Nodal Lymphangiogenesis and Metastasis

Role of Tumor-Induced Lymphatic Vessel Activation in Extramammary Paget’s Disease

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Nodal lymphangiogenesis promotes distant lymph node (LN) metastasis in experimental cancer models. However, the role of nodal lymphangiogenesis in distant metastasis and in the overall survival of cancer patients remains unknown. Therefore, we investigated mechanisms that might facilitate regional and distant LN metastasis in extramammary Paget’s disease (EMPD). We retrospectively analyzed the impact of tumor-induced lymphatic vessel activation on the survival of 116 patients, the largest cohort with EMPD studied to date. Nodal lymphangiogenesis was significantly increased in metastatic, compared with tumor-free, LNs (P = 0.022). Increased lymphatic invasion within regional LNs was significantly associated with distant metastasis in LN (P = 0.047) and organs (P = 0.003). Thus, invasion within regional LNs is a powerful indicator of systemic tumor spread and reduced patient survival in EMPD (P = 0.0004). Lymphatic vessels associated with tumors expressed stromal cell-derived factor-1 (SDF-1), whereas CXCR4 was expressed on invasive Paget cells undergoing epithelial-mesenchymal transition (EMT)-like process. A431 cells overexpressing Snail expressed increased levels of CXCR4 in the presence of transforming growth factor-β1. Haptotactic migration assays confirmed that Snail-induced EMT-like process promotes tumor cell motility via the CXCR4-SDF-1 axis. Sinusoidal lymphatic endothelial cells and macrophages expressed SDF-1 in subcapsular sinuses of lymph nodes before Paget cell arrival. Our findings reveal that EMT-related features likely promote lymphatic metastasis of EMPD by activating the CXCR4-SDF-1 axis. (Am J Pathol 2009, 175:2235–2248; DOI: 10.2353/ajpath.2009.090420)

The metastatic spread of cancer cells from a primary site generally occurs in sentinel lymph nodes (LNs). Thus, the presence and extent of LN metastasis determines staging and prognosis in most human malignancies and often guides therapeutic decisions. Although surgical resection of primary tumors and their regional LN metastases can cure several types of cancer, distant LN and organ metastases represent a significant therapeutic concern due to the absence of effective antimetastatic therapies.

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The mechanisms of tumor cell metastasis to regional and distant LNs have remained unclear, mainly due to the absence of lymphatic-specific markers and lack of insight into the molecular mechanisms mediating tumor cell entry and persistence within the lymphatic system.

Recent studies have identified novel lymphatic-specific markers, including the mucin-type glycoprotein podoplanin, the lymphatic vascular endothelial cell hyaluronan receptor-1 (LYVE-1), and the homeobox transcription factor Prox1. Moreover, several lymphangiogenic growth factors have also been identified. Among these, vascular endothelial growth factor (VEGF)-C and VEGF-D activate VEGF receptor-3 (VEGFR-3) that is predominantly expressed by lymphatic endothelial cells (LECs). More recently, VEGFR-3 has been shown to be expressed on tumor-associated blood vascular endothelial cells. The angiogenesis factor VEGF-A also promotes lymphangiogenesis in the skin. Importantly, VEGF-C, VEGF-D, or VEGF-A overexpression in experimental tumor models induces lymphatic vessel growth associated with primary tumors, leading to enhanced tumor metastasis to regional LNs. Moreover, a blockade of VEGFR-3 signaling inhibits tumor lymphangiogenesis and LN metastasis in rodent models of tumor metastasis.

We recently found that targeted VEGF-A or VEGF-C overexpression in the skin of a multistep chemically induced model of skin carcinogenesis promotes the lymphangiogenesis of primary tumors, which increases tumor metastasis to regional LNs and beyond. More importantly, metastatic tumors that overexpress VEGF-A or VEGF-C also induced new lymphatic vessel growth within the regional LNs, probably contributing to enhanced distant LN metastasis. Thus, primary tumors in the skin induced LN lymphangiogenesis even before they metastasized, thereby preparing the lymphvascular niche, a tumor-conditioned microenvironment that serves as a future metastatic site within the regional LNs.

Recent studies have shown that stromal cell-derived factor (SDF)-1, a ligand for the chemokine receptor CXCR4, is required for the formation of vascular niches that maintain hematopoietic stem cells in murine bone marrow. Furthermore, CXCR4 is induced in several types of invasive cancers. In fact, several clinico-pathological studies have found that increased CXCR4 levels are associated with the survival of cancer cells, increased regional LN metastasis and/or reduced patient survival, and vascular niches comprising tumor-associated blood vessel capillaries were found to serve as therapeutic targets in an experimental brain tumor model in mice. However, the mechanisms that induce CXCR4 in invasive tumor cells remain unknown, and it has remained unclear whether the CXCR4-SDF-1 axis plays a significant role in the creation of the tumor-associated lymphvascular niche.

