Heterogeneous Nuclear Ribonucleoprotein B1 as Early Cancer Biomarker for Occult Cancer of Human Lungs and Bronchial Dysplasia

Eisaburo Sueoka, Naoko Sueoka, Yuri Goto, Satoru Matsuyama, Hitoshi Nishimura, Masami Sato, Shigefumi Fujimura, Hiroshige Chiba, and Hirotaka Fujiki

Saitama Cancer Center Research Institute [E. S., N. S., Y. G., S. M., H. F.] and Saitama Cancer Center Hospital [H. N.], Saitama 362-0806; Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Toho University, Sendai 980-8575 [M. S., S. F.]; and Department of Oral and Maxillofacial Surgery, Tokyo Medical University, Shinjuku, Tokyo 160-8402 [Y. G., H. C.], Japan

ABSTRACT

Heterogeneous nuclear ribonucleoprotein (hnRNP) B1 is a RNA-binding protein of Mr, 37,000. We previously reported that hnRNP B1 was specifically overexpressed in the nuclei of human lung cancer cells, particularly in squamous cell carcinoma (E. Sueoka et al., Cancer Res., 59: 1404–1407, 1999). We extended this study to determine whether hnRNP B1 was overexpressed in roentgenographically occult cancers of the lungs and premalignant lesions of squamous cell carcinomas, such as bronchial dysplasia. The additional object of our study was to examine the usefulness of hnRNP B1 as a potential diagnostic marker for squamous cell carcinoma of various organs, such as the oral cavity and esophagus in humans. Surgically resected specimens of bronchial dysplasia, lung cancers, and various human squamous cell carcinomas, collected at two hospitals in Japan, were subjected to immunohistochemical staining with anti-hnRNP B1 antibody. Overexpression of hnRNP B1 protein was observed in 100% of stage I lung cancer tissues, but it was not found in normal bronchial epithelium. Squamous cell carcinoma of the lungs showed stronger staining than other histological types, and elevation of hnRNP B1 was found in both roentgenographically occult lung cancers and bronchial dysplasia. Furthermore, cytological examination with anti-hnRNP B1 antibody detected cancer cells in sputum, suggesting the potential of hnRNP B1 protein as a new biomarker for the very early stage of lung cancer in humans. Because strong staining of hnRNP B1 was also observed in various squamous cell carcinomas of oral and esophageal tissues as shown in our recent reports, overexpression of hnRNP B1 seems to be a common event in the carcinogenic processes of squamous cell carcinoma. These results suggest that hnRNP B1 protein could be a useful diagnostic biomarker for both the very early stages of lung cancer and various squamous cell carcinomas in humans.

INTRODUCTION

In Japan, lung cancer is the leading cause of cancer-related death among males (21.4%) and the second leading cause among females (12.3%). Frequency of death from the disease has increased remarkably (1), with 50,000 people dying of lung cancer in 1998 (2). In an effort to combat this, technology of roentgenographic examinations, such as helical computed tomography and magnetic resonance imaging, has progressed enormously, and the population-based mass screening test for lung cancer detection using sputum cytology and chest X-ray examination is now widely distributed in Japan. Even with these, however, the overall survival rate of lung cancer is still only 40% 5 years after diagnosis (2). In addition, patients who are treated for a primary cancer are likely to experience recurrence or development of a second primary cancer during the long life span (3). Thus, to extend survival of patients, early detection of lung cancer is urgently needed, in light of evidence that the 5-year survival of stage I patients is over 80% (1). Conventional cytological diagnosis of sputum is not sufficient for clinical use because of its lack of efficacy (4): what is required is a new molecular diagnostic marker visualizing the early events of lung carcinogenesis in humans. In this study, we considered how the early events of lung cancer, such as roentgenographically occult cancer or bronchial dysplasia, might be diagnosed for improvement in lung cancer survival. If a new molecular diagnostic marker could reliably detect both occult cancer and dysplasia, the underlying molecular mechanisms of that marker would certainly be worthy of investigation.

