Prognostic Impact of Lymphangiogenesis and Lymphatic Invasion (CD105 Expression) in Small Cell Lung Carcinoma

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Abstract. *Background: Lymphangiogenesis, an essential process in the metastasis of malignant tumors, has not been thoroughly studied. The possibility of using it to define subsets of patients with different prognosis in cancer could be of vital clinical importance. Materials and Methods: Fifty patients (5 women, 45 men; mean age, 64.47 years) with SCLC were retrospectively studied. Tumor specimens were stained for CD105, and intratumoral lymphatic microvessel density (ILMVD) and lymphatic invasion were determined. Results: Twenty-five patients were diagnosed with limited and 25 with extensive SCLC. All patients received chemotherapy and 32.7% radiation therapy. A direct association between ILMVD (CD105 expression) and lymphatic invasion was observed ($p<0.046$). CD105 expression was significantly associated with the stage of the disease ($p=0.004$) and the presence of metastasis ($p=0.05$). Conclusion: CD105 expression and lymphatic invasion correlated significantly with the clinical parameters and patient outcome, therefore, constituting an important prognostic role in SCLC.*

Lung cancer is the most common cause of cancer death in the United States exceeding deaths from colon, breast and prostate cancer together and being responsible for approximately 563,100 deaths in 1999 (1). The estimated number of new cases and deaths from both non-small cell (NSCLC) and small cell lung carcinomas (SCLC) in the US in 2006 was 174,470 and 162,460, respectively (2).

Recent studies have demonstrated that tumor angiogenesis plays a pivotal role in tumor growth, maintenance and metastatic potential in lung, colorectal and breast, as well as head and neck squamous cell carcinomas (3-8). However, the relevance of tumor lymphangiogenesis (the growth and production of new lymphatic vessels) to tumor spread and its importance in the overall prognostic context of lung neoplasms has not been thoroughly studied.

CD105 (endoglin) is expressed on the cell surface as a 180 kDa homodimeric transmembrane protein that can bind transforming growth factor-β1 and transforming growth factor-β3 (9-11). Studies performed in different laboratories using various antibodies to CD105 have revealed CD105 up-regulation in a wide range of tumour endothelia including that within colon, breast, prostate and lung, as well as head and neck cancer compared to that in normal tissues, thus suggesting the possible involvement of CD105 in tumour angiogenesis (12-15).

The present study aims to evaluate the prognostic impact of lymphangiogenesis in SCLCs. Therefore, we assessed the monoclonal antibody anti-CD105 (mAb) expression by means of calculating the intratumoral lymphatic microvessel density (ILMVD-CD105) and lymphatic invasion in SCLCs and testing them for association with the clinical evolution of the disease.

Materials and Methods

A retrospective study was undertaken of 50 consecutive small cell lung carcinomas investigated in the Department of Pathology of the Ioannina University Medical School, Greece, during the period 2003-2005. The surgical specimens consisted of bioptic material and one lobectomy. A representative formalin-fixed embedded block of tumor tissue was selected from each case on the basis of containing viable tumor and surrounding non-neoplastic lung parenchyma, avoiding areas with necrosis. Regarding all these patients, the inpatient medical records were reviewed at the Pneumonology Clinic of the University. Follow-up of the clinical course following the diagnosis was performed on an outpatient medical record basis and by telephone inquiries. Staging was established according to the American Joint Committee on Cancer standards (AJCC 2002) (16, 17) and grading was performed according to the World Health Organization (WHO) criteria (18).

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Table I. *Significant statistical association between CD105 expression and stage of the disease (p=0.004; Fisher's two-tailed exact test).*

			Stage		Total
			Extensive	Limited	
CD105	High expression	No. of specimens	23	14	37
		%	62.2%	37.8%	100.0%
	Low expression	No. of specimens	2	11	13
		%	15.4%	84.6%	100.0%
Total		No. of specimens	25	25	50
		%	50.0%	50.0%	100.0%

Table II. *CD105 expression is strongly related to the presence of metastasis (p=0.05; Fisher's two-tailed exact test).*

			Metastasis		Total
			Absent	Present	
CD105	High expression	No. of specimens	6	31	37
		%	16.2%	83.8%	100.0%
	Low expression	No. of specimens	6	7	13
		%	46.2%	53.8%	100.0%
Total		No. of specimens	12	38	50
		%	24.0%	76.0%	100.0%

