesis of clear cell carcinoma.

Focusing on tumor angiogenesis, many conducted studies described targeted antibodies as therapeutic shield toward the production of tumor vessels [10]. Classical examples of target therapy consist monoclonal antibodies against vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (PDGF), and angiopoietin/Tie2 receptor complex [11]. Therapeutic philosophy depends on adjunction of monoclonal antibodies with growth factors, in order to prohibit tumor angiogenesis and infiltration. The emphasis in this procedure reflects the significant chemoresistance and poor prognosis of the lesion. The results of this target therapy remain controversial, justifying the significance of therapeutic strategy (**Figure 4**).

All therapeutic strategies consisted of overall survival, patient's quality of life and, in young ages with early stage lesion, the fertility-sparing surgery [12]. There are extreme selected indications, performing this surgical dissection.

Nasioudis et al. using the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) database managed to perform the safety of fertility-sparing surgery in stages IA and IC of ovarian clear cell carcinoma [13]. The comparison, in patients with stage I ovarian clear cell carcinoma with preservation of the uterus and ovaries with general survival outcome, did not lead to statistical conclusion. However, further conducted studies are mandatory, in order to establish this type of surgical strategy in young female patients with stage IA or IC ovarian clear cell carcinoma.

Besides understanding the carcinogenesis of the lesion, the biochemical pathways, and the effort of fertility-sparing surgery in young female patients, we must mention the advanced metastatic opportunity of the lesion.

Lymphatic, hematogenic, and endoperitoneal infiltration of the lesion can lead to advanced metastatic possibilities. First of all, the lesion can penetrate the local anatomic organs: the salpinx, round ligament, uterus, peritoneal wall, colon, or even the omentum [14].

The most common, premature, and characteristic route of infiltration consists of the endoperitoneal [15]. All neoplasmatic cells are deafened, entering the peritoneal cavity. Through respiratory movements, endoperitoneal fluid with neoplasmatic cells finally reaches all epithelial

Gene	Gene type	Change	Pathways affected	Roles in tumor development
ARID1A	Tumor suppressor	Mutation in ~50%	SWI/SNF chromatin complex	Modulate accessibility of transcription factors to promoters
PIKECA	Oncogenic	Mutation in ~40%	PI3K/AKT/mTOR	Proliferation/survival
PPP2R1A	Oncogenic	Mutation in 7%	AKT/MAPK	Proliferation/survival
KRAS	Oncogenic	Mutation in 5%	AKT/MAPK	Proliferation/survival
BRCA1/BRCA2	Tumor suppressor	Mutation in 6%	DNA repair	Genomic instability
PTEN	Tumor suppressor	Mutation in 5%	PI3K/AKT/mTOR	Proliferation/survival
CDKN2A/CDKN2B	Tumor suppressor	Deletion in 9%	CDK inhibitors (p15/p16)	Cell cycle progression
ZNF217	Oncogenic	Amplification in 36%	ZNF217	Antiapoptosis
PPM1D	Oncogenic	Amplification in 10%	P53-mediated apoptosis	Antiapoptosis
AKT2	Oncogenic	Amplification in 14%	AKT/mTOR	Proliferation/survival
MET	Oncogenic	Amplification in 37%	AKT/MARK	Proliferation/survival

ARID1A, AT-rich interactive domain 1A; BRCA, breast cancer; CDK, cyclin-dependent kinase; CDKN, cyclin-dependent kinase inhibitor; MAPK, mitogen-activated protein kinases; mTOR, mammalian target of rapamycin; PIK3CA, phosphatidylinositol-45-bisphosphate 3-kinase catalytic subunit alpha; PI3K, phosphatidylinositol 3-kinase; PPM1D, protein phosphatase 1D; PPP2R1A, protein phosphatase 2 regulatory subunits 1A; PTEN, phosphatase and tensin homolog; SWI/SNF, SWItch/sucrose non-fermentable; ZNF217, zinc finger protein 217.

Table 2. Mabuchi S, Sugiyama T, Kimura T. Clear cell carcinoma of the ovary: molecular insights and future therapeutic perspectives. J Gynecol Oncol. 2016May; 27(3); e31.

areas and especially the hemidiaphragms. Final result, building of metastatic lesions as metastatic plaque or in advanced lesion as neoplasmatic "cake" (**Figure 5**). Through the right hemidiaphragm, the lesion can be spread in the pleura area, provoking hydrothorax or reaching the subclavian lymph nodes.

Usual distant organs with signs of infiltration are liver, lungs, and lymph nodes beyond the pelvic and para-aortic chains. Lymphatic spread of this lesion is common. The spread route follows the lymphatic vessels of ligamentum teres uteri or the lymphatic vessels of the right hemidiaphragm. The most common areas are pelvic lymph nodes with less frequent inguinal, axillary, or subclavian lymph nodes.