Increasing evidence indicates that tumor lymphangiogenesis arises in different types of human cancers, and that tumor-associated lymphatic vessel expansion is associated with enhanced rates of sentinel LN metastasis and reduced patient survival. However, the mechanisms of tumor lymphangiogenesis and its relative importance to cancer metastasis and the survival of patients with different types of cancer have remained controversial. Therefore, the function(s) of tumor-associated lymphatic vessels need to be clarified so that interactions between tumor-associated LECs and invasive tumor cells within primary sites and the subsequent formation of LN metastasis can be understood in more detail.

Extramammary Paget’s disease (EMPD) is a cutaneous adenocarcinoma that is characterized by the presence of vacuolated Paget cells. The condition usually develops in genital and/or axillary skin and appears as an erythematous plaque at the early stages that is characterized by slow intraepidermal growth. During tumor progression, EMPD can develop nodules and ulcerations associated with local tissue invasion. Subsequently, tumors metastasize to regional LNs and distant organs, leading to a poor outcome. Although these clinical features indicate that EMPD progression is associated with tumor lymphangiogenesis and angiogenesis, the occurrence and the pathogenetic role of vascular activation in EMPD have not been studied. Moreover, little is understood about the mechanisms through which Paget cells acquire the invasive phenotype that spreads to LNs and beyond.

The epithelial-mesenchymal transition (EMT), which plays a key role in promoting embryonic development, induces the striking transformation of epithelial cells to adopt the features of mesenchymal cells such as the expression of N-cadherin and loss of E-cadherin. Recent studies have further proposed that cancer cells can acquire EMT-like phenotypes such as loss of cell polarity, loss of cell-cell adhesion, and/or loss of keratin expression and considerable expression of vimentin during tumor progression. Among the molecular regulators of EMT-like processes in the tumor microenvironment, the transcription factor Snail initiates the down-regulation of E-cadherin expression in several tumor cells of epithelial origin, enabling these cells to detach from the tumor mass and to invade the surrounding stroma toward tumor-associated blood vessels to metastasize to distant organs. The pleiotropic cytokine transforming growth factor (TGF)-β1 promotes EMT but whether EMT-like behavior by tumor cells enhances their invasion of lymphatic vessels in primary sites remains obscure.

The present study investigates tumor angiogenesis, lymphangiogenesis, lymphatic invasion, and EMT-like phenotypes in 116 patients with EMPD, representing the largest cohort of this type analyzed in a single study. We investigated whether nodal lymphangiogenesis and/or lymphatic invasion within regional LNs promotes distant LN metastasis and examined the molecular mechanisms promoting lymphatic cancer spread in EMPD. Overall, we identified lymphatic invasion of regional LNs as a novel, significant prognostic indicator of metastasis and survival of patients with EMPD. Moreover, we found that invasive Paget cells undergoing EMT-like process express CXCR4, whereas its ligand SDF-1 is produced by tumor-associated lymphatic vessels and lymphatic sinuses of draining lymph nodes. Thus, the SDF1 axis plays an important role in mediating EMPD LN metastasis, and
CXCR4 expression by Paget cells predicts tumor metastasis and patient survival.

Materials and Methods

Patient Population

Patients with EMPD in the groin area were retrospectively identified through a review of survival data from the Graduate Schools of Medicine at Ehime and Osaka Universities. Surgical samples from primary skin tumors were collected from 116 patients and tissue samples of regional LNs were obtained from 45 of them. H&E staining of primary tumors in the skin identified carcinoma in situ (CIS) in 73 patients and invasive growth of primary tumors in the dermis of 43 of them. Regional LN metastasis was confirmed at the time of diagnosis in 20 of these patients and 23 had none. We also obtained representative sections from 17 patients with Bowen's disease, 42 patients with malignant melanoma in situ, and 15 normal skin samples from the surgical margin. This retrospective study was approved by the institutional review boards of the Graduate Schools of Medicine at Ehime and Osaka Universities.

Classification and Staging

Table 1 shows the clinical and pathological features used to diagnose EMPD. N-factors categorized no evident metastasis as N0, unilateral regional LN metastasis as N1, or bilateral regional LN metastasis as N2. We specifically categorized M-factors as no distant metastasis evident (M0), metastasis in distant LN(s) beyond regional LN(s), or metastasis in visceral organs (lung, liver, bone). Distant LN and organ metastases were detected by computer-assisted tomography, ultrasound examination, or skeletal scintigraphy. The Tumor Necrosis Metastasis classification for EMPD was designed and standardized by the Japanese Skin Cancer Society.

Immunostaining

Primary tumors or LNs were fixed in buffered formalin, or embedded in OCT compound (Sakura Finetek, Torrance, CA) and snap-frozen. Paraffin (5 μm) or cryostat sections were immunostained as previously described, using the primary antibodies shown in Table 2. The respective secondary antibodies were labeled with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR). Antigens were usually retrieved in paraffin sections by incubation with citrate buffer (pH 6.0 for 30 minutes at 95°C) before immunostaining. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Molecular Probes). Sections were also immunohistochemically stained using a 3-amino-9-ethylcarbazole peroxidase substrate kit (Vector Laboratories, Burlingame, CA). Respective control IgG was stained as a specificity control. Sections were examined, and digital images were captured using a confocal laser scanning microscope LSM510 (Carl Zeiss, Jena, Germany) or A1 (Nikon, Tokyo, Japan).

