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European Journal of Cardio-thoracic Surgery 33 (2008) 723–727

EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY

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Tumor type M2-pyruvate-kinase levels in pleural fluid versus plasma in cancer patients: a further tool to define the need for invasive procedures☆

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Received 13 July 2007; received in revised form 19 December 2007; accepted 16 January 2008; Available online 7 February 2008

Abstract

Objective: Pleural effusion is a common diagnostic problem and a challenge to the thoracic surgeon. The analysis of serum and body fluids for tumor markers is an established diagnostic procedure. Among various markers, tumors are linked to the overexpression of a glycolytic isoenzyme, M2-pyruvate-kinase (M2-PK). This preliminary study evaluated this enzyme as a tumor marker to differentiate malignant from benign pleural effusion. **Methods**: The tumor M2-PK concentration was measured in the EDTA-plasma and pleural fluid of 34 patients with an established diagnosis of cancer, either primary of the chest (18) or secondary to chest (16) and in 34 controls with benign effusion. The concentration was quantitatively determined by an enzyme-linked immunosorbent assay. The cut-off level between negative and positive values of the tumor M2-PK was defined as the benign group's mean + 2SD (95% percentile). True-positives, false-positives, true-negatives, and false-negatives, were determined with 'positive' referring to histologically proven malignant effusion and 'negative' referred to as nonmalignant effusions. Sensitivity, specificity, positive predictive value, and negative predictive value were assessed. **Results**: The cut-off value was established at 7.61 U/ml for plasma and 32.9 U/ml for pleural fluid. Both plasma and pleural fluid levels of tumor M2-PK were significantly higher in patients with known chest malignancy, either primary or metastatic, compared to nonmalignant effusions ($p < 0.001$). Sensitivity in pleural fluid was significantly higher compared to plasma (85.7% vs 76.2%; $p < 0.01$). Moreover, negative predictive value was higher for pleural fluid compared to plasma (79.4% vs 70.8; $p < 0.01$) **Conclusions**: Tumor M2-PK marker is useful in differentiating malignant from benign pleural effusions. Moreover, its sensitivity and NPV in pleural fluid are significantly higher compared to plasma. The usefulness of such a test is not strictly diagnostic but aims at excluding poorly performing patients from further invasive procedures. Thus, the inclusion of M2-PK within a panel of well-known tumor markers such as CEA, MCA, Ca 125 and Ca 19-9, may help in increasing the overall sensitivity and specificity.

Keywords: Pleural effusion; Tumor markers; M2-pyruvate-kinase; Thoracoscopy; Enzyme immunoassay; Thoracic malignancies; Diagnostic procedure

1. Introduction

Pleural effusion is a frequent clinical presentation and is due to malignancy in about 20% of cases. A malignant pleural effusion (MPE) is a common and debilitating complication of various advanced malignant diseases. It may be the first presenting sign of cancer in 10–50% of patients, suggestive of recurrent or advanced disease [1].

The main recognized cause of MPEs is lung cancer together with malignant pleural mesothelioma followed by breast cancer in women. Yet, a wide variety of malignancies, such as cancer of stomach, ovary, kidney, may also determine the onset of a MPE during the course of the disease. About 10% of MPEs are due to lymphoma and the incidence of MPE in lymphoma patients ranges between 5% and 33% [2]. In approximately 15% of patients with pleural effusions a definite diagnosis is not achieved despite invasive procedures and in 5–10% of MPEs, no primary tumor is identified [3].

Since the presence of MPE is suggestive of end stage disease with very short life expectancy, the evacuation of the pleural fluid and prevention of its re-accumulation are the main goals of management. Nonetheless, the achievement of a definite diagnosis is appropriate to address further treatment.

