Serum Fragment of Cytokeratin Subunit 19 Measured by CYFRA 21-1
Immunoradiometric Assay as a Marker of Lung Cancer

Jean-Louis Pujol,1 Jean Grenier, Jean-Pierre Daurès, Alain Daver, Henri Pujol, and François-Bernard Michel

ABSTRACT

Cytokeratin 19 is a subunit of cytokeratin intermediate filament expressed in simple epithelia and their malignant counterparts. Therefore, it is expressed by respiratory epithelium cells and has been detected in lung cancer specimens. An immunoradiometric assay was used to detect a fragment of the cytokeratin 19, referred to as CYFRA 21-1, in the serum of 165 patients with histologically proved lung cancer (128 non-small cell and 37 small cell lung cancers). This prospective study was conducted to evaluate the reliability of this immunoradiometric assay and to identify the relationship between serum CYFRA 21-1 and different features of lung cancer including prognosis. The minimal detectable concentration detected by this assay was 0.06 ng/ml. The reliability of the immunoradiometric assay was demonstrated by the linear relationship between CYFRA 21-1 measurement and dilution of the serum, the reproducibility of the dosage in intraassay and interassay, and the high sensitivity of the method in discriminating low CYFRA 21-1 concentrations. Using a threshold of 3.6 ng/ml, sensitivity and specificity were 0.52 and 0.87, respectively. The sensitivity of the marker was highest in squamous cell carcinoma and lowest in small cell carcinoma. In non-small cell lung cancer patients, the marker varied significantly according to both stage of the disease (Kruskal-Wallis, 13.7; P < 0.005) and performance status (Kruskal-Wallis, 9.16; P < 0.05) inasmuch as a high serum CYFRA 21-1 level was associated with advanced stages, mediastinal lymph nodes, and poor performance status. Consequently, the marker was significantly lower in patients who were operated upon when compared with unresectable ones. Lung cancer patients with serum CYFRA 21-1 over 3.6 ng/ml proved to have a significantly shorter overall survival than those with a normal serum level (log rank, P = 0.007; Wilcoxon, P = 0.001). The negative prognostic effect of CYFRA 21-1 was highly significant in squamous cell carcinomas whereas it was nonsignificant for the other histologies. In Cox’s model analysis, performance status, stage grouping, and CYFRA 21-1 were the only significant determinants of survival. This study supports the use of the serum fragment of cytokeratin subunit 19 CYFRA 21-1 as an independent prognostic marker of squamous cell carcinoma of the lung.

INTRODUCTION

Treatment of lung cancer is probably one of the great challenges of medical oncology owing to an increasing incidence in both men and women and poor prognosis (1, 2). SCLC2 have neuroendocrine biological properties usually associated with a high sensitivity to chemotherapy (2–4). Surgery offers the best probability of cure for NSCLC but it cannot be proposed for locally advanced and metastatic stages; therefore, other modality treatments such as radiotherapy-chemotherapy association have been proposed (5). Because metastatic disease is the first cause of failure for both SCLC (6) and NSCLC (7), research for new treatment modalities is an important goal. Such an experimental approach requires an accurate definition of groups of patients and prognosis. The three main prognostic factors able to help treatment decision are histology (mainly SCLC versus NSCLC), stage of disease, and performance status (8, 9). The search for new clinical or biological variables as putative independent prognostic factors may potentially improve the management of lung cancer patients.

Cytokeratins are intermediate filaments which are part of the cytoskeleton of both normal epithelia and their malignant counterparts (10). Immunohistochemical studies using broad-spectrum cytokeratin antibodies have demonstrated the expression of cytokeratins in both SCLC and NSCLC suggesting a common endodermal lineage (11, 12). In contrast to other intermediate filaments, cytokeratins are a family of polypeptides, some of them having cell type specificity (10). Cytokeratin 19 is an acidic (type I) subunit expressed in all simple epithelia and in carcinomas such as lung cancer which arise from them.

