Molecular Complexity of Lymphovascular Invasion: The Role of Cell Migration in Breast Cancer as a Prototype

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Short tittle: Molecular Complexity of LVI in Cancer: The Role of Cell Migration in BC as a Prototype

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ABSTRACT

Lymphovascular invasion (LVI) is associated with poor outcome in breast cancer (BC); however, its underlying mechanisms remain ill-defined. LVI in BC develops through complex molecular pathways involving not only the interplay with the surrounding microenvironment along with endothelial cells lining the lymphovascular spaces but also changes in the malignant epithelial cells with the acquisition of more invasion and migration abilities. In this review, we focus on the key features that enable tumour cell detachment from the primary niche, their migration and interaction with the surrounding microenvironment as well as the crosstalk with the vascular endothelial cells, which eventually lead to intravasation of tumour cells and LVI. Intravascular tumour cells emboli survival and migration, their distant site extravasation, stromal invasion and growth are part of the metastatic cascade. Cancer cell migration commences with loss of tumour cells' cohesion initiating the invasion and migration processes which are usually accompanied by the accumulation of specific cellular and molecular changes that enable tumour cells to overcome the blockades of the extracellular matrix, spread into surrounding tissues and interact with stromal cells and immune cells. Thereafter, tumour cells migrate further via interacting with lymphovascular endothelial cells to penetrate the vessel wall leading ultimately to intravasation of cancer cells. Exploring the potential factors influencing cell migration in LVI can help in understanding the underlying mechanisms of LVI to identify targeted therapy in BC.

INTRODUCTION

The outcome of breast cancer (BC) has significantly improved in recent years [1, 2]. However, approximately 30% of early stage BC patients develop distant metastasis within 10 years following surgical resection of the primary tumours [3]. Lymphovascular invasion (LVI), which is defined as the presence of malignant cells within vascular or lymphatic spaces, is the major prerequisite for cancer progression and distant metastasis development [4-6]. Although LVI is strongly associated with advanced large-sized tumours, it is also observed in small early stage BC and tumours with varying degrees of differentiation. There is a strong correlation between lymph node and distant metastasis and clinically detectable LVI in the primary BC [7]. This suggests that LVI may occur early in the process of carcinogenesis but the more common clinically detectable LVI, which is often observed in larger tumours, occurs late following the accumulation of molecular alterations more than those required for invasion of the ducto-lobular basement membrane. This observation supports the hypothesis that LVI could be targeted in BC despite the presence of stromal invasion. However, the challenges of targeting LVI in BC stem from being multifactorial i.e. it includes complex molecular mechanisms that drive LVI such as; tumour cell invasion and migration and the interplay between BC cells and various cells and structures in the surrounding tumour microenvironment including extracellular matrix, stromal cells, immune cells and endothelial cells. However, understanding the role of cancer cell invasion and migration in the development of LVI and differentiation of LVI-related molecular alterations from these driving tumour cell invasion and migration as an early mechanism associated with malignancy is a 'difficult' but 'beatable' challenge. Among various LVI-related pathways, this review focuses on the key features of cellular invasion, migration and their interaction with the surrounding microenvironment as principal factors leading to LVI.

The liberation of cancer cells from their parent tumours is a primary and essential event for daughter tumour cells to colonise and resettle in a secondary site [8]. It has been proposed that the detachment of cancer cells from the primary tumour in breast cancer may be initiated by several genetic modifications in a sub-population of cells which enable them acquiring migratory potential [9]. In addition, several factors have been reported to influence tumour cell detachment such as enzyme activity, the rate of tumour growth and stress on cell release [10]. Beside the changes in cancer cell traits toward loss of epithelial and gain of certain mesenchymal markers, release of extracellular matrix (ECM)-degrading enzymes including matrix metalloproteinases (MMPs) accelerates the release of cells from the primary tumour tissue and subsequent invasion into tumour-adjacent tissues such as epithelial cell stratums and ultimately lymphatic vessels [11].

Structurally, lymphatic vessels have exiguous cellular junctions between the lining endothelial cells (i.e. fenestrated) and are surrounded by occasional pericytes and lack basement membranes which typically surround capillary vessels. This fenestrated structure facilitates access of BC cells into lymphatic vessels [12]. Tumour cells release growth factors and angiogenic factors which play a crucial role in tumour progression, angiogenesis and LVI. Lymphatic endothelial cells, in turn, interact with tumour cells resulting in the secretion of small leucine-rich repeat proteoglycan, thus stimulating tumour cells to metastasise [13, 14]. However, the key molecular mechanisms underlying LVI, whether the alterations are in the tumour cells, in the endothelial cells or in both remain to be deciphered. Thus far, HER2 positive tumour cells have been found to be a promotor for invasion and migration in BC resulting in highly aggressive tumours with poor outcome [15, 16]. HER2 has been strongly associated with LVI via disrupting the architecture of tumour cells and encouraging their migratory activity and epithelial mesenchymal transition (EMT) to lose their adhesion [17, 18]. Although the current standard therapeutic option is anti-HER2

therapy such as Trastuzumab [15], Pertuzumab [19] or Lapatinib plus Capecitabine [16] have markedly improved the survival of HER2-positive breast cancer, the window is still open for further discovery of potential therapeutic targets such as those that can suppress LVI thereby preventing distant metastasis and improve patient outcome.

