Cancer Science
The official journal of the Japanese Cancer Association

Morphophenotypic characteristics of intralymphatic cancer and stromal cells susceptible to lymphogenic metastasis

Yuki Matsumura,[1,2] Genichiro Ishii,[1,3] Keiju Aokage,[1,2] Takeshi Kuwata,[1] Tomoyuki Hishida,[2] Junji Yoshida,[2] Mitsuyo Nishimura,[2] Kanji Nagai[2] and Atsushi Ochiai[1,3]

[1]Pathology Division, Research Center for Innovative Oncology, Chiba; [2]Division of Thoracic Surgery, National Cancer Center Hospital East, Chiba, Japan

(Received December 19, 2011/Revised March 2, 2012/Accepted March 12, 2012/Accepted manuscript online March 16, 2012/Article first published online April 23, 2012)

The intravessel microenvironment has significant effects on cancer metastasis. The aim of the present study was to determine how the morphologic and immunophenotypic features of cancer cells and infiltrating stromal cells within the permeated lymphatic vessels are associated with lymphogenic metastasis. A total of 137 primary lung adenocarcinoma patients with extratumoral lymphatic permeations were examined. Morphologically, the floating cancer nests within the permeated lymphatic vessels were divided into two types: Type A, consisting of a single large cancer nest; and Type B, consisting of multiple small cancer nests. We compared the clinicopathologic characteristics and the immunophenotypes of the cancer cells and infiltrating stromal cells between the Type A and Type B nests. Eleven of 54 Type A patients (20%) had intrapulmonary metastases, compared with 36 of 83 Type B patients (43%; $P = 0.006$). Immunohistochemically, Type B cancer cells expressed significantly higher levels of CD44 than Type A cancer cells (mean scoresAUTHOR: Scores - what is this score? Is it the number of cells expressing CD44 or the concentration of CD44 or some other type of scoring system? 43.0 *vs* 20.5, respectively) and E-cadherin (60.5 *vs* 31.5, respectively), but lower levels of Geminin (11.9% *vs* 20.3%, respectively) and cleaved caspase 3 (2.4% *vs* 7.8%AUTHOR: 11.9% vs 20.3%, respectively) and cleaved caspase 3 (2.4% vs 7.8%, – what do the percentages here refer to? The number of cells expressing geminin and caspase 3? The levels of these factors? Please clarify., respectively). Moreover, a significantly larger number of CD204-positive macrophages were present within the cancer-permeated lymphatic vessels in Type B patients than in Type A patients (mean number 9.5 *vs* 4.6, respectively). The present study reveals that intralymphatic cancer cell and stromal cell phenotypes are susceptible to lymphogenic metastasis, suggesting that lymphogenic metastasis may be affected by the intralymphatic microenvironment they create. (*Cancer Sci* 2012; 103: 1342–1347)

The cause of death in most cancer patients is the development of metastases from the primary tumor. The metastatic process includes various complex steps. The process starts with the separation of cancer cells from the primary lesion, followed by the permeation of these cells into vessels. In permeated vessels, the cancer cells survive apoptosis (a process known as anoikis), proliferate, and then transmigrate to the metastatic site. Thereafter, the tumor cells adhere to the endothelial cells and extravasate to the connective tissues surrounding the vessels. By interacting with the stromal cells present in that location, they finally invade the target organ parenchyma, followed by the development of metastatic lesions. We recently reported that, after extravasation from lymphatic vessels, cancer cells undergo a dynamic phenotypic change associated with the epithelial–mesenchymal transition (EMT) within the connective tissue of the vessel wall.[1] The cellular and molecular mechanisms involved in each process have been the topic of constant debate and have inspired extensive research.

One study focusing on the location of lymphatic permeation in resected non-small cell lung cancers found that extratumoral lymphatic permeation was an independent prognostic factor.[2] That study report implied that extratumoral lymphatic vessels are an important metastatic route. Sakuma *et al.*[3], using lung adenocarcinoma cell lines, reported that tumor cells floating in lymphatic vessels resist anoikis by expressing phosphorylated (p-) Src. Conversely, recent reports have revealed the presence of circulating stromal cells that associate with cancer cells and play a role in cancer cell survival, proliferation, and invasion. Ishii *et al.*[4] advocated that the blood in the vicinity of human lung cancers contains fibroblast progenitor cells that have the capacity to migrate into the cancer stroma and differentiate into stromal fibroblasts. Duda *et al.*[5] also revealed that tumor-associated stromal cells shed from the primary tumor together with accompanying cancer cells survive in the blood circulation, as well as at secondary sites, and proliferate within the metastatic nodules. Together, these results suggest that the metastatic process may be affected not only by the characteristics of floating cancer cells, but also by those of circulating stromal cells.