A fragment of cytokeratin subunit 19 can be measured in serum by a new immunoradiometric assay, the CYFRA 21-1 using two mouse MoAb KS 19-1 and BM 19-21. Thus, this cytokeratin 19 fragment is referred to as CYFRA 21-1. The aims of the present prospective study are (a) to evaluate the reliability of serum CYFRA 21-1, (b) to identify any relationship between the marker and other clinical and biological parameters in both SCLC and NSCLC and (c) to analyze the prognostic significance of a high CYFRA 21-1 level at time of diagnosis.

MATERIALS AND METHODS

Patients. One hundred sixty-five consecutive lung cancer patients referred to the Montpellier University Hospital between February 1990 and February 1992 were prospectively entered in the study (Table 1). All patients had pathologically confirmed lung cancer. Among them were 37 SCLC, 85 SQC, 28 adenocarcinomas, and 15 large cell carcinomas as defined by the WHO classification (13). Performance status was estimated according to the Eastern Cooperative Oncology Group and the percentage of weight loss during the previous 4 months was recorded. Staging was carried out by exhaustive procedure according to the 4th edition of the Union Internationale Contre le Cancer tumor-nodes-metastasis classification (14) and the American Thoracic Society map of regional pulmonary nodes (15). Staging of NSCLC was established according to International’s stage grouping (16); for SCLC, limited disease was defined as disease confined to one hemithorax including mediastinal lymph nodes and/or ipsilateral supraclavicular lymph nodes; extensive disease was defined as disease more advanced than limited SCLC. For all patients staging procedure included clinical examination, standard chest roentgenography, computer tomographic scan of chest and upper abdomen, fiberoptic bronchoscopy, liver sonography, and bone scanning. Brain computed tomographic scan was done systematically for SCLC and if clinically required for NSCLC.

Controls. The serum CYFRA 21-1 level was measured in 104 patients with nonmalignant pulmonary diseases (20 infectious diseases, 75 chronic obstructive pulmonary diseases, and 9 miscellaneous).

Treatment Decision. Each patient was discussed by a medical panel composed of thoracic surgeons, chest physicians, radiotherapists, and medical oncologists. NSCLC patients with stage I or II disease or with moderate locally advanced NSCLC (IIIa with resectable node metastasis) underwent surgery in an attempt to achieve complete resection. Other NSCLC patients with performance status 2 and distant metastasis (stage IV) or gross mediastinal involvement (stage IIIb and stage IIIa with more than 2 ipsilateral mediastinal lymph

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1To whom requests for reprints should be addressed.

2The abbreviations used are: SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; MoAb, monoclonal antibodies; SQC, squamous cell carcinomas; MDC, minimal detectable concentration; ROC, receiver operating characteristic; St, cytoskeleton standard; LDH, lactate dehydrogenase; Nq, nodal stage x.
node metastases) were eligible for chemotherapy. Best supportive care, including palliative radiation therapy when needed, was given to patients with advanced stage and poor performance status. SCLC patients underwent chemotherapy (usually an alternating regimen containing cisplatin (17)). Treatment was decided upon according to routine clinical and biological findings and without knowledge of the serum CYFRA 21-1 level. Hence, treatment was not considered as a prognostic variable in this study.

CYFRA 21-1 Immunoradiometric Assay. A blood sample was taken from each patient at presentation, and the serum was separated and stored at -190°C until tested.

CYFRA 21-1 (Centocor Diagnostics, Malvern, PA; Cis Biointernational, Gif/Yvette, France) is a solid-phase immunoradiometric assay based on the two-site sandwich method. In this method the cytokeratin 19 was recognized with MoAb KS 19-1-coated polystyrene spheres were incubated with 200 μl of patient serum, control serum, or standard curve (composed of the following concentrations of cytokeratin 19: 0, 3, 8, 25, and, 50 ng/ml), for 20 h at 2-8°C. Afterwards, the solid phase was washed with distilled water and then incubated with 0.85 μCi/ml of 125I-labeled BM 19-21 for 3 h at 2-8°C. Finally, the solid phase was washed again with distilled water in order to cancel the nonfixed labeled reagents.

Radioactivity was counted in a well-type gamma counter (Auto-Gamma; Packard Instrument Company, Downers Grove, IL) and expressed in cpm. The calculated concentration of cytokeratin 19 was expressed in ng/ml.

