

Lymphatics-associated genes are downregulated at transcription level in non-small cell lung cancer

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Abstract. The present study aimed to verify a possibility of ongoing lymphangiogenesis in non-small cell lung cancer (NSCLC) via examination of mRNA levels of a number of lymphangiogenesis-associated genes in tumors. It was hypothesized that transcriptional activation of these genes would occur in tumors that stimulate new lymphatic vessel formation. The study was performed on 140 pairs of fresh-frozen surgical specimens of cancer and unaffected lung tissues derived from NSCLC stage I-IIIa patients. mRNA levels were evaluated with the reverse transcription-quantitative polymerase chain reaction method and expressed as fold change differences between the tumor and normal tissues. Possible associations between expression and patient clinicopathological characteristics and survival were analyzed. In the NSCLC tissue samples, vascular endothelial growth factor (VEGF) C, VEGFD, VEGFR3, VEGFR2, VEGFR1, lymphatic vessel endothelial hyaluronan receptor 1, integrin subunit α 9, FOX2, neuropilin 2, fibroblast growth factor 2 genes were significantly downregulated ($P < 0.001$ for all) compared with matched normal lung tissues, whereas mRNA levels for VEGFA, spleen associated tyrosine kinase, podoplanin, and prospero homeobox 1 genes were similar in both tissues. Neither lymph node status, nor disease pathological stage influenced expression, whereas more profound suppression of gene activities appeared to occur in squamous cell carcinomas compared with adenocarcinomas. The VEGFR1 mRNA expression level was significantly connected with patient survival in the univariate analysis, and was an independent prognostic factor for overall survival in the multivariate Cox's proportional hazards model (HR 2.103; 95% confidence interval: 1.005-4.401; $P = 0.049$). The results support a hypothesis of absence of new lymphatic

vessel formation inside growing NSCLC tumor mass, however do not exclude a possibility of lymphangiogenesis in narrow marginal tumor parts.

Introduction

The lymphatic system forms an extensive network of low shear force vessels that penetrates almost all organs of the human body. It plays a key role in the maintenance of tissue-fluid homeostasis and is essential for the immune system functioning (1). Lymphatic vasculature has long been considered one of the main routes of solid tumors metastatic dissemination to distant organs (2,3). Highly-permeable and comparatively wide lymphatic capillaries seem to be well accommodated to tumor cell transport from the primary tumor mass into the blood circulation. Sentinel lymph nodes that directly drain primary tumors are usually the first sites of detectable metastases. Histological examination of these and nearby lymph nodes is routinely used for determining the stage of disease progression and for prediction of patients' survival (4). Moreover, it has become clear that lymphatics profoundly affects cancer progression (5). Growing evidence indicates that direct modulation of immune cell functions by lymphatic endothelial cells (LECs) may be essential for both antitumor immune response at early stages of tumor progression and subsequent cancer-induced immunosuppression (6). Based on these assumptions, it has been proposed that tumors may stimulate formation of new lymphatic vessel via process of lymphangiogenesis in a manner analogous to tumor angiogenesis, thereby promoting both tumorigenesis and lymphagenous metastasis (3,5,7).

Evidence for ongoing lymphangiogenesis inside growing tumors was initially provided from animal studies. In experimental models of cancer, forced formation of intratumor lymphatic vasculature increased tumor aggressiveness and facilitated metastatic spread (8-11), while inhibition of the lymphangiogenesis prevented lymph node and distant metastases without significantly affecting primary tumor growth (12,13). In agreement with these data, numerous clinical studies demonstrated an association between tumor expression of lymphatic-specific growth factors or lymph vessel density and tumor progression or poor patient survival (14-16). However, a lack of the correlation as well

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as an absence of proliferating LECs in the primary tumors were reported by others (17,18). Moreover, detailed histological analyses of various solid tumors frequently failed to reveal lymphatic vessels throughout tumor masses except the periphery of these tumors (19-21), suggesting a lack of ongoing lymphangiogenesis. Besides, growing evidence suggests that lymphatics suppression might be favorable for tumor growth at early stages of cancer progression due to anti-tumor immune response weakening (22).

Thus, formation of new lymphatic vessels in growing human tumors remains an unresolved question. In order to evaluate a probability of lymphangiogenesis induction in non-small cell lung cancer (NSCLC), we performed a comprehensive analysis of the transcriptional activity of 15 genes encoding lymphatics regulators or markers (23-26). Using a comparative quantitative polymerase chain reaction (qPCR) method we examined the expression at mRNA level of the vascular endothelial growth factors: VEGFA, VEGFC, and VEGFD/FIFG, their receptors: VEGFR1/FLT1, VEGFR2/KDR, and VEGFR3/FLT4 and co-receptors neuropilin 2 (NRP2) and integrin $\alpha 9$ subunit (ITG9), basic fibroblast growth factor 2 (FGF2), transcription factors: prospero-related homeobox domain 1 (PROX1) and Forkhead box C2 (FOXC2), lymphatic-specific membrane proteins: lymphatic vessel hyaluronan receptor 1 (LYVE1) and glomerular podocyte mucoprotein podoplanin (PDPN), spleen protein kinase (SYK) and key component of desmosomal plaque proteins: desmoplakin (DSP). A brief characteristics of the analyzed factors is presented in Table I. Transcript levels were evaluated by comparison to those in non-malignant lung tissue and analyzed in terms of patients' clinicopathological characteristics.

