Molecular Mechanisms Underlying Lymphovascular Invasion in Invasive Breast Cancer

Mohammed A. Aleskandarany Sultan N. Sonbul Abhik Mukherjee
Emad A. Rakha

Division of Cancer and Stem Cells, School of Medicine, The University of Nottingham, and Nottingham City Hospital, Nottingham University Hospitals NHS Trust, Nottingham, UK

Key Words

Breast carcinoma · Epigenetics · Epithelial-mesenchymal transition · Extracellular matrix · Invasion · Lymphangiogenesis · Lymphovascular invasion · Migration · Tumorigenesis

Abstract

Lymphovascular invasion (LVI), the presence of malignant cells within lymphovascular channels, is a crucial step in the invasion-metastasis cascade. LVI, when identified morphologically in the peritumoural area, is regarded as an indicator of metastatic potential and is strongly associated with a poor prognosis in many solid tumours, including breast cancer (BC). Although molecular mechanisms associated with the development of LVI have been extensively studied, details of driver genes, and molecular pathways and mechanisms involved in its development in BC, remain poorly defined. Although invasive BC cells have the ability to invade surrounding stroma, only those that can interact with endothelial cells, penetrate the vascular wall and withstand the intravascular stress will develop LVI and complete metastatic dissemination. Identification of additional molecular events associated with LVI in the primary tumour and characterisation of the contribution of the tumour micro-environment to

modulating biological processes leading to LVI in BC remain a challenging task. This stems not only from the complexity of the molecular alterations in the primary tumour and the interactions with different components of its micro-environment but also from the subjective nature of LVI assessment in human BC. In this review, we discuss the clinicopathological features and the current knowledge of the molecular mechanisms underlying LVI in BC.

© 2015 S. Karger AG, Basel

Introduction

Breast cancer (BC) is recognised as a heterogeneous disease with varied presentation, morphology, behaviour and response to therapy. Although a minority of BC patients (<10%) present with metastatic disease, 20–30% of patients presenting with early-stage disease will develop distant metastases over time [1, 2]. Distant metastases, which are considered as a prognostically poor event in cancer, develop through coordinated and highly selective biological processes involving several related mechanisms, including cell proliferation, cell-matrix interactions, stromal inva-

M.A.A. and S.N.S. contributed equally to this work.

sion, cellular migration, lymphovascular invasion (LVI) and evasion of host immune responses [3–5].

LVI denotes the presence of tumour cells within lymphatic spaces, blood vessels, or both, at the peritumoural area and is identified morphologically by microscopic examination of the primary tumour with or without endothelial-specific markers. Detection of LVI in the primary tumour is a marker of metastatic potential. The prognostic value of LVI has been demonstrated by several independent studies [6–14]. In a previous study of 3,812 cases of BC [7], it has been demonstrated that LVI is not only an independent prognostic variable in the whole series but also in the various prognostic subgroups, including the lymph node-negative cohort. In this subgroup, LVI could be used as a high-risk criterion conferring survival disadvantage equivalent to that provided by involvement of one or two lymph nodes and to that provided by one higher size category (pT1 to pT2) [7]. At the molecular level, there are several biochemical and biophysical interactions that cancer cells utilise to facilitate their metastatic progression through vascular and lymphatic channels [4, 15–17]. Exploration of these signalling pathways would be crucial to find novel molecules controlling critical steps of LVI and could be relevant therapeutic candidates. The hallmarks of cancer have been extensively elaborated, and they all pointed to the dominant intrinsic and extrinsic qualities of the tumour cells that govern their genetic regulation [18]. Elevated activity of protein expression, resistance to apoptotic signals, increased proliferation rate, adhesion, invasion and cellular motility leading ultimately to cellular migration are some characteristics of malignant cells [19]. Although these criteria are not directly related to the occurrence of LVI and can be identified in LVI-negative and LVI-positive cancers, they are critically required traits for its development, and the molecular mechanisms controlling these processes must remain active, at least in some cellular clones, throughout the carcinogenic pathway from initiation to dissemination through vascular channels. In this review, clinicopathological features of LVI in BC and molecular alterations characteristics of invasion and LVI are discussed.

Morphological Assessment of LVI

The morphological assessment of LVI has been traditionally performed in the peritumoural areas. This is based predominantly on the morphological ease of detecting LVI outside tumorous tissue, avoiding the controversy regarding the existence of intratumoural lymphat-

ics, presence of tumour retraction artefacts, the prognostic significance of peritumoural rather than intratumoural LVI and the rarity of pure intratumoural LVI [20–22]. In our own laboratory, we have also observed LVI both in peritumoural and intratumoural vessels [22]. In LVI-positive cases, three quarters had peritumoural LVI and almost one quarter had intratumoural LVI. Of the intratumoural LVI-positive cases, only a handful (~4%) had LVI in the intratumoural compartment alone. Such small numbers makes conclusions regarding the significance of intratumoural LVI tenuous. This leaves us with the conundrum whether intratumoural LVI is important or not. Probably in cases where there is intratumoural LVI alone but with positive lymph node status, the intratumoural LVI may explain the metastatic cascade. However, when such node involvement is not apparent, care should be taken before disregarding the value of intratumoural LVI. The pragmatic approach would be to acknowledge that the tumour has found its mechanisms of spreading into the vasculature and is therefore worth clinical vigil.

There are several lines of evidence to indicate the occurrence of LVI in breast tumours lacking morphological evidence of LVI such as the demonstration of circulating tumour cells [23], bone marrow micrometastases [24] and lymph node positivity. This may indirectly influence our ability to understand the molecular mechanisms driving LVI in BC since identification of metastatic potential in cases lacking LVI will be challenging. In addition, there may be a possibility that molecular alterations identified in LVI-positive tumours when compared to LVI-negative cases are a reflection of quantity and not quality of the biological processes. In other words, LVI in BC may comprise a biological continuum akin to other morphological surrogates of tumour biology, such as tumour grade; at one end of the spectrum are cases lacking LVI and at the other end cases with extensive LVI (fig. 1). This, in addition to the complexity of the molecular mechanisms underlying LVI and their overlap with the metastatic cascade of invasion and the lack of suitable models to investigate BC cell intravasation, complicates the identification of driver molecules, pathways and potential therapeutic targets associated with LVI in BC.