Computer-Assisted Morphometric Vessel Analyses

Representative sections obtained from 116 patients with primary EMPD, 17 with Bowen's disease, 42 with malignant melanoma in situ, and from 15 normal skin samples were double-stained by differential immunofluorescence for podoplanin and von Willebrand factor, and then analyzed using a LSM 510 microscope (Carl Zeiss). Computer-assisted morphometric analyses of blood and lymphatic vessels on captured images proceeded using IP-LAB software (Scanalytics, Billerica, MA) as described previously. Data are displayed as box and whisker plots.

Culture of LECs and Enzyme-Linked Immunosorbent Assays

Primary human dermal LECs were isolated from neonatal foreskins as described previously. LECs (1 × 10⁴) were seeded onto triplicate fibronectin-coated culture dishes.
and propagated in endothelial cell growth medium containing 0.5% fetal bovine serum (Invitrogen, Grand Island, NY). Conditioned media were collected at days 1, 3, or 5. Human SDF-1 or CCL21 levels were subsequently measured using enzyme-linked immunosorbent assays (ELISAs; Quantikine M; R&D Systems, Minneapolis, MN). Data were normalized to cell number at each time point.

Flow Cytometry and Haptotactic Cell Migration Assays

Recombinant human TGF-β1, human SDF-1α and anti-human CXCR4 monoclonal antibody 12G5 were purchased from R&D Systems. A431 cells stably transfected with mouse Snail or control pcDNA 3.1 (Invitrogen, Carlsbad, CA) vector were incubated with or without 10 ng/ml of recombinant human TGF-β1. Single cell suspensions were prepared using cell dissociation buffer (Invitrogen). These cells were stained with the biotinylated anti-CXCR4 antibody 12G5, and then positively and negatively labeled with streptavidin-conjugated Alexa Fluor 488 (BD Biosciences Pharmingen) and propidium iodide, respectively. Stained cells (10,000 cells/sample) were analyzed by flow cytometry using a FACScan and a FACS-Calibur (BD Biosciences) and analyzed by FlowJo software (Tree Star, San Carlos, CA). For migration assays, cells were seeded in serum-free Dulbecco’s modified Eagle’s medium containing 0.2% delipidized bovine serum albumin into the upper chambers of 24-well FluoroBlok inserts (BD Biosciences) in the presence of human CXCR4-neutralizing monoclonal antibody 12G5 (10 μg/ml), or corresponding control IgG, and incubated for 3 hours at 37°C in the presence of SDF-1α in the bottom chambers. Cells on the undersides of inserts were stained with DAPI (Molecular Probes), and migrated cells were counted by computer-assisted image analysis of three random fields per well. Three independent experiments were performed for each assay.

Table 2. Antibodies Used for Immunofluorescence and Immunohistochemistry

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<th>Antibody</th>
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<th>Source</th>
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<td>Y Kato</td>
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<td>Lymphatics</td>
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<td>Goat polyclonal</td>
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<td>Panvascular</td>
<td>Dako</td>
<td>Rabbit polyclonal</td>
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Statistical Analyses

Across-group comparisons were performed by one-way analysis of variance when appropriate, followed by t-tests for pairwise comparisons. P values were adjusted using Bonferroni’s method. Trends were analyzed by linear regression adjusted for age and gender. Overall survival after the date of surgery was defined as the primary endpoint. The Kaplan-Meier product limit method was applied and comparisons according to markers were examined using the log-rank test. We defined P < 0.05 as statistically significant.

Results

Increased Tumor Lymphangiogenesis and Angiogenesis in EMPD

The macroscopic appearance of EMPD is characterized by a red patch, usually in the groin region, indicating a high level of vascularization (Figure 1A). Routine H&E stain showed a few number of small vessels in normal skin (Figure 1B). In contrast, histology in EMPD revealed a hyperplastic epidermis and prominent enlargement of numerous vessels within the dermis (Figure 1C) as compared with normal skin (Figure 1B). The majority of epidermal cells are composed of round Paget cells with clear and abundant cytoplasm with hyperchromatic nuclei (Figure 1C, inset). Within a hyperplastic epidermis, tumors occasionally invaded the vasculature during tumor progression (Figure 1D), indicating the metastatic potential of invasive Paget cells.