In 1988, Mulshine’s group (5) raised lung cancer-specific antibodies against extracts of human lung cancer cell line by mouse immunization. One antibody, 703D4, recognized tumor cells in all examined squamous cell carcinomas of the lungs and premalignant lesions of squamous cell carcinomas, collected at two hospitals in Japan, were subjected to immunohistochemical staining with anti-hnRNP B1 antibody. Overexpression of hnRNP B1 protein was observed in 100% of stage I lung cancer tissues, but it was not found in normal bronchial epithelium. Squamous cell carcinoma of the lungs showed stronger staining than other histological types, and elevation of hnRNP B1 was found in both roentgenographically occult lung cancers and bronchial dysplasia. Furthermore, cytological examination with anti-hnRNP B1 antibody detected cancer cells in sputum, suggesting the potential of hnRNP B1 protein as a new biomarker for the very early stage of lung cancer in humans. Because strong staining of hnRNP B1 was also observed in various squamous cell carcinomas of oral and esophageal tissues as shown in our recent reports, overexpression of hnRNP B1 seems to be a common event in the carcinogenic processes of squamous cell carcinoma. These results suggest that hnRNP B1 protein could be a useful diagnostic biomarker for both the very early stages of lung cancer and various squamous cell carcinomas in humans.

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2 To whom requests for reprints should be addressed, at Saitama Cancer Center, Ina, Kitaadachi-gun, Saitama 362-0806, Japan. Phone: 81-48-722-1111, ext. 4611; Fax: 81-48-722-1739; E-mail: hfujiki@cancer-c.pref.saitama.jp.

3 The abbreviation used is: hnRNP, heterogeneous nuclear ribonucleoprotein.
oral tissue and the esophagus (12, 13). Here, we report the usefulness of hnRNP B1 as a potential biomarker for early detection of various human cancers and we discuss the molecular significance of dysplasia in cancer diagnosis.

**MATERIALS AND METHODS**

**Tissue Samples.** Advanced lung cancer tissues were obtained from patients who had undergone surgery at Saitama Center Hospital. Roentgenographically occult lung cancer and dysplastic lesion of the lung were surgically resected or obtained by transbronchial lung biopsy from patients at the Institute of Development, Aging and Cancer Hospital, Tohoku University. Sputum samples were obtained from a well-organized population-based lung cancer screening center in Miyagi Prefecture, Japan. Resected surgical specimens were fixed by formalin and then embedded in paraffin. Histological diagnosis and clinical stages were determined by the criteria of WHO and the International Union Against Cancer. Informed consent was obtained from all patients.

**Immunohistochemical Staining with Anti-hnRNP B1 Antibody.** Two sets of 5 μm-thin tissue sections (one set from the center of the tumor, and the other from the margin) were subjected to immunohistochemical staining with anti-hnRNP B1 antibody by the standard method as reported previously (11). In brief, deparaffinized 5 μm-thin tissue sections were put in a microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for two periods of 5 min each. The tissue sections were then treated with anti-hnRNP B1 antibody at a dilution of 1:200 overnight at 10°C, followed by visualization with DAKO ENVISSION system (DAKO Corp., Carpinteria, CA). Counter-staining was performed with hematoxylin. Immunohistochemical examination of staining with anti-hnRNP B1 antibody was evaluated by the following criteria: staining of over 50% of cancer cell nuclei in a whole tissue section, with no remarkable staining observed in parenchymal cells, was taken as positive. Positive staining was confirmed by two different tissue sections obtained from one patient, and the intensity of staining was determined by three investigators (E. S., N. S., and Y. G.).

**Sputum Cytology Using Anti-hnRNP B1 Antibody.** Samples for sputum cytology were obtained from a population-based lung cancer screening in Miyagi Prefecture, Japan. Sputum samples were subjected to the modified Saccomanno method (fixed by 2% polyethylene glycol and 50% ethanol). Before immunocytochemistry, the samples were treated with 100% methanol for 30 min, followed by 4% paraformaldehyde with 0.2% Triton X-100 treatment for 10 min. The samples were then heated in microwave oven (750 W) for two periods of 5 min each. After blocking endogenous peroxidase with 0.3% hydrogen peroxide, anti-hnRNP B1 antibody was evaluated by the following criteria: staining of over 50% of cell nuclei in a whole tissue section, with no remarkable staining observed in parenchymal cells, was taken as positive. Positive staining was confirmed by two different tissue sections obtained from one patient, and the intensity of staining was determined by three investigators (E. S., N. S., and Y. G.).

**Statistical Analysis.** The association between the positivity of immunostaining and clinicopathological parameters was examined by use of the χ² test for contingency tables, taking P < 0.05 as the criterion of significance.

**RESULTS**

**Overexpression of hnRNP B1 in Human Lung Cancers.** We recently reported that hnRNP B1 was significantly overexpressed in nuclei of human lung cancer cells (11). To investigate the significance of hnRNP B1 as an early detection marker for lung cancer, we next determined expression levels of hnRNP B1 in various clinical features of 43 patients, classified by histological type and clinical stage (Table 1). The 43 lung cancers included 15 squamous cell carcinomas, 24 adenocarcinomas, 3 large cell carcinomas, and 1 small cell carcinoma; the patients were 14 stage I, 11 stage II, 16 stage III, and 2 stage IV. Strength of staining with anti-hnRNP B1 antibody was divided into four groups (negative, weak, moderate, and strong) and compared with the histological and clinical types mentioned above.