CANCER CELL MIGRATION FROM A PRIMARY SITE TOWARDS LYMPHOVASCULAR SYSTEM

Cancer cell migration is an early process in carcinogenesis and all invasive tumours exhibit migration ability. Cell migration is essential for the disease progression and can be considered the initial stage of both LVI and tumour metastasis. Studies by our group as well as others have shown the association between LVI and genes related to cancer cell migration in BC (Table 1) [20, 21]. Although these genes appear to play a role in cancer cell migration and invasion, their precise role in LVI as an additional biological event remains difficult to be summarised and the discrepancy of invasion/migration driving genes and those driving LVI remains blurred. Furthermore, the currently ongoing mechanistic studies including 3D culture and co-culture with endothelial cells which allow investigating the intricate cell-cell and cell-matrix interactions may further help identifying the candidate genes or pathways driving LVI in BC.

From a temporal standpoint, cell migration in BC is divided into three successive stages: 1) migration of the malignant epithelial cells (of the *in situ* disease) through breast duct basement membrane (invasion), 2) migration through tumour stroma and interstitium (local spread), and 3) migration through the lymphovascular wall (intravasation into lymphovascular spaces). With the success of the latter stage, tumour cells present within vascular spaces initiate the process of distant metastasis (Figure 1). As in a complex molecular mechanism, various factors, pathways and processes contribute in each step such

as EMT of the invading tumour cells that might undergo changes in tumour cell motility and the interaction between tumour cells and microenvironment.

During the stromal and ECM infiltration process, two distinct migratory patterns for tumour cells with potentially different molecular mechanisms have been documented: "single cancer cell migration" and "collective cancer cell migration" [22, 23].

Single Cell Migration

In single cancer cell migration, individual cancer cells detach from the primary tumour mass and lose epithelial polarity, acquire morphological characteristics of mesenchymal cells through the process of EMT [24]. The physical process involved in single cell migration occurs when cancer cells migrate towards the lymphovascular channels through protease-dependent mesenchymal migration or protease-independent amoeboid-like migration. In the former, cells migrate based on the protease activity that cleaves ECM and facilitates cell entry into the vessels. In protease-independent amoeboid-like migration, cancer cells initiate mechanical forces that lead to promotion of cell permeation allowing them to cross the ECM instead of cleaving it [23]. Protease dependent mesenchymal migration is said to lead to slower tumour cell migration compared to protease-independent amoeboid-like migration [25].

Collective Cancer Cell Migration

In collective cancer cell migration, malignant cells destined for migration retain cellular cohesion to one another and migrate as detached clusters or remain connected to the primary tumour [26]. Force generation is a major prerequisite for collective cell migration where tumour cells advance by means of new protrusions. Clustering cancer cells develop integrin-mediated focal adhesions and membrane protrusions, which bind to actin filaments and aid tumour migration. ECM degradation involves the creation of an invasion path by the leading cells in the infiltrating tumour border through β1 integrin-mediated focal attachment to ECM

components such as fibronectin [27]. In addition, cytoskeletal adaptor proteins, including talin, paxillin, cortactin and vinculin, play a role in cancer associated fibroblasts (CAF) by mediating intracellular singling pathways and communication with tumour cells that promote cancer metastasis [23, 28] .

Each of these migration patterns has specific characteristics and occurs through different biological mechanisms influenced by intrinsic vessel structures [22]. Collective cell migration is based only on the initial physical forces (protrusions) that are created by tumour cells themselves [22]. Furthermore, it usually executes vascular invasion through lymphatic spaces due to presence of several fenestrations between cellular junctions within the lymphatic vessel structure which facilities intravasation as compared to blood vessels [23]. On the other hand, single cell migration can utilise both biological (mesenchymal and/or ameoboid) and physical forces, and therefore can invade lymphatic vessels [23]. Vascular invasion assessment with collective cell migration has better performance due to the difficulty in identifying single tumour cell invasion [25]. This difficulty may be also attributed to the lower chance of single cells surviving as compared to collective cell migration, which produces a unique form of cortical actin filaments congregation around the cell junctions [23]. Proteins involved in the migration process are summarised in Table 2.