We hypothesized that the intralymphatic microenvironment created by tumor cells and stromal cells has a considerable influence on the metastatic process and that the phenotypes of the tumor cells and infiltrating stromal cells in extratumoral lymphatic vessels may be informative for research into the mechanism of cancer metastasis, including intrapulmonary metastasis. We noted the high prevalence of intrapulmonary metastasis among patients with extratumoral lymphatic permeation of lung adenocarcinoma. The aim of the present study was to determine how the morphologic and immunophenotypic features of tumor cells and infiltrating stromal cells within permeated lymphatic vessels are associated with intrapulmonary metastasis.

Materials and Methods

Patient selection. Between July 1992 and May 2010, 2016 patients with primary lung adenocarcinoma underwent surgical resection at the National Cancer Center Hospital East, Chiba, Japan. After reviewing the medical records, 137 patients with extratumoral lymphatic permeations were chosen for as subjects for the present study.

Histological studies. Surgical specimens were fixed in 10% formalin or 100% methyl alcohol. The specimens were sliced

[3]To whom correspondence should be addressed.
E-mail: gishii@east.ncc.go.jp; aochiai@east.ncc.go.jp

doi: 10.1111/j.1349-7006.2012.02275.x

through the largest diameter of the primary tumor and all sections were embedded in paraffin. We also identified all peripheral subsegmental bronchi and obtained sections containing subsegmental bronchi even in sections without the primary tumor. These sections were also embedded in paraffin. The median number of tissue blocks was 21 pieces for each case. All serial 4-μm sections were stained using H&E, the Alcian blue–periodic acid-Schiff (AB-PAS) method for the detection of cytoplasmic mucin production, or the Elastica van Gieson (EVG) or Victoria–blue van Gieson (VVG) methods for the detection of elastic fibers. All histological materials included in this series were reviewed by two pathologists (YM and GI) to ascertain the presence of extratumoral lymphatic permeation and intrapulmonary metastasis (PM), as well as to assess the histopathological features of both the primary and metastatic tumors. A PM was defined as an independent tumor having the same histopathological features, including the growth pattern, cell size, and nuclear atypia, as the primary tumor and was differentiated from synchronous multiple primary lung cancer.[6] Metastatic nodules that had apparently occurred as a result of aerogenous spreading were excluded from the present study. As a result, 47 of 137 patients were found to have PM. The pathological stage was determined based on the TNM classification of the International Union Against Cancer (renamed Union for International Cancer Control [UICC]).[7] Histological typing of the primary tumors was performed based on World Health Organization classification of cell types,[8] and the types were divided into five subtypes as follows: bronchioloalveolar carcinoma (BAC) (non-mucinous BAC or mucinous BAC), acinar, papillary, solid adenocarcinoma with mucin production, and mixed subtype.[8] Lymphatic permeation was confirmed in all 137 patients based on findings for both H&E- and D2-40-stained sections, with the latter used to evaluate lymphatic endothelial cells. Extratumoral lymphatic permeation was defined as tumor cells existing in the lymphatic vessels outside the primary tumors. Vascular invasion was considered to be present when tumor cells in the blood vessels were identified on EVG- or VVG-stained sections. Pleural invasion was also evaluated based on whether the tumor cells had invaded the visceral pleura of the lung on EVG- or VVG-stained sections.

Morphological assessment of tumor cell nests within lymphatics. Microscopic examinations revealed that tumor nests within extratumoral lymphatic vessels can be divided into two morphologic patterns based on the structure of the nest: (i) "Type A", defined as tumor cells in the permeated lymphatic vessels forming a single large mass without separation (Fig. 1a); and (ii) "Type B", defined as tumor cells in the permeated lymphatic vessels arranged in multiple small clusters (Fig. 1b). More concretely, we defined Type A as having one large cluster consisting of 20 or more combined cancer cells within permeated lymphatic vessels. Even if many cancer cells exist in the lymphatic vessels, small clusters of <20 cancer cells were classified as Type B. When both morphologic patterns were observed in the same section, we adopted the dominant one.

Antibodies and immunohistochemical staining. The markers of cell proliferation and anoikis resistance used in the present study were Gemini (clone EM6; Novocastra, Newcastle-upon-Tyne, UK), cleaved caspase 3 (polyclonal; Cell Signaling Technology, Danvers, MA, USA), and p-Src ($pTyr^{416}$, polyclonal; Calbiochem, Darmstadt, Germany). The markers of adhesion-related molecules were CD44 (clone DF1485; Novocastra), E-cadherin (clone 36; BD Biosciences, San Jose, CA, USA), and fibronectin (clone 568; Leica Microsystems, Newcastle, UK). To evaluate growth factors, we used epithelial growth factor receptor (EGFR; clone H11; Dako Cytomation, Glostrup, Denmark) and insulin-like growth factor receptor (IGFR; Chemicon, Temecula, CA, USA). To evaluate T lymphocytes, B lymphocytes, and activated macrophages, we used CD3 (Leica Microsystems), CD79a (Leica Microsystems), and CD204 (clone SRA-E5; Trans Genic, Hyogo, Japan), respectively. Podoplanin (clone D2-40; Signet, Princeton, NJ, USA) was used as a lymphatic endothelial marker and carbonic anhydrase IX (CA-IX, polyclonal; Novus Biologicals, Littleton, CO, USA) was used as a marker of hypoxia. Immunostaining was performed on 4-μm paraffin-embedded tissue sections. Slides were deparaffinized in xylene and dehydrated in a graded ethanol series, and endogenous peroxidase was blocked with 3% hydrogen peroxide in absolute methyl alcohol. After epitope retrieval, the slides were washed with PBS and incubated overnight with primary antibodies. The reaction products were stained with diaminobenzidine and counterstained with hematoxylin.