Immunohistochemistry. Immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections by means of EnVision System (DAKO, Glostrup, Denmark) as formerly mentioned. Consecutive 2-μm-thick sections were re-cut from each study block and used for the immunohistochemical study. Briefly, sections were deparrafinized and rehydrated in distilled water. Then they were placed in citrate buffer (pH=6) and underwent microwave treatment at 300 W twice (15 minutes each time) (antigen retrieval). Afterwards, they remained in room temperature for 15 minutes. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide in absolute methanol for 30 minutes at room temperature. Washing with distilled water preceded a 10-minute wash with Tris-buffered saline (TBS) (DAKO). The primary antibody used was CD105 (1:30 dilution) (DBS, California, USA) at room temperature for 24 hours. The optimal dilutions were defined on the basis of a titration experiment. After washing with TBS for 10 minutes, the application of the secondary monoclonal antibody against CD105 protein was performed for 30 minutes (Envision Kit, DAKO). Washing with TBS for 10 minutes and DAB application (Envision Kit, DAKO) for 2-3 minutes with simultaneous microscopical control followed. Finally, counterstaining with hematoxylin 10% was performed and the slides were rinsed in running tap water, dehydrated, mounted and coverslipped.

Assessment of CD105 expression. Two experienced pathologists examined the slides without any knowledge related to the clinicopathological data. Examination was simultaneously performed by means of a double-headed light microscope. Both pathologists had to reach a consensus on what constituted a single lymphatic vessel prior to its inclusion in the count. The three most vascular areas (hot spots) within a section were selected for quantification of lymphangiogenesis (Olympus BX-51 microscope at x40 magnification) and lymphatic vessels labeled with the anti-CD105 mAb were counted under light microscopy at x200 magnification. The average counts were recorded as the CD105-ILMVD. The score was defined as follows: ≤25, low expression of lymphangiogenesis, >25, high expression. There was more than 95% agreement between the two investigators regarding the CD105 evaluation. Lymphatic invasion was additionally assessed by identification of neoplastic emboli within the tumor cells stained by CD105.

Statistical analysis. All statistical manipulations were performed using the SPSS 13.0 for Windows system (Statistical Package for Social Science) (SPSS, Inc., Chicago, IL, USA).

Table III. *Association between lymphatic invasion and metastasis was not assessed as being statistically significant (χ^2=0.26, p=0.6, Chi-square test).*

			Metastasis		Total
			Absent	Present	
Lymphatic invasion	Absent	No. of specimens	10	29	39
		%	25.6%	74.4%	100.0%
	Present	No. of specimens	2	9	11
		%	18.2%	81.8%	100.0%
Total		No. of specimens	12	38	50
		%	24.0%	76.0%	100.0%

The Chi-square (χ^2) test and Fisher's two tailed exact test were applied to assess the association between categorical variables. Student's *t*-test was used to compare continuous variables in subgroups when the former followed a normal distribution. Survival function and recurrences were calculated from the time of histopathological diagnosis to the last date of follow up by means of the Kaplan-Meier estimate. The log-rank test was applied to compare survival functions. A *p*-value less than 0.05 was considered statistically significant.

Results

The group of patients studied comprised 45 (90%) men and 5 (10%) women having a mean age of 64.47 years (range 48-80 years). According to AJCC standards (16), 25 (50%) patients were staged with limited SCLC and 25 (50%) patients with extensive SCLC. All patients received chemotherapy, while 30% (15/50) underwent radiotherapy and 4% (2/50) surgery, mainly for diagnosis.

High expression of CD105 (CD105-ILMVD>25) (Figure 1) was detected in 37/50 (74%) specimens and low CD105 expression (ILMVD≤25) was detected in 13/50 (26%) (Figure 2). Significant association was found between CD105 expression and the stage of the disease (χ^2=8.4; df=1; p=0.004). In this context, 62.2% (23/37) of

