The value of matrix metalloproteinase-9 and vascular endothelial growth factor receptor 1 pathway in diagnosing indeterminate pleural effusion†

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Abstract

OBJECTIVES: Our aim was to determine the diagnostic value of the matrix metalloproteinase-9/vascular endothelial growth factor receptor-1 pathway in differentiating pleural effusions (PE) of varying origin.

METHODS: In the last two years, 55 consecutive patients with exudative PE have been enrolled. In all patients, we measured PE levels of vascular endothelial growth factor receptor-1 (VEGFR-1) in soluble form, through enzyme-linked immunosorbent assay (ELISA) (results expressed in pg/ml) and western blot, and of matrix metalloproteinase-9 (MMP-9), through ELISA (results expressed in ng/ml). The values recorded were then statistically compared with the etiologic diagnosis of the PEs.

RESULTS: Between the PEs analysed, 40 were found to be malignant and 15 to be benign. VEGFR-1 in soluble form (sVEGFR-1) was significantly higher in malignant than in benign effusions ($P<0.0001$), using ELISA; the same was shown by the western blot analysis method. MMP-9 levels results also indicated significantly more malignant than benign effusions ($P<0.0001$). VEGFR-1 in soluble form showed a sensitivity and specificity of 92% and 93%, respectively, (cut-off value >852; AUC: 0.9) in predicting the malignant nature of a PE. Sensitivity and specificity of MMP-9 in predicting the malignant nature of a PE were, respectively, 95% and 73% (cut-off value >639; AUC: 0.8). In the pleural fluids, the values of the two markers were significantly related to each other ($r=0.5$; $P<0.0001$). Eighteen patients with malignancies, diagnosed by pleural biopsy, had negative cytological findings. Of these patients, sixteen (89%) presented elevated levels of both markers.

CONCLUSIONS: Our data suggest that the VEGFR-1/MMP-9 pathway is significantly increased in malignant—rather than in benign—pleural effusions; thus, the measurement of their levels in the pleural effusion could be useful, throughout the diagnostic work-up, to select which cases would warrant a pleural biopsy.

Keywords: Matrix metalloproteinase-9 • Vascular endothelial growth factor receptor-1 • Pleural effusion

INTRODUCTION

Pleural effusion (PE) is a common complication of several benign and malignant diseases. In patients with exudative PE, the differential diagnosis between benign and malignant etiologies is often a clinical challenge and, in about one-third of the cases, the cause remains unknown, despite many investigations [1]. This could be due to the poor comprehension of the mechanism by which fluid accumulates within the pleural space.

Increased vascular permeability plays a principal role in the development of exudative PE. Vascular endothelial growth factor (VEGF) is the most potent and specific growth factor involved in the phenomena of neovascularization, vascular permeability increase and haemorrhage that occur during both inflammatory processes and tumour progression [2]. Recently, we showed that...
concentrations of VEGF in PE was significantly higher in exudative than transudative effusions and that the greatest levels were observed in patients with malignancy, corresponding to other reports [3–7]. However, considering the multifactorial pathogenesis of PE, it is unlikely that a single mediator plays a key role in all types of exudative PE.

A VEGF-specific tyrosine kinase receptor, VEGF receptor-1 (VEGFR-1/Flt-1), has been found in the vascular endothelial cells of tumour vessels and a soluble form of VEGFR-1 (sVEGFR-1) has been found to be secreted into the circulation [8, 9]. Soluble VEGFR-1 could originate not only from vessels but also from tumour cells; it appears to be a crucial, intrinsic, negative regulator of VEGF, having thus a key role in tumour angiogenesis [10–12].

Matrix metalloproteinases (MMPs), are photolytic enzymes involved in the turnover of extracellular matrix proteins. Of these, MMP-9 plays a role in chronic inflammation and is associated with tumour aggressiveness and metastatic potential [13, 14]. Yet MMP-9 is thought to be a tumour angiogenic factor that signals through the VEGF–VEGFR system and recent publications have showed that MMP-9 induction by VEGFR-1 is involved in lung-specific metastases [15, 16]. Thus, the aim of the present paper is to determine the diagnostic value of the MMP-9/VEGFR-1 pathway evaluation in differentiating exudative PEs of varying origin.