Hematogenic infiltration is strongly connected with advanced stages of the lesion. In these cases, the most common is liver and lung infiltration. In extreme advanced stages of the lesion, there are cases of skin or brain infiltration.

Nam et al. reported skin metastases in ovarian clear cell carcinoma as severe advanced metastatic area of the lesion [16]. Infiltration of these organs reflects severe decrease of disease-free survival, overall survival, and quality of life of the patient.

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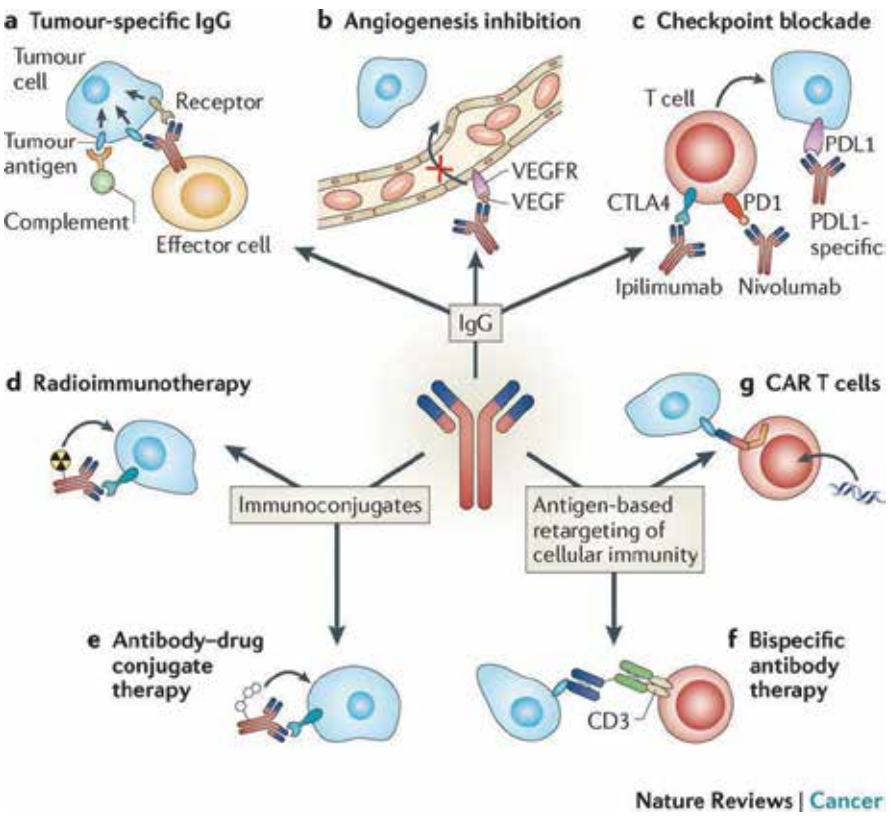

Figure 4. Weiner Building better monoclonal antibody-based therapeutics. Nature Reviews Cancer 15,361–370 (2015).

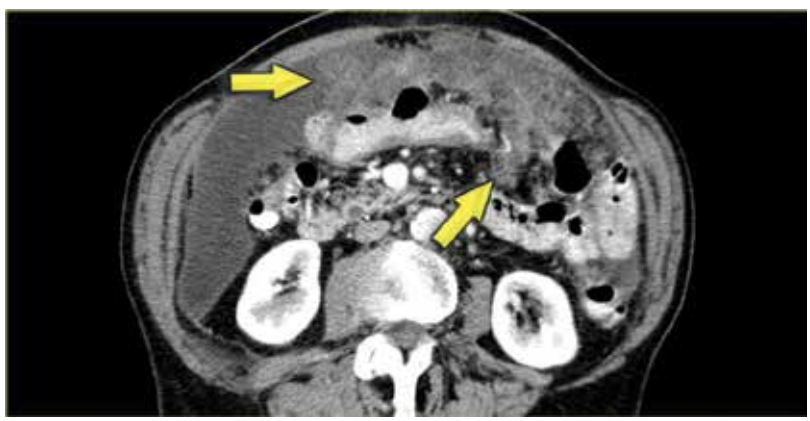

Figure 5. Omental cake (arrows) and ascites in a patient with peritoneal metastases derived from ovarian cancer. Levy Angela. Chief Gastrointestinal Radiology, Department of Radiologic Pathology, Armed Forces Institute of Pathology, Washington DC, Associate Professor of Radiology, Uniformed Services University of the Health Sciences, Bethesda, MD.

Postoperative treatment of clear cell ovarian carcinoma deviates, representing a distinct entity from other epithelial ovarian carcinomas. Reflecting a chemoresistant phenotype, the final prognosis of the lesion is poor, decreasing the quality of life of the patient. In cases of clear cell

carcinoma, gold standard combination with paclitaxel and carboplatin consists a not promising therapeutic strategy. Irinotecan hydrochloride, a topoisomerase I inhibitor, reflects an alternative solution regarding the postoperative treatment of clear cell carcinoma [17] (first-line chemotherapy for clear cell carcinoma) (**Table 3**).