Because VEGF-A and VEGF-C have been identified as tumor lymphangiogenesis factors in experimental mouse models, we investigated their expression in EMPD by immunohistochemistry. Levels of VEGF-A were high in both Paget cells and in inflammatory cells in various grades of primary tumors (Figure 1G) but not in normal skin (Figure 2238 Hirakawa et al AJP November 2009, Vol. 175, No. 5
Double-immunofluorescence staining for VEGF-A and CD68 revealed that a subpopulation of immune cells producing VEGF-A in fact consisted of macrophages (Figure 1, I–K). VEGF-C was exclusively expressed in Paget cells within the epidermis (Figure 1H) and was undetectable in normal skin (Figure 1F). These results suggest that Paget cells induce both tumor lymphangiogenesis and angiogenesis via the secretion of VEGF-A and VEGF-C.

We then investigated the presence of tumor-associated lymphatic and blood vessels, using specific antibodies against podoplanin for lymphatic vessels and against von Willebrand factor for blood vessels. Differential immunofluorescence analyses revealed lymphatic and blood vessel formation at the early stage of CIS (Figure 1M), as compared with normal skin obtained from surgical margins (Figure 1L). Tumor lymphangiogenesis and angiogenesis were increased throughout the successive stages of EMPD tumor progression, as compared with normal skin. Computer-assisted morphometric analyses confirmed that relative tissue areas occupied by lymphatic vessels that were podoplanin-positive and by blood vessels that were positive for von Willebrand factor were increased in the peritumoral areas of CIS, as compared with normal skin (Figure 1, M and N; \( P < 0.0001 \), respectively). We then compared neovascularization in EMPD with other skin tumors such as Bo-
w en’s disease or malignant melanoma in situ that historically assume a pagetoid pattern within the epidermis (Figure 1, N and O). Tumor-associated angiogenesis and lymphangiogenesis were most obviously increased in EMPD, as compared with all other types of skin tumors (P < 0.05), indicating that EMPD is the most highly vascularized type of skin tumor. Levels of tumor angiogenesis and lymphangiogenesis in CIS were comparable with those in invasive EMPD in the absence (N0) or presence (N1,2) of regional LN metastases (Figure 1, M and N).

Differential immunofluorescence analysis of primary EMPD tumors for the proliferation marker Ki-67 and podoplanin revealed proliferating LECs in the peritumoral area, indicating active tumor lymphangiogenesis (Figure 1P). All podoplanin-positive vessels also expressed the lymphatic-specific transcription factor Prox1, confirming their lymphatic identity (Figure 1Q).

**Tumor-Associated LECs Attract Aggressive Paget Cells via the CXCR4-SDF-1 Axis**

Although the assessment of tumor-associated lymphatic vessel growth did not significantly contribute to estimating an increased frequency of regional LN metastasis in EMPD, we postulated that tumor-induced lymphatic vessels are functionally distinct from normal lymphatic vessels in the skin. In fact, a subpopulation of podoplanin and LYVE-1-positive, tumor-associated lymphatic vessels expressed large amounts of VEGFR-3 (Figure 2, A–C and D–F, arrows) whereas other lymphatic vessels did not, according to immunofluorescence staining (Figure 2, A–C and D–F, arrowheads). Furthermore, neuropilin-2 was highly induced in a subpopulation of podoplanin-positive tumor-associated LECs (Figure 2I, arrow). Therefore, these results suggest that tumor-associated LECs are functionally activated in EMPD and indicate that tumor-induced lymphatic vessels might positively mediate tumor cell invasion toward those lymphatic vessels and metastasis to regional LNs.

To define the potential role of specific chemokine-receptor interactions in promoting contact between Paget cells and LECs, we investigated whether SDF-1, a specific ligand for CXCR4, is expressed by lymphatic vessels associated with primary skin tumors. Double-immunofluorescence staining revealed that tumor-associated LYVE-1-positive lymphatic vessels were positive for SDF-1, whereas lymphatic vessels in normal skin were not (Figure 2, J–O). Furthermore, ELISAs showed that cultured LECs expressed marked levels of SDF-1, whereas LECs constitutively expressed CCL21, a known lymphoid tissue chemokine, in conditioned media (Figure 2P), indicating that SDF-1 may be secreted by LECs toward CXCR4-expressing cells.

To further elucidate the role of CXCR4 in promoting LN metastasis in EMPD, immunofluorescence staining revealed that highly invasive Paget cells indeed expressed CXCR4 (Figure 2Q). In contrast, Paget cells in CIS did not express detectable levels of CXCR4 (Figure 2R). Kaplan-Meier analysis revealed that the expression of CXCR4 on Paget cells represented a significant prognostic factor for reduced overall patient survival (P < 0.0001; Figure 2S).

Taken together, these results suggest that tumor-associated LECs can chemotact CXCR4-positive Paget cells toward lymphatic vessels within primary sites, and might play a crucial role in promoting LN metastasis, leading to a patient poor outcome.

