Complement factor H is elevated in bronchoalveolar lavage fluid and sputum from patients with lung cancer

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**Running title:** Factor H in BAL and sputum from patients with lung cancer

**Key words:** Tumor markers, lung cancer, bronchoalveolar lavage, sputum, complement factor H, diagnosis
Abstract

**Background:** Cytologic examination of specimens obtained from the respiratory tract is a lung cancer diagnostic procedure with high specificity, but moderate sensitivity. The use of molecular biomarkers may enhance the sensitivity of cytologic examination in the detection of lung cancer.

**Methods:** Complement factor H, a protein secreted by lung cancer cells, was quantified in a series of bronchoalveolar lavage supernatants from lung cancer patients and patients with non-malignant respiratory diseases. Albumin, total protein content, and hemoglobin were also analyzed. Results were validated in independent sets of bronchoalveolar lavage and sputum supernatants.

**Results:** There was a significantly higher concentration of factor H in bronchoalveolar lavage samples from lung cancer patients. The sensitivity and specificity of the factor H test was 82% and 77%, respectively. These results were validated in an independent set of patients with nearly identical results. Furthermore, 70% and 45% of bronchoalveolar lavage fluids from central and peripheral tumors, respectively, reported as cytologically negative were classified as positive using this marker. Finally, the test was evaluated in a series of sputum supernatants from lung cancer patients and controls. The sensitivity and specificity of the factor H test in this series was 80% and 88%, respectively.

**Conclusion:** Factor H is elevated in bronchoalveolar lavage and sputum from lung cancer patients.

**Impact:** Measurement of molecular biomarkers, such as complement factor H, may be used in the future as an adjunct to cytology in the diagnosis of malignant pulmonary diseases.
Introduction

Lung cancer is the leading cause of cancer death in developed countries (1). In patients with suspected lung cancer, a clear diagnosis is mandatory to determine the treatment strategy. Flexible fiberoptic bronchoscopy is a safe and relatively easy procedure that has proved extremely useful in the diagnosis and management of many pulmonary diseases (2). In fact, bronchoscopy can be considered as the primary diagnostic tool in patients with suspected centrally located pulmonary carcinoma. Bronchoscopic procedures for the diagnosis of lung cancer include cytologic examination of specimens from bronchial biopsy, bronchial brushing and bronchial washing/bronchoalveolar lavage. These tests are less invasive than other tissue procurement methods, carry a small risk of complications, and have high specificity. However, sensitivity is relatively low, especially for peripheral lesions that are beyond the visual segmental bronchi (3). It is expected that new approaches, such as the detection of molecular markers, could be used in the future to enhance the sensitivity of cytologic examination in the diagnosis of bronchial fluids. In fact, some molecular markers have already been shown to be differentially increased in bronchial fluids from lung cancer patients. These include: proteins, such as carcinoembryonic antigen, tumor polypeptide antigen, neuro-specific enolase or telomerase (4-7); cell-free nucleic acids (8-10); chromosome and locus-specific changes (11-14); and epigenetic modifications (15, 16). We have previously reported that complement factor H is expressed and secreted by many non-small cell lung cancer (NSCLC) tumors (17). Factor H is an inhibitor of complement activation and promotes tumor growth in vivo (18). The aim of the present study was to explore whether factor H levels were detectable in bronchial fluids of lung cancer patients, and to evaluate its potential diagnostic usefulness. Our results demonstrate that bronchoalveolar lavage fluids from subjects with lung cancer present...
a significantly higher expression of factor H than those from patients with non-malignant pulmonary diseases. Unexpectedly, levels of factor H in bronchoalveolar samples correlated with levels of albumin, suggesting that the cause of factor H accumulation in bronchial fluid is not its direct secretion from tumor cells but the exudation of plasma proteins to the bronchial lumen. Finally, sputum samples from patients with NSCLC also had higher levels of factor H than sputum samples from healthy donors.

