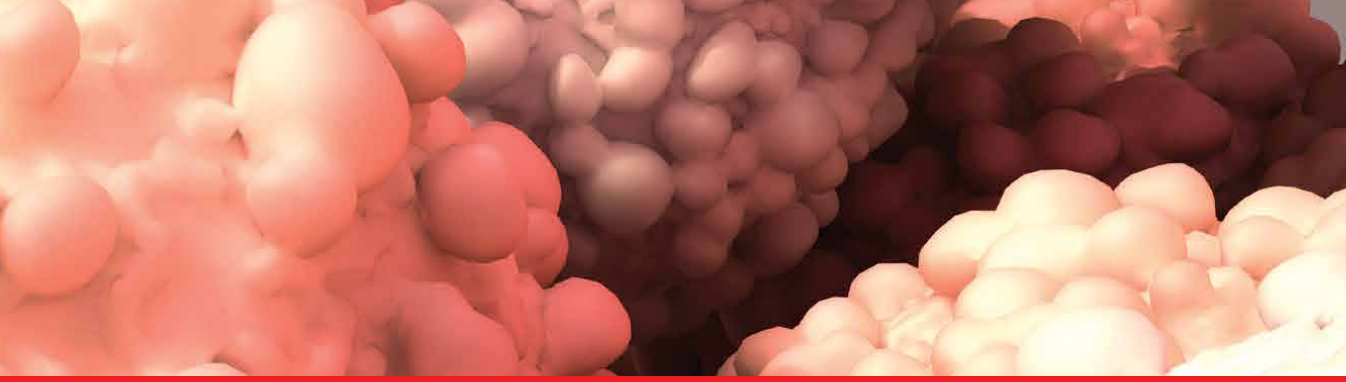

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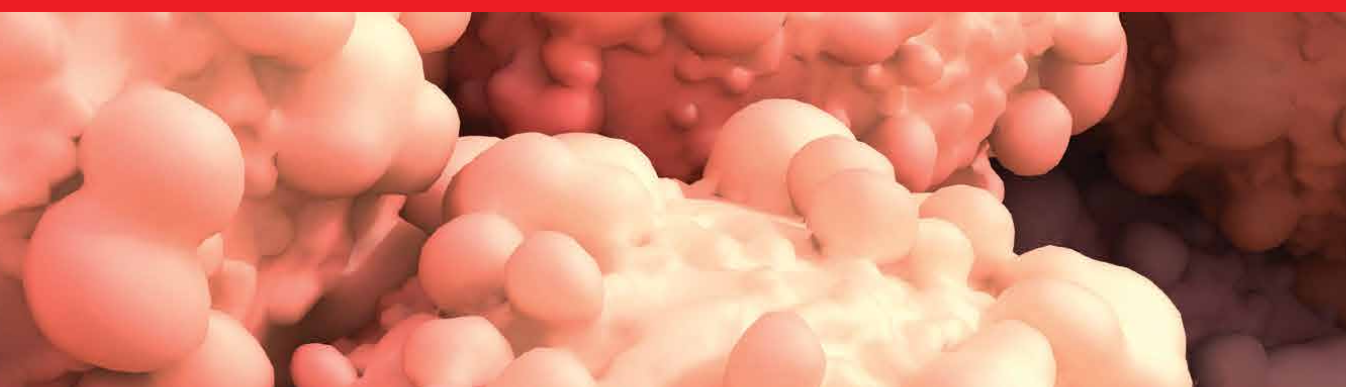
# Cancer Metastasis

## Molecular Mechanism and Clinical Therapy

*Edited by Yusuf Tutar and Lütfi Tutar*



# Cancer Metastasis - Molecular Mechanism and Clinical Therapy

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Published in London, United Kingdom

Cancer Metastasis - Molecular Mechanism and Clinical Therapy
http://dx.doi.org/10.5772/intechopen.100926
Edited by Yusuf Tutar and Lütfi Tutar

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First published in London, United Kingdom, 2023 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, 5 Princes Gate Court, London, SW7 2QJ, United Kingdom

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

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

Cancer Metastasis - Molecular Mechanism and Clinical Therapy
Edited by Yusuf Tutar and Lütfi Tutar
p. cm.
Print ISBN 978-1-80356-161-5
Online ISBN 978-1-80356-162-2
eBook (PDF) ISBN 978-1-80356-163-9

# Meet the editors

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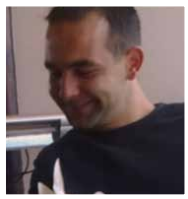

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# Contents

# Preface

Cancer mortality and morbidity are primarily brought on by metastasis. The ability of neoplastic cells to spread and colonize distant tissues is their most dangerous trait. Most malignancies are curable when they are detected early and have not spread beyond the original tissue. However, cancer is frequently incurable when tumor cells have created colonies elsewhere.

Thus, this book's content provides comprehensive information on cancer metastasis with reference to specific cancer cases. The first section covers the formation of metastatic cells through the perturbation of cell-cell and cell-matrix adhesion and through matrix degradation, as well as the sub-topics of motility, intravasation, extravasation, metastatic colonization and angiogenesis. The next section considers the molecular mechanism of metastasis, discussing stromal cells and extracellular vesicles, and epigenetics in cancer metastasis. Factors affecting the molecular basis of cancer metastasis are discussed. The third section covers the molecular mechanism of breast cancer metastasis, and the link between molecular mechanisms and novel therapeutic approaches in non-small cell lung cancer brain metastasis. The final section of the book reviews current palliative therapies for bone cancer metastasis.

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Section 1

# Prelude

Chapter 1

# Introductory Chapter: Molecular Mechanism of Cancer Metastasis

*Yusuf Tutar*

## 1. Introduction

Neoplastic cells' capacity to spread and colonize distant tissues is their most dangerous trait. Most malignancies are curable when detected early and have not spread outside of the original tissue. However, cancer is frequently incurable when tumor cells have created colonies elsewhere. Tumor progression is the transformation of a healthy cell into a metastatic cancer cell that poses a threat to life. Neoplasia is a cellular illness, and research has been done to better understand the molecular mechanisms underlying the early stages of the progression that lead to the formation of cancer. The molecular mechanisms underpinning the behavioral changes that distinguish a metastatic cell from cells that are still at the site of tumor formation are now understood to reflect a fraction of cells that have left the primary tumor and are known as metastases [1, 2].

Tumor cells go through predominant steps and reach metastasis: invasion, intravasation, delivery, extravasation, and metastatic colonization. Further, tumor cells communicate with the surrounding microenvironment or tumor-associated stroma [3]. Tumor microenvironment affects tumor cells' metastatic ability and subpopulation of cancer stem cells, angiogenic vascular cells, cancer-associated fibroblasts, and infiltrating immune cells, the process additionally affects primary tumor metastatic capacity to distant locations [2, 4].

The spread of malignant cells to distant or disjointed secondary sites, where they multiply to create a mass, is known as metastasis. For almost every characteristic that is measured, tumor heterogeneity exists [1–3]. Positional, temporal, and genetic heterogeneity are the three forms that can exist inside a tumor. The accessibility of a cell to heterogeneous extrinsic stimuli influences positional heterogeneity. Regarding alterations in cells brought on by cycle signals, temporal heterogeneity is important. Genetic diversity is a result of the characteristics that tumor cells have by nature. Single-cell clone isolation proves that there are fundamental variations among the subpopulations that make up a single tumor mass [1].

## 2. Formation of metastatic cell

### 2.1 Invasion by perturbing cell: Cell and cell matrix adhesion

Invasion initiates as a result of tumor cell breaking of the basement membrane and penetrate underlying stroma. Tissues architecture forms from epithelium, basement membrane, and stroma. Catherins adhere cells to each other through catenins inside the cell. Integrin receptors attach cells to fibronectin at the extracellular matrix and

fibronectin attaches to collagen. Normally epithelial cells are maintained by cell–cell anchoring junctions: tight junctions- adherent junctions attached to actin and keratin, respectively, whereas cell matrix anchoring junctions hemidesmosomes attached to keratin intermediate filaments like cell-anchoring factor desmosomes. Changes in cell–cell and cell-matrix adhesion are necessary for invasion; these changes must be coordinated with matrix breakdown and cellular mobility. All these molecules provide cell integrity, and therefore, cell adhesion proteins are the target of oncogenes as well as the tumor suppressor proteins that regulate the signaling pathways.

Cadherin functions as tumor suppressor and suppresses tumor cell metastasis at distant sites. Integrins pin cells to basement membrane—extracellular membrane, and cells break free from the binding site during metastasis. Integrins affect the cytoskeleton by binding to actin and key kinases like FAK (Focal Adhesion Kinase). Actually, FAK mediates cell motility and activates the RAS pathway. Therefore, enhancing integrin expression in tumor cells induces mobility and invasion of metastasizing cells. Degradation of extracellular matrix and stroma for invasion of tumor cells to the nearby tissue also depends on proteases [1, 3].

Epithelial-mesenchymal transition involves changes in shape and confers metastatic properties and this process is accompanied by enhancing mobility, invasion, and resistance to apoptotic stimuli. The change provides cells to migrate to distant sites. Epithelial-mesenchymal transition associates with loss of E-cadherin from the adherens junctions and a switch from the expression of keratins to the mesenchymal intermediate-filament vimentin [5].

### 2.2 Invasion through matrix degradation

One of the hallmarks of the malignancy is the disruption of basement membrane and enzymes extruded from tumor cells degrade matrix for invasion. These enzymes form a diverse family, including serine/cysteine proteinases, cathepsin, disintegrin, ADAM metalloproteinases, and matrix metalloproteinases (MMP). Increased MMPs are considered poor prognosis in several cancer types and correlate to invasion and metastasis. Cathepsins, proteinase inhibitors, and cysteine proteinase inhibitors regulate proteolysis. Both tumor and stromal cells play roles in the inhibitory mechanism [1, 5].

### 2.3 Motility

Actin filament assembly and treadmilling through coordinated polymerization and depolymerization provide cellular locomotion. However, tumor cells stimulate motility through lysophospholipase D in an autocrine fashion. *C-met* and hepatocyte growth factor interact and induce invasive epithelial cells chemokinetic activity. Chemotactic/haptotactic effect correlates to directional motility [1, 6]. Structures so called invadopodia determined in invading cells and represent the physical convergence of adhesion, proteolytic, and motility components of invasion. Therefore, invapodia is the essential structure for cancer invasion; however, if a tumor cell can not complete subsequent steps, it cannot go through metastasis [1].

### 2.4 Intravasation

Entry of a tumor cell into either blood or lymphatic vessels by serine and metalloproteinase action is called intravasation. After proteinase activity, tumor cells pass

from endothelial cells into the bloodstream. Tumor cells may travel either alone or as emboli (clumps with platelets) within the direction of blood flow.

Once in the vessels, most of the tumor cells are killed by monocytes or natural killer cells. Larger size of the tumor cells at the capillaries encounters a problem—hemostatic shear force. Smaller vessels break tumor cells by shear forces due to hydrostatic pressure.

Further, when tumor cells bind to endothelium via E-selectin, the cells are attached/overlapped. In this case, arrested tumor cells can go through apoptosis. This attachment may lead tumor cells to release NO and the process also drives the cells to apoptosis [2, 7].

### 2.5 Extravasation

The escape of a tumor cell from the vessels is named as extravasation. Tumor cells invade from the interior of a vessel into the organ parenchyma. There is a debate in the literature about whether extravasation is necessary for metastases process. One key evidence for this dilemma originates from lung endothelium-attached tumor cells. The cells survive and grow intravascularly; therefore, further experiments are required to elucidate the molecular mechanism [1, 3].

### 2.6 Metastatic colonization

Metastatic colonization is an inefficient metastatic cascade step in which progressively growing tumor forms at distant ectopic sites. This process involves the formation of new blood vessels to provide nutrients and oxygen. Micrometastasis contrast with colonization do not constantly grow but stays dormant for longer times. Metastatic colonization is the rate-limiting step of metastasis [3].

### 2.7 Metastasis and angiogenesis

Formation of new blood vessels from pre-existing vessels, angiogenesis, and augment metastatic colonization. Angiogenesis is essential for metastasis so that tumor cells get oxygen and nutrients as tumor cells exceed a minimum size, nutrients and oxygen can no longer reach through diffusion. By the same token, metabolism end products (lactate, ammonia, and lactate) cannot diffuse easily [3].

## 3. Conclusion

Elucidating the molecular mechanism of metastasis can improve efficient drug design and therapies. Currently, no distinguishable cellular behavior detected between normal and metastatic cells. Further, invasion is not a unique property for cancer cells. However, invadopodia may provide some insights and metastatic cells also proliferate without differentiating. Plus, the colonization stage of metastasis provides therapeutic opportunities as the cells are proangiogenic for long periods. All these differences may provide targets for both drug design and therapeutic approaches. In spite of all these differences, it is relatively easy to compare two distinct stages/properties of the tumor and may provide plethora of data.

## Conflict of interest

The author declares no conflict of interest.

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Section 2

# Molecular Mechanism

Chapter 2

# Stromal Cells and Extracellular Vesicles

*Arinzechukwu Ude, Emmanuel Ogbodo and Kelechi Okeke*

## Abstract

Stromal cells are stem cells in the bone marrow microenvironment that can 'talk' with neighbouring and distant cells within the bone marrow microenvironment. Stromal cells propagate this intercellular communication via cytokines, growth factors as well as small extracellular vesicles. The interaction between stromal cells and the haematopoietic stem cells, is crucial in the regulation of haematopoiesis. Aberration in this regulatory process will lead to the development of various diseases, including cancer. These stromal cells also play important role in the patient's response to cancer therapy. As a result, these stromal cells may be crucial in the development and metastasis of cancer within the bone marrow microenvironment. In this chapter, we will explore the role of these stromal cells in carcinogenesis and cancer metastasis.

**Keywords:** stromal cells, extracellular vesicles, cancer, metastasis, tumour, microenvironment, bone marrow, therapy

## 1. Introduction

The bone marrow (BM) consists of multilineage cell types, most especially the haematopoietic and mesenchymal lineage. The interaction between the haematopoietic and mesenchymal cell lineages are crucial in the maintenance of haematopoiesis [1, 2]. As a result, BM is the major site of haematopoiesis, which is the lifelong process of blood cells formation. Within the BM microenvironment or stroma, the haematopoietic and mesenchymal progenitor cells give rise to different cells such as immune cells, osteoclasts endothelial cells, stromal cells (mesenchymal stromal cells; MSC) and nerve cells [1, 3].

These stromal cells are characterised *in vitro* by the International Society for Cellular Therapy (ISCT) by three main qualities; (i) ability to adhere to plastic in standard culture conditions; (ii) expression of CD105, CD73 and CD90 surface molecules but lack expression of CD45, CD34, CD14 or CD11b, CD79a, or CD19, and HLA-DR proteins; (iii) multilineage differentiation potential into osteoblasts, adipocytes, fibroblasts and chondroblasts [4–6]. These stromal cells offer haematopoietic support, immunomodulation, and bone remodelling via cell-to-cell contact and/or secretion of soluble factors.

During carcinogenesis, the BM stroma goes rogue and enables cancer cells to recruit supporting cells from the tissue stroma needed to promote critical steps in

tumour formation and thus, constitute a vital cog of the tumour microenvironment (TME) [5–7]. These stromal cells are recruited into the TME via secretion of different biomolecular factors such as cytokines, extracellular vesicles (EVs), chemokines, and growth factors. These stromal cells play important roles in all steps of cancer metastasis such as extracellular matrix (ECM) remodelling, migration, invasion, intravasation, circulation, survival, extravasation, and colonisation of distant secondary tumour sites [8–10].

Metastasis refers to the process of dissemination of cancer cells from its point of origin (primary site) to a distant disconnected part of the body, forming macroscopic secondary foci which constitutes a metastatic cancer [11, 12]. Metastasis was coined from the two Greek prefixes "meta" (alteration or change) and "stasis" (an equilibrium state), to represent both a process and its outcome. Despite the advances in cancer treatment, evidence from clinical experience and biologic inferences, show that metastasis is responsible for about 90% of cancer morbidity and mortality, with over two-thirds (66.7%) of deaths originating from solid tumours [13]. Metastasis is one of the hallmarks of cancer have been shown to occur as a complex, sequential but inter-related cell-biological events called the invasion-metastasis cascade [14].

Depending on the tumour type, stromal cell composition within the tumour microenvironment often varies and usually includes mesenchymal stem/stromal cells, pericytes, fibroblasts, adipocytes, vascular endothelial cells, stellate cells, and immune cells such as macrophages, T-cells, and natural killer (NK) cells [15–17]. Once recruited, these stromal cells undergo tumoral education and transform into tumour stroma. These damaged stromal cells are also vulnerable to cancer aggression either via direct contact with each other, through gap junctions thereby resulting in transfer of material from stromal cells to cancer cells [9, 15–17]. These lead to promotion of tumour growth, angiogenesis, proliferation, invasion, metastasis and chemoresistance once recruited to the tumour microenvironment [18].

## 2. Stromal cells and cancer metastasis

Normally, cells in the human body undergo continuous cellular division to ensure proliferation and differentiation of cells, and removal of damaged/worn-out cells (apoptosis) to ensure balance in the cellular system. Cancer arises when there's uncontrolled growth and/or proliferation of cells in the body without apoptosis. Cancer can emanate anywhere in the human body, and these cancer cells can be benign or malignant. In addition, cancer cells can metastasize or spread into, or invade nearby tissues and can travel to distant places in the body to form new tumours [19, 20].

Cancer cells spread either by invasion of nearby tissues or by movement through the lymphatic and blood vessels. Although different cancers are more likely to spread to downstream organs and lymph nodes close to its primary sites than others, most common metastatic areas include the liver, lung, and bone [19, 20]. Most of the cancers that separate from the original tumour do not survive as they also require the capacity to adhere to the blood or lymph vessels, grow and thrive in the new site as well as evade the attacks from the immune system [20].

However, it is noteworthy to mention that not all cancer cells are metastatic and not all cells within the metastatic tumour have the potential to metastasize [21]. The essential hallmarks of metastasis can be difficult to ascribe since they are super-imposed by that of cancer itself, however, these five qualities have been reported

and includes: dissemination (detachment) and invasion, intravasation, circulation, extravasation, and colonisation [12, 22].

## 3. Endothelial cells

Endothelial cells are crucial in the promotion of cancer cell migration, invasion, and metastasis. During tumorigenesis, gaseous exchange and nutrient transport occur by passive diffusion however an increase in the volume of the tumours (1–2 $mm^3$) leads to insufficient oxygen and a build-up of metabolic waste in the tumour microenvironment [6, 7, 16, 23]. This makes the tumour microenvironment to become hypoxic and acidic thereby highlighting a need for the tumours to develop their own blood supply to overcome this.

The vascular endothelium, a thin layer of endothelial cells, aids in orchestrating the separation of circulating blood from tissues, delivery of water, oxygen and nutrients, movement and adhesion of leucocytes, and formation of blood vessels within the tumour microenvironment [6, 7, 23]. The vascular endothelium is highly organised and hierarchical in structure, and this enables the interaction between stromal and non-stromal cells to provide support and stability for the blood vessels.

Tumours co-opt existing blood vessels and induce growth of new blood vessels by a mechanism known as vessel sprouting [7, 23]. Abnormal sprouts are characteristic of the tumour vasculature along with intercellular gaps and no hierarchical arrangement. These vascular endothelial cells within the tumour microenvironment interact with tumour cells and other stromal cells to promote tumorigenesis and metastasis.

The hypoxic tumour microenvironment leads to expression and activation of hypoxia-inducible factors (HIFs) that co-ordinate cellular response to low oxygen levels. These HIFs then instruct the endothelial cells to secrete and release proangiogenic factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) thereby initiating vessel sprouting [6, 7, 23, 24]. These proangiogenic factors, especially VEGF then promote vascular permeability and angiogenesis by stimulating the migration of endothelial cells to form new blood vessel lumen in an autocrine and paracrine fashion. Activation of VEGF receptors (VEGFR) on endothelial cells also activate several downstream signalling pathways including the mitogen-activated protein kinases and extracellular signal-regulated kinases (MAPK/ERK) and phosphatidylinositol-3 kinases (PI3K/Akt) pathways involved in the regulation of cell survival, cell cycle progression, cell growth and angiogenesis [23].

The endothelial cells secrete proteins to form new basement membranes, which are often immature and fail to reach final stages of maturation thus resulting in a leaky vasculature [6, 7, 23]. Endothelial cells communicate with the basement membrane and the ECM through integrin proteins (collagen, elastin, fibronectin and fibrillin) and proteoglycans for mechanical and physical support [25]. The basement membrane degrades to activate stroma thus allowing activated stroma to have a direct contact with tumour cells during tumorigenesis [26]. This induces alterations such as enhanced vascularity and increased ECM production, which are all essential for invasion.

During tumour metastasis, following cell detachment, tumour cells first undergo intravasation by first escaping the primary tumour site and enter the vasculature [6, 7, 23, 27]. Upon entering the vasculature, the tumour cells then adhere to endothelial cells during intravasation thereby changing the endothelial

barrier, which allows the tumour cells to migrate between two endothelial cells. This signifies that the interaction between the endothelial cells and tumour cells is reciprocal. The tumour cells can differentiate into endothelial cells within the tumour microenvironment to support and sustain tumour growth. Endothelial cells can also change cell fate and often undergo endothelial-mesenchymal transition (EMT), organised by TGF-β, EGF and bone morphogenetic protein (BMP), to cancer-associated fibroblasts (CAFs) during tumour progression [6, 24, 28]. This leads to a loss in cell-to-cell connection, detachment and elongation, enhanced migration, and a loss of endothelial properties. Cell adhesion establishes a tight a tight connection between cells as well as between cells and ECM thus activating cell proliferation and survival pathways [7]. Therefore, this loss in cell-to-cell connections enables cancer cells to transverse the vasculature (extravasation) and interact with pre-metastatic niches that permits cell proliferation and colonisation at the secondary sites [7, 24].

Furthermore, vascularization and endothelial cell expansion enhance tumour initiation and self-renewal properties of cancer stem cells within the tumour microenvironment. Endothelial cells secrete soluble factors that aid in the maintenance of stem cell properties in neural stem cells and activate cancer cells thereby promoting tumour growth [6]. Endothelial cells also secrete cytokines such as IL-8, which promote characteristics of cancer stem cells in glioblastoma including their migration and invasion abilities [6]. In a positive feedback loop of IL-8 mediated signalling, glioblastoma cells induce endothelial cell migration toward the tumour bulk thereby promoting brain tumour growth. In oesophageal cancer, epiregulin (EREG) overexpression is induced by endothelial cells and this leads to an increase in actin rearrangement, spheroid formation and enrichment of cancer stem cells [6].

## 4. Cancer-associated fibroblasts

CAFs are heterogenous populations of cells within the tumour microenvironment that have different phenotypic characteristics even within the same type of cancer. The origin of the cells is diverse; usually arise from tissue-resident fibroblasts but can also be derived from adipocytes, endothelial cells, pericytes, stellate cells and bone-marrow derived mesenchymal stem cells [7]. The role of these CAFs in the tumour microenvironment is to shape the tumour microenvironment via tumour proliferation, neoangiogenesis, invasion, metabolic reprogramming, extracellular matrix remodelling, immunosuppression, and metastasis [6, 7].

These cells facilitate the crosstalk between cancer cells and the tumour microenvironment. In the tumour microenvironment, a crosstalk between cancer cells and stromal cells leads to the secretion of factors such as TGF-β, PGDF, connective tissue growth factor (CTGF), hepatocyte growth factor (HGF) and fibroblast growth factor 2 (FGF2), which initiates the conversion of fibroblasts into cancer-associated fibroblasts (CAFs) [6, 7, 23, 29, 30]. Usually, their activation is via the NF-κB and JAK-STAT signalling pathways and is dependent on the secretion and release of signalling molecules such as TGF-β, stromal cell-derived factor-1 (CXCL12/SDF-1), platelet-derived growth factor α/β (PDGF α/β), basic fibroblast growth factor (b-FGF), RTK ligands, IL-1β and IL-6 by cancer or immune cells [6, 31]. The activation of CAFs is a common feature in tumorigenesis and these CAFs are perpetually activated unlike in normal tissues.

