Diagnostic value of cytokeratin fragment 19 (CYFRA 21-1) in bronchoalveolar lavage fluid in lung cancer

M. J. CREMADES*, R. MENÉNDEZ*, A. PASTOR*, R. LLOPIS† AND J. AZNAR†

*Servicio de Neumología, y †Laboratorio de Bioquímica, Hospital Universitario La Fe, Valencia, Spain

The aim of this study was to evaluate the diagnostic value of a new tumour marker, cytokeratin fragment 19 (CYFRA 21-1), in bronchoalveolar lavage fluid (BALF) for the diagnosis of lung cancer. The cross-sectional study included 36 patients with lung cancer, 19 with benign lung diseases and 13 control subjects. In the group with cancer, BAL was performed in the cancer-involved lung and in the opposite lung. Results in BALF were expressed both as absolute concentrations (ng ml⁻¹) and referred to total protein (TP) (ng mg⁻¹ TP), and results in plasma were expressed in ng ml⁻¹. In BALF, there was no significant difference between cancer and control groups. Using the 95th percentile of levels obtained in benign lung disease in BALF (specificity 95%) as the cut-off point, the sensitivity of CYFRA 21-1 was 13%. Positive and negative predictive values (PPV and NPV) at different pretest probabilities, and positive and negative gains were obtained applying a Bayesian analysis. Results showed low positive gains for PPV (maximal increase of 22%) and almost none for NPV (negative gains <5%). In plasma, CYFRA 21-1 provided a sensitivity of 65%. The combination of BALF and plasma tumour marker levels showed a sensitivity of 69%. Therefore, measurement of CYFRA 21-1 in BALF has poor diagnostic value in lung cancer.

Introduction

Determinations of tumour markers (TMs) have been used in neoplastic diseases to evaluate their potential as diagnostic tests. However, research on lung cancer, performed in plasma or serum, has proven that their value is limited because they lack sensitivity as screening tests and are not specific enough to discriminate between neoplastic and benign conditions (1–3).

A new approach to improving the diagnostic yield consists of assaying TMs in fluid recovered from washing the involved organs or from tissue cultures. In lung cancer, obtaining bronchoalveolar lavage fluid (BALF) makes it possible to assay TMs from airways where neoplastic changes take place. Although some previous reports performed in BALF showed better results than in serum with carcinoembryonic antigen (CEA) and squamous cell carcinoma antigen (SCC), posterior studies did not seem to confirm these findings (4–6). CYFRA 21-1, a new TM assayed in lung cancer, is a cytokeratin present in all histological types of lung cancer, and distributed in simple or pseudostratified epithelium such as the surface of tracheobronchial epithelial airway (7). This TM has proven to be superior to CEA and SCC in blood samples (8–10), but prior to the present study, it had not been studied in BALF. The aim of the present study was to investigate the diagnostic value of CYFRA 21-1 in lung cancer, using a Bayesian analysis (11). The authors hypothesized that CYFRA 21-1 assayed in BALF, as this was obtained in the airway where the tumour is located, would offer more diagnostic information. For this purpose, the levels of TMs were determined in BALF and plasma in three groups: (a) patients with unilateral lung cancer; (b) patients with benign lung disease; and (c) control subjects without bronchopulmonary disease. In the cancer group, TMs in BALF from the opposite lung were also measured, in order to compare the results with those from the cancer-involved lung.

Subjects and Methods

SUBJECTS

The authors designed a prospective cross-sectional study on three groups of subjects, recruited from inpatients and outpatients who were scheduled to have a diagnostic bronchoscopy exploration. Thirty-six patients had lung cancer, 19 had benign lung disease, and 13 were control subjects, smokers and non-smokers. The demographic characteristics of the groups are shown in Table 1. Informed written consent was obtained from all subjects to undergo BAL for
TABLE 1. Demographic characteristics of the groups

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age (M:F)</th>
<th>Sex</th>
<th>Non-smoker</th>
<th>Smoker</th>
<th>Ex-smoker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>36</td>
<td>61 ± 9</td>
<td>35:1</td>
<td>2</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>BLD</td>
<td>19</td>
<td>54 ± 15</td>
<td>13:6</td>
<td>8</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>CG</td>
<td>13</td>
<td>59 ± 7</td>
<td>12:1</td>
<td>2</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± sd. BLD, benign lung disease; CG, control group.

research purposes during fibre optic bronchoscopy. Patients without a definitive diagnosis of cancer or benign lung disease were excluded from the study. Patients with severe renal or/and liver failure were also excluded because TM concentrations can increase in blood (7). Plasma specimens were obtained at the time of bronchoscopy.