**EMT-Like Phenotypes Are Novel Prognostic Parameters for Reduced Survival in EMPD**

To further characterize Paget cell motility toward lymphatic vessels within the primary sites, we initially investigated whether EMT-like process plays a significant role in promoting stromal invasion in EMPD. Immunofluorescence staining revealed that Paget cells in CIS do not express large amounts of E-cadherin on the cell membrane, in particular at the tumor-stroma interface, unlike normal epidermal keratinocytes (Figure 3, A, B, D, and E). The expression of E-cadherin was further altered in invasive Paget cells (Figure 3, C and F), and cytoplasmic E-cadherin expression was detectable by the early stages of tumor progression in a subpopulation of patients with a poor outcome (Figure 3F, arrowheads). Therefore, we calculated the overall survival of patients with or without detectable cytoplasmic E-cadherin expression using Kaplan-Meier analysis. The results showed that the expression of cytoplasmic E-cadherin was a significant prognostic factor for reduced overall survival in EMPD (P = 0.0068; Figure 3J).

Paget cells did not express N-cadherin and/or vimentin (mesenchymal markers) at the early stages of CIS (Figure 3, E and H) like normal epidermal keratinocytes (Figure 3, D and G) but did so in invasive EMPD (Figure 3, F and I). Kaplan-Meier analyses revealed that N-cadherin or vimentin expression in EMPD significantly correlated with reduced overall survival (P < 0.0001; Figure 3J), indicating that EMT-like process plays a key role in promoting tumor invasion within the primary sites.

**EMT-Like Process Promotes Enhanced Chemotaxis to SDF-1 by Inducing CXCR4 in A431 Cells**

Double-immunofluorescence staining showed that invasive Paget cells located at the edges of tumors expressed both N-cadherin and CXCR4 (Figure 4, A–C), indicating that EMT-related process in Paget cells might induce CXCR4 that enables invasive tumor cells to migrate toward tumor-associated SDF-1-secreting lymphatic vessels. To determine whether EMT-associated pathway increases CXCR4 expression on the surface of cancer cells, we investigated A431 cells that were stably transfected with Snail, a key transcription factor that induces EMT-like phenotypes in tumor cells. The expression of CXCR4 was considerably increased on the surface of transfected cells compared with mock-transfected A431 cells (Geo Mean: mock A431, 7.4%; Snail A431, 15.1%; Figure 4D). In addition, TGF- β1 further enhanced CXCR4 on Snail- but not on mock-transfected A431 cells (Geo Mean: mock A431, 9.5%; Snail
A431, 36.6%; Figure 4E), indicating that EMT-like process promotes CXCR4 expression in A431, a vulval epidermoid tumor cell line, particularly in the presence of TGF-β1. Haptotactic migration assays further revealed enhanced chemotaxis toward SDF-1 in the lower chamber by TGF-β1-treated Snail-transfected A431 cells, compared with mock-transfected A431 cells in the upper chamber. In contrast, neutralization of CXCR4 on A431 cells significantly inhibited the enhanced chemotaxis toward SDF-1 (Figure 4F). Therefore, the CXCR4-SDF-1 axis plays a key role in promoting A431 cell motility undergoing EMT-like processes.

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Figure 3. Epithelial-mesenchymal transition-like phenotypes as novel prognostic parameters for reduced survival in EMPD. A–C: H&E staining of representative normal skin (A), CIS (B), and invasive EMPD tumors (C). D–F: Double-immunofluorescence staining for E-cadherin (green) and for N-cadherin (red) shows typical E-cadherin expression on the surface of normal epidermal keratinocytes (D). In contrast, intraepidermal Paget cells do not express high levels of E-cadherin (E). Cytoplasmic E-cadherin expression is obvious in invasive EMPD tumors (F, arrowheads). The mesenchymal marker N-cadherin is induced in invasive Paget cells (F). G–I: Immunohistochemical staining shows vimentin expression in highly invasive Paget cells from patients with advanced disease (I, red) but not in normal epidermis (G) or CIS (H), suggesting that EMT-like phenotypes correlate with invasive feature of Paget cells. J: Kaplan-Meier survival analyses shows that the EMT-related markers N-cadherin and vimentin, and cytoplasmic E-cadherin expression are significantly associated with poor survival in EMPD. Scale bars = 50 μm (A–I). Nuclei are stained blue (DAPI or hematoxylin stain).
Regional LNs during Tumor Metastasis

We and others have shown in experimental animal tumor models that primary tumors can promote metastatic spread by the induction of lymphangiogenesis within draining lymph nodes. Therefore, to investigate whether nodal LECs are involved in human EMPD metastasis, we assessed lymphangiogenesis and the formation of a pre-metastatic niche within regional LNs. Lymphangiogenesis in LNs containing metastatic Paget cells was obviously induced within the metastases (Figures 5C and 6, A and B). Surprisingly, lymphatic vessel growth in regional LNs was already induced in patients with invasive EMPD before the tumors had metastasized (Figure 5B), whereas such changes were undetectable in the regional LNs of patients with CIS (Figure 5A). Quantitative image analysis of LN sections stained for podoplanin and von Willebrand factor and logistic regression analysis confirmed that lymphatic vessel growth within the regional LNs was progressively enhanced throughout the metastatic process ($P < 0.001$; Figure 5D). Computer-assisted morphometric analysis revealed that areas of lymphatic vessels in the LNs of group N1 and N2 tumors ($n = 19$) were significantly more extensive than in the group with N0 ($n = 14$) tumors ($P = 0.022$). Taken together, these results indicate that EMT-related features actively promote tumor cell invasion into tumor-associated lymphatic vessels, and that active lymphatic invasion by Paget cells undergoing EMT-like process probably promotes the successive progression of regional LN metastasis, leading to an increased risk for patient survival in EMPD.