CAFs are a rich source of growth promoting molecules and proangiogenic factors as well as extracellular components such as growth factors, cytokines, and

extracellular matrix components. During cancer progression and metastasis, these cells secrete VEGF and TGF-β, which are crucial in angiogenesis and epithelial-mesenchymal transition (EMT) respectively [6, 7]. EMT is a vital step in metastasis via epigenetic changes, and it involves the loss of cell polarity and cell-to-cell adhesions by epithelial cells. In turn, these cells gain migratory and invasive phenotypes.

CAFs provide the physical scaffolding of cells and facilitate the migration of cancer cells through the tumour microenvironment by altering three-dimensional structure of ECM via the secretion of plasminogen activator protein and matrix metalloproteinase 3 (MMP3) that degrades E-cadherin to promote cancer cell invasion [9, 32, 33]. CAFs also migrate together with epithelial cancer cells thereby suggesting these cells play an important role in intravasation and extravasation of epithelial cells in metastasis by enhancing transmigration of cancer cells through endothelial cell layers [9, 32, 33]. In invasion of squamous cell carcinoma and breast cancer, CAFs create tracks through the ECM that cancer cells could not create on their own through Hippo-signalling dependent remodelling of ECM [9, 32, 33]. Activated fibroblasts also secrete elevated levels of ECM-degrading proteases such as matrix metalloproteases 2 and 9 (MMP2 and MMP9) [23]. Increase in ECM remodelling and degradation is associated with increase in metastasis.

Following activation by TGF-β, CAFs also modulate immune cells through factors such as monocyte chemoattractant protein-1 (MCP-1) and IL-1 leading to a pro-inflammatory microenvironment [23]. Activated CAFs also regulate collagen structure in the stroma of multiple solid tumours, including breast cancer. The cross-linking and alignment of collagen are associated with poor prognosis in cancer thus regulating invasion and metastasis [23]. CAFs also interact with the epithelium in breast cancer thus enhancing breast cancer progression and metastasis. In addition, CAFs also express programmed death ligand 1 (PD-L1) that leads to the suppression of $CD8^+$ T-cell immune responses and thence, progression of colon cancer [23].

## 5. Adipocytes

Adipocytes are specialised cells in the body that synthesise and store excess energy as fat thus regulating energy balance. Adipocytes are divided into lipid storing white adipocytes and thermogenic brown adipocytes [7, 23]. These cells secrete and release metabolites, enzymes, hormones, growth factors and cytokines through which they exert their effects on the tumour microenvironment. Adipocytes become activated when located near a growing tumour thus supplying pro-tumorigenic factors that will stimulate cancer cell invasion [7, 23]. These adipocytes are termed cancer-associated adipocytes (CAA).

In cancer progression and metastasis, these CAA form a crucial yet reciprocal relationship with the tumour cells. For example, in tumour microenvironment, adipocytes play very important role as the breast tissue is largely composed of white adipose tissue [7, 23]. This white adipose tissue enhances metastasis of breast cancer cells to the liver and lungs via paracrine signalling. Under the stimulation of breast cancer cells, adipocytes undergo lipolysis to breakdown lipid stores and make free fatty acids available for cellular uptake by cancer cells in response to local ECM remodelling [7, 23, 28, 32]. Cancer cells then use up these free fatty acids for respiration (energy production), formation of cell membrane, lipid bioactive molecules and/or package them into extracellular vesicles such as exosomes [7].

Adipose tissues play a vital role in the formation of mammary duct and vasculature by providing growth factors such as VEGF [23]. Therefore, adipocytes regulate angiogenesis and epithelium function. White adipocytes are also important in the production and secretion of hormones, especially leptin, oestrogen, and IGF-1 [6, 7, 23]. As a result, adipocytes directly promote tumour progression by releasing leptin that regulates food intake thus helping the body to maintain its weight. In breast cancer, leptin signalling enhances breast cancer cells by increasing receptor expression levels and activating different signalling pathways such as Notch, Wnt, HER2, AKT and NF-κB that have been implicated in tumorigenesis and tumour invasion [6]. Elevated levels of leptin in the BM microenvironment supports the proliferation and migration of cancer cells and protects them from cellular damage by suppressing caspase-3 activity [7, 23]. Most cancer patients are overweight making obesity a major risk factor for different types of cancer such as breast, pancreatic and ovarian. In addition, obesity-associated fatty acid binding protein (FABP4) is elevated in patients with breast cancer [6]. FABP4 increases tumour volume, tumour-initiating frequency and stemness markers via IL-6/STAT3/ALDH1 signalling pathway [7]. Breast cancer cells also interact with adipocytes via secretion of inflammatory factor IL-6 that plays a key role in maintaining cancer stemness. Adipocyte-secreted IL-6 also play important roles in Notch/Wnt/TGF-β signalling pathways by upregulating ALDH1A1 and LEF1 and AXIN2 gene expression in the Wnt pathways to promote invasion, angiogenesis, and metastasis of breast cancer [6]. Adipocytes also increases the metastasis of breast cancer cells via upregulation of PLOD2 expression. Elevated levels of IL-6 in the tumour microenvironment also regulates Bcl-xl and OCT4 expression in ovarian cancer through the regulation of STAT3, which contributes to chemoresistance [6]. Tumour-secreted soluble factors such as IL-6 and parathyroid hormone-related peptide (PHRP) stimulate browning (trans-differentiation of white to brown adipocytes) thereby resulting in an increase in energy expenditure of adipose tissues that contribute to cancer-associated cachexia [23]. Cancer-associated cachexia is a muscle wasting condition that negatively impacts patient quality life and as a result, is associated with poor prognosis.

Adipocytes also promote tumour progression indirectly, by activating macrophages. These tumour-associated macrophages (TAM) release growth factors, cytokines, inflammatory mediators, and proteolytic enzymes that mediate tumour growth, tumour cell migration and invasion [22]. Finally, adipocytes secrete metalloproteases such as MMP1, MMP7, MMP10, MMP11 and MMP14 that are important in modifying and degrading ECM [22, 34, 35]. These MMPs and serine proteases (such as urokinase plasminogen activator; uPA) are the major enzymes responsible for ECM degradation [29, 36, 37]. Both MMPs and serine proteases are involved in all stages of tumour progression such as angiogenesis, stroma invasion, intravasation, regulation of inflammation and metastasis [22, 29, 36, 37].

## 6. Extracellular vesicles and cancer metastasis

Extracellular vesicles (EVs) are nanoparticles released by different types of cells that contain a lipid bilayer structure [38, 39]. There are three major types of EVs: namely exosomes, apoptotic bodies and microvesicles [39, 40]. However, other types such as oncosomes, cytoplasts and exomeres have also been identified. These subtypes are characterised based on their sizes, biogenesis, origin (tumour-derived, stromal cell-derived etc), functions (immune-suppressing/stimulating-EVs, pro-apoptotic EVs etc)

and surface markers ($CD63^+$, $CD9^+$, $CD81^+$, or $EpCAM^+$ EVs) [38, 41]. Despite this heterogeneous population of EVs, each EV is unique thus the dynamic function of EVs is due to their highly heterogeneous characteristics, which makes it difficult to accurately differentiate these EV subtypes [42].

Apoptotic bodies are the largest EV in size and are produced by dying cells [38]. These EVs contain many intercellular materials such as intracellular fragments, cellular organelles, and cytosolic contents [38, 40]. Microvesicles are the second largest EV in size and originate from the outward budding or fusion of the cytoplasm membrane and are later released into the extracellular space [43]. They majorly contain lipids such as sphingolipids, cholesterol, and phosphatidylserine [40, 44]. Both apoptotic bodies and microvesicles are sometimes collectively called ectosomes and often originate via direct outward budding or blebbing of the plasma membrane [40]. Lastly, exosomes are bilayered membrane small extracellular vesicles of 40–200 nm size that are derived from the fusion of multivesicular bodies (MVB) into the plasma membrane and resulting release of intraluminal vesicles (ILVs) into the extracellular space through exocytosis [45, 46]. Therefore, any factor that may affect the plasma membrane may positively or negatively influence formation of these EVs.

These membrane-bound organelles function as important mediators of intercellular communication mechanism and often harbour bioactive molecules such as metabolites, proteins, RNA, DNA, and lipids that often reflect the parent cell [39, 47]. The lipid membrane of these EVs serves a protective shield for enclosed nucleic acids thereby protecting them from degradation by extravesicular nucleases [48]. Much of the RNA composition are from miRNAs, a class of non-coding RNAs that mediate post-transcriptional gene silencing in many biological processes [47, 49]. Once released, these vesicles are taken up by recipient cells and could influence the pathological and physiological functions in the recipient cells by activating different signalling pathways [49–52]. These EVs deliver genetic information to recipient cells, which affect signalling transduction pathways thereby regulating target gene expression and determining the function and fate of recipient cells such as apoptosis, growth, cell cycle, migration, and differentiation [49, 53, 54]. Internalisation of these vesicles into the recipient cells occur by endocytic process via phagocytosis, fusion with the cell membrane and interaction with receptors on the cell membrane [48, 55].

During tumorigenesis, the bidirectional cell-to-cell communication between tumour and healthy cells within the TME is one of the mechanisms that enable cancer progression and metastasis, and EVs mediate this intercellular communication [56]. EVs released by cancer cells are increasingly found circulating in body fluids such as blood, urine, saliva, ascitic fluid and milk whereby they enhance the proliferation and invasion of tumour cells in autocrine and paracrine manner [44, 55]. The hypoxic or metastatic status of the tumours plays an important role in sorting the loading of composition of EVs, which affects the functions of tumour-derived EVs in the TME [24, 56]. EVs shuttle regulatory molecules, including lipids, nucleic acids and proteins that induce the reprogramming and remodelling of the stroma by facilitating the development of a tumour-supportive environment [39, 47, 57, 58]. These tumour-derived EVs within the hypoxic microenvironment also drive Warburg effect thereby driving conversion of glucose mainly into lactate to meet energy requirements to ensure tumour survival [57, 58]. They also regulate the metabolism of lipids and amino acids by cancer cells to build biomass and provide more energy.

This leads to immunogenic stress thereby initiating immune changes within the TME and influencing cancer progression. Tumour-derived EVs inhibit immune response, promote the transformation of CAFs, and reprogram endothelial cells

function thus creating an anti-tumoral environment. Tumour-derived EVs interact with the host immune system and cause functional and phenotypic changes in immune cells such as natural killer (NK) cells, macrophages, T-cells, and B-cells thereby affecting the immune system homeostasis [57, 59]. EVs released by tumour cells also induce immunosuppressive or tumour-associated macrophages by NF-κB mediated metabolism and secretion of VEGF, IL-6, TNF-α and G-CSF thereby leading to cancer metastasis [60, 61]. Tumour-derived EVs also increase neutrophil mobilisation and activate regulatory T-cells that protects the tumour from $CD8^+$ T-cell mediated killing [59, 62, 63]. In addition, tumour-derived EVs activate or suppress NK cells depending on the type of tumour and express FasL and TRAIL on their membrane thereby directly influencing the apoptosis of $CD8^+$ cells [59, 62, 63]. However, tumour-derived EVs can also activate dendritic cells via delivery of tumour-derived antigens and stimulate a $CD8^+$-mediated anti-tumour response.

Tumour-derived EVs also regulate the pro-tumoral function of endothelial cells by sustaining the constant delivery of nutrients and oxygen from the vascular endothelium [58, 60, 64]. Under hypoxic conditions, tumour-derived EVs also promote the regulation of endothelial cell proliferation, migration, sprouting, branching, as well as tubular-like structure formation via delivery of miRNAs, mRNAs, and proteins hence tumour-derived EVs promote angiogenesis in different types of cancer, including hepatocellular carcinoma, colorectal cancer, cervical cancer, nasopharyngeal carcinoma, glioma, and lung cancer. Neoangiogenesis, secretion of growth factors and EVs, and inflammatory cells recruitment induce the formation of pre-metastatic niches, where new tumour cells extravasate, get arrested or colonise [43, 57, 58, 61]. This further ensures tumour metastasis. In addition to pre-metastatic niches, EVs are also involved in other processes of tumour metastasis such as EMT and organ-specific metastasis.

Under hypoxic conditions, tumour-derived EVs stimulate the transition of stromal cells into CAFs via TGF-β, which in turn increase shedding of EVs and induce ECM remodelling, angiogenesis, migration, and invasion of cancer cells via different signalling pathways [58, 60, 64]. Tumour-derived EVs enhance the ability of CAFs in response to metabolic environment by activating MYC signalling pathway in stromal cells resulting in rapid tumour growth. These EVs-bound factors modify the phenotype of cancer cells or tumour stromal cells to support the aggressive phenotype and tumour progression. CAFs regulate tumour microenvironment and transfer proteins, metabolites such as tricarboxylic acid (TCA) intermediates and lipids utilised by cancer cells via EVs to facilitate and promote tumour growth under nutrient deprivation conditions [15, 31, 59, 65]. CAFs-derived EVs also enhance EMT via release of factors such as fibronectin and vimentin that trigger the loss of tumour cell adhesion, as well as differentiation of osteoblasts and proliferation of osteoclasts, which regulate the microenvironment of bone metastasis.

## 7. Stromal cells and clinical therapy

Stromal cells have therapeutic potential in cancer treatment and targeting stromal components in combination with cancer cells may increase the efficacy of cancer therapy [4, 6]. Stromal signatures characteristic of different cancer subtypes may have clinical relevance and may even serve as a prognostic marker of the disease.

Previously, chemotherapeutic agents were used to target all cells within the tumour microenvironment however, efficacy of these therapies is reduced by the development of drug resistance [7, 59]. Drug resistance occurs primarily by activation or mutation of signal transducers downstream of the targeted molecule or secondarily when neoplastic cells originally sensitive to these drugs lose their response to drugs [6, 8, 59]. In recent years, advancement in therapeutic targeting of the tumour microenvironment has led to specific targeting of cells within the tumour microenvironment. Poorly vascularised stroma supports tumorigenesis and simultaneously forms a barrier for chemotherapeutic drugs making it as an attractive drug target [6, 8]. Since tumours require endothelial cells to form new blood vessels to help relieve oxygen deprivation and accumulate metabolic wastes, angiogenesis is one of the mechanisms targeted by chemotherapy.

Most of these drugs such as bevacizumab, aflibercept, sorafenib and ramucirumab target the VEGF-VEGF signalling pathway in diverse ways as this is associated with tumour progression and poor prognosis in breast cancer [7, 8, 51, 58]. Bevacizumab acts a neutralising antibody to VEFG that reduces vascular permeability thus affecting the first step of tumour stroma development however aflibercept acts a decoy receptor for VEGF. Sorafenib acts a tyrosine kinase inhibitor and ramucirumab acts as an antibody that blocks VEGF from binding to its receptor. However, these chemotherapeutic agents have shown limited success when administered to patients as a single agent. Most patients develop resistance or do not respond to this anti-angiogenic therapy. Metastatic tumour cells have a striking feature/ability to plastically adapt to different microenvironmental conditions and overcome a single-drug treatment [7, 8, 51, 58, 66].

To enhance success within the clinical settings, combination of these drugs or other drugs/approaches may likely prove to be beneficial. For example, combination of bevacizumab and PDL1 proved to be a success in the treatment of hepatocellular carcinoma and renal cancer [7]. Combination therapies targeting thyroid cancer cells and stroma may also offer treatment alternatives as there have been no convincing clinical studies that show the efficacy of tumour stroma inhibition in the most aggressive forms of thyroid cancer.

In addition, an antibody that blocks IL-8 has also been trialled to target the tumour-promoting effect of endothelial cells in glioblastoma with success [6, 23, 31]. This led to a marked reduction in tumour size. Other researchers have also shown that inhibition of IL-8 re-sensitised tumour cells to chemotherapeutic agents, cisplatin, and paclitaxel [6, 31]. Furthermore, CCL5 and IL-6 have also been shown to be associated with acquisition of chemoresistance [6, 31]. These suggest that these cytokines as well as other ligands of CXC chemokine receptors 1 and 2 could be very important in the induction of chemoresistance via recruitment of MSCs around the tumour. In addition, EGF secreted by endothelial cells has been associated with drug resistance in squamous cell carcinoma [6]. Nevertheless, there are very few existing FDA-approved treatments with limited efficacy, but new therapeutic targets and strategies will be identified as researchers continue to understand how the tumour microenvironment contributes to tumour progression and metastasis. There is potential for the use of chimeric antigen receptor natural killer cells, liver stellate cells and fibroblasts [7, 51].

In addition, CAFs may be novel and attractive targets for cancer therapy. CAFs also show the strongest expression level of the stem/mesenchymal transcription subtype of cancer. The crosstalk between CAFs and cancer stem cells is a convincing strategy for immune suppression, drug resistance, metastasis and stemness of

cancer cells [6, 23]. CAFs secrete TGF-β and HGF that contribute to drug resistance in tumour cells, including tamoxifen-associated resistance in breast cancer cells [6, 23]. As a result, some novel drugs target the interaction between CAFs and breast cancer cells as it is believed that CAFs increase interstitial pressure within the tumour thereby reducing the efficacy of drug delivery [6, 23, 66]. Also, pirfenidone, which is an anti-fibrotic agent with multiple functions including anti-TGF-β activity, was combined with doxorubicin to inhibit tumour growth and metastasis in a preclinical triple-negative breast cancer (TNBC) model [6, 23].

Targeting CAFs may affect other stromal cells such as polarising tumour-associated macrophages (TAM) and cause suppression of the cytotoxic activities of NK cells since CAFs are involved in promoting immunosuppression [23]. Partial depletion of stroma using CD40-activated macrophages has shown to improve patient survival and increase drug delivery into the tumour [6]. CAFs-induced EMT causes resistance to cisplatin in non-small-cell lung carcinoma [6, 28, 51]. Therefore, a build of CAFs in the tumour microenvironment is associated with poor prognosis in many cancers, including lung adenocarcinoma, squamous cell carcinoma and colorectal cancer, where it is associated with diseases reoccurrence [6, 23, 51]. However, these cells are associated with improved prognosis and overall survival in small lung cell carcinoma. Some researchers have illustrated that targeting Hedgehog-activated CAFs results in improved survival, chemosensitivity and reduced metastatic burden in breast cancer [6, 67].

However, depleting CAFs is not always beneficial and has been associated with increased angiogenesis and enhanced cancer cell properties in pancreatic cancer with shorter patient survival. Hence, these suggest that therapeutic targeting of these CAFs may ameliorate some cancers. Furthermore, the expression of CD44 on CAFs can be functional target for destroying cancer cells in the TME and TGF-β signalling mediated by CAFs plays a role in regulating cancer cells in gastric cancer. Inactivating CAFs or lowering the level of infiltrating CAFs in the TME are potential therapeutic strategies for reducing cancer stemness. Targeting myofibroblast-like CAFs using focal adhesion kinase (FAK) inhibitor resulted in a reduction of pancreatic cancer cells [6]. CAFs can also be targeted by inhibiting their activation by using drugs to target CAF-associated proteins such as fibroblast activation protein (FAP) and DNA methyltransferase 1 (DMNT1) [6]. Sibrotuzumab, a FAP-targeting antibody has been tested in the treatment of Phase II metastatic colorectal cancer whilst combination of DNMT1 and DNMT1 and Janus Kinase (JAK) signalling resulted in the normalisation of fibroblasts, but these failed to demonstrate efficacy [6, 8, 31]. Thus, it is noteworthy to mention that identifying and targeting fibroblasts is problematic due to heterogeneity of markers found on these cells. This, identifying CAFs aid define activate stroma borders and may even affect clinical response to treatment.

Furthermore, interaction between adipocytes and cancer cells has been therapeutically targeted using BMS309403, a FABP4-specific inhibitor in breast cancer [6]. The results revealed a reduction in tumour growth with changes in secretion of IL-6 and ALDH1 expression. Another drug, anti-leptin blocking peptide, impeded the migration of ovarian cancer cells thereby suggesting antibodies against leptin may be an effective therapy for different cancers, including breast cancer [6]. An agonist of Farnesoid X, GW4064, also decreases the signalling of leptin whilst doxorubicin and pirfenidone have been combined to reduce the progression and motility of tumours in the ECM components by inhibiting the production of collagen [6]. Decreased collagen production has also been induced by vaccination, which sensitises fibroblasts to CD8 T-cell attack thereby significantly increasing the uptake of chemotherapeutic drugs.

Furthermore, stromal cells also play an important role in regenerative therapy as well as haematopoietic stem cell transplantation (HSCT), which is the major treatment for cancer where they enhance HSC engraftment and prevent graft-versus-host disease (GVHD) [4, 8]. GVHD is a major complication of HSCT in the treatment of haematological malignancies. GVHD is caused by an attack on recipient tissues by transplanted immune cells.

## 8. Extracellular vesicles and clinical therapy

Since EVs reflect the physiological and pathological states of the parent cell, and control the energy production machinery of tumour cells, developing EVs as therapeutic strategy and drug delivery system is a promising clinical therapeutic strategy. In cancer, tumour-derived EVs have been identified in various types of body fluids of cancer patients and reflect the characteristics of the tumour cells [46, 62, 64]. Once internalised, alter the metabolism of recipient cells. Thus, EVs can act as biomarkers in disease prognosis, diagnosis, and treatment. Studies have shown the value of EV-derived proteins and miRNAs as prognostic and diagnostic markers in different types of cancer [44, 57, 64, 68].

Tumour-derived EVs have been shown to play vital roles in the resistance of tumour cells to anti-cancer therapy such as chemotherapy and radiotherapy [58, 62, 69]. This may be due to EVs' ability to mediate the transfer of miRNA, lncRNA and proteins associated with drug resistance to recipient cells. Proteins such as transient receptor potential channel 5 (TrpC5) and annexin-6 as well as miRNAs such as miR-310a and miR-17-92 family are highly expressed or upregulated in EVs released from patients with a poor response to chemotherapy and/or radiotherapy [40, 56, 57, 60]. Chemotherapy and radiation affect the function of EVs of target cells. Irradiated and drug-treated cells released EVs that confer a drug-resistant phenotype and reduce sensitivity of recipient cells to the chemotherapy/radiotherapy [57, 58].

However, EVs-derived biomolecules are also used as drug targets for cancer treatment. For instance, miRNAs found in EVs promote glycolysis of CAFs and are involved in pre-metastatic niche formation [53, 54, 64]. As a result, miRNA inhibitors have been used to target and reverse this effect. Fas ligand (FasL) found in EVs of activated T-cells also induce cancer metastasis upon interaction between cancer cells and FasL positive EVs [44, 54]. To ameliorate this effect, several studies have focused on using GW4869 to inhibit the secretion and release of EVs from cells with promising results [40, 54]. Thus, GW4869 might serve as a useful therapeutic strategy to inhibit communication different cells within the TME.

The cargo of EVs can also be useful as a drug delivery system in cancer treatment as EVs deliver bioactive molecules through the plasma membrane barriers with low cytotoxicity. In recent years, various molecules such as miRNAs, siRNAs and therapeutic molecules are incorporated into EVs to cross the blood-brain barrier to treat different types of tumours including brain tumours more efficiently [53, 64]. EVs have also been used to deliver chemotherapeutic drugs such as cisplatin and paclitaxel to increase concentration of these drugs in specific cells or organs [40, 53, 64]. Red blood cells-derived EVs have also been used to deliver drugs in liver cancer treatment through a macrophage-dependent manner [53, 54]. However, it is important to explore the process of cargo selection in the formation of EVs to focus the treatment strategy on specific molecules transported by EVs

from tumour cells or other cells within the TME. There are still discrepancies and difficulties surrounding the methods of isolation and purification of EVs from multiple body fluids [39, 42]. Thus, developing standard methods to isolate EVs may provide the gateway to further explore the possibility of targeting bioactive molecules in EVs and using EVs as a delivery system to carry therapeutic drugs to cells within the TME for cancer treatment.

## 9. Conclusion

Cancer metastasis is the leading cause of cancer morbidity and death. Stromal cells such as endothelial cells, cancer-associated fibroblasts and adipocytes are all involved in cancer development, progression, and metastasis by aiding the spread of cancer from the point of origin to a distant disconnected part of the body. In recent years, clinicians have focused on these stromal cells to provide clinical therapy to patients with cancer. However, this field is relatively new and further research into the roles of these cells in cancer metastasis and the molecular mechanisms should be explored. This will provide a molecular understanding of different types of cancer, and lead to the development of different therapies that will enhance patient survival.