MATERIALS AND METHODS

Study design

All consecutive patients with exudative PE, presented to our unit in the last two years, were enrolled in this study. Thoracentesis procedure was performed for diagnostic or therapeutic purposes and PE was collected at the time of first tapping. MMP-9 (ng/ml) and sVEGFR-1 levels (pg/ml) were measured using enzyme-linked immunosorbent assay (ELISA); sVEGFR-1 was also evaluated by western blot analysis. At the time of sample collection, none of the patients had received any immune-stimulating agent, anti-cancer treatment, anti-tuberculosis treatment, corticosteroid or other non-steroidal anti-inflammatory drug treatment. The sVEGFR-1 and MMP-9 laboratory tests were performed independently from clinical diagnosis and treatment. The readers of the index tests were blind to the results of the other tests. Only after the completion of all experimental tests and clinical data collection were the different distributions of sVEGFR-1 and MMP-9 levels among benign and malignant effusions—and their correlation with the final diagnosis of PE—statistically evaluated. The protocol of this study was approved by the Hospital Ethics Committee of the Second University of Naples and written, informed consent was obtained from all cases before being included in the study.

Patients

In the last two years, 69 consecutive patients with PE presented to our hospital. The PE was classified as exudates or transudates, according to Light’s criteria as described in literature [17]. A PE was defined as an exudate when the ratio of pleural fluid/serum proteins was higher than 0.5, pleural fluid/serum LDH >0.6, or pleural fluid LDH <2/3 of the upper normal limit in serum LDH. PEs were then categorized as benign or malignant according to definitive diagnosis. A parapneumonic PE was defined as one associated with bacterial pneumonia, including empyema. A PE was categorized as tubercular if mycobacterium tuberculosis was found in pleural fluid, sputum, bronchial lavage fluid or pleural biopsy specimen (positive smear or culture), or if pleural biopsy revealed granuloma and other granulomatous diseases were excluded. PEs were considered malignant if malignant cells were found on cytological examination or in biopsy specimen. Malignant PEs were divided into three groups as follows: pleural metastasis of primary lung cancer, mesothelioma and pleural metastasis of extrathoracic cancer.

Sample processing

Pleural effusions were collected via diagnostic thoracentesis. They were immediately centrifuged at 1500 rpm for 7 min at 4°C and the supernatant was stored at −70°C awaiting analysis of sVEGFR-1 and MMP-9.

Measurements of sVEGFR-1 and MMP-9 in PE

The levels of sVEGFR-1 (pg/ml) and MMP-9 (ng/ml) were determined using a ‘sandwich’ ELISA kit (R&D Systems Inc, Minneapolis, MN, USA) according to the manufacturer’s guidelines. The minimum detectable levels of sVEGFR-1 and of MMP-9 were less than 8 pg/ml and 1.2 ng/ml, respectively.

Western blot analysis of sVEGFR-1

To evaluate the expression of sVEGFR-1 protein, supernatants were harvested from pleural effusion by centrifuging at 1500×g and then incubated with radiolabeled precipitation assay buffer [50 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l EDTA, 5 Ag/ml aprotinin, 5 Ag/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and homogenized by sonication (4–15 s) in an ice bath, incubated on ice for 30 min and centrifuged (15 000×g for 30 min). The supernatants corresponding to the protein extracts were then collected for protein analysis. The following antibody and working dilution were used for western blotting: rabbit monoclonal antibody against VEGFR-1 (55B11) (1:2000, Cell Signalling Technology Inc., Danvers, MA, USA). The protein-antibody complexes were detected, using an enhanced chemiluminescence kit (Amersham Pharmacia) according to the manufacturer’s recommended protocol, and exposed to photographic film.

