Pro-cathepsin D as a diagnostic marker in differentiating malignant pleural effusion from benign pleural effusion: A retrospective cohort study

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Abstract

Background

Malignant pleural effusion (MPE) is a common complication of lung cancer and intrathoracic spreading or metastasis of extra-thoracic malignancy. The aim of the present study was to evaluate the levels of pro-cathepsin D from plasma and pleural fluid in patients with MPE and those in patients with benign pleural effusion (BPE) including pleural tuberculosis and parapneumonic effusion.

Methods

This study included 81 patients with pleural effusion who underwent thoracentesis and pleural biopsy. Pleural fluid and serum were collected as a standard procedure for all individuals at the same time. The level of pro-cathepsin D was measured by the sandwich enzyme-linked immunosorbent assay method.

Results

Though there were no significant differences in plasma pro-cathepsin D between the two groups, the level of pleural fluid pro-cathepsin D was significantly higher in the MPE group than the BPE group (0.651 versus 0.590 pg/mL, P = 0.034) (Table 1). In addition, there were no differences in pleural fluid pro-cathepsin D level according to causative malignancy of MPE. On receiver operating characteristic curve analysis, the optimal discrimination point between the MPE group and BPE group was defined as a cut-off value of 0.5960 pg/mL for pleural fluid pro-cathepsin D (81.0% sensitivity; 53.0% specificity) and 0.4335 pg/mL for plasma pro-cathepsin D (71.4% sensitivity; 61.7% specificity).

Conclusions

We found that the level of pleural fluid pro-cathepsin D was significantly higher in the MPE group than the BPE group. Pro-cathepsin D could be a novel and potential biomarker to discriminate MPE from BPE.

Background

Malignant pleural effusion (MPE) is a common complication of lung cancer and intrathoracic spreading or metastasis of extra-thoracic malignancy [1–3]. It is encountered as advanced malignancy at the time of diagnosis, progression of primary disease despite anti-neoplastic treatment, or recurrence. MPE is usually found in patients with advanced malignancy and is accompanied by dyspnoea, pleuritic chest pain, cachexia, and physical inactivity [1]. Thus, a rapid and accurate diagnosis of MPE is essential for adequate management of patient symptoms and prognosis [3]. The definite diagnosis of MPE is determined by pleural fluid cytology, once or several times, or sometimes by pleural biopsy [1]. Although pleural fluid cytology is a simple method for diagnosis, its diagnostic yield is approximately 60% and depends on the underlying pathologic type of primary malignancy [1, 4]. Moreover, MPE can be mimicked by other common causes of exudative pleural effusion such as pleural tuberculosis and parapneumonic...
effusion [5]. Thus, there is an increasing need to discover non-invasive biomarkers to diagnose MPE accurately and efficiently in clinical practice.

To avoid an invasive pleural biopsy, several serum or pleural fluid biomarkers have been studied for diagnosis of MPE, either alone or in combination [1, 6, 7]. Pro-cathepsin D, the inactive precursor of lysosomal aspartyl proteinase cathepsin D, is overexpressed and secreted by several types of cancer cells such as breast, liver, and lung cancer and cancerous cell lines [8–11]. The role of pro-cathepsin D has not been completely elucidated; however, it has been suggested to be involved with tumour growth and invasion by intercellular communication [10]. Several previous studies showed the level of pro-cathepsin D to be associated with progression of primary cancer [8]. Thus, MPE, another form of primary cancer progression that can be difficult to diagnose, may be aided by novel biomarker pro-cathepsin D in diagnosis.

The aim of the present study was to evaluate the levels of plasma and pleural fluid pro-cathepsin D in patients with MPE and those in patients with benign pleural effusion (BPE) including pleural tuberculosis and parapneumonic effusion. In addition, we aimed to investigate the value of pro-Cathepsin D in differentiating MPE from BPE.