Control of the Immunoradiometric Assay. The sensitivity of the method was assessed by determining the MDC. This method used two cytokeratin 19 standards containing, respectively, 0 (cytokeratin free, St0) and 3 ng/ml (St1); these 2 standards were measured in 6 different assays (Table 2); in each of the 6 assays, the same total radioactivity was introduced for both standards. The bound/total radioactivity was calculated for St0 and St1. The MDC in ng/ml was determined as:

\[
X = \frac{x}{y} \quad Y = y
\]

where \(x\) is MDC in ng/ml; \(X\) is [standard St1] - [standard St0] = 3 ng/ml; \(Y\) is mean St1 (cpm) - mean St0 (cpm); and \(y = 2\) SD of St0 (cpm).

The control of the reliability of the method of measurement was done using the following tests: (a) test of linearity of the relationship between consecutive dilutions of a serum of known high cytokeratin 19 level and results of the titration; (b) assessment of intraassay reproducibility by measuring 7 different sera twice during the same assay; (c) assessment of interassay reproducibility by measuring 2 different sera in 10 different assays; (d) analysis of the standard curve in the low values in order to evaluate the difference of signal given by the St0 and the St1.

Total LDH assays were done following the Deutsch Chemical Society recommendations by measuring its activity using pyruvate as a substrate (Bio-Mérieux, France).

The upper limits of normal values were as follows: LDH, 330 units/liter; alkaline phosphatase, 220 units/liter; leukocytes 8000/μl. The lower limits of normal values were 32 g/liter for albumin and 135 mmol/liter for serum sodium.

All sera samples were assayed blind of clinical information.

Statistics. ROC curves were constructed using both patient and control subject serum CYFRA 21-1 levels in an attempt to establish a sensitivity-specificity relationship; areas under the ROC curve were calculated (18). The serum tumor marker was not distributed normally; thus, for each patient subset, results were expressed as median, and variation was expressed as interquartile range. Nonparametric statistical analyses were used: differences between two independent groups were determined by means of the Mann-Whitney U test; differences between more than two groups were determined by means of the Kruskal-Wallis one-way analysis of variance; \(P < 0.05\) was considered as significant. Survival was defined as the time from the date of sampling to the date of death. Survival data were updated in February 1992 and none of the patients were lost from sight during follow-up. Probability of survival was estimated by the method of Kaplan and Meier (19). Single variable survival analyses were done by means of Wilcoxon and log rank tests and multivariate regression was done with the Cox’s model (20). There were 12 variables in the model. Serum CYFRA 21-1 was examined as normal (≤3.6 ng/ml) or elevated (>3.6 ng/ml) as were the other biological variables (LDH, serum sodium, alkaline phosphatase, albumin, and leukocytes) according to their respective upper normal limits. Age groups were defined as less than or equal to and over 50 years old. Other variables were stage of the disease, sex, history, weight loss, and performance status. Survival was analyzed using the SAS software package.

RESULTS

Sensitivity and Reliability of the Immunoradiometric Assay.

According to the data in Table 2 the calculated MDC was 0.06 ng/ml. The test of linearity showed a linear relationship between the titrations of CYFRA 21-1 and the dilutions of a serum sample with a high cytokeratin 19 concentration (Fig. 1). Seven sera were measured twice during the same assay (intraassay). The results of the first and second measurements were, respectively (ng/ml): 12, 11.4; 27, 30; 6, 7.5; 3, 2; 4.4, 4.8; 45, 41; 3.4, 3.2. The coefficients of variation of 2 second measurements were, respectively (ng/ml): 12, 11.4; 27, 30; 6, 7.5; 3, 2; 4.4, 4.8; 45, 41; 3.4, 3.2. The coefficients of variation of 2 sera tested in 10 different assays (intraassays) were 7 and 9.8%, respectively.

Analysis of the standard curve in the low values demonstrated that the difference between 0 and 3 ng/ml was 700 cpm, a value sufficient to prevent overlap of results.