Cytological examination in PE

After thoracentesis, each pleural fluid specimen was sent for pathology in a clean container within 4 h of the procedure. Ten millilitres of the fluid were transferred into a centrifuge tube and spun for 10 min at 1500–2000 rpm. The supernatant was decanted and the sediment smeared to make four slides. These slides were fixed in 95% ethyl alcohol and stained with Papanicolaou stain.
Statistical analysis

Data are presented as median and interquartile range (IQR). Comparisons between different groups were performed using Mann–Whitney U-tests. To evaluate correlations between variables, we used the Spearman’s rank correlation test. The accuracy of variables to distinguish malignant from benign PEs was calculated with receiver operating characteristics (ROC). The optimum cut-off point from the ROC analysis was established by selecting the value that provides the greatest sum of sensitivity and specificity. For the optimum cut-off point provided by ROC analysis, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. A value of \( P < 0.05 \) was considered statistically significant. MedCalc® statistical software was used for analysis.

RESULTS

Patient characteristics

Among 69 patients with PE, 11/69 (16%) patients presented a transudative pleural effusion and were thus excluded from the study. Of 58/69 (84%) patients with exudative pleural effusion, three were excluded because the pleural fluid collection and processing were inadequate to determine sVEGFR-1 and/or MMP-9. Thus, our study population included 55 patients. A flow chart of the study design is shown in Fig. 1. The median age was 68 years (range 23–87). There were 40/55 (73%) malignant and 15/55 (27%) benign PEs. Among the malignant group (\( n = 40 \)), 14 (35%) PEs were secondary to a pleural invasion by lung cancer; 19 (47.5%) to extrathoracic cancer (11 breast cancer, 4 colic cancer, 1 gastric cancer, 1 lymphoma, 2 kidney cancer) and 7 (17.5%) to mesothelioma. The 15 patients with parapneumonic effusions suffered from bacterial pneumonia (\( n = 12 \); 80%), empyema (\( n = 2 \); 13%), and tuberculosis (\( n = 1 \); 7%).

Pleural fluid cytology was positive in 22/40 (55%) at the initial thoracentesis. In the remaining 18/40 (45%) cases, the malignant demonstration was obtained by multiple biopsy, performed during thoracoscopy. The diagnosis of the study group is summarized in Table 1.

\[ \text{sVEGFR-1 levels in pleural fluid} \]

The sVEGFR-1 levels were significantly higher in malignant PE than in benign PE (1187 [1095–1445] vs 130 [103–168]; \( P < 0.0001 \); Fig. 2A). Among malignant PEs, the patients with lung cancer had higher median VEGF levels (1310 [1052–1430]) than the patients with extrathoracic cancers (1187 [1092–1455]), and those with mesothelioma (1156 [382–1470]); however, there was no significant difference in sVEGFR-1 levels in malignant PEs of differing origin (lung cancer vs extrathoracic cancer \( P = 0.7 \); lung cancer vs mesothelioma: \( P = 0.8 \); extrathoracic cancer vs mesothelioma: \( P = 0.6 \); Fig. 2B).

\[ \text{Expression of sVEGFR-1 on western blot analysis} \]

Consistent with the results obtained from ELISA analysis, western blot analysis revealed that sVEGFR-1 was increased in the PE of patients with malignant disease, as compared with those with benign disease. All malignant samples demonstrated sVEGFR-1 protein expression at different levels, except three cases which presented no increased sVEGFR-1 on western blot. An example is reported in Fig. 3.

\[ \text{MMP-9 levels in pleural fluid} \]

The MMP-9 levels were significantly higher in malignant PE than in benign forms (1200 [979–1423] vs 171 [145–872]; \( P < 0.0001 \); Table 1).

\[ \text{Table 1. Study population} \]