Materials and Methods

Patients

Seventy eight subjects undergoing diagnostic bronchoscopy at the Clinica Universidad de Navarra were consecutively included in the study. Based on the diagnosis, bronchoalveolar lavage samples were divided into four groups (Supplement Table S1): samples from patients with non-malignant lung disease (group I), samples from patients with lung malignancies in which the cytologic examination of the bronchoalveolar lavage revealed malignant cells (group II), samples from patients with centrally located, endoscopically visible, lung malignancies in which cytologic examination of the bronchoalveolar lavage did not revealed malignant cells (group III), and samples from patients with peripheral, non-visible, lung malignancies in which malignant cells were not identified (group IV). Table 1 summarizes the characteristics of the four study groups, regarding age, gender and smoking status. The validation set consisted of 26 bronchoalveolar lavage supernatants from patients with benign lung disease and 25 bronchoalveolar lavage supernatants from patients with lung cancer, collected at Charité-Universitätsmedizin, Berlin. Sputum samples were collected from 103
healthy individuals and 68 patients with histologically proven lung cancer, treated in the Lung Cancer Clinic of Catalan Institute of Oncology, Barcelona. All protocols were approved by the Institutional Ethical Committees, and all patients gave informed consent.

**Bronchoscopy procedure**

Local anesthesia was achieved with 2% lidocaine. Bronchoscopy was performed using a fiberoptic bronchoscope (Olympus Exera BF 240) after intravenous administration of 2-5 mg midazolam. After routine inspection of the tracheobronchial tree, the bronchoscope was wedged into a segmental or subsegmental bronchus and bronchial lavage fluid was obtained by instilling 160 ml of saline solution (four 40-ml aliquots) into the bronchus and withdrawing by hand aspiration using the attached syringe. In control patients with diffuse disease, the lavage was performed in the medial lobe or in the lingula. In patients with localized pulmonary disease, the sample was obtained from a bronchus of the affected area or as close as possible. Aliquots were pooled and divided into two fractions, one used for cytology and the other for marker determination (which was stored frozen at -80°C until the time of analysis).

**Conventional cytologic examination**

Cytologic smears were prepared using Saccomanno solution as a fixative. The specimens were stained following the Papanicolaou method and evaluated by an experienced cytopathologist (M.D.L.). Histological diagnosis and classification of the specimens were defined based on WHO criteria (19).

**Sputum collection**
Spontaneous or hypertonic saline solution-induced sputa were collected in a sterile specimen cup. To increase the probability that material of deep lung origin was obtained, subjects received detailed instructions by study personnel at the participating institution. Sputum samples were extensively vortexed after adding 10 ml of saline. Samples were stored at -80°C until analysis.

**Marker measurements**

Samples were spun down at 300 g for 10 min, and the supernatants were collected. For factor H quantification, a polystyrene 96-well plate (Corning Incorporated, Corning, NY) was coated with 50 ng/well of purified monoclonal anti-factor H antibodies (in 50 μl of 50 mM sodium bicarbonate, pH 8.3) during 1 hour at room temperature. Purified monoclonal anti-factor H antibodies were obtained from the OX-24 mouse hybridoma (European Collection of Cell Cultures, Salisbury, UK) as previously described (17). The plate was then washed and blocked overnight at 4°C with blocking buffer: Tris buffered saline (25 mM Tris, 150 mM NaCl, pH 7.4) with 1% BSA and 0.1% Tween-20). After washing, 50 μl of samples diluted in blocking buffer (1:20 or 1:40 for bronchoalveolar lavage samples and 1:5 for sputum samples) or standards (ranging from 1.5 to 400 ng/ml) were added and incubated for two hours at room temperature. To prepare the standards, factor H (Sigma-Aldrich, Saint Louis, MO) was used. After washing, 50 μl of an anti-factor H rabbit antibody (1:1000, Serotec, Raleigh, NC) was added, and after 30 minute incubation at room temperature the assay was developed using a donkey anti-rabbit antibody coupled to horseradish peroxidase (1:2000, Amersham Biosciences, Piscataway, NJ) and o-phenylenediamine dihydrochloride (Sigma-Aldrich). The plate was read at 450 nm. Quantitative determinations of albumin and hemoglobin were carried out by ELISA following manufacturer’s instructions (Immun Diagnostik, Bensheim, Germany).
Germany). For the determination of albumin, prior to the assay, bronchoalveolar lavage and sputum samples were diluted 1:1000 and 1:100, respectively, in phosphate buffered saline: 10 mM phosphate, 150 mM NaCl, pH 7.4. For hemoglobin determination, bronchoalveolar lavage samples were diluted 1:10. Total protein was determined using the BCA protein assay (Pierce, Rockford, IL), with bovine serum albumin as the standard.

