Heterogeneous Nuclear Ribonucleoprotein A2/B1 Up-Regulation in Bronchial Lavage Specimens: A Clinical Marker of Early Lung Cancer Detection

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ABSTRACT

This study examines the use of a new epithelial marker in the detection of early lung cancer in bronchial lavage samples. The monocular antibody 703D4 recognizes the heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, and its overexpression/up-regulation was assessed and compared with routine diagnostic cytology. One hundred and three individuals were recruited into a prospective study. These individuals were referred to a chest physician with a request to examine for possible lung cancer, and a full clinical work-up was undertaken, including bronchoscopy and radiological investigations. In this study, we analyzed hnRNP expression in individuals with metaplastic bronchial epithelial cells or tumor cells in the bronchial lavage specimens, in a blinded study. The results from 103 bronchial lavage specimens indicate that hnRNP overexpression was more accurate in detecting evidence of a neoplasia than routine cytological examination. Twenty-two of 23 specimens in which malignant cells were identified cytologically demonstrated overexpression of hnRNP A2/B1. However, in the 80 specimens that were reported as cytologically negative, 41 of 80 demonstrated hnRNP overexpression, and 29 of these individuals were shown to have a lung neoplasm based on radiological findings and/or the biopsy taken at the bronchoscopy. An additional 4 of these 41 patients were shown to have a lung neoplasm within 8 months of the initial bronchoscopy. In conclusion, detection of hnRNPA2/B1 in bronchial lavage specimens that contain metaplastic bronchial epithelial cells or cancer cells predicts the presence of a neoplasm with a sensitivity of 96%, 82%, specificity.

INTRODUCTION

Lung cancer is a major contributor to overall cancer mortality, accounting for 78,500 deaths per year worldwide. It is the most common malignancy in males in the United Kingdom (1). The highest incidence rates of lung cancer for both males and females are found in the Merseyside region in the northwest of England (2). Clinical detection of lung cancer usually occurs late in the disease, when it is often beyond effective treatment; consequently, there is a high mortality rate. Detection at the earlier stage would influence both the mortality and morbidity rates.

hnRNP has been characterized as a Mₐ 31,000 protein (3) responsible for posttranscriptional regulation of gene expression by capping, splicing, polyadenylation, and cytoplasmic transport of mRNAs (4). Overexpression of hnRNPA2/B1 is critical in certain stages of mammalian lung development (5). Tockman et al. (6) reported that the tumor-associated monoclonal antibody 703D4 may be used as an early detection marker for lung cancer. They demonstrated the efficacy of this antibody in archival sputum specimens from a high-risk cohort (7) with a 90% accuracy in identifying individuals 2 years before a clinical diagnosis of lung cancer. Tockman et al. have also undertaken two prospective studies; a study on Stage 1 resected non-small cell lung cancer patients in the United States with a high risk of developing second primary tumors and also a study on tin miners in Yunnan China, who are at a high-risk of developing lung cancer. The results of these two studies predicted 67 and 69%, respectively, of those with hnRNP up-regulation in the exfoliated cells from their sputum would develop lung cancer in the first year of follow-up, compared with background risks of 2.2 and 0.9%, respectively (8–11).

In this study, we have posed the question as to whether hnRNP up-regulation in BL might be used to identify individuals with lung cancer in a group of patients referred to a lung cancer clinic in Liverpool, United Kingdom. The objective was to assess the potential use of the hnRNPA antibody 703D4 in identifying lung cancer patients and to determine its utility as an adjunct to present clinical diagnostic techniques.

MATERIALS AND METHODS

Patient Selection and Clinical Samples. We have collected BL from individuals with suspected lung cancer who

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The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; BL, bronchial lavage; NMCS, no malignant cells seen; MCS, malignant cells seen.
have been referred to the Cardiothoracic Center in Liverpool.