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Number of patients (( n = 55 ))</th>
<th>sVEGFR-1 (pg/ml)</th>
<th>MMP-9 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>15 (27%)</td>
<td>130 [103–168]</td>
<td>171 [145–629]</td>
</tr>
<tr>
<td>Bacterial pneumonia</td>
<td>12</td>
<td>128 [96–144]</td>
<td>154 [134–174]</td>
</tr>
<tr>
<td>Empyema</td>
<td>2</td>
<td>656</td>
<td>1030</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>1</td>
<td>853</td>
<td>910</td>
</tr>
<tr>
<td>Malignant</td>
<td>40 (73%)</td>
<td>1187 [1095–1445]</td>
<td>1200 [979–1423]</td>
</tr>
<tr>
<td>Lung</td>
<td>14</td>
<td>1310 [1052–1430]</td>
<td>1100 [980–1320]</td>
</tr>
<tr>
<td>Extrathoracic</td>
<td>19</td>
<td>1187 [1092–1455]</td>
<td>1200 [975–1323]</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>7</td>
<td>1156 [382–1470]</td>
<td>1450 [964–1456]</td>
</tr>
</tbody>
</table>

Data are presented as median and interquartile range.
Fig. 4A). Among malignant PEs, patients with mesothelioma had higher median VEGF levels (1450 [964–1456]) than the patients with extrathoracic cancers (1200 [975–1323]), and those with lung cancer (1100 [980–1320]), but there was no significant difference in MMP-9 levels in the PEs of different origin (lung cancer vs extrathoracic cancer: \( P = 0.6 \); lung cancer vs mesothelioma: \( P = 0.5 \); extrathoracic cancer vs mesothelioma: \( P = 0.2 \); Fig. 4B).

ROC curve for the presence of malignant effusion

Using logistic regression models, we calculated sensitivity and specificity of such markers to predict malignant effusion for each possible threshold value. ROC curves were constructed to visualize the relationship between sVEGFR-1 and MMP-9 levels in pleural fluid. PE sVEGFR-1 reached a sensitivity of 92%, a specificity of 93%, a PPV of 97% and an NPV of 82%, when a cut-off value of 852 pg/ml was applied (Fig. 5A). MMP-9 reached a sensitivity of 95%, a specificity of 73%, a PPV of 90% and an NPV of 84% when a cut-off value of 639 ng/ml was applied (Fig. 5B). In the ROC curve analysis, sVEGFR-1 (0.930) had higher area under the ROC curve than that of MMP-9 (0.853) (Fig. 5C), but the difference was not significant.

The correlation of sVEGFR-1 with MMP-9 levels in pleural fluid

In the pleural fluids, levels of sVEGFR-1 were significantly correlated with those of MMP-9 (\( r = 0.5 \); \( P < 0.0001 \), Fig. 6).

sVEGFR-1, MMP-9 and cytological examination

Of malignant PEs, fluid cytology was positive in 22/40 (55%) at the initial thoracentesis. In the remaining 18/40 cases, the malignant demonstration was obtained by multiple biopsy, performed
during thoracoscopy. Of these 18 patients with negative cytology results but malignancy diagnosed by pleural biopsy, 16/18 (89%) presented elevated levels of VEGFR-1 (>852 pg/ml) and of MMP-9 (>639 ng/ml) in PE. Thus, the combined use of cytological examination with VEGFR-1 and MMP-9 measurements increased the detection rate of malignancy when compared to cytological examination only (38/40 patients (95%) vs 22/40 (55%), respectively).

**DISCUSSION**

Exudative PE often remains a diagnostic problem after biochemical and cytological analysis of the pleural fluid. More invasive procedures, such as blind or open pleural biopsies, are frequently required to establish a diagnosis. It would be helpful to have new fluid markers to speed up the diagnostic process and identify, at an earlier stage, patients who require invasive procedures.

The sVEGFR-1 receptor plays an important role in the pathogenesis of several diseases. Most of the available data on sVEGFR-1 in humans was obtained in a gynaecological context, where serum and amniotic fluid levels of sVEGFR-1 were related to pregnancy [10]. Little is known about the role of sVEGFR-1 in human cancers and only two studies investigated its involvement in pleural effusions. Tomimoto et al. [18] found higher levels of sVEGFR-1 in exudative than in transudative effusions; Hooper et al. [19] showed that high levels of sVEGFR-1 in PEs were strongly associated with a poor outcome in patients with small-cell lung cancer.