Materials and methods

Patients and samples. The study was performed on 140 pairs of tumor and matched unaffected lung tissue specimens obtained from I-IIIa stage NSCLC patients who underwent a curative surgery at the Bialystok Medical University Hospital between 2000 and 2010. Disease staging was performed according to the seventh edition of the tumor-nodes-metastasis system (TNM) for lung cancer (27). None of the patients received chemo- or radiotherapy before the surgery. All of them gave the written informed consent for specimen collection and clinicopathological data processing. The study design was approved by the Ethics Committee of the University.

Tissue samples were collected intraoperatively and processed immediately after surgical removal according to the systematic biobanking quality (28). After the macroscopic visual assessment, the tumors were divided into two sections. One of them was fixed in formalin followed by paraffin embedding, and the other was divided into small pieces (approximately 0.5 cm in diameter) and frozen in liquid nitrogen followed by storage at -80°C . Unaffected lung parenchyma specimens were dissected from the same lobe or lung of the patient at an area at least 5 cm distant from the tumor and processed similarly to tumor specimens. Prior to RNA extraction, the cross-sections of frozen tissue samples were stained with hematoxylin-eosin and evaluated by an experienced pathologist (L.C.) to confirm the suitability of cell content. Namely, tumor specimens with the highest percentage of the malignant cells (but at least

60% of tumor cells on a microscopic section) and normal lung epithelium without metaplasia or dysplasia were used for further processing.

RNA extraction. Total RNA was isolated from tissue specimens by magnetic extraction method on EasyMag machine (bioMerieux, Marcy l'Étoile, France) according to the producer's protocol. The resulting RNA was transcribed into cDNA in a reaction with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the producer's recommendations.

mRNA expression level. For an mRNA level evaluation a TaqMan Low Density Array analysis was used: For each sample, amplification of all the analyzed transcripts was performed simultaneously in the MicroFluid Cards (Applied Biosystems; Thermo Fisher Scientific, Inc.) that contained manufactory loaded and dried commercially available primers/probe sets for gene expression examination (Assays-on-Demand; Applied Biosystems; Thermo Fisher Scientific, Inc.). Gene symbols and Assay-on-Demand accession numbers are summarized in Table I. Ribosomal 18S RNA (18SrRNA) gene with a relatively low level of expression variability in lung cancer cell lines and clinical specimens (29) was used to normalize for the differences in the input cDNA concentration. Each channel of a card was loaded with 100 μl of the reaction mixture containing 50 μl 2X TaqMan Gene Expression Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 20 μl of a cDNA solution (corresponding to 100 ng of total RNA). The amplification was performed with ABI PRISM 7900HT Sequence Detection System equipped with the SDS v.2.4 software for baseline and C_q calculations. The cycling conditions were as follows: 50°C for 2 min followed by 95°C for 10 min hold, 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Each sample was analyzed in triplicate. The raw C_q data for each mRNA (C_q) was normalized as follows: $\Delta C_q = C_q - C_{q \text{ ref}}$, where $C_{q \text{ ref}}$ equaled the C_q value of the reference 18SrRNA gene. Tumor-associated fold-changes (FC) in gene activities (relative expression) were calculated as follows: $\text{FC} = 2^{-\Delta\Delta C_q}$, where $\Delta\Delta C_q$ equaled the differences between normalized expressions of the analyzed gene in tumor (ΔC_{qT}) and nonmalignant lung tissue (ΔC_{qN}) from the same patient ($\Delta\Delta C_q = \Delta C_{qT} - \Delta C_{qN}$) (30). To examine possible associations between gene activity and patients' clinicopathological characteristics or survival, $\log_2\text{FC}$ values were used. For survival analysis a median $\log_2\text{FC}$ for each gene was used as a cutoff and the expression was categorized as high (equal or higher than the median) or low (lower than the median).

Statistical analysis. The differences in mRNA expression levels between the tumor and unaffected lung tissues were analyzed with paired Wilcoxon rank-sum test. The Wilcoxon rank-sum or Kruskal-Wallis rank tests were used to analyze the associations between clinicopathological characteristics and mRNA expression levels. OS was calculated and plotted with Kaplan-Meier method with the log-rank test for comparison between the groups. Cox proportional hazards method was used to evaluate the effect of clinicopathological and molecular variables on OS. $P < 0.05$ was considered to indicate a statistically significant difference. All the statistical

Table I. Brief characteristics of the analysed genes.