The Role of Cellular Protrusions in Tumour Cell Migration

Malignant cell protrusions, which consist of filopodia, lamellipodia and invadopodia, play a critical role in cancer cell migration via enhancing the physical forces leading to loss of adhesion between tumour cells (Figure 2). Tumour cell migration, which occurs as a result of re-modulation in cell-cell and cell-matrix adhesion, involves reorganisation of actin cytoskeleton and F-actin rich membrane protrusions at the tumour edge. Tumour protrusions are of two varieties namely, invasive and non-invasive protrusion. Non-invasive protrusion is fundamentally associated with protease-dependent mesenchymal migration such as in the

bespoke EMT [23]. The invasive protrusion is involved in invadopodia which plays important roles in collective cancer cell migration and LVI in BC via upregulation of podoplanin without activation of EMT as reported in a recent clinical study recently [23, 29].

Non-invasive protrusion involving lamellipodia and filopodia usually occur in association with single cell migration. Lamellipodia play an important role in cancer cell migration through loss of epithelial tissue cohesion with resultant tumour cell release from the primary tumour mass. Lamellipodia are produced by tumour cells where movement is initiated by forming new lamellipodia whilst promoting the loss of existing lamellipodia through forward contraction of the newly synthesised lamellipodia. This process helps tumour cells lose connection to the main tumour mass [30]. The focal adhesion adaptor proteins Scr and paxillin are used by non-invasive protrusion involved in lamellipodia and filopodia for ECM attachment via focal adhesion [31]. On the other hand, invadopodia affects the adhesion of cancer cells to the ECM which is crucial for invasion of lymphatic vessels, and the invasion of tumour cells to the vascular system [32]. Formation of invadopodia has been observed in various cancer cell lines including BC [27]. This structure is formed when the degradation of ECM and cell adhesion junctions occur concomitantly. An invadopodium is composed of three main parts: proteolytic, invasive and adhesive domains. The proteolytic domain largely contains proteases such as serine proteases e.g. A Disintegrin and Metalloproteinase (ADAM) and MMPs [23]. The invasive domain is located inside the connection between invadopodial protrusion and ECM and is responsible for the regulation of actin as well as actin-linked proteins including mammalian Ena (MENA) and cortactin [33]. MENA is involved in invadopodium maturation and migration via the regulation of cortactin dephosphorylation [33]. Protein interactions occurring inside the invasive domain cause polymerisation of actin filaments leading to generation of mechanical forces which drive

the cell towards the lymphatic vessels. Lastly, the adhesive domain is present at the tumour periphery connected to the ECM and functioned by integrin-mediated adhesion [23].

FACTORS ATTRACTING TUMOUR CELLS TOWARD THE LYMPHOVASCULAR SYSTEM

Before discussing the interplay between tumour cells and ECM detachment from the primary mass, some factors that may direct or attract the tumour cells towards the endothelial cells whether in lymphatic are considered. It has also been reported that the dysfunctional lymphatic network leads to elevation of the interstitial fluid pressure which in turn would be responsible for the directional locomotion of the migratory tumour cells toward the lymphatics at the tumour periphery [34]. By this mechanism, the tumour cells would be presented nearby the lymphatic system and passively increasing the opportunity of their intravasation into lymphatic channels. Nevertheless, attracting malignant cells towards both types of capillaries can also occur by active molecular mechanisms [35]. For example, vascular endothelial growth factor receptor-3 (VEGFR3) is solely expressed on lymphatic endothelial cells, and by binding to its ligand vascular endothelial growth factor (VEGF)-C, which is highly expressed in tumour cells, it may act as an attracting way which can also facilitate the tumour cells to leak into the vessels [35]. Previous reports have suggested that ECM also plays an important role in the process of cancer cell migration. MMPs are produced by stromal cells and are crucial for the progression of tumour cells owing to their capacity for ECM degradation remodelling and activation of matrix molecules and cytokines [36]. For instance, MMPs degrade matrix proteins and guide the tumour cells to lymphatic vessels causing generation of chemoattractant mitogen-like hepatocyte growth factor (HGF), which in turn activates MMP2 and MMP9 [37]. Tumour cells are attracted by HGF along a gradient, which leads to rearrangements in the actin cytoskeleton via Rho and Ras like GTPases. These GTPases subsequently regulate the assembly and organisation of protrusions made from actin

cytoskeleton [38]. The role of HGF in this directionality has been also verified *in vitro*, where tumour cells and macrophages, when seeded together, show a random bi-directional migration pattern [39]. However, sustained directionality of tumour cells akin to what observed *in vivo* was retrieved *in vitro* when human umbilical vein endothelial cells were added to the culture within the same assay. These endothelial cells secrete the HGF required for the chemotactic gradient responsible for sustained directionality. However, when inhibiting the HGF signalling pathway between endothelial cells and tumour cells, the directional streaming of tumour cells become defective again [39].