Tumour Histological Features and LVI

Certain tumour morphological features are associated with LVI, including tumour type, grade and size. One of the characteristic features of inflammatory BC (IBC) is

Fig. 1. A case of invasive breast carcinoma of no special type with extensive LVI showing tumour cell emboli within vascular and lymphatic spaces in the peritumoural area. H&E.

extensive LVI, particularly the involvement of dermal lymphatics [25]. This extensive lymphatic spread of IBC is associated with high lymphatic endothelial cell proliferation [26]. It was reported that tumour lymphovascular emboli in an IBC human xenograft mouse model form cohesive spheroids that resembled embryonal blastocyst [27]. Cells in these spheroids co-express E-cadherin along with the stem cell phenotype $CD44^{+}/CD24^{-/low}$ and were able to generate tumour nodules exhibiting florid LVI in vivo with subsequent development of metastases irrespective of their site of injection and molecular subtype [27]. Interestingly, different authorities reported the co-operative role of E-cadherin overexpression and marked reduction in sialyl-Lewis X/A antigen by the intravascular emboli, rendering these emboli both coherent to each other yet poorly adherent to the E-selectin of the vascular endothelium. Accordingly, the tumour cell-endothelial cell aversion contributes towards the passive propagation of IBC spheroids in metastatic dissemination [28, 29]. Invasive micropapillary carcinoma is another rare subtype of BC characterised by frequent development of LVI. Gruel et al. [30] have recently identified, through exome and RNA sequencing of invasive micropapillary carcinoma, somatic mutations in genes playing key roles in cell polarity (*FMN2* and *SEC63*), ciliogenesis (*DNAH9*) and

cytoskeletal organisation (*ZFYVE26*) [30]. It is also important to note that invasive micropapillary carcinomas show a high frequency of LVI regardless of the proportion of micropapillary components in the invasive tumour [31] suggesting an intrinsic biological feature of this tumour type that promotes the development of LVI. Contrasting this, invasive lobular carcinoma (ILC) is known to show a low frequency of LVI, even when LVI was detected using lymphatic endothelial-specific biomarkers [32], despite having equal frequency of nodal involvement and a tendency to metastasise to more nodes than grade-matched invasive ductal carcinoma [33]. The disproportionate balance between the metastatic rate and the frequency of morphologically detected LVI in ILC could be explained by the discohesive single-cell infiltration pattern of ILC with the inherited loss of E-cadherin resulting in intratumoural or peritumoural intravasation of single small uniform cells rather than the easily identifiable cohesive clusters of invasive ductal carcinoma cells. In the rare pleomorphic variant of ILC, LVI is more detectable, although it shares the same molecular alterations with the classical ILC [34, 35]. Benign papillary lesions of the breast are a unique type of benign breast tumours which may show LVI. This phenomenon is likely to be related to their inherent friability resulting in epithelial

intravascular displacement following needle instrumentation procedures rather than biological features associated with metastatic potential [31].

There is a direct correlation between LVI and tumour grade and primary tumour size. For instance, LVI is rarely seen in grade 1 tumours while up to one half of grade 3 tumours are associated with LVI [7, 12–14]. Similar associations were reported with tumour size [36]. Although infrequent, detection of LVI in small BC is an independent predictor of node metastases and a poor outcome [11, 36], which may reflect the aggressive nature of these tumours. There is also a correlation between LVI and BC molecular subtype. Triple-negative or basal-like molecular classes showed the least association with LVI despite their poor prognosis and higher tendency to local recurrence [10]. Contrasting this, luminal and HER2-positive classes showed the highest incidence of LVI. One explanation attributed this difference to the lower expression of claudin tight junction protein and the higher expression of proteins that are crucial to transform the malignant epithelial cell to the mesenchymal form in triple-negative and basal-like tumours compared with luminal and HER2 BC classes [37, 38]. LVI is more frequent in symptomatic as compared to screen-detected BC and in young premenopausal BC patients [7, 13]. Therefore, studies of the molecular determinants of LVI should endorse the appropriate choice of cohorts as well as the control samples for more reliable identification of LVI-controlling genes.

Vascular or Lymphatic Invasion

BC can disseminate through blood vessels or lymph vessels; however, current evidence indicates that lymph vessels rather than blood vessels play the major role in the initial dissemination of BC, which may be related to the intrinsic tropism of the malignant cells towards lymphatic channels [14, 22]. Structural differences between lymphatics and blood vessels expectedly determine the initial route of spread, and, in this regard, lymphatic capillaries lack the tight junctions typically present between endothelial cells lining blood capillaries. Moreover, they lack the surrounding layers of pericytes, myocytes and basement membranes [39]. Accordingly, this renders lymphatics leakier than blood vessels, and, therefore, easing tumour cell intravasation. In other words, peritumoural lymphatic vessels represent the primary route for the intravasation pathway for BC cells incapable of overcoming the physical restrictions of vascular endothelial cells [40].

It has also been suggested that the interstitial fluid pressure is responsible for the directional motility of the metastatic malignant cells toward the lymphatics at the tumour periphery, thus passively increasing the chance of their intravasation into lymphatic channels [41]. Nevertheless, active molecular mechanisms attracting malignant cells more towards either type of vessel is another likely explanation [42]. Expression of lymphangiogenic factors may influence the route of metastatic spread through the lymphatic vessels due to the specific molecular profile of the latter [43, 44]. For instance, vascular endothelial growth factor receptor-3 (*VEGFR3*) is exclusively expressed on lymphatic endothelial cells, and its ligand vascular endothelial growth factor (VEGF)C, which is overexpressed by malignant cells, influences the process of lymphangiogenesis. However, some studies did not find significant associations between VEGFC and LVI or node metastasis, but this may be attributed to the probable role of multiple factors besides VEGFC and VEGFD and the heterogeneity of the study samples [45].

