

## RESEARCH ARTICLE

# Clinical Impact and Reliability of Carbonic Anhydrase XII in the Differentiation of Malignant and Tuberculous Pleural Effusions

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### Abstract

**Objective:** To assess the practical utility of pleural fluid carbonic anhydrase XII (CAXII) quantification for differential diagnosis of effusions. **Materials and Methods:** Fluid was collected prospectively from fifty patients presenting with lymphocytic pleural effusions for investigation and CAXII was quantified by ELISA. **Results:** Pleural fluid CAXII concentrations were significantly higher in lung cancer patients (n=30) than in tuberculous controls (n=20). The sensitivity and specificity of this biomarker were 60% and 75%, respectively. CAXII measurement was not inferior to cytological examination in the diagnosis and exclusion of pleural effusions from lung cancer patients (sensitivity 60% vs. 57%; specificity 75% vs. 100%; positive predictive value 77%; negative predictive value 54%). In patients with negative cytology, it offered a sensitivity of 54%. **Conclusions:** Pleural fluid CAXII is elevated in pleural effusions from lung cancer patients. Measurement of CAXII may be used in the future as a valuable adjunct to cytology in the diagnostic assessment of patients with pleural effusions related to lung cancer, especially when cytological examination is inconclusive.

**Keywords:** Carbonic anhydrase XII - lung cancer - tuberculosis - pleural effusion

*Asian Pacific J Cancer Prev*, 14 (1), 351-354

### Introduction

Malignant and tuberculous pleurisy are two main causes of pleural effusion (PE) in a high tuberculosis prevalence region (Liam et al., 2000; World Health Organization, 2011). There are significant difficulties in the differential diagnosis between malignant and tuberculous pleural effusions. Although cytologic examination of PE is an informative diagnostic procedure with high specificity, its sensitivity ranges between 40%-80% (Giazza et al., 1990). At present there are evidences to demonstrate that pleural fluid mesothelin, MMP-7 and MMP-10 may provide additional value over pleural fluid cytology in patients with an undiagnosed pleural effusions (Davies et al., 2009; Cheng et al., 2012). Unfortunately, conventional tumor markers had already failed to show adequate sensitivity and specificity for their routine clinical use, including carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), and cytokeratin 19 fragments (CYFRA 21-1) (Alataş et al., 2001; Porcel et al., 2004; Shitrit et al., 2005).

Carbonic anhydrase XII (CAXII) is a transmembrane enzyme, which catalyzes the reversible hydration of carbon dioxide to form bicarbonate ( $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ ). The role of CAXII in tumor growth is to regulate acidification of the extracellular milieu of

tumors, creating a suitable microenvironment for rapid tumor growth (Ivanov et al., 2001; Whittington et al., 2001; Chiche et al., 2009). The possibility of CAXII overexpression has been demonstrated in resectable non-small cell lung cancers (NSCLC) (Ilie et al., 2011). Serum CAXII levels are significantly higher in lung cancer patients than healthy controls (Kobayashi et al., 2012). And there is no allegation at present to show that it is overexpression in tuberculosis patients. CAXII produces locally and releases directly into pleural cavity. Hence its measurement in pleural fluid (PF) offers a theoretical advantage over serum measurement and has attracted attention as a diagnostic assay.

The objective of present study is to explore whether CAXII levels is detectable in pleural fluids and to evaluate its potential diagnostic usefulness and its reliability.

### Materials and Methods

#### Patients

Fifty patients presenting with lymphocytic exudative pleural effusions were consecutively included in the study. Thirty pleural fluids from patients with lung cancer were collected at Shandong Cancer Hospital and Institute, Jinan, China. Twenty tuberculous pleural fluids were collected at Shandong Chest Hospital, Jinan, China. Metastatic

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pleural effusion from lung cancer was confirmed by cytologic and/or histologic testing. Tuberculous pleural effusion was diagnosed with a smear or culture positive for *Mycobacterium tuberculosis* from pleural effusion and/or histology showing a caseating granuloma. Samples were divided into three groups (Table 1): samples from patients with tuberculous pleural effusion (group I); samples from patients with lung cancer in which the cytologic examination of pleural effusion revealed malignant cells (group II); samples from patients with lung cancer in which the cytologic examination of pleural effusion did not reveal malignant cells (group III) (A thoracoscopic pleura biopsy confirmed the diagnosis of metastatic disease).