As shown in Table 1, all four histological types of lung cancer tissues showed positive staining with anti-hnRNP B1 antibody, ranging from 70.8–100% (Table 1 and Fig. 1). The strength of staining for squamous cell carcinoma was 66.7% moderate and 26.7% strong staining; that of adenocarcinoma was 29.1% negative, 16.7% weak, and 54.2% moderate staining. As for control, normal bronchial epithelial cells and alveolar epithelial cells of adjacent noncancerous tissue did not show any positive staining with anti-hnRNP B1 antibody (Fig. 1C). In data not shown, the lung of bacterial pneumonia and interstitial pneumonia did not express hnRNP B1 protein, indicating that hnRNP B1 expression is not associated with inflammatory reactions.

**Elevated Expression of hnRNP B1 in Roentgenographically Occult Cancer.** Next, we determined the expression level of hnRNP B1 protein in 43 roentgenographically occult cancers that were not detected by roentgenographic diagnosis, such as chest X-ray or computed tomography. The clinical features of the cancer patients, such as invasive grade, mean age, mean smoking index, pathological diagnosis, and positive staining of hnRNP B1 in the tissues, are summarized in Table 2. Invasive grade of the occult cancers was divided into three types: carcinoma in situ (11 cases), intrabronchial invasion (14 cases), and extrabronchial invasion (18 cases). Pathological diagnosis revealed that all 43 occult cancers were squamous cell carcinomas.

Fig. 2A shows a representative occult cancer, with a very small lesion (<5 mm in diameter) of early lung cancer tissue. Of the 43 occult cancer patients, 58.1% showed positive staining of hnRNP B1: intra- and extrabronchial invasions showed 71.4% and 61.1% positivity, respectively, considerably higher than carcinoma in situ, 36.4% (Table 2). The results suggest that overexpression of hnRNP B1

![Table 1: Positivity of immunohistochemical staining with anti-hnRNP B1 antibody in lung cancer patients](https://example.com/table1.png)

- **No.** Number of patients with tumor of given cell type or stage.
- **Positive** was defined as nuclear staining observed in over 50% of >300 cells examined for each case.
begins in the early stage of the lung cancer and is also associated with cancer progression.

**Elevated Expression of hnRNP B1 in Bronchial Dysplasia.** Eleven patients with bronchial dysplasia were diagnosed by transbronchial lung biopsy. The clinical features of these patients were then summarized by age, smoking index, pathological diagnosis, and positive staining of hnRNP B1 in the lesions (Table 3). The mean age was 60 years. Pathological diagnosis of all dysplasia showed three mild, three moderate, and five severe dysplasias.

Fig. 2B shows a representative bronchial dysplasia: positive hnRNP B1 staining for dysplasia patients was 63.6% in total (100% (three of three) for mild, 33.3% (one of three) for moderate, and 60.0% (three of five) for severe dysplasia); adjacent noncancerous tissue did not show any positive staining. Whether positively stained bronchial
Fig. 2. Overexpression of hnRNP B1 in roentgenographically occult cancer and bronchial dysplasia. Immunohistochemical staining of roentgenographically occult cancer (A) and bronchial dysplasia (B) was performed. The hnRNP B1 protein was overexpressed in nuclei of cancer cells and bronchial dysplasia (arrowhead), but not in those of normal bronchial epithelial cell adjacent to dysplasia. C: H&E staining of the same case in B.
dysplasia and nonstained bronchial dysplasia represent different carcinogenic processes remains to be investigated. However, the results suggest the presence of qualitative differences in the carcinogenesis of bronchial dysplasia. For population-based lung cancer screening approaches, anti-hnRNP B1 antibody staining will provide useful diagnostic information for early lesions of human lung cancer.

Detection of Tumor Cells by Sputum Cytology Using Anti-hnRNP B1 Antibody.
To confirm the usefulness of hnRNP B1 in early diagnosis of lung cancer, we applied anti-hnRNP B1 antibody in sputum cytology, first developing the optimum condition of immunocytochemistry for sputum staining with anti-hnRNP B1 antibody. Fig. 3 shows positive staining of adenocarcinoma cells contained in sputum samples obtained from an adenocarcinoma patient: no positive staining was observed in normal bronchial epithelial cells or oral squamous