Patients with Lung Cancer

Thirty-six consecutive patients with a lung tumour or infiltrative mucosal appearance were selected. Only unilateral and proven neoplastic diseases were included. Histologic cell types comprised 19 squamous cell carcinomas, six adenocarcinomas, six small cell carcinomas, three large cell carcinomas and two undifferentiated carcinomas.

Patients with Benign Lung Diseases

Nineteen patients with benign lung disease and without prior neoplastic disease were included. They had: pneumonia (3), bronchiectasis (3), idiopathic interstitial pneumonitis (3), sarcoidosis (2), tuberculosis (2), chronic obstructive pulmonary disease (2), hypersensitivity pneumonitis (1), eosinophilic pneumonia (1), pneumoconiosis (1) and thromboembolic pulmonary disease with pulmonary infarct (1). Patients with different benign processes were selected because these diseases can be associated with lung cancer or can mimic it, thus making a differential diagnosis necessary.

Control Subjects

Thirteen matched subjects undergoing fibre-optic bronchoscopy for persistent cough and previous mild haemoptoic sputum were included. None showed evidence of concomitant infections or neoplastic disease on the chest roentgenogram, routine analysis or endoscopic exploration, and their spirometric parameters were normal. There were no endobronchial lesions or cases of active haemoptysis. Nine subjects had haemoptysis because of acute bronchitis, and four had psychogen cough.

METHODS

Local anaesthesia of the nasopharynx and larynx was performed using aerosolized lignocaine. A fibre-optic bronchoscope (Olympus BF10; Olympus Corp, NY, U.S.A.) was introduced transnasally and wedged under direct vision. In patients with lung cancer, the bronchoscope was wedged into the airway at the tumour site with the tumour visible, and into the corresponding bronchus of the opposite lung to do the BAL. Only patients in whom the bronchial caliber permitted BAL were used in this study. In patients with focal parenchymal lung disease, the bronchoscope was placed in a bronchus leading to the involved segment. In patients with homogeneous diffuse abnormalities and in controls, the bronchoscope was wedged into the right middle lobe. Two 50-ml aliquots of sterile normal saline solution, at room temperature, were then instilled and aspirated gently by mechanical suction after each instillation, and the recovered fluid was collected in a siliconized vessel. All aliquots, including the first one, were used. Bronchoalveolar lavage was performed in all subjects prior to brushing or biopsy to avoid contamination with blood.

The lavage fluid was immediately filtered through a nylon gauze (Nytal tissue), centrifuged for 10 minutes at 500 g to obtain a cell pellet, and the supernatant was decanted and stored at −70°C until CYFRA 21-1 analysis. All samples, BALF and plasma specimens, were labelled with a number code blinded to the laboratory personal. Measurements of CYFRA 21-1 and total proteins were carried out simultaneously at the end of the study.

Tumour Marker Assays

The CYFRA 21-1 concentrations in BALF and plasma were measured by a specific ELISA immunoassay called Enzymun-Test CYFRA 21-1 (Boehringer Mannheim, Mannheim, Germany). The test is based on a two-step sandwich assay using streptavidin-biotin technology and performed at 25°C on ES 600 multibatch analysers. Cytokeratin 19 is recognized by two mouse antibodies, KS 19-1 and MB 19-21, directed against two different epitopes of a fragment of cytokeratin subunit 19, which is referred to CYFRA 21-1. Sample, 35 and 700 µl of incubation solution, together with biotinylated antibody (MAK 19-1), are incubated in streptavidin-coated polystyrene tubes for 30 min. After aspiration and washing, incubation solution is added together with antibody-horseradish peroxidase conjugate (BM 19-21). After 30 min incubation time, the tubes are aspirated and washed again. Finally, ABTS (2,2-azino-bis[3-ethyl-benzthiazoline-sulphonic acid-(6)] di-ammonium salt)-substrate solution is added and incubated for 60 min. Absorbance is read at 422 nm and the CYFRA 21-1 concentration is calculated from the standard curve.
The sensitivity of the assays was 0.3 ng ml$^{-1}$. None of the samples were concentrated for determination. Total protein (TP) content in BALF was determined by the Lowry method in order to correct the dilution factor. CYFRA 21-1 in BALF was expressed both as ng ml$^{-1}$ BALF and as ng mg$^{-1}$ TP.