INTERACTION WITH THE SURROUNDING MICROENVIRONMENT

Although the initial molecular alterations that lead to invasion and metastasis begin in transformed BC cells, these processes cannot be completed without sustenance from and interaction with cells in the microenvironment which can either promote or suppress tumour cell migration and invasion. Subsequently, BC cells interact with various types of stromal and immune cells in their vicinity such as stromal mesenchymal cells, fibroblasts, myofibroblasts, adipocytes, immune inflammatory cells, endothelial cells and pericytes lining lymphovascular spaces for intravasation [22, 40, 41].

The interplay between tumour cells and tumour microenvironment is complex and the effect on cancer cell migration and invasion is mutual; being driven by both tumour cells and various components of tumour microenvironment. Many molecules secreted by non-malignant cells in the microenvironment could influence tumour cell behaviour and promote tumour migration specifically toward lymphatic vessels. These molecules include C-C chemokine receptor-7 dependent paracrine effect and macrophages-derived Vascular Endothelial Growth Factor-C (VEGF-C) acting through autocrine mechanisms [42].

The Role of Myoepithelial Cells and Fibroblasts

Typically, myoepithelial cells are considered natural tumour suppressors in breast tissue through their action as tumour gatekeepers preventing cancer cell proliferation, survival, invasion, migration and metastatic spread [43]. However, following interaction with BC cells which are capacitated for invasion, these stromal and immune cells become modified and execute divergent function which promote BC cell migration and invasion. For instance, evidence indicates that CAFs ; modified stromal fibroblasts/myofibroblasts [44], promote tumour growth, migration and invasion via enhancing Wnt/β-catenin signalling, with subsequent activation of MMPs which degrade the vessel walls, thereby, facilitate BC cells' migration towards vessels [45]. CAFs also express podoplanin and VEGF-C, which affect intratumoral micro-vessels, regulate cancer cell migration, influence malignant, inflammatory and endothelial cells and play pivotal roles in tumourigenesis and invasion [29]. In LVI, podoplanin positivity is more frequent than CD31 or CD34 positivity and is associated with the development of nodal metastasis and poor clinical outcome [29, 46]. CAFs are also a major source of N-cadherin in BC, thus affecting the tumour cells' migration by prompting them to leave the primary site and start the migration process [47]. Furthermore, stromal fibroblast transformation into CAFs could be promoted through the loss of interleukin (IL)-6 hence, promoting BC cell migration and invasion [48].

The Role of Immune Cells and Inflammatory Mediators

Inflammatory cells populating the tumour microenvironment play a critical role during LVI by enhancing cancer cell proliferation, migration, and invasion [49]. In invasive cancer microenvironment, tumour-associated angiogenesis and lymphangiogenesis tend to produce an interconnecting vascular organisation of lymphatics and blood vessels which facilitates tumour interaction with other cell types such as lymphoid, haematopoietic and mesenchymal cells, resulting in remodelling of the ECM [50, 51]. During tumour progression, malignant

cells produce certain chemokines and cytokines that are mitogenic and/or chemoattractant for immune cells [50]. Infiltrating inflammatory cells, in turn, are activated to produce certain chemokines, cytokines and proteolytic enzymes which prompt tumour migration, invasion and survival, and also stimulate endothelial cells for neoangiogenesis and lymphangiogenesis [50, 51]. There is an increasing interest in the role of immune cells and tumour infiltrating lymphocytes (TILs) and their interaction with cancer cells to control tumour behaviour and response to therapy. Manipulation of this interaction is a fast growing field and several immune checkpoint inhibitors have been described in several solid cancers including BC which show highly promising results for therapy [52, 53]. There is sufficient evidence to demonstrate that moderate and intense inflammation is associated with improved prognosis in triple negative (TN) and HER2-positive BC [54-56]. TILs comprise several types of immune cells including tumour promoting and tumour suppressing cells. Cytotoxic T-cells (CD8+ T-cells) and natural killer T cells attack cancer cells and are considered to be tumour suppressor cells, whereas regulatory T-cells (CD4+, and FOXP3+ T-cells) inhibit immune responses to cancer cells and therefore can be considered as cancer-promoting cells. Conversely, CD4+ T lymphocytes are able to promote invasion via activation of the epidermal growth factor receptor (EFGR) signalling pathway [57]. Moreover, a CD4+ T-cells fraction (Tregs) can inhibit anti-tumour immune response by suppressing effector T-cells and is responsible for producing several members of immunosuppressive cytokines family, including transforming growth factor-β and IL-10 [58]. Therefore, CD4+ T-cells enhance the migration of tumour cells and facilitate LVI by inhibiting the anti-tumour response through significant chemokine production.