Immunohistochemical scoring. All stained tissue sections were scored semiquantitatively and evaluated independently under a

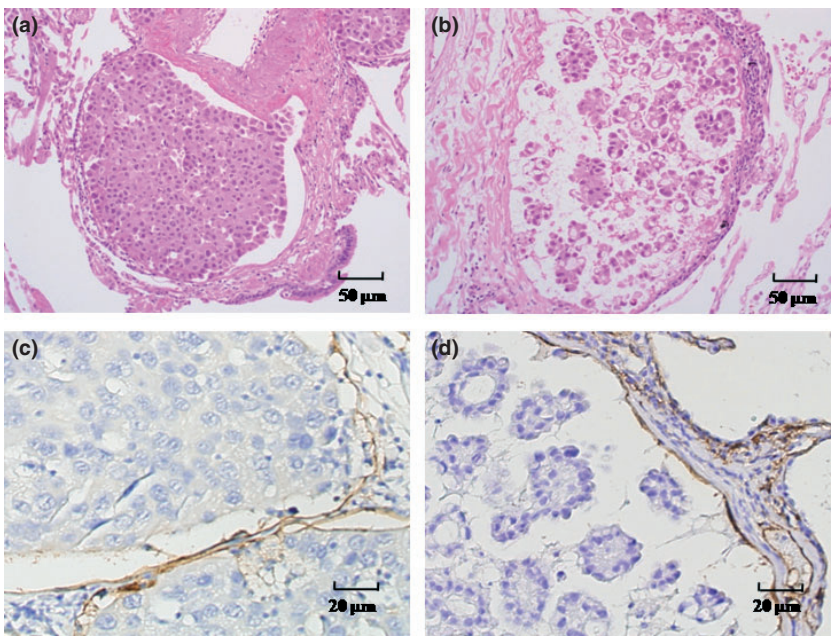

Fig. 1. Morphologic patterns of cancer cell nests within lymphatic vessels. (a,b) Hematoxylin–eosin staining of a "Type A" nest (a) and a "Type B" nest (b). In the Type A nest, tumor cells within the lymphatic vessels form a single large mass without separation, whereas in the Type B nest the tumor cells within the lymphatic vessels are arranged in multiple small clusters. (c,d) Staining using an anti-D2–40 antibody to evaluate Type A (c) and Type B (d) tumor cell nests within lymphatic vessels.

light microscope by two pathologists (YM and GI) who had no knowledge of the patients' clinicopathological data. The labeling scores for the tumor cells within extratumoral lymphatics were calculated by multiplying the percentage of positive tumor cells per each lesion (0–100%) by the staining intensity level (0 = negative; 1 = weak; 2 = strong). For Geminin and cleaved caspase 3, the number of positive tumor cells per 100 tumor cells was counted. For CD3, CD79a and CD 204, the number of positive infiltrating cells was counted under a microscopic at × 400 (area = 0.0625 mm^2). We confirmed that positive control tissues were stained by each antibody and we also performed negative control studies without the primary antigen for all antibodies. When the antibody evaluations differed, the observers discussed the results, re-examining the slides if necessary, until an agreement was reached.

Statistical analysis. The significance of differences between two groups was evaluated using the Mann–Whitney *U*-test or Fisher's exact test. All *P*-values reported are two sided, and the significance level was set at <0.05. Analyses were performed using Dr. SPSS II for Windows, standard version 11.0 (SPSS, Chicago, IL, USA).

Results

Clinicopathologic findings of patients with extratumoral lymphatic permeation. The clinicopathologic characteristics of 137 patients who had adenocarcinoma with extratumoral lymphatic permeation (Ly^{ext}(+)) and the other 1879 patients are given in Table 1. Men, ex or current smokers, patients with high serum carcinoembryonic antigen (CEA) levels, those with pathological T2–3, and pleural invasion were significantly more likely to be Ly^{ext}(+). Furthermore, significantly more cases with lymph node metastasis (pN1–2) and PM were found among the Ly^{ext}(+) patients.