Gene symbol ^a	Brief characteristics of the encoded protein role in lymphatic system development and functioning	Assay ID	(Refs.)
<i>VEGFC</i>	Vascular endothelial growth factor C, a VEGF family member, is the most potent inducer of lymphatic endothelial cell migration and sprouting, is a ligand for the receptor tyrosine kinases VEGFR2 and VEGFR3	Hs01099203_m1	(5,25)
<i>VEGFD/FIGF</i>	Vascular endothelial growth factor D, a VEGF family member, is an inducer of lymphatic sprouting	Hs01128657_m1	(5,25)
<i>VEGFA</i>	Vascular endothelial growth factor A, a founder of VEGF family, a key regulator of tumor angiogenesis, but also essential for lymphatic vessel formation	Hs00900055_m1	(23,33)
<i>VEGFR3/FLT4</i>	Vascular endothelial growth factor receptor 3, fms-like tyrosine kinase 4, the main receptor for VEGFC, also binds VEGFD, is expressed by lymphatic endothelial cells, on some blood vessels and stem cells	Hs01047677_m1	(5,25)
<i>VEGFR2/KDR</i>	Vascular endothelial growth factor receptor 2, is found in blood vessels and in a subset of lymphatic vessels, binds vascular growth factors VEGFA and VEGFC	Hs00911700_m1	(23,33)
<i>VEGFR1/FLT1</i>	Vascular endothelial growth factor receptor 1, fms-like tyrosine kinase 1, VEGFA receptor	Hs01052961_m1	(23,33)
<i>NRP2</i>	Neuropilin-2-VEGFR3 co-receptor is found on lymphatic vessels, binds the lymphangiogenic growth factors VEGFC and VEGFD, also expressed on veins	Hs00187290_m1	(60,61)
<i>ITGA9</i>	Integrin a9, cell-matrix adhesion receptor, is critical for lymphatic valve maturation	Hs00979865_m1	(59)
<i>LYVE1</i>	Lymphatic vessel hyaluronan receptor, is strongly expressed on the surface of lymphatic endothelial cells of growing vessels during lymphangiogenesis, and also on some blood vessels and macrophages; participates in cell migration and differentiation	Hs00272659_m1	(35,36)
<i>PDPN</i>	Podoplanin-glomerular podocyte mucoprotein, is expressed on lymphatic but not on blood vessel endothelium, osteoblasts, renal podocytes, lung alveolar cells; participates in cell motility	Hs00366766_m1	(38,54)
<i>PROX1</i>	Prospero-related homeobox domain 1 transcription factor, plays a key role for lymphatic endothelial cell differentiation and maintenance of their identity	Hs00896293_m1	(40,41)
<i>FOXC2</i>	Forkhead box C2 transcription factor, is essential for the normal development of the lymphatic system	Hs00270951_s1	(37)
<i>FGF2</i>	Fibroblast growth factor 2, is important for tumor angiogenesis but also promotes lymphangiogenesis via an indirect mechanism involving VEGFC/VEGFR3 signaling	Hs00266645_m1	(55)
<i>SYK</i>	Spleen tyrosine kinase, possible indirect role through inhibition of cell motility and enhancement of cell-cell interactions	Hs00895377_m1	(58)
<i>DSP</i>	Desmoplakin, a key component of desmosomal plaque proteins, may contribute to vessel formation	Hs00950591_m1	(56,57)

^aAccording to HUGO Gene Nomenclature Committee.

analyses in this study were performed using STATA/SE 11.1 software (Stata Corporation, College Station, TX, USA).

Results

Patient characteristics. A total of 140 NSCLC patients, aged from 39 to 79 years (mean 62, standard deviation 8.0 years),

were included in the study. The majority of the patients (117 out of 140, 84%) were males. Among the patients, 57 (41.4%) had lung adenocarcinoma (ADC), 66 (47.1%) had squamous cell carcinoma (SCC), and the remaining 17 (11.4%) had large cell lung carcinoma (LCC). Forty-five tumors were recognized as highly differentiated (grade 1 or 2), and fifty-five were lowly differentiated ones (grade 3 or 4). Lymph node metastasis was

Table II. Gene expression at mRNA level in tumor and non-tumor lung tissue [$\log_2(\Delta Cq)$] and the difference in the log-FC between the paired tissues [$\log_2(FC)$].

Gene symbol	N	mRNA level [$\log_2(\Delta Cq)$]		P-value	Difference in mRNA level between tumor and normal lung tissues [$\log_2(FC)$] Me (25-75%)
		Tumor tissue Me (25-75%)	Normal lung tissue Me (25-75%)		
<i>VEGFC</i>	136	17.67 (16.33-18.54)	16.58 (15.39-17.84)	<0.0001	-0.92 (-1.88-0.06)
<i>VEGFD</i>	136	18.75 (16.37-21.74)	15.57 (14.27-16.55)	<0.0001	-2.72 (-5.92-0.61)
<i>VEGFR3</i>	136	18.72 (17.65-19.53)	17.39 (16.79-18.27)	<0.0001	-0.89 (-1.95-0.16)
<i>LYVE1</i>	136	18.91 (17.15-20.23)	16.69 (15.31-17.68)	<0.0001	-2.08 (-3.37-0.48)
<i>ITGA9</i>	136	16.06 (15.16-17.34)	15.43 (14.45-16.22)	<0.0001	-1.02 (-1.86-0.14)
<i>PDPN</i>	136	15.71 (14.45-16.96)	15.75 (14.50-16.81)	0.640	-0.13 (-1.15-1.30)
<i>DSP</i>	137	13.81 (11.93-15.59)	16.66 (15.12-17.52)	<0.0001	2.58 (0.44-4.39)
<i>PROX1</i>	136	20.53 (18.27-21.80)	20.47 (18.95-22.09)	0.611	0.17 (-1.21-1.70)
<i>FOXC1</i>	136	15.27 (14.04-16.44)	13.96 (13.06-15.03)	0.0003	-1.45 (-2.29-0.41)
<i>NRP2</i>	136	14.16 (12.78-15.29)	13.97 (12.61-14.99)	0.021	-0.16 (-1.12-0.55)
<i>VEGFA</i>	136	13.34 (11.86-14.77)	13.37 (11.97-14.76)	0.861	0.03 (-0.81-0.80)
<i>FGF2</i>	137	19.57 (17.46-20.50)	18.05 (16.86-19.05)	0.0002	-1.10 (-2.41-0.24)
<i>VEGFR1</i>	136	16.99 (15.48-18.40)	16.83 (14.73-17.42)	0.0002	-0.92 (-1.78-0.15)
<i>VEGFR2</i>	136	16.13 (14.65-17.85)	15.11 (13.63-16.56)	<0.0001	-1.16 (-2.18-0.10)
<i>SYK</i>	138	16.27 (15.28-17.40)	16.16 (15.02-17.50)	0.387	-0.30 (-1.11-1.61)