Previous studies have demonstrated an association between LVI and tumour immune cell infiltration. For instance, LVI-positive squamous cell carcinoma of the tongue showed less CD4+ and CD8+ T-cells, and lower CD4+/FOXP3+ T-cell and CD8+/FOXP3+ T-cell ratios,

indicating that tumour progression is associated with a shift towards a more immunosuppressive environment [59]. CD8+ T-cells induced by pre-existing tumour–antigen, are downregulated by PD-1/ PD-L1 interactions [60]. Inflammatory factors are also involved in tumour migration through the release of pro-migratory factors. For instance, IL-1β plays an important role in BC migration, adhesion to lymphatic endothelial cells and transmigration by activating unique chemokines and cytokines using the IL-1R [61]. IL-17A also promotes BC cell migration and invasion by activating certain intracellular signalling pathways [62].

Macrophages have been shown to promote tumour progression in BC [63]. Tissue macrophages play a pivotal role in tumour angiogenesis through their inherent characteristics such as flexibility and mobility in enhancing endothelial tip-cell anastomosis and by regulating excessive vessel sprouting. Thus they are suited to stimulate endothelial cells in different vessel segments to initiate contacts with other microenvironment molecules [64, 65]. Macrophages have been described to have both oncogenic and tumour suppressing functions. Once CD8+ T cells interact with tumour cell antigens, they produce interferon gamma that is responsible to perform a tumouricidal activity via inducing macrophage tumour killing activity and/or antigens [66], thereby suppressing LVI by decreasing their lymphatic microvascular density by producing extracellular vesicles which breaks down neighbouring mesenchymal tumour stroma [66, 67]. Contrasting this, macrophages also play a key role in promoting migration and invasion of tumour cells through production of epidermal growth factor which activates migration and invasiveness of malignant mammary epithelial cells [68]. Evidence from recent preclinical research studies has demonstrated that tumour-associated macrophages (TAMs) are one of the principal factors controlling tumour progression in many tumour types including BC [51]. TAMs are reported as having the greatest migratory capacity amongst haematopoietic cells [69]. TAMs have M2 macrophage phenotype (alternatively activated macrophages) in BC

which requires CD4$^+$ T-cell derived IL-4 for activate ion [70]. Once activated, TAMs can enhance angiogenesis via IL-1, MMP2 and VEGFs [64]. These proteases and growth factors released by macrophages have the ability to instigate tumourigenesis and promote tumour progression [71]. Moreover, in BC stroma, TAM-derived cytokines can stimulate tumour invasiveness by enhancing their adhesion to the ECM [72]. VEGF-C expression is strongly associated with TAM expression in BC and is strongly correlated with developing lymphovascular tumour density and LVI [73]. Macrophage migration inhibitory factor (MIF), a pleiotropic inflammatory cytokine, promotes the migration of BC cells via controlling CD74 expression and hypoxia-inducible factor-1 (HIF-1) [74]. Zhang et al, have indicated that MIF can regulate the level of VEGF-C in BC cells through activation of the MAP-kinase signalling pathway [75]. This activation increases the invasive capacity of cancer cells by promoting TNF-α, leading to increased protease levels, and allowing tumour cells' access into lymphatic vessels [76].

The Role of Hypoxic Microenvironment on LVI

Hypoxia is also associated with cancer cell migration. HIF-1α drives tumour cell migration into a favourite 'less hypoxic' environment. This promotes tumour progression by enhancing tumour cell survival, invasion, anaerobic metabolism and angiogenesis [77]. VEGF, which is strongly associated with angiogenesis, is also regulated by HIF-1α [78]. Further, Schoppmann *et al* have suggested that HIF-1α is strongly associated with BC LVI together with VEGF-C/D and their receptor VEGFR-3 [77].

CELLULAR ADHESION IN LVI

Loss of tumour cell-cell adhesion is an essential step and early event in tumour cell migration, stromal invasion and intravasation. Most tumour cells lose their adhesion to the primary tumour cell mass and migrate by undergoing EMT. Conversely, several mechanisms including EMT and mesenchymal epithelial transformation (MET) are involved in tumour cell adhesion to

lymphatic and or vascular endothelial cells [79]. MET is reversed EMT involving upregulation of E-cadherin and downregulation of N-cadherin. N-cadherin regulates the expression of vascular endothelial (VE)-cadherin in aggressive BC and hence promote tumour progression [79]. Several changes including upregulation of E-cadherin are responsible for N-cadherin paralysis that leads to downregulation of several mesenchymal markers and changes in several morphological features thus facilitating tumour cells' adhesion to endothelial cells (due to reversal EMT to MET) [79]. Alternatively, VE-cadherin paralysis, which is a cadherin subtype produced by endothelial cells as a consequence of N-cadherin downregulation, affects epithelial markers and endothelial cell-cell junctions without influencing E-cadherin or MET process. Moreover, VE-cadherin is located on the cell membrane due to the disruption of VE-cadherin clusters which activates VE-ectodomain encouraging its clustering at intracellular contracts and segregation of stable and mature junction [80]. Therefore, these effects lead to actin polymerisation or actomyosin inactivation resulting in changing the formation of inter-epithelial adherens junctions. According to this evidence, the cytoskeleton regulates VE-cadherin clustering and VE-cadherin regulates cytoskeletal organisation and barrier stability alternately [80]. Thus, VE-cadherin can assist tumour cells to adhere to lymphatic vessels via loss of lymphatic permeability control and through its effect on cytoskeletal integrity.