Yet, MPE is a challenging problem since pleural fluid cytology findings are positive in only 60% of cases on average [4]. An additional biopsy adds only 7–13% of positive findings

☆ This study was partially financed by the Italian Ministry of University research funds (MURST 60%).
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1010-7940/$ – see front matter
doi:10.1016/j.ejcts.2008.01.014

to cytology-negative cases [5–7]. On the other hand thoracoscopy or video-assisted thoracic diagnostic procedures will achieve diagnosis in about 95% of cases but they are not always available at all facilities and often too invasive for poorly performing patients [8].

A significant number of tumor markers have been evaluated for use in the diagnosis of MPE, but to date all studies have not clearly defined their role in clinical practice. Among the most common found to be of diagnostic significance are carcinoembryonic antigen (CEA), cancer antigen (CA) 15-3, and cytokeratin fragment (Cyfra 21-1) while CA 19-9 and CA 125 are usually not contributory [9]. CEA is highly specific for lung adenocarcinoma and is typically negative in benign, reactive and malignant mesothelioma cells but its immunoreactivity varies considerably depending on the type of antibody clone used and the range of tumor tested. The use of polyclonal CEA in effusion cytology results in a significant background stain that impairs interpretation and this is why some authors routinely omit this marker from their panel [10]. A combination of tumor markers (CA 549, CEA, and CA 15-3) has been shown to have a sensitivity of 0.65, a specificity of 0.99, and an accuracy of 0.85 to differentiate malignant from benign pleural effusions [11]. Higher levels of CEA are seen in squamous cell cancer and adenocarcinoma of lung, while higher levels of CA 15-3 are observed in breast cancer [4,12,13]. Based on such findings it is rational to hypothesize that the addition of any tumor marker assay would further improve the diagnostic value of cytology.

Pyruvate-kinase (PK) is a key enzyme in the glycolytic pathway and among its functions controls nucleotide triphosphate generation. Different tissue-specific isoforms of this enzyme exist and are homotetramers in their activate state. In tumor cells, the isoenzyme M2-PK is shifted to a dimeric form which is overexpressed during multi-step carcinogenesis and present both in blood and other body fluids, presumably released from tumor cells by necrosis and cell turnover [14–16]. To our knowledge there are no studies so far that have investigated the presence of M2-PK in pleural effusion. Therefore, the aim of this study is to determine the usefulness of quantification of M2-PK in serum and in samples of pleural fluids for the detection of neoplastic pleural effusion and to determine whether this tumor marker may be an indicator for a more invasive procedure to establish the diagnosis of MPE.

2. Materials and methods

Pleural effusions and plasma samples were prospectively collected from 34 consecutive patients with known primary or metastatic chest malignancy between 1 January 2006 and 31 December 2006. All effusions had a reliable diagnosis previously obtained by cytology and/or histology after pleural biopsy performed by videothoracoscopy. Effusions from 34 patients with nonmalignant disease caused by infection or cardiac failure were collected in the same period and served as controls. Among the 34 malignant effusions, 20 were cytologically negative. Once collected, EDTA-blood specimens were centrifuged and the supernatant plasma was removed. All samples were frozen at −20 °C and stored until processing for a maximum of 2 months. Pleural fluid samples were centrifuged at 3000 rpm for 10 min to remove cellular components. Supernatant was stored at −20 °C until the performance of the tumor marker assay.

The tumor M2-PK concentrations were determined in duplicate both in plasma and pleural fluid by means of a sandwich enzyme-linked immunosorbent assay (ELISA) using two monoclonal antibodies specific for tumor M2-PK (Sche-Bo®Tech, Giessen, Germany).

The cut-off level between negative and positive values of the tumor M2-PK was defined as the benign group's mean + 2SD (95% percentile).

Sensitivity was calculated as the number of patients with cancer who tested positive for tumor M2-PK (true-positives) divided by the total number of histologically confirmed tumor patients (true-positives plus false-negatives), expressed as a percentage. Specificity was calculated as the number of controls who tested negative for tumor M2-PK (true negatives) divided by the total number of control subjects (true-negatives plus false-positives), expressed as a percentage.