**Statistical analysis**

Box plots were used to present the results. In these plots, the bottom and top of the boxes represent the lower and upper quartiles, the band inside the box is the median, and the whiskers the minimum and maximum of all the data. SPSS 15.0 software was used for statistical analysis. The Mann-Whitney U test was used to compare values between lung cancer and control groups. Relationship between variables was quantified using Spearman’s rank correlation. Receiver operating characteristic (ROC) curves were used to define the best cut-off points for the diagnostic markers, and to describe and compare their performance. Area under the ROC curve, as well as the 95% confidence interval, was also calculated. A p value of less than 0.05 was considered as statistically significant.

**Results**

_Evaluation of factor H in bronchoalveolar lavage fluids_

Quantification of complement factor H was first performed in samples from patients with non-malignant lung disease (group I in Supplement Table S1) and samples from patients with lung malignancies in which the cytologic examination of the bronchoalveolar lavage sample had revealed malignant cells (group II in Supplement Table S1). Bronchoalveolar lavage
supernatants from patients with lung cancer presented significantly higher levels of factor H than those from non-cancer patients (p=0.002) (Table 2; Fig. 1A). There were no statistical differences in the levels of this molecular marker between smokers and non-smokers within the control group, ruling out the possibility that the differences between control and lung cancer patients were due to a higher proportion of smokers in the latter group. In the cancer group, no association was found between factor H levels and tumor histology. In fact, both NSCLC and small cell lung cancer (SCLC) patients had high levels of factor H in their bronchoalveolar fluids. We then evaluated the possibility that this protein extravasated from blood to bronchial lumen. For this aim, we examined the concentration of albumin in the clinical samples. There was a significantly higher concentration of albumin in bronchoalveolar lavage samples of the lung cancer group than in those of the control group (p=0.006) (Table 2; Fig. 1B). Albumin concentration was highly correlated with factor H concentration (r=0.867; p<0.001). These results strongly suggest that lung cancer patients present a significant increase in the concentration of plasma proteins in bronchoalveolar fluids. In fact, there was also a significant increase in the amount of total protein in bronchoalveolar lavage supernatants from patients with lung cancer as compared with the control group (p=0.001). To exclude the presence of blood due to bleeding during the bronchoscopy procedure, the amount of hemoglobin in the samples was determined. Hemoglobin levels were similar between the control group and the lung cancer group (p=0.912) (Table 2). Besides, there was no correlation between factor H and hemoglobin (r=0.210; p=0.219).

A ROC curve was generated for factor H (Fig. 1C) and albumin (Fig 1D). The area under the curve was 0.79 (95% confidence interval, 0.63-0.94) for factor H and 0.76 (95% confidence interval, 0.60-0.92) for albumin. Cut-off points were established in order to provide a
sensitivity of at least 80%. The sensitivity and specificity of the factor H test was 82% and 77%, respectively (cut-off 1 μg/ml). The sensitivity and specificity of the albumin test was 82% and 71%, respectively (cut-off 17 μg/ml). Five false-positives, common to the two biomarkers, were observed. They corresponded to patients diagnosed with hamartoma, bronchiectasis, pneumonia, sarcoidosis, and diffuse interstitial lung disease. A non-cancer patient with hemoptysis was also misclassified with the albumin test.

We next evaluated whether the determination of plasma proteins in bronchoalveolar lavage fluids may complement conventional cytology in the diagnosis of lung cancer. For that propose, we used bronchoalveolar lavage samples from patients in which cytologic examination was insufficient to diagnose the malignancy. These patients were divided into two groups: patients with centrally located tumors visible by bronchoscopy at the moment of the lavage (group III in Supplement Table S1), and patients with peripheral tumors not endoscopically visible (group IV in Supplement Table S1). We used the cut-off points previously established (1 μg/ml for factor H and 17 μg/ml for albumin). In group III, 13 out of 19 cases were classified as positive using both determinations (Fig. 2A). Therefore, the tests were positive in almost 70% of bronchial samples from patients with centrally located tumors initially classified as negative by cytology. In group IV, positivity was found in 45% (9/20) of cases using the factor H determination and in 55% (11/20) of cases using the albumin determination (Fig. 2B).