Enhanced Nodal Lymphangiogenesis in Regional LNs during Tumor Metastasis

We and others have shown in experimental animal tumor models that primary tumors can promote metastatic spread by the induction of lymphangiogenesis within draining lymph nodes. Therefore, to investigate whether nodal LECs are involved in human EMPD metastasis, we assessed lymphangiogenesis and the formation of a pre-metastatic niche within regional LNs. Lymphangiogenesis in LNs containing metastatic Paget cells was obviously induced within the metastases (Figures 5C and 6, A and B). Surprisingly, lymphatic vessel growth in regional LNs was already induced in patients with invasive EMPD before the tumors had metastasized (Figure 5B), whereas such changes were undetectable in the regional LNs of patients with CIS (Figure 5A). Quantitative image analysis of LN sections stained for podoplanin and von Willebrand factor and logistic regression analysis confirmed that lymphatic vessel growth within the regional LNs was progressively enhanced throughout the metastatic process ($P < 0.001$; Figure 5D). Computer-assisted morphometric analysis revealed that areas of lymphatic vessels in the LNs of group N1 and N2 tumors ($n = 19$) were significantly more extensive than in the group with N0 ($n = 14$) tumors ($P = 0.022$). Taken together, these results indicate that the activation of tumor-associated sinusoidal lymphatic vessels is induced before metastasis, and enhanced by metastatic Paget cells within regional LNs.

Therefore, we investigated whether increased lymphatic vessel areas (LVAs) in regional LNs could predict

with invasive EMPD to determine whether active invasion of lymphatic vessels by Paget cells is induced by EMT-like features and/or CXCR4 and to predict the subsequent incidence of regional LN metastasis. Double-immunofluorescence staining for cytokeratin 7 (expressed by tumor cells) and for podoplanin identified active invasion of the tumor-associated lymphatic vessels by Paget cells (Figure 4G). Expression of the EMT-related markers vimentin and N-cadherin, and of cytoplasmic E-cadherin, closely correlated with the incidence of lymphatic invasion in primary skin tumors ($P = 0.0003$, 0.0036, and 0.012, respectively). Furthermore, expression of CXCR4 was strongly associated with the incidence of lymphatic invasion within the primary sites ($P < 0.0001$). Importantly, presence of those EMT-associated markers closely correlated with the expression of CXCR4 by invasive Paget cells ($P < 0.01$, respectively), revealing a potential induction of CXCR4 by EMT-related process in invasive Paget cells. Moreover, lymphatic invasion by Paget cells was significantly increased at stages N1 and N2, compared with N0 ($P = 0.002$ and 0.003, respectively; Figure 4H). Multivariable linear regression analysis adjusted for age and gender confirmed a remarkable increase of lymphatic invasion throughout N-grade progression ($P = 0.002$; Figure 4H). Taken together, these results indicate that EMT-related features actively promote tumor cell invasion into tumor-associated lymphatic vessels, and that active lymphatic invasion by Paget cells undergoing EMT-like process probably promotes the successive progression of regional LN metastasis, leading to an increased risk for patient survival in EMPD.
distant LN metastasis. We found that LVAs in regional LNs were increased in patients with distant LN metastasis (LVA without versus with regional LN metastasis; 0.88 ± 1.26% versus 3.10 ± 3.50%; \( P = 0.1204 \)), indicating that lymphatic vessel enhancement in regional LNs could predict the presence of distant LN metastasis.

We also found that sinusoidal LECs and macrophages within the subcapsular sinuses of non-metastatic regional LNs draining EMPD tumors expressed abundant SDF-1 (Figure 5, E–L). Thus, the subcapsular sinuses that comprise primary sites of tumor metastasis in the LNs might form premetastatic niches\(^{45,46} \) by promoting the migration and retention of CXCR4-positive Paget cells. In fact, 8 of 9 and 6 of 11 patients had bilateral and unilateral regional LN metastases, respectively, but Paget cells in primary invasive tumors expressed high levels of CXCR4 in only 2 of 95 patients without regional LN metastases. Importantly, CXCR4 was expressed within primary sites in 13 of 15 patients with distant LN metastasis. Indeed, distant LN metastasis was significantly associated with CXCR4 expression by invasive Paget cells in primary tumors, compared with its absence (\( P < 0.0001 \)). Therefore, these results suggest that the CXCR4-SDF-1 axis contributes to the increase of distant LN metastasis in EMPD.