FC, fold-change; VEGF, vascular endothelial growth factor; LYVE1, lymphatic vessel hyaluronan receptor 1; ITGA9, integrin a9; PDPN, podoplanin; DSP, desmoplakin; PROX1, prospero-related homeobox domain 1; FOXC1, Forkhead box C1; NRP2, neuropilin 2; FGF2, fibroblast growth factor 2; SYK, spleen protein kinase.

detected in 60 (42.9%) patients. Fifty-seven (40.8%) patients had TNM stage I disease, 66 (47.1%) had stage II disease, and 17 patients (12.1%) had stage III disease.

Differential gene expression between tumor and non-tumor lung tissues. Ten out of 15 analyzed genes (*VEGFC*, *VEGFD*, *VEGFR3*, *VEGFR1*, *VEGFR2*, *FGF2*, *SYK*, *LYVE1*, *ITGA*, and *FOXC2*) showed a significantly lower mRNA level in tumors compared with non-tumor tissues. Four genes (*PROX1*, *PDPN*, *NRP2*, and *VEGFA*) had similar expression levels in the tumors and in the normal samples, and only for one gene (*DSP*) an increase in expression in tumors was observed (Table II).

Associations between transcript level and clinicopathological characteristics. The analysis of the effect of patients' clinicopathological features on gene expression revealed a relatively limited and differentiated influence on the fold-change values. In particular, tumor-associated downregulation of the expression for *VEGFC* (P=0.049), *VEGFR3* (P=0.107), *VEGFR2* (P=0.028), and *ITGA* (P=0.011) genes was higher in SCC than in ADC or LCC, and two genes (*PROX1* and *VEGFA*) were downregulated in SCC but not in non-squamous histological types (P=0.005 and P=0.012 for *PROX1* and *VEGFA*, respectively) (Fig. 1A-F). In larger tumors, suppression of *VEGFR3* and *LYVE1* activity was more significant than those in smaller ones (P=0.034 and P=0.50 for *VEGFR3* and *LYVE1*, respectively), whereas the opposite relation was revealed for *PDPN* and *NRP2* genes (P=0.019 and P=0.019, respectively)

(Fig. 2A-D). However, we failed to find associations between the analyzed mRNA levels and lymph node metastases or disease stage. *PDPN* (P=0.049), *SYK* (P<0.001) and *FGF2* (P=0.041) transcriptional downregulation was more significant in high-graded tumors (G3 or G4) compared with low-graded ones (G1 or G2) (Fig. 3A-C). Although unchanged in the whole cohort of our patients or in men, *VEGFA* expression was upregulated in tumors derived from women (P=0.020) (Fig. 4A). In addition, more significant suppression of *FGF2* (P=0.012), *VEGFR2* (P=0.045), and *ITGA* (P=0.005) transcription was observed in men compared to women (Fig. 4B-D).

The effects of gene expression level on patients' survival. The median follow-up time was equal to 54.6 months (ranged from 2 to 86 months). During the follow-up, 64 (45.6%) patients had disease recurrence and all of them had died. In the Kaplan-Meier curve analysis, none of the analyzed parameters influenced OS, except *VEGFR1* expression. The OS rate of the patients with low *VEGFR1* expression was significantly shorter than that of the patients with high expression level (P=0.045). In multivariate analysis by Cox's proportional hazards method, low *VEGFR1* expression was an independent prognostic factor for a poor OS time (HR 2.103; 95% CI: 1.005-4.401; P=0.049) (Table III).

Discussion

NSCLC remains one of the most life-threatening human malignances (31), mostly due to early metastasis