The differences between E-cadherin, N-cadherin and VE-cadherin can be ascribed due to the different localisation of β-catenin. N-cadherin and VE-cadherin are expressed in the nucleus and cytoplasm respectively, while E-cadherin is expressed on the cell membrane. Therefore, E-cadherin levels are elevated in the presence of reduced N-cadherin expression leading to the repression of EMT regulators and transforming the invasive mesenchymal phenotype to an epithelial phenotype prompting tumour cells to adhere to lymphatic vessels and establish LVI as revealed in a recent clinical study [79].

Vimentin is related to the adhesion and migration properties of tumour cells [81]. Gilles et al have observed the accumulation of nuclear and cytoplasmic vimentin and β-catenin have a dual functional role in tumour cell migration and invasion by promoting a β-catenin/T-cell factor pathway in BC [82]. Moreover, vimentin expression is transactivated as a result of the interaction between β-catenin and T-cell factor (TCF)/ lymphoid enhancer factor-1 (LEF-1) transcription factor family. In mammary carcinoma cell lines where β-catenin is found on the plasma membrane, vimentin is downregulated while its level remains unchanged in the cytoplasmic and nuclear allocation of β-catenin [82]. Therefore, β-catenin acts as an EMT/MET regulator during cell migration. Liu et al have demonstrated that vimentin participates in the focal adhesion and cytoskeleton organisation and also regulates cancer cells mechanical homeostasis [83]. Hence, vimentin contributes to an aggressive tumour phenotype via LVI, because of its ability to enhance motility, change the configuration of cancer cells and downregulate tumour cell adhesiveness [84].

TUMOUR CELL INTRAVASATION

Intravasation is the process whereby malignant cells cross the endothelial barrier and invade lymphatics across the basement membrane [26]. The rate of tumour cell intravasation is highly influenced by several factors involved in tumour cell invasion and migration and the interplay with the surrounding structures as described above [26]. Interaction between tumour microenvironment and tumour cells at the intravasation site can activate the reciprocal secretion of EGF. EGF executes its action through EGF receptor to promote tumour cell migration and invasion into the lymphatic channels via transactivation of EGFR signalling by EP4 receptor pathway which is involved in invadopodia formation [85]. Some authors have suggested that tumour cell intravasation plays a significant role in BC metastasis via attachment of endothelial cells to fibroblast-derived CXCL12 [86] where TGFβ augments intravasation by amplified penetration of microvessel walls [87]. Type IV collagenase, which can actively

digest the ECM and basement membrane, enables malignant epithelial cells to migrate through the interstitial tissue and into lymphatic vessels facilitating the development of LVI [88]. Collection of BC cells can directly penetrate lymphatic vessels through the action of Cyclooxygenase (COX) 12 or COX15, which metabolises arachidonic acid to 12(S)-hydroxy-eicosatetraenoic acid (12(S)-HETE). Exposure to 12(S)-HETE transiently reduces their VE-cadherin expression resulting in the migration of these cells [89]. *In vivo* BC murine models have shown that the transcription factor Twist enhances the process of intravasation, acting as an EMT-inducing transcription factor and promoting the rate of haematogenous intravasation and bone metastasis [90]. Despite the documented importance of tumour intravasation into lymphatic/vascular spaces, the driving mechanisms controlling the process are still incompletely characterised and therefore warrants further studies. VE-cadherin regulates VEGFs and affects cancer cell migration through regulating actin-driven junction- associated intermittent lamellipodia [91]. Certain actions of VEGFs activate the C-C motif chemokine ligand 21 (CCL21), which interacts to C-C motif chemokine receptor 7 (CCR7) that is located on the vascular endothelial surface. The secretion of CCL21 is strongly affected by the upstream signalling of its receptor VEGFR3 and VEGF-C secretion by malignant cells [42]. *Kim et al* suggested that the Let-7a targeting of CCL21/CCR7 singling is a promising approach to prevent BC migration and invasion [92].

VEGFs are also involved in tumour cell migration as regulators of BC proliferation enhancing and tumour cell escape from apoptotic signals. This characteristic makes VEGFs a potential target candidate in BC patients in the neoadjuvant setting [93]. Arai et al suggested that VEGFs also play an essential role in Trastuzumab therapy by minimising the allocation of tumour cells to lymphatic vessels in HER2-positive BC [94].