Morphological patterns of tumor nests within extratumoral lymphatic vessels. The tumor nests within the lymphatic vessels were divided morphologically into two groups, Type A and Type B. Fifty-four patients were found to have "Type A" tumor nests (i.e. tumor cells formed a large solid nest within the permeated lymphatic vessels; Fig. 1a,c). Eighty-three patients were found to have "Type B" tumor nests (i.e. tumor cells were arranged in multiple small clusters within the permeated lymphatic vessels; Fig. 1b,d). When both morphologic patterns were observed in the same section, we adopted the dominant one. Figure S1, available as Supplementary Material to this paper, shows the distribution of cases with extratumoral lymphatic permeation according to the rate of Type A and Type B tumor nests. There were 36 patients with 100% Type A tumor nests, 25 with 100% Type B tumor nests, and 76 patients with mixed Type A and Type B tumor nests. Patients with 50% of both type (n = 7) were classified as having Type B tumor nests. Table 2 gives the clinicopathologic characteristics of patients with Type A and Type B tumor nests. In the group in which Type B tumor nests were dominant, significantly more patients had a papillary predominant pattern and PM, whereas fewer patients had a solid predominant pattern, compared with the Type A group.

Clinicopathologic factors related to PM. The clinicopathologic characteristics of cases with or without PM are summarized in Table 3. No significant differences in gender, age, smoking history, CEA, primary tumor size, pathological N status, or pleural invasion were observed between the two groups, but

Table 1. Clinicopathological characteristics of all patients

	Ly^{ext}(+) (n = 137)	Ly^{ext}(−) (n = 1879)	*P*-value
Gender			
Men (*n*)	92	961	<0.001
Women (*n*)	45	918	
Age range (years)	41–84	20–92	
Median age (years)	66	65	
Smoking habit			
Never smoked (*n*)	45	842	0.009
Ex or current smoker (*n*)	92	1018	
Carcinoembryonic antigen (ng/mL)			
≤5 (*n*)	71	1231	0.0017
>5 (*n*)	66	640	
Median tumor size (cm)	3.0	2.5	0.006
T status			
pT1 (*n*)	32	981	
pT2–4 (*n*)	105	898	<0.001
N status			
pN0 (*n*)	21	1382	<0.001
pN1–2 (*n*)	110	423	
Pleural invasion			
No (*n*)	53	1341	<0.001
Yes (*n*)	85	538	
Intrapulmonary metastasis			
No (*n*)	90	1763	<0.001
Yes (*n*)	47	116	

Ly^{ext}, extratumoral lymphatic permeation.

Table 2. Clinicopathological characteristics of Type A- and Type B-dominant patients

	Type A dominant (n = 54)	Type B dominant (n = 83)	*P*-value
Gender			
Men (*n*)	40	52	0.20
Women (*n*)	14	31	
Age range (years)	42–84	42–82	0.58
Median age (years)	65	64	
Smoking habit			
Never smoked (*n*)	14	31	0.20
Ex or current smoker (*n*)	40	52	
Carcinoembryonic antigen (ng/mL)			
≤5 (*n*)	30	41	0.49
>5 (*n*)	24	42	
Tumor size (cm)	1–16	1.2–12	0.29
Median size (cm)	3.2	2.8	
Predominant subtype			
Lepidic growth pattern (*n*)	2	3	0.65
Acinar (*n*)	12	17	0.48
Papillary (*n*)	9	48	<0.01
Micropapillary (*n*)	0	0	–
Solid (*n*)	31	15	<0.01
Pathological T status			
pT1 (*n*)	14	18	0.35
pT2 (*n*)	27	30	
pT3–4 (*n*)	13	35	
Pathological N status			
pN0 (*n*)	12	14	0.51
pN1–2 (*n*)	42	69	
Pleural invasion			
No (*n*)	20	33	0.86
Yes (*n*)	34	50	
Intrapulmonary metastasis			
No (*n*)	43	47	<0.01
Yes (*n*)	11	36	

Ly^{ext}, extratumoral lymphatic permeation.

doi: 10.1111/j.1349-7006.2012.02275.x

the group with PM contained significantly more cases with a Type B morphologic pattern than a Type A pattern. These results suggest that the morphologic characteristics of the tumor cell nests within the extratumoral lymphatics are closely related to PM.

Immunophenotypic characteristics of tumor cells forming cancer cell nests within extratumoral lymphatics. Based on the morphological analysis, we postulated that tumor cells forming "Type B" clusters may exhibit a metastatic preference for the lung parenchyma via a lymphatic route. To examine the biological characteristics of these tumor cells, we compared the immunophenotypes of the tumor cells forming "Type A" and "Type B" clusters (Fig. 2). Twenty cases from each of the "Type A" and "Type B" groups were selected. Although their clinicopathological characteristics are not shown, the only significant difference between the two groups was in the number of PM. The staining scores are summarized in Table 4.