Regardless of the nature of the involved vessels, whether blood or lymphatic, native or newly formed, it is imperative to make it clear that neither angiogenesis nor lymphangiogenesis is equivalent to LVI despite their strong associations. Angiogenesis is the development of neovasculature to sustain tumour growth and promote invasion, whereas LVI is the process where tumour emboli lodge into vascular/lymphatic channels early in the metastatic cascade. Although the presence of the vasculature is necessary to provide the fertile ground for LVI, it does not equate to LVI itself.

Tumour Cell Invasiveness, Tumour Microenvironment and LVI

Before BC cells get access into the vascular lumina, they have to detach themselves from the growing tumour cell mass, followed by invasion and migration through the extracellular matrix (ECM) towards the vascular walls [46]. These steps are, therefore, considered prerequisites for the LVI process. Malignant cells infiltrate the surrounding frontline of ECM as single cells or as clusters of malignant cells. BC cells on the tumour boundaries up-regulate genes like *SNAIL*, *SLUG* and *ZEB1*, and other transcriptional factors that accelerate potential transformation of the malignant epithelial cells to the mesenchymal-like phase through repression of E-cadherin and other cell-cell adhesion molecules, which, hence, acquire more motility [47].

The tumour micro-environment controls the mechanobiological interactions between malignant cells and the components of the stroma [48]. The interaction, through genetic and epigenetic regulations, between malignant cells and the stromal components of the tumour micro-environment has been reported to alter adhesion and promote invasion and migration of malignant cells through the adjacent ECM with subsequent increase in their penetration into vascular channels [18, 49]. Stromal cells assist in proteolytic degradation via matrix metalloproteinase (MMP) dictated by the epigenetic regulations from malignant cells. Strong correlations have been reported between LVI and MMP9 [50, 51] and MMP1 expression [52]. Overexpression of cancer cell-associated membrane type-1 (MT1)-MMP by BC cells has been reported to induce degradation of the vascular basement membrane and cancer cell intravasation [53]. Perentes et al. [54] have demonstrated that MT1-MMP expression in cancer cells induces its expression in vascular endothelial cells, and leads to vascular basement membrane remodeling and blood vessel invasion but not lymphatic vessel invasion. They suggested that MT1-MMP should be explored as a predictor and therapeutic target of haematogenous metastasis in triple-negative BC patients [54].

Podoplanin, a glycoprotein that is encoded by the *PDPN* gene, is expressed in lymphatic endothelium. Together with other markers such as LYVE-1 and Prox-1, podoplanin is considered as a specific lymphatic endothelial marker. However, podoplanin is also expressed by cancer-associated fibroblasts (CAFs), epithelial cells and endothelial cells, is known to boost cellular motility and reduces cellular adhesion. Some studies have reported an association between podoplanin expression by CAFs in BC and aggressive features including LVI, node metastasis and poor outcome [55]. Podoplanin expression in CAFs is also correlated with BC cell VEGFC expression and intratumoural microvessel count [56]. Interestingly, high podoplanin expression at the invasive front of BC plays an alternative role in invasion and metastasis by forming invasive protrusions and filopodia without activation of endothelial-mesenchymal transition [55]. This phenomenon could be considered as a potent synergism between metastasis-related paracrine signalling from CAFs and epigenetics from malignant cells to facilitate lymphatic vessel invasion.

Probably one of the best examples illustrating the concert played by malignant cells, vascular endothelium and dendritic cells in LVI is the C-C chemokine receptor 7 (CCR7), a lymphatic-homing chemokine receptor. It has been reported that CCR7 and VEGFC expression by tu-

mour cells acts synergistically to promote LVI [57]. The transcellular lymphatic drainage that is caused by the elevated interstitial flow of lymphatic fluids may also polarise malignant cells toward the nearest lymphatic vessel through the autologous secretion of CCR7 [58]. The ligand of CCR7 (CCL21) is located on the vascular endothelial surface. Under the influence of VEGFC secreted by malignant cells and the upstream signalling of its receptor VEGFR3, the expression of CCL21 by vascular endothelium is significantly elevated. Therefore, CCR7+ malignant cells will actively migrate toward lymphatic vessels and the chances of LVI increase significantly. In other words, this illustrates how the malignant cells can intensify and utilise the chemokinetics of lymphangiogenesis to direct themselves to the preferential invasion target.

Although the presence of macrophages in primary tumours is associated with increased metastatic potential and development of LVI, the mechanistic basis for this observation remains unknown. Wyckoff et al. [59] have reported that the interaction between tumour cells and macrophages facilitates the migration of carcinoma cells. Epidermal growth factor (EGF) and/or colony-stimulating factor 1 (CSF-1) expression was able to stimulate collection of tumour cells and macrophages into microneedles whilst inhibition of either EGF or CSF-1-stimulated signalling reduces the migration of both cell types. Overexpression of EGF receptor by mammary tumour cells renders cells chemotactic to EGF released by macrophages lining blood vessels [59]. This work provides evidence for a synergistic interaction between macrophages and tumour cells during cell migration in vivo and could explain, at least in part, the increased tendency of basal-like BCs, which overexpress EGF receptor, to be more node negative and show haematogenous rather than lymphatic dissemination to viscera and bone [60, 61]. Macrophages are well known to produce interleukin-10 and angiogenic factors [62]. Within the hypoxic environment, growth factors that promote angiogenesis and lymphangiogenesis are up-regulated and secreted by malignant cells [63] and serve as chemo-attractants for macrophages. Robinson et al. [64] have demonstrated an association between perivascular macrophages and malignant cell intravasation. Recruited macrophages promote invasion and metastasis through a dual-direction reaction loop of paracrine signalling with malignant epithelial cells. Therefore, intravasation into vascular channels and migration through the lumen of vessels is facilitated [65]. Accordingly, the use of immunomodulatory drug therapy may interfere with tumour-induced immuno-

suppression mechanisms and may provide useful adjuncts to non-specific cytotoxic drugs [66].