**Methods**

**Patients and pleural fluid collection**

The present study included 81 patients with pleural effusion who underwent thoracentesis and pleural biopsy. Patients with pleural effusion had not received any kind of systemic treatment. These patients were selected based on pathologic reports of pleural biopsy. Clinical and pathology data, including tumour type, were acquired for all patients, with approval from the Institutional Review Board (IRB) at Hallym University, and informed consent was obtained from all patients (IRB application no. 2014-18). Pleural fluid and serum were collected at the same time as a standard procedure for all individuals. Obtained pleural fluid and blood samples were immediately centrifuged at 2000 g for 10 min, and the supernatants were stored at – 80°C until assayed.

**Analysis of Pro-Cathepsin D**

For analysis, 96-well microtiter plates were coated by applying 100 ul/well of anti-cathepsin D monoclonal antibody clone 6410, Abcam, Cambridge, UK) at 5 ug/ml in 100 mM sodium carbonate, pH 9.6 incubated overnight at room temperature (RT). Plates were washed with PBS and blocked with 2% BSA and 10% lactose in PBS prior to use. Next, 100 ul of standard or sample diluted in PBS with 4% BSA or in PBS with 4% BSA and 0.7% NP40 was added to each well and incubated overnight at RT. Plates were washed 6 times with wash buffer (10 mM phosphate, pH 7.5, 150 mM NaCl, 0.05% Tween-20), and 100 ul of anti-pro-cathepsin D rabbit polyclonal detector antibody (4 ug/ml) was added and incubated for 1 hr at RT. Plates were washed 6 times as before, followed by addition of 100 ul of goat anti-rabbit HRP conjugate (KPL) at 0.25 ug/ml. After 30 min at RT, the plates were again washed 6 times, and 100 ul of O-
phenylenediamine substrate (Dako, 1 mg/ml in 100 mM citrate buffer, 0.03% hydrogen peroxide) was added. Development proceeded for 1 hr at RT in the dark and was stopped by addition of 100 ul of 4N N$_2$SO$_4$. Absorbance was measured at 490 nm using a Biotek EL 309 autoreader.

Statistical analysis

The data are presented as median and IQR (interquartile range) for continuous variables and as number (percentage) for categorical variables. Data were compared using the Mann–Whitney U test for continuous variables and Pearson's chi-square test or Fisher's exact test for categorical variables. Spearman's test was used to assess correlations between variables. The receiver operating characteristic (ROC) curves were analysed to determine the optimal cut-off value, calculated using the highest sum of sensitivity and specificity, and to compare the diagnostic accuracies of pro-cathepsin D. All tests were two-sided, and a P-value < 0.05 was considered significant. Data were analysed using IBM SPSS Statistics version 24 (IBM Corp., Armonk, NY, USA).

Results

Characteristics of study participants

In total, 81 cases with pleural effusion were enrolled in this study. The demographic and clinical characteristics of the study populations are shown in Table 1. Of these, 21 (25.9%) had MPE, and 60 (74.1%) had BPE. With respect to the clinical characteristics, the patients with MPE were older than those with BPE (68.0 versus 58.0 years, $P$ = 0.016). Of the 21 cases with MPE, 19 (90.5%) were lung cancer, and the other two (9.5%) were pleural metastasis of extra-thoracic malignancy. All cases with MPE were positive for malignant cells in the cytologic examination of pleural fluid. Pleural fluid white blood cell counts were lower in the MPE group compared with those of the BPE group (450 versus 1,160 /µl, $P$ = 0.003). In addition, patients with MPE demonstrated significantly higher glucose (114.0 versus 95.5 mg/dL, $P$ = 0.037) and lower adenosine deaminase (17.0 versus 83.0 IU/L, $P$ = 0.001) levels than those with BPE.
Table 1
Clinical characteristics of the two patient groups.