As a summary, Table 3 shows the sensitivity and specificity of cytologic examination and factor H and albumin tests in all the subjects consecutively included in this initial series.
Validation in bronchoalveolar lavage and sputum supernatants from independent cohorts

Content of complement factor H was also assessed in an independent series of bronchoalveolar lavage fluids from unselected patients with lung cancer (n=25) and patients with benign lung diseases (n=26). Factor H was significantly elevated in bronchoalveolar lavage samples from lung cancer patients (Fig. 3A). Factor H levels were $2.65 \pm 2.20$ μg/ml in samples derived from cancer patients vs. $0.41 \pm 0.46$ μg/ml in control samples (p<0.001). A ROC curve was also generated (Fig. 3B). The area under the curve was 0.86 (95% confidence interval, 0.75-0.96).

Finally, complement factor H, albumin and total protein were determined in a series of sputum samples from NSCLC patients (n=68) and healthy donors (n=103). There was a significant increase in the amount of factor H in sputum samples from patients with lung cancer as compared with those from healthy volunteers (Fig. 4A). Factor H levels were $0.42 \pm 0.49$ μg/ml in samples derived from cancer patients vs. $0.04 \pm 0.03$ μg/ml in control samples (p<0.001). No differences were observed between stages or histological types (Fig. 4B and 4C). A ROC curve for factor H showed an area under the curve of 0.93 (95% confidence interval, 0.89-0.96) (Fig. 4D). Using a cut-off of 70 ng/ml, sensitivity and specificity were 80% and 88%, respectively. Although there was a weak correlation between the levels of factor H and albumin ($r=0.534$; p<0.001), no differences were found in the concentration of albumin between samples obtained from cancer patients and samples obtained from controls ($76.36 \pm 60.01$ μg/ml vs. $60.70 \pm 40.31$ μg/ml, respectively, p=0.250). Neither was any difference found in the levels of total protein between patients and controls ($509.78 \pm 337.92$ μg/ml vs. $459.06 \pm 431.42$ μg/ml, respectively, p=0.376).
Discussion

Cytologic examination of bronchoscopy material has a very low false-positive rate. However, there are still a substantial number of patients in whom bronchoscopy fails to provide a diagnosis. Alternative methods, based on the detection of molecular biomarkers differentially expressed in bronchial samples, may be used to enhance the sensitivity of cytologic examination in the detection of lung cancer. Data presented in this study show that some proteins, such as complement factor H, are significantly increased in bronchial fluids from patients with lung cancer when compared with a heterogeneous group of patients with non-malignant lung diseases. We initially hypothesized that factor H would be increased in these fluids due to its production and secretion by tumor cells. However, our results strongly suggest that tumor cells are not the source of the molecule. Firstly, patients with tumor types which do not express factor H, such as SCLC (17), have high levels of factor H in their bronchial fluids. Secondly, there is a high correlation between factor H and albumin. The most plausible explanation is that these markers appear in the bronchial lumen as a result of exudation of plasma proteins. An alternative explanation would have been that bleeding of the epithelium or the tumor at the time of bronchoscopy causes the accumulation of plasma proteins in the bronchial lumen. However, we discarded this possibility since hemoglobin levels were not increased in bronchoalveolar lavage samples from lung cancer patients and did not correlate with factor H levels. Therefore, we propose that the interstitial entry of large plasma proteins, such as factor H, reflects an increase in microvascular permeability of the tumor circulation. In agreement with these results, Charokopos et al. showed that bronchial washings from patients with lung carcinoma present an increased concentration of albumin (20). The authors found
that the determination of albumin in bronchial washings had a high sensitivity and specificity for the diagnosis of lung cancer and suggested that the presence of albumin in the bronchial fluid was caused by its exudation to the bronchial lumen. In fact, the increased extravasation of macromolecules due to hyperpermeability is an established property of tumor vessels and is the consequence of an intense inflammation combined with tumor angiogenesis (21, 22). It is also thought that hyperpermeability of tumor blood vessels contributes to tumor progression (22, 23). Interestingly, this may make plasma exudation in cancer more intense than in other inflammatory conditions, which would explain why patients with other lung inflammatory disorders show lower levels of plasma proteins in bronchial fluid. The observed absence of correlation between the markers and smoking history is supported by previous work in which no differences in albumin and total protein were found in bronchial secretions from smokers and non-smokers (24). We neither found any correlation between the concentration of the marker and lung cancer histology. Therefore, it appears that high permeability and plasma protein exudation is a common feature for lung tumors, regardless of its histology or its specific molecular alterations. It is attractive to suggest that the use of protein markers in bronchial material would overcome the limitations of cancer heterogeneity.