Vasculogenic mimicry or vascular mimicry (VM) was so termed to emphasise the de novo generation of microvascular channels without participation by endothelial cells independent of angiogenesis. VM was initially demonstrated in vitro in highly aggressive and primary and metastatic melanoma cells [67]. The generation of these highly patterned microvascular channels by genetically deregulated aggressive tumour cells occurs in the absence of endothelial cells and fibroblasts [68]. It was subsequently identified in xenograft IBC model and cell lines [69, 70]. The presence of VM in tumour tissues has been reported to be associated with a poor clinical outcome and node metastases of patients [71]. This poor prognostic impact of VM was attributed to the possible advantage of survival of aggressive tumour cells, and the potential facilitation for metastatic tumour cells to get access into the bloodstream and subsequently distant organs [72]. Diverse molecular biomarkers have been reported to be overexpressed in VM BC cells, including VEGF, COX2, TWIST2 and molecules conferring the cancer stem cell phenotype and those undergoing endothelial-mesenchymal transition [73].

Gene Expression Profiling, Gene Signatures and LVI

Identification of genes differentially expressed between LVI-positive and -negative BC may help identify key genes and pathways driving LVI. With the advancement in high-throughput molecular and bioinformatic techniques, it has been possible to investigate genes associated with LVI at a large scale [74]. One of the main problems in the identification of LVI-associated genes in genome-wide gene profiling studies was the nature of the cohorts investigated as described above and the use of archival frozen tissue with inaccurate or incomplete data on LVI. In a previous study of endometrial carcinoma, 18 genes were identified to be associated with LVI (LVI gene signature) [75] and subsequently applied to BC utilising 11 open access gene expression data sets comprising data on more than 2,400 BC patients [76]. This 18-gene signature was found to be significantly associated with morphologically detected LVI and other poor prognostic features in BC, including high grade, advanced stage, hormone receptor negativity, HER2 positivity and basal-like phenotype [76]. Candidate genes included 7 up-regulated and 11 down-regulated genes, both spanning a diverse array of genes controlling ECM

collagens, ECM proteolysis, interleukins, angiopoietin, tumour necrosis factor and fibroblast growth factor receptors, for example (table 1). Although this gene signature is associated with LVI in BC, it appears to reflect features characteristic of tumour cell aggressiveness and interactions with the surrounding micro-environment in general rather than specific molecular mechanisms driving the process of LVI. Identification of genes using such an approach is limited by the number of genes included in the predefined gene set that was identified in a different tumour type (endometrial carcinoma). Another study [77] investigated 214 hepatocellular carcinomas to develop the gene signature that predicts the presence of LVI in patients with surgically treated hepatocellular carcinoma. Interestingly, they identified 14 genes up-regulated and 21 genes down-regulated with LVI, but none of them overlap with the 18-gene signature described above by Mannelqvist et al. [76], though this could be due to different cancer types. Some of the genes, e.g. *CD24*, *CDKN3*, *UBE2C* and *PIK3R1*, play a role in BC development and progression [77]. In a recent study, Fidalgo et al. [78] applied array-CGH, RT-qPCR and cDNA micro-arrays to identify DNA copy number aberrations and the gene expression profile linked to LVI in a cohort of invasive BC. Although they did not find a significant association between LVI and increased genomic instability considering the total number of chromosomal alterations, they identified an increased frequency of specific alterations associated with LVI, including gains at 5p, 16p, 17q12 and 19, and losses at 8p, 11q, 18q and 21. They also detected three novel small-scale rearrangements in LVI-positive grade 3 tumours that harbour putative BC genes, including amplicons at 11p11.2 (harbouring *HSD17B12*), 20q13.2 (harbouring *ZNF217*), 19q12 (harbouring *CCNE1*) and 4q13.3 (harbouring *ADAMTS3*), and a deletion at 12p12.3 (harbouring *RERGL* and *PIK3C2G*). Gene expression analysis revealed networks highlighting *MMP1*, *MED1* and *S100A8* as potential candidate genes involved in LVI-positive grade 3 tumours [78]. Genes that showed a concordant pattern of up-regulated gene expression and copy number gains/high gains in LVI-positive samples were *UMOD*, *ARSG*, *MYCBPAP* and *MED1* [78]. Despite the promising results of this study, it is important to note that the cohort of BC was small ($n = 57$), and frozen tissue was utilized with no further validation of LVI status. The cohort included a high proportion of LVI-positive cases (44%) with more lymph node-positive (65%) and oestrogen receptor (ER)-negative (42%) cases, which may influence the interpretation of the final results. Although data

Table 1. Genes/proteins associated with LVI in invasive BC

Ref. No.	Gene/protein	Association with LVI	Type of study	Patient numbers
76	Up-regulated <i>MMP3; TNFAIP6; FPR2; IL8; ANGPTL4; SERPINE1; COL8A1</i>	Positive	This signature was originally identified in endometrial carcinomas using carcinomas by gene micro-array and qPCR analysis	Analysis of 11 open access gene expression data sets for BC collectively holding information on 2,423 BC patients
	Down-regulated <i>OGN; ATCAY; MAMDC2; COL4A6; C1orf114; KLHL13; OSR2; ALDH1A2; SEMA5A; FGFR2; ITIH5</i>	Negative		
52	Up-regulated <i>S100A8, MMP1; MCM10; IMUP; LGALS7B; MED1;</i> Down-regulated <i>RERG</i>	Positive association with LVI in grade 3 BC	Array CGH and gene expression array	Array CGH (n = 57) Gene expression micro-array (n = 32)
27, 28	CD44 ⁺ /CD24 ^{-/low} phenotype E-cadherin overexpression Reduction of sialyl-Lewis X/A	Positive	In vivo xenograft IBC model and in vivo assay	
86	Calpastatin	Negative	Gene expression array, PCR and validated on IHC	91 cases
95	Podoplanin	Positive	IHC; RT-PCR	117 full-face sections
50	MMP9 protein expression	Positive	Gene expression analysis of IHC	300 prepared as TMA
54	<i>MT-1-MMP</i>	Positive	In vitro work on transfected human MDA-MB-231 and MDA-MB-435S cell lines and in vivo invasion assay, mouse model for in vivo metastasis assay, and IHC of frozen mouse tumour tissues	
89	ER, PR expression	Negative	IHC	123 full-face FFPE sections
88	pERK1/2	Inverse association in ER+ subgroup only	IHC	1,300 prepared as TMA
90	KU70/KU80	Positive	IHC	1,302 prepared as TMA
91	<i>PIAS4</i>	Positive	IHC	1,494 prepared as TMA
	<i>PIAS1; UBC9</i>	Negative		

PR = Progesterone receptor; TMA = Tissue micro-array; FFPE = formalin fixed paraffin embedded.

about the role of microRNA (miRNA) in BC is conflicting, some authors have demonstrated an association between LVI and levels of some miRNAs such as miR-9-3 [79], miRNA-205 [80] and miR-10b [81].