Tumor Marker Distribution. The median and interquartile range interquartile range, of serum CYFRA 21-1 was significantly higher in

| Table 2 Results of 6 different immunoradiometric assays performed in order to determine the minimal detectable concentration |
|---|---|---|---|---|
| **Assay** | **St0** | **St1** |
| **T (cpm)** | **cpm** | **B/T** | **cpm** | **B/T** |
| 1 | 226,473 | 35 | 0.02 | 1,947 | 0.99 |
| 2 | 219,399 | 89 | 0.04 | 2,648 | 1.20 |
| 3 | 208,757 | 76 | 0.04 | 1,939 | 0.93 |
| 4 | 241,441 | 71 | 0.03 | 1,773 | 0.73 |
| 5 | 207,643 | 83 | 0.04 | 1,776 | 0.85 |
| 6 | 198,408 | 64 | 0.03 | 1,633 | 0.82 |

* B, bound radioactivity; T, total radioactivity.
level differed when the histological type was considered (Kruskal-Wallis test KW = 7.69; P < 0.05). Serum CYFRA 21-1 level was significantly higher in SQC when compared with each of the other histologies (Mann-Whitney U test, P < 0.05).

**CYFRA 21-1 and Clinical Variables at Presentation.** In NSCLC, the CYFRA 21-1 level, was significantly higher in patients with a metastatic disease (7.4; interquartile range, 3.0–21.0 ng/ml) in comparison with the limited disease patient subgroup (3.8; interquartile range, 1.9–8.5 ng/ml; Mann-Whitney U test, P < 0.001). When the marker was analyzed according to stage grouping, a significant increase of the serum level was observed from stage I to stage IV (Kruskal Wallis, 13.7; P < 0.05; Fig. 4) but there was no significant difference when stage IIIa and stage IIIb were compared (Mann-Whitney U test, 500; nonsignificant). CYFRA 21-1 levels were lower in patients who were operated upon when compared with inoperable patients (2.4; interquartile range, 1.25–5.0 versus 5.8; interquartile range, 2.7–13.0 ng/ml; Mann-Whitney U test, P < 0.001). In addition,

### Table 3 Sensitivity and specificity of CYFRA 21-1 using a 3.6-ng/ml upper limit of normal values

<table>
<thead>
<tr>
<th>Subsets</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-small cell</td>
<td>0.56</td>
<td>0.89</td>
</tr>
<tr>
<td>SQC</td>
<td>0.63</td>
<td>0.91</td>
</tr>
<tr>
<td>Small cell</td>
<td>0.46</td>
<td>0.89</td>
</tr>
<tr>
<td>All</td>
<td>0.52</td>
<td>0.87</td>
</tr>
</tbody>
</table>

**CYFRA 21-1 and Histology.** The median (interquartile range) serum CYFRA 21-1 for SCLC, SQC, adenocarcinomas, and large cell carcinomas were 3.4 (1.8–7.0), 5.6 (2.6–13.0), 2.9 (1.85–7.10), and 3.8 (1.8–7.0) ng/ml, respectively (Fig. 3). The serum CYFRA 21-1 cancer patients (4.3; interquartile range, 2.3–9.5) when compared with control subjects (1.05; interquartile range, 0.8–1.8; Mann-Whitney U test, P < 0.001). Areas under ROC curves were 0.83 ± 0.03, 0.80 ± 0.03, and 0.74 ± 0.05 for the SQC, NSCLC, and SCLC groups, respectively (Fig. 2). Using ROC curves, a threshold of 3.6 ng/ml was chosen as the upper limit of normal values. The sensitivity and specificity of this cutoff in the different patient subsets are shown in Table 3.

**Fig. 1.** Dilution test of a serum with a high level of cytokeratin 19.

**Fig. 2.** Receiver operating characteristic curve for serum CYFRA 21-1. ○, squamous cell carcinoma, area under the concentration curve = 0.83 ± 0.03 (SE); ●, non-small cell lung cancer, area under the concentration curve = 0.80 ± 0.03.

**Fig. 3.** CYFRA 21-1 distribution according to histology. ——, median value; columns, interquartile range. ADE, adenocarcinoma; LCC, large cell carcinoma.