### Table 1
hnRNP expression assessed with the 703D4 antibody in the study group of patients

<table>
<thead>
<tr>
<th>Cytological diagnosis</th>
<th>703D4 staining</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant cells seen</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>(3)</td>
<td>(1)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>(16)</td>
<td>(0)</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>(3)</td>
<td>(0)</td>
</tr>
<tr>
<td>NMCS</td>
<td>41</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td>40</td>
</tr>
</tbody>
</table>

The criteria for referral to the Cardiothoracic Center included: unresolved chest infection, abnormal chest X-ray, cough (for >4 weeks), nonspecific weight loss, stridor, persistent (>3 weeks) hoarse voice, and other suspicious features that would prompt referral to the lung cancer clinic. Each patient underwent a full clinical work-up for lung cancer, including a chest X-ray, spirometry, and bronchoscopy. BL specimens were obtained from all of these patients; the choice of site was based on bronchoscopic findings within the large airways, in which saline was introduced via the bronroscope and then aspirated. One-half of the resulting fluid was placed in a sterile container and sent to the Department of Pathology, Royal Liverpool University Hospital, for routine cytological assessment. The remainder was placed in an equal volume of saccomannos fixative (2% polyethylene glycol in 50% ethanol) and sent to Roy Castle International Center for Lung Cancer Research.

The selection of patients was undertaken on the basis of an adequate cytology preparation containing endobronchial cells and alveolar macrophages. To make this study comparable with that of Tockman et al., we included only BL specimens that contained metaplastic or tumor cells. (It should be noted that specimens containing metaplastic cells alone are reported as NMCS, i.e., no malignant cells seen). One hundred and three patients who met these criteria were recruited into this project, and hnRNP staining was undertaken. The age of the patients ranged between 38 and 89 years. Forty-five of the individuals were females, and 58 were males. Smoking data were available for 95 individuals (87 smokers, 8 nonsmokers).

In this study, 195 individuals were assessed by the consultant cytopathologist, who found 23 specimens to contain malignant cells (MCS) and 172 to have no malignant cells. No metaplastic cells were seen in 47 of 172 NMCS, and 45 of 172 NMCS were not suitable for immunostaining because they contained a very high proportion of polymorphonucleocytes or were obscured by blood. Thus 80 of the 172 NMCS specimens met the criteria, together with the 23 individuals with MCS, giving the 103 individuals reported in this study.

The cytology reports were performed prospectively (by L. T.). The immunohistochemical analysis with hnRNP was undertaken in batches and blinded to the assessors (P. F. and L. T.). The comparison of the cytology findings and the immunohistochemical and clinical outcome was performed only when the assessors agreed on the staining results.

### Immunostaining
Four cytocentrifuge preparations were made from each BL using 3-aminopropyltriethoxy-silane coated slides. One slide from each case was stained with H&E to determine the presence of metaplastic squamous cells. Two slides from each case were stained with the monoclonal antibody 703D4. A negative control was also included for each patient. Before staining, the polyethylene glycol from the Saccomannos fixative was removed in graded alcohols. The cytocentrifuge preparations were pretreated with 0.5 N hydrochloric acid at 37°C before nonspecific protein blocking with goat serum in PBS (Biogenex). Slides were then incubated overnight at 4°C with the 703D4 antibody. The reaction product was detected using a Supersensitive StrAviGen multilink alkaline phosphatase kit (Biogenex) and disclosed using the chromogen Fast red (Biogenex). All of the slides were counterstained with hematoxylin. Nonimmune mouse serum was substituted for the 703D4 antibody in the negative controls. Positive control slides were made from American Type Culture Collection human bronchogenic cancer cell lines HTB58 (squamous cell carcinoma) and Calu-3 (adenocarcinoma), mixed with normal BL material, placed in Saccomannos fixative, and processed as above. All of the immunostained slides were screened using a Nikon E800 microscope and assessed as positive if metaplastic squamous cells showed cytoplasmic staining.

Three or more metaplastic or tumor cells were scored per specimen. The staining of hnRNP was assessed as positive or negative by two independent cytologists (L. T. and P. F.). The elimination of background staining was, thus, a prerequisite for a slide to be considered acceptable. The staining intensity had to be comparable with that of the positive control to be scored positive. If less than three cells stained positive, then additional samples were prepared and examined; and only if a total of three positive cells were found, was the sample considered ‘positive’.