Table 3. Clinicopathological characteristics of patients exhibiting extratumoral lymphatic permeation, with or without intrapulmonary metastasis

	Ly^{ext}(+) and PM(−) (n = 90)	Ly^{ext}(+) and PM(+) (n = 47)	P-value
Gender			
Men (n)	62	30	0.57
Women (n)	28	17	
Age range (years)	41–82	44–84	0.06
Median age (years)	65	67	
Smoking habit			
Never smoked (n)	27	18	0.34
Ex or current smoker (n)	63	29	
Carcinoembryonic antigen (ng/mL)			
≤5 (n)	49	22	0.47
>5 (n)	41	25	
Tumor size (cm)	1.0–16	1.3–12	
Median size (cm)	2.9	3.5	0.15
N status			
pN0 (n)	18	8	0.82
pN1–2 (n)	72	39	
Pleural invasion			
No (n)	37	16	0.46
Yes (n)	53	31	
Vascular invasion			
No (n)	24	8	0.29
Yes (n)	66	39	
Morphologic pattern of intralymphatic tumor cells			
Type A (n)	43	11	0.006
Type B (n)	47	36	

Ly^{ext}, extratumoral lymphatic permeation; PM, intrapulmonary metastasis.

Cell proliferation and anoikis resistance markers. The mean (±SE) positive ratio for Geminin in the Type A and Type B groups was 20.3 ± 2.6% and 11.9 ± 2.5%, respectively. Mean (±SE) staining scores in the Type A and Type B groups were 7.8 ± 3.3% and 2.4 ± 0.5%, respectively, for cleaved caspase 3 and 33.5 ± 9.9 and 22.5 ± 6.2, respectively, for p-Src. The expression of Geminin and cleaved caspase 3 was significantly lower in the Type B group than in the Type A group (P = 0.008 and P = 0.01, respectively), but there was no significant difference in p-Src expression between the two groups (P = 0.78).

Adhesion-related molecules. The mean (±SE) staining scores in the Type A and Type B groups were 20.5 ± 9.6 and 43.0 ± 10.4, respectively, for CD44; 31.5 ± 6.9 and 60.5 ± 9.3, respectively, for E-cadherin; and 18.5 ± 6.5 and 9.5 ± 3.6, respectively, for fibronectin. The expression of CD44 and E-cadherin was significantly higher in the Type B than Type A group (P = 0.04 and P = 0.02, respectively), but there was no significant difference in fibronectin levels between the two groups (P = 0.50).

Growth factor receptors. The mean (±SE) staining scores in the Type A and Type B groups were 9.0 ± 5.4 and 25.5 ± 9.0, respectively, for EGFR; 72.5 ± 11.5 and 92.0 ± 13.3, respectively, for IGFR; and 49.8 ± 5.4 and 47.3 ± 10.3, respectively, for c-Met. The expression of EGFR was significantly higher in the Type B than Type A group, but there were no significant differences in the expression of IGFR or c-Met between the two groups (P = 0.29 and P = 0.86, respectively).

Stromal cells around the cancer nest. The mean (±SE) number of cells in the Type A and Type B groups was 4.6 ± 1.4 and 9.5 ± 1.0, respectively, for infiltrating CD204-positive macrophages; 3.8 ± 3.4 and 8.7 ± 7.5, respectively, for CD3-positive T lymphocytes; and 1.3 ± 1.7 and 0.2 ± 0.2, respectively, for CD79a-positive B lymphocytes. No significant differences were observed between the two groups in the expression of CD3 and CD79a (P = 0.14 and P = 0.72, respectively), but the expression of CD204 was significantly higher in the Type B group than in the Type A group (P = 0.007).

Others. The mean (±SE) staining score for CA-IX, a marker of hypoxia, in the Type A and Type B groups was 17.0 ± 4.0 and 16.5 ± 4.7, respectively. There was no significant difference in CA-IX expression between the two groups.

Immunophenotypic characteristics of tumor cells among the primary tumors. In the same 40 cases described above, we also evaluated the immunophenotypes of the tumor cells among the primary tumors. (Note, the staining scores are not shown.) In this examination, only the expression of cleaved caspase 3