The protocol and procedure for obtaining informed consent were approved by the Institutional Ethical Committees, and all patients who agreed to participate in this study were required to sign consent forms.

#### Pleural fluid collection

A diagnostic pleural fluid sample should be gathered with a fine bore (21G) needle at the bedside and 50ml syringe were adequate for diagnostic pleural taps. The sample were divided into four sterile pots to be sent for microbiological, biochemical, cytological analysis and biomarker measurement (which was centrifuged at 1,000×g for 20 minutes, and supernatants were stored frozen at -80°C until assay) (Hooper et al., 2010).

#### Conventional cytologic examination

The specimen was centrifuged and a cytospin slide was prepared. The prepared slide was stained following the Papanicolaou method and evaluated by an experienced cytopathologist. Histologic diagnosis and classification of the specimens were defined according to the relevant World Health Organization International Histological Tumor Classification System (Travis et al., 1999).

#### Quantification of CAXII

CAXII concentrations were measured with a commercially available ELISA (Uscn, China) according to the manufacturer's instructions. All samples (undiluted) were encrypted and analyzed by a lab technician blinded to the patients' diagnoses.

#### Statistical analysis

All analyses were performed with SPSS 18.0 software, and a P value of less than 0.05 was regarded as statistically significant. Box plots were used to present the results. Intergroup comparisons were analyzed by one-way analysis of variance (ANOVA). A receiver operating characteristic (ROC) curve was used to define the best cutoff point for the diagnostic biomarker and to calculate the sensitivity and specificity of CAXII. The spearman's rank correlation coefficient (rs) was used to determine correlations between nonparametric variables.

## Results

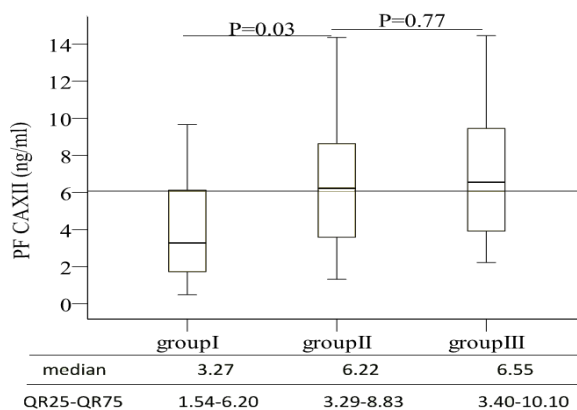
#### Pleural fluid CAXII levels

Quantification of CAXII was first done in specimens

**Table 1. Baseline Characteristics of Patients in the Initial Series of Pleural Fluids**

Group*(n)	Age, y (mean±SD; range)	Gender (males/females)	Histological type (AD/SCLC/SCC)
I (20)	37.52±10.51	14; 6	—
II (17)	59.11±12.95	8; 9	14; 2; 1
III (13)	53.75±15.14	7; 6	5; 4; 4

\*I, tuberculous pleural effusion; II, cytology-proven malignant pleural effusion associated with lung cancer; III, cytology-negative malignant pleural effusion associated with lung cancer. AD, adenocarcinoma; SCLC, small cell lung cancer; SCC, squamous cell carcinoma



**Figure 1. The levels of CAXII in Pleural Effusions from Patients with Tuberculous, Cytologically Positive and Cytologically Negative Pleural Fluids Associated with Lung Cancer**

from patients with tuberculous pleural effusion (group I in Table 1) and specimens from patients with lung cancer in which the cytologic examination of pleural fluids was positive (group II in Table 1). The median concentration was significantly higher in lung cancer patients with cytologically positive pleural effusions compared with the tuberculous control group: median (IQR25-IQR75) values were 6.22 (3.29-8.83) versus 3.27 (1.54-6.20) ng/ml, respectively (P=0.03) (Figure 1). No statistically significant difference was observed in the concentrations of pleural fluid CAXII between males and females within the tuberculous group, ruling out the possibility that this biomarker levels differed due to a large proportion of males in the latter group (group I) (P=0.647). There were no statistical differences in tumor marker levels between cytologically positive and negative pleural fluids associated with lung cancer: median (IQR25-IQR75) values were 6.22 (3.29-8.83) and 6.55 (3.40-10.10) ng/ml, respectively (P=0.77) (Figure 1).