The value of the analysis of MMP-9 levels in pleural fluid, as a means of diagnosing malignant PEs, remains controversial. Hurewitz et al. [20] reported that MMP-2 and MMP-9 pleural fluid levels did not appear predictive of malignant neoplasms. Jin et al. [21] showed that VEGF and MMP-9 pleural fluid levels were significantly increased in patients with tuberculosis, and higher in patients with lung cancer than in those with liver cirrhosis. Vatansever et al. [22] reported that overproduction of MMP-9 and TIMP-2 was associated with the development of pleural effusion in malignancies.

To the best of our knowledge, no studies investigated the combined role of VEGFR-1 and MMP-9 in differentiating PEs of differing origin, as in the present work. Firstly, we found that sVEGFR-1 pleural fluid levels are significantly higher in malignant PEs than in benign PEs. Such a finding seems to indicate a specific response of the pleura to the presence of malignant cells, as suggested by Tomimoto et al. [18]. The sVEGFR-1 production is likely to take place in thoracic and extrathoracic malignancies metastasized to the pleura, as well as in mesothelioma. Supporting this assertion is the fact that we did not observe significant differences in pleural fluid sVEGFR-1 levels between malignant pleural effusions of different origin.

Pleural fluid MMP-9 levels were also significantly higher in malignant PEs than in benign PEs. This discovery is in agreement with other reports that confirm an elevated concentration of sVEGFR-1 in malignant PEs [19]. Such a finding seems to indicate a specific response of the pleura to the presence of malignant cells, as suggested by Tomimoto et al. [18]. The sVEGFR-1 production is likely to take place in thoracic and extrathoracic malignancies metastasized to the pleura, as well as in mesothelioma. Supporting this assertion is the fact that we did not observe significant differences in pleural fluid sVEGFR-1 levels between malignant pleural effusions of different origin.

Pleural fluid MMP-9 levels were also significantly higher in malignant PE than in benign PE. Even if pleural effusions of inflammatory origin are rich in MMPs, the higher levels of MMP-9 found in malignant effusions may be due to the relevant MMP-9 role in the neoplastic diffusion to the pleura.

Secondly, both markers were useful in differentiating benign from malignant effusion, with a sensitivity of 92% and 95% for sVEGFR-1 and MMP-9, respectively, and a specificity of 93% and 73% for sVEGFR-1 and MMP-9, respectively.

Two patients with malignant effusion presented pleural fluid MMP-9 levels less than the cut-off value of 639. Conversely, between patients with benign PEs, three (one with tuberculosis effusion and two with empyema) had MMP-9 levels

**Figure 5:** sVEGFR-1 reached a sensitivity of 92%, a specificity of 93%, a PPV of 97%, and a NPV of 82% (cut-off value: 852 pg/ml, AUC: 0.930; (A)); and MMP-9 a sensitivity of 95%, a specificity of 73%, a PPV of 90%, and a NPV of 84% (cut-off value: 639 ng/ml, AUC: 0.69; (B)) in distinguishing malignant from benign disease. In the ROC curve analysis, sVEGFR-1 had higher AUC compared with that of MMP-9 (0.930 vs 0.853, respectively), but the difference was not significant (C).

**Figure 6:** In the pleural fluids, levels of sVEGFR-1 were significantly correlated with those of MMP-9 ($r = 0.5$; $P < 0.0001$: Spearman correlation test).

where serum and amniotic fluid levels of sVEGFR-1 were related to pregnancy [10]. Little is known about the role of sVEGFR-1 in human cancers and only two studies investigated its involvement in pleural effusions. Tomimoto et al. [18] found higher levels of sVEGFR-1 in exudative than in transudative effusions; Hooper et al. [19] showed that high levels of sVEGFR-1 in PEs were strongly associated with a poor outcome in patients with small-cell lung cancer.