### RESULTS
The 103 patients fulfilling the entry criteria into this study were assessed for hnRNP expression with the 703D4 antibody. The hnRNP immunostaining assay was prospectively applied sequentially to all of the patients who fulfilled the criteria outlined in the “Materials and Methods” over a specific time period (1995–1997). Details of the clinical diagnosis of these patients are given in Table 1.

Twenty-three of the 103 specimens examined cytologically were reported to have malignant cells present. However, only one of these, an adenocarcinoma, failed to stain with the 703D4 antibody. All of the squamous cell carcinomas (16 of 23) and the small cell carcinomas (3 of 23) demonstrated positive staining with the 703D4 antibody. In the remaining specimens (80 of 103) no malignant cells were seen (NMCS) on the specimens reported by the cytologists. However, 41 of these 80 specimens demonstrated positive staining with the 703D4 antibody (Table 1; Fig. 1).

Forty-one patients overexpressed hnRNP but were reported cytologically as NMCS; 29 of these had a lung neoplasm based on radiological findings and/or the biopsy taken at the bronchoscopy. An additional four patients were shown to have a lung neoplasm within 8 months of the initial bronchoscopy (Table 2). An additional 2 of these 41 patients had a previous lung tumor and another 2 patients had a carcinoma elsewhere. The remaining four patients had no evidence of a neoplasm and have been
discharged from the clinic. None of these patients have as yet presented with a lung neoplasm (Table 3). The 39 patients who were hnRNP-negative and cytologically negative were also investigated. Thirty-seven of these 39 patients have had no evidence of lung neoplasia. Of the two remaining patients, one had a poorly differentiated squamous cell lung carcinoma and the remaining patient had had a previous adenocarcinoma but had been disease-free for over 2 years. The latter patient was classified as falling into the ‘no carcinoma’ group in this study (Table 4).

Fifty-seven patients in the study were shown to show clinical evidence of lung cancer, based on cytology, radiology, or histology reports. Fifty-five (96%) of these 57 patients demonstrated hnRNP overexpression with the 703D4 antibody. Forty-six patients were found to have no clinical evidence of lung cancer and 8 (17%) of 46 of these individuals demonstrated hnRNP overexpression. The sensitivity and specificity calculations are based on the current follow-up data, but four of these 8 individuals have been followed-up only for an average of 20 months. The remaining four patients have been followed for 36–42 months.

The sensitivity of 703D4 positive staining was found to be 96%, whereas the specificity was 82%, positive predictive value was 87%, false negative rate was 3.5%, false positive rate was 8.6%, and negative predictive value was 95% (Table 5).

**DISCUSSION**

There is a need to develop molecular and cellular markers for early detection of lung cancer at a stage at which the disease...
is potentially reversible. It is most probable that a set of biomarkers will be required to achieve this objective; however, potential molecular and cellular biomarkers have to be assessed in high-risk populations to justify their inclusion in large population-based validation studies.

The utility of overexpression of hnRNP has been studied in one prospective and two ongoing retrospective studies (12). It has been demonstrated that hnRNP overexpression may be used to detect lung cancer before any clinical diagnosis, and that it is superior to routine cytology and radiology. Indeed, the predictive power of hnRNP overexpression in sputum specimens from the two prospective studies [i.e., monitoring for second primary lung cancers (12) and screening for lung cancer in the Yunnan China Tin miners (9, 10)] was found to be 67 and 69%, respectively. In the current study of 103 individuals referred to a lung cancer clinic in the northwest of the United Kingdom for a full clinical work up for ‘query lung cancer’, hnRNP overexpression was found in 22 (95%) of 23 of the BL specimens from patients shown to have a malignancy from their original cytology report. Forty-one patients had hnRNP overexpression in metaplastic bronchial epithelial cells, but the cytology report indicated NMCS. On follow-up, 29 of these 41 patients were found to have a lung neoplasm based on radiological findings and/or the biopsy taken at the bronchoscopy. An additional four patients were diagnosed within 8 months. (Tables 2 and 3).