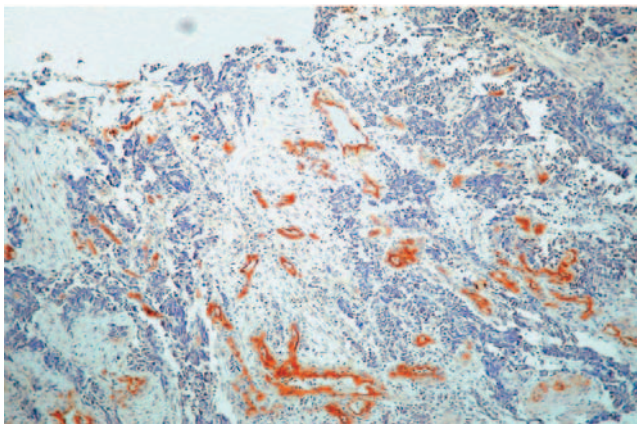

Figure 1. *Immunohistochemical staining with DAB (original magnification x10). SCLC presenting with high CD105 expression (CD105-ILMVD>25).*

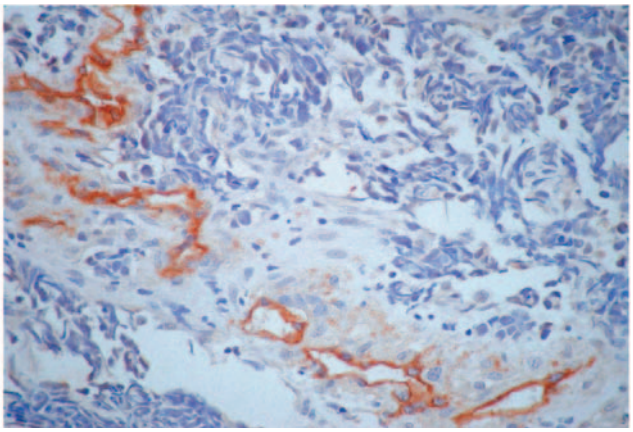

Figure 2. *SCLC presenting with low CD105 expression (CD105-ILMVD≤25) (original magnification x40).*

patients presenting high CD105 expression were clinically classified as extensive stage SCLC (Table I). CD105 expression in SCLC was directly correlated with the presence of lymphatic invasion. Eleven of 37 SCLC specimens presenting high CD105 expression exhibited simultaneously lymphatic invasion whereas no specimen with low CD105 expression presented lymphatic invasion (p=0.046; Fisher's two-tailed exact test). CD105 expression and the presence of metastasis displayed a borderline statistically important association (p=0.05; Fisher's two-tailed exact test) according to which 83.3% of patients having a high CD105-ILMVD (>25) presented with metastases in target organs (liver, adrenals, brain and bones) (Table II).

No significant correlation was assessed between CD105 expression and survival (log-rank=0, 12; p=0.72). Mean survival for high ILMVD patients was 3.16±0. 6 months (average±typical error) whereas for those having low ILMVD was lower (2.69±0.93 months) (Figure 3).

CD105 expression and demographic data in terms of gender and age were devoid of any significant association between them (p=0.3, Fisher's two-tailed exact test and t=1.7, p=0.1, respectively). Likewise, there was no significant association between the lymphatic invasion and patients' sex (p=1, Fisher's two-tailed exact test) whilst patients presenting lymphatic invasion were older (mean age 67.9±1.77 years) than those without (mean age 63.3±1.28 years).

Lymphatic invasion and stage of the disease, as well as the presence of metastases, were also associated although not to a significant degree (χ^2=0.18, p=0.73 and χ^2=0.26, p=0.6, respectively) (Table III). Lymphatic invasion and survival were also not significantly correlated (log-rank=0.06, p=0.8) (Figure 4).

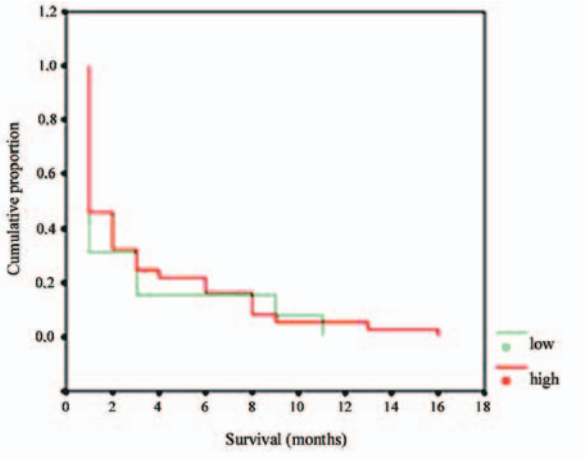

Figure 3. *Kaplan-Meier survival curve for SCLC patients presenting high and low ILMVD.*