Applying bioinformatic analytical techniques to identify target genes to a well-selected and empowered BC cohort stratified based on strict histopathological criteria for LVI may be a promising approach. Only cas-

es with definite LVI should be considered, and, as a control, the LVI-negative group includes cases with no morphological or immunohistochemical (IHC) evidence of LVI or biological evidence of LVI such as circulating tumour cells or positive lymph nodes ideally in discovery and validation sets. Candidate genes should be analysed based not only on their differential expression but also on their biological function, their functional interac-

tions and specific molecular pathway involvements. Correlation with gene copy number alterations may help to identify driver genes. This approach is likely to narrow down the number of potential genes that can be investigated in detail using *in vivo* models and *in vitro* functional assays.

Proteomics and LVI

Correlations of proteins to LVI have been mostly indirect or incidental till date. In an IHC-based analysis [82] of 177 paraffin-embedded BC sections stained with VEGFA, VEGFC, VEGFD, podoplanin and CD34, increased expression of VEGFA and VEGFC, but not VEGFD, was associated with significantly higher lymph vessel and microvessel density, nodal and distant metastases, and a shorter survival. This provides indirect evidence that high VEGFA and VEGFC augur a poor prognosis by angiogenesis and lymphangiogenesis, and are related to LVI. In another IHC-based study [83] on 77 patients, VEGFC and VEGFD expression in BC was significantly correlated with peritumoural lymph vessel density, which was significantly correlated with LVI and nodal metastases. Other authors [84] have built a more holistic picture in that with VEGFC and COX2 IHC on 70 BCs, elevated VEGFC and COX2 expression were not only associated with higher lymph vessel density, but also D2-40-positive LVI. Hence, in spite of other contrasting reports [45], there is enough indirect and direct evidence of the importance of the VEGF pathways in the early metastatic cascade and indirectly to LVI. Autocrine functions of VEGF on tumour cells include adhesion, survival, migration and invasion [85], and deconstruction of the pathway and its interacting members will further aid in understanding the LVI biology.

However, some studies have attempted to discover novel proteins associated with LVI taking leads from gene analysis [86]. The ANN (Artificial Neural Network) approach to supervise a gene expression array of 91 BC patients identified 89 transcripts associated with LVI. Low expression of one transcript, calpastatin, an inhibitor of the calpain proteases, was correlated with intratumoural LVI on PCR and validated on IHC platforms. Calpain-1 and calpain-2, which are involved in tumorigenesis and the proteolysis of substrates, such as inhibitors of nuclear factor- κ B (I κ B) and focal adhesion proteins, on the other hand, were not correlated with LVI [87]. Thus, the inhibitor of calpains, calpastatin, is the key regulator of LVI within this pathway.

Other protein correlates of LVI have been incidental findings while investigating the roles of novel proteins in BC in general. For example, in a study investigating members of the mitogen-activated protein kinases in BC [88], pERK1/2 was associated with a negative LVI status. This correlation was also true for the ER-positive subgroup in this series. Traditionally, LVI has been shown to have an inverse relationship to ER and progesterone receptor expression [89], and molecules such as pERK1/2 may provide the clues to the underlying biology. Investigation into DNA repair proteins in BC has also revealed correlations with LVI. For example, high KU70/KU80 expression correlated with a positive LVI status [90]. Members of the SUMO (small ubiquitin-like modifier protein) family have shown correlations with LVI; positive LVI was associated with loss of nuclear expression but positive cytoplasmic expression of PIAS1 and UBC9, and with the expression of PIAS4 [91]. This exemplifies the importance of the SUMO pathways in LVI. On the other hand, PARP1 [poly(ADP-ribose)polymerase-1], a key facilitator of DNA repair, is not related to LVI in either cleaved or non-cleaved forms [92] nor is the CHK1 (checkpoint kinase-1), a key member of the DNA damage-activated checkpoint signalling responses [93]. The aforementioned findings, though incidental, provide a picture of the DNA response pathways in relation to the LVI-positive phenotype. As more and more proteins are investigated in BC in relation to invasion, migration, apoptosis and proliferation, more relationships with LVI will be unearthed.

The Unified Plot of LVI

The different key mechanisms underlying LVI collaborate progressively under the dictation of tumour genetic, transcriptomic and proteomic profiles, and the resulting biological interactions are the guarantor of cancer cells to invade vascular spaces and metastasise. Initially, ECM is the field of the vibrant interactions between malignant cells and stromal non-tumoural cells in the event of LVI, and when malignant cells induce significant molecular modifications in the ECM, they will secure their progressive pathways of invasion and, hence, metastasis. Those remodelling actions are the synthesis, alignment, cross-linking and proteolysis of the ECM, and malignant cells will select a combination of remodelling actions to set up the ECM micro-environment to their optimum conditions to invade the targeted vessels and disseminate to distal sites of the body [94]. Fibroblasts, macrophages and

other immune cells, and endothelial cells are perceived as LVI mediators due to their different contributions of LVI mechanisms. Angiogenesis and lymphangiogenesis are crucial biological processes that are governed by several chemokines and cytokines in the tumour micro-environment. Hypoxia, the growing tumour mass and high interstitial fluid pressure are reasons that the formation of new lymphatic/blood vessels is not only needed to keep the accessibility to the nourishment supplies on and to replace damaged intratumoural vessels, but also increase contact surface areas and subsequently the chances of LVI.

Conclusions

Several molecular factors governing the occurrence of LVI have been unravelled, although other major contributors/players still await discovery. Questions around molecular mechanisms driving BC tumour cells to invade vascular spaces and to disseminate remain largely ambiguous. Therefore, large-scale genomic and transcriptomic profiling of histologically validated LVI in BC may potentially yield a high throughput of genes and pathways within which LVI master regulators could exist. The candidate genes composing these signatures and pathways could be further scrutinised using different research methodologies to identify novel therapeutic targets.