### Lymphatic Invasion within Regional LNs Predicts Distant LN and Organ Metastasis

We previously showed in mouse models of experimental carcinogenesis that LN lymphangiogenesis, which might be a target for metastatic tumor cells, positively mediates distant LN and distant organ metastasis.\(^{18,20} \) Therefore, we examined nodal lymphangiogenesis and metastatic Paget cells within tumor-associated lymphatic vessels in the regional LNs of 23 patients with dermal invasion in primary sites. Routine histology stains revealed metastatic tumor cell foci within the regional LNs but tumor cell-lymphatic vessel interactions could not be analyzed in detail (Figure 6A). Immunofluorescence stains for podoplanin and von Willebrand factor demonstrated not only new lymphatic vessel growth within the regional LNs (Figure 6B), but also the presence of metastatic Paget cells within these metastasis-associated lymphatic vessels (Figure 6, C and D).

Therefore, we investigated Paget cell invasiveness toward tumor-associated sinusoidal lymphatic vessels within regional LNs. We examined the expression of cytokeratin 7 in metastatic Paget cells and in sinusoidal lymphatic vessels by double-immunofluorescence staining (Figure 6, E and F). Lymphatic invasion by Paget cells within regional LNs significantly correlated with distant LN metastasis (metastasis in distant LN(s) beyond regional LN(s), \( n = 12 \)) (\( P = 0.0472 \); Figure 6G) and with visceral organ metastasis (metastasis in visceral organs, \( n = 11 \)) (\( P = 0.0033 \); Figure 6H). We also found that distant LN metastasis was a significant prognostic parameter for reduced overall survival in patients with EMPD (\( P = 0.0004 \); Figure 6H). Taken together, these findings indicate that lymphatic invasion within regional LNs promotes distant LN and organ metastasis in patients with EMPD and leads to a poor outcome.

### Discussion

The present study analyzed the largest cohort of patients with EMPD known to date, and identified novel pathomechanisms that promote regional and distant LN metastasis. Tumor lymphangiogenesis was induced not only in primary tumors, but also in regional LNs draining invasive EMPD tumors. Our results revealed that lymphatic invasion by metastatic Paget cells in regional LNs indicates a high risk of distant metastasis and of poor survival for patients with EMPD. Invasion of metastasis-associated lymphatic vessels by Paget cells within regional LNs significantly correlated with distant LN and distant organ metastasis, indicating that active lymphatic invasion in regional LNs is a novel risk marker for the systemic
Figure 6. Lymphatic invasion within regional LNs is associated with distant LN metastasis. A: Routine H&E stains of metastatic regional LNs. Normal LN structure is visible on left side. Metastatic Paget cells occupy right side of LN. Tumor-associated nodal lymphangiogenesis was detected by immunofluorescence staining for podoplanin (B, green), as compared with nonmetastatic regions of LN. C and D: High power magnification of metastatic LNs confirms that metastatic Paget cells invade podoplanin-positive lymphatic vessels. Blood vessels stained red for von Willebrand factor (B and D). Double-immunofluorescence staining of metastatic regional LNs for cytokeratin 7 (red) and for podoplanin (green) with poor (F) or successful (E) clinical outcome. Metastatic Paget cells were identified in regional LNs using anti-cytokeratin 7 antibody, whereas these Paget cells did not invade tumor-associated podoplanin-positive lymphatic vessels (E). In contrast, highly aggressive Paget cells invaded adjacent lymphatic vessels (F), likely enabling them to further metastasize beyond regional LNs. Nuclei are stained blue (DAPI stain). Scale bars = 100 (A, B, E, and F) and 50 (C and D) mm. G: Lymphatic invasion scores within regional LNs are significantly increased in patients with distant LN (MLN; n = 12) or visceral organ (MVO; n = 11) metastasis compared with those without metastasis (P = 0.047 and P = 0.003, respectively). Data are expressed as means ± SD. H: Distant LN metastasis/MLN is significantly associated with reduced patient survival (Kaplan-Meier survival analyses, P = 0.0004). MLN, metastasis in distant LN(s) beyond regional LN(s); MVO, metastasis in visceral organs (lung, liver, bone).
spread of EMPD. Thus, we discovered that nodal lymphangiogenesis, which is a crucial target for metastatic tumor cells, plays a significant role in augmenting the metastatic spread of tumors in cancer patients. Moreover, we very recently identified that intrametastatic lymphatic invasion occurs also in human mammary carcinoma metastasis (manuscript in preparation).

We found that tumor lymphangiogenesis in EMPD was induced during successive stages of tumor progression. Both tumor and inflammatory cells including macrophages produced abundant VEGF-A or VEGF-C, which led to enhanced tumor lymphangiogenesis in EMPD. Other studies have found that VEGF-A overexpression in the skin promotes lymphangiogenesis as well as angiogenesis. The characteristic inflammatory reaction associated with EMPD might thus contribute to more pronounced lymphangiogenesis and angiogenesis in these compared with other types of skin tumors such as those of Bowen's disease and malignant melanoma in situ.