Statistical evaluation was done by means of computer analysis with MedCalc software (MedCalc, Mariakerke, Belgium) according to Mann–Whitney test. This study was approved by the institutional ethics committee.

3. Results

A total of 68 patients were studied between January 2006 and December 2006. They were divided by diagnosis in two groups, 34 patients with cancer, either primary or metastatic, and 34 controls with known diagnosis of benign pleural effusion due to inflammatory disease or cardiac failure. Table 1 shows the demographic and cancer primary sites of the MPE of the neoplastic patient population. The upper normal limit for tumor M2-PK established on the basis of the best cut-off, discriminating patients with cancer from controls, was 7.61 U/ml for plasma and 32.9 U/ml for pleural fluid. Both plasma and pleural fluid levels of tumor M2-PK were significantly higher in patients with known chest malignancy, either primary or metastatic, compared to nonmalignant effusions ($p < 0.0001$) (Figs. 1 and 2). Evaluated diagnostic parameters are shown in Table 2. Sensitivity in pleural fluid was significantly higher compared to plasma (85.7% vs 76.2%; 95% CI 63.6–96.8 and 52.8–91.7;

Table 1
Demographic of the patient population, and site of malignancy of 34 patients with malignant pleural effusion (MPE)

Mean age	66 ± 13.6 (range 42–78)
Sex	
Male	21
Female	13
Primary	
Lung	16
Pleura	2
Metastatic	
Breast	7
Kidney	2
Seminoma	2
Non-Hodgkin lymphoma	2
Unknown	3

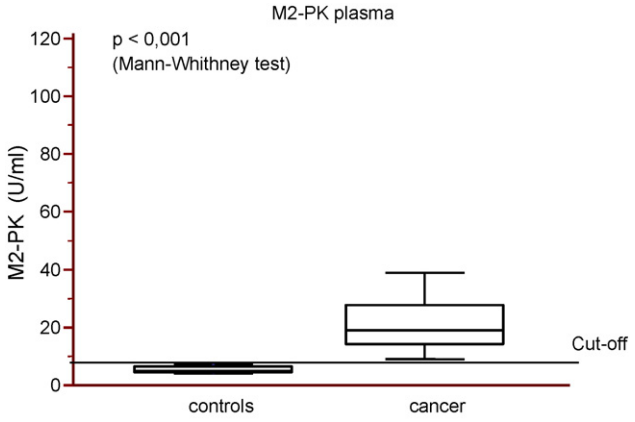

Fig. 1. Tumor M2-PK concentration in plasma of patients with nonmalignant (controls) and malignant chest diseases.

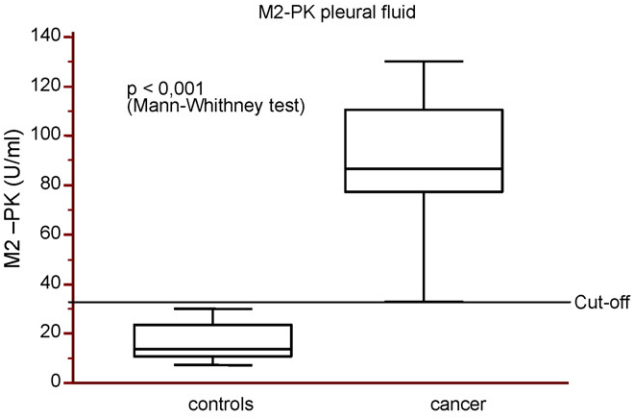

Fig. 2. Tumor M2-PK concentration in pleural fluid of patients with nonmalignant (controls) and malignant chest diseases.

$p < 0.01$) while specificity was the same (92.3%; 95% CI 63.9–98.7). Moreover, the negative predictive value (NPV) was significantly higher in pleural fluid compared to plasma samples (79.5% vs 70.8%; $p < 0.01$).

Normality of distribution was verified by means of the Kolmogorov–Smirnov test and ROC curve analysis was performed for both pleural fluid and plasma levels of M2-PK with a comparison of ROC curves.