**Fig. 4.** CYFRA 21-1 distribution according to stage grouping in non-small cell lung cancer. ——, median value; columns, interquartile range.
patients who presented mediastinal lymph nodes (N2 or N3) demonstrated higher serum CYFRA 21-1 levels (5.0; interquartile range, 2.6–12.5 ng/ml), when compared with patients without mediastinal lymph nodes (N0 or N1; 3.2; interquartile range, 1.75–8.0 ng/ml; Mann-Whitney $U$ test, $P < 0.05$).

In NSCLC, the distribution of CYFRA 21-1 varied significantly according to the performance status of NSCLC patients (Kruskal-Wallis 9.16; $P < 0.05$; Table 4). In SCLC, CYFRA 21-1 did not significantly differ between limited (3.0; interquartile range, 1.8–5.4 ng/ml) and extensive disease (4.55; interquartile range, 2.1–11.5 ng/ml; Mann-Whitney $U$ test, $P = 0.07$).

Survival. Univariate analysis showed that lung cancer patients with serum CYFRA 21-1 over 3.6 ng/ml proved to have a significantly shorter overall survival than those with a normal serum level (log rank, $P = 0.007$; Wilcoxon, $P = 0.001$; Fig. 5). The negative prognostic effect of CYFRA 21-1 was highly significant in SQC (log rank, $P < 0.001$; Wilcoxon. $P = 0.001$; Fig. 5). The negative prognostic effect of stage group, weight loss, performance status, serum albumin, LDH, and CYFRA 21-1 (Table 5). With Cox’s model analysis, performance status, stage grouping, and CYFRA 21-1 were the only significant determinants of survival (Table 6).

**DISCUSSION**

New strategies of chemotherapy intensification are under evaluation in SCLC patients in an attempt to circumvent secondary chemoresistance (3, 4). NSCLC is a group of different histologies having a lower chemosensitivity when compared with SCLC. Surgery of stage I and II NSCLC offers the best probability of cure (5, 21) but in the more advanced stages combined modality treatments are required owing to the frequency of microscopic metastatic disease (7, 22–24). Thus, the treatment of both SCLC and NSCLC lung cancer is still an experimental approach. In this setting, an awareness of significant variables able to predict metastatic spread and poor prognosis is recommended (25).

Some important prognostic factors of lung cancer have already been reported. For NSCLC, the two main variables which determine survival are the stage of the disease and the performance status (8). In several studies, tumor markers such as tissue polypeptide antigen (26, 27), LDH (8), or carcinoembryonic antigen (26, 27) have been demonstrated as significant prognostic factors in univariate analyses. Other biological properties of NSCLC have been investigated as putative indicators of prognosis. In different multivariate studies aneuploidy (28), lack of expression of blood group A antigen (29) or expression of the neural cell adhesion molecule (30) have been independently demonstrated as prognostic factors; however, correlation and interactions between these variables are not known. For SCLC, several studies have demonstrated the univariate influence of disease extent, performance status, LDH, and alkaline phosphatase on survival (9, 31–33); moreover, some authors have suggested that the poorest prognosis is seen in SCLC patients with bone marrow metastases (34), elevated serum neuron specific enolase (35), or carcinoembryonic antigen (26). Intermediate filaments are part of the cytoskeleton which provide important information on the cell origin (36). They are classified into five tissue specific protein families. The cytokeratin family is expressed by all epithelial cells (10) including endocrine cells of the dispersed neuroendocrine system (37) and, it appears, therefore, to be a general specific and useful marker of the epithelial differentiation. Interestingly, the expression of cytokeratins is not lost by epithelial cells during malignant transformation (10, 36), a phenomenon which

<table>
<thead>
<tr>
<th>Stage</th>
<th>Median survival (days)</th>
<th>Wilcoxon</th>
<th>Log rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and II</td>
<td>407</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>IIIa</td>
<td>274</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>IIIb</td>
<td>148</td>
<td>191</td>
<td></td>
</tr>
</tbody>
</table>