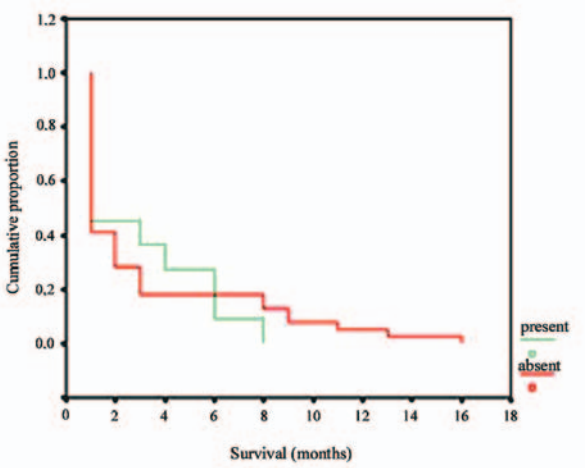

Figure 4. *Comparison of survival in SCLC patients presenting lymphatic invasion and those without by means of Kaplan-Meier survival curve.*

Discussion

Angiogenesis comprises an essential process in the progression of malignant tumours and is associated with patient outcome in several malignancies. Metastasis of malignant tumours to regional lymph nodes is one of the early signs of cancer spread in patients and is associated with high mortality rates. In certain types of cancer, such as breast cancer, lymphatic metastasis is one of the predominant routes of cancer spread (19, 20). There have been a number of studies evaluating the prognostic significance of microvessel density in several neoplasms and demonstrating the superiority of CD105 (Endoglin) expression to other neoangiogenetic factors. Dales *et al.* reported that CD105 expression is of a prognostic significance in paraffin sections of breast carcinomas in terms of overall survival ($p<0.01$), whereas CD31 on paraffin sections did not equally correlate with patients' overall survival (21, 22).

Interestingly, Mineo *et al.* reported the significance of CD105, CD34 and VEGF expression in neoangiogenesis in NSCLCs (stage IB-IIa) and their correlation with poor survival rate, but CD105 was not finally selected by the statistical model and was excluded at the multivariate analysis (23). This finding contrasts with the results of Tanaka *et al.*, who found that CD105 expression was the best marker and significant prognosticator of disease- free survival (24) in NSCLC. A possible explanation for this apparent contradiction would be the stricter relation of CD34 and tumour vessel invasion to the metastatic process than neoangiogenesis itself.

In colorectal cancer, CD105 expression was related with stage and overall poor prognosis (25, 26) and prostate cancer seems to follow the same pattern (27). On the contrary, CD105 did not provide significant prognostic information in hepatic neoplasms (28).

The purpose of our study was to assess CD105 expression and lymphatic invasion in SCLC and to evaluate their prognostic impact on patient outcome. To the best of our knowledge, this is the first study dealing with the expression of lymphangiogenesis and its importance in clinical practice in SCLC.

Our results suggested a statistically significant association between ILMVD and the stage of the disease in SCLCs as well as with the presence of metastasis in the most commonly affected target organs (liver, adrenals, brain, bones). There was a direct association between ILMVD (CD105 expression) and lymphatic invasion. Patients exhibiting increased ILMVD presented significantly more neoplasmatic emboli within the CD105-labeled tumor cells (lymphatic invasion) than those exhibiting low ILMVD. These findings ascertain the association of lymph-angiogenesis with tumor spread in SCLC.

ILMVD, lymphatic invasion and survival were not statistically correlated to a significant degree. The present observation could be attributed to the use of chemotherapy and radiation according to the individuality of each clinical case and not on a general basis, thus constituting a fact not being thoroughly examined in the present study as it does not comprise part of its aims.

The present study demonstrated the validity of CD105 as a marker of lymphangiogenesis in SCLC. Nevertheless, its direct implication in lymphangiogenesis is still vague; present studies focus on the discovery of intracellular molecules interacting with endoglin (CD105) (15).

The results of the current study suggest that CD105 expression (by means of ILMVD calculation) and lymphatic invasion are capable of constituting a significant prognostic role in the overall aspect of SCLC prognosis by being associated with the clinical parameters and patient outcome. Our observations regarding the prognostic significance of endoglin should be confirmed in larger series of SCLCs from the same anatomic location and patients should be subjected to the same treatment modalities before the incorporation of the determination of CD105 into clinical practice.

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