#### Diagnostic value of CAXII measurements

Using ROC curve analysis, CAXII offered an area under the curve (AUC) of 0.71 between lung cancer cases and the tuberculous group (95% confidence interval, 0.564-0.856; P=0.013). The diagnostic sensitivity and specificity of pleural fluid CAXII test, at a optimal cutoff value of 6.07 ng/ml, were 60% and 75%, respectively (Figure 2).

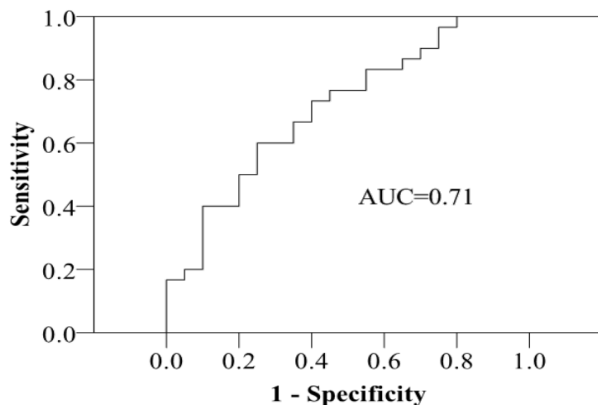
#### Diagnostic value of CAXII measurements in cytology-negative pleural effusions associated with lung cancer

We next evaluated whether the determination of pleural

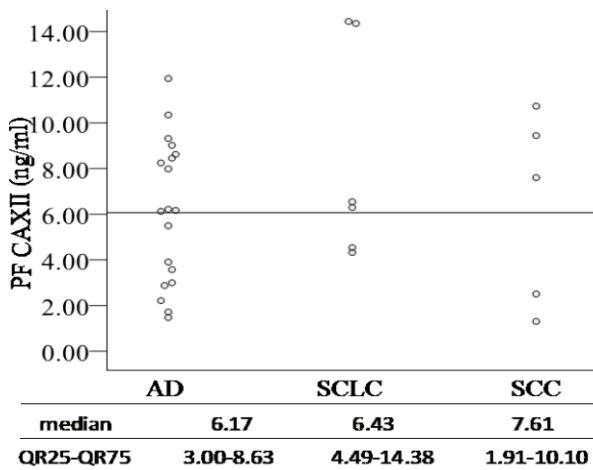
**Table 2. Sensitivity and Specificity of Conventional Cytology and the CAXII Assay in Samples from 50 Patients Included in This Study**

Group	Cytology	CAXII
Lung cancer patients	17/30(57%)	18/30(60%)
Tuberculosis patients	20/20(100%)	15/20(75%)

The optimal cutoff value used was 6.07 ng/ml



**Figure 2. ROC Curve of PF CAXII Levels for Diagnosis of Lung Cancer.** The area under the curve is 0.71 (95% confidence interval, 0.564-0.856;  $P=0.013$ )



**Figure 3. CAXII Concentrations in Pleural Fluids from Patients with Different Histological Types of Lung Cancer**

fluid CAXII may complement conventional cytology in diagnosis of pleural effusions with lung cancer. For that propose, we used pleural fluid samples from patients with lung cancer in which the cytologic examination of pleural effusion did not reveal malignant cells (group III in Table 1). When the previously established cutoff point (6.07 ng/ml) was applied, 7/13 cases were classified as positive by using this determination. Therefore, the assay was positive in almost 54% of pleural fluid samples from patients in whom cytologic examination was insufficient to diagnose the malignancy.

#### CAXII versus cytology

As a single test, a pleural fluid CAXII concentration exceeding 6.07 ng/ml was not inferior to cytological examination in the diagnosis and exclusion of malignant pleural effusion (sensitivity 60% vs. 57%; specificity

75% vs. 100%, respectively). In this group of patients, an elevated pleural fluid CAXII level provided a 77% positive predictive value (PPV) and a 54% negative predictive value (NPV).

The summary provided the sensitivity and specificity of cytologic examination and CAXII test in all the subjects consecutively included in the study (Table 2).