References

- 1 Early Breast Cancer Trialists' Collaborative Group (EBCTCG): Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; 365:1687–1717.
- 2 Kennecke H, Yerushalmi R, Woods R, et al: Metastatic behavior of breast cancer subtypes. *J Clin Oncol* 2010;28:3271–3277.
- 3 Sun Y, Wang X, Zhou Q, et al: Inhibitory effect of emodin on migration, invasion and metastasis of human breast cancer MDA-MB-231 cells in vitro and in vivo. *Oncol Rep* 2015;33:338–346.
- 4 Carmeliet P, Jain RK: Angiogenesis in cancer and other diseases. *Nature* 2000;407:249–257.
- 5 Bidard FC, Pierga JY, Vincent-Salomon A, Poupon MF: A 'class action' against the microenvironment: do cancer cells cooperate in metastasis? *Cancer Metastasis Rev* 2008;27: 5–10.
- 6 Debled M, de Mascarel I, Brouste V, Mauriac L, MacGrogan G: Re: Population-based study of peritumoral lymphovascular invasion and outcome among patients with operable breast cancer. *J Natl Cancer Inst* 2010;102:275–276, author reply 276–277.
- 7 Rakha EA, Martin S, Lee AH, et al: The prognostic significance of lymphovascular invasion in invasive breast carcinoma. *Cancer* 2012;118:3670–3680.
- 8 Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG: Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat Rev Cancer* 2014;14:159–172.
- 9 Lee AK, DeLellis RA, Silverman ML, Heatley GJ, Wolfe HJ: Prognostic significance of peritumoral lymphatic and blood vessel invasion in node-negative carcinoma of the breast. *J Clin Oncol* 1990;8:1457–1465.
- 10 Ugras S, Stempel M, Patil S, Morrow M: Estrogen receptor, progesterone receptor, and HER2 status predict lymphovascular invasion and lymph node involvement. *Ann Surg Oncol* 2014;21:3780–3786.
- 11 Choi SH, Barsky SH, Chang HR: Clinicopathologic analysis of sentinel lymph node mapping in early breast cancer. *Breast J* 2003;9:153–162.
- 12 Song YJ, Shin SH, Cho JS, Park MH, Yoon JH, Jegal YJ: The role of lymphovascular invasion as a prognostic factor in patients with lymph node-positive operable invasive breast cancer. *J Breast Cancer* 2011;14:198–203.
- 13 Truong PT, Yong CM, Abnoui F, et al: Lymphovascular invasion is associated with reduced locoregional control and survival in women with node-negative breast cancer treated with mastectomy and systemic therapy. *J Am Coll Surg* 2005;200:912–921.
- 14 Lauria R, Perrone F, Carlomagno C, et al: The prognostic value of lymphatic and blood vessel invasion in operable breast cancer. *Cancer* 1995;76:1772–1778.
- 15 Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG: Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat Rev Cancer* 2014;14:159–172.
- 16 Michor F, Liphardt J, Ferrari M, Widom J: What does physics have to do with cancer? *Nat Rev Cancer* 2011;11:657–670.
- 17 Suresh S: Biomechanics and biophysics of cancer cells. *Acta Biomater* 2007;3:413–438.
- 18 Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000;100:57–70.
- 19 Bray K, Gillette M, Young J, et al: Cdc42 overexpression induces hyperbranching in the developing mammary gland by enhancing cell migration. *Breast Cancer Res* 2013;15:R91.
- 20 Nathanson SD, Kwon D, Kapke A, Alford SH, Chitale D: The role of lymph node metastasis in the systemic dissemination of breast cancer. *Ann Surg Oncol* 2009;16:3396–3405.
- 21 Kandemir NO, Barut F, Bektas S, Ozdamar SO: Can lymphatic vascular density be used in determining metastatic spreading potential of tumor in invasive ductal carcinomas? *Pathol Oncol Res* 2012;18:253–262.
- 22 Mohammed RA, Martin SG, Mahmood AM, et al: Objective assessment of lymphatic and blood vascular invasion in lymph node-negative breast carcinoma: findings from a large case series with long-term follow-up. *J Pathol* 2011;223:358–365.
- 23 Rack B, Schindlbeck C, Juckstock J, et al: Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. *J Natl Cancer Inst* 2014;106:dju066.
- 24 Braun S, Vogl FD, Naume B, et al: A pooled analysis of bone marrow micrometastasis in breast cancer. *N Engl J Med* 2005;353:793–802.
- 25 Robertson FM, Bondy M, Yang W, Yamauchi H, et al: Inflammatory breast cancer: the disease, the biology, the treatment. *CA Cancer J Clin* 2010;60:351–375.
- 26 Van der Auwera I, Van den Eynden GG, Colpaert CG, et al: Tumor lymphangiogenesis in inflammatory breast carcinoma: a histomorphometric study. *Clin Cancer Res* 2005;11: 7637–7642.
- 27 Xiao Y, Ye Y, Yearsley K, Jones S, Barsky SH: The lymphovascular embolus of inflammatory breast cancer expresses a stem cell-like phenotype. *Am J Pathol* 2008;173:561–574.
- 28 Alpaugh ML, Tomlinson JS, Kasraeian S, Barsky SH: Cooperative role of E-cadherin and sialyl-Lewis X/A-deficient MUC1 in the passive dissemination of tumor emboli in inflammatory breast carcinoma. *Oncogene* 2002;21: 3631–3643.
- 29 Tomlinson JS, Alpaugh ML, Barsky SH: An intact overexpressed E-cadherin/alpha,beta-catenin axis characterizes the lymphovascular emboli of inflammatory breast carcinoma. *Cancer Res* 2001;61:5231–5241.

