

RESEARCH ARTICLE

Role of VEGF-stromal cell–derived factor-1 α /CXCL12 axis in pleural effusion of lung cancer

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Abstract

Context and objective: It has been suggested that stromal cell–derived factor-1 α (SDF-1 α) or CXCL12, both transcripts, TR1 and TR2) and its cognate receptor CXCR4 may regulate cancer metastasis. We have investigated the role of vascular endothelial growth factor (VEGF), angiopoietins (Ang-1 and Ang-2) and the biological axis of CXCL12—CXCR4, in patients with malignant pleural effusions (PEs).

Material and methods: Twenty five patients, seven with transudative PEs due to heart failure and 18 with exudative malignant PEs (7 with small cell lung cancer (SCLC) and 11 with nonsmall cell lung cancer (NSCLC)) were included in the study. Expression analysis of the mediators was performed in pleural fluid pellet using real-time reverse transcription–PCR. Protein expression has been evaluated by western blot analysis.

Results: SDF-TR1 ($P=0.02$) but not SDF-TR2 ($P=0.23$) or CXCR4 levels ($P=0.23$) were higher in malignant PEs than in transudates. SDF-TR1 ($P=0.04$) and SDF-TR2 levels ($P=0.04$) but not CXCR4 levels ($P=0.123$) were higher in SCLC PEs than in heart failure PEs. SDF-TR1 ($P=0.03$) but not SDF-TR2 levels ($P=0.6$) and CXCR4 levels ($P=0.4$) were higher in NSCLC PEs than in transudates. Ang-1 has not been expressed in PEs, whereas no significant difference has been detected in VEGF and Ang-2 expression between malignant PEs and transudates. However, protein expression showed increased VEGF and SDF expression in malignant PEs.

Conclusions: These results suggest that elevated SDF-1 α /CXCL12 levels would be suggestive of a link to metastasis and may participate in pleural trafficking in lung cancer.

Keywords: Lung cancer; pleural effusion; metastasis; chemokines; angiogenesis

Introduction

Lung cancer ranks as the leading cause of death from malignancies worldwide. Most patients present with locally advanced (37%) or metastatic (38%) disease at the time of diagnosis (1). The average 5-year survival rate of these patients remains extremely poor despite advances in chemotherapy. Malignant pleural effusion (PE) is the most often caused by lung adenocarcinoma, because this type often forms a primary tumor in the periphery of the lung and invades the pleural cavity (2). Malignant PE indicates a poor prognosis in patients with advanced lung cancer, being associated with high morbidity and

mortality in nonsmall cell lung cancer (NSCLC) (1–3). The therapies which are commonly used to control malignant PE do not extend the survival of these patients.

Malignant PEs can develop as a direct consequence of cancer cell dissemination into the pleural space, however the exact mechanisms are not fully understood (4). Increased vascular permeability and leakage play a principal role in the development of exudative pleural PEs. However, the role of angiogenesis in the pathogenesis of PE has not been extensively studied. Angiogenic cytokines, such as vascular endothelial growth factor (VEGF) has been shown to play an important role in the pathogenesis of malignant PEs; however, VEGF

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expression substantially overlaps between malignant and nonmalignant effusions (5–8). Our group has recently demonstrated that although VEGF is one of the main mediators in exudative malignant PEs, this effect is not mediated through the angiogenetic pathway of angiopoietins' receptor Tie-2 (7). In addition, it has been shown that angiopoietin-2 (Ang-2) along with VEGF, but not Ang-1, participate in pleural inflammation (8). However, the role of the above biological axis of VEGF and angiopoietins needs further investigation, particularly in malignant PEs.