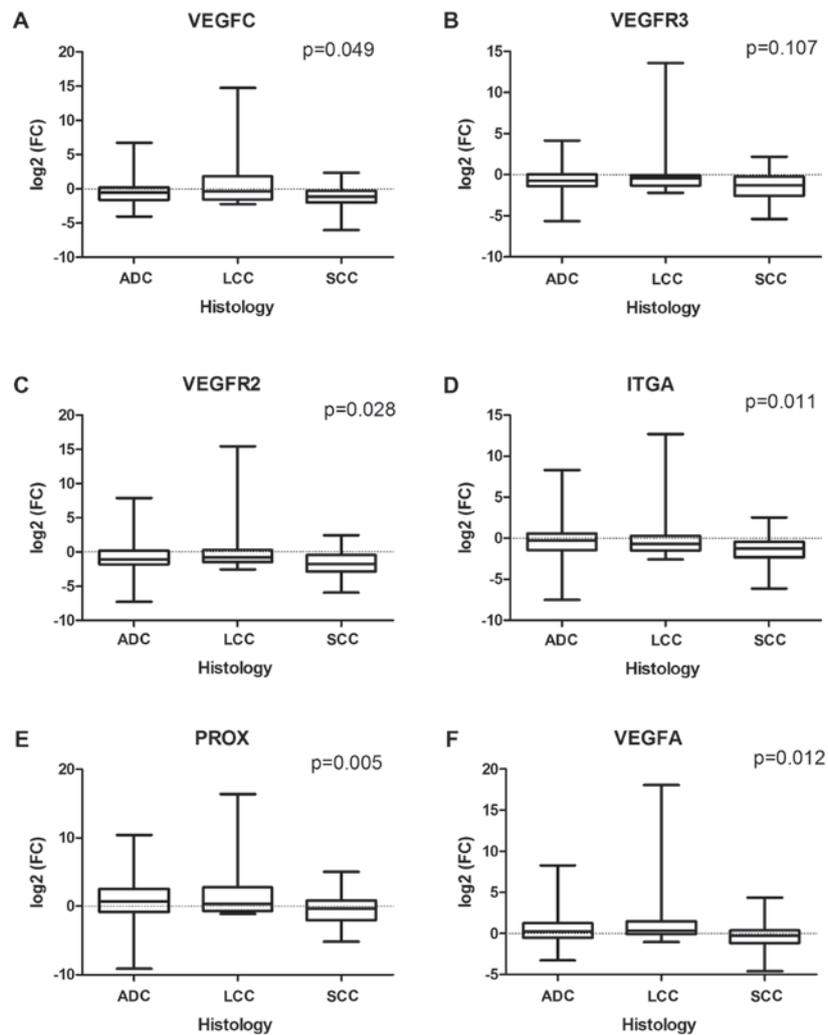


Figure 1. Associations between NSCLC histological type and (A) *VEGFC*, (B) *VEGFR3*, (C) *VEGFR2*, (D) *ITGA*, (E) *PROX1* and (F) *VEGFA* mRNA expression level, defined as $\log_2(\text{FC})$. NSCLC, non-small cell lung cancer; VEGF, vascular endothelial growth factor; ITGA9, integrin $\alpha 9$; PROX1, prospero-related homeobox domain 1; ADC, adenocarcinoma; LCC, large cell carcinoma; SCC, squamous cell carcinoma; FC, fold-change difference in mRNA level.

occurrence (32). Although lymphatic system has long been considered one of the main routes of cancer cell dissemination to distant organs (2,3), an issue of new lymphatic vessel formation in solid tumors, including lung cancer, remains unresolved (33). The aim of the present study was to examine a possible impact of lung cancer cells on lymphangiogenesis induction within lung tumor mass. To do that we, firstly, analyzed mRNA expression level of well-established lymphangiogenesis inducers and markers (namely, VEGFC, VEGFD, VEGFR3, LYVE1, PDPN) and also of a number of pleiotrophic factors with reported contribution to the process (VEGFA, FGF2, NRP2, PROX1 and others). Secondly, although we did not perform tissue microdissection to exclude the influence of nonmalignant stromal cells on the analyzed parameters, we used lung cancer tissue specimens enriched in malignant cells (a median cancer cell content was 80%, ranged from 60 to 100%). Thirdly, we compared the expression level of the examined genes in tumors with that in the nonmalignant lung tissue derived from the same patient. We assumed that transcriptional activation (an increase in transcript level in tumors compared with paired unaffected lung tissues) of the genes essential for lymphatic

vessel formation, reorganization and maintenance had to be observed in lymphangiogenesis-inducing tumors.

Despite expectations, none of the analyzed genes, except *DSP*, was activated in tumor tissue. Moreover, in malignant tissues, a statistically significant decrease in transcript level was observed for growth factors VEGFC and VEGFD and their receptor VEGFR3 that are thought to be the most potent inducers of lymphatic vessel formation (10,34,35), and transcripts for lymphatics-specific markers LYVE1 (36,37) and FOXC2 (38). The expression levels of other well-estimated lymphatic molecules PDPN (39,40) and PROX1 (41,42) were similar to those in nonmalignant tissue. Moreover, neither lymph node status, nor disease stage influenced transcript level for these genes, while more significant suppression of gene activity seemed to occur in SCC, compared to ADC or LCC. Also, no impact of aforementioned genes on patients' survival was observed. Thus, our results do not confirm a hypothesis of lymphangiogenesis induction in NSCLC, but instead seem to indicate a possible transcriptional suppression of the process.

Similar results were recently published by Sanmartín *et al* (43), who analyzed the mRNA expression of all the VEGF family members, their receptors and co-receptors