Many conducted studies managed to express the synergic effects of the combined therapeutic strategy of irinotecan and cisplatin (**Table 4**).

In cases of recurrent clear cell carcinoma, therapeutic mapping is very disappointed. Even in cases of sensitive platinum disease, the use of antineoplasmatic agents offers a response rate not up to 10% [18] (second-line chemotherapy for clear cell carcinoma).

Regimen	Author	Year	Response/number of patient, response rate
Conventional platinum based	Goff (28)	1996	1/6, 17%
	Sugiyama (29)	2000	3/27, 11%
	Ho (30)	2004	4/15, 27%
	Takano (9)	2006	5/30, 17%
Taxane-platinum	Ecomoto (31)	2003	2/9, 22%
	Ho (30)	2004	9/16, 56%
	Utsunomiya (32)	2006	8/15, 53%
	Takano (9)	2006	9/28, 32%
Irinotecan-cisplatin	Takano (9)	2006	3/10, 30%

Takano et al. Clear cell carcinoma of the ovary: Is there a role of histology-specific treatment? Journal of Experimental & Clinical Cancer Research 2012, 31:53

Table 3. Response rates of primary chemotherapy for clear cell carcinoma.

Regimen	Author	Year	Response/number of patient, response rate
Megestrol acetate	Malailak (45)	2001	2/10, 20%
Cyclophosphamide + cisplatin	Takano (46)	2008	1/9, 11%
Irinotecan + platinum	Sugiyama (29)	1998	1/3, 33%
	Takano (46)	2008	2/15, 13%
Etoposide + platinum	Takano (46)	2008	2/13, 15%
Paclitaxel + carboplatin	Utsunomiya (32)	2006	3/13, 23%
	Crotzer (43)	2007	2/7, 29%
Gemcitabine	Crotzer (43)	2007	1/9, 11%
	Yoshino (47)	2012	1/5, 20%
Docetaxel + irinotecan	Yoshino (47)	2012	1/11, 9%
Temsirolimus	Takano (46)	2011	1/5, 20%

Takano et al. Clear cell carcinoma of the ovary: Is there a role of histology-specific treatment? Journal of Experimental & Clinical Cancer Research 2012, 31:53.

Table 4. Response rates of salvage chemotherapy for recurrent or refractory clear cell carcinoma.

The main objective of the previous study was the presentation and implementation of an epithelial-type ovarian carcinoma with specific metastatic pathways, prohibiting especially episodes of target therapy. New scientific keys, in the near future, will unlock unknown biochemical mechanisms and give answers to many questions, concerning the understanding of carcinogenesis of this lesion.

3. Conclusion

Ovarian clear cell carcinoma represents a rare histological entity with extreme chemoresistance and poor prognosis in correlation with overall survival and quality of life of the patient. Better understanding of metastatic and biochemical pathways of the lesion could schedule a proper therapeutic mapping. Further conducted studies are needed, in order to establish such strategy.

Conflict of interest

The author declares any financial interest with respect to this manuscript.

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Chapter 8

Genetic Mutations and Ubiquitination in Melanoma Growth and Metastasis

Anushka Dikshit and Jennifer Zhang

Additional information is available at the end of the chapter

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Abstract

Upon neoplastic transformation, melanoma is intrinsically prone to metastasis, which marks the most dangerous aspect of the disease and dubs it one of the most challenging cancers to treat. BRAF/MEK oncokinase inhibitors and immunotherapies have shown considerable promise in some patients, but the clinical benefits are often short-lived due to rapid development of resistance. Recently, ubiquitination enzymes have emerged as potential therapeutic targets. These enzymes can be targeted to increase expression of tumor suppressors and impede activation of oncogenic signaling pathways mediating cell proliferation and tissue invasion. This chapter describes some of the common genetic mutations in melanoma, ubiquitinating and deubiquitinating enzymes that are linked to melanoma progression, metastasis, and therapeutic resistance.

Keywords: A20, BAP1, BRAF, CDKN2A, CYLD, DUB, E-cadherin, ERK, melanoma, N-cadherin, Snail1, TRAF6, UBE2S, ubiquitination, USP

1. Introduction

Melanoma is the most aggressive form of skin cancer. The 5-year survival rate for metastatic melanoma is less than 20%. The incidence of melanoma is on the rise especially among the young population. The NIH SEER program estimated that 87,110 people were diagnosed with melanoma in the United States in 2017, accounting for 5.2% of all new cases of cancer, which is 1.2% higher than the melanoma cases reported in 2007. About 11% of the newly diagnosed patients would succumb to the disease due to uncontrollable metastatic tumor growth [1, 2]. This chapter describes the common genetic mutations and posttranslational modifications crucial for melanoma growth, survival and dissemination, with a particular focus on enzymes regulating ubiquitination.