Tumor cell migration and metastasis share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors (9,10). Chemokines direct various subsets of hematopoietic cells to home-specific anatomical sites through interaction with their G protein-coupled receptors (11,12). Much attention has been paid to one particular member of the chemokine receptor family, termed CXCR4, because of its key role in HIV infection (13). Stromal cell-derived factor-1 α (SDF-1 α /CXCL12) is a member of the CXC chemokine family that has been found to recruit CD34⁺ hematopoietic progenitor cell, megakaryocytes, B cells, and T cells (12–14). The SDF-1 α /CXCL12 was expressed on target tissues (10). Although, most chemokine receptors bind several chemokines, CXCR4 is a specific chemokine receptor because it only interacts with SDF-1 α /CXCL12 (15). The involvement of CXCR4 and SDF-1 α /CXCL12 in these processes makes this chemokine receptor pair of particular interest in both tumor metastasis and angiogenesis (16). Recently, many studies have demonstrated that the metastatic propensity of tumors from several different types of cancer including lung, breast, ovarian, renal, and prostate is related to the expression of the chemokine receptor CXCR4 (10,15–17). Furthermore, in both NSCLC and breast cancer it has been shown that the ligand for CXCR4, SDF-1 α /CXCL12, exhibited peak levels of expression in organs that were the preferred destination for their respective metastases (17,18).

A better understanding of the molecular mechanisms that are involved in cancer cell dissemination is required to understand the process of malignant PE formation and to find ways to design new and effective therapies. Therefore, in this study, we hypothesized that interaction of SDF-1 α /CXCL12/CXCR4 axis and VEGF through angiopoietins' expression may be involved in the formation of malignant PEs.

Material and methods

Between September 1, 2007 and February 28, 2008, we prospectively studied 25 consecutive patients with PEs. The study was approved by the Ethics Committee of our

hospital, and before the thoracentesis all patients signed an informed consent.

Pleural fluid was obtained by routine thoracentesis in 25 patients. Seven showed transudates due to congestive heart failure and 18 malignant exudates: 7 from SCLC and 11 from NSCLC. PEs were categorized as exudates or transudates according to the criteria of light (19). A PE was attributed to heart failure when it was transudative, the patient had symptoms and signs of left ventricular failure, a heart ultrasound study revealed systolic or diastolic dysfunction of the left ventricle, and the PE responded to the appropriate therapy. A malignant PE was diagnosed if the PF cytology was positive for malignant cells. The characteristics of the subjects and PEs are shown in Table 1.

RNA isolation and reverse transcription

Total RNA was extracted from each specimen using a power homogenizer and the TRIzol reagent (Invitrogen, Carlsband, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the Strascript reverse transcriptase kit (Stratagene, La Jolla, CA, USA) as previously described (20).

Real-time reverse transcription-PCR

Peptide growth factor mRNA expression was measured using a real-time reverse transcription-PCR assay with SYBR-Green I. Primers were designed to span introns (20). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, in order to normalize VEGF and angiopoietins, SDF-1 or CXCL12, transcript 1 and 2/CXCR4 expression levels (Table 2). Specifically, 1 μ l cDNA from pathological or control samples was amplified in a PCR reaction containing 2 \times Brilliant SYBR-Green I QPCR Master Mix, 300 nM of each primer and 30 μ M ROX passive reference dye, in a final volume of 20 μ l. After an initial denaturation at 95°C for 10 min, the samples were subjected to 40 cycles of amplification, comprised of denaturation at 95°C for 30 s, annealing at appropriate temperature for each

Table 1. Patients and of pleural effusions' characteristics.

Patients	Transudates	SCLC	NSCLC
Number	7	7	11
Male/Female	6/1	7/0	11/0
Age (years)	76 (70–84)	71 (65–80)	70 (59–82)
RBCs ($\times 10^3$ cell/ μ L)	1.1 (0.5–1.6)	2.7 (0.7–9.9)	2.1 (0.7–6.0)
LDH (IU/L)	118 (61–243)	218 (176–664)	204 (151–429)
Protein (g/dL)	2.3 (1.0–2.8)	2.7 (2.2–3.2)	2.5 (1.4–3.6)
Glucose (mg/dL)	102 (90–156)	124 (88–222)	101 (67–206)
ph	7.42 (7.40–7.45)	7.43 (7.39–7.49)	7.46 (7.13–7.49)

Values are given as the median (IQR).