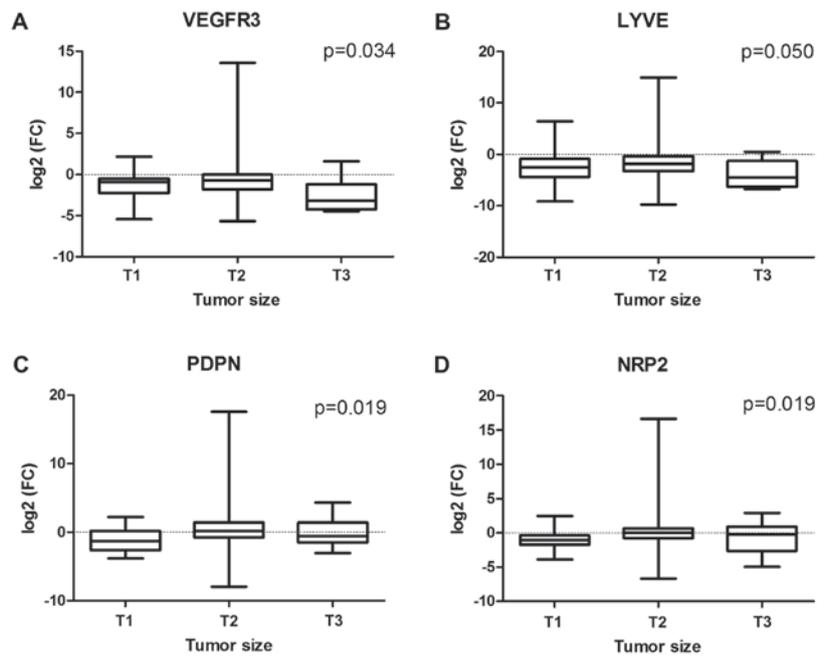


Figure 2. Associations between NSCLC tumor size and (A) *VEGFR3*, (B) *LYVE1*, (C) *PDPN* and (D) *NRP2* mRNA expression level, defined as $\log_2(\text{FC})$ NSCLC, non-small cell lung cancer; VEGF, vascular endothelial growth factor; LYVE1, lymphatic vessel hyaluronan receptor 1; PDPN, podoplanin; NRP2, neuropilin 2; FC, fold-change, difference in mRNA level between tumor and normal lung tissues.

NRP1 and NRP2 in early-stage NSCLCs. The authors applied a similar methodological approach for mRNA evaluation and indicated significantly lower levels of *VEGFD*, *VEGFR2*, and *VEGFR3* mRNA in tumors, especially remarkable in the case of *VEGFD* transcripts. Unfortunately, no information about the remaining analyzed genes has been reported by authors (43). Lower *VEGFC* and similar *VEGFR3* mRNA expression levels in NSCLC tissues compared with normal lung tissues were also indicated by Takizawa *et al* (44). However, in another study, a differentiated *VEGFC* and *VEGFD* expression across tumor mass was indicated. In this analysis, a significantly reduced *VEGFC* and *VEGFD* mRNA expression was indicated in central tumor regions compared with the corresponding non-tumor lung tissues. However, in external tumor marginal regions, the mRNA level was found to be similar (for *VEGFC* transcripts) or even higher (for *VEGFD* transcripts) than those in non-tumoral tissues. Immunohistochemical examination confirmed these data. Moreover, the number of D2-40-immunostained lymphatic vessels was much higher at tumor periphery than in the central zone, and correlated with *VEGFC* and *VEGFD* mRNA levels (45). These results suggest that formation of new lymphatic vessels in NSCLC may be restricted to the peripheral tumor zones. In the present study, we did not analyze separately internal and external tumor zones. Instead, specimens of bulk tumor mass enriched in malignant cells were used for transcript evaluation. In our opinion, our results do not confirm an induction of new lymphatic vessels formation in NSCLC.

We also failed to indicate associations between *VEGFC*, *VEGFD* or *VEGFR3* mRNA expression and lymph node metastasis or patients' prognosis. Our data are partially consistent with previously reported observations, although in terms of the expression at mRNA level, limited and opposite data have also been reported. Thus, no associations between

VEGFC and *VEGFR3* expression and lymph node status or patients' survival were indicated by Maekawa *et al* (46), whereas Takizawa *et al* (44) and Li *et al* (47) reported similar data for *VEGFC* and *VEGFR3* expression, respectively. In contrast, Takizawa *et al* (44) indicated significantly lower *VEGFR3* mRNA levels in the node-positive group and an inverse relation in terms of *VEGFC/VEGFR3* expression ratios. In respect to *VEGFD*, a negative correlation was found between *VEGFD* mRNA under-expression in NSCLC and lymph node metastasis (43,46). In contrast, Feng *et al* (45) indicated a positive correlation between *VEGFC* or *VEGFD* mRNA expression and lymph node metastases, but only in terms of the invasive marginal tumor regions.

Although studies on *VEGFC*, *VEGFD*, and *VEGFR3* expression at mRNA level are limited, protein expression in NSCLC cells has been examined extensively by immunohistochemistry. A number of recent meta-analyses that summarize the results of these clinical investigations preferentially indicate positive *VEGFC/D* and *VEGFR3* immunostaining in tumor cells and a positive correlation between the expression level and lymph node involvement or disease progression (48,49). Similar data were obtained for breast, colorectal and esophageal cancer patients (50-52). However, in all the reports, significant discrepancies across particular studies have been highlighted. In our opinion, currently, there is no data to clearly support or oppose new lymphatic vessel formation in NSCLC.