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Chapter 3

# Deciphering and Targeting Epigenetics in Cancer Metastasis

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## Abstract

Once cancer metastasizes to distant organs like the bone, liver, lung, and brain, it is in an advanced stage. Metastasis is a major contributor to cancer-associated deaths. Countless molecules and complex pathways are involved in the dissemination and colonization of cancer cells from a primary tumor at metastatic sites. Establishing the biological mechanisms of the metastatic process is crucial in finding open therapeutic windows for successful interventions. Emerging evidence suggested a variety of epigenetic regulations were identified to regulate cancer metastasis. Here we summarize the procedures and routes of cancer metastasis as well as the roles of epigenetics including ncRNA, DNA methylation, and histone modifications in common metastases. Then we further discuss the potentials and limitations of epigenetics-related target molecules in diagnosis, therapy, and prognosis.

**Keywords:** non-coding DNA, epigenetics, cancer metastasis, DNA methylation, histone modifications

## 1. Introduction

Cancer is the leading cause of death all over the world, accounting for nearly 10 million deaths in 2020 according to the World Health Organization (WHO). Metastatic cancer, the main contributor to high mortality, results in more than 90% of cancer death. This is because when cancer metastasizes to distant organs, especially the bone, liver, lung, and brain, this secondary tumor is formed. And this kind of tumor is difficult to remove despite the various systemic treatments including chemotherapy, screening, and immunotherapy. Efforts from doctors, researchers, and other aspects to promote cancer killing over the past years have paid off in some countries and in some cancers. Since 1991, the cancer death rate has fallen continuously in the United States. Up to 2018, the total mortality rate fell by 31%. Yet the mortality rate has been increasing in other places, such as China [1]. In most cases, many patients with metastatic cancer will face death within 5 years after their diagnosis, which is a horrible thing. Therefore, knowing the mechanism of cancer metastasis to treat metastasis is meaningful for patients, and is a challenging project for oncologists and clinical investigators.

Exploring the physiological mechanisms of the metastatic process is the foundation to find successful interventions. In the beginning, body fluids were thought to be responsible for tumor metastasis. In 1929, James Ewing proposed a theory that

believed the anatomical structure of the vascular system contributes to metastasis and dissemination of cancer cells [2]. This view prevailed for decades. Nonetheless, the most classic and now popular is the "seed and soil" hypothesis proposed by Stephen Paget in 1889 [3]. Over the next few decades, cancer scientists gradually enhanced our knowledge of this mechanism based on molecular and cellular aspects. During this process of metastasis, countless molecules, and complex pathways, including epigenetic regulations, are involved in the dissemination and colonization of cancer cells from a primary tumor at metastatic sites. Epigenetics, a reversible process, refers to the study of heritable changes in gene expression without DNA sequence changes. Increasing studies of epigenetic regulation suggest that such regulations without altering the DNA sequence are critical for the normal physiological activities and the maintenance and development of tissue-specific gene expression in mammals [4]. The location of modified residue and the degree of methylation determines whether the transcriptional activation or repression. For example, the trimethylation of lysine 4 on histone H3 (H3K4me3) can be observed at the promoters of activated genes transcriptionally, yet trimethylation of H3K9 (H3K9me3) and H3K27 (H3K27me3) is enriched at repressed gene promoters transcriptionally [5].

Moreover, the importance of epigenetic changes in early tumorigenesis and cancer metastasis also has been shown, including non-coding RNA (ncRNA), DNA methylation, and histone modifications. Some such examples are increased N6-methyladenosine (m6A) modification of c-Myc mRNA enhances tumor cell growth, invasion, and tumorigenesis in animal models [6]. Upregulated Lysine Demethylase 6B (KDM6B) facilitates lung metastasis in osteosarcoma by modulating the H3K27me3 demethylation level of lactate dehydrogenase (LDHA) [7]. In addition, the enhancer of zeste homolog 2 (EZH2), the histone methyltransferase (HMT) of H3K27, is increased in cancers and promotes tumor metastasis [8, 9]. overexpressed long non-coding RNA (lncRNA) H19 enhances the migration of malignant cells and promotes the occurrence of epithelial to mesenchymal transition (EMT) in endometrial cancer [10].

Given the distinguished functions of epigenetics in cancer progression, and numerous crucial pathways and key biomarkers discovered by researchers, various potent and specific inhibitors targeting biomarkers have been studied and applied in clinics for treating cancer, since azacytidine, the first epigenetic drug approved by Food and Drug Administration (FDA) in 2004. In addition, as inhibitors of DNA methyltransferase (DNMT) enzymes (also termed hypomethylating agents), decitabine (5-aza-2′-deoxycytidine) and guadecitabin are the most extensively applied epigenetic therapies to kill various cancer cells, such as mutated monocyte in acute myeloid leukemia (AML) [11]. Several histone deacetylase (HDAC) inhibitors also have been extensively applied to anticancer (*i.e.,* vorinostat, romidepsin, panobinostat, and belinostat), gaining the approval of FAD for hematological malignancies based on the activity of the single drug [12]. What's more, histone methyltransferase (HMTs), like EZH2, protein arginine methyltransferase 6 (PRMT6), SET domain bifurcated 1 (SETDB1), SUV39H1, and disruptor of telomeric silencing 1-like (DOT1L), also are the targets of cancer treatment. Note that, several small-molecule inhibitors of EZH2 (*i.e.,*tazemetostat, SHR2554, MAK683) and DOT1L (i.e., EPZ-5676) have entered into clinic phases [13].

Based on the increasing knowledge about the mechanism of metastasis and drug development, the prognosis and survival in patients with cancer will gain an effective improvement in clinical outcomes. That is because using the vulnerabilities of

metastatic cancer cells and the properties of metastatic tumor microenvironments are a great entry point to prevent cancer metastasis. As a result, several related drugs involved in cancer metastasis to treat cancer came into being. For instance, gefitinib and erlotinib are tyrosine kinase inhibitors (TKIs) that target activating epidermal growth factor receptor (EGFR) mutations and can improve overall survival by inhibiting metastasis in non-small cell lung cancer (NSCLC) [14, 15]. In general, cancer metastasizes to bone, liver, lung, and brain at an advanced stage, which is difficult for clinicians to destroy the secondary tumor. This is an urgent task and of great significance to patients. Thus, cancer biologists are working to deepen their understanding of epigenetic mechanisms in cancer metastasis to develop better therapy.

Although these drugs mentioned above contribute to clinical improvements in cancer patients, there exist some challenges. The first obstacle is that tumor heterogeneity, one of the characteristics of malignant tumors, which leads to the differences in immune characteristics, growth rate, aggressive ability, sensitivity to drugs, prognosis, and other phenotypic aspects after taking the same drugs. That means precision medicine and personalized medicine are the points of future medical development. Besides, the vast majority of genetic changes of epigenetics are inactivating mutations that are inherently difficult to treat, even though cancer biologists are designing drugs to interfere with adaptive mechanisms. Epigenetics provides a novel insight for researchers to improve the prognosis and survival of patients.

In this review, we summarize the procedures and routes of cancer metastasis as well as the roles of epigenetics including lncRNA, DNA methylation, and histone modifications in common metastases including bone, liver, lung, and brain, followed by discussing about potentials and limitations of epigenetics-related molecules in diagnosis, therapy, and prognosis.

## 2. Cancer metastasis

At present, metastasis is known as the result of a complex multistep cell-biological process collectively known as the invasion-metastasis cascade. It involves the changes of cancer cells in the physical position from the primary tumor to distant or adjacent sites of dissemination, and the colonization of the "seed cells" by adapting to the alien tissue microenvironments. More specifically, during metastatic progression, the first change is the cellular adhesion and morphology of cancer cells are reduced by EMT. Then, cancer cells improved the capability of invading the normal tissue surrounding (local invasion). Next, cancer cells make a way into (intravasation) and out of (extravasation) systemic circulation such as the lymphatic or circulatory system to land at distant sites. In this step, surviving cancer cells are termed circulating tumor cells. Lastly, the cancer cells proliferate and colonize in an unknown tissue microenvironment of different distant organs (**Figure 1**).

### 2.1 The invasion-metastasis cascade

#### *2.1.1 Local invasion*

The local invasion of cancer cells is the foundation for metastatic cancer process. Local invasion refers to the entry of cancer cells into the surrounding tumor-associated stroma, subsequently entering the adjacent normal parenchymal tissue.

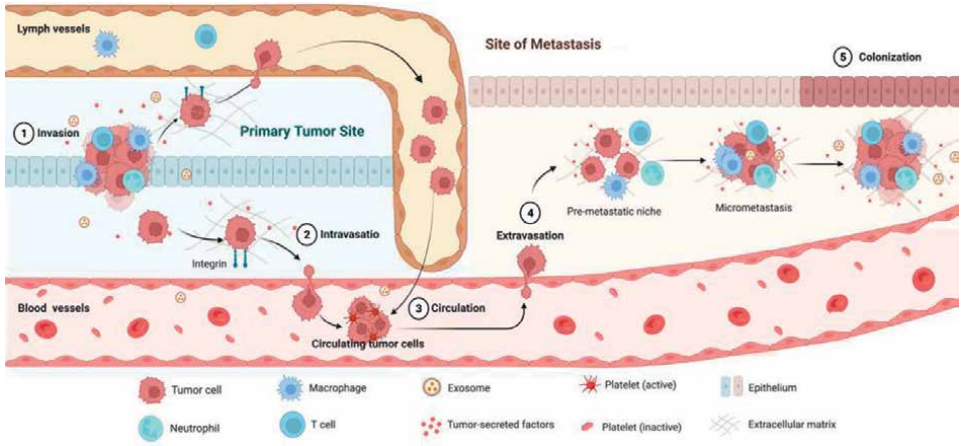


**Figure 1.**
*Overview of metastatic Cascade. During metastatic progression, the first change is that the cellular adhesion and morphology of cancer cells are reduced by epithelial-mesenchymal transition (EMT). Then, the ability of cancer cells to invade the surrounding normal tissue (local invasion) is increased. Next, cancer cells make a way into (intravasation) and out of (extravasation) systemic circulation such as the lymphatic or circulatory system to land at a distant site. In this step, surviving cancer cells are termed circulating tumor cells. Lastly, the cancer cells proliferate and colonize an unknown tissue microenvironment of different distant organs. This figure was created with BioRender.com.*

To invade the stroma, cancer cells must first break the basement membrane (BM) located at the interface of the epithelial tissue and connective tissue. The specialized extracellular matrix (ECM) can exchange material and regulate tissue growth, differentiation, and regeneration [16]. When these cancer cells invade the stroma, they have to be confronted with diverse cancer-associated stromal cells, including myofibroblasts, fibroblasts, adipocytes, endothelial cells, and plenty of bone marrow-derived cells (BMDCs) (i.e., macrophages, mesenchymal stem cells) [17]. Then, these stromal cells are able to enhance the aggressiveness of cancer cells through various cytokines. Secretion of interleukin-6 (IL-6) by cancer-associated fibroblasts (CAFs), stimulates the migration and invasiveness of colorectal cancer cells by the STAT3-LRG1 axis [18]. Increased IL-4 in endothelial cells can lead to enhanced invasiveness of liver cancer cells via the ERK-AKT signaling axis [19]. Besides, colorectal cancer cells secret IL-4 to promote M2-like tumor-associated macrophage (TAM) polarization [20]. These findings suggest there exists a positive-feedback loop in the tumor microenvironment, which is that cancer cells maintain high inflamed surroundings, and these stromal cells further enhance the malignant characteristics of cancer cells.

Researchers have observed various patterns of invasion when cancer cells infiltrate the substrates of adjacent tissues. Due to the dissemination of cancer cells, as individuals and collectives, these researchers divide migrations into individual cell migration and collective cell migration. Both types of migration are simultaneously present in many cancers [21]. In cancer progression, the plastic changes of numerous cancer cells are shown by morphological and phenotypical conversions, such as EMT and its reverse process the mesenchymal-epithelial transition (MET) [22], the collective-amoeboid transition (CAT) [23], the mesenchymal-amoeboid transition (MAT) [24]. Among these conversions, EMT has been increasingly

considered a crucial and indispensable stage in the cancer metastatic process over the last decade [22], despite the studies of Seyfried et al. in the VM mouse model of systemic metastasis suggesting that EMT is unnecessary for the initial cancer metastasis [25, 26].

EMT is a cellular process activated by master transcription regulators, including-ZEB1, ZEB2, Twist, Slug, and Snail, which enhance cell motility and migration ability to invade stroma. Besides, transforming growth factor (TGF)-β has proved to be a strong inducer of EMT by collaborating with other signaling pathways, especially the RAS-MAPK cascade [27]. Moreover, increasing emerging evidence shows the potent roles in invasion and EMT of many long noncoding RNAs (lncRNAs), such as lncRNA MEG3, lncRNA PNUTS, and lncRNA MIR100HG [28–30]. During this process, cells lost epithelial characteristics and markers like E-cadherin and cytokeratin, instead of gaining mesenchymal characteristics and markers like vimentin, N-cadherin, and fibronectin [22]. In addition to cancer metastasis, EMT has been involved in different cancer stages, including cancer initiation, malignant progression, cancer stemness, and drug resistance [31].

### *2.1.2 Intravasation*

It is a vital and indispensable step for cancer cells to disseminate to distant organs during which cancer cells infiltrate into the vascular or lymphatic wall and then enter circulation, becoming circulating tumor cells (CTCs) and potential metastatic seeds. The formation of new blood vessels around cancer cells has a great influence on cancer cells entering the circulatory system, thus understanding the various mechanisms of neoangiogenesis stimulated by cancer cells in local microenvironment will help us comprehend intravasation. Vascular endothelial growth factor (VEGF), a highly bioactive functional glycoprotein, promotes blood vessel growth and lymphatic vessels, which plays an irreplaceable role in angiogenesis. However, the neo-vasculatures generated by cancer cells increase capillary permeability compared with the blood vessels produced by normal cells and tissues [32]. During the lung metastasis of breast cancer, VEGF/VEGF receptor 2 (VEGFR2) and its target proteins such as ERK1/2, Src, and FAK regulate neo-angiogenesis and blood vessel permeability to enhance metastasis [33].

On the other hand, a bunch of studies reveal intravasation can be improved by boosting the penetrability of cancer cells to pass the barrier of endothelial cells. For example, secretion of epidermal growth factor (EGF) by TAMs enhances the intravasation of breast cancer cells [34]. Additionally, the TGF-β enhances mammary cancer intravasation by increasing carcinoma cell penetration of micro-vessel walls or more generally strengthening invasiveness [35]. What's more, in melanoma, the migration of cancer cells to endothelial cells and intravasation are promoted via endothelial-derived SLIT2 protein and its receptor ROBO1 [36]; activated Notch1 receptors (N1ICD) can promote neutrophil infiltration into the tumor, the intravasation of cancer cells and postsurgical metastasis [37]. In the study of Wei et al., increased IL-6 from TAMs is observed and can promote the invasiveness of cancer cells through the STAT3/miR-506-3p/FoxQ1 axis, then increases CCL2 level to boost the recruitment of macrophages. Besides, the authors suggested that there exists a feedback loop between TAMs and cancer cells, which was essential for the EMT and intravasation into the blood vessels [38].

### *2.1.3 Circulation*

Once cancer cells have successfully entered lymph and blood, these malignant cells have the chance to disseminate throughout the body. In blood and lymphatic vessels, these cancer cells must escape the killing of immune cells and physical damage from hemodynamic shear forces to survive. In general, CTCs are in a dormant state that can cause relapse and poor prognosis for patients. This is because conventional surgery, radiotherapy, and chemotherapy are powerless against these CTCs in the blood, lymph, and body fluids, as well as dormant cancer cells, further leading to a decrease in immunity and the rapid growth and metastasis of hidden CTCs.

Many studies have verified the prognostic role and value of CTCs in the early and metastatic stages of cancer by measuring biomarkers [39]. An informative meta-analysis including 1847 patients with colorectal cancer under chemotherapy studied by Huang et al., demonstrated the high expression of CTCs in the bloodstream has a positive correlation with decreased progression-free survival (PFS) (hazard ratios = 2.500, 95% CI [1.746–3.580], $P < 0.001$) [40]. Moreover, CTCs in blood samples of 100 patients with head and neck squamous cell carcinoma were enriched and isolated and the PFS and overall survival of these patients were observed and recorded. The result showed a worse prognosis like decreased PFS and overall survival in CTCs-high patients [41]. Rink et al. also observed patients with ≥1CTCs C per 7.5 ml of blood in distant metastatic bladder cancer shortened the time of disease recurrence and cancer-specific death, resulting in worse clinical outcomes [42]. With the improvement of technologies and the depth of research, plenty of CTCs-related biomarkers are uncovered. At present, a set of biomarkers has been applied to detect CTCs in various cancers. Lin D et al. summarized the CTC-related biomarkers in different cancers [43]. EpCAM as the most common marker can be found in most cancer (i.e., breast cancer, liver cancer, prostate cancer, kidney cancer, melanoma, bladder cancer), which is because most cancers originate from the epithelium [44]. Just like EpCAM, human epidermal growth factor receptor-2 (HER-2), estrogen receptor (ER), prostate-specific membrane antigen (PSMA), and folate receptor (FR) also have been applied to detect CTCs in some cancers, with outstanding clinical significance [45–49].

In addition to CTCs-related biomarkers, the mechanism by which CTCs escape the detrimental shear stress and anoikis in the circulatory system is becoming clearer. There is evidence that CTCs in the blood can stay away from immune cells' killing to increase survivability by bounding tightly to blood constituents like neutrophils, myeloid-derived suppressor cells (MDSCs), CAFs, or platelet [50, 51]. A few years ago, Szczerba et al. found the concentration of CTCs and neutrophils have a significant correlation in animal models and patients with breast cancer, which displays greater metastatic potential and higher gene expression involving cell proliferation. They thought the binding of CTC and neutrophil is possibly mediated by vascular cell adhesion molecule [52]. Besides, Spicer et al. suggested neutrophils could directly adhere to CTCs by the neutrophil Mac-1/ICAM-1, which becomes a bridge between cancer cells and the liver to accelerate CTCs extravasation and colonization [53]. Neutrophils can also enhance metastasis in an indirect manner by trapping CTCs in the circulation through neutrophil extracellular traps (NETs) [54]. In several in vivo experiments, liver or lung NETs were found to collect cancer cells to promote distant metastases by a transmembrane protein named coiled-coil domain

containing 25 (CCDC25) to activate the ILK-β-parvin pathway, leading to enhance cell motility [55].

TAMs play crucial roles in the mechanical adhesiveness and endurance of CTCs, which contribute to the formation of protective cell clusters and the resistance to shear stress [56]. Liu et al. proposed that CTCs interacting with adhesive immune cells like MDSCs could create a defensive shield to allow evasion of immune surveillance, facilitating distant metastatic lesions [57]. Sprouse et al. found reactive oxygen species (ROS) from MDSCs could activate the Notch pathway in CTCs, promoting CTCs proliferation [58]. In addition, CAFs could protect CTCs from the fluid shear forces in the peripheral blood via intercellular contact and soluble derived factors in prostate cancer [59]. As an important component in blood, platelet also supports the survival and metastasis of CTCs in a CTCs-platelet cluster manner. Platelets have been shown to help CTCs evade attack by NK cells by creating a surface shield and normal MHC-I [60], or by downregulating natural killer group 2 member D (NKG2D) and its ligands, further stimulating glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) to exert functions in NK cells [61–63]. Furthermore, platelets involve the adhesion process of endothelial cells. The attachment between platelets and CTCs is enhanced by integrin αIIbβ3 and P-selectin (platelet adhesion receptors), in which supports the strong adherence of CTCs to the endothelial wall [64–66].

### *2.1.4 Extravasation*

Cancer cells extravasate from a vascular lumen into tissues such as the lung, liver, and brain by passing through the endothelial cell and pericyte layers. Extravasation is comparable to intravasation in that it is morphologically similar to invadopodia but mechanistically different. Certain cell types in the primary tumor microenvironment, such as TAMs [34], can initiate intravasation, but these same cells do not have the same promoting function in the extravasation process of disseminated CTCs. Indeed, macrophage phenotype and function differ between primary and metastatic tumor locations. For example, macrophage seeding at distant places is VEGFR+, CCR2+, CXCR4-, Tie2-, and the subpopulation of macrophage at perivascular macrophage is phagocytic [67].

CTCs must overcome the physical barriers of the microvascular wall to extravasate. According to some studies, primary tumors have been shown to release substances that interfere with these distant microenvironments and cause vascular hyperpermeability. Secreted protein angiopoietin-like-4 (Angptl4), as well as the pleiotropically active proteins like NOX4, MMP-1, and MMP-9, disrupt pulmonary vascular endothelial cell-cell junctions, allowing colorectal cancer cells to extravasate into the lungs [68]. Angiopoietin2 (Angpt2), MMP-3, MMP-10, placental growth factor, and VEGF, all of which are secreted by many types of primary tumors, can induce pulmonary hyperpermeability prior to the arrival of cancer cells in the lungs, allowing CTCs to extravasate more easily [69, 70]. Finally, by secreting VEGF, inflammatory monocytes recruited to pulmonary metastases via CCL2-dependent processes increase breast cancer cell extravasation in the lung [71].

Interestingly, whereas Anglptl4 improved the extravasation of breast carcinoma cells in the lung, it did not increase the extravasation or intravasation efficiency of these same breast cancer cells in the bone [72]. As a result, Anglptl4 selectively and only increases extravasation within the lung tissue environment.

*2.1.5 Colonization and metastatic growth*

Cell-nonautonomous mechanisms required to transform a foreign microenvironment into a more friendly niche may be required for disseminated tumor cells to emerge from hibernation and begin active proliferation. For example, the growth of other inactivated disseminated tumor cells might need to stimulate BMDCs to enter the circulation system, as well as the followed recruitment of these cells to the metastatic location; in some situations, the process may be activated through systemic signals such as osteopontin (OPN) or SDF-1 produced by cancer cells, [73, 74].

Alternatively, because the body is in a constant state of homeostasis, dormant cancer cells could continue to proliferate without a net increase. The reasons driving such high rates of attrition are unknown, however, a lack of disseminated tumor cells to initiate neoangiogenesis has been hypothesized as one possible explanation. Prostate tumor cell-secreted prosaposin (Psap) may limit metastatic colonization by increasing the expression of the anti-angiogenic factor thrombo-spondin-1 in stromal cells, which is consistent with this theory [75]. Angpt2, on the other hand, promotes the metastatic colonization of breast and pancreatic cancer by improving the infiltrating capability of myeloid cells to support the vascularization of metastatic nodules [76].

Numerous genes promote the metastatic colonization of cells in breast cancer to bone, lung, brain, or liver, which have recently been discovered. These genes are able to adapt and overcome incompatibilities between the special development procedure of disseminated cancer cells and the demands from foreign tissue milieu, in parallel, researchers come up with the idea that these genes could control organ-specific metastatic tropism. The osteoclastic cytokine IL-11 is an excellent example of this, IL-11 works through a receptor activator for nuclear factor kB (RANK), which disrupts the normal crosstalk between osteoclasts and osteoblasts [77]. Moreover, it strengthens metastatic tumor growth in breast cancer and osteolysis by JAK1/STAT3/c-myc signaling pathway rather than in a RANKL-dependent manner [78].

Similarly, the Notch ligand Jagged1 enhances the osteolytic bone metastases in breast cancer cells by boosting osteoclast activity through IL-6 released by osteoblasts [79]. By encouraging osteoclast action, IL-11 and Jagged1 are able to cause osteolysis and release the rich deposits of growth factors from the bone matrix. The fact that genes identified as candidate mediators of breast cancer cell metastatic colonization in bone, lung, brain, or liver show very little overlap, illustrates the idea that different tissue microenvironments are needed to be organ-specific for metastatic colonization.