- 30 Gruel N, Benhamo V, Bhalshankar J, et al: Polarity gene alterations in pure invasive micropapillary carcinomas of the breast. *Breast Cancer Res* 2014;16:R46.
- 31 Ueng SH, Mezzetti T, Tavassoli FA: Papillary neoplasms of the breast: a review. *Arch Pathol Lab Med* 2009;133:893–907.
- 32 Laser J, Cangiarella J, Singh B, et al: Invasive lobular carcinoma of the breast: role of endothelial lymphatic marker D2-40. *Ann Clin Lab Sci* 2008;38:99–104.
- 33 Fernandez B, Paish EC, Green AR, et al: Lymph-node metastases in invasive lobular carcinoma are different from those in ductal carcinoma of the breast. *J Clin Pathol* 2011;64:995–1000.
- 34 Bentz JS, Yassa N, Clayton F: Pleomorphic lobular carcinoma of the breast: clinicopathologic features of 12 cases. *Mod Pathol* 1998;11:814–822.
- 35 Buchanan CL, Flynn LW, Murray MP, et al: Is pleomorphic lobular carcinoma really a distinct clinical entity? *J Surg Oncol* 2008;98:314–317.
- 36 Guarneri A, Neri A, Correale PP, et al: Prediction of lymph node status by analysis of prognostic factors and possible indications for elective axillary dissection in T1 breast cancers. *Eur J Surg* 2001;167:255–259.
- 37 Sabatier R, Finetti P, Guille A, et al: Claudin-low breast cancers: clinical, pathological, molecular and prognostic characterization. *Mol Cancer* 2014;13:228.
- 38 Holliday D, Speirs V: Choosing the right cell line for breast cancer research. *Breast Cancer Res* 2011;13:215.
- 39 Leak LV: The structure of lymphatic capillaries in lymph formation. *Fed Proc* 1976;35:1863–1871.
- 40 Alitalo A, Detmar M: Interaction of tumor cells and lymphatic vessels in cancer progression. *Oncogene* 2012;31:4499–4508.
- 41 Dadiani M, Kalchenko V, Yosepovich A, et al: Real-time imaging of lymphogenic metastasis in orthotopic human breast cancer. *Cancer Res* 2006;66:8037–8041.
- 42 Wong SY, Hynes RO: Lymphatic or hematogenous dissemination: how does a metastatic tumor cell decide? *Cell Cycle* 2006;5:812–817.
- 43 Partanen TA, Paavonen K: Lymphatic versus blood vascular endothelial growth factors and receptors in humans. *Microsc Res Tech* 2001;55:108–121.
- 44 Alitalo K, Carmeliet P: Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* 2002;1:219–227.
- 45 Ran S, Volk L, Hall K, Flister MJ: Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology* 2009;17:229–251.
- 46 Pantel K, Brakenhoff RH: Dissecting the metastatic cascade. *Nat Rev Cancer* 2004;4:448–456.
- 47 Hajra KM, Chen DY-S, Fearon ER: The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res* 2002;62:1613–1618.
- 48 Balkwill FR, Capasso M, Hagemann T: The tumor microenvironment at a glance. *J Cell Sci* 2012;125(pt 23):5591–5596.
- 49 Hanahan D, Weinberg RA: Hallmarks of cancer: the next generation. *Cell* 2011;144:646–674.
- 50 Yousef E, Tahir M, St-Pierre Y, Gaboury L: MMP-9 expression varies according to molecular subtypes of breast cancer. *BMC Cancer* 2014;14:609.
- 51 Wu Q-W, Yang Q-M, Huang Y-F, et al: Expression and clinical significance of matrix metalloproteinase-9 in lymphatic invasiveness and metastasis of breast cancer. *PLoS One* 2014;9:e97804.
- 52 Fidalgo F, Rodrigues T, Pinilla M, et al: Lymphovascular invasion and histologic grade are associated with specific genomic profiles in invasive carcinomas of the breast. *Tumor Biol* 2015;36:1835–1848.
- 53 Hotary K, Li XY, Allen E, Stevens SL, Weiss SJ: A cancer cell metalloprotease triad regulates the basement membrane transmigration program. *Genes Dev* 2006;20:2673–2686.
- 54 Perentes JY, Kirkpatrick ND, Nagano S, et al: Cancer cell-associated MT1-MMP promotes blood vessel invasion and distant metastasis in triple-negative mammary tumors. *Cancer Res* 2011;71:4527–4538.
- 55 Pula B, Wojnar A, Werynska B, et al: Impact of different tumour stroma assessment methods regarding podoplanin expression on clinical outcome in patients with invasive ductal breast carcinoma. *Anticancer Res* 2013;33:1447–1455.
- 56 Pula B, Wojnar A, Witkiewicz W, Dziegiel P, Podhorska-Okolow M: Podoplanin expression in cancer-associated fibroblasts correlates with VEGF-C expression in cancer cells of invasive ductal breast carcinoma. *Neoplasma* 2013;60:516–524.
- 57 Issa A, Le TX, Shoushtari AN, Shields JD, Swartz MA: Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype. *Cancer Res* 2009;69:349–357.
- 58 Shields JD, Fleury ME, Yong C, Tomei AA, Randolph GJ, Swartz MA: Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling. *Cancer Cell* 2007;11:526–538.
- 59 Wyckoff J, Wang W, Lin EY, et al: A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* 2004;64:7022–7029.
- 60 Cheang MC, Voduc D, Bajdik C, et al: Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res* 2008;14:1368–1376.
- 61 Huo Z, Gao Y, Zuo W, Gang Z, Kong R: Metastases of basal-like breast invasive ductal carcinoma to the endometrium. A case report, with a review of the literature. *Thorac Cancer* DOI: 10.1111/1759-7714.12195.
- 62 Joimel U, Gest C, Soria J, et al: Stimulation of angiogenesis resulting from cooperation between macrophages and MDA-MB-231 breast cancer cells: proposed molecular mechanism and effect of tetrathiomolybdate. *BMC Cancer* 2010;10:375.
- 63 Cursiefen C, Chen L, Borges LP, et al: VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* 2004;113:1040–1050.
- 64 Robinson BD, Sica GL, Liu YF, et al: Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. *Clin Cancer Res* 2009;15:2433–2441.
- 65 Cheung KJ, Ewald AJ: Illuminating breast cancer invasion: diverse roles for cell-cell interactions. *Curr Opin Cell Biol* 2014;30:99–111.
- 66 Alizadeh D, Trad M, Hanke NT, et al: Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. *Cancer Res* 2014;74:104–118.
- 67 Maniotis AJ, Folberg R, Hess A, et al: Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* 1999;155:739–752.
- 68 Kirschmann DA, Seftor EA, Hardy KM, Seftor RE, Hendrix MJ: Molecular pathways: vasculogenic mimicry in tumor cells: diagnostic and therapeutic implications. *Clin Cancer Res* 2012;18:2726–2732.
- 69 Shirakawa K, Tsuda H, Heike Y, et al: Absence of endothelial cells, central necrosis, and fibrosis are associated with aggressive inflammatory breast cancer. *Cancer Res* 2001;61:445–451.
- 70 Shirakawa K, Kobayashi H, Heike Y, et al: Hemodynamics in vasculogenic mimicry and angiogenesis of inflammatory breast cancer xenograft. *Cancer Res* 2002;62:560–566.
- 71 Liu T, Sun B, Zhao X, et al: OCT4 expression and vasculogenic mimicry formation positively correlate with poor prognosis in human breast cancer. *Int J Mol Sci* 2014;15:19634–1949.
- 72 Paulis YWJ, Soetekouw PMMB, Verheul HMW, Tjan-Heijnen VCG, Griffioen AW: Signalling pathways in vasculogenic mimicry. *Biochim Biophys Acta* 2010;1806:18–28.
- 73 Qiao L, Liang N, Zhang J, et al: Advanced research on vasculogenic mimicry in cancer. *J Cell Mol Med* 2015;19:315–326.
- 74 Rakha EA, El-Sayed ME, Reis-Filho JS, Ellis IO: Expression profiling technology: its contribution to our understanding of breast cancer. *Histopathology* 2008;52:67–81.
- 75 Mannelqvist M, Stefansson IM, Bredholt G, et al: Gene expression patterns related to vascular invasion and aggressive features in endometrial cancer. *Am J Pathol* 2011;178:861–871.