4. Discussion

Pleural effusion is a diagnostic challenge often unsolved after clinical and laboratory assessment. The differential diagnosis may include a variety of disease such as malignancy, congestive heart failure, tuberculous pleurisy and empyema. The etiology of a pleural effusion can be established in most cases with a careful history, physical examination, evaluation of pleural fluid obtained by thoracentesis including fluid cultures, cytology and TBC testing [17]. Approximately 20% of pleural effusions are caused by neoplastic processes [1,2].

The diagnosis of effusion acts directly on the prognosis and treatment of the disease as the presence of tumor cells in effusions points out a poor prognosis and often implies an aggressive treatment such as talc pleurodesis followed by chemotherapy. Therefore, it is of utmost importance to detect with the greatest specificity and sensitivity any cancer cells in the fluid. The search for cancer cells in serous effusions is somewhat difficult due to their scarceness or the many problems in differentiating them from reactive mesothelial or inflammatory cells [18]. Cytology is undoubtedly the gold standard method for the diagnosis of serous effusion but routine cytomorphology reaches 50–60% sensitivity and a specificity of about 90% at the most while some studies have shown that positive and negative predictive values for detection of malignancy by cytomorphology are 89.3% and 69.4%, respectively [19]. Moreover, a significant morphologic overlap exists between malignant mesothelioma cells and metastatic carcinoma cells [10]. In conclusion there is a grey zone where the cytopathologist cannot determine if the cells are reactive, atypical, certainly malignant, or coming from primary or metastatic cancer [19,20].

Table 2
Sensitivity (S), specificity (s), negative predictive value (NPV) and positive predictive value (PPV) of tumor marker M2-PK in plasma and pleural fluid of 34 patients with known malignant pleural effusion (MPE)

	S	s	NPV	PPV
Plasma	76.2	92.3	70.8	94.6
Pleural fluid	85.7	92.3	79.5	94.1

Values are expressed in percentage (%).

Therefore, diagnosis is usually carried out by more invasive techniques which even though performed by minimally invasive procedures are not always available at all facilities and tolerable by immunocompromised patients [1,8]. Thoracoscopy is the established method in the diagnosis of pleural diseases since it is highly sensitive for detecting pleural malignancy when pleural fluid cytology is negative and in the diagnosis of tuberculosis [21,22]. The American Thoracic Society has stated the indications for performing thoracoscopy that include the evaluation of exudative effusions of unknown cause and suggest that in cases of undiagnosed exudative effusions with a high clinical suspicion for malignancy some clinicians may proceed directly to thoracoscopy if the facilities are available [23]. Thus, many additional methods to cytology have been assessed to improve the diagnostic accuracy and avoid invasive diagnostic techniques such as thoracoscopy whenever possible.

Polymerase chain reaction (PCR) techniques in serous effusions have been shown as a beneficial adjunct to conventional procedures for they increase the detection sensitivity of tumor cells [18].

Inflammatory parameters and growth factors such as vascular endothelial growth factor (VEGF) in MPE are a new and not yet widely established diagnostic tool. VEGF is an important mediator of angiogenesis and vascular permeability thus contributing to developing pleural effusion. The VEGF level has been found to be higher in pleural effusions secondary to breast cancer, mesothelioma, and non-small cell lung cancer [2].

To increase the sensitivity of the pleural fluid study, several tumor markers have been analysed, among them the

carcinoembryonic antigen being the most commonly assessed. Notwithstanding its greater accuracy in pleural effusion compared to other tumor markers high false-positive results have been reported [4,12,24].

One alteration consistently found during tumor formation, including gastrointestinal tumors, is the upregulation of glycolytic enzymes. This upregulation takes place at the RNA and protein level, as well as at the level of enzymatic activities. In addition, in the case of the glycolytic enzyme pyruvate kinase, a loss of the tissue-specific isoenzymes and expression of the pyruvate kinase isoenzyme type M2 (M2-PK) is described in all tumors investigated thus far [25].