**2.2 Routes of cancer metastasis**

*2.2.1 The circulatory system*

Despite the fact that lymphatic diffusion of cancer cells is a key prognostic marker for cancer progression, spreading through the blood circulation seems to be the main mechanism of dispersal of metastatic carcinoma cells. Based on intravital imaging studies, tumor cells can travel toward blood arteries. Li et al. injected metastasized breast cancer cells in mice and found that these cells move toward arteries, illustrating that metastatic cells have the ability of directional migration toward blood streams [80]. Morphologically, compared with non-metastatic cells, metastatic cells are more round,

and this kind of morphology boost both their ability to spread, enter, and colonic tumor vasculature [81, 82]. These findings show that tumor cells with an elongated morphology may need to change their shape to become more rounded in order to successfully intravasate and endure shear forces within blood arteries. It's tempting to think that higher cortical acto–myosin contraction, which promotes the rounded morphology, also allows the cortical cytoskeleton to withstand more mechanical stress. Tumor cells have been found to form part of blood vessel walls in other imaging studies. GFP-labeled tumor cells have been shown to constitute part of the lumen of blood arteries using in situ imaging [80]. This behavior is likely to be linked to tumor cells expressing genes that are normally restricted to endothelial cells. In many circumstances, entering the bloodstream could be as simple as separating tumor cells from the walls of blood vessels.

*2.2.2 The lymphatic system*

Tumor cell entry into the lymphatic system is another major mechanism for tumor cell dissemination. Even in the early phases of tumor formation, changes in lymphatic artery architecture have been seen, under the aid of VEGFC lymphangiogensis can be established quickly using these cells [83]. The lymphatic vasculature can be examined and the interaction of tumor cells with lymphatics can be seen in live tissue by injecting dyes or fluorescent tracers [84]. Cancer cells may extend protrusions via holes in lymph vessel walls before entering the vessels, according to electron microscope photos [85, 86]. To learn more about the cytoskeletal architecture of intravasation cells and their interactions with the lymphatic endothelium, time-lapse imaging should be possible. Interstitial pressure within the tumor affects lymphatic outflow [87]. Interstitial flow can produce autocrine gradients that signal through CC chemokine receptor 7 (CCR7) to induce cell migration in the same direction as the interstitial flow, according to in vitro research. In vivo, however, it is uncertain whether lymphatic channel movement is influenced by interstitial pressure. Trapped breast cancer cells can be discovered in the subcapsular region of lymph nodes after entering lymphatic channels [88]. However, clinically, lymph node metastases might stay in this place or spread to other parts of the node. The admission of tumor cells into the central sections of lymph nodes, as well as their interactions with immune cells, has yet to be captured in high resolution, but when it is, it will likely provide fascinating discoveries.

## 3. Epigenetics in cancer metastasis

Due to the devilishness of cancer metastasis, understanding how cancer cells acquire and maintain metastatic characteristics is critical. However, metastasis-specific genetic alterations cannot be discovered in most exome or genome sequencing investigations. Reversible epigenetic pathways control important phases in metastasis, which can be targeted to prevent and treat metastatic illness in increasingly emerging data. ncRNA, DNA methylation, and histone changes are only a few of the epigenetic processes that have been discovered to modulate the cancer metastasis process. Large-scale chromatin structural changes, such as enhancer reprogramming and chromatin accessibility to transcription factors, have been revealed to be a possible driving force of cancer metastasis in diverse malignancies in recent years. Given that numerous

researchers have reviewed the function of epigenetic markers in different stages of metastasis [89–91], we will concentrate on well-defined particular metastatic locations such as bone, liver, lung, and brain in various malignancies over the last 5 years.

### 3.1 Epigenetics in bone metastasis

Bone is one of the most common sites of metastasis for a variety of solid tumors, including lung, liver, breast, and prostate, with bone metastases being seen in 70% of metastatic prostate and breast cancer patients [92]. Unfortunately, once cancer has progressed to the bone, it is seldom treated and is associated with countless complications such as discomfort, increased fracture risk, and hypercalcemia. This finding has prompted scientists interested in bone and cancer biology to investigate the bone, revealing a number of mechanisms, including epigenetically related elements that promote cancer spread to the bone (**Table 1**).

| Cancer types | Epigenetic types | Biomarkers | Pathways | References |
|---|---|---|---|---|
| Lung cancer | ncRNA | lncRNA-SOX2OT | miRNA-194-5p/RAC1 signaling axis | [93] |
| | | miRNA-660-5p | miR-660-5p/SMARCA5/ RANKL axis | [94] |
| | | miR-574-5p, miR-328-3p, and miR-423-3p | Wnt/β-catenin pathway | [95] |
| | | miR-106a | miR-106a/TP53INP1 | [96] |
| | | miR-139-5p | miR-139-5p/Notch 1 | [97] |
| | | miR-17-5p | PTEN/PI3K/Akt | [98] |
| | | miR-365 | NKX2-1/EGFR/PI3K | [99] |
| | | miR-192-5p | miR-192-5p/ TRIM-44 | [100] |
| | | miR-886-3p | miR-886-3p-PLK1/TGF-β1 pathway | [101] |
| | DNA methylation | DNMTs | miR-886-3p-PLK1/TGF-β1 pathway | [101] |
| | | DNMTs | WIF-1 | [102] |
| Prostate cancer | ncRNA | lncRNA NEAT1 | CYCLINL1/CDK19/ NEAT1-1 | [103] |
| | | miR-940 | miR-940/ARHGAP1 and FAM134A | [104] |
| | | miR-181b-5p | miR-181b-5p/ Oncostatin M axis | [105] |
| | | lncRNA HOXA11-AS | HOXB13/lncRNA HOXA11-AS/ IBSP | [106] |
| | | lncRNA PCAT6 | PCAT6/IGF2BP2/IGF1R axis | [107] |
| | | lncRNA NEAT1 | miR-205-5p/RUNX2/ SFPQ/PTBP2 axis | [108] |
| | | miR-378a-3p | miR-378a-3p/ Dyrk1a/ Nfatc1/Angptl2 | [109] |

| Cancer types | Epigenetic types | Biomarkers | Pathways | References |
|---|---|---|---|---|
| | | lncRNA MAYA | ROR1-HER3-lnc RNA MAYA/Hippo-YAP pathway | [110] |
| | | miR-214 | miR-214/TRAF3 | [111] |
| | | miR-124 | miR-124/IL-11 axis | [112] |
| | | miR-218 | miR-218/COL1A1 | [113] |
| | | miR-125b | miR-125b/ HIF1A/PTGS2 | [114] |
| | | miR-429 | miR-429/CrkL/MMP-9 | [115] |
| | | miR-21 | miR-21/PDCD4 | [116] |
| | | miR-19a | miR-19a/IBSP | [117] |
| | | lncRNA SNHG3 | miR-1273 g-3p/BMP3 axis | [118] |
| | DNA methylation | DNMTs | HGF/Met receptor signaling; E-cadherin, Twist transactivation | [119] |
| Liver cancer | ncRNA | lncRNA 34a | PHB2/DNMT3a/ miR-34a; TGF-β/Smad4 pathway | [120] |
| | | lncRNA H19 | H19/p38MPAK/OPG; H19/ miR200b-3p/ZEB1 | [121] |

*Notes: ncRNA: non-coding RNA; TP53INP1: tumor protein 53-induced nuclear protein 1; NKX2-1: NKX homeobox-1, EGFR: epidermal growth factor receptor, PI3K: phosphoinositide-3-kinase; TRIM-44: tripartite motif-44; PHB2: Prohibitin 2; TGF-β: transforming growth factor β; MPAK: mitogen-activated protein kinase; OPG: osteoprotegerin; ROR1: receptor tyrosine kinase (RTKs)-like orphan receptor-1; YAP: yes-associated protein; TRAF-3: TNF receptor-associated factor-3; COL1A1: collagen type I alpha 1 chain; PTGS2: prostaglandin-endoperoxide synthase 2; HIF1A: hypoxia-inducible factor 1 alpha subunit; CrkL: v-crk avian sarcoma virus CT10 oncogene homolog-like; PDCD4: programmed cell death 4; IBSP: integrin-binding sialoprotein; RUNX2: runt-related transcription factor 2; SFPQ: splicing factor proline- and glutamine-rich; and PTBP2: polypyrimidine tract-binding protein 2.*

**Table 1.**
*Epigenetic biomarkers in bone metastases of various cancers.*

### *3.1.1 Lung cancer*

In the last year, Ni et al. extracted exosomes from the plasma of non-small cell lung cancer (NSCLC) patients with or without bone metastasis. They found exosomal lncRNA-SOX2OT enhanced bone metastasis of NSCLC by targeting the miRNA-194-5p/RAC1 signaling axis in osteoclasts [93]. In organ-specific metastatic lung cancer cells, Ai et al. observed miR-660-5p involved tumor progression and bone-specific metastasis by nm23-H1/miR-660-5p/SMARCA5/RANKL axis [94]. Yang et al. identified an exosomal microRNA cluster that has an association with bone metastasis. Specifically, in this cluster miR-574-5p was down-regulated, miR-328-3p and miR-423-3p were up-regulated in patients with bone metastasis, which suppressed or activated the Wnt/β-catenin pathway [95]. Han et al. observed that upregulated miR-106a promoted bone metastasis by targeting tumor protein 53-induced nuclear protein 1 (TP53INP1), including cell migration, death, and EMT [96]. Xu et al. found miR-139-5p was downregulated in serum to facilitate lytic bone metastasis by targeting Notch1 [97]. In addition, miR-17-5p promotes osteoclastogenesis through the PI3K/Akt pathway via targeting PTEN [98]. Liu et al. revealed that miR-365 was reduced in patients with bone metastasis of NSCLC, and miR-365 could suppress lung metastasis via NKX2-1/EGFR/PI3K axis [99]. Zou et al.

demonstrated that increased miR-192-5p in patient serum inhibited lung cancer metastasis, possibly by reducing TRIM44 [100]. Loss of miR-886-3p expression was mediated by DNA hypermethylation of its promoter in both cultured small cell lung cancer (SCLC) cells and tumor samples. What's more, upregulated miR-886-3p greatly inhibited bone metastasis [101]. The downregulation of Wnt inhibitory factor 1 (WIF-1) expression was linked to hypermethylation of its promoter, which increased lung metastasis [102].

*3.1.2 Liver cancer*

In liver cancer bone metastasis, Zhang et al. revealed the molecular function of lncRNA 34a regulated bone metastasis. Mechanistically, lncRNA 34a epigenetically suppressed miR-34a level via the recruitment of DNMT3a by Prohibitin 2 (PHB2) to methylate miR-34a promoter and histone deacetylase (HDAC) 1 to promote histones deacetylation. On the other hand, miR-34a regulated Smad4 through the transforming growth factor-β (TGF-β) pathway, impacting the downstream genes (i.e., connective tissue growth factor (CTGF) and IL-11) associated with bone metastasis [120]. Huang et al. identified lnRNA H19/p38 mitogen-activated protein kinase (MPAK)/ osteoprotegerin (OPG) and lncRNA H19/miR200b-3p/ZEB1 axes contributed to hepatocellular carcinoma bone metastasis [121].

*3.1.3 Breast cancer*

In breast cancer, the Hippo-YAP pathway was controlled by a ROR1-HER3-LncRNA signaling axis to govern bone metastases [110]. Both osteoclastic miR-214/ TNF receptor-associated factor-3 (TRAF-3) pathway and dysregulated miR-124/ IL-11 axis were devoted to the understanding of breast cancer metastases to the bone [111, 112]. A study pointed to a concept in which cancer-derived miR-218 impairs osteoblast function by directly targeting collagen type I alpha 1 chain (COL1A1) and regulating inhibin βA expression [113]. miR-125b may reduce the effect of hypoxia-inducible factor 1 alpha subunit (HIF1A), which is known to enhance metastatic spread by upregulating prostaglandin-endoperoxide synthase 2 (PTGS2) [114]. To investigate the influence of miR-429 on the metastatic bone environment in vivo, Zhang et al. created an orthotopic bone degradation model and a left ventricle implantation paradigm. The levels of V-crk sarcoma virus CT10 oncogene homolog-like (CrkL) and MMP-9 were negatively influenced by miR-429 [115]. Exosomal miR-21 generates from breast cancer cells promotes osteoclastogenesis by modulating the levels of the protein programmed cell death 4 (PDCD4). Furthermore, the amount of miR-21 in breast cancer patients with bone metastases is considerably greater in serum exosomes [116]. Exosomal miR-19a and integrin-binding sialoprotein (IBSP) are highly increased and secreted from bone-tropic estrogen receptor-positive (ER+) breast cancer cells, resulting in a milieu conducive to colonization in the bone [117]. Teng et al. identified many key lncRNAs such as lncRNA RP11-317-J19.1 related to bone metastasis in breast cancer [122]. By influencing the miR-1273 g-3p/BMP3 axis, LncRNA SNHG3 regulates BMSC osteogenic development in breast cancer bone metastases [118].

In breast cancer, the level of DNA methylation is increased to further regulate Wwox, following to stimulate HGF/Met receptor signaling and E-cadherin, down-regulating Twist transactivation, leading to bone metastasis [119].

*3.1.4 Prostate cancer*

Wen et al. analyzed the m6A status using patient samples and bone metastatic patient-derived xenografts (PDXs) with prostate cancer through m6A high-throughput sequencing, and they found 4 credible m6A sites on lncRNA NEAT1-1. Besides, NEAT1-1 acted as a bridge to strengthen the combination between CYCLINL1 and CDK19 and promoted the Pol II ser2 phosphorylation in the promoter of RUNX2, leading to the development of bone metastatic prostate cancer [103]. By targeting ARHGAP1 and FAM134A, miR-940 boosted osteogenic differentiation of human mesenchymal stem cells [104]. miR-181b/Oncostatin m axis also contributes to prostate cancer bone metastasis by altering osteoclast differentiation [105]. To promote the bone-specific metastasis of prostate cancer, HOXA11-AS controlled the expression of chemokines, integrins, and associated genes like IBSP in collaboration with HOXB13 [106]. lncRNA PCAT6 enhances prostate cancer bone metastasis and tumor growth by upregulating IGF1R expression via increasing IGF1R mRNA stability through the PCAT6/IGF2BP2/IGF1R pathway [107]. lncRNA NEAT1/miR-205-5p/RUNX2/SFPQ/PTBP2 axis and miR-378a-3p/ Dyrk1a/Nfatc1/Angptl2 axis are also devoted to bone metastasis [108, 109].

## 3.2 Epigenetics in liver metastasis

*3.2.1 Esophageal squamous cell carcinoma*

Tang et al. explored the function of lncRNA LOC146880 in esophageal squamous cell carcinoma (ESCC) progression. The result of in vivo and in vitro experiments showed LOC146880 sponged miR-328-5p to regulate fascin actin-bundling protein 1 (FSCN1) activating MAPK signaling pathway, resulting in liver metastasis [123] (**Table 2**).

*3.2.2 Breast cancer*

In breast cancer, the TGF network in liver metastasis can be explained by the ZEB1-miR-190-SMAD2 axis [124]. miR-1204 inhibits vitamin D receptors (VDR), which promotes epithelial-mesenchymal transition and metastasis [125]. As a ceRNA of miR-1299, circular RNA ciRS-7 promotes lung and liver metastases by targeting MMPs [126]. Wang et al. found circROBO1 was upregulated to boost tumor development and liver metastasis in vivo. Further research revealed the mechanism that circROBO1 upregulated KLF5 by sponging miR-217-5p, allowing KLF5 to activate FUS transcription, hence promoting circROBO1 back splicing [127].

## 3.3 Epigenetics in lung metastasis

*3.3.1 Osteosarcoma*

LncRNA-CASC15 promotes lung metastasis in osteosarcoma by regulating EMT via the Wnt/β-catenin signaling pathway [139] (**Table 3**). MIR205HG also can drive lung metastatic osteosarcoma via regulating the axis of miR-2114-3p/twist family bHLH transcription factor 2 (TWIST2) [140]. miR-485-3p regulated by lncRNA MALAT1 inhibites osteosarcoma glycolysis and lung metastasis by directly suppressing c-MET

| Cancer types | Epigenetic types | Biomarkers | Pathways | References |
|---|---|---|---|---|
| Liver metastases | | | | |
| ESCC | ncRNA | lncRNA LOC146880 | LOC146880/miR-328-5p/ FSCN1/MAPK axis | [123] |
| Breast cancer | ncRNA | miR-190 | ZEB1-miR-190-SMAD2 axis | [124] |
| | | miR-1204 | miR-1204/VDR | [125] |
| | | cirRNA ciRS-7 | ciRS-7/miR-1299 | [126] |
| | | circROBO1 | circROBO1/ KLF5/FUS | [127] |
| Colorectal cancer | ncRNA | miR-221; miR-222 | — | [128] |
| Brain metastases | | | | |
| Breast cancer | ncRNA | lncRNA XIST | EMT and MSN/c-Met | [129] |
| | | miR-10b | — | [130] |
| | | miR-576-3p | — | [131] |
| | | lncRNA BCBM | lncRNA BCBM /JAK2/STAT3 | [132] |
| | | circBCBM1 | circBCBM1/miR-125a/BRD4 axis | [133] |
| | | lncRNA-CCRR | lncRNA-CCRR/connexin 43 | [134] |
| | | miRNA let-7d | PDGF/PDGFR axis | [135] |
| | | miR-802-5p; miR-194-5p | — | [136] |
| | | miR-132-3p; miR-199a-5p; miR-150-5p; miR-155-5p | — | [137] |
| | | miR-211 | SOX11/NGN2 axis | [138] |

*Notes: ESCC: esophageal squamous cell carcinoma; FSCN1: fascin actin-bundling protein 1; VDR: vitamin D receptor; KLF5: Kruppel like factor 5; bHLH transcription factor 2; CDK6: Cyclin Dependent Kinase-6; STAT3: signal transducer and activator of transcription-3; HDAC: histone deacetylase 1; ZEB1: Zinc Finger E-Box Binding Homeobox 1; ROCK1: Rho associated coiled-coil containing protein kinase 1; CRYAB: αB-crystallin; DNMTs: DNA methyltransferase; ERα: estrogen receptor alpha; TET2: ten-eleven translocation 2; IRX1: iroquois homeobox 1; PDK1: phosphoinositide-dependent kinase-1; ST7L: suppression of tumorigenicity 7 like; and BRD4: bromodomain containing 4.*

**Table 2.**
*Epigenetic biomarkers in liver and brain metastases of various cancers.*

and AKT3/mTOR signaling, meanwhile, MALAT1 also facilitated lung metastasis of osteosarcomas through miR-202 sponging [141, 142]. Besides, Chen et al. also observed that the LOC100129620/miR-335-3p/CDK6 signaling promoted the lung metastasis of osteosarcoma by mediating the osteosarcoma cells proliferation, macrophage polarization, and angiogenesis [144]. The lncRNA NEAT1/miR-483/STAT3 axis also exerts a crucial role in regulating the lung metastasis process in osteosarcoma, especially in EMT [145]. miR-326 inhibited by SP1/HDAC1 has a great impact on proliferation and metastasis of osteosarcoma through stimulating SMO/Hedgehog pathway [146]. The miR-19a/RhoB/AKT1 network and miR-491/αB-crystallin (CRYAB) axis also may help us to better know the lung metastatic mechanism of osteosarcoma [147, 149]. In Ewing sarcoma, miR-130b directly targets ARHGAP1 to activate a lung metastatic CDC42-PAK1-AP1 positive feedback loop [157].

Additionally, Lillo et al. found estrogen receptor alpha (ERα) was not expressed in osteosarcoma due to promoter DNA methylation. They took Decitabine, a DNA

| Cancer types | Epigenetic types | Biomarkers | Pathways | References |
|---|---|---|---|---|
| Osteosarcoma | ncRNA | lncRNA-CASC15 | Wnt/β-catenin signaling | [139] |
| | | MIR205HG | MIR205HG/miR-2114-3p/ TWIST2 axis | [140] |
| | | lncRNA MALAT1 | miR-485-3p/c-MET; miR-485-3p /AKT3/mTOR signaling; lncRNA MALAT1/ miR-202; miR-129-5p/RET/ PI3K-Akt axis | [141, 142, 143] |
| | | lncRNA LOC100129620 | LOC100129620/miR-335-3p/ CDK6 signaling | [144] |
| | | lncRNA NEAT1 | lncRNA NEAT1/miR-483/ STAT3 axis | [145] |
| | | miR-326 | Sp1/HDAC1/miR-326/SMO/ Hedgehog axis | [146] |
| | | miR-19a | miR-19a/RhoB/AKT1 | [147] |
| | | lncRNA DANCR | miR-335-5p and miR-1972/ ROCK1 | [148] |
| | | miR-491 | miR-491/CRYAB | [149] |
| | DNA methylation | DNMTs | ERα | [150] |
| | | DNMTs | SPARCL1/ WNT/β-catenin signaling | [151] |
| | DNA demethylation | — | TET2/IL-6 | [152] |
| | | — | IRX1/ CXCL14/NF-κB signaling | [153] |
| Breast cancer | ncRNA | linc-ZNF469-3 | miR-574-5p-ZEB1 axis | [154] |
| | | cirRNA ciRS-7 | ciRS-7/ miR-1299 | [126] |
| | | lncRNA MIR31HG | miR-575/ ST7L | [155] |
| Colorectal cancer | Histone acetylation | CBP | CBP-DOT1L/ RNF8/H3K79 | [156] |
| Ewing Sarcoma | ncRNA | miR-130b | miR-130b-AP-1/ CDC42-PAK1-AP1 axis | [157] |
| Gastric cancer | ncRNA | lncRNA GMAN | ephrin A1 | [158] |
| | | Lnc RNA MIR17HG | Wnt/β-catenin signaling | [159] |
| | | lncRNAs AC093818.1 | PDK1 | [160] |

*Notes: TWIST2: twist family bHLH transcription factor 2; CDK6: Cyclin Dependent Kinase-6; STAT3: signal transducer and activator of transcription-3; HDAC: histone deacetylase 1; ZEB1: Zinc Finger E-Box Binding Homeobox 1; ROCK1: Rho associated coiled-coil containing protein kinase 1; CRYAB: αB-crystallin; DNMTs: DNA methyltransferase; ERα: estrogen receptor alpha; TET2: ten-eleven translocation 2; IRX1: iroquois homeobox 1; PDK1: phosphoinositide-dependent kinase-1; and ST7L: suppression of tumorigenicity 7 like.*

**Table 3.**
*Epigenetic biomarkers in lung metastases of various cancers.*

methyltransferase (DNMTs) inhibitor to activate ERα, further inhibiting osteosarcoma growth and lung metastasis [150]. In primary osteosarcoma cells, increased IL-6 expression regulated by DNA demethylation of the promoter of ten-eleven translocation 2 (TET2) promotes lung metastasis in osteosarcoma [152]. Secreted

protein acidic and rich in cysteine (SPARCL1) downregulated by epigenetic promoter DNA methylation in osteosarcoma promotes lung metastasis via canonical WNT/β-catenin signaling activated through stabilization of the WNT–receptor complex [151]. Hypomethylation of iroquois homeobox 1 (IRX1) in osteosarcoma cell lines substantially affected metastatic behavior in vitro, including migration, invasion, and resistance to anoikis and influenced lung metastasis in animal models by upregulating CXCL14/NF-B signaling, according to another study [153].

*3.3.2 Breast cancer*

In the study by Wang et al. they showed that linc-ZNF469-3 accelerated lung metastasis of triple-negative breast cancer (TNBC) via miR-574-5p-ZEB1, which may be acted as a potential and promising prognostic marker for TNBC patients [154]. MIR31HG, a long noncoding RNA that sponges miRNA-575 to control ST7L expression, suppresses hepatocellular carcinoma proliferation and metastasis [155].

*3.3.3 Colorectal cancer*

Colostrum basic protein (CBP), a histone acetyltransferase (HAT), mediates DOT1L K358 acetylation and has a positive correlation with colorectal cancer stages. DOT1L acetylation confers DOT1L stability by blocking RNF8 binding to the protein and subsequent proteasomal degradation, but it has no effect on the enzyme's activity. DOT1L can catalyze the H3K79 methylation of genes involved in epithelial-mesenchymal transition, such as SNAIL and ZEB1, once stabilized [156].

*3.3.4 Gastric cancer*

GMAN, a long non-coding RNA, is upregulated in stomach cancer patients and is linked to overall survival and metastasis process. It inhibits the translation of ephrin-A1 mRNA by binding to GMAN-AS in a competitive manner [158]. Interferon regulatory factor-1 (IRF-1) suppresses gastric cancer spread by suppressing Wnt/−catenin signaling and downregulating the MIR17HG-miR-18a/miR-19a axis to inhibit gastric cancer lung metastasis [159].