Table 2. Primer sequences used for quantitative real-time RT-PCR.

Growth factor or cytokine	Primer pair Sequence (5'-3')	Annealing temperature	Product size
VEGF	ATGACGAGGGCCTGGAGTGTGCCTATGTGCTGGCCTGGTGAG	60°C	91
SDF1-TR1	TGAGAGCTCGCTTTGAGTGACACCAGGACCTTCTGTGGAT	55°C	233
SDF1-TR2	CTAGTCAAGTGCCTCCACGAGGACACACCACAGCACAAAC	55°C	221
CXCR4	GGTGGTCTATGTTGGCGTCTTGGAGTGTGACAGCTTGAG	55°C	229
ANGPT1	TTAATGGACTGGAAGGGAACCAGGGGCCACAAGCATCAA	55°C	235
ANGPT2	AGCCGGCAAATAAGCAGCATCGGTTGTGGCCTTGAGCGAATAGC	59°C	238
GAPDH	GGAAGTGAAGGTCGGAGTCAGTCATTGATGGCAACAATATCCACT	60°C	101

primer pair for 30 s and elongation at 72°C for 30 s, followed by a melt curve analysis, in which the temperature was increased from 55 to 95°C at a linear rate of 0.2°C/s. Data collection were performed both during annealing and extension, with two measurements at each step, and at all times during melt curve analysis. In each PCR reaction two nontemplate controls were included. All PCR experiments were conducted on the Mx3000P real-time PCR thermal cycler using the software version 2.00, (Stratagene, La Jolla, CA, USA). To verify the results of the melt curve analysis, PCR products were analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide and photographed on a UV light transilluminator. Primer sequences, annealing temperatures and PCR products length for all the growth factors analyzed, as well as for GAPDH, are described in Table 2.

All reactions were run in triplicates, and peptide growth factor transcript levels were calculated and normalized to each specimen's house keeping gene mRNA (GAPDH) as well as the appropriate calibrators, using the $\Delta\Delta C_t$ method for relative quantification. Specifically, after amplification, standard curves were constructed from samples used in a series of consecutive dilutions, for both the gene of interest (GF) and the internal control (GAPDH). Growth factor and GAPDH amplification efficiencies were the same, reaching 100%. Exudates or transudates PE data were first normalized against variation in sample quality and quantity. Normalized values to GAPDH, $\Delta C_{t\text{ sample}}$, were initially calculated using the following equation: $\Delta C_{t\text{ sample}} = C_{t\text{ GF}} - C_{t\text{ GAPDH}}$. The $\Delta\Delta C_t$ was then determined using the formula: $\Delta\Delta C_t = C_{t\text{ transudates}} - C_{t\text{ exudates}}$, and the expression of the normalized (to GAPDH) genes of interest in malignant PEs compared to the mean of the transudates as a calibrator equals $= 2^{-\Delta\Delta C_t}$. Twofold increased (a value ≥ 2) or decreased (a value ≤ 0.5) value was considered gene mRNA overexpression or downregulation respectively, in that malignant PE sample.

Western blot

Proteins extracts (20 μg) were electrophoresed through a 10% polyacrylamide gel, transferred onto nitrocellulose membranes and incubated with an anti-VEGF antibody (Cat. No. MAB293, R&D systems, UK) at a dilution of 1:500,

an anti-SDF antibody (Cat. No. MAB350, R&D systems, UK) at a dilution of 1:500 and with an antiactin antibody (sc-47778, Santa-Cruz biotechnology, USA) at a dilution 1:1000. Antibody binding was revealed by a peroxidase labeled secondary antibody. Bands were visualised using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA), according to the manufacturer's protocol. The analysis was performed twice for each sample (normal or tumor). The film was photographed with the Alpha Imager system (Alpha Innotec Corp., USA). VEGF and SDF protein levels in transudates and exudates were quantified using Alpha Innotec image analysis software, with the β -actin protein levels of each sample used as an internal control.