In terms of the remaining genes examined in the present study, it is difficult to compare our results to previously reported data. Protein products of these genes have been demonstrated to be implicated in lymphatic system development, reorganization and maintenance in both physiological and pathological conditions (24-26,34) and are widely used as markers for microscopic imaging of lymphatic vessels (40,53). However, in addition to lymphatics, these protein are expressed in various

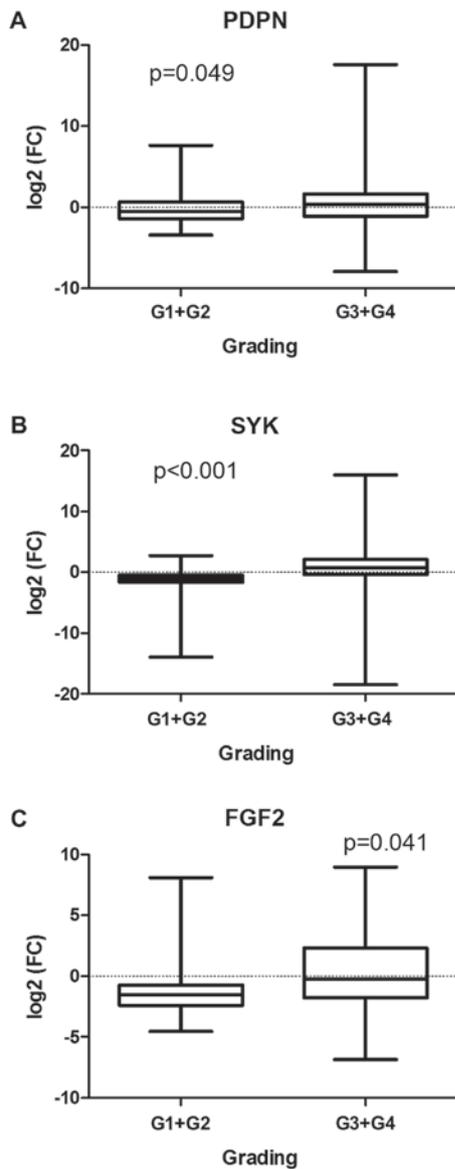


Figure 3. Associations between NSCLC grading and (A) *PDPN*, (B) *SYK* and (C) *FGF2* mRNA expression level, defined as $\log_2(\text{FC})$. NSCLC, non-small cell lung cancer; *PDPN*, podoplanin; *SYK*, spleen protein kinase; *FGF2*, fibroblast growth factor 2; FC, fold-change, difference in mRNA level between tumor and normal lung tissues.

cell types and contribute to multiple molecular processes, including those in malignancies, as it has been demonstrated in a number of recent comprehensive reviews (54-62). This may provide an explanation for inconsistent data on the expression of analyzed proteins in cancer and their impact on tumor progression and clinical outcome (63-77).

For one of the genes, namely *DSP*, encoded for desmoplakin, an increase in mRNA level in NSCLC has been demonstrated. Desmoplakin is one of the main components of desmosomes that confer strong cell-cell adhesion and tissue resistance against mechanical stress but are also involved in cell proliferation, differentiation migration, morphogenesis and apoptosis (57,58). A body of evidence indicates that desmosomal proteins are deregulated in various cancers and the deregulation contributes to cancerogenesis (58). Although a tumor-suppressive function of desmosomal proteins has

mainly been postulated, discrepant data in the literature indicate that differential changes in their expression in tumor tissue may occur and possibly have different consequences (58).

Among the genes we examined here, there were those for growth factor VEGF and their receptors VEGFR1 and VEGFR2. VEGFA/VEGFR1-2 signaling is considered a key inducer of physiological and tumor-associated angiogenesis (78). Recently, VEGFA and VEGFR2 have also been implicated in tumor lymphangiogenesis (3,5,33,34). Of interest, a number of clinical NSCLC studies demonstrated a positive correlation between high tumor cell VEGFA expression and lymph node metastasis (79,80) and an inverse association in terms of stromal cell VEGFA expression (80). In our study, we failed to demonstrate VEGFA/VEGFR1-2 signaling up-regulation in NSCLC, and these data seem to be discordant with a widely accepted view on angiogenesis induction in cancers (81). However, a gross of other factors have been found to stimulate new blood vessel formation, and tumors with VEGFA-independent angiogenesis (82,83) or those co-opting preexisting vessels have been frequently indicated (84,85).

In our study, *VEGFR1* mRNA expression level seemed to be linked to patients' survival ($P=0.049$). However, further investigations on larger patients cohort are needed to confirm this possibility. VEGFR1 is an alternative VEGFA receptor which also binds VEGFB and placental growth factor PIGF (78,86). The prognostic value of this receptor expression in NSCLC remains controversial. In several recent studies, an unfavorable effect of high VEGFR1 expression on NSCLC patient' survival has been demonstrated (87,49), whereas others found no correlation between the expression and the prognosis of the disease (88). To resolve discrepancies in the results further investigations are needed.

An important conclusion raising from our analysis reveals possible differences between NSCLC histological types in lymphangiogenesis regulation which are known to exist regarding new blood vessel formation and are taken into account in targeted antivascular therapy. We indicated a significantly lower VEGFC, VEGFR2, VEGFR3, and PROX1 mRNA expression in SCC compared with non-squamous NSCLC histological types, that suggests a more profound suppression of lymphangiogenesis in SCC and is in line with Takizawa *et al* data according to *VEGFC* and *VEGFR3* mRNA levels (44).