- 76 Mannelqvist M, Wik E, Stefansson IM, Akslen LA: An 18-gene signature for vascular invasion is associated with aggressive features and reduced survival in breast cancer. *PLoS One* 2014;9:e98787.
- 77 Minguez B, Hoshida Y, Villanueva A, et al: Gene-expression signature of vascular invasion in hepatocellular carcinoma. *J Hepatol* 2011;55:1325–1331.
- 78 Fidalgo F, Rodrigues TC, Pinilla M, et al: Lymphovascular invasion and histologic grade are associated with specific genomic profiles in invasive carcinomas of the breast. *Tumour Biol* 2015;36:1835–1848.
- 79 Iorio MV, Ferracin M, Liu CG, et al: MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005;65:7065–7070.
- 80 Fiannaca A, La Rosa M, La Paglia L, Rizzo R, Urso A: Analysis of miRNA expression profiles in breast cancer using biclustering. *BMC Bioinformatics* 2015;16(suppl 4):S7.
- 81 Ma L, Teruya-Feldstein J, Weinberg RA: Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 2007;449:682–688.
- 82 Mohammed RA, Martin SG, Gill MS, Green AR, Paish EC, Ellis IO: Improved methods of detection of lymphovascular invasion demonstrate that it is the predominant method of vascular invasion in breast cancer and has important clinical consequences. *Am J Surg Pathol* 2007;31:1825–1833.
- 83 Zhao YC, Ni XJ, Wang M-H, Zha X-M, Zhao Y, Wang S: Tumor-derived VEGF-C, but not VEGF-D, promotes sentinel lymph node lymphangiogenesis prior to metastasis in breast cancer patients. *Med Oncol* 2012;29:2594–2600.
- 84 Zhang X-H, Huang D-P, Guo G-L, et al: Co-expression of VEGF-C and COX-2 and its association with lymphangiogenesis in human breast cancer. *BMC Cancer* 2008;8:4.
- 85 Perrot-Applanat M: VEGF isoforms. *Cell Adh Migr* 2012;6:526–527.
- 86 Storr SJ, Mohammed RA, Woolston CM, Green AR, et al: Calpastatin is associated with lymphovascular invasion in breast cancer. *Breast* 2011;20:413–418.
- 87 Storr SJ, Carragher NO, Frame MC, Parr T, Martin SG: The calpain system and cancer. *Nat Rev Cancer* 2011;11:364–374.
- 88 Jerjees DA, Alabdullah M, Alkaabi M, Abduljabbar R, et al: ERK1/2 is related to oestrogen receptor and predicts outcome in hormone-treated breast cancer. *Breast Cancer Res Treat* 2014;147:25–37.
- 89 Marinho V, Metze K, Sanches F, Rocha G, Gobbi H: Lymph vascular invasion in invasive mammary carcinomas identified by the endothelial lymphatic marker D2-40 is associated with other indicators of poor prognosis. *BMC Cancer* 2008;8:64.
- 90 Alshareeda AT, Negm OH, Albarakati N, Green AR, et al: Clinicopathological significance of KU70/KU80, a key DNA damage repair protein in breast cancer. *Breast Cancer Res Treat* 2013;139:301–310.
- 91 Alshareeda AT, Negm OH, Green AR, Nolan C, et al: SUMOylation proteins in breast cancer. *Breast Cancer Res Treat* 2014;144:519–530.
- 92 Green AR, Caracappa D, Benhasouna AA, Alshareeda A, et al: Biological and clinical significance of PARP1 protein expression in breast cancer. *Breast Cancer Res Treat* 2015;149:353–362.
- 93 Al-Kaabi MM, Alshareeda AT, Jerjees DA, et al: Checkpoint kinase1 (CHK1) is an important biomarker in breast cancer having a role in chemotherapy response. *Br J Cancer* 2015;112:901–911.
- 94 Nguyen-Ngoc K-V, Cheung KJ, Brenot A, et al: ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium. *Proc Natl Acad Sci USA* 2012;109:E2595–E2604.
- 95 Pula B, Jethon A, Piotrowska A, et al: Podosplanin expression by cancer-associated fibroblasts predicts poor outcome in invasive ductal breast carcinoma. *Histopathology* 2011;59:1249–1260.