Table 3  Clinical details of patients with positive 703D4 staining but negative cytology

<table>
<thead>
<tr>
<th>Clinical details</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No carcinoma lung</td>
<td>4</td>
<td>37</td>
<td>41</td>
</tr>
<tr>
<td>Carcinoma lung RCO</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous carcinoma</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>6</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous lung carcinoma (negative on follow up)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous carcinoma site elsewhere (negative for lung cancer)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* RCO, radiological confirmation only.

Table 4  703D4 staining results for patients without cytological evidence of lung cancer

<table>
<thead>
<tr>
<th>Clinical details</th>
<th>703D4-positive</th>
<th>703D4-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No carcinoma lung</td>
<td>4</td>
<td>37</td>
<td>41</td>
</tr>
<tr>
<td>Carcinoma lung</td>
<td>33</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>Previous carcinoma lung</td>
<td>2</td>
<td>1*</td>
<td>3</td>
</tr>
<tr>
<td>Previous carcinoma elsewhere</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>39*</td>
<td>80</td>
</tr>
</tbody>
</table>

* Previous cancer of the lung <6 years ago, this patient is classified as negative for lung cancer, thus giving 38 703D4 cancer-negative specimens.

The eight remaining patients who overexpressed hnRNP but had no evidence of lung cancer require further discussion: two individuals had a previous lung cancer, the BL samples being taken 6 months and 1 year after resection of the primary cancer. The patient with the 6-month postresection sample developed an adenocarcinoma of the prostate 9 months after the collection of the BL specimen. The individual with the BL taken one-year postresection has had no evidence to date of lung cancer. Indeed, two of eight patients with positive hnRNP expression who had previous carcinomas of the breast and bowel may be considered to be at risk of developing lung cancer but currently have no clinical evidence of disease. The remaining four patients in this group of eight with hnRNP overexpression have now been followed up for an average of 20 months and show no evidence of cancer. Thus, in the calculation of sensitivity and specificity of hnRNP expression, these eight individuals are considered as false positives. However, a longer follow-up may indicate that some of these individuals do develop lung cancer.

The findings of this study indicate immunostaining with an antibody to hnRNP gives a 96% sensitivity and 82% specificity, levels greater than found previously by Tockman et al. (12). Furthermore, the predictive value of 87% with a 3.5% false negative rate indicate that this marker should be considered a strong contender in planning population-based early detection studies in lung cancer.

In this study, we scored hnRNP expression visually by two independent cytologists, and, because there was no background staining, the positive staining slides were equivocal. This investigation was based on BL specimens, whereas Tockman et al. (12) have used induced sputum for their analysis. It has to be noted that there are less squamous epithelial cells in BL compared with that of sputum, thus the reason for setting the minimum number of metaplastic or tumor cells to three in our analysis. The selection of patients for this study was based on individuals referred to a rapid-access lung cancer clinic, whereas the three patient groups of Tockman and coworkers—a National Cancer Institute collaborative early-lung-cancer detection trial at The John Hopkins University (JHLP; Ref. 6), Yunnan tin miners in China (10), and stage-1 resected non-small cell lung cancer patients (12)—all have had different referral or inclusion criteria.

The results of this study further support the hypothesis that hnRNP overexpression may be considered a potential early detection marker. Future population-based studies will have to determine the most appropriate panel of biomarkers for inclusion in risk model assessment. To date, the forerunners in early lung cancer molecular diagnostics, apart from hnRNP, are ras mutations (13, 14), p53 mutation/expression (15, 16), and genomic instability (17–21). The developing bio-chip technologies will allow a much greater range and number of putative markers to be considered for this important diagnostic tool.
genes involved in carcinogenesis to be assessed. However, although this technology will provide a wealth of information, the interpretation of results will be complex and will require a level of informatics that has not as yet been developed and tested. Thus, the potential power of markers that we have available today, such as hnRNP, should be used in the determination of high-risk individuals.

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