In summary, our results demonstrate that the expression of the lymphangiogenesis-promoting factors in NSCLC cells seem to be suppressed at mRNA level early in cancer progression and more profoundly in SCC compared with ADC or LCC. These findings are in accordance with a recent hypothesis of absence of ongoing lymphangiogenesis inside a growing tumor mass, but do not exclude a possibility of lymphangiogenesis in narrow marginal tumor regions and a contribution of this lymphatics to lymph node metastasis. On the other hand, in the light of current knowledge on crosstalk between lymphatic and immune cells, our data may suggest a possibility of repression of active lymphatic function by tumor cells in order to reduce anti-tumor immunity. Of course, the some factors we had analyzed in the present study, are not limited only to lymphatic system development and functioning, but may play other multiple roles in both tumor and stromal cells, and alterations in their expression may depend on tumor biological characteristics and progression stage.

Table III. Univariate and multivariable analysis of the prognostic effect of patients' clinicopathological characteristics and gene mRNA level [defined as $\log_2(\text{fold-change})$ difference between NSCLC and non-tumor lung tissues] on overall survival (Cox proportional hazards model).

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	P-value	95% confidence interval	Hazard ratio	P-value	95% confidence interval
Age	1.448	0.138	0.888-2.361			
Sex	1.570	0.234	0.747-3.297			
Histology	0.979	0.873	0.759-1.263			
Grading	1.169	0.587	0.665-2.054			
Tumor size	1.749	0.036	1.037-2.948	1.264	0.435	0.701-2.280
Lymph node metastasis	2.258	0.001	1.376-3.704	0.836	0.642	0.392-1.780
TNM	2.414	<0.001	1.713-3.402	2.542	0.001	1.486-4.346
VEGFC	0.824	0.445	0.502-1.353	0.557	0.259	0.201-1.539
VEGFD/FIGF	0.967	0.893	0.592-1.578	1.480	0.481	0.498-4.401
VEGFA	1.322	0.275	0.800-2.184	1.143	0.810	0.382-3.428
VEGFR1/FLT1	2.110	0.046	1.012-4.392	2.103	0.049	1.005-4.401
VEGFR2/KDR	0.874	0.553	0.533-1.435	0.805	0.684	0.284-2.285
VEGFR3/FLT4	0.970	0.905	0.590-1.595	1.179	0.761	0.409-3.411
NRP2	1.084	0.754	0.656-1.791	1.156	0.800	0.376-3.553
ITGA9	1.052	0.839	0.642-3.663	0.924	0.868	0.364-2.347
FGF2	1.845	0.080	0.929-3.663	2.161	0.094	0.878-5.334
PROX1	0.806	0.394	0.491-1.323	0.829	0.686	0.335-2.052
FOXC2	0.599	0.155	0.297-1.212	0.569	0.222	0.230-1.406
LYVE1	0.934	0.806	0.572-1.544	1.277	0.663	0.425-3.837
PDPN	1.156	0.569	0.703-1.901	1.952	0.261	0.608-6.267
SYK	1.345	0.397	0.677-2.671	1.297	0.605	0.843-3.481
DSP	0.855	0.530	0.525-1.393	0.458	0.068	0.197-1.060

NSCLC, non-small cell lung cancer; TNM, tumor-nodes-metastasis; VEGF, vascular endothelial growth factor; FLT, fms-like tyrosine; NRP2, neuropilin 2; ITGA9, integrin a9; FGF2, fibroblast growth factor 2; PROX1, prospero-related homeobox domain 1; FOXC2, Forkhead box C2; LYVE1, lymphatic vessel hyaluronan receptor 1; PDPN, podoplanin; SYK, spleen protein kinase; DSP, desmoplaki.

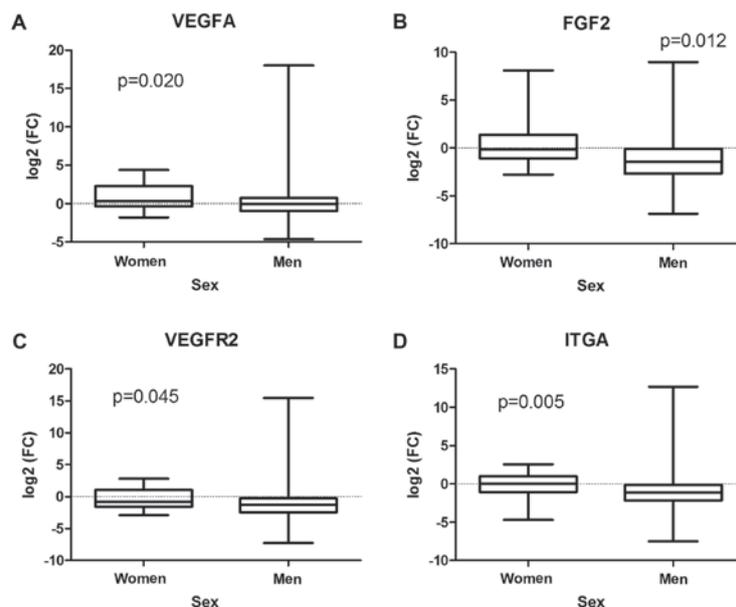


Figure 4. Associations between NSCLC patient sex and (A) *VEGFA*, (B) *FGF2*, (C) *VEGFR2* and (D) *ITGA* mRNA expression level, defined as $\log_2(\text{FC})$ NSCLC, non-small cell lung cancer; VEGF, vascular endothelial growth factor; FGF2, fibroblast growth factor 2; ITGA, integrin a9; FC, fold-change, difference in mRNA level between tumor and normal lung tissues.

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