Statistical analysis

Peptide growth factor and cytokine mRNA levels were first evaluated by the one-sample Kolmogorov-Smirnov goodness of fit test, in order to determine whether they follow a normal distribution pattern. Based on the results, the nonparametric Spearman rank correlation was used to examine their relation pair-wise. The Mann-Whitney U and Kruskal-Wallis H test, used when indicated by the analysis, were used to examine SDF1-TR1, SDF1-TR2 and CXCR4 expression status after stratification for transudates or malignant PEs. All statistical analyses were performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95% level (P value < 0.05).

Results

Chemokines-growth factor mRNA transcript levels in malignant PEs vs. controls

Angiogenic cytokines (SDF-1 α /CXCL12-CXCR4) and growth factor (VEGF) that studied exhibited mRNA expression in all samples (malignant PEs and controls). Ang-2 has been expressed in 40% of transudates, 29% of SCLC and 27% of NSCLC. Ang-1 has not been expressed in any PEs.

PF SDF-TR1 ($P=0.02$) but not SDF-TR2 ($P=0.23$) or CXCR4 levels ($P=0.23$) were higher in exudates than in

transudates (Table 3A). VEGF and Ang-2 mRNA levels are not statistically significant different between exudates and transudates (Table 3A) (Figure 1).

PF SDF-TR1 ($P=0.04$) and SDF-TR2 levels ($P=0.04$) but not CXCR4 levels ($P=0.123$) were higher in SCLC PEs than in transudates. PF SDF-TR1 ($P=0.03$) but not SDF-TR2 levels ($P=0.6$) and CXCR4 levels ($P=0.4$) were higher in NSCLC PEs than in transudates. The expression levels of SDF-1 α /CXCL12-CXCR4 did not differ significantly between SCLC and NSCLC PEs (Table 3B).

A significant positive correlation between VEGF and SDF1 TR1 ($P=0.04$), SDF-TR2 ($P=0.02$), CXCR4 ($P=0.03$) was found in malignant exudates. We have also detected a significant correlation between SDF-TR1 and SDF1 TR2 mRNA levels ($P=0.0001$), between SDF1 TR1 and CXCR4 mRNA levels ($P=0.001$) and between SDF1 TR2 and CXCR4 mRNA levels, ($P=0.017$). There is a positive correlation between VEGF mRNA levels and LDH, in the malignant PEs (SCLCs and NSCLCs) ($P=0.013$).

Similar Ang-2 mRNA levels were expressed in all patients and controls. Ang-1 mRNA is not expressed in the PEs, whereas Ang-2 is expressed but not in all PEs (transudates 40%, SCLC 29%, NSCLC 27%). There is no correlation between Ang-2 and pleural pH, GLU, LDH, total protein, albumin.

Table 3. mRNA expression in trasudates (control PEs) and exudates (SCLCs and NSCLCs).

A

	Transudates (N=7)	Exudates (SCLCs & NSCLCs) (N=18)	P value*
VEGF	27.5 ± 14.3	1.65 ± 0.76	NS
SDF1-TR1	0.70 ± 0.16	(11.2 ± 8.8) × 10 ²	0.020
SDF1-TR2	0.89 ± 0.15	(16.8 ± 9.9) × 10 ²	NS
CXCR4	5.61 ± 2.76	(21.8 ± 16.2) × 10 ²	NS
ANGPT2	33.5 ± 22.1	0.20 ± 0.13	NS

Data are presented as mean ± SEM.

*Mann-Whitney test.