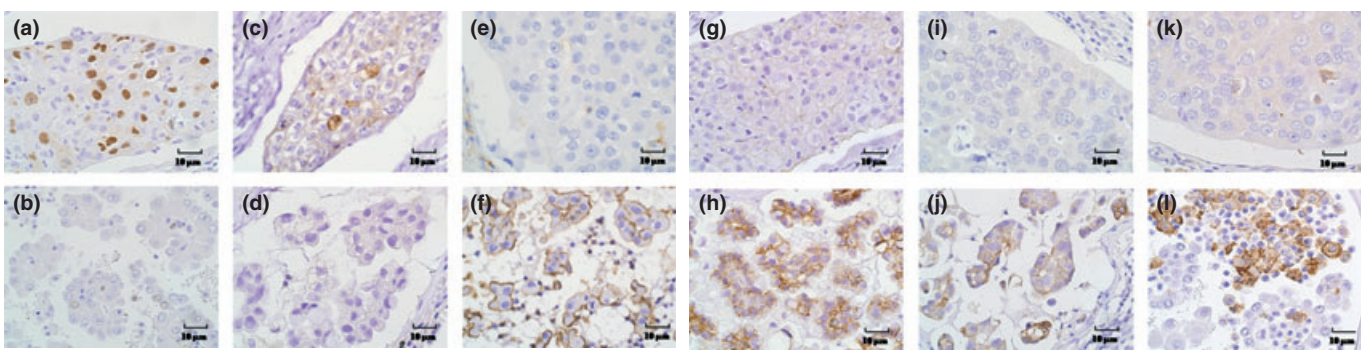

Fig. 2. Immunohistochemical staining of (a,c,e,g,i,k) Type A and (b,d,f,h,j,l) Type B tumor cell nests within permeated lymphatic vessels. (a,b) Geminin, (c,d) cleaved caspase-3, (e,f) CD44, (g,h) E-cadherin, (i,j) epidermal growth factor receptor, and (k,l) CD204.

Table 4. Immunohistochemical staining scores of tumors in lymphatic vessels

Antibodies and their purpose	Type A dominant (n = 20)	Type B dominant (n = 20)	P-value
Proliferation and anoikis resistance			
Geminin	**20.3 ± 2.6**	**11.9 ± 2.5**	**0.008**
Cleaved caspase 3	**7.8 ± 3.3**	**2.4 ± 0.5**	**0.01**
p-Src	33.5 ± 9.9	22.5 ± 6.2	0.78
Adhesion-related molecules			
CD44	**20.5 ± 9.6**	**43.0 ± 10.4**	**0.04**
E-Cadherin	31.5 ± 6.9	60.5 ± 9.3	0.02
Fibronectin	18.5 ± 6.5	9.5 ± 3.6	0.50
Laminin-5γ3	21.5 ± 6.7	29.7 ± 9.0	0.47
Growth factor receptors			
EGFR	**9.0 ± 5.4**	**25.5 ± 9.0**	**0.04**
IGFR	72.5 ± 11.5	92 ± 13.3	0.29
c-Met	49.8 ± 9.4	47.3 ± 10.3	0.86
Stromal cells			
CD3	3.8 ± 3.4	8.7 ± 7.5	0.14
CD79a	1.3 ± 1.7	0.2 ± 0.2	0.72
CD204	**4.6 ± 1.4**	**9.5 ± 2.0**	**0.007**
Others			
CA-IX	17.0 ± 4.0	16.5 ± 4.7	0.78

Data are the mean ± SD. Bolded typeface indicates significant differences. EGFR, epidermal growth factor receptor; IGFR, insulin-like growth factor receptor; CA-IX, carbonic anhydrase IX.

differed significantly between the two groups, being lower in the Type B than Type A group.

Discussion

During the early phase of metastatic tumor development, floating cancer cells proliferate within the vessel lumen, adhere to the endothelium, extravasate from the vessel lumen, migrate into the connective tissue surrounding the vessels, and invade the target organ parenchyma. During each of these processes, cancer cells, accompanied by the surrounding stromal cells, organize their peculiar microenvironment. The present study is the first to show that pulmonary metastasis is correlated with the morphologic and immunophenotypic characteristics of intralymphatic cancer cells and infiltrating stromal cells.

E-Cadherin is now regarded as a key marker for EMT. In the field of carcinogenesis, EMT has been recognized as a phenomenon during which tumor cells in primary lesions invade the surrounding stroma, repressing the transcription of the adherens junction protein E-cadherin.[9–11] Recently, many studies have postulated that the loss or repression of E-cadherin is strongly linked with cancer invasiveness, metastasis, and patient prognosis.[9,12] In contrast with these reports on the repression of E-cadherin and tumor invasiveness, the expression E-cadherin in the present study was elevated in the Type B group, even though this group exhibited more PM than the Type A group. Wells *et al.*[11] discussed the role of E-cadherin in the cancer-associated mesenchymal-epithelial reverting transition (MErT) at distant metastatic sites and speculated that micrometastases may undergo a transition to become E-cadherin positive, just as the critical EMT event is the downregulation or silencing of E-cadherin. Others[13,14] have also reported that the expression of E-cadherin at the metastatic site is higher than that at the primary site, consistent with the results of the present study.

Epithelial growth factor receptor is a receptor tyrosine kinase that plays essential roles under both normal physiological and cancerous conditions. Ligand binding to the EGFR leads to epithelial cell migration away from cohesive masses secondary to E-cadherin downregulation.[11] Some studies[15,16] have reported that inhibition of the autocrine EGFR loop results in E cadherin re-expression and cell–cell cohesion. These reports are in contrast with our results, in which the expression of EGFR was higher in the Type B group than in the Type A group, although the expression of E-cadherin was also higher in the Type B group. These findings suggest that these morphologic and immunophenotypic differences in the tumor cells within lymphatic vessels are not associated with EMT.