**Statistical Analysis**

Data were introduced in a database (DBASE III plus) and all calculations were made using a statistical package (SPSS) for personal computers. The Kolmogorov-Smirnov test was used to evaluate the distribution of CYFRA 21-1. Data on CYFRA are expressed as medians or percentiles because they were not normally distributed. Therefore, non-parametrical statistical analysis was used to compare the results between groups (12). Comparisons of the cancer-involved and non-involved lungs were made with the T-Wilcoxon test for paired samples. Comparisons between the three groups studied were assessed by a Kruskal-Wallis test. A probability value of less than 0.05 was considered significant. Spearman’s ranks correlation test was used to evaluate a possible association between CYFRA 21-1 in plasma and in BALF.

The sensitivity of TM was calculated with a 2 x 2 contingency table, using the 95th percentile of levels obtained in benign lung disease as the cut-off point (specificity of 95%). A Bayesian analysis (12) was performed to obtain positive and negative predictive values (PPV and NPV) according to the following formulas:

\[
PPV = \frac{\text{sensitivity} \times \text{prevalence}}{\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})}
\]

\[
NPV = \frac{\text{specificity} \times (1 - \text{prevalence})}{(1 - \text{sensitivity}) \times \text{prevalence} + \text{specificity} \times (1 - \text{prevalence})}
\]

Positive and negative gains, at different prevalences, are calculated. Positive gains are (PPV – prevalence), and negative gains are [NPV – (1 – prevalence)].

**Results**

Demographic data are expressed in Table 1. The results of CYFRA 21-1 are expressed as percentiles (medians), both in BALF and plasma (Table 2). Plasma levels were significantly higher in the cancer group ($P<0.0001$).

**Absolute Concentrations in BALF**

The absolute concentrations of CYFRA 21-1 in BALF are expressed in Table 2. There were no statistical differences between the three groups. However, the tumour-involved lung showed significantly higher levels than the opposite lung ($P<0.008$).

**Relative Concentrations in BALF**

CYFRA 21-1 levels referred to the TP concentration of BALF (ng mg$^{-1}$ TP) are shown in Table 2. Total protein showed significant differences between the three groups with higher levels in patients with benign lung disease and in tumour-involved lungs ($P<0.03$). In lung cancer group, TP was higher in tumour-involved lung with respect to the opposite one ($P<0.001$). CYFRA 21-1 did not show statistical differences between the three groups. However, patients with cancer did not have significantly higher levels in their tumour-involved lung than in the opposite lung ($P>0.05$) (Fig. 1).

**Diagnostic Value of CYFRA 21-1 in Lung Cancer**

With the 95th percentile of the benign lung disease as the cut-off value in BALF (CYFRA 21-1: 446 ng mg$^{-1}$), the sensitivity was 13% for CYFRA 21-1 (95% confidence interval 4.28%) at a specificity of 95% (95% confidence interval 74-99%). The sensitivity in plasma was 65% at 95% specificity. Using both BALF and plasma levels, the sensitivity increases to 69%. Positive (PPV) and negative predictive values (NPV), at different pretest probabilities, and positive and negative gains are shown in Fig. 2.
Low positive and negative gains were obtained for PPV (maximal increase of 22%) and almost none for NPV (negative gain <5%). There was no significant correlation between CYFRA 21-1 levels in BALF and plasma.

There was no significant difference when the results of each histologic cell type were compared. Even CYFRA 21-1, which showed a sensitivity of 10% for the squamous cell carcinoma, remained basically the same. When considering the TNM stage of cancer, no significant correlation was found either.