2. Melanoma staging and diagnosis

Melanoma treatment plans are designed based on the stage of the disease. In 2017, the American Joint Committee on Cancer (AJCC) published the 8th edition of the tumor, lymph node, and metastasis (**TNM**) system. The **T** recognizes tumor thickness or the depth of the cancer in the skin and characterizes the tumor as being ulcerated or non-ulcerated. The **N** is used to establish whether the cancer has spread to the proximal lymph nodes and finally, the **M** stands for metastasis and gives information if the cancer has spread to distant lymph nodes or other organs [3]. Using the TNM numbers along with elaborate clinical and pathological assessments, the cancer is assigned a stage. Further histological analyses allow pathologists to assign a 'grade' to the tumor which is an indication of the abnormality of the tumor cells. Both staging and grading are crucial to determine the course of treatment and give an overall prognosis for the disease. Although it is under debate whether a linear progression is the primary theme, cutaneous melanomas can progress from a precursor lesion, namely benign nevi which becomes dysplastic, to melanoma in situ, and finally to invasive melanoma. A nevus is a benign aggregation of melanocytes that is formed at the junction of the dermis and epidermis or within the dermis. When the nevus shows signs of cytological atypia and change in growth, it becomes dysplastic. Melanoma in situ, also called as the stage (0) melanoma, is when the transformed melanocytes are still within the epidermis or the dermal/epidermal junction. Lastly, when the transformed melanocytes invade the dermis and gain access to other cell tissues and/or the vascular system, they turn into metastatic melanoma [4]. The development of melanoma involves multifactorial and heterogeneous biologic processes that are controlled at the genetic, transcriptional, and posttranslational levels [5, 6].

3. Genetic mutations

3.1. Germline mutations in melanoma

Certain genetic changes predispose an individual to an increased risk of melanoma. These changes include point mutations, amplifications, deletions or translocations. CDKN2A is one of the first genes linked to the familial atypical mole melanoma (FAMM). Deletion or loss of heterozygosity of CDKN2A increases patient susceptibility to developing melanoma [7]. Consistently, germline deletion of Cdkn2a in mice sensitizes animals to developing cutaneous melanoma when coupled with an activating HRAS mutation in the melanocytes [8]. While the CDKN2A gene codes for two proteins, p16 INK4A and p15 ARF, p16 INK4A loss-of-function is responsible for spontaneous and carcinogen induced melanoma [9]. CDK4 and RB1 are two other genes with germlines mutations linked to familial susceptibility to melanoma. CDK4, a kinase involved in regulating cell cycle, is amplified or mutated in human melanomas with some studies showing 100% of all the affected family members harboring a specific CDK4 mutation [10, 11]. Inactivation of RB1 tumor suppressor causes hereditary retinoblastoma and increases the risk of developing melanoma by about 80-fold [12]. Variants of the melanocortin-1 receptor (MC1R) are associated with fair pigmentation and melanoma risk [13]. The

presence of a CDKN2A mutation gave a hazard ratio of 13.35, and the MC1R variants increase the hazard ratio by 3.72-fold [14].

3.2. Somatic mutations in melanoma

Ultraviolet (UV) radiation induces somatic gene mutations and is the major risk factor for melanoma [6, 15]. The RAS/RAF/MEK/ERK signaling pathway is commonly mutated in melanoma. Among the RAS GTPase family members, NRAS is the most frequently mutated protein with activating mutations detected in up to 56% of benign nevi and 26% of metastatic melanoma [16, 17]. Activating mutations of BRAF are found in about 70% of cutaneous melanomas, and 90% of these mutations are of the BRAF(V600E) (valine to glutamic acid substitution) [18, 19]. There is a well-established correlation between sun-exposure and the development of BRAF mutations [15, 20]. BRAF(V600E) mutation is observed in the benign melanocytic nevi, indicating that this mutation alone is not sufficient for malignant transformation [19, 21]. Other genetic changes such as CDKN2B loss are required for the progression from benign melanocytic nevus to melanoma [22]. Random mutagenesis screens have revealed that ERK point mutations impart resistance to RAF and MEK inhibitors, although such mutations are infrequent [23].

4. Melanocyte origin and its intrinsic effects on melanoma metastasis

Melanoma is intrinsically prone to metastasis [4]. Melanocyte precursors are derived from highly migratory neural crest cells, which give rise to a number of differentiated cells including the melanocytes. During this process, they express several canonical neural crest markers such as SOX8, SOX9, SOX10, Snail1, and Snail2. The strong metastatic potential of melanoma can be attributed to the plethora of mutations acquired over the development of the disease and to the aberrant reactivation of some of the transcriptional factors such as Sox5, Sox10, and Snail1 that are crucial for the melanocyte differentiation program [24–26].