Complement factor H was also found significantly elevated in sputum samples from lung cancer patients. Sputum is a very useful source of biomarkers for lung cancer because it can be obtained in an easy, noninvasive, and inexpensive way. Sputum cytology has a 66% sensitivity for the detection of lung cancer (3). Our data suggest that the quantification of factor H on sputum samples offers a comparable detection rate. In contrast, the levels of albumin or total proteins were not increased in lung cancer patients, suggesting that sputum samples are more heterogeneous than bronchoalveolar lavage fluids and may contain proteins from sources other...
than the tumor microenvironment. Furthermore, the presence of factor H in samples from all lung cancer stages suggests that plasma protein exudation is an early change in the bronchial epithelium and may be used for early detection.

A diagnostic molecular biomarker needs to be clinically useful and competitive. In this sense, we have demonstrated the usefulness of quantifying complement factor H in bronchial fluids for the identification of patients with lung cancer after a false-negative diagnostic bronchoscopy, suggesting that the determination of factor H may increase the yield of conventional cytologic analysis. This simple test could be considered as part of the diagnostic work-up of the patient. For example, a positive molecular test would represent additional information supporting the presence of a tumor. In any case, more standardized studies prospectively evaluating the performance characteristics of the test will be required to elucidate the potential utility of this marker in the clinical practice.

In conclusion, we have shown that bronchoalveolar lavage and sputum samples from patients with lung cancer contain high levels of complement factor H. Quantification of markers such as factor H may be used in the future as a sensitive and reliable molecular diagnostic technique to implement cytologic examination in the diagnosis of lung cancer.
References


Figure legends

Figure 1. A, Box plot for the quantification of complement factor H (A) and albumin (B) in bronchoalveolar lavage supernatants from patients with a benign respiratory disease and patients with lung cancer. Patients with lung cancer had a cytologic confirmation of the presence of malignant cells in the lavage. The levels of factor H and albumin were significantly higher in the lung cancer group than in the control group (**, p<0.01). A ROC curve was generated for factor H (C) and albumin (D). Area under the curve was 0.79 (95% confidence interval, 0.63-0.94; p=0.003) for factor H and 0.76 (95% confidence interval, 0.60-0.92; p=0.007) for albumin.

Figure 2. Concentration of complement factor H and albumin in bronchoalveolar lavage fluids with negative cytology obtained from patients with centrally located lung cancers (A), or peripheral lung cancers (B). The cut-off point used for each marker is indicated in the figures.

Figure 3. A, Concentration of complement factor H in the validation set of bronchoalveolar lavage supernatants from lung cancer patients and patients with non-malignant pulmonary diseases. The levels of factor H were significantly higher in the lung cancer group than in the control group (***, p<0.001). B, ROC curve for factor H in the bronchoalveolar lavage samples. The area under the curve was 0.86 (95% confidence interval, 0.75-0.96; p<0.001).

Figure 4. A, Box plot for complement factor H in sputum samples from lung cancer patients and healthy volunteers. Levels of factor H were significantly higher in the lung cancer group compared with the control group (***, p<0.001). B, Box plot for factor H in sputum samples separated by tumor stage: I (n=9), II (n=6), III (n=21), IV (n=32). C, Box plot for factor H in sputum samples separated by histological type of the tumors: SCLC, small cell lung cancer
(n=6); ADC, adenocarcinoma (n=28); SCC, squamous cell carcinoma (n=16); LC, large cell carcinoma (n=4); NOS, not otherwise specified (n=16). D, ROC curve for factor H in the sputum samples from lung cancer patients and healthy volunteers. The area under the curve was 0.93 (95% confidence interval, 0.89-0.96; p<0.001).
Fig. 1. Pio et al.