B

	Transudates (N=7)	SCLCs (N=7)	NSCLCs (N=11)	P value*
VEGF	27.5 ± 14.3	1.34 ± 0.74	1.8 ± 1.2	NS
SDF1-TR1	0.70 ± 0.16	(2.7 ± 2.2) × 10³	(1.0 ± 1.0) × 10 ²	0.04
SDF1-TR2	0.89 ± 0.15	(3.56 ± 2.5) × 10³	(4.8 ± 2.0) × 10 ²	0.04
SDF1-TR2	0.89 ± 0.15	(3.56 ± 2.5) × 10 ³	(4.8 ± 2.0) × 10²	0.03
CXCR4	5.61 ± 2.76	(4.8 ± 4.1) × 10 ³	(5.06 ± 1.76) × 10 ²	NS
ANGPT2	33.5 ± 22.1	0.33 ± 0.29	0.25 ± 0.13	NS

Data are presented as Mean ± SEM.

*Mann-Whitney test performed between the groups highlighted with bold.

VEGF and SDF protein levels in malignant PEs vs. controls assessed by western blot analysis

VEGF and SDF1 α /CXCL12 protein were evaluated by western blot analysis in 17 and 14 pleural fluid protein lysates respectively. VEGF protein was expressed in 88% (15/17) specimens. In detail, VEGF protein was detected in 12 cancer cases (six SCLCs and six NSCLCs) and three controls. SDF1 protein was present in all examined cases (14/14, 100%). Specifically SDF1 protein was detected in 11 cancer cases (four SCLCs and seven NSCLCs) and three controls (Figure 2).

Higher mean SDF protein levels were observed in SCLC (0.42 ± 0.16) and NSCLC (0.38 ± 0.19) specimens respectively compared to transudates cases (0.16 ± 0.06). Similarly, higher VEGF protein levels were observed in SCLC (0.76 ± 0.22) and NSCLC (0.81 ± 0.38) specimens, respectively compared to control PEs (0.26 ± 0.17); however the statistical significance could not be evaluated due to the limited number of samples in each category (<10).

VEGF protein levels were not correlated with its mRNA levels in malignant PEs. Interestingly, SDF-1 α /CXCL12 protein expression was negatively correlated with SDF-TR1 mRNA levels in malignant PEs (NCLCs and NSCLCs) ($P=0.012$). Moreover, SDF-1 α protein expression was found to be significantly correlated with pleural glucose ($P=0.019$) and serum LDH ($P=0.043$). Furthermore, VEGF and SDF1 protein levels were found to be correlated ($P=0.019$).

Discussion

The aim of this study was to investigate the interaction of the biological axis VEGF-angiopoietins with SDF-1 α /CXCL12/CXCR4, in order to contribute to the better understanding of the angiogenetic mechanisms in malignant PEs. To the best of our knowledge, this is the first study to discuss the aforementioned angiogenetic pathway in pleural fluid with two distinct methodologies. Our major finding is the significant increase in SDF-1 α expression in both mRNA and protein level in malignant exudates in comparison to controls. In addition, we detected a positive correlation between post-transcriptional expression protein levels of VEGF and SDF-1 α /CXCL12 in cancer pleural samples. Furthermore, we found that these findings were correlated with functional pleural fluid characteristics like LDH and/ or glucose. These findings could suggest that SDF-1 α /CXCL12 along VEGF-Ang-2 expression is involved in the dissemination of malignant cells into pleural space. Disseminated cancer cells can block the drainage of pleural space and this eventually leads to PE.