The Wnt signaling pathway plays a major role in melanocyte differentiation and migration mainly through the activation of the microphthalmia-associated transcription factor (MITF) [27]. MITF promotes melanocyte differentiation and melanin production, in addition, MITF is often upregulated in melanomas associated with poor prognosis [28]. β-catenin dependent MITF activation induces proliferation of melanoma cells and inhibition of β-catenin leads to a cell cycle arrest along with downregulation of MITF [29]. Hyperactivated Wnt signaling has been reported in about 30% of melanoma samples [30] and is shown to promote melanomagenesis. On the other hand, there are also reports that demonstrate loss of β-catenin decreases melanoma cell proliferation, but promotes invasion and predicts poor prognosis [31]. This discrepancy is predominantly because the Wnt proteins utilize three distinct signaling pathways with different mediators [29]. The canonical Wnt signaling pathway entails activation of the frizzled surface receptors by the Wnt ligand, leading to the formation of a vast protein complex on the cell surface. This leads to a cascade of events, resulting in the nuclear translocation of β-catenin which then acts as a co-activator to regulate target gene expression [32]. A non-canonical Wnt signaling pathway involves activation of GTPases such as RAC1 and

RHOA, and MAP kinase such as JNK, downstream of the frizzled receptor. A second noncanonical pathway includes activation of phospholipase C (PLC) that promotes the release of intracellular Ca^{2+}, leading to the expression of target genes involved in cell migration and inflammation [33]. The noncanonical Wnt signaling is shown to stimulate cytoskeletal remodeling, increase cell survival, and promote invasive characteristics [34, 35]. Both the canonical and the noncanonical Wnt signaling pathways are involved in the promotion of melanoma cell proliferation and epithelial-mesenchymal translation (EMT) [29, 36].

5. Ubiquitination in melanoma

Posttranslational modification (PTM) is a reversible and dynamic process that regulates protein function at a posttranslational level and plays crucial roles in signal transduction, gene regulation, vesicle transportation, and protein degradation. PTMs include phosphorylation, ubiquitination, sumoylation, methylation, acetylation, glycosylation and N-nitrosylation. Dysregulation of PTM results in pathogenesis including cancer [37]. Phosphorylation is catalyzed by protein kinases and is the core mediator of signal transduction. Oncogenic mutations of the protein kinases, most notably BRAF(V600E), result in constitutive phosphorylation and activation of the downstream targets such as the MEK and ERK kinases [38]. Kinase-mediated signals are also regulated by other PTMs including ubiquitination.

Ubiquitination (Ub) is a rather complex process involving a substantial series of variable components. Mono-Ub involves a covalent attachment of a 76 amino acid ubiquitin polypeptide to a lysine residue of a target protein. Poly-Ub involves attachment of additional ubiquitin moieties to one of the seven lysine (K) residues (K6, K11, K27, K29, K33, K48 and K63) or the methionine residue (M1) of the proceeding ubiquitin, forming structurally and functionally distinct polymers. If more than one K-residues are involved, it is called a heteropolymeric chain and, if a single K-residue is involved, it is called as a homopolymeric chain, such as K48-Ub and K63-Ub [39]. Different Ubs carry out distinct functions, ensuring the robust control of essentially every cellular process spanning from signal transduction, DNA-repair, vesicle transportation, cell division, differentiation, and migration [40]. While K48-Ub generally marks protein for proteasomal degradation, K63-Ub regulates signal transduction, RNA splicing, protein sorting, DNA repair and immune response [41–43].

Ubiquitination generally requires a three-step process: the E1 enzyme catalyzes the first step by activating an ubiquitin moiety in the presence of ATP and the E2 conjugase carries the ubiquitin via covalent bonding, and works together with an E3 ligase to complete ubiquitin ligation with the target protein [44]. There are two known E1 enzymes, UBA1 and UBA6 [45]. The canonical UBA1 is characterized as a potential target for the treatment of hematologic malignancies [46]. The noncanonical UBA6 suppresses epithelial-mesenchymal transition of mammary epithelial cells [47]. The role of UBA1 and UBA6 in melanoma is not well-understood.

5.1. Ubiquitin conjugases in melanoma

There are about 50 known E2 conjugating enzymes. Each of them contains a conserved cysteine residue that accepts the ubiquitin molecule activated by the E1 enzyme. A number of E2

enzymes have been implicated in melanoma. For example, Rad6 promotes melanoma development and progression by inducing nuclear translocation of β-catenin [48]. Overexpression of E2-EPF UCP (ubiquitin carrier protein) promotes melanoma metastasis, while its downregulation decreases tumor invasion [49]. UBE2S targets VHL protein for proteasomal degradation via a K48-Ub-mediated process. UBE2S is overexpressed in metastatic melanoma cell lines and mediates the degradation of VHL and a consequent upregulation of HIF-1α and VEGF proteins to promote distant metastasis and angiogenesis [50].