A

B

C

D

Factor H (μg/ml)  

Albumin (μg/ml)  

Sensitivity  

1-Specificity  

Factor H  

Albumin
Fig. 2. Pio et al.
Fig. 3. Pio et al.

A

B

Factor H (μg/ml) vs Sensitivity

Non Tumor Tumor

***

1-Specificity

Factor H
Fig. 4. Pio et al.

A

B

C

D

Factor H (μg/ml)

Non Tumor  Tumor

Stage

Factor H (μg/ml)

I  II  III  IV

Histology

Factor H

Sensitivity

1-Specificity

0 0.2 0.4 0.6 0.8 1.0

0 1

0 1 2 3

0 1 2 3 4

0 1 2 3

0 1 2 3
Table 1. Characteristics of the study groups in the initial series of bronchoalveolar lavage fluids

<table>
<thead>
<tr>
<th>Group*</th>
<th>Age</th>
<th>Gender</th>
<th>Ever smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td>(yr: mean ± SD; range)</td>
<td>(males/females)</td>
<td></td>
</tr>
<tr>
<td>I (22)</td>
<td>58.2 ± 14.9; 30-82</td>
<td>14/8</td>
<td>11</td>
</tr>
<tr>
<td>II (17)</td>
<td>60.5 ± 12.7; 38-83</td>
<td>12/5</td>
<td>14</td>
</tr>
<tr>
<td>III (19)</td>
<td>60.4 ± 11.2; 39-79</td>
<td>16/3</td>
<td>14†</td>
</tr>
<tr>
<td>IV (20)</td>
<td>63.7 ± 10.0; 45-81</td>
<td>17/3</td>
<td>16†</td>
</tr>
</tbody>
</table>

* I: No malignancy; II: Lung malignancy with diagnostic cytology; III: Endoscopically visible lung malignancy with negative cytology; IV: Distal lung malignancy with negative cytology.
† No smoking history was available from one patient in the group.
Table 2. Quantification of the molecular markers in the initial series of bronchoalveolar lavage fluids†

<table>
<thead>
<tr>
<th>Group ‡</th>
<th>Factor H (μg/ml)</th>
<th>Albumin (μg/ml)</th>
<th>Hemoglobin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.17 ± 1.50</td>
<td>37.2 ± 56.7</td>
<td>6.10 ± 3.90</td>
</tr>
<tr>
<td>II</td>
<td>2.61 ± 1.88**</td>
<td>262.7 ± 451.6**</td>
<td>5.39 ± 2.32</td>
</tr>
<tr>
<td>III</td>
<td>2.38 ± 2.30</td>
<td>126.0 ± 274.4</td>
<td>8.82 ± 4.65</td>
</tr>
<tr>
<td>IV</td>
<td>2.15 ± 2.52</td>
<td>221.9 ± 432.4</td>
<td>5.94 ± 3.32</td>
</tr>
</tbody>
</table>

† Data represent mean ± SD. Statistically significant differences between the control group and the lung cancer group were evaluated with the Mann-Whitney U test. **, p<0.01.
‡ I: No malignancy; II: Lung malignancy with diagnostic cytology; III: Endoscopically visible lung malignancy with negative cytology; IV: Distal lung malignancy with negative cytology.
Table 3. Sensitivity and specificity of cytologic examination and the factor H and albumin tests in the bronchoalveolar lavage fluids from the 78 subjects consecutively included in the study*

<table>
<thead>
<tr>
<th>Group</th>
<th>Cytology†</th>
<th>Factor H</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patients</td>
<td>18/56 (32%)</td>
<td>35/56 (62%)</td>
<td>38/56 (68%)</td>
</tr>
<tr>
<td>Non-cancer patients</td>
<td>22/22 (100%)</td>
<td>17/22 (77%)</td>
<td>15/21 (71%)‡</td>
</tr>
</tbody>
</table>

* The cut-off values used were 1 μg/ml for the factor H test and 17 μg/ml for the albumin test.
† Correctly classified cases / total number of cases (% of correctly classified cases).
‡ One sample could not be analyzed.
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