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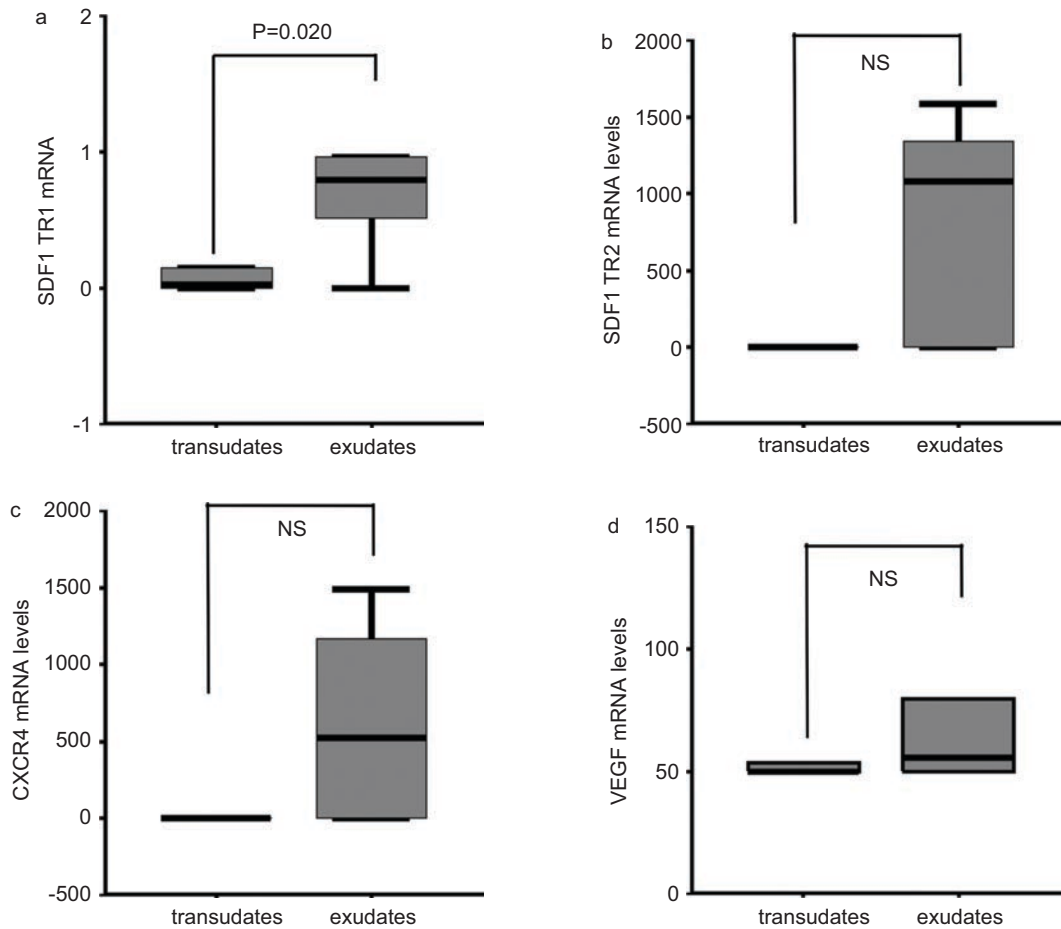


Figure 1. mRNA expression levels of angiogenic chemokines in exudates and transudates.

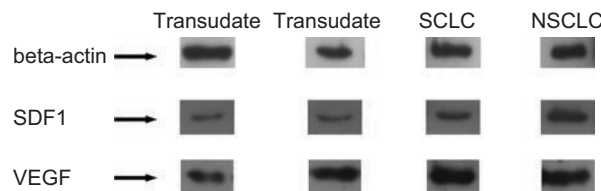


Figure 2. Western blot analysis of transudates and cancer samples.

Lung cancer is the leading cause of malignant PEs. At least 25% of all patients with lung cancer will develop malignant PE (2). Treatment of malignant PE consists of drainage by chest tube and induction of pleural sclerosis by injection of antibiotics, antiseptics and antineoplastics (21,22). The results of the treatment are variable, because these procedure do not prolong survival and prediction of recurrence is also difficult (21). SDF-1 α exerts pleiotropic effects regulating processes essential to tumour metastasis such as locomotion of malignant cells, their chemoattraction and adhesion, as well as plays an important role in tumour vascularization (23). This implies that new therapeutic strategies aimed at blocking the SDF-1 α -CXCR4 axis could have

important therapeutic applications by modulating the trafficking of hemato/lymphopoietic cells and inhibiting the metastatic behaviour of tumour cells as well (23). More in detail, CXCR4 antagonists, such as Plerixafor (AMD3100) and T140 analogues (TN14003/ BKT140), disrupt CXCR4-mediated SCLC cell-adhesion to stromal cells. Therefore, in stromal cell cocultures, CXCR4 antagonists also sensitize SCLC cells to cytotoxic drugs, and thereby antagonize cell adhesion-mediated drug resistance (24).