5.2. Ubiquitin ligases in melanoma

There are over 600 putative E3 Ubiquitin ligases in the human genome. These enzymes display substrate specificity, and have attracted tremendous attention for therapeutic targeting. The SCF ubiquitin ligases constitute the largest family of E3 ligase enzymes characterized by an F-box component that interacts with the substrate. Among the SCF family members, β-TrCP (beta transducing repeats containing protein) is upregulated in melanomas with BRAF(V600E) mutation and a corresponding increase of NF-κB activity [51]. Another SCF family member, SCF-Skp2 is shown to promote melanoma cell cycle progression by degradation of CDK inhibitors such as p27 and p57 [52]. FBXW7, a component of the SCF-FBW7 E3 complex, is mutated in 8.1% of melanoma patients and some of these mutations interfere with its substrate binding, leading to oncogenic activation of substrates including Notch1 [53, 54]. FBXW7 inactivating mutations are detected in melanomas without the typical BRAF(V600E) and NRAS(G12D/G13R/Q61K/L/S) mutations [55]. Knockdown of FBXW7 in melanoma cell lines leads to the upregulation of NOTCH1, HEY1, and the downstream effectors Cyclin E, Aurora A and Myc, resulting in increased tumorigenesis [56–58].

The E3 ligase MDM2 regulates p53 and its expression in melanoma correlates with increased malignancy, tumor thickness and invasion [59]. TRAF6 is a K63-Ub ligase overexpressed in primary and metastatic melanoma, and it promotes tissue invasion by stimulating MMP9 activation [60]. TRAF6 recruitment and activation is regulated by EGFR through the oncoprotein, DCBLD2 (discoidin, CUB, and LCCL domain-containing protein) in a number of cancers including melanoma [61]. TRAF6 together with p62 also regulates mTOR activation via K63-Ub which is important for the mTORC1 and mTORC2 complex formation [62]. TRAF2 mediates K63-Ub of GβL (mTOR LST8 homolog) thereby preventing mTORC1 complex formation. Mutations in the K63-Ub site of GβL are shown to promote chemoresistance of melanoma cells in vitro and in the xenograft mouse model [63].

The E3 ubiquitin ligase RNF125 negatively regulates the retinoic acid-inducible gene I (RIG-I) signaling pathway by targeting RIG-I for proteasomal degradation [64]. It also regulates p53 and innate immune adaptor protein TRIM14 [65, 66]. Deletion and missense mutations in RNF125 are linked to the overgrowth syndrome [67]. RNF125 is regulated by MITF and SOX10 transcription factors and its downregulation elevates JAK1 and EGFR signaling, and underlies resistance of melanoma cells to the BRAF inhibitor, vemurafenib [68].

Oncogenic RAS and BRAF mutants drive tumor growth through hyperactivation of ERK1/2 kinases and concomitantly induce ERK-dependent negative feedback. Relief of this feedback inhibition by RAF inhibitors contributes to the attenuation of the therapeutic potency in BRAF mutant melanomas [69]. Ubiquitination-dependent degradation of BRAF constitutes for one of

the negative feedback mechanisms [70]. The $APC^{/C}$ E3 ligase complex activator FZR1 but not FBXW7 tumor suppressor controls BRAF oncogene function [70, 71]. FZR1 as a direct target of ERK and CyclinD1/CDK4 kinases and its phosphorylation inhibits APC^{FZR1}, leading to increased expression of a cohort of oncogenic APC^{FZR1} substrates important for melanomagenesis [71].

The E3 ubiquitin ligase Trim7 is activated by the Ras/RAF/MEK/ERK pathway via MSK1-mediated phosphorylation, and consequently mediates K63-Ub of the AP-1 co-activator RACO-1, leading to RACO-1 protein stabilization and increased AP-1-dependent gene expression [72]. The c-Jun/RHOB/AKT pathway confers resistance to BRAF mutant melanoma cells from BRAF and MEK inhibitors [73]. Trim7 may represent a potential target for combination therapies to mitigate therapeutic resistance.

5.3. Deubiquitinating enzymes in melanoma

Ubiquitination is a reversible process and a group of enzymes called the deubiquitinases (DUBs) cleave the isopeptide linkage between the polyubiquitin chains and the target proteins. There are five major families of deubiquitinases: ubiquitin-specific proteases (USP), ubiquitin carboxyl-terminal hydrolases (UCH), Jab1/MPN domain associated metalloisopeptidase domain proteins, Machado-Joseph Domain (Josephin domain) containing proteins (MJD) and Otubain/Ovarian tumor domain containing proteins (OTU) [74]. Major functions of DUBs involve rescuing incorrectly ubiquitinated proteins from degradation and modulating target protein function. DUBs play important roles in DNA repair, apoptosis, cell proliferation, kinase activation, and chromatin remodeling, and they can function as tumor suppressors as well as oncogenes [75].