Table 5 Significant prognostic factors of in the entire population

<table>
<thead>
<tr>
<th>Factor and level</th>
<th>Median survival (days)</th>
<th>Wilcoxon</th>
<th>Log rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performance status</td>
<td>1-2</td>
<td>191</td>
<td>0.0049</td>
</tr>
<tr>
<td>&gt;2</td>
<td>213</td>
<td>0.007</td>
<td></td>
</tr>
</tbody>
</table>

Table 6 Regression analysis results: estimated relative risk for significant variables ($n = 165$)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient ($\beta \pm SE$)</th>
<th>$P$</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performance index</td>
<td>1.03 ± 0.22</td>
<td>&lt;0.0001</td>
<td>2.81</td>
</tr>
<tr>
<td>Stage of disease</td>
<td>0.90 ± 0.29</td>
<td>0.0024</td>
<td>2.47</td>
</tr>
<tr>
<td>CYFRA 21-1</td>
<td>0.49 ± 0.21</td>
<td>0.021</td>
<td>1.64</td>
</tr>
</tbody>
</table>

Fig. 5. Probability of survival of all patients with normal and elevated pretreatment serum CYFRA 21-1 level.
contrasts with the well-known phenotypic instability of cancer cells (38). Antibodies raised against some common antigenic determinants of all cytokeratins are therefore useful in typing malignant tumors with poor histological features of differentiation (36). Immunohistochemical studies using these broad-spectrum cytokeratin antibodies have clearly demonstrated that all four histological types of lung cancer expressed cytokeratins (11, 12, 39); in particular, the classic SCLC type which have been shown to coexpress both cytokeratin and neurofilament whereas the variant type only express cytokeratin (40). This supports the hypothesis of a common epithelial lineage of all histological types of lung cancer. A cytokeratin is a heterotetramer of protofilaments composed of two polypeptides: one acidic type I subunit; and one basic type II subunit. Each type of epithelium and their malignant counterpart express a specific cytokeratin polypeptide pattern (10, 41); simple epithelia, including pseudostratified epithelia, express cytokeratins 7, 8, 18, and 19. Selective antibodies raised against simple epithelium type of cytokeratin have been shown to react with all histologies of lung cancers (42, 43). Although cytokeratins are part of the cytoskeleton some fragments might be released in the serum owing to cell lysis or tumor necrosis, which gives support to the present research on cytokeratin subunit 19 fragment as a tumor marker of lung cancer.

The immunoradiometric assay described in this study is able to detect the serum fragment of cytokeratin subunit 19 referred to as CYFRA 21-1, with both sensitivity and reliability. The threshold of 3.6 ng/ml was determined using the ROC curve analysis which showed that this threshold allowed the best sensitivity-specificity relationship. Our study has demonstrated a correlation between serum CYFRA 21-1 and stage of the disease in NSCLC but not in SCLC. Moreover, in NSCLC, the marker varied significantly according to nodal status. These data suggest that CYFRA 21-1 might be considered as a marker of tumor mass. Thus, patients who present a high level of serum CYFRA 21-1 might require a careful search of metastases. Despite these results it is not possible to predict operability of NSCLC using the serum level of the marker owing to the important overlap of the distribution in stage IIIa (usually resectable) and stage IIIb (unresectable) NSCLC groups. The reason for this result might be that the distinction between stage IIIa and stage IIIb mainly depends on anatomic extension rather than tumor mass. The low incidence of a high serum CYFRA 21-1 level in the stage I–II group clearly indicates that the marker is of no help in screening or early diagnosis of the disease. Because CYFRA 21-1 is correlated with both disease extent and performance status it is not surprising that univariate analysis showed that patients with a high serum level proved to have a significantly shorter survival. The negative effect of CYFRA 21-1 on survival was particularly significant in SQC whereas this effect was not demonstrated in the other histologies. Interestingly, Cox’s model demonstrated that the prognostic significance brought by CYFRA 21-1 is independent and adds information to the well-known and above-mentioned prognostic factors. This study supports the use of the serum fragment of cytokeratin subunit 19 CYFRA 21-1 as an independent prognostic marker of squamous cell carcinoma of the lung.

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