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To the best of our knowledge, no studies investigated the combined role of VEGFR-1 and MMP-9 in differentiating PEs of differing origin, as in the present work. Firstly, we found that sVEGFR-1 pleural fluid levels are significantly higher in malignant PEs than in benign PEs. This discovery is in agreement with other reports that confirm an elevated concentration of sVEGFR-1 in malignant PEs [19]. Such a finding seems to indicate a specific response of the pleura to the presence of malignant cells, as suggested by Tomimoto et al. [18]. The sVEGFR-1 production is likely to take place in thoracic and extrathoracic malignancies metastasized to the pleura, as well as in mesothelioma. Supporting this assertion is the fact that we did not observe significant differences in pleural fluid sVEGFR-1 levels between malignant pleural effusions of different origin.

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Two patients with malignant effusion presented pleural fluid MMP-9 levels less than the cut-off value of 639. Conversely, between patients with benign PEs, three (one with tuberculosis effusion and two with empyema) had MMP-9 levels
higher than the cut-off value of 639. This result is not surprising: Jin et al. [21] and Iglesias et al. [23], in fact, found higher levels of MMP-9 in tuberculosis pleural fluids and in empyemas, than those seen in other exudates and in uncomplicated pleural effusions.

This finding could be explained, assuming a role of the MMP-9 in the escalation of the pleural inflammatory and fibrotic reaction to an advanced stage of disease.

Three patients with malignant PE had pleural fluid sVEGFR-1 levels lower than the cut-off value of 852, while two patients with benign effusion presented pleural fluid sVEGFR-1 levels > 852.

The low pleural levels of sVEGFR-1 in patients with malignant effusion could be due to the fact that not all types of human cancers induce the expression of sVEGFR-1.

Thirdly, in malignant PEs, a significant correlation was found between sVEGFR-1 and MMP-9 levels (P < 0.0001), suggesting that such biomarkers might perform an important role in tumour-dependent pleural exudation. In a mouse model, Hiratsuka et al. [15] showed that MMP-9 expression, induced by primary tumours in endothelial cells and macrophages of the lung, promoted the neoplastic invasion. Furthermore, the same authors [15] demonstrated that the MMP-9 induction was achieved though VEGFR-1-linked tyrosine kinase.

However, we are conscious that the simple observation of elevated VEGFR-1 and MMP-9 levels in malignant PEs—and of a significant correlation between such markers—is insufficient evidence of any specific role in the pathogenesis of malignant effusions. Therefore further studies are needed to demonstrate the pathogenic role of these molecules in promoting cancerous invasion of the pleural cavity.

Fourthly, in patients with malignancies, pleural fluid cytology was positive in 22/40 cases (55%) at the initial thoracentesis, but negative in the remaining 18 cases. In the patients with malignancies diagnosed by biopsy samples and with negative cytology, we found elevated pleural fluid levels of VEGFR-1 (>852 pg/ml) and of MMP-9 (>639 ng/ml). Possible explanations for this finding include: (1) presence in the pleural fluid of a small number of tumour cells: too low for a cytological diagnosis, but sufficient to induce an increase of MMP-9 and sVEGFR-1 levels; (2) surface metastasis secreting MMP-9 and sVEGFR-1 in the pleural cavity; (3) tumour cell death or destruction with a residual presence of MMP-9 and sVEGFR-1 in the pleural fluid. Therefore, the combined execution of cytological examination and of VEGFR-1 and MMP-9 analysis on PEs of patients with suspected malignancy may increase the detection rate of malignancies in comparison with cytological examination alone. Even if histological confirmation is always required for diagnosing malignant PEs, the measurement of MMP-9 and sVEGFR-1 levels on pleural fluid may help in selecting which patients undergo the more invasive procedures, such as thoracoscopic biopsy. The analysis of MMP-9 and sVEGFR-1 levels on pleural fluid would be particularly useful in patients with negative thoracentesis for neoplastic cells but with a high suspicion of malignancy in the light of clinical history and of radiological findings. On the other hand, the presence of low MMP-9 and sVEGFR-1 levels in the pleural fluids of patients with low probability of malignancy, may serve to avoid performing a more invasive procedure, thus saving costs.