#### Histological types

The histological type of lung cancer significantly influenced expression of CAXII: the median concentration was highest with squamous cell carcinoma (SCC), followed by small cell lung cancer (SCLC) and then adenocarcinoma (AD) (7.61[1.91-10.10] vs. 6.43 [4.49-14.38] vs. 6.17[3.00-8.63] ng/ml, respectively) (Figure 3). The positive rate of pleural fluid CAXII in SCC, SCLC and AD were 60%, 67% and 58% (The optimal cutoff value used was 6.07 ng/ml).

#### Correlation with conventional biochemical parameters

There was no significant correlation between pleural fluid CAXII concentrations and pleural fluid total protein ( $rs = -0.148$ ,  $P = 0.727$ ), albumin ( $rs = -0.260$ ,  $P = 0.534$ ), glucose ( $rs = -0.214$ ,  $P = 0.612$ ), lactate dehydrogenase ( $rs = -0.328$ ,  $P = 0.428$ ) values.

#### Discussion

Differentiating malignant from tuberculous pleural fluids is an important issue in clinical practice. Cytologic examination of pleural effusion has a very low false-positive rate. However, there are still a substantial number of patients with an undiagnosed pleural effusion. Alternative methods, the use of biomarkers may be used to enhance the sensitivity of cytologic examination in the diagnosis of pleural effusion. Although blood is the biological fluid traditionally used in biomarker studies, pleural fluid may be a new source of tumor markers, more specific for lung cancer. Biomarkers are produced locally and released directly into the pleural space.

Data presented in this study showed that pleural fluid CAXII levels were significantly elevated in patients with lung cancer compared to patients with tuberculosis. We also found correlation between the concentration of the marker and lung cancer histology. The median concentration was highest with squamous cell carcinoma, followed by small cell lung cancer and then adenocarcinoma. However, numbers of such cases included in this study were small.

The most plausible explanation was CAXII levels would be increased in these fluids due to its production and secretion by tumor cells. In agreement with this explanation, Ilie et al. (2011) reported an immunohistochemical study of the expression of CAXII in lung cancer. CAXII is highly expressed in 19% of NSCLC. They also find significantly higher expression of CAXII in SCC ( $P < 0.001$ ) and well-differentiated tumors ( $P = 0.015$ ). Furthermore, high CAXII expression is also significantly associated with better overall and disease-specific survival of patients with resectable NSCLC. Kobayashi et al. (2012) demonstrated that the CAXII protein flows out into the sera and its levels in patients with lung cancer are significantly

higher than in healthy controls in both the training set ( $P < 0.0001$ ) and validation set ( $P = 0.03$ ). In lung cancers, the CAXII expression levels are significantly higher in patients with SCC than with AD ( $P = 0.035$ ). In addition, expression levels of CAXII are significantly higher in well- and moderately differentiated SCCs than in poorly differentiated ones ( $P = 0.027$ ).

CAXII is induced under hypoxic conditions in specialized cells within normal tissues, several different tumor types and cultured tumor cells, such as renal, cervical, ovarian, breast carcinomas (Türeci et al., 1998; Parkkila et al., 2000; Ivanov et al., 2001; Wykoff et al., 2001; Hynninen et al., 2006; Kim et al., 2006). It is likely that CAXII might play an important role in tumor development and differentiation (Kim et al., 2006).

A diagnostic biomarker needs to be clinically competitive and useful. In this sense, we had showed that measurement of CAXII offered a valuable adjunct in the diagnostic assessment of patients with pleural effusions related to lung cancer, especially when cytological examination was inconclusive. Likewise, the CAXII level did not correlate with results of conventional pleural fluid indicators of inflammation, such as LDH. This test could be recommended as part of the diagnostic work-up of patients with lung cancer. For example, a positive CAXII test may offer a valuable adjunct to cytology in order to rule in malignancy as a probable diagnosis, thus guiding the selection of patients who might benefit from further invasive procedures. In conclusion, we demonstrated pleural effusions from lung cancer patients contained higher levels of CAXII than tuberculous pleural effusions. Quantification of biomarkers such as CAXII may be used in cases of differentiation between lung cancer-related and tuberculous effusions. And this study seemed to offer a great opportunity to compare CAXII levels in other non-malignant conditions as well as tuberculous related effusion – levels in pleural infection / other malignancies would be worth assessing.

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