In tumor cells, pyruvate kinase isoenzyme M2 is strongly overexpressed and shifted into dimeric state and it has been demonstrated that the amount of type M2 pyruvate kinase extracted from neoplastic tissues increases with tumor size and metastases. For this reason, the dimeric form of M2-PK has been termed tumor M2-PK and is linked to an increased nucleic acid synthesis [15].

Tumor M2-PK is released from tumor cells into the blood and from gastrointestinal tumors also into the stool of tumor patients most likely by tumor necrosis and cell turnover. Immunohistological studies revealed a heterogeneous distribution of tumor M2-PK, in the primary tumors and a homogeneous large amount in metastatic cancer. In accordance with such studies the tumor M2-PK increases in the EDTA-plasma of patients with renal, pancreatic, lung, breast, cervical and gastrointestinal cancer. These results indicate that tumor M2-PK is an organ-unspecific marker, which reflects the metabolic activity of the tumors [14]. Moreover, the concentration of tumor M2-PK in EDTA-plasma correlates with tumor stage and size, thus suggesting a link with tumor load that may be used for disease and therapy monitoring [25].

It has been previously reported that tumor M2-PK determination in the circulation provides a good discrimination of benign disease from malignancy and may correlate with the stage of disease [14]. Schneider et al. [25] found that a significant rise of tumor M2-PK levels in lung cancer patients during treatment indicated tumor progression.

To our knowledge no data are available on determination of tumor M2-PK in the pleural effusion. We aimed at evaluating an ancillary test that could improve sensitivity of cytological analysis for cancer diagnosis in MPE.

In the present study the tumor M2-PK concentrations were significantly increased both in plasma and pleural fluid, in all patients with cancer. Moreover, sensitivity and NPV were higher in pleural fluid compared to plasma. Thus, the evaluation of this marker in pleural fluid showed a significant discrimination of tumor patients from non-neoplastic patients.

Some authors argue that the presence of elevated levels of a tumor marker in the pleural fluid can only serve as an indicator for a more invasive procedure to establish the diagnosis of MPE but should not be used to establish the diagnosis [17]. This is true when we face patients that we know to be suitable for thoracoscopy. Moreover, the same authors admit that other procedures such as cutting needle pleural biopsy may only reach 17% of diagnostic yield in patients with known malignancy. Even taking into consideration those factors indicated by Ferrer et al. [24] as predictive for malignancy in patients with pleural effusions, such as a symptomatic period of >1 month, absence of fever, serosanguineous fluid, and chest CT scan suggestive for malignancy, a precise diagnosis will not be achieved unless histological proof is obtained, which sometimes is not possible.

Previous studies on pleural fluid involved heterogeneous pathologic conditions and several tumor markers with different cut-off values, which made comparison of results difficult and sometimes unreliable. This study evaluated the diagnostic utility of a relatively new single marker strictly involved in the carcinogenetic cascade, comparing its concentrations both in plasma, as already reported in a controversial fashion [14–16,25] and pleural fluid (first report to our knowledge).

Tumor M2-PK is detected with an ELISA that can be easily performed in every routine laboratory. Therefore, the detection of tumor M2-PK levels in the pleural effusion might provide an interesting screening tool for cancer. The main advantages are that it detects a metabolic state which is specific for cancer cells, it can be easily measured both in blood and in pleural fluid, the results are highly reproducible and, finally, its specificity and sensitivity are more than acceptable. The usefulness of such a test is not strictly diagnostic but aims at excluding poorly performing patients from further invasive procedures. Moreover, the association of M2-PK with other tumor markers such as CEA, MCA, Ca 125 and Ca 19-9, may help in increasing the overall sensitivity and specificity. A larger cross-sectional study is warranted to further validate this new test in a larger cohort of patients and include this marker among the useful diagnostic tools to distinguish malignant from benign pleural effusions.

Acknowledgement

We thank Dr Andrea Pierantozzi for the statistical evaluation of data.

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