5.3.1. Deubiquitinating enzymes acting as tumor suppressor

5.3.1 a **BAP1** (BRCA-associated protein 1) is a deubiquitinase belonging to the ubiquitin C-terminal hydrolases family (UHCs). BAP1 binds to BRCA1, and acts a tumor suppressor. Loss of function of BAP1 due to germline mutations leads to the tumor predisposition syndrome which increases the risks of uveal and cutaneous melanomas, and malignant mesotheliomas. Individuals who carry the mutated BAP1 gene develop melanocytic lesions later in their life and some of those benign lesions can transform into cutaneous melanomas [76]. In addition to its deubiquitinase function, BAP1 possess a nuclear localization signal that allows it to translocate to the nucleus and interact with proteins such as HCF-1 to regulate cell growth [77].

5.3.1 b **A20** (TNFAIP3) is a deubiquitinase commonly induced by inflammatory cytokines via NF-κB. It has an innate deubiquitinating activity imparted by the OUT zinc finger domain that allows it to interact with ubiquitinated substrates and maintain specificity. A20 inhibits auto-ubiquitination of the K63-Ub E3 ligase TRAF6, and consequently inhibits IKK/NF-κB activation [78]. Independent of its deubiquitinating functions, A20 disrupts the interaction between the E2 conjugase (UBE2N) and the E3 ligase (TRAF2/TRAF6), and consequently promotes K48-Ub and proteasomal degradation of these enzymes [79]. A20 is characterized as a potent tumor suppressor in non-Hodgkin's lymphomas including diffuse large cell lymphoma, mantel cell lymphoma and ocular marginal zone B-cell lymphoma [80], but its role in melanoma is not at all clear. Increased expression of A20 in $CD8^+$ T cells results in impaired

anti-tumor immunity in a melanoma animal model, while knockdown of A20 in $CD8^+$ T cells increases cytokine production and decreases melanoma tumor burden [81].

5.3.1 c **CYLD** is a deubiquitinase that preferentially removes K63-Ub and M1-Ub from target proteins [82, 83]. CYLD has been identified as a tumor suppressor on account of its loss-of-function correlating to a number of cancers including melanoma [53, 84]. In addition to gene deletion and mutation, CYLD is downregulated by Snail1 at the transcriptional level and by microRNAs including mir-186 and mir-767 at the post-transcriptional level [85, 86]. Furthermore, CYLD is subject to proteolytic inactivation by MALT1, a paracaspase known to promote melanoma growth and metastasis through JNK/c-Jun signaling pathway [87]. CYLD exhibits anti-oncogenic effects by regulating cell proliferation, angiogenesis, and tumor cell differentiation [88].

CYLD inhibits K63-Ub of a plethora of target proteins. Among these are Bcl3, TRAF2/6, Tak1, plk1, lck, HDAC6, Dvl, and c-Jun/c-Fos AP1 subunits [89–93]. In the absence of CYLD, Bcl3 along with NF-κB is recruited to the Cyclin D1 promoter to facilitate its transcription to induce G1 to S cell cycle progression. CYLD inhibits JNK activation and expression of the β1-integrin in non-melanoma and melanoma cells with a corresponding decrease in cell proliferation and increase in apoptosis [88, 89]. On the other hand, CYLD regulates cell motility via inhibition of HDAC6-mediated deacetylation of tubulin and cortactin, by directly binding to the catalytic domain of HDAC6 [94, 95].

EMT and metastasis are orchestrated by an array of gene regulators, such as TWIST1/2, SNAIL1/2, ZEB1/2 and FOXC2 [96]. Snail1-mediated suppression of CYLD is crucial for melanoma progression and metastasis [97]. CYLD regulates EMT by facilitating the maintenance of E-Cadherin expression and inhibiting N-Cadherin expression in melanoma [84]. E-cadherin is a crucial protein facilitating cell-cell adhesion, maintaining cytoskeletal stability and regulating cell polarization. Germline mutations in E-cadherin predispose individuals to a higher risk of breast and gastric cancers [98]. E-cadherin appeared downregulated in melanoma predominantly due to the alterations in the tumor microenvironment rather than genetic mutations as reported in some gynecological cancers [99]. Loss of E-cadherin expression/function increases melanocyte proliferation due to impaired interaction with keratinocytes [100]. When melanocytes are cultured in vitro, in the absence of the basal keratinocytes, they not only display increase in doubling time but also begin expression of melanoma-related markers such as β3-integrin, MUC18, melanotransferrin, and other growth factor receptors. These melanocytes regain their normal phenotype when co-cultured with keratinocytes [100]. E-cadherin also regulates the activation of β-catenin, c-Myc and Cyclin D1.

N-cadherin is expressed in melanoblasts during embryonic development, which helps cell migration from the neural crest to the epidermis by the way of interacting with fibroblasts and endothelial cells. In adults, upregulation of N-cadherin potentiates the ability of melanoma cells to invade through the stroma and interact with the vascular endothelial cells and fibroblasts, which facilitates migration of cancer cells. N-cadherin has also been demonstrated to stabilize β-catenin thereby promoting anti-apoptotic proteins and inhibiting pro-apoptotic proteins such as Bad [101]. Normal melanocytes do not express N-Cadherin, while melanoma cells display moderate to strong expression of N-cadherin with a corresponding decrease in the expression of E-cadherin. This phenomenon termed as the 'cadherin switching' is a hallmark of EMT [102].