**3.4 Epigenetics in brain metastasis**

Through activation of EMT- and MSN-mediated up-regulation of c-Met, the loss of lncRNA XIST increases breast cancer brain metastasis by boosting both stemness and aggressiveness of tumor cells [129] (**Table 2**). Yoo et al. proved the therapeutics function of miRNA-10b by targeting the brain metastases process in breast cancer [130]. JAK2-binding lncRNA BCBM promotes breast cancer brain metastasis by regulating STAT3 [132]. circBCBM1 is involved in breast cancer brain metastasis via circBCBM1/miR-125a/BRD4 axis [133]. By modulating connexin 43 expression, dysregulation of lncRNA-CCRR contributes to breast cancer brain metastases through intercellular coupling [134]. Loss of miRNA let-7d and active hypoxia- inducible factor-1 (HIF1) signaling enhances breast cancer brain metastasis via platelet-derived growth factor (PDGF), while pharmacologic inhibition of PDGF receptor (PDGFR) inhibits brain metastasis, implying new therapeutic possibilities [135]. miR-132-3p, miR-199a-5p, miR-150-5p, miR-155-5p, miR-802-5p and miR-194-5p from breast cancer cells also were identified the important role in brain metastasis [136, 137].

In triple-negative breast cancer, miR-211 regulates brain metastatic selectivity via the SOX11/NGN2 axis [138].

## 4. Therapeutic potentials and limitations

Epigenetic drugs are chemicals that alter DNA and chromatin structure, promoting the disruption of transcriptional and post-transcriptional modifications, primarily by regulating the enzymes required for their establishment and maintenance, and reactivating epigenetically silenced tumor-suppressor and DNA repair genes [161]. The development of treatment techniques incorporating epigenetic medicines, which focus on the cancer epigenome to generate pharmacological molecules that could restore a "normal" epigenetic landscape, is a developing field of drug discovery [161]. Epigenetic medicines target the enzymes that are required for the maintenance and establishment of epigenetic alterations, with the inhibition of DNMTs and HDACs being the most common technique [161]. The epigenetic alterations caused by these medications can regulate the temporal and spatial expression of genes [162], and they have ramifications for the regulation and dysregulation of physiological and pathological processes. Because epigenetic markings are tightly linked to the type of tumor and stage of disease, as well as individual genetic variation, such as in personalized medicine [163, 164], they have a lot of promise to give molecular biomarkers for diagnosis and treatment alternatives for cancer therapy [165].

The FDA has approved six new epigenetic medicines and multi-drug regimens for use in clinical cancer treatment. Some side effects will happen, so novel epigenetic therapeutic compounds are continually being tested in preclinical research, as well as clinical trials for the development and release of new medicines, for cytotoxicity, and pharmacological characteristics, and to better understand their mechanism of action. The majority of epigenetic medication studies are focused on cancer detection, therapy, and prognosis.

NcRNAs have shown new promise and insight as therapeutic targets for cancer treatment and preventing cancer metastasis in vivo preclinical models of metastatic illness. Research has shown that lncMAYA, MALAT1, and lncARSR have all been targeted for in vivo suppression using ASOs in mice models to alleviate the burden of metastatic disease [110, 166–168]. When targeting lncRNAs with ASO therapies, however, it will be vital to ensure minimal off-target effects [169, 170], which could offer additional challenges given the decreased quantity of lncRNA transcripts in vivo. ASOs disrupt target RNAs by premature transcriptional termination [171, 172], in addition to RNase H-mediated destruction of mature RNA, according to new findings, which should be taken into account when estimating the efficacy of ASO therapies. The biggest problem is the species conservatism in ncRNA, especially lncRNAs. Animal testing is also required before conducting clinical trials.

## 5. Conclusions

Cancer metastasis is a common cause of death. The role of epigenetics in the etiology of metastases cannot be ignored. To overcome cancer and its metastasis, many methods and technologies are applied to developing new drugs. In parallel with the development of specific and potent small-molecule inhibitors, some novel and cutting-edge technologies like proteolysis-targeting chimeras (PROTACs), RNA interference (RNAi),

clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome editing, and artificial intelligence (AI)-based drug design, in chemical biology show huge potentials for cancer treatment, which allows to screening therapeutics targeting almost all kinds of molecules, like proteins and epigenetic regulators [173].

## Acknowledgements

This section is supported by the Natural Science Foundation Council of China (81700780 and 81922081), the Department of Education of Guangdong Province (2021KTSCX104), and the Guangdong Basic and Applied Basic Research Foundation (2022A1515012164), and the Science, Technology, and Innovation Commission of Shenzhen (JCYJ20210324104201005).

We thank the administrative assistant (Ms. Yufang Zuo) for providing help and support.

## Conflict of interest

The authors declare no conflict of interest.

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Section 3

# Metastasis

Chapter 4

# Molecular Mechanisms of Breast Cancer Metastasis

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## Abstract

Breast cancer (BC) is one of the most frequently occurring diseases with high morbidity and mortality rates in the world today. BC cells live under stress with altered pathway signaling, chromosome and microsatellite instability, aneuploidy, hypoxia, low pH, and low nutrient conditions. In order to survive and reproduce in these stressful environments, BC cells rapidly undergo adaptive mutations, rearrange their chromosomes, and repress tumor suppressor genes while inducing oncogene activities that cause the natural selection of cancer cells and result in heterogeneous cancer cells in the tumor environment. Unfortunately, these genetic alterations result in aggressive BC cells that can not only proliferate aggressively but also migrate and invade the other tissues in the body to form secondary tumors. In this review, molecular mechanisms of metastasis of BC subtypes are discussed.

**Keywords:** breast cancer, metastasis, heterogeneity, luminal A and B, TNBC, HER2+

## 1. Introduction

The breast tissue is made up of lobes, adipose tissue, ligaments, cavities (sinuses), glands, and milk ducts. Breast cancer (BC), which develops as a result of excessive cell growth in the breast tissue, is one of the leading causes of mortality after heart and vascular disorders. Although male BC is uncommon, female BC is the most frequent cancer. BC, like all cancers, causes a multitude of DNA abnormalities in healthy cells. As a result, cells begin to multiply uncontrollably. Cancerous cells reproduce and replicate more than healthy cells, and they live much longer. A tumor is defined as an aggregation of cells that results in the creation of a mass [1]. This syndrome frequently occurs in BC as a result of the fast growth of transformed cells in the milk ducts or mammary glands in the breast tissues. Cancer cells that grow in these locations generate a mass known as tumors. BC tumors can be in non-cancerous benign or in cancerous malignant forms. Both forms have varying impacts on the body. Cell growth, which leads to a malignant tumor, is generally gradual in the beginning and does not cause symptoms [2]. Despite advancements in early diagnosis and treatment strategies, metastatic BC continues to be an incurable disease [1].

The spread of malignant BC cells to different human tissues and organs is referred to as BC metastasis. Angiogenesis, invasion, migration, extravasation,

and proliferation are a few of the multistep, intricate, and interconnected chains of events that contribute to the development of cancer. By inducing the growth of new blood arteries, tumor cells first break their interactions with surrounding cells and detach from the underlying tumor tissue which is called Epithelial to Mesenchymal Transition (EMT). A primary tumor is one that has formed at the initial location of the tumor. A variety of specific transcription factors, "Epithelial-to-Mesenchymal Transition (EMT) inducers," are at least partially responsible for the complex genetic changes required to achieve EMT-associated phenotypic changes. Snail, Slug, SIP-1, δEF1, E12/E47, and Twist are included in transcription factors that induce EMT. These factors act as transcriptional repressors of E-cadherin in various cell types [1]. In addition, it has been reported to promote EMT by affecting many genes such as matrix metalloproteinase 9 or SPARC in metastasis and cancer invasion. In several cancer models, activation of the transforming growth factor—(TGF) signaling pathway and subsequent upregulation of the EMT inducers Snail, Slug, Twist, and ZEB have been reported to result in EMT [2]. In addition, FOXC2 is a transcriptional factor that promotes EMT and metastasis in vivo. It has been reported that this factor is associated with basal-like cancers [3].

Ten percent of cancer-related deaths are caused by original tumors, but 90% of cancer deaths are caused by metastases, which are secondary cancers that have grown outside the primary tumors. After exiting the main tumor tissue, BC cells move into the extracellular matrix where they advance and either occupy nearby tissues or enter the circulatory system to migrate to distant tissues. Lymph and blood arteries carry them from the main tumor's development site to the metastatic areas. As a result, they survive and reproduce themselves [1, 2]. Because metastasis is a multistep and complex process that includes different steps, due to the heterogeneity of BC, the mechanism of metastasis may diverge from the genetic background of the BC cells. BC has a lot of morphological and molecular heterogeneity not just across tumors, but even within a single tumor. Gene expression profiling allows the identification and classification of major subgroups with varying clinical characteristics and therapeutic responses. The difference between subtypes is caused by three tumor markers: estrogen (ER), progesterone hormone receptors (PR), and human epidermal growth factor receptor 2 (HER2). High levels of hormone receptor expression are observed in luminal A tumors. In addition, the subtype has HER2-negative, ER and/or PR positive, and has low levels of proliferation-related genes. This subtype not only has a modest growth rate, but also a favorable prognosis [1]. BC can show differences in the expression of the hormonal receptors as the result of different genetic alterations and rearrangements within the cell. These differences result in tumor subtypes of BC that show different strategies to survive and invade. Different genomic backgrounds in BC result in genetic variation that shows different mechanisms in cancer progression, especially in metastasis. In this review, metastatic features of BC subtypes are discussed.

## 2. Mechanism of metastasis in breast cancer subtypes

BC is a very common cancer type that can arise either genetically or environmentally. It is divided into different subtypes according to its genetic overcomes (**Figure 1**). Each subtype has its specific genomic character. Therefore, their inner signaling pathways' roles in metastasis are quite distinct.

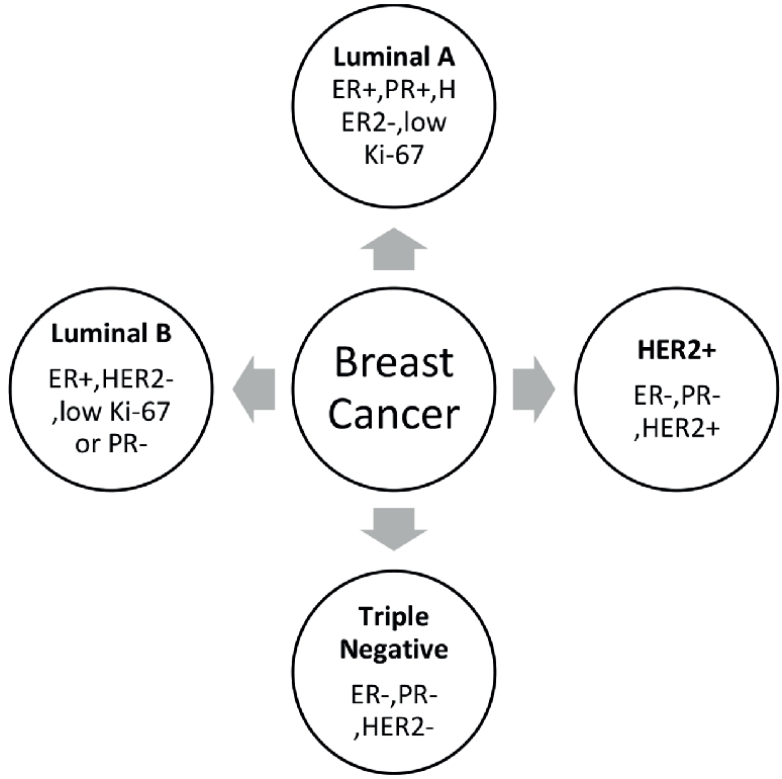


**Figure 1.**
*Breast cancer is heterogenous cancer that includes 4 major subtypes according to their mutations. Estrogen receptors (ER) and progesterone (PR) receptors are specialized proteins found in certain cells in the body. Estrogen, progesterone, and female hormones circulating in the blood bind to these receptors and promote new cell growth and division. HER2 is a growth-promoting protein found outside of all breast cells (–, no expression; +, high expression).*

### 2.1 Luminal A and B

ER-positive BC is divided into two types: luminal A and luminal B. It is shown to have different gene expression patterns, prognoses, and therapeutic responses. When compared to luminal A tumors, luminal B tumors have lower levels of ER or estrogen-regulated genes, lower or no expression of the PR higher tumor grade, higher expression of proliferation-related genes, and activation of growth factor receptor signaling pathways like IGF-1R and PI3K/AKT/mTOR [2]. Luminal B cancers, like luminal A tumors, are expected to have reduced endocrine sensitivity but have increased chemotherapy sensitivity [3].

Extravasation or lymphatic, local invasion, intravasation, colonization, and blood vessel migration are all steps in the tumor metastasis process. These processes then lead to metastases to distant organs [4]. The connection between tumor cells and the tumor microenvironment, which includes noncancerous cells such as immune cells, fibroblasts, adipocytes, and endothelial cells and as well as extracellular matrix (ECM), is crucial for organ-specific colonization [5]. The parallel progression model is more prevalent in breast tumors than the linear metastasis model. This suggests that BC cells spread early in the tumor's formation and that cancer cell spread may not be

dependent on tumor progression [6]. Several investigations have demonstrated that the genetic modifications of BC bone metastasis cells are not necessarily the same as those of their primary tumors. Distinct BC subtypes have been demonstrated to favor different metastatic sites, which are influenced by different molecular pathways. The molecular characteristics of BC and target organs appear to validate the organotropism of metastasis. All BC subtypes are prone to bone metastasis when compared to other subtypes; however, the luminal A subtype is an extraordinarily high-risk factor for bone metastasis. In addition, the prevalence of bone metastasis in luminal subtype malignancies is significantly higher (80.5%) than in HER2+ tumors (55.6%) or basal-like tumors (41.7%) [7]. Not only proliferation and metastatic capacities of BC subtypes are different, but also metabolic genotypes and phenotypes, vary with subtype. Nonetheless, metabolic alterations may differ not just within BC subtypes, but also depending on how tumor cells interact with their microenvironment [8]. This section highlights current knowledge about the association between metabolic programming, epigenetic modifications, and the metastatic process in BC. Understanding the metabolic processes that induce BC spread may lead to the development of new anticancer drugs.

Normal cells engage several signaling pathways in response to external growth signals and regulate glycolysis, oxidative phosphorylation (OXPHOS), and anabolic metabolism. Furthermore, unlike normal cells, which make adenosine triphosphate (ATP) largely by OXPHOS via the TCA cycle, most cancer cells rely on glycolysis for energy during aerobic conditions. The reverse Warburg effect, also known as metabolic coupling, is a metabolism that some tumor cells have. This mechanism not only results in chemotherapy resistance but also explains why some tumor cells have a high rate of mitochondrial respiration but a low rate of glycolysis [9, 10]. Moreover, the research identified a link between the luminal subtype and metabolically inactive reverse-Warburg/null phenotypes, whereas triple-negative breast cancer (TNBC) was linked to metabolically active Warburg/mixed phenotypes [11].

The expression of glucose transporter proteins (GLUTs) varies in BC and is connected to different clinical phases. In BC cells, GLUT1-5 and GLUT12 are active, although GLUT1 is the most important [12]. The pentose phosphate pathway (PPP) produces fructose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADPH), and ribose phosphate in addition to glycolysis and the TCA cycle [13]. Proteins involved in PPP are expressed in diverse ways in different molecular subtypes of BC. For instance, the HER2 subtype has greater expression of 6-phosphogluconolactonase and glucose-6-phosphate dehydrogenase than other BC subtypes, indicating a more active PPP [14]. Transketolase and G6PD expression have been associated with a worse overall and relapse-free survival rate in BC patients [15].

Glutathione and nicotinamide adenine dinucleotide (NADH) are the intermediates of glutamine and aid tumor cell proliferation and development by providing energy, supplementing glucose metabolism, and helping cells survive oxidative stress. Furthermore, certain tumor cells have developed an "addiction to glutamine", meaning that when there is no glutamine, they cannot survive [16]. Oncogenic transcription factors c-MYC and RAS can raise the metabolic activity of glutamine in tumor cells. At the same time, they can also upregulate some glutamine transporters including alanine-serine-cysteine transporter 2 (ASCT2) and enzymes involved in glutamine-to-glutamate conversion like glutaminase (GLS-1) [17]. Recent studies revealed that a greater glutamate-to-glutamine ratio particularly in ER-negative tumors was observed in breast tumor tissues. Glutaminase-1 (*GLS-1*), glutamate dehydrogenase (*GDH*), and *ASCT2* were found to be more strongly expressed in

HER2+ BC than in other subtypes. This indicates that HER2+ BC has the greatest glutamine metabolism activity. Importantly, the lowest expression of stromal *GLS1* and *GDH*, tumoral *ASCT2*, and serine hydroxymethyltransferase 1 were found in the luminal A subtype [18].

Amino acid biosynthesis and degradation, *de novo* nucleotide biosynthesis, reductive metabolism, and methylation are all involved in one-carbon metabolism. This metabolism has long been assumed to play a key role in sustaining tumor cells' high proliferation rate [19]. Additionally, folate (vitamin B9), and other B vitamins like B6 and B12, play an important role in one-carbon metabolism. Although the link between folic acid consumption and the risk of BC is still debated, a recent study found that increasing folate intake reduced the risk of ER-, ER-/PR- [20]. Immunity and tolerance are manipulated by tryptophan and arginine, which are frequently unregulated in malignancies. In BC contexts, the activity of arginase, the primary enzyme that catalyzes L-arginine, is increased, creating an adverse environment for T cell adaptability [21].

The development and progression of BC are dependent on lipid and fatty acids (FAs) metabolism [8]. By enhancing lipid and lipoprotein absorption or increasing cholesterol and lipid synthesis, cancer cells maintain a high rate of proliferation, displaying active lipid and cholesterol metabolisms [22]. Furthermore, the synthesis of FAs causes cancer cells to grow and proliferate faster. Fatty acid synthase (FASN) is a critical enzyme for FAs. When it is overexpressed, cancer proliferation occurs and a poor prognosis is observed in BC. That is why enhanced FAs activity is required for BC progression [23]. SREBP-1, a lipogenic transcription factor, can influence FASN expression by interacting with the *FASN* promoter region. *FASN* expression has also been shown to be influenced by the phosphatidylinositol-3-kinase (PI3K)/AKT/mTOR and mitogen-activated protein kinase (MAPK) pathways [24]. The *FASN* expression is increased in BC cells because AKT and Sterol Regulatory Element-Binding Protein 1 (*SREBP-1*) are activated under hypoxic environments. Finally, inhibiting the MAPK pathway or using the mTOR inhibitor rapamycin can lower the expression of *FASN* in BC cells [25].

The signaling pathway of PI3K/AKT/mTOR is important for the cell cycle and metabolism in cancer development. Signals from growth factors, nutrients, energy signals, and various stress signals under hypoxia or DNA damage that provide growth and division of cells are integrated by the mammalian target of rapamycin complex 1 (mTORC1). The 110 genes in PI3K/AKT/mTOR pathway are often mutated in luminal BC. The most common *PI3K* mutations are found in around 40% of cases in luminal subtypes [26].

The alpha catalytic subunit of PI3K (PIK3CA) has been identified as the site of the bulk of *PI3K* mutations in ER-positive tumors. The most prevalent somatic mutation in BC is *PIK3CA*, which is seen in 36% of individuals with hormone receptor-positive, HER2-negative (HR+/HER2−) BC [27]. Crosstalk between the ER and the PI3K/AKT/mTOR signaling pathways has been proposed to be present during BC progression. Estrogens activate the PI3K/AKT/mTOR pathway, which allows ER cancers to migrate and invade distant tissues. mTOR signaling regulates the expression and activity of ER-α (one of two isoforms) in a reciprocal manner [8]. A recent study suggests that inhibition of the PI3K pathway activated the histone-lysine N-methyltransferase 2D (KMTD2), resulting in ER activation in BC cells [28]. Importantly, when AKT/mTOR signaling is activated by PI3K antagonists, the activation of energy-active mitochondria to the cortical cytoskeleton of cancer cells occurs. In this way, tumor cell invasion is increased. Inhibitors of the PI3K pathway slow cancer development, however, they

may promote tumor invasion by reprogramming mitochondrial transport, OXPHOS, and boosting cell motility [29].

ER-positive tumors exhibit lower levels of glycine, lactate, and glutamate (high glutamine), as well as reduced glutaminolysis. Therefore, this suggests that ER is involved in tumor metabolic control. Through interaction with many important regulators and pathways, including PI3K/AKT/mTOR, TP53, c-MYC, and Ras/Raf/MAPK, ER plays a crucial role in metabolic control, allowing tumors to reprogram their metabolism to match diverse sorts of environments [29]. By activating ER-α, 17b-estradiol can increase insulin receptor expression while lowering the lipogenic activity of lipoprotein lipase in adipose tissue. Furthermore, Estradiol (E2) and ER-α can both control how the metabolism is reprogrammed in the presence of glucose. E2 promotes glycolysis by upregulating AKT kinase activity and inhibits TCA cycle activity in high glucose situations. On the other hand, in low glucose conditions, E2 activates the TCA cycle by upregulating PDH activity and suppresses glycolysis to meet the tumor cell's energy needs [30]. Importantly, recent research revealed that E2 appeared to promote glycolysis whereas tamoxifen inhibited it. E2 can upregulate *GLUT1* transcriptionally and so enhance glycolysis [31]. The other form of ER is ER-b. In high-grade BC, ER-b expression is downregulated or absent. ER-b, like ER-α, appears to boost glycolysis while suppressing OXPHOS in glucose metabolism. Multiple glycolysis-related pathways are elevated in ER-b-activated mammospheres, suggesting that ER-b plays a major role in regulating BC stem cell metabolism [32]. Epigenetic alterations are mostly enzymatic and possibly reversible. Methylation of DNA, acetylation of histone proteins, and changes in miRNA expression are all epigenetic alterations that affect protein synthesis patterns [33].

In mammalian cells, DNA methylation is one of the essential epigenetic changes. While it controls gene expression in normal development and growth, it is dysregulated in cancer. DNA methyltransferases (DNMTs) such as DNMT1, DNMT3a, and DNMT3b catalyze the methylation of CpG islands in DNA. DNMT1 is critical for methylation to be maintained during DNA replication in normal cells during mitosis. Its absence can result in hypomethylation. *De novo* methylation patterns are thought to be generated by DNMT3a and DNMT3b. DNMT1, DNMT3a, and DNMT3b expression levels are higher in BC than in normal breast tissue. When compared to DNMT1 and DNMT3a, the DNMT3b gene has the largest range of expression [34]. This suggests that DNTM3b is the primary actor in BC. Studies have shown that there are nearly 70% of methylated-CpG islands in the human genome and are found in closely packed core regions of DNA, where they affect gene silence and chromosomal integrity.

On contrary, unmethylated CpG islands are present in relaxed, the open state typically promoter regions of DNA. In this way, transcription factors and other regulatory proteins can access housekeeping and regulatory genes for expression. Normal cells are transcriptionally active. Because CpG islands that are present in the promoters of tumor-suppressor genes are frequently unmethylated in normal cells. On the other hand, in malignant tumors, hypermethylation of CpG islands that are found in promoters of tumor-suppressor genes is observed. Several studies have found and analyzed DNA methylation patterns and their association with breast cancer development and progression throughout the last decade. Cell cycle regulation (Ras Association Domain Family Member 1 -*RASSF1A*), Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*), Cyclin-Dependent Kinase Inhibitor 1B (*CDKN1B*), Cyclin D2 (*CCND2*), DNA repair *(*BRCA1 DNA Repair Associated-*BRCA1*), MutL homolog 1 (*MLH1*), O-6-Methylguanine-DNA Methyltransferase (*MGMT*), cell

detoxification (glutathione S-transferase pi 1-*GSTP1*), apoptosis (Homeobox protein Hox-A5—*HOXA5*), the target of methylation-induced silencing (*TMS1*), cell adhesion and invasion (Twist-related protein—*TWIST*), Cadherin-1 (*CDH1*), metalloproteinase 3 (*TIMP3*), hormone receptors (*ESR1* and progesterone—*PGR*) are among the genes that methylated and thus are silenced [35]. The most important genes for breast cancer, *BRCA1*, and *BRCA2* are tumor suppressor genes that maintain genomic stability by participating in homologous recombination repair and gene conversion of double-stranded DNA breaks. Mutations in BRCA1 and BRCA2 tend to develop breast cancer. Loss of BRCA function due to pathogenic mutations in BRCA causes a lack of homologous recombination. *BRCA1* tumors are high-grade and negative for hormone receptors as well as have a high proliferation rate. Also, *BRCA1* tumors are positive for some cell cycle promoter genes. *BRCA2* tumors, on the other hand, present an opposite phenotype to *BRCA1* tumors but are very similar to sporadic tumors except for *BRCA2*. A research proposed that BRCA1 carriers may be more likely to develop triple-negative cancers and also develop invasive ductal carcinomas of high nuclear, histological grade, and hormone receptor-positive tumors are more common in *BRCA2* mutation carriers [35].