Firstly, our study involved a limited number of patients. Our results should therefore be corroborated by further, larger studies. In addition to this, in our population, the prevalence of malignant PE is higher than of benign (73% vs 27%, respectively). This could result in a selection bias.

Secondly, our paper is unable to demonstrate that MMP-9/VEGFR-1 pathway mediates or regulates the pathogenesis of PE, since our clinical results are not supported by functional in vitro and/or animal studies.

Thirdly, in the present study we investigated the diagnostic role of such markers. No other aspect of everyday thoracic surgery practice was evaluated: for example, we did not assess whether such molecules may predict the success of pleurodesis and their relationship with the clinical outcome. We hope to solve these issues in a second phase of the study.

CONCLUSIONS

Our data suggest that MMP-9/VEGFR-1 pathway is significantly increased in malignant pleural effusion, compared to benign PE. If our data is corroborated by future larger studies, pleural fluid sVEGFR-1 and MMP-9 levels analysis—combined with that of the other routine markers—may be applied to clinical practice in order to rule out malignancies as a probable diagnosis, and thus to guide the selection of patients who would benefit from further invasive procedures.

Conflict of interest: none declared

REFERENCES


Study limitations

We are conscious that our paper presents several limitations, as follows:
APPENDIX. CONFERENCE DISCUSSION

Dr A. Turna (Istanbul, Turkey): My question is about methodology. In order to be sure that a benign effusion is indeed benign, you have to follow the patient for a considerable period of time. My question is, did you follow the patients with so-called benign aetiology and did you perform some other invasive procedures, such as VATS or thoracotomy, rarely required but sometimes necessary, in order to be sure that they are benign?

Dr Fiorelli: In some patients, yes, we performed invasive procedures when a malignancy was suspected in the light of clinical history and/or radiological findings, for example. If we did not find malignancy in the biopsy specimen, these patients were followed up. If we have reduced effusion, we considered that as benign.

In younger patients with low risk of malignancy according to data from the clinical history, we commenced medical therapy and followed up these patients; if the pleural effusion resolved, we classified it as benign and no further invasive procedures were undertaken. But in all patients we performed thoracentesis to measure MMP9 and VEGFR1.

Although it is a prospective study, the measurement of VEGFR1 and MMP9 didn’t have any consequence on the routine diagnostic work-up of these patients. After completion of all diagnostic examinations the different distributions of sVEGFR-1 and MMP9 were statistically correlated with the final diagnosis of pleural effusion.

Dr D. Branscheid (Bielefeld, Germany): Another question about the method. What did you do with the pleural effusion? Did you centrifuge it? Did you just perform one puncture and remove 5 or 6 or 10 ml? Did you use the pellet of it or did you use just the liquid? How did you do that? What was your concept?

Dr Fiorelli: The question is how I preserved the samples to do the MMP9 and the VEGFR1 measurements. In the design of this study, all patients underwent thoracentesis, and pleural effusions were collected via diagnostic thoracentesis. We put a little sample in our product and performed centrifugation. Afterwards, samples were stored at -70°C. Levels of sVEGFR1 and MMP9 were determined using ELISA, the analysis being undertaken when a sufficient number of samples were accumulated in order to reduce the number of ELISA kits used with consequent cost savings.

Dr M. Lanuti (Boston, MA, USA): How do you envision applying this? Are you going to perform routine cytology first and then use this as a reserve assay?

Dr Fiorelli: Can you repeat because I don’t understand.

Dr Lanuti: How will you apply this new technology, assessment of MMP9 and VEGFR1, in someone with a pleural effusion? Will you obtain cytology and VEGFR1, in someone with a pleural effusion? Will you obtain cytology?