Podoplanin, which is expressed by lymphatic endothelial cells, induces the formation of a glycoprotein and is used as a specific lymphatic endothelial marker [95]. Podoplanin

encourages migration, proliferation rate and invasiveness of malignant cells via increasing the RhoA activity [96]. Neri et al have shown that tumours with high expression of podoplanin have higher local migration and invasion than those with low podoplanin [97], therefore, suggesting podoplanin as a potential therapeutic target through preventing migration, using a podoplanin monoclonal antibody (NZ-1) and lectin (MASL) inhibition [98]. Knockdown studies with podoplanin siRNA have resulted in decreased cell proliferation and cell motility. Previous research study has shown that podoplanin could be a potential therapeutic target to prevent cancer cell migration via synthesis of phosphatase modular that may prevent cancer cell migration [99].

THE ROLE OF CANCER STEM CELL IN LVI

Cancer stem cells (CSCs) play an important role in BC progression by regulating tumourigenesis and resistance to therapeutic regimens. CSCs are high malleability with reciprocal directions between stem and non-stem (differentiation) states [100, 101] with activation of the classical mesenchymal phenotype [102] and enhancement of the protrusive activation, which is a known migration hallmark, compared to differentiated cells [101]. Therefore, CSCs seem to control the alterations of the focal adhesion signaling dynamics that allow them to perform a rapid switching between migration/phenotype modes and that contributes in tumour cell movement through fibrous matrices and hence, initiate a symbiotic environment that increases metastatic potency [101]. Indeed, EMT plays a critical process underlying the role CSCs in the ability of cancerous cells to migrate, cause LVI and metastasise [103]. CSCs in *in situ* breast carcinomas can transform into migrating cancer stem cells (MCS), which have the ability to disseminate and form metastatic colonies, by EMT [104]. It has been demonstrated that some CSC biomarkers such as ALDH1, CD44, and CD133 promote lymphatic metastasis of cancer cells [105, 106]. A recent *in vivo* study demonstrated that CSCs play a key role in BC tumour progression by creating a permissive environment for the

collective cancer migration and invasion processes via controlling E-cadherin and N-cadherin expression, and guide the invasion and metastasis [107]. It has also been demonstrated that in hypoxic conditions, CSCs can indirectly enhance angiogenesis and lymphangiogenesis by expression of angiogenetic and lymphangiogenic factors such as VEGFR2. VEGFR2 also alters the morphology of CSCs to elongated endothelial-like cells [101]. Another *in vitro* BC study [108] has indicated that CSCs expressed endothelial markers CD31, VEGFR2 and FVIII when cultured with the presence of VEGF. This data provides further support to the role of CSCs in the development of LVI.

KEY LVI-RELATED BIOMARKERS

There have been several studies attempting to identify target genes that play an essential role in LVI. Our group has recently published a bioinformatics study using RNA expression data to identify differentially expressed genes in LVI-positive and LVI-negative cases. Following a stringent approach, 99 genes were demonstrated as significantly associated with BC LVI whether being upregulated or downregulated [109].

In their study, Klahan et al [90] identified 86 differentially expressed genes with LVI in BC, including 37 downregulated and 49 upregulated genes. Among these genes, EPAS1 which stimulates the production of VEGF, and TNFSF11 which is a receptor activator of nuclear factor kappa-B ligand (RANKL); both enhanced mammary cell invasion, migration, and metastasis. Other genes included TNFSF11 and IL6ST that play important roles in cytokine-receptor interaction, which is the most enriched pathway related to LVI.

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is associated with a plethora of singling pathways fundamental to cell cancer migration, proliferation, death and tumour survival [110]. According to Golubovskaya et al, high FAK levels correlated strongly with aggressive BC subtypes such as TNBC as well as with LVI, hence FAK might be a target for