A total of 220 different DNA methylation sites in malignancies were examined. It is demonstrated that with these loci, normal and benign tissues of BC are distinct [36]. Genome-wide researches on breast tumors demonstrated that large number of genes have hypermethylation patterns, known as the "CpG island methylator" phenotype. This phenotype has some advantages [37]. For instance, it is protective, with a specific epigenomic profile linked to reduced metastatic risk and longevity. In contrast, a significant risk of metastatic disease and mortality is observed in the absence of this phenotype. In addition, DNA methylation patterns can be different in BC subtypes [38]. Luminal B tumors are more commonly methylated than basal-like or TNBC [39]. As a result, it is clear that methylation has a substantial role in distinct subgroups of BC and it will be crucial to elucidate the mechanisms in the methylation states. In this way, BC may be targeted therapeutically. Last, but not least, the DNA methylation pattern in endocrine-resistant cancer might give precise indicators to identify and predict the response to therapy. Thus, drugs that target particular enzymes that have crucial roles in epigenetic alterations are being developed and evaluated [38].

Ubiquitination, phosphorylation, and SUMOylation are all examples of post-translational modifications of histone tails. However, acetylation/deacetylation and methylation are well-studied modifications to the expression of genes. The acetyl groups from ε-amino groups of lysine residues are removed by histone deacetylases (HDACs). In this way, chromatins are compacted into well-ordered nucleosomes, preventing transcription factors from accessing DNA. Histone acetyltransferases (HATs) acetylate the lysines, loosening chromatin and facilitating transcription factor binding. When histones are methylated, the genes are generally turned off. On the other hand, when histones are demethylated, the genes are turned on by loosening histone tails. In a summary, histone methylations prevent DNA to be bound by transcription factors, therefore controlling the activity of genes. HDACs and HATs are divided into various groups, each of which catalyzes a different biological process [40].

Based on their structure, HDACs are divided into two groups: zinc-dependent class I, IIa, IIb, and IV, and zinc-independent class III. According to their chemical structure, HDAC inhibitors are classified into four classes: hydroxamic acids, cyclic peptides, short-chain FAs, and benzamides. Some of them can inhibit cancer cell proliferation and promote apoptosis by repressing silenced genes. Vorinostat and

other HDAC inhibitors including entinostat and panobinostat (LBH-589) are being studied in several Phase I and II clinical trials for the treatment of BC. Moreover, their use in combination with standard cytotoxic (paclitaxel) and endocrine (tamoxifen) therapies, as well as therapies targeting HER2 (Herceptin; trastuzumab) or Vascular endothelial growth factor (VEGF), (Avastin; bevacizumab). A combination therapy that uses HDAC inhibitors and DNMT inhibitors works together to re-express suppressed genes, causing apoptosis and reducing tumor metastasis [41].

Lysine (K) and arginine (R) residues restrict histone methylation, with lysins being the most prevalent. Lysine methyltransferases and demethylases reverse the process. Active transcription is linked to methylation of histone H3 lysine 4 (H3K4), H3K36, or H3K79, while gene silencing is linked to methylation of H3K9, H3K20, or H3K27 [42]. Enhancer of zeste homolog 2 (EZH2) is a highly conserved histone methyltransferase that acts as a transcriptional repressor and methylates H3K27. Overexpression of the *EZH2* is linked to aggressive and metastatic BC tumors. The EZH2 inhibitor 3-Deazaneplanocin (DZNep) promotes apoptosis in BC cells although this is not the case in normal ones. Tanshindiols are EZH2 inhibitors that also have an anticancer effect in a variety of tumor cell lines. Last but not least, inhibitory EZH2 peptides have been developed, one of which, SQ037, has been verified and found to have significant anti-EZH2 potency. These reagents show how specificity may be tailored to create medications that specifically target epigenomic enzymes and have the desired effect with minimum adverse effects [43].

The methyltransferase *SMYD3*, which is overexpressed in various tumors, including BCs, targets H3K4. The use of short interfering RNAs to silence *SMYD3* decreases the development of cancer cells. Novobiocin suppresses the proliferation and migration of MDA-MB-231 BC cells via inhibiting *SMYD3* expression. Tranylcypromine is another powerful H3K4 methylase. This tiny chemical demethylation inhibitor inhibits the transcription of key target genes, including the pluripotent stem cell marker *OCT4* [44]. LSD1 demethylates H3K4, as well as nonhistone proteins including p53 and DNMT1. This indicates that it has a wide range of biological roles. When histone-modifying enzymes like LSD1 and EZH2 are overexpressed, they silence essential genes like tumor suppressor genes. Inactivation of these proteins is suspected to have a role in the development of BC and other cancers. However, because LSD1 is abundantly expressed in ER-breast tumors and is a hallmark of aggressiveness, its control in malignancies needs further investigation [45].

In metastasis, miRNAs perform a unique role: while overexpression of a few miRNAs leads to metastasis, the expression of some miRNAs suppresses metastasis. Inflammation and BC metastasis suppressor 1 (*BRMS1*)-mediated metastasis suppression are both controlled by miR-146. Overexpression of miR-146a/b in MDA-MB-231 cells resulted in a substantial drop in epidermal growth factor receptor (*EGFR*) expression, as well as decreased migration, invasion, and metastasis to the lungs [46]. Additionally, in human BC cells, expression levels of miR-335 and miR-206 decreased as the metastatic potential increased. Although a decrease in the expression of these miRs in cancer cells reduced lung and bone metastases, the initial tumor size had no effect. miR-335 inhibits metastasis via regulating the expression of *SOX4* [47].

In BC cells, studies show a negative association between miR-142-3p and the migration of cells. When the miR-142-3p expression is suppressed, the expression of proteins such as zinc finger E-box binding homeobox 1 (*ZEB1*) and Ras-related C3 botulinum toxin substrate 1 (*RAC1*), that allow for the development of an invasive phenotype increases. Additionally, recent research has indicated that overexpression of miR-142-3p has been linked to the suppression of BACH-1, MMP9, chemokine

receptor CXCR4, and vascular endothelial growth factor receptor (VEGFR) protein expression in BC cells [48]. miR-17-5p has been shown to have a unique antimetastatic action in recent investigations. Suppressing miR-17-5p resulted in increased pro-metastatic gene expression and increased metastasis to the lungs. On the other hand, intratumoral delivery of miR-17-5p mimics decreased lung metastasis considerably. Moreover, reduced miR-1179 expression in BC was linked to advanced clinical stage and metastases to the lymph node, according to a clinicopathological study [49]. When miR-1179 is upregulated, it suppresses BC cell proliferation and metastasis by regulating the expression of *NOTCH1*, *NOTCH4*, and its downstream modulators, *HES1* [50]. Last but not least, miR-21 overexpression in MDA-MB-231 cells decreases tumor invasive and metastatic characteristics. On the contrary, reduced miR-21 expression enhanced these cells' migration and invasion capabilities [51].

### 2.2 HER2(+) breast cancer

Human Epidermal Growth Factor Receptor 2 (Her2) protein is a transmembrane receptor tyrosine kinase, which is a member of the EGFR family and plays an important role in mitogen signaling. Amplification of this receptor plays an important role in BC. Overexpression of the Her2 protein results from the Erb-B2 Receptor Tyrosine Kinase 2 (*ERBB2*) gene amplification of all BC tumors (known as HER2-positive BC) [52]. *Her2* overexpression is caused by overactivation of the downstream phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt), *Phospholipase* C, gamma 1 *(PLC-γ),* and mitogen-activated protein kinase (*Mapk)* pathways, leading to increased tumor cell growth, survival, motility, and invasion [53]. *HER2* amplification and/or overexpression causes its conversion from a protoncogene to an oncogene. This has important effects on the metastasis of BC. Clinical studies show that amplification of *HER2* has a significantly worse prognosis in BC patients compared to patients with unamplified *HER2* [54]. In addition to the presence of HER2 in the membrane, it is also found in the nucleus at a lower rate than in the membrane. Despite low HER2 in the nucleus, it is thought to have important roles in the nucleus and chromatin [55]. HER2 is transported to the nucleus by endocytosis via importin β1 and the nuclear pore protein (NUP358) [56]. When HER2 enters the nucleus, it joins forces with PR and Activator protein 1 (AP-1) to activate the transcription factor Signal transducer and activator of transcription 3 (STAT3) in a complex [57, 58]. HER2 interacts with RNA pol I and actin, which enhanced growth by increasing the transcription of the rRNA gene [59]. In another study, it was reported that HER2 binds to the Cyclooxygenase (COX) promoter, which is associated with several malignant tumors found in SK-BR-3 (Skbr3) and BT-474 BC cell lines [60]. In a sizable cohort of BC patients, Dillon et al. [61] showed a correlation between *COX-2* expression and *HER2,* and *HER2* predicted poor disease-free survival in patients receiving endocrine therapy.

Tumor cells may become more vulnerable to further genetic harm and develop extensive instability in the tumor genome as a result of early genetic alterations that de-regulate tumor suppressors and oncogenes. Another research reported that comparative genomic hybridization to measure global copy number alterations discovered that HER2-amplified tumors had considerably greater levels of aberrations than HER2-negative tumors, indicating that these cancers were genetically more progressed [62]. The positive correlation between chromosomal changes at chromosomes 11q13.1, 16q22-q24, and 18q21 and HER2 amplification suggests that genes in these regions may be involved in the pathogenesis of HER2+ tumors in addition to the high levels of overall genomic instability associated with *HER2* amplification [63].

Depending on the metastatic stage, cancer cells may employ one or several metabolic pathways [64]. In addition, depending on where they metastasis, cancer cells may adopt a particular metabolic pattern [65]. Cytoplasmic and mitochondrial nuclear crosstalk can regulate the metabolism of BC. Metabolites in the cytoplasm and mitochondria dictate gene transcription and DNA methylation. Numerous transcription factors shuttle between the nucleus and mitochondria to ensure that genes that regulate metabolism are transcribed [66]. *HER2*-mediated signals regulate lactate dehydrogenase-A levels, 6-Phosphofructo-2-kinase levels, and lactate accumulation in tumors because they promote glucose utilization [66–69]. It has been reported that *HER2* can be replaced by the heat shock protein associated with mitochondria (mtHSP70), both in patient samples and in many cell lines. *HER2* in mitochondria has a negative regulatory effect by indirectly promoting glycolysis of oxygen consumption [70]. In another study, higher levels of glycine, succinate, creatinine, and glutamine were observed in *HER2*+ tumors compared to *HER2*- tumors, while a decrease in alanine levels was reported [71]. *HER2* promotes the RAS-ERK-RSK pathways, ensuring cell survival. HER2+ inhibitors of HER2 in the mammary gland cause a decrease in the activity of these pathways. This, in turn, inhibits the survival of the cell. Another way is the AKT-mTOR pathway. This pathway causes cell proliferation. The HER2 inhibitor causes a decrease in the activity of this pathway. The rapamycin and rapalogs inhibit the activity of mTOR, such as in HER2+ BC, and prevent the phosphorylation of S6K, the process inhibits cell proliferation and reduces aerobic glycolysis as the result of the downregulation of glycolytic enzymes. In addition, the glucose analog 2-deoxy-D-glucose (2-DG) inhibits mTOR signaling by activating PI3K signaling, suppressing aerobic glycolysis, and phosphorylating AMPK on T172 [72].

*HER2* expression in epithelial-like BC cells is significantly higher than in mesenchymal-like BC cells. This is because of the open/active chromatin of the *ERBB2* gene in epithelial-like cells, as well as the closed/inactive chromatin of the *ERBB2* gene in mesenchymal-like BC cells. The chromatin-based epigenetic silencing of the *ERBB2* gene in the EMT of HER2+ BC cells causes inhibition of *HER2* expression, which in turn leads to the emergence of resistance to anti-*HER2* monoclonal antibodies such as trastuzumab [73]. In this study, the H3K9ac and H3K27me3 epigenetic profiles and microanalysis of genes enriched with the promoter h3k9ac chip revealed epi-promoter regions of genes modified by mark at HER2+ and TNBC tumors. The H3K9ac modification has been reported to induce downregulation of most of the related genes in *HER2*-amplified tumors [74]. Descriptive investigations of the HER2/neu epigenetics in BC support the repression of *Her2/neu* by increased H3K9me2. Lim et al. demonstrated that the histone demethylase Kdm1, which removes the methyl groups from dimethylated H3K9, directly targets *Her2/neu*. In this instance, siRNA mediates *Kdm1* knockdown and reduces *Kdm1* accumulation on the *Her2/neu* promoter, which increases H3K9 methylation, decreases *Her2/neu* expression, and inhibits the proliferation of the treated BC cell lines [75]. In another study, it was determined that LAQ824 treatment caused the activation of Her2/neu transcriptional repressor, and acetylation of HSP90, on the other hand, it caused phosphorylated mitogen-activated protein kinase levels and hyperacetylation of HSP 90 with a labile chaperone complex. LAQ824 indirectly marked the Her2/neu protein for proteasomal degradation [45]. For several primary cancers, endocrine organs are metastatic targets. Primary tumors can spread directly or metastasize through the lymphatic and arterial routes. Melanomas, breast, and lung carcinomas are the primary tumors that metastasize to the adrenals most frequently. These tumors can cause adrenal insufficiency, especially

when both adrenals are affected. The most typical primary malignancies that metastasize to the pituitary are breast and lung tumors, which cause pituitary dysfunction in around 30% of cases [76].

Further, cyclin-dependent kinase pathways of PI3K/AKT may lead to endocrine resistance in treatments. Estrogen activity at the molecular level can induce activation of the PI3K/AKT and MAPK pathways at the cell surface and decrease ER and PgR expression [77]. Upregulation of the PI3K/AKT/mTOR pathway contributes to anti-estrogen resistance by promoting survival, tumor cell growth, motility, and metabolism. In this case, the ER promotes transcriptional activity [78]. The intrinsic properties of tumor cells, both soluble factors and ECM proteins from the microenvironment influence the response to Her2-targeted lapatinib or neratinib (TKI). In a study of growing cells on microenvironment microarrays (MEMA), both soluble and ECM factors from various microenvironments were reported to reduce responses to Her2-targeted TKIs. In addition, resistance-conferring factors differed between luminal-like (L-*Her2*+) and basal-like (*Her2E*) *Her2*+ subtypes as defined (Cancer Genome Atlas Network, 2012). Microenvironment-mediated resistance was reversed when pertuzumab-treated L-*Her2*+ cells co-treated with crizotinib in *HER2E* cells. Hepatocyte growth factor and neuregulin1–1 conferred resistance in *HER2E cells*, but not vice versa, in L-*Her2*+ subtype cells. These varied responses to microenvironmental variables are the result of basic variations in the design and wiring of the signaling networks between the two subtypes. In *L-HER2*+ cells and *HER2E* cells, co-treatment with crizotinib and pertuzumab successfully restored the microenvironment-mediated resistance. The findings in this study were consistent with studies that showed that *HER2E* and *L-HER2*+ represent different diseases. The results suggest that *Her2*+ subtype-specific approaches to block resistive microenvironmental signals may enhance clinical management of *Her2*+ BC with *Her2*-targeted TKIs lapatinib and neratinib [79].

### 2.3 TNBC (HER-, ER-, PR-)

TNBC forms 10–15% of all BCs [80]. The cells from this subtype test as negative for the receptors of estrogen and progesterone hormones and also for HER2 protein [80]. When compared to other types that are hormone receptor-positive and HER2+, TNBC is generally more aggressive while being difficult to treat with its insufficient treatment options since hormonal therapy medicines or medicines that target HER2 protein is not available for this situation [81]. TNBC also shows to have a worse prognosis due to the development of metastasis in secondary organs like the brain, lungs, and bone [82]. The complexity in the metastatic process when combined with the lack of targeted therapy makes this disease a harder one to cure. Besides these problems, it is also found to be more likely to reoccur. But its symptoms, staging, diagnosis, and survival are similar to other invasive ductal carcinomas [83].

The basal-like subtype of BC is characterized by high proliferation, high histological grade, and poor prognosis. And this subtype can be triple negative although not all of the basal-like cancers resemble the forms that express ER and HER2 [82]. By gene expression profiling, TNBC is also identified with seven subtypes: two basal-like (BL1 and BL2), a mesenchymal (M), a mesenchymal-stem cell-like, an immunomodulatory, a luminal androgen receptor/luminal-like, and an unclassified type. And each subtype shows unique ontologies and different responses to standard-of-care chemotherapy [84]. Besides the differences, TNBC is generally found to be responding less to conventional chemotherapy while the patients carry a bigger risk of recurrence and relapse [85].

Non-coding RNAs (ncRNAs) may have a role in the progression of BC cases and the metastasis process. And in the process of forming miRNAs, Heterogeneous nuclear ribonucleoproteins A2/B1 (*HNRNPA2B1*) interacting with a component of the DROSHA complex is known to stimulate the processing of pri-miRNA to pre-miRNAs [86]. Also, *HNRNPA2B1* transcript and protein expression were found to be high in BC cells and tumors when compared to nontransformed cell lines and normal breast tissue. With the TNBC subtype, it is shown that MDA-MB-231 TNBC cells with *HNRNPA2B1* knockout have reduced tumor growth but were stimulated in metastasis when injected into mice. So, the role is not clear with *HNRNPA2B1* but it is essential to research deeply to understand its role in BC metastasis. Also, using the sublines of MDA-MB-468 TNBC cells, drivers of metastasis are identified as IL11 and VEGF-D in *in vivo*. They activate the effector neutrophils and promote metastatic niche [87]. In this case, chemotherapy may have a negative effect by increasing the metastatic potential if the cells with innate resistance are selected.

MetastamiRs are the miRNAs have a pro- or anti-metastatic effect [88]. Pro-metastatic miRNAs are expressed higher in breast tumors showing a link to reduced disease-free survival (DFS) and survival by miR-9-5p is one of them as a pro-metastatic oncomiR and found to be at a higher level in TNBC than other subtypes. miR-373, miR-29a/b/c, and miR-19a are all found to be pro-metastatic and with a high level in TNBC subtypes. miR-206, and miR-31-5p are listed as anti-metastatic with a lower expression in TNBC subtypes. But miR-20a-5p which also is listed as anti-metastatic has a higher expression in TNBC compared to other types [87]. All of the miRNAs affect the regulation of the metastasis process. The increased expression of miR-520c-3p is observed in MDA-MB-231 TNBC cells. And it is found to be inhibiting TGF-β signaling which can be related to inhibiting phosphorylation of suppressors against decapentaplegic 2 (SMAD2) and decapentaplegic 3 (SMAD3) and decreases target genes *ANGPTL3*, *PTHLH*, and *SERPINE1* (*PAI-1*) [89]. miR-373 is another miRNA that is pro-metastatic and with its increased expression, MDA-MB-435 cell migration and invasion are induced in *in vitro*. It has also been found to be promoting tumor metastasis observed via tail vein injection mouse model [87]. Some genes are found to be linked with TNBC metastasis. In a study, 26 hub genes were identified as metastasis-associated candidate genes [90]. In-depth studies with four of them, Immunoglobulin Superfamily Member 10 (*IGSF10*), Runt-related transcription factor 1 translocation partner 1 (*RUNX1T1*), X-inactive specific transcript (*XIST*), and transcription factor teeshirt zinc finger homeobox 2 (*TSHZ2*) indicated that they were downregulated in TNBC tissues and these genes have prognostic and diagnosis values in TNBC [90]. *IGSF10* is an immunoglobulin superfamily member 10 normally associated with developmental processes and differentiation [91]. By whole-exome sequencing it was found to be a potential cancer-related gene *RUNX1T1* is a member of the mind the gap (*MTG*) family. It was already known to be reported in many cancer types as a novel biomarker or as being vital for tumorigenesis. *XIST* plays a role in the inactivation of the X chromosome. It was also known to have a relationship with cancer cases since its expression was observed to be dysregulated in some cases. *TSHZ2* is a member of the TSHZ family and like the others, its expression was observed in other cancer types and was found to have a downregulation in some cancers [90].

With the studies done in TNBC, it is found that this subtype is possess more therapy-resistant Cancer Stem Cells (CSC) when compared to other subtypes. The difference in mortality and recurrence rates with the therapy failures may be the result of this difference in CSC enrichment in TNBC. So, studying cellular signaling

pathways and transcription factors that contribute to stemness can show an insight into this subtype. Notch signaling is a developmental pathway triggered by Notch ligands binding to Notch receptors. Its expression in BC CSCs promotes self-renewal and metastasis. TNBC is significantly deregulated compared to the other BC subtypes. Also, it is found that hypoxia which is a hallmark of TNBC can induce Jagged1, a Notch ligand, expression in TNBC CSCs and lead to metastasis and self-renewal. PKD3 is part of the protein kinase D (PKD) family and is shown to have a role in increasing TNBC metastasis, proliferation, and stemness. Another pathway that is linked to the increased risk of metastasis in TNBC is the Interleukin/Janus kinase-2/signal transducer and activators of transcription-3 (IL-6/JAK2/STAT3) pathway and it is preferentially activated in TNBC CSCs comparing to the other BC subtypes. SRY-box transcription factor 2 (*SOX2*) is a stem cell pluripotency regulator which is effective in embryonic development and it is over-expressed in TNBC. This expression level of *SOX2* is associated with increased proliferation and metastasis. Further, Lipase H (*LIPH*) is found to regulate *SOX2* so it is another gene to promote metastasis in TNBC CSCs. *NANOG* is known to be a self-renewal and pluripotency regulator and it is also found to be a key driver of metastasis too. Ubiquitin carboxyl-terminal hydrolase 1 (*USP1*) and Protocadherin-7 (*PCDH7*) were also found to be promoting metastasis in TNBC CSCs. And finally, under the list of epigenetic regulation, histone methyltransferase *EZH2* was found to maintain metastasis in TNBC CSCs [85].

## 3. Future perspective

BC signaling pathway is complex and in certain immune subtypes and at different stages, the pathways may cross-talk. CSCs enhance the cellular environment and the heterogeneity burden of anticancer treatment. Therefore, elucidating molecular mechanisms is complicated. Further, clinical drugs may cause resistance, and patients may not give a similar response to cancer treatment. However, computer-based algorithms and designing similar compound patterns with modified side chains smooth drug design studies. Omics technologies highlight molecular correlations and coupling the knowledge with drug design provides innovative solutions. Non-coding elements also help our understanding of the molecular mechanism. Altogether, new perspectives in anti-cancer treatment may provide comprehensive and contemporary solutions.

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Chapter 5

# Non-Small Cell Lung Cancer Brain Metastasis: The Link between Molecular Mechanisms and Novel Therapeutic Approaches

*Shiva Najjary, Dana A.M. Mustafa and Johan M. Kros*

## Abstract

The prognosis of patients suffering from non-small cell lung carcinomas (NSCLC) worsens significantly when brain metastasis occurs. Seeding to the brain usually happens relatively early in the course of disease and therefore, new therapies anticipating this complication would result in considerable improvement in outcomes. In this review, we address recent molecular data of NSCLC with a focus on the risk of the formation of brain metastasis. Included is new data on the involvement of miRNAs and lncRNAs in the rise of the cerebral seeding of NSCLC. We summarize novel therapeutic approaches developed in the light of these recent molecular discoveries.