We found that primary tumors in patients with distant organ metastasis and poor survival exhibited EMT-like phenotypes, which facilitated invasion by malignant Paget cells. We found that the EMT-related markers N-cadherin and vimentin could serve as novel prognostic markers of reduced survival among patients with EMPD. The EMT-like features of Paget cells were closely associated with lymphatic invasion in primary tumors. Since no cell line of Paget cell origin has been established thus far, we investigated the A431 cell line, a genital epidermoid tumor cell line. Our in vitro observations revealed that A431 cells transfected with Snail expressed increased amounts of CXCR4 and that chemotaxis to its ligand SDF-1 was enhanced. These results are in accordance with a recent study identifying the induction of CXCR4 by oral squamous cell carcinoma cell lines undergoing EMT. Furthermore, our present study revealed that tumor-associated LECs abundantly express SDF-1, an inducible chemokine, indicating a crucial role for the CXCR4-SDF-1 axis in tumor cell invasion of the lymphatic endothelium. Overall, our results suggest that EMT-like process contributes to the induction of lymphatic invasion within primary sites, and thus to the further development of regional LN metastasis in EMPD.

We also found that the CXCR4-SDF-1 axis might promote lymphatic invasion by Paget cells in primary tumors, as well as the induction and maintenance of premetastatic lymphvascular niches in the regional LNs of EMPD patients. Invasive Paget cells expressed CXCR4, whereas LECs associated with tumors expressed high levels of SDF-1 and LECs of lymphatic vessels in normal skin did not. Our previous lineage-specific gene profile revealed that LECs can potently produce SDF-1 as compared with blood vascular endothelial cells. The present study confirmed that cultured LECs secrete increased levels of SDF-1 as well as CCL21, a chemokine that is constitutively produced by the lymphatic endothelium. Therefore, the CXCR4-SDF-1 axis might play a pivotal role in promoting the chemoattraction of tumor-associated SDF-1-secreting LECs toward CXCR4-positive Paget cells and their subsequent lymphatic invasion. These results are in agreement with the recent discovery that lymphatic invasion by cutaneous malignant melanoma cells promotes sentinel LN metastasis and reduces patient survival.

Our results also suggest that lymphatic invasion within primary tumors requires the functional activation of both tumor cells and LECs. Indeed, neuropilin-2 expression was induced by tumor-associated LECs in an experimental mouse model, and neuropilin-2 was identified as a therapeutic target for the prevention of LN metastasis. Our present study confirmed that neuropilin-2 is induced in subpopulation of tumor-associated LECs within the primary site in EMPD. Furthermore, a novel gene profile has recently provided a specific gene expression pattern of tumor-associated LECs induced by VEGF-C in a mouse syngeneic tumor model. Moreover, we and others have generated vascular lineage-specific gene profiles of cultured human LECs that express high levels of SDF-1 transcripts, as compared with mRNA levels in blood vascular endothelial cells. Therefore, functional analyses of tumor-associated LECs might reveal additional targets for the prevention of lymphatic cancer metastasis.

We found that the subcapsular sinuses of regional LNs serve as a major source of SDF-1, which is probably required for the formation of a premetastatic niche since invasive Paget cells up-regulate CXCR4 expression that promotes efficient migration toward lymphatic vessels and metastasis to LNs. Whereas SDF-1 production by LNs has been identified in isolated human LN-derived mesenchymal cells, the present findings reveal that both sinusoidal lymphatic endothelium and LN-resident macrophages represent a potent source of SDF-1. Furthermore, parenchymal invasion by tumor cells may begin with a specific contact to sinusoidal LECs in LNs. Therefore, CXCR4-positive Paget cells and SDF-1-expressing sinusoidal LECs likely promote the formation of tumor metastasis in regional LNs, although sinusoidal LECs may be fewer in number as compared with resident macrophages. Moreover, we found significant induction of new lymphatic vessel growth within regional LNs before tumor arrival in EMPD. These novel findings within LNs indicate that metastatic foci modulate structural and functional changes that encourage the formation of lymphvascular niches for the preferential initiation and progression of LN metastasis in patients with EMPD.

Stephan Paget proposed the "seed-and-soil" hypothesis over a century ago, indicating that inherent organ-specific characteristics are responsible for the preferential metastasis of distinct tumors to organs. The present study found that CXCR4-positive Paget cells ("seed") can actively induce a SDF-1-rich tumor microenvironment, as well as lymphatic vessel growth in primary tumors and in draining LNs ("soil") to promote their metastatic spread. The molecular mechanisms that promote interactions between metastatic tumor cells and activated, tumor-associated lymphatic endothelium in primary tumors and draining LNs should be investigated in more detail for the prevention and treatment of human cancers.
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