**Discussion**

In the present study, the main finding was the low diagnostic value for lung cancer of CYFRA 21-1 in BAL, despite the fact that a better diagnostic value in blood was also confirmed (7,10,13). To the authors' knowledge, this is the first study performed with CYFRA 21-1 in this fluid in lung cancer. In the valuation of the diagnostic value, two control groups (benign lung diseases and control subjects), and the opposite lung as internal control, were used.

In a prior report (14), CYFRA 21-1, CEA and SCC were determined in blood in patients with lung cancer; higher sensitivity was obtained for CYFRA 21-1 than for CEA and SCC. Unfortunately, in the present report, the levels of CYFRA 21-1 in BALF are similar in lungs bearing a cancer and in benign lung disease. Moreover, CYFRA 21-1 in BALF showed no better profile in squamous cell carcinoma, while it was demonstrated in blood. When considering the TNM stage of cancer and CYFRA 21-1 levels in BALF, no significant correlation was found either. However, since not many cases of each histological type are included, a possible beta error might be considered. To calculate sensitivity, a high specificity was chosen and only the benign disease group was selected to avoid overestimation of specificity. The authors chose 95% specificity because when a disease is suspected, a test with very high specificity is more adequate. In fact, when cancer is suspected, the highest specificity must be recommended. These are the reasons for selecting the 95th percentile of the levels obtained in benign lung disease as the cut-off value (specificity of 95%), and not a ROC curve, another method to obtained the cut-off point. All calculations were based on relative values as usually made (15-18). The combination of BALF and blood results for CYFRA 21-1 proved to have a higher sensitivity, but this increase was mainly at the expense of the blood results.

Previous studies in the literature have demonstrated disappointing results with other TMs, such as CEA and SCC, in BALF (19-22). Several authors have even found higher or similar CEA levels in BALF from smokers,
chronic bronchitis or benign lung diseases than in lung cancer (23–27). They speculated that this increase might be due to the release of TMs by the airway cells as a consequence of inflammation and epithelial damage associated with several injuries on the airway, infection or merely a smoking habit. CYFRA 21-1 is present in the surface of tracheobronchial epithelial airway, but in the presence of cellular necrosis, a fragment of CYFRA can be released into the blood (28,29). Curiously, significantly higher blood CYFRA 21-1 levels were found in patients with lung cancer than in the benign lung diseases group, but not in BALF. In fact, no correlation between the blood and BALF levels was detected, and the authors have no explanation for these results. However, it may be speculated that the presence of inflammation caused by benign diseases might promote the release of this cytokeratin into the airway, and even self instillation of saline solution could contribute to its liberation by the airway epithelium.

Interestingly, in another biological fluid, pleural fluid, Romero et al. (30) found that CYFRA 21-1 showed a poor diagnostic value in neoplastic diseases. These authors preclude the use of CYFRA 21-1 in this fluid as a TM because it was also raised in several benign conditions as expression of a local inflammatory or repairing reaction.

When applying a Bayesian analysis to the present results, as expected, information about diagnostic value was not improved. At difference pretest probabilities, the predictive values (positive and negative) reached by CYFRA 21-1 in BALF were poor. That is, after a test result, CYFRA 21-1 levels in BALF are not useful either to confirm or to discard the existence of lung cancer.

Another way to evaluate the usefulness of TMs in BALF in lung cancer is by comparing results between the lung bearing a tumour and the opposite lung. When this was done, CYFRA 21-1 only showed statistical significance when the results were not referred to total protein. Stockley et al. (19), in a similar study, found that CEA levels were raised in the cancer involved lung with respect to the opposite lung in patients with cancer. On the other hand Wesselius et al. (4) reported no differences in CEA and BALF between neoplastic and non-neoplastic lungs, but the BALF from the cancer-involved lung was obtained first, hence fluid could enter into the opposite lung.

In summary, a poor diagnostic value for CYFRA 21-1 in BALF was found, despite better results obtained in blood, with very low sensitivity when a high specificity is required. Also, no correlation with the tumour stage or histological cell types and CYFRA 21-1 levels was found. Bayesian analyses have demonstrated small predictive values at several prevalences.

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