**Keywords:** brain metastasis, lung cancer, blood-brain barrier, microRNAs, targeted therapy, immunotherapy

## 1. Introduction

Lung carcinoma is among the deadliest cancers and its treatment is an important challenge for oncologists. Approximately 16–20% of patients with lung cancer develop brain metastasis, regarded as the most life-threatening complication of the disease. Population-based incidence proportions for brain metastasis are highest for lung cancer (20% against 9.6% for all common cancers) [1]. The frequency of the diagnosis of lung cancer brain metastasis (LCBM) has increased and the reasons for this are not entirely clear. Certainly, advances in radiology have resulted in increased sensitivity for tracing brain metastatic sites. In addition, metastatic tumor cells behind the blood-brain barrier (BBB) are less vulnerable to chemotherapeutic agents ("pharmacologic sanctuary"). Further, there are effects of the increasing age of the population [2]. The incidence and severity of the cerebral symptoms vary from minimal to severely debilitating. Less than 4% of patients with metastatic NSCLC live longer than five years after the diagnosis [3–5]. Obviously, protecting patients from developing brain metastases would significantly alleviate the disease burden and improve outcomes. Knowledge of the subsequent steps tumor cells need to take before growing as metastases in the brain is essential. In this review, we will summarize

current knowledge on the genes and pathways operative in the development of brain metastasis of NSCL and summarize the application of targeted drugs.

## 2. Brain metastasis development and the blood-brain-barrier

A significant part of the disease burden and death of cancer is caused by the seeding of tumor cells to the brain [6]. The stages of the development of brain metastasis include the detachment of cancer cells from the primary tumors and their penetration of the BBB followed by extravasation, colonization, and macro-metastatic growth [7]. The detachment of tumor cells from the primary tumor mass depends on cell adhesion molecules (CAMs) including immunoglobulins (IgCAMs), selectins, integrins, and cadherins [8–10]. The tumor cells require the loss of functional E-cadherin (CDH1) in order to increase their motility, and close relation between reduced E-cadherin expression and poor outcome due to tumor spread in NSCLC exists [11]. CDH1 regulates EGFR activity through receptor tyrosine kinases (RTKs) and provides functions in intracellular signaling. Subsequent events include the epithelial-mesenchymal transition (EMT), a crucial phenomenon in the dissemination and motility of cancer cells [12, 13]. The process of EMT relays on proteases such as secreted matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM) components including proteoglycans, collagen, fibronectin, and laminin, and modify the structural and mechanical features of the ECM [14]. MMPs also break down cell-ECM - and cell-cell connections by cleaving CDH1 and CD44. MMP-1, MMP-2, and MMP-9 are particularly associated with metastases of lung cancer [15]. Once detached and motile, the tumor cells enter the circulation to become circulating tumor cells (CTCs). Some CTCs resist the forces of the blood flow and by using surface receptors adhere to the endothelial cells. Subsequently, the cells will migrate through the endothelial layer by the expression of selectins, integrins, and chemokines. This process is accompanied by the creation of a permissive immune microenvironment through the activation of integrins and the release of cytokines such as vascular endothelial growth factor (VEGF) [16]. VEGF is vital in the process of neovascularization and takes part in the creation of high endothelial venules, to increase lymphocyte extravasation and infiltration in the perivascular niches (PVN) at the metastatic sites [17]. The altered microenvironment promotes further migration of CTCs to the brain parenchyma by secreting site-specific chemokines such as CXCR4 and its ligand, CXCL12 [17, 18]. There is high expression of CXCR4/CXCL12 in brain metastases of NSCLC and, together with integrins, CXCR4 enhances further tumor cell invasion. The metastatic cells in the PVNs activate tumor-associated macrophages (TAMs) and microglia. TAMs play a role in the survival of CTCs and induce extravasation and colonization by expressing survival factors such as epidermal growth factor (EGF) [19]. While the TILs try to combat the tumor cells, the microglia switches from the M1 (anti-tumor) phenotype to the M2 (anti-inflammatory) phenotype by factors secreted from tumor cells [20] and display tumor-supporting activity. The M2 microglia counteracts TILs activity via the induction of immunosuppressive factors including programmed cell death protein 1(PD1) /programmed death-ligand 1 (PD-L1) [21]. Also, activated astrocytes promote the proliferation and brain invasion of the tumor cells [22]. Obviously, cell types and pathways that initially are activated to counter-act the metastatic process become collaborators in progressive colonization of the brain later on. So far, therapeutic interventions aimed at the elimination of the tumor cells growing in the brain.

Future therapeutic strategies may target any of the preceding events, including CTC trafficking and penetration of the BBB.

## 3. Genetic alterations in NSCLC associated with brain metastasis

Targeted therapies that successfully combat tumor cells outside the brain may fail to be effective behind the BBB. There are several reasons for this, one of which are differences in genetic alterations between the primary tumors and their metastases [23]. Patients suffering from NSCLC have been classified according to the genetic changes in the primary tumor, which include epidermal growth factor receptor (EGFR), Kirsten rat sarcoma (KRAS), and anaplastic lymphoma kinase (ALK). NSCLC brain metastasis-specific mutations can be detected in the cerebrospinal fluid (CSF) and can also be used to evaluate the presence of disease and response to therapy [24].

### 3.1 EGFR mutations

EGFR is a receptor for extracellular growth factors such as epithelial growth factor (EGF) and tumor growth factor-α (TGFα). Binding of these factors causes a structural change and activation of the receptor complex, resulting in the activation of signaling pathways that promote cell proliferation, motility, and survival. Dysregulation of the receptor is associated with various human cancers. The prevalence of EGFR mutations is dependent on a variety of factors, including ethnicity, gender, smoking, tumor heterogeneity, and tumor progression. EGFR is often overexpressed in NSCLC and the two most frequent EGFR mutations encountered involve exon 19 (deletions) and 21 (L858R mutations) [25–27]. There is data supporting that CNS metastases of NSCLC are promoted by EGFR-activated mesenchymal–epithelial transition (MET) through mitogen-activated protein kinases (MAPK) signaling. EGFR activates signal transducer and activator of transcription 3 (STAT3) via the expression of interleukin-6 (IL-6) which would increase the risk of BM [28]. NSCLC patients with EGFR mutations at the time of diagnosis or in the early stages of the disease seem to have two times higher risk of brain metastasis [29–31]. In a series of 30 primary tumor/metastasis series, there was discordance between EGFR status as measured by IHC of one-third of sample pairs and a little less by FISH [32]. In 14 out of 54 paired samples of lung adenocarcinomas, EGFR alterations of EGFR were restricted to the brain metastases [33]. In a recent paper by Haim et al., the EGFR mutational status of brain metastasis could be predicted with an accuracy of almost 90% by using clinical, radiological, and molecular data for deep learning strategies [34]. Obviously, the presence of CNS metastases leads to poorer outcomes (viz., 11.6 months vs 18.7 months) as shown in a study on 101 EGFR positive metastatic NSCLC previously treated with either combination chemotherapy or oral TKI [35]. The progression of the cerebral lesions is also relatively high during treatment in these patients and there is a connection between the EGFR mutations and EMT-related tumor invasion [36, 37].

### 3.2 KRAS mutations

The K-Ras protein is encoded by the KRAS gene and is part of the RAS/MAPK pathway, where it transfers signals to proliferate and divide from extracellular into the nucleus. A single substitution of a nucleotide may serve as an activator of the signaling pathway turning tissue hyperplasia into invasive cancers. Although it is believed

that KRAS and EGFR mutations are mutually exclusive [38, 39], yet cases of simultaneous occurrence were found [40–42]. Nearly 15–30% of NSCLCs have activating mutations in the KRAS gene that are associated with adenocarcinoma initiation and clinical aggressiveness [38, 43, 44]. There is a clear connection between KRAS mutations and smoking history [45, 46]. In a study of 482 lung adenocarcinomas (LADC), it was found that KRAS mutations also occur in patients who had never smoked, but the mutations differ from those in the tumors of smokers. For instance, transition mutations (G > A) prevail in those who never smoked while transversion mutations (G > T or G > C) are typical for NSCLCs in smokers [47]. The relation between KRAS mutations in NSCLC and propensity for brain metastasis is still unknown and need to be further studied [36, 42]. Approximately 25% of brain metastatic tumors with KRAS mutations were observed in smokers [44]. Other mutations, including ROS proto-oncogene 1, liver kinase B1 (LKB1), and hepatocyte growth factor receptor (HGFR), were associated with the development of lung carcinoma [48–50], but their relations with brain metastasis of lung cancer is also unknown. LKB1 is inactivated in nearly 30% of all NSCLCs [46] and its effects are synergistic with those of KRAS mutation on the progression of lung cancer and the development of metastases in general [51, 52]. In a study of 154 patients with NSCLC, Zhao et al. concluded that KRAS mutations in combination with low LKB1 copy numbers (CNs) are related to a 20-fold increase in brain metastasis [53]. So far, therapeutic KRAS targeting has been unsuccessful.

### 3.3 ALK translocations

ALK gene mutations, copy number changes, or fusion with other genes have oncogenic effects. Similar to EGFR mutations, translocations of ALK are predictive of response to Tyrosine Kinase Inhibitors (TKIs) [54]. ALK testing is mostly recommended for non-squamous cell lung cancers lacking EGFR mutations. The fusion between ALK and EML4 (echinoderm microtubule-associated protein-like 4) produces molecular variants with diverse biological functions and affects various signaling pathways [55, 56]. The incidence of cerebral metastases in NSCLC with ALK mutations is high and ALK translocations of primary tumors and their brain metastases are often similar. Interestingly, the progress of brain metastases of tumors with ALK mutations slows down significantly when treated with targeted therapy: over 45% of patients with BM had overall survival rates of three years [57]. Because nearly 45% of patients with ALK-positive NSCLC have developed BM at death [58], cerebral seeding is an important clinical challenge for developing strategies for personalized care in NSCLC [59].

### 3.4 MET and RET mutations

The large variety of mutations in EMT: (mesenchymal epithelial transition factor) affects a range of cancers, including NSCLC. The MET gene codes for a tyrosine-kinase receptor that plays role in developmental processes and wound healing. Hepatic growth factor/scatter factor (HGF/SF) and their splice isoforms NK1 and 2 are the only known ligands of the MET receptor. In cancer, abnormal MET activation triggers proliferation, angiogenesis, and metastasis. The MET pathways interfere with the key oncogenic pathways RAS, P13K, STAT3, and beta catenin. In general, mutations consist of duplications of mutant alleles, intronic splice site alterations, and mutations affecting the receptor downstream targets. In NSCLC BM, the majority of

MET mutations found at metastatic sites affect the extracellular SEMA: Semaphorin superfamily domain of the receptor [60–62]. Remarkably, mutations in MET occur more frequently in CNS metastasis from NSCLC than in their primary tumors.
RET chromosomal rearrangements have been detected in 1–2% of all patients with NSCLC, particularly in patients with adenocarcinoma. The rearrangements are mutually exclusive with EGFR, ALK, or RAS mutations [63]. Importantly, NSCLC with RET rearrangement is associated with an increased risk of BMs [64].

## 4. The role of MicroRNAs in NSCLC brain metastasis

MicroRNAs (miRNAs) are conserved short endogenous RNA molecules (21–25 nt) that play critical roles in gene expression patterning by interfering with target mRNAs [65]. MiRNAs regulate cellular functions including cell growth, cell differentiation, and cell death. About half of the miRNAs participate in tumorigenesis [66]. The expression of miRNAs may lead to the rise of tumors by activating the pathways implicated in carcinogenesis. The function of miRNAs in the development of tumor metastasis to brain has recently attracted attention and various studies have addressed the role of miRNAs in the progression of brain metastases of lung cancers in particular (**Table 1**) [83, 84]. The effects of miRNAs vary widely, depending on the expressional cascades they influence.

In **Table 1**, a summary of currently known miRNA associations with NSCLC and their brain metastases is presented. MiRNA-184 and miRNA-197 are highly expressed in EGFR-mutant NSCLCs of patients with cerebral metastases and may serve as biomarkers for the risk of cerebral seeding [67]. Expression of miRNA-9 and miRNA-1471 has also been found in lung cancer with brain metastasis. Up-regulation of miRNA-145 inhibits the proliferation of human tumor cells in lung adenocarcinomas via targeting of c-Myc and EGFR [79, 85, 86]. MiRNA-146a is overexpressed in NSCLC and is associated with down-regulation of heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 and up-regulation of β-catenin, resulting not only in tumor cell invasion and migration but also in the metastatic potential to brain [87, 88]. Also, MiRNA-95-3p is upregulated in lung adenocarcinoma but overexpression of this MiRNA seems to suppress the formation of brain metastasis via down-regulation of cyclin D1 [75]. MiRNA-378 is overexpressed in NSCLC and their brain metastases and increases tumor growth and metastasis via the upregulation of MMP-7, VEGF, and MMP-9 [74]. Also, MiRNA-328 is overexpressed in NSCLC and allegedly promotes the formation of brain metastases via PRKCA and urokinase-type plasminogen activator (uPA) [71]. PRKCA mediates the expression, resulting in the migration of the cancer cells [89]. Lastly, increased miRNA-21 levels suppress cell death and promote the proliferation and invasion of NSCLC and lung adenocarcinoma cells [68, 90].

Some miRNAs are downregulated in the context of cerebral seeding of lung cancer. MiRNA-768-3p is downregulated in lung cancer cells co-cultured with astrocytes, leading to increased KRAS expression, tumor outgrowth, and propagation of brain metastasis [91]. MiRNA-375 is another miRNA that reportedly is down-regulated in primary NSCLC and reduced levels of miRNA-375 are associated with NSCLC brain metastasis [72]. In tumors in which miRNA-375 was downregulated MMP9 and VEGF were found overexpressed [72]. Reduced miRNA-145 levels also seem to promote brain metastasis in lung adenocarcinoma, while overexpression reduces tumor dissemination [69].

| miRNA | Tissue | Target | Tumor suppressor/ Oncogene | Effect | References |
|---|---|---|---|---|---|
| miR-184, miR-197 | EGFR-mutant lung tumors | | | | [67] |
| miR-21 | *In vivo* | SPRY2, TIMP3, CDKN1A, SERPINB5 and PTEN | Oncogene | Initiating cell proliferation promoting brain metastasis- | [68] |
| miR-145-5p | Brain and lung tumors | TPD52 | Suppressor | Inhibited cell invasion and migration | [69] |
| miR-142-3p | TCGA data | TRPA1 | Suppressor | Suppressing NSCLC progression | [70] |
| miR-328 | Brain and lung tumors | PRKCA | Oncogene | Increasing cell migration | [71] |
| miR-375 | Brain and lung tumors | VEGF and MMP-9 | Suppressor | | [72] |
| miR-590 | Lung tumors | ADAM9 | Suppressor | Suppressing tumorigenesis and invasion | [73] |
| miR-378 | Brain and lung tumors | MMP-2, MMP-9 and VEGF | Oncogene | Promoting migration, invasion, and angiogenesis | [74] |
| miR-95-3p | *In vivo* | Cyclin D1 | Suppressor | Inhibiting invasion and proliferation | [75] |
| miR-330-3p | Lung tumors | GRIA3 | Oncogene | Promoting growth, tumor invasion, and migration. | [76, 77] |
| miR-490-3p | Brain tissues | PCBP1 | Oncogene | Promoting proliferation, invasion, and migration | [78] |
| miR-145 | Brain and lung tumors | | Suppressor | Inhibiting cell proliferation | [79] |
| miR-423-5p | Lung tumors | MTSS1 | Oncogene | Promoting cell invasion and migration. | [80] |

| | | | | | |
|---|---|---|---|---|---|
| miR-15a, miR-210, miR-214 | Lung tumor | | | Predicting brain metastasis in patients with lung adenocarcinoma | [81] |
| miR-4317 | Lung tumors | FGF9 and CCND2 | Suppressor | Inhibiting proliferation, migration, colony formation, and invasion | [82] |

*TPD52: tumor protein D52; TRPA1: transient receptor potential ankyrin 1; GRIA3: glutamate receptor, ionotropic, AMPA 3; PRKCA: protein kinase C-α; MMP: matrix metalloprotease; ADAM9: a disintegrin and metalloproteinase 9; PCBP1: poly r(C)-binding protein 1; MTSS1: metastasis suppressor protein 1; FGF9: fibroblast growth factor 9; CCND2: cyclin D2; and TCGA: The Cancer Genome Atlas.*

**Table 1.**
*MicroRNAs associated with brain metastasis from NSCLC.*

Taken together, miRNAs appear to have great potential for cancer diagnosis, prognosis, and treatment at the molecular level, but the use of miRNAs for the clinical treatment of brain metastases requires further investigation. Many studies focused on the identification of alterered expression patterens of miRNAs after outgrowth in the brain microenvironment, but validation of data in larger groups of tumor samples is needed [31].

## 5. Role of lncRNAs in NSCLC brain metastasis

Long non-coding RNAs (lncRNAs) are non-coding transcripts comprising > 200 nucleotides that have substantial functions in various physiological and pathological pathways. Similar to miRNAs, lncRNAs also regulate a variety of molecular targets by various mechanisms. Recently, the effective role of lncRNAs in tumorigenesis was shown [92]. lncRNAs are important regulators of lung cancer progression. Some lncRNAs serve different functions in various types of cells [93]. MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1) is a large non-coding RNA gene that is highly conserved in mammals and regulates gene expression via splicing-independent mechanisms in NSCLC metastasis [94]. MALAT1 is located on chromosome 11q13 and increased MALAT1 levels were recently discovered in patients with NSCLC who had developed cerebral metastasis, while not in patients without brain locations [95, 96]. In addition, functional studies revealed that overexpression of MALAT1 leads to overexpression of vimentin in highly invasive metastatic lung cancer cell lines while silencing MALAT1 affects EMT programming and suppresses metastasis of the lung cancer cells [96]. Moreover, RNAi-mediated repression of MALAT1-RNA has a negative impact on the migration and outgrowth of human NSCLC cell lines. Overexpression of MALAT1 in NIH/3T3 fibroblasts significantly enhanced migration [97] and stimulated cell motility via the regulation of related genes [98]. The oncogene c-MYC influences cerebral metastasis of NSCLC by inducing the overexpressing of Non-coding RNA BCYRN1 (brain cytoplasmic RNA 1) in NSCLC cells [99, 100]. c-MYC-activated BCYRN1 induces NSCLC metastasis by the expression of MMP9 and MMP13, members of the matrixin subfamily that behave as ECM-degrading enzymes [101–103]. HOTAIR (HOX transcript antisense RNA) is a lncRNA that is highly expressed in NSCLCs with brain seeding [104]. *In vitro* studies have revealed that HOTAIR expression enhances

tumor cell migration and outgrowth [105]. At this point, the relationship between MALAT1 and HOTAIR in NSCLC brain metastasis is still unknown [104].

## 6. Novel therapeutic approaches

For many years, the rise of brain metastases of lung cancer has been considered the final stage of the disease. Patients were treated with standard therapeutic options such as palliative care or whole brain radiotherapy (WBRT). However, since the discovery of new systemic and targeted therapies, additional effective treatments for lung cancer were introduced with the aim to enhance local control and survival [106].

### 6.1 Targeted systemic therapy

The BBB is an obstacle to enter the brain for many agents and has limited the application of drugs used for systemic therapy [107]. The application of drugs targeting EGFR and ALK has heightened the interest in utilizing systemic agents to treat brain metastases [108–111]. In **Table 2** clinical trials of targeted therapy for NSCLC brain metastases are listed.

### 6.2 EGFR tyrosine kinase inhibitors

Patients with tumors harboring EGFR mutations are prone to develop brain metastases [112, 113]. Although the efficacy of EGFR-TKIs for NSCLCs with EGFR mutations has been proven, its effectiveness is not clear in patients with brain metastases since they were excluded from controlled clinical trials. In a prospective study, 41 patients with unselected NSCLC brain metastasis were treated with Gefitinib resulting in 10% intracranial partial responses (PR) with an average response period of 13.5 months [114]. However, most information on the efficacy of TKIs in patients with brain metastases was obtained from retrospective studies [110]. Firstly, it appeared that recorded concentrations of Afatinib, Erlotinib, and Gefitinib in cerebrospinal fluid (CSF) clearly exceeded those needed to inhibit the growth of cells with EGFR mutations *in vitro*. In patients with lung adenocarcinoma, about 70% intracranial tumor response was obtained with Gefitinib or Erlotinib as first-line treatment [115]. Other retrospective clinical studies revealed that patients with brain metastases from EGFR-mutant NSCLC have more favorable responses to WBRT or TKI therapy than patients with brain metastases from EGFR–wild-type NSCLC [116]. The progression periods were 11.7 months for patients with EGFR-mutant NSCLCs treated with Erlotinib and 5.8 months for patients with EGFR-wild-type NSCLCs, respectively [117]. The potent EGFR-TKI, AZD3759 showed significant penetration of the BBB in pre-clinical models for the treatment of EGFR-mutant NSCLC with brain metastasis [118]. Moreover, the third-generation EGFR inhibitors osimertinib and rociletinib targeting the T790M-EGFR resistance mutation in NSCLC appeared effective in treating patients with NSCLCs with these mutations [119, 120]. Unfortunately, a phase 3 trial conducted by RTOG using WBRT plus SRS with Temozolomide and Erlotinib in unselected patients with a maximum of three brain metastases was closed prematurely because of low accrual [121]. No significant benefit of adding Gefitinib to WBRT in phase 2 trials in patients with unselected NSCLC with brain metastasis was recorded [122].

| Targeted agent | Target | Pretreatment with radiotherapy | Progression-free survival (month) | Phase | Status | NCT identifier |
|---|---|---|---|---|---|---|
| Alectinib, bevacizumab | ALK, VEGF | No | NA | I/II | Recruiting | NCT02521051 |
| AT13387, Crizotinib | c-MET, ALK, ROS1, Hsp90 | No | NA | I/II | Completed | NCT01712217 |
| MK-3475 | PD-L1 | No | NA | II | Completed | NCT02085070 |
| Sunitinib | VEGF, KIT, PDGF, FLT-3 | Yes | 2.1 | II | Completed | NCT00372775 |
| GRN1005 | - | Yes | NA | II | Completed | NCT01497665 |
| Dasatinib | BCR-ABL | Yes | NA | II | Completed | NCT00787267 |
| Cetuximab | EGFR | Yes | NA | II | Completed | NCT00103207 |
| Certinib | ALK | No | NA | II | Active, not recruiting | NCT02513667 |
| Erlotinib | EGFR | Yes | 1.6 | III | Completed | NCT01887795 |
| Afatinib | HER2, EGFR, HER4 | No | NA | III | Completed | NCT02044380 |
| Osimertinib | EGFR | No | NA | IIIb/IV | Completed | NCT03790397 |

*c-MET: tyrosine-protein kinase Met, hepatocyte growth factor receptor; ROS1: Proto-oncogene tyrosine-protein kinase ROS; Hsp90: heat shock protein 90; KIT: Proto-oncogene c-KIT; PDGF: Platelet-derived growth factor; FLT-3: fms like tyrosine kinase 3, CD135; HER2: human epidermal growth factor receptor 2; and HER4: human epidermal growth factor receptor 4.*

**Table 2.**
*Clinical trials of targeted therapy for the treatment.*

At present, there is not sufficient data to draw conclusions on TKI therapy plus CNS-directed radiation therapy for patients with NSCLCs with EGFR mutations.

### 6.3 ALK tyrosine kinase inhibitors

ALK rearrangements are found in about 4–8% of NSCLCs, representing a distinct subgroup [110]. ALK-TKIs are active against CNS metastases and novel drugs are effective in treating brain metastases, even in patients with multiple intracranial tumors [123]. Crizotinib (Xalkoric) was the first ALK-TKI for metastatic ALK-positive NSCLCs. In phase 3, randomized clinical trial with a single-arm of crizotinib for patients with NSCLC with cerebral metastases, better intracranial response was obtained for patients who were also treated with RT [124] and median survivals of almost 50 months were recorded for patients with ALK-positive tumors [58]. Second-generation ALK-TKIs including brigatinib, ceritinib (Zykadia), and alectinib (Alecensa) have shown a better BBB penetration and activity against BM in crizotinib-resistant tumors [125]. Ceritinib appeared to be a powerful drug for patients with metastatic ALK-positive NSCLCs in whom treatment with crizotinib was not effective anymore. Ceritinib also showed activity against crizotinib-resistant tumors in the mouse models [126]. Patients with ALK-positive tumors with CNS lesions were treated with alectinib against crizotinib in the ALEX trial. The results showed that patients treated with alectinib had a longer progression-free survival (PFS) rate than patients treated with crizotinib [123]. In addition, nearly half of patients with ALK-positive NSCLCs with cerebral metastasis improved significantly upon treatment with alectinib. Taken together, these results indicate that Alectinib can be used as an effective treatment option for patients with NSCLC-positive ALK with cerebral metastasis [123].