therapeutic intervention in these patients [111]. Using pre-clinical mouse models of BC, a significant reduction in tumour growth was achieved by an anti-proliferative activity using a chemical inhibitor of FAK (BI 853520). Moreover, *in-vitro* and *in-vivo* suppression of FAK signalling in combination with chemotherapies improved chemo-cytotoxicity [112]. McCall et al showed that astaxanthin (ASX), a potent antioxidant, could play a critical role in preventing LVI by inhibiting tumour cell proliferation and migration through promoting apoptosis in BC cells compared with normal breast epithelial cells. ASX inhibits migration in the aggressive TN BC cell line [113] by decreasing the inflammatory mediators within the tumour microenvironment such as TNF-α, IL-6 and IFN-γ through NFκβ inhibition, therefore, rendering ASX a potential target to prevent LVI in BC [113]. Blocking migration would have a significant positive impact on patient outcome. Inhibiting the early events of cell migration from primary tumours into the circulatory or lymphatic systems will help reduce distant metastasis. Forkhead transcription factor family (FOXO) which are tumour suppressor regulating cell proliferation and promoting apoptosis in BC. Recent *in vitro* studies have demonstrated that expression of FOXO6 promotes BC migration and invasion by aiding EMT [114]. Elevated FOXO6 in BCs could be, at least in part, due to reduced activity of the PI3K/AKT pathway. Normal adult tissues exhibit decreased FOXO6 expression levels in contrast to BC which showed increased expression, thus it may be a potential candidate for BC therapy [115]. Others have indicated that FOXO3a is strongly associated with adverse clinicopathological parameters including EMT and poor patient outcome in TNBC, and plays an essential role in enhancing tumour cell migration via TGFβ1 triggered hepatocyte growth factor (HGF)-induced and MET-dependent migration *in vitro* [116]. The subcellular localisation of FOXO3a is important where phosphorylation of FOXO3a leads to its translocation from the nucleus to the cytoplasm [117]. For instance, in T-cell acute lymphoblastic leukaemia (T-ALL), FOXO3 is an indirect target of BMS-345541, selective

IKK inhibitor. FOXO3a was reported to act as an EGFR inhibitor, and also increases chemosensitivity of cancer cells to lapatinib [118]. Consequently, FOXO6 and FOXO3a could be potential therapeutic targets to prevent LVI in BC.

The current proteomic, transcriptomic and genetic evolution is not enough to draw a precise molecular roadmap for LVI. Although this difficulty may reflect the complexity of the whole process, this review has highlighted some important biological players at the molecular level to further assist deciphering the mechanistic overlap between the cancer cell migration and the surrounding microenvironment in BC LVI.

FUTURE DIRECTIONS AND CONCLUSION

Investigating a large cohort early-stage metastasis free (both node metastasis and distant metastasis) BC that contains subgroups of LVI negative and LVI extensive tumours using multiplatform high throuhput molecular techniques looking at differentiational expressed genes and pathways could help eliminating the impact of metastasis on the candidate gene lists. Although LVI is considered an essential prerequisite for tumour metastasis, there are multiple LVI associated genes/proteins that do not generate metastasis. HER2-positive BC has been investigated in playing a significant role in LVI by enhancing the tumour microenvironment to support the tumour cell growth, stimulating invasion LVI and metastasis [119]. There are several pathways related to cancer cell migration which are essential to the prevention of metastasis, such as P120 Catenin, FAK, RhoA, Paxillin and P130CAS pathways. Furthermore, other pathways which affect cancer cell angiogenesis (STAT3 pathway), survival (PI3K pathway) and proliferation (RAS pathway) might be candidates for preventing BC LVI/metastasis. An in depth understanding of these pathways will facilitate the exploration of those genes responsible for LVI in BC, thus could be helpful pinpointing the target genes for therapeutic strategy and thus preventing BC metastasis.

In conclusion, invasion and migration are important phenomena required for the process of LVI, are highly complex and overlapping, therefore, further mechanistic evaluation is necessary to explore the inter-relationship of these two processes in BC. This review has summarised the key factors associated with BC cell invasion and migration and the interaction with the microenvironment. We propose that further exploration of the candidate genes/proteins will be a tool to interrogate the metastatic cascade in BC and encourage a better understanding of LVI molecular mechanisms. Focusing on the migratory capacity and cancer cells spreading could be useful for tailoring the treatment regimens for aggressive BC subtypes.

Figure Legends:

Figure 1: A schematic diagram for breast cancer cell migration process.

The diagram shows the single and collective migration processes. In single migration process, individual tumour cells are detached and migrate through the surrounding environment and then invade the lymphovascular channels. This process in controlled mainly through epithelial mechanical transition process (EMT). In collective migration/invasion process; the tumour grows in nests and sheets that protrude through the surrounding tumour stroma and invade the blood channels in adherent tumour nests. EMT and mesenchymal epithelial transition (MET) factors play major roles in this process. Various tumour microenvironmental factors such as cancer associated fibroblasts (CAFs), podoplanin, tumour infiltrating lymphocytes (TILs), tumour associated macrophages (TAMs) and hypoxia inducible factor (HIF). Other endothelial related factors such as CXCL12, epidermal growth factors (EGFs), tumour growth factor beta (TGF-β), and COX15 are elevated during the process of intravasation.

Figure 2: A schematic diagram illustrating types of cellular protrusions

Filopodia and lamellipodia are non-invasive protrusions mechanisms which contribute in single cell migration by changing tumour cell cytoskeleton via initiating cell-physical forces resulting in loss of tumour cell-epithelial tissue adhesion. Invadopodia are invasive protrusions associated with cluster cell migration.

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