Snail1 also regulates the expression of Notch-4, MMP-2, TIMP-1, SPARC, and T-PA, among others, in melanoma cells [103]. Notch4 and SPARC expression can be directly regulated by Snail or indirectly via E-cadherin [103]. MMP-2 is a matrix metalloprotease that is involved in remodeling of the extracellular matrix through degradation of cell adhesion proteins and in turn facilitating tumor cell migration and invasion [104]. SPARC is a potent inducer of MMP-2, and downregulation of Snail1 decreases the expression of MMP-2 and SPARC [103, 104], as well as T-PA, a protease that converts plasminogen to active zymogen [105], leading to reduced degradation of the extracellular matrix and subsequent invasion. Notch signaling mediates melanoma-endothelial cell and melanoma-keratinocyte communications, facilitating melanoma cell migration and metastasis [106, 107]. Notch 4 expression in melanoma is stimulated by Snail, independent of the repression of E-cadherin [103]. Notch inhibition enhances the efficacy of ERK and ERBB inhibitors for melanoma growth arrest [108, 109].

5.3.2. Deubiquitinating enzymes required for melanoma growth and metastasis

Deubiquitinases (DUB) have a role in stem cell maintenance and tumor growth [110]. Among the 89 DUBs examined in over 300 different tumor samples of breast, colon, lung, stomach, kidney, prostate, non-Hodgkin's lymphoma, and melanoma, 22 DUBs are significantly dysregulated in at least one tumor type [111]. Specifically, three DUBs, USP10 (ubiquitin specific peptidase 10), USP11 and USP22, are expressed significantly higher in metastatic melanoma compared to benign nevi and primary tumor. In addition, expression of USP10 and USP22 is significantly correlated to the presence of ulceration and the Breslow index, a prognostic parameter indicating the depth of tumor invasion. USP10, USP11 and USP22 regulate deubiquitination of target proteins crucial for melanoma transformation and metastasis [111]. For example, USP22 deubiquitinates chromosomal binding proteins H2A-Ub1 and H2A-Ub2, and consequently activates transcription factors and induces epigenetic modifications favorable for cancer growth and metastasis [112].

USP7 mediates degradation of tumor suppressors such as p53, MDM2, FOXO and PTEN [110]. USP14 is expressed at increased levels in melanoma cells compared to melanocytes, and its high expression correlates with melanoma progression and poorer survival. Knockdown or pharmacological inhibition of USP14 impairs viability of melanoma cells irrespective of the mutational status of BRAF, NRAS and TP53, and overcomes resistance to MAPK inhibitors. This was accompanied by accumulation of poly-ubiquitinated proteins, mitochondrial dysfunction, ER stress, and a ROS production [113].

USP9X, another member of the USP family, is responsible for attenuating the degradation of Mcl-1, an anti-apoptotic protein in melanoma and other cancers [53]. USP9X deubiquitinates, and stabilizes ETS-1, a transcription factor involved in regulation of angiogenesis, cell migration, proliferation and cellular differentiation in melanoma [114]. Phenethyl ITC, an inhibitor of USP9X, is currently in a Phase I trial for the treatment of melanoma and leukemia and Phase II trials for oral and lung cancer [53]. WP1130 (degrasyn) is another agent that inhibits USP9X and promotes the accumulation of protein-ubiquitin conjugates, leading to formation of aggresomes and apoptosis. This agent is shown to decrease melanoma cell growth both in vitro and in vivo melanoma models [115].

6. Conclusion

Melanoma is an aggressive disease intrinsically prone to metastasis. Germline and sporadic mutations are responsible for the initiation and progression of the disease and PTMs such as ubiquitination play dominant roles in melanoma growth and metastasis, and offer wealth of opportunities for therapeutic targeting. With better mechanistic understating of specific functions of the ubiquitinating enzymes as well as the deubiquitinases, new targeted therapies are expected to emerge in the foreseeable feature.

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Metastasis of cancer cells from primary tumor site to secondary locations is considered a late event in multistep tumorigenesis, and causes most cancer-related mortality. The process from the spreading of cancer cells to the seeding of newly formed tumor colonizations is governed by sequential events, including local invasion, intravasation into stroma and blood vessels, survival in circulation, extravasation, and colonization at secondary tumor sites. Cancer research provides information on the fate of metastatic cancer cells in each sequential movement or heterogeneous tumor microenvironment. However, the complexity of this mechanism remains the most stringent concept of cancer management. This book provides information for cancer researchers on metastatic phenotypes of cancer cells, and diverse promoting factors and molecular mechanisms of metastasis.

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