CD44 is an integral membrane glycoprotein that functions as a receptor for the extracellular matrix glycan hyaluronan. The expression of CD44 in the Type B group was higher than that in the Type A group, similar to the results for E-cadherin. These findings indicate that an adhesive ability may be deeply associated with the intrapulmonary metastatic process. Some studies[17–20] have also reported that the expression of CD44 is associated with cancer metastasis. Tomlinson *et al.*[21] examined cell lines and human cancers and reported that, as a result of homophilic and homodimeric interactions among E-cadherin, the E-cadherin/α,β-catenin axis-associated adhesive network mediates tumor cell–tumor cell aggregates that are characteristic of the lymphovascular emboli observed during lymphovascular invasion.

The lower expression of Geminin and cleaved caspase 3 in the Type B group indicates that lower proliferative and apoptotic activities are present in this group. In a review, Wells *et al.*[11] reported that metastatic seed cancer cells may inertly become part of the ectopic tissue and therefore surmount the metastatic inefficiencies to which most disseminated cancer cells succumb. Many other studies have also suggested that tumor cells with metastatic potential have a repressed proliferative activity and the ability to resist apoptosis,[3,10,22] also known as anoikis.

The tyrosine kinase Src is one of the key molecules that play a critical role in the development of resistance to apoptosis, known as anoikis.[23,24] However, in present study no significant differences in the expression of p-Src were observed between the Type A and Type B groups. This result does not coincide with the results for cleaved caspase 3, which indicated a lower level of apoptosis in the Type B group. The environment within the lymphatic vessels was inappropriate for tumor cells, so the anoikis resistance process via p-Src may be fully activated in both the Type A and Type B groups. Thus, another mechanism underlying apoptotic resistance within lymphatic vessels may exist.

Regarding stromal cells around cancer nests, Ohtaki *et al.*[25] recently suggested that CD204-positive macrophages clearly reflect the tumor-promoting phenotype of tumor-associated macrophages in lung adenocarcinoma. In the present study, the number of infiltrating CD204-positive macrophages around and within the cancer nests was significantly higher in the Type B group than in the Type A group. This implies that not only tumor cells, but also stromal cells (such as CD204-positive macrophages) may contribute to changing the intralymphatic microenvironment. Zhang *et al.*[26] also advocated that the intravascular microenvironment is a critical staging area for the development of metastases that subsequently invade the parenchyma.

We hypothesized that there may be hypoxic area in the center of the Type A cluster, so we performed CA-IX immunostaining, However, there were no significant differences in CA-IX staining scores between the Type A and Type B groups.

Conversely, in the primary tumors, no significant differences were observed in immunohistochemical staining scores between Type A and Type B groups, except for that of cleaved caspase 3. These differences in staining scores between the

doi: 10.1111/j.1349-7006.2012.02275.x

intralymphatic cancer nests and primary tumors may be explained by the possibility that primary tumors are composed of a strongly heterogeneous cell population phenotypically and functionally. Conversely, intralymphatic tumors may be comprised of a relatively less heterogeneous cell population. Alternatively, cancer cells that have already permeated into lymphatic vessels perceive signals from circulating stromal cells, which lead to phenotypic changes to the cancer cells. However, we could not find any apparent differences in lymphatic vessel morphology and characteristics between Type A and Type B groups in the present study.

In conclusion, the present study clearly shows an association between the morphological and immunophenotypic features of tumor cells and stromal cells within lymphatics and pulmonary metastasis. From this finding, metastasis is predicted to proceed through dynamic changes in the intralymphatic microenvironment, demonstrating that the lymphatic vessels are far from merely a conduit connecting the primary site with the metastatic site. A more mechanistically oriented experimental approach is required to elucidate the key regulator(s) of the intralymphatic microenvironment, possibly leading to useful therapeutic options in the years to come.

Acknowledgments

The authors thank Shinya Yanagi for technical support. This work was supported by a Grant-in-Aid for Cancer Research (19–10) from the Ministry of Health, Labour, and Welfare Programs; the Foundation for the Promotion of Cancer Research, 3rd-Term Comprehensive 10-Year Strategy for Cancer Control; Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation; and JSPS KAKENHI (20590417).

Disclosure

All work reported herein was performed at the National Cancer Center Hospital East, Kashiwa, Chiba, Japan. The research was approved by the Internal Review Board of the institution. No patient consent was required as the research is a retrospective chart review and no personally identifiable information was included in the manuscript. The authors have no conflict of interest to disclose.