<table>
<thead>
<tr>
<th></th>
<th>Malignant pleural effusion (n = 21)</th>
<th>Benign pleural effusion (n = 60)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>68.0 (59.0–82.5)</td>
<td>58.0 (35.3–73.8)</td>
<td>0.016</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>14 (66.7)</td>
<td>41 (68.3)</td>
<td>0.888</td>
</tr>
<tr>
<td>Diagnosis of MPE</td>
<td></td>
<td></td>
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<tr>
<td>LCA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Adenocarcinoma</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>Cholangiocarcinoma</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>Diagnosis of BPE</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tuberculous pleural effusion</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parapneumonic effusion</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleural fluid findings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG</td>
<td>1.020 (1.015–1.020)</td>
<td>1.020 (1.015–1.020)</td>
<td>1.000</td>
</tr>
<tr>
<td>pH</td>
<td>7.5 (7.5–7.5)</td>
<td>7.5 (7.5–7.5)</td>
<td>0.870</td>
</tr>
<tr>
<td>WBC (total cells)</td>
<td>450.0 (288.0–710.0)</td>
<td>1169.0 (397.5–2124.0)</td>
<td>0.003</td>
</tr>
<tr>
<td>Neutrophil, %</td>
<td>70.0 (60.0–82.5)</td>
<td>30.0 (20.0–54.0)</td>
<td>0.526</td>
</tr>
<tr>
<td>Lymphocyte, %</td>
<td>30.0 (17.5–40.0)</td>
<td>76.0 (46.0–80.0)</td>
<td>0.526</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>114.0 (106.5–151.0)</td>
<td>95.5 (69.3–139.3)</td>
<td>0.037</td>
</tr>
<tr>
<td>Protein, g/dL</td>
<td>4.2 (3.7–5.0)</td>
<td>4.6 (2.9–5.4)</td>
<td>0.845</td>
</tr>
<tr>
<td>Albumin, g/dL</td>
<td>2.3 (2.0–2.9)</td>
<td>2.4 (1.6–2.7)</td>
<td>0.551</td>
</tr>
<tr>
<td>LDH, IU/L</td>
<td>417.0 (235.5–548.0)</td>
<td>447.0 (211.0–881.0)</td>
<td>0.552</td>
</tr>
</tbody>
</table>

Data are presented as the median (interquartile range) or no. (%).

MPE, malignant pleural effusion; BPE, benign pleural effusion, LCA, lung cancer; SG, specific gravity; WBC, white blood cell; LDH, lactate dehydrogenase; ADA, adenosine deaminase.
Malignant pleural effusion | Benign pleural effusion | P-value
---|---|---
(n = 21) | (n = 60)
ADA, IU/L | 17.0 (14.0–24.0) | 83.0 (17.8–109.2) | 0.001
Pro-cathepsin D
Plasma, pg/mL | 0.469 (0.421–0.554) | 0.455 (0.405–0.549) | 0.528
Pleural fluid, pg/mL | 0.651 (0.601–0.716) | 0.590 (0.511–0.692) | 0.034

Data are presented as the median (interquartile range) or no. (%).

MPE, malignant pleural effusion; BPE, benign pleural effusion; LCA, lung cancer; SG, specific gravity; WBC, white blood cell; LDH, lactate dehydrogenase; ADA, adenosine deaminase.

### Level of pro-cathepsin D and diagnostic accuracy

For all study cases, a significant positive correlation between pleural fluid pro-cathepsin D level and plasma pro-cathepsin D level was shown (Spearman's $r = 0.8704$, 95% confidence interval = 0.8027 to 0.9159, $P < 0.0001$) (Fig. 1). Though there were no significant differences in plasma pro-cathepsin D between two groups, the level of pleural fluid pro-cathepsin D was significantly higher in the MPE group than the BPE group (0.651 versus 0.590 pg/mL, $P = 0.034$) (Table 1). In addition, there were no differences in pleural fluid pro-cathepsin D level according to causative malignancy of MPE (Fig. 2).

On ROC curve analysis, the optimal discrimination point between the MPE group and other BPE groups was defined as a cut-off value of 0.5960 pg/mL for pleural fluid pro-cathepsin D (81.0% sensitivity; 53.0% specificity) and 0.4335 pg/mL for plasma pro-cathepsin D (71.4% sensitivity; 61.7% specificity) (Fig. 3). The area under the curve (AUC) values for pleural fluid and plasma pro-cathepsin D were 0.656 and 0.546, respectively.

### Discussion

Pleural fluid pro-cathepsin D was significantly higher in patients diagnosed with MPE and can be used to discriminate between MPE and BPE. In addition, a cut-off level for pleural fluid pro-cathepsin D of 0.5960 pg/ml was predictive of MPE in patients with exudative pleural effusion with moderate sensitivity and specificity.