It has been recently shown that SDF-1 α /CXCL12 is highly expressed in breast cancer cells and in organs representing the major areas of breast cancer metastasis, respectively and that SDF-1 α /CXCL12 promotes

metastasis by stimulating the migration of breast cancer cells (18). For the first time in lung cancer metastases, Phillips and coworkers hypothesized that the CXCL12-CXCR4 biological axis is important in mediating NSCLC metastases by evaluating lung cancer tumor specimens and cell lung cancer cell lines (17). Their data suggested that the SDF-1 α /CXCL12-CXCR4 biological axis is involved in regulating the metastasis of NSCLC through enhanced extracellular signal-related kinase-1/2 phosphorylation without change in either proliferation or apoptosis (17). Additional studies supported the findings of the previous study and strengthen the possibility that the SDF-1 α /CXCL12-CXCR4 axis contributes to the pathogenesis of NSCLC metastases (25). The SDF-1 α /CXCL12 levels in malignant PE were significantly higher than those in transudate PE and showed a significant positive correlation with PE volumes (25). Furthermore, cancer cells in malignant PE expressed CXCR4, and mesothelial cells of the pleura stained positive for SDF-1 α (24).

However, there are no studies in the current literature to support the aforementioned involvement of SDF-1 α /CXCL12-CXCR4 in malignant PE of SCLC. Our findings are in agreement with the above studies (17,25), whereas we found similar data with different-modern methodologies directly in pleural fluid cells of patients with malignancy. In addition, we are of the opinion the pathophysiology of metastasis in SCLC could be similar with NSCLC, as we did not detect differences between mRNA expression.

On the other hand, we did not detect any increase of the CXCR4 expression between malignant cells and controls by real-time PCR. Kijima and coworkers reported that the CXCR4 receptor is functionally expressed in SCLC cell lines and regulates their migration, adhesion and morphologic change in cooperation with the stem cell factor/c-kit pathway (26). Additional studies confirm the involvement of CXCR4 in lung cancer metastases (27,28), however, by different techniques and not directly in pleural fluid cells. In addition, it has been recently demonstrated a differential expression of CXCR4 associated with the metastatic of human NSCLC (29). This hypothesis raises the possibility that blockade of CXCR4/SDF1 interaction may lead to novel therapeutic molecules (29).

Our novel finding is the interaction between VEGF and SDF-1 α /CXCL12 expression at protein level in malignant exudates, probably showing the involvement of a cardinal angiogenetic mediator not only in inflammatory pathway of exudation but also in trafficking of cancer cells and metastases pathophysiology. The increased VEGF expression in malignant PEs has been recently shown by us and other investigators (7,8). Furthermore, we confirmed with more advanced methodology, a previous study (8) showed that only Ang-2 and not Ang-1 is implicating in

this angiogenetic pathway VEGF-Tie-2-angiopoietins in the formation of exudative malignant PE. However, further investigation is needed in this field as we have to confirm the above finding in larger samples.

In conclusion, SDF-1 α /CXCL12 but not CXCR4 levels, are elevated in malignant exudative PEs, and they correlate with PF VEGF levels and markers of pleural inflammation. Thus, it is possible that VEGF along with SDF-1 α /CXCL12 participate not only in pleural inflammation and formation of exudative PEs, but also in lung cancer metastases' pathophysiology. Our observations call for further *in vivo* studies to elucidate the role of angiogenetic chemokines in the pathogenesis of pleural disease in lung cancer patients.

Acknowledgements

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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