### 6.4 MET inhibitors

In recent years, several MET inhibitors have been approved and have entered clinical trials. There are limited data available on the role and efficacy of monoclonal antibodies that inhibit MET in brain metastasis [127]. The effectiveness of Sym015, which consists of two monoclonal antibodies targeted to non-overlapping epitopes of MET, was high in inhibiting MET-amplified tumors as compared to emibetuzumab, a humanized monoclonal antibody developed for patients with NSCLC [128]. Among the new small inhibitors, cabozantinib, an inhibitor of MET, RET, and VEGFR2, appeared effective in radiation-resistant MET-mutated BM in renal cell carcinoma [129]. In addition, cabozantinib yields rapid responses in crizotinib-resistant NSCLC harboring a MET exon 14 alteration [130]. Simultaneous activation of the MET receptor and the ALK fusion gene in NSCLC yielded effective responses to crizotinib in patients with brain metastases [131]. The oral administered selective MET inhibitor capmatinib came with controllable toxicity profiles in treatment-naive patients with MET-exon14 positive NSCLC. Preliminary studies in mice that were injected with human BM cells from NSCLC showed that capmatinib is able to cross the BBB and is active in the brain. In *in vivo* models, the combination of capmatinib and afatinib was found to suppress tumor growth [132]. Recently it was demonstrated that bozitinib, another novel orally administered PLB-1001 compound, better penetrated the BBB as compared to other MET inhibitors in MET-mutated glioblastoma [133]. These preliminary results raise hopes for the effectiveness of PLB-1001 in the treatment of secondary brain lesions from various primary sites.

### 6.5 RET inhibitors

Cabozantinib and vandetanib are oral multi-kinase, non-selective RET inhibitors that have a modest advantage but significant toxicity. Cabozantinib is effective in RET-rearranged NSCLC and has limited activity against RET, while vandetanib more effectively targets RET. No specific activity against CNS seedings of NSCLC has been reported for these drugs [134, 135]. Selpercatinib and pralsetinib are small highly selective RET inhibitors approved by the FDA for the treatment of NSCLC with RET fusion [136, 137]. Selpercatinib (LOXO-292) is an oral tyrosine-kinase inhibitor specifically targeting the RET kinase domain. Its activity profile and clinical safety were evaluated in phase I/II clinical trial LIBRETTO-001. The study included patients with advanced RET-positive NSCLC who had progressed disease after platinum-based chemotherapy in patients who were treatment naïve. In the phase II trial, 105 patients were pretreated with platinum-based chemotherapy. The ORR was 64% (95% confidence interval (CI): 54% to 73%) with a median duration of response of 17.5 months. A major advantage was observed among the 39 treatment naïve patients, with an ORR of 85% (95% CI: 70% to 94%). Selpercatinib was also designed to have an effect on the CNS. Eleven patients with BM participated and Intracranial responses were observed in 10/11 patients with response rates of 91% (95% CI: 6.7% to NE) [138]. The FDA granted to accelerate the approval of selpercatinib for treating patients with metastatic RET-positive NSCLC, regardless of specific treatment strategy. The RET kinase domain inhibitor Pralsetinib (BLU-667) is currently applied in a multicenter phase I/II ARROW trial. Based on the results of this trial, the FDA approved the efficacy of pralsetinib in patients with RET alteration-positive NSCLC with/without prior therapy. Patients with asymptomatic BM were allowed to be included in this trial. In total, 79 patients participated, and the majority were pretreated primarily with chemotherapy (76%) and immunotherapy (41%). CNS metastasis at the baseline observed in 39% of patients. Efficacy was based on 57 patients, all of whom had at least one follow-up evaluation [139, 140].

### 6.6 Immune therapy

Although the immune system plays a role in all stages of the development of cerebral metastasis, so far therapeutic interference was limited to the immune response around the tumor cells present in the brain. The inflammatory microenvironment of brain metastases mainly consists of infiltration by tumor-infiltrating lymphocytes (TIL) expressing immunosuppressive factors like programmed death-1 (PD-1) ligand (PD-L1). Immunotherapeutic agents include anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), anti- PD-1, and PD-L1 monoclonal antibodies (mAbs). There are limited data available on the efficacy and safety of immunotherapy for patients with NSCLC brain metastasis. Approximately, 15% of patients participated in studies and all had stable BM or had been treated for BM, while patients with symptomatic BM were excluded from trials [16]. The available data were derived from single-arm phase I/II trials [141–143], pre-arranged analyses of phase III trials [143–145], and expanded access programs [146, 147]. In the phase I multi-cohort CheckMate 012 study of the tolerability and safety of nivolumab in patients with NSCLC with BM only twelve patients were included. Their median PFS and OS were 1.6 months and 8.0 months, respectively, and no more than two intracranial responses were observed [143].

In a phase 2 trial, the PD-1 blockade by pembrolizumab was studied in patients with advanced NSCLC with untreated brain metastases. Forty-two patients were treated with Pembrolizumab. The cohort with PD-L1 ≥1% 1, 29.7% of patients had a BM response,

while the patients with PD-L1 <1% did not show a response. The median OS and PFS of patients in cohort 1 was 9.9 months and 1.9 months, respectively¸ confirming that pembrolizumab activity in CNS metastasis is limited to NSCLC with higher PD-L1 expressions. Moreover, the PD-L1 expression was associated with long-term OS [142]. In two nivolumab EAP studies conducted in Italy and France, 409 and 130 patients respectively, were included with advanced NSCLC and asymptomatic and stable BM. Part of the patients received corticosteroids and the other part underwent concomitant brain radiotherapy. The OR was 17% in the Italian study and 12% in the French study; the OS was 8.6 and 6.6 months, respectively [146, 147]. In another pooled analysis of larger trials on pembrolizumab monotherapy (KEYNOTE 001, 010, 024, and 042) and pembrolizumab combined with chemotherapy (KEYNOTE 021, 189 and 407), the OS of patients who received pembrolizumab alone or with chemotherapy was better as compared to patients who received chemotherapy alone [144, 145].

## 7. Conclusions

Brain metastasis of NSCLC is most life-threatening for patients and the treatment is a major challenge. Traditional therapies do not eradicate cerebral cancer cells and recurrent disease is common. A significant obstacle in treating patients with brain metastases is the BBB, which prevents chemotherapeutic agents from entering the brain. Due to this obstacle and the failure of conventional therapies, novel therapeutic approaches are being explored. Despite recent advances in lung cancer treatment, a better understanding of the molecular mechanisms and pathways implicated in lung cancer is essential to identify appropriate targets to prevent brain metastasis. It is undeniable that many factors in the tumor microenvironment contribute to the outgrowth of tumor cells, not only at the primary site but also at the sites of seeding in distant organs. The formation of brain metastases is largely the result of tumor-microenvironment interactions. The brain micro-environment not only contributes to colonization by tumor cells but also affects the results of therapeutic interventions. Obviously, detailing the entire spectrum of genomic alterations and molecular mechanisms involved in lung cancer brain metastasis is important to develop effective treatments. Specifically scrutinizing the mechanisms by which cancer cells cross the BBB is important for establishing preventive brain metastases strategies.

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Section 4

# Palliative Therapy

Chapter 6

# Palliative Therapy of Bone Metastases

*Saman Dalvand*

## Abstract

This chapter overviews palliative treatment modalities for patients with bone metastases. In the introduction section, the origin of bone metastases and complication of metastatic patients have been discussed. Then, the main body explains treatment modalities including pain relievers, bisphosphonates, surgery, external beam radiotherapy, and targeted radionuclide therapy for pain palliation of patients with bone metastases.

**Keywords:** bone metastases, pain palliation, palliative therapy

## 1. Introduction

### 1.1 Bone metastasis

Bone is one of the most common sites of metastasis in cancer patients [1]. Bone tissue consists of living cells located in an extracellular matrix composed of minerals (**Figure 1**). This extracellular matrix is composed of organic matter, mainly type 1 collagen, and inorganic matter, including calcium and phosphate. Calcium and phosphate combine to form hydroxyapatite crystals in bone tissue [3]. Bone cells include three types of cells: osteoblasts, osteoclasts, and osteocytes. Osteoblasts, known as bone-forming cells, are located along the surface of the bone and play a role in bone formation. Osteoclasts, also known as bone-eating cells, contain multinucleated cells that are formed from hematopoietic stem cells under the influence of several factors and play the role of bone resorption. The location of these cells is also on the surface of the bone. Osteocytes contain 90–95% of bone cells derived from osteoblast cells that are surrounded by extracellular matrix and play a structural role [2, 4].

Bone metastasis occurs due to a complex pathophysiological process between cancer cells and bone cells that stimulates bone formation or resorption activity. Bone metastasis occurs in people with cancer that started outside the bone. In this case, the cancer cells are isolated from the original site and reach the peripheral areas mainly through venous blood flow, and if the conditions are provided for the growth and proliferation of these cells in the target tissue, they metastasize there [5].

Communication between tumor cells and hematopoietic stem cells is essential for the formation of bone metastases. Bone is the third most common tissue that hosts cancer cells from other tissues. Liver and lung metastases usually do not cause

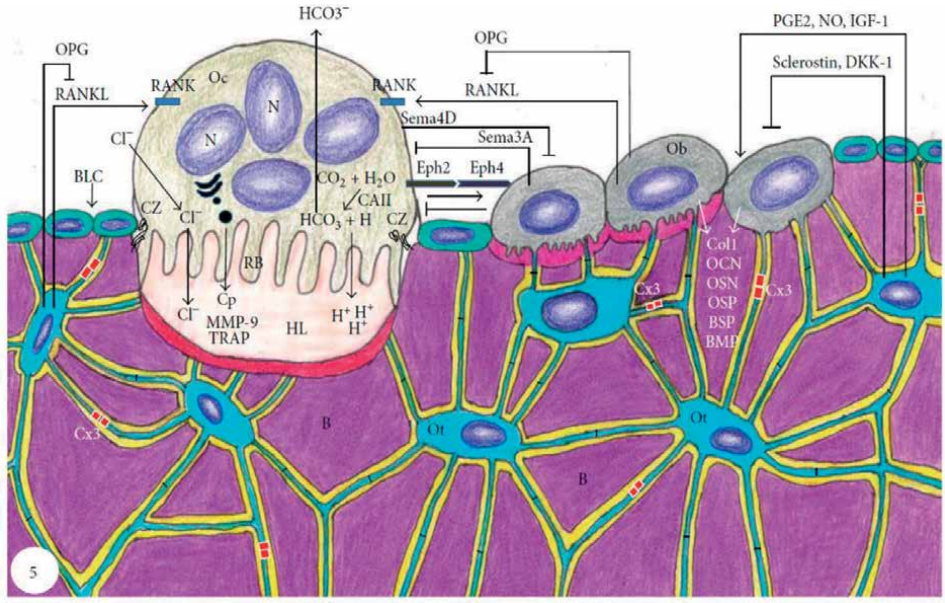


**Figure 1.**
*Cells located in the bone matrix (B): Osteoblast (Ob), osteoclast (Oc), and osteocyte (Ot) cells [2].*

symptoms until the patient is advanced. However, bone metastases in patients are very painful and are usually diagnosed earlier. The source of most bone metastases is breast, prostate, lung, thyroid, and kidney cancers [6–8].

The risk of bone metastasis varies in different cancers. For example, 70% of patients with breast and prostate cancer develop bone metastases, while the rate of bone metastases in patients with gastrointestinal cancer is between 3 and 15% [4]. Also, the most common sites of bone metastasis include the bones of the spine, pelvic, ribs, humerus, and femur [9].

### 1.2 Bone remodeling

Bone tissue is normally constantly renewed and replaced, meaning that osteoclasts absorb old bone and osteoblasts build new bone. This process is called bone remodeling. Normally, these two processes are in balance with each other. Any abnormality in the function of osteoblast and osteoclast cells can cause this balance to be lost, resulting in a change in the resorption or formation of bone by these cells. Bone metastasis is one of the factors that upset this balance. Bone metastases are divided into three types: osteoblastic, osteolytic, or a combination of both [10–12].

### 1.3 Osteoblastic and osteolytic metastases

The origin of various osteoblastic and osteolytic metastases is not yet well understood. Osteoblastic metastasis occurs when, under certain factors, the bone formation activity of osteoblast cells exceeds the bone-eating activity of osteoclast cells, and bone formation becomes greater than bone resorption. Osteolytic metastasis also occurs when the activity of osteoclasts exceeds that of osteoblasts and bone resorption becomes greater than bone formation [11, 12].

Most metastases are osteolytic. Patients with primary prostate cancer develop osteoblastic metastases. Also, most of the metastases that are the primary source of breast, thyroid, and kidney cancers are osteolytic. Although patients with breast cancer are more likely to develop osteolytic metastases, between 15% and 20% of patients develop osteoblastic metastases or a combination of both.

### 1.4 Complications of bone metastases

Patients with bone metastases are at risk for bone symptoms and complications such as severe pain, hypercalcemia, bone fractures, pressure, and damage to nerve structures such as the spinal cord [13, 14].

Many bone metastases are asymptomatic and are often discovered accidentally on initial examination or follow-up. In symptomatic cases, pain is the most common symptom. The quality of pain varies from point pain to shooting pain. Involvement or invasion, stretching, or pressure on pain-sensitive structures such as nerves, arteries, and small fractures can lead to pain. Pain due to bone metastases can also be due to mechanical instability in the weakened bone or high intraosseous pressure [15]. Although many factors can cause pain in bone metastases, the major part of the pain is related to bone resorption by osteoclast cells in osteolytic metastases. The pain often intensifies at night and during activity, but direct local invasion and fractures cause persistent pain. Pathological fractures are often seen in osteolytic metastases. Hypercalcemia occurs in 10% of patients and is more common in breast and lung cancers [16].

## 2. Palliative therapy of bone metastases

### 2.1 Treatment modalities of bone metastases

Because people with bone metastases live longer than other visceral metastases, finding the right treatment and dealing with the complications of bone metastases are very important in oncology. The goals of treatment for bone metastases include maximizing pain control, maintaining limb function, maintaining bone stability, and local control of the tumor. There are several treatments for bone metastasis and its associated effects, including the use of pain relievers, bisphosphonates, surgery, external beam radiotherapy, and targeted radionuclide therapy [13, 17].

### 2.2 Pain relievers

Treatment with pain relievers is the first line of treatment for bone metastases. Common medications for pain relief from bone metastases are based on World Health Organization (WHO) guidelines. If the pain is mild, the main treatment is non-steroidal anti-inflammatory drugs or acetaminophen. Tramadol can be used in the second line of treatment in cases of moderate pain, and in the next step, if the pain is not controlled, drugs can be used. Drugs relieve pain with short-term effects such as oxycodone and hydromorphone [18].

### 2.3 Surgery

Another treatment for bone metastases is surgery. Most people with bone metastases without bone fractures do not need surgery. If a pathological fracture occurs, the first

step in surgery is to fix the fracture site. Preventive surgery is mostly used in metastases with a high probability of fracture in tall bones that support the weight of the body. Vertebroplasty is another method of reducing pain for those patients with vertebral fractures that do not put pressure on the spinal cord but have severe pain [19].

The affected bone should be radiographed and scanned with radionuclides before surgery. Radiotherapy is used to treat every other metastatic lesion that may develop into a pathological fracture after these measures are taken. If methylmethacrylate is used to fix a plate or nail in the bone, a pathological fracture will be more difficult to treat than if there were no implant.

Usually, radiotherapy is the only modality likely to restore mobility and relieve pain in pathological fractures of long bones. For primary internal stabilization of long bones, radiotherapy is the treatment of choice. Even though radiotherapy might control local tumors, it is unlikely that a pathological fracture will heal without treatment. A large area of bone destruction could result in an insufficient matrix for adequate fracture healing, as radiotherapy inhibits chondrogenesis.

### 2.4 Bisphosphonates

Another treatment is the use of bisphosphonates. Bisphosphonates prevent bone destruction by causing apoptosis in osteoclast cells and inhibiting the activity of these cells in osteolytic metastases. In patients with bone metastases with increased blood calcium, the use of bisphosphonates with adequate hydration is standard treatment. Bisphosphonates are excreted by the kidneys and should not be used by kidney patients [20, 21]. The bisphosphonate zoledronic acid induces apoptosis of osteoclasts and reduces the risk of skeletal-related events. As a result of large randomized controlled trials, bisphosphonates have become the standard of care for treating and preventing skeletal complications associated with bone metastases in patients with solid tumors or multiple myeloma [22]. In these studies, the primary endpoint was how bone-targeted treatment affected the number of patients experiencing skeletal-related events (SREs), the rate of SREs, and the time before the first SRE.

Patients with malignant bone diseases can benefit significantly from early-generation bisphosphonates, such as sodium clodronate and disodium etidronate. The bisphosphonates are metabolized by osteoclasts into non-hydrolyzable, cytotoxic ATP analogs, which have the effect of directly inducing apoptosis and impair mitochondrial function.

Bisphosphonates containing nitrogen inhibit the enzyme farnesyl diphosphate synthase, in contrast to the early-generation bisphosphonates. As a result, osteoclasts cannot function properly and are less able to resorb bone. There are several nitrogen-containing bisphosphonates, including disodium pamidronate, alendronic acid, ibandronate sodium, risedronate sodium, and zoledronic acid. As a result of their introduction in clinical trials, these agents showed dramatic improvements in therapeutic activity [23, 24].

### 2.5 External radiotherapy

In relieving painful bone metastases, external beam radiotherapy is the most common palliative method used among oncology treatments and provides a very effective treatment to reduce the symptoms of local pain. Radiation therapy helps control pain by destroying tumor cells and eliminating existing inflammation, as well as increasing the ossification of osteolytic lesions. However, the most important disadvantage of

external radiotherapy is the exposure of healthy tissues to radiation and unnecessary absorbed dose in these tissues, which can lead to acute complications [25]. In terms of relieving bone pain, local irradiation is without doubt effective. Approximately 85% of patients report complete relief of pain, with half reporting complete relief. Within 1–2 weeks, more than half of responders experience pain relief. Improvement in pain is unlikely to occur if it hasn't been achieved by 6 weeks or more after treatment [26]. It is common to use single treatments or short fractionation schedules in Canada and Europe rather than prolonged fractionated treatments in the United States. Treatment techniques and doses have varied considerably throughout history, with prolonged fractionated treatments preferred in the United States. Fractionation schedules have been compared in a number of randomized trials. The pain relief offered by each approach was not superior. According to several studies, fractionated vs. single treatments do not differ in toxicity, pain response, analgesic consumption, adverse effects, or quality of life when compared with single treatments, including a large Dutch study of 1157 patients with painful bone metastases. 76 No statistically significant differences were found for pain response, analgesic consumption, treatment adverse effects, or quality of life [27].

After a meta-analysis comparing single fraction versus multiple fractions, it was revealed that both single fraction and multiple fractions achieved similar symptomatic responses; 1011 of 1391 (73%) receiving a single fraction and 958 of 1321 (73%) receiving multiple fractions. thus, for many patients suffering from painful bone lesions, single-fraction radiotherapy is a viable treatment option [28].

### 2.6 Targeted radionuclide therapy

Systemic therapy with appropriate radionuclide has been accepted as a common treatment modality for patients with various bone metastases [29]. In this treatment, radionuclides are combined with targeted agents with the aim of specific uptake into bone tissue. Radiopharmaceuticals have several advantages over local radiotherapy over topical radiation therapy: they can be administered intravenously, they can target very small metastases (micro-metastases), they can treat several separate affected areas at the same time, and they have fewer side effects, including they cause nausea, vomiting, diarrhea, and tissue damage [30]. However, in this type of treatment, the bone marrow as a critical organ receives a dose, and the absorbed dose of the bone marrow needs to be considered as a limiting factor in treatment [31, 32].

Beta-emitters were found to have a response rate of 70% in a systematic review that included 57 studies. Not only does radionuclide-targeted therapy alleviate pain, but it reduces or defers the incidence of skeletal-related events (SRE). Ionizing radiation is delivered to areas with increased osteoblast activity using these agents, which substitute calcium or bind to hydroxyapatite in bones. Radiation should be targeted at metastatic foci while sparing non-affected tissues to the maximum extent possible. In order to achieve the best results, many radiopharmaceuticals have been studied.

Physicians choose appropriate radiopharmaceuticals based on a number of factors, such as metastatic disease extent, renal function, bone marrow reserve, and availability.

### 2.7 Radionuclides used to relieve bone metastases

Common radionuclides for the treatment and relief of pain from diffuse bone metastases include Phosphorus-32, Strontium-89, Samarium-153, Rhenium-186,

Rhenium-188, Lutetium-177, and Radium-233. The first radionuclide used for this purpose was Phosphorus-32, which is currently rarely used due to the high energy and consequent high range of emitted beta-particles and the unacceptable absorbed dose in the bone marrow. Rhenium-186 has been approved in Europe, while Rhenium-188 and Luteinium-177 are still being studied as promising radionuclides [33, 34]. Currently, Cerium-141 is being studied as a new promising beta-emitter radionuclide [35, 36].

Radium-223 has also recently been approved by the Food and Drug Administration (FDA) as an alpha radionuclide for those patients with bone metastases without visceral metastases [37, 38]. Patients treated with Ra-223 survived 3.6 months longer and experienced reduced skeletal morbidity compared with those treated with a bisphosphonate. There were no significant side effects associated with Ra-223, which improved QOL. Ra-223 is now being investigated in both endocrine and cytotoxic combinations, after it was approved for use in late-stage disease [39, 40]. Today, Samarium-153 and Strontium-89 are the most widely used radionuclides for bone metastases that are approved by the Food and Drug Administration and have decades of clinical use [41, 42].

Radionuclides are routinely administered in clinical practice as monotherapies. However, radionuclides have been studied in conjunction with other therapies, specifically for prostate cancer. An early study reported an 11-month increase in survival rates if Strontium-89 was added to chemotherapy with doxorubicin [43]. A follow-up study combined beta-emitting bone agents with chemotherapy made in response to this encouraging result. Cancer patients who are castrate-resistant to chemotherapy may benefit from docetaxel because it relieves their pain and improves their quality of life [44]. Strontium-89 combined with docetaxel was found to be a safe combination for concomitant administration in a phase I study [45]. According to Fizazi et al. [46], Sm-153-EDTMP was studied in combination with docetaxel in a single-arm phase II trial. Comparing this study with reference data, the authors reported that the treatment was well tolerated and resulted in an improved overall survival rate. Monoclonal antibodies are used to treat osteoclastic diseases including denusomab. The combination of denusomab with Ra-223 was found to be more effective than Ra-223 alone in reducing symptoms of skeletal events [47].

### 2.8 New targeted agents for palliative therapy of bone metastases

A number of new targeted agents are being developed as we gain a deeper understanding of the signaling mechanisms between bone cells and tumor cells. The agents include cathepsin K inhibitors (an osteoclast-derived enzyme that is involved in bone resorption), an antibody against PTHrP, Src kinase inhibitors (a key molecule in osteoclastogenesis), and various anabolic agents. As time goes on, it will be possible to learn how these agents can be used to prevent and treat bone metastases.

## 3. Conclusions

Patients' quality of life is greatly affected by bone metastases. Therefore, prevention and treatment of skeletal metastases require new strategies. New therapeutic strategies can be expected as our understanding of bone metastases evolves. It is possible to further reduce the clinical burden of metastatic bone disease by combining bone-targeted therapies.

## Conflict of interest

The author declares no conflict of interest.

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*Edited by Yusuf Tutar and Lütfi Tutar*

Cancer mortality and morbidity are primarily brought on by metastasis. The ability of neoplastic cells to spread and colonize distant tissues is their most dangerous trait. Most malignancies are curable when they are detected early and have not spread outside of the original tissue. However, cancer is frequently incurable when tumor cells have created colonies elsewhere. Thus, the book content This book provides comprehensive information on cancer metastasis, along with references to specific cancer cases. The topics discussed include invasion by perturbation of cell-cell and cell-matrix adhesion, matrix degradation, motility, intravasation, extravasation, metastatic colonization, metastasis and angiogenesis. Molecular mechanisms, the metastatic process, and palliative bone metastasis therapy are also considered.

Published in London, UK


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ISBN 978-1-80356-163-9