Pleural fluid cytology is usually used for diagnosing MPE; however, its diagnostic yield was only about 50% in previous reports [5, 12]. Furthermore, even when the cytology results are negative, a thoracoscopic pleural biopsy is not feasible in most patients with an advanced stage of cancer. Thus, various biomarkers have been investigated, and pro-cathepsin D is one of the potential candidates for diagnosing MPE. Pro-cathepsin D, which is a proform of lysosomal aspartic peptidase cathepsin D, was overexpressed in breast cancer, lung cancer, and hepatocellular carcinoma [9, 11, 13, 14]. In agreement
with previous reports, our study showed that pro-cathepsin D was significantly higher in patients with MPE than those with BPE. The reason why we chose pro-cathepsin D rather than cathepsin D as a potential diagnostic marker was that previous studies have suggested that mature cathepsin D participates in intracellular protein catabolism, hormone and antigen processing, and the apoptotic pathway, which also occur in non-neoplastic cells [15, 16]. On the other hand, the proform pro-cathepsin D was correlated with enhanced proliferation and neoplastic transformation [17, 18]. Thus, we aimed to investigate the diagnostic role of pro-cathepsin D in MPE. This study showed the correlation of serum and pleural fluid pro-cathepsin D and its diagnostic performance in MPE with moderate sensitivity and specificity.

Regarding underlying mechanisms of pro-cathepsin D, previous studies suggested that they are involved in multiple stages of tumour progression including proliferation, invasion, metastasis, angiogenesis, and apoptosis [19, 20]. From this perspective, pro-cathepsin D might be used as a prognostic marker as well as a diagnostic marker. Though this study could not demonstrate the association of pro-cathepsin D level and patient prognosis due to its small sample size, Y.-J. Qi and colleagues suggested its role as a candidate biomarker associated with hepatocellular carcinoma development and progression [11]. Future study with a larger study population is needed to establish its role as a prognostic marker, which will provide invaluable information to clinicians and patients.

There are several potential limitations to our study. First, this study is retrospective and investigated a small number of patients. Second, we could not provide sufficient evidence to replace previous diagnostic methods with pleural fluid pro-cathepsin D. Third, laboratory facilities are necessary to measure pleural fluid pro-cathepsin D, which limits its application to other institutions. Fourth, patients with MPE were enrolled only when cancer cells were observed on cytology, although malignant cells may not be visualized on cytology in the real world.

In conclusion, our study suggests that pleural fluid pro-cathepsin D could be a potential novel biomarker to discriminate between MPE and BPE. In addition, a cut-off level for pleural fluid pro-cathepsin D of 0.5960 pg/ml was predictive of MPE in patients with exudative pleural effusion with moderate sensitivity and specificity.

**Abbreviations**

MPE: malignant pleural effusion; BPE: benign pleural effusion; RT: room temperature; IQR: interquartile range; ROC: receiver operating characteristic; AUC: area under the curve

**Declarations**

**Acknowledgments**

None.
Authors’ contributions
HC: acquisition and interpretation of data and article writing. YK: interpretation of data, statistical analysis, and article revising. CYL: design of the work, acquisition and interpretation of data, and article revising. We state that the manuscript has been read and approved by all authors. This manuscript has not been published and is not under consideration for publication elsewhere.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval and consent to participate
Clinical and pathology data, including tumour type, were acquired for all patients, with approval from the Institutional Review Board (IRB) at Hallym University, and informed consent was obtained from all patients (IRB application no. 2014-18).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


Correlation of plasma pro-cathepsin D and pleural fluid pro-cathepsin D levels in study participants (n = 81; Spearman's r = 0.8704, 95% CI = 0.8027 to 0.9159, p < 0.0001)
Figure 2

Comparisons of pleural fluid pro-cathepsin D level according to pathologic type of malignant pleural effusion. AD, adenocarcinoma; SQ, squamous cell carcinoma; SC, small cell carcinoma.

Fig. 3
Figure 3

Receiver operating characteristic curves of pleural fluid pro-cathepsin D and plasma pro-cathepsin D for differentiation of malignant pleural effusion from other causes of pleural effusion. The areas under the curve values were 0.656 and 0.546, respectively.