References

1 Aokage K, Ishii G, Ohtaki Y *et al.* Dynamic molecular changes associated with epithelial–mesenchymal transition and subsequent mesenchymal–epithelial transition in the early phase of metastatic tumor formation. *Int J Cancer* 2010; **128**: 1585–95.
2 Saijo T, Ishii G, Ochiai A *et al.* Evaluation of extratumoral lymphatic permeation in non-small cell lung cancer as a means of predicting outcome. *Lung Cancer* 2007; **55**: 61–6.
3 Sakuma Y, Takeuchi T, Nakamura Y *et al.* Lung adenocarcinoma cells floating in lymphatic vessels resist anoikis by expressing phosphorylated Src. *J Pathol* 2010; **220**: 574–85.
4 Ishii G, Ito TK, Aoyagi K *et al.* Presence of human circulating progenitor cells for cancer stromal fibroblasts in the blood of lung cancer patients. *Stem Cells* 2007; **25**: 1469–77.
5 Duda DG, Duyverman AM, Kohno M *et al.* Malignant cells facilitate lung metastasis by bringing their own soil. *Proc Natl Acad Sci USA* 2010; **107**: 21677–82.
6 Girard N, Deshpande C, Lau C *et al.* Comprehensive histologic assessment helps to differentiate multiple lung primary nonsmall cell carcinomas from metastases. *Am J Surg Pathol* 2009; **33**: 1752–64.
7 Union for International Cancer Control (UICC). *TNM Classification of Malignant Tumours*, 6th edn. Geneva: UICC, 2002.
8 Travis W, Coblby T, Corrin B, Shimosato Y, Brambilla E. *Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*, 3rd edn. Berlin: Springer Verlag, 1999.
9 Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 2008; **68**: 3645–54.
10 Thiery JP. Epithelial–mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; **2**: 442–54.
11 Wells A, Yates C, Shepard CR. E-Cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* 2008; **25**: 621–8.
12 Ono K, Sugio K, Uramoto H *et al.* Discrimination of multiple primary lung cancers from intrapulmonary metastasis based on the expression of four cancer-related proteins. *Cancer* 2009; **115**: 3489–500.
13 Imai T, Horiuchi A, Shiozawa T *et al.* Elevated expression of E-cadherin and alpha-, beta-, and gamma-catenins in metastatic lesions compared with primary epithelial ovarian carcinomas. *Hum Pathol* 2004; **35**: 1469–76.
14 Kowalski PJ, Rubin MA, Kleer CG. E-Cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast Cancer Res* 2003; **5**: R217–22.
15 Yates CC, Shepard CR, Stolz DB, Wells A. Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. *Br J Cancer* 2007; **96**: 1246–52.
16 Mamoune A, Kassis J, Kharait S *et al.* DU145 human prostate carcinoma invasiveness is modulated by urokinase receptor (uPAR) downstream of epidermal growth factor receptor (EGFR) signaling. *Exp Cell Res* 2004; **299**: 91–100.
17 Kargi HA, Kuyucuoglu MF, Alakavuklar M, Akpinar O, Erk S. CD44 expression in metastatic and non-metastatic non-small cell lung cancers. *Cancer Lett* 1997; **119**: 27–30.
18 Sheridan C, Kishimoto H, Fuchs RK *et al.* CD44 + /CD24− breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* 2006; **8**: R59.
19 Singleton PA, Salgia R, Moreno-Vinasco L *et al.* CD44 regulates hepatocyte growth factor-mediated vascular integrity. Role of c-Met, Tiam1/Rac1, dynamin 2, and cortactin. *J Biol Chem* 2007; **282**: 30643–57.
20 Valentiner U, Valentiner FU, Schumacher U. Expression of CD44 is associated with a metastatic pattern of human neuroblastoma cells in a SCID mouse xenograft model. *Tumour Biol* 2008; **29**: 152–60.
21 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**: 5231–41.
22 Al-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A, Muschel RJ. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. *Nat Med* 2000; **6**: 100–2.
23 Wei L, Yang Y, Zhang X, Yu Q. Altered regulation of Src upon cell detachment protects human lung adenocarcinoma cells from anoikis. *Oncogene* 2004; **23**: 9052–61.
24 Windham TC, Parikh NU, Siwak DR *et al.* Src activation regulates anoikis in human colon tumor cell lines. *Oncogene* 2002; **21**: 7797–807.
25 Ohtaki Y, Ishii G, Nagai K *et al.* Stromal macrophage expressing CD204 is associated with tumor aggressiveness in lung adenocarcinoma. *J Thorac Oncol* 2010; **10**: 1507–15.
26 Zhang Q, Yang M, Shen J, Gerhold LM, Hoffman RM, Xing HR. The role of the intravascular microenvironment in spontaneous metastasis development. *Int J Cancer* 2010; **126**: 2534–41.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The rate of Type A and Type B cancer cell nests in patients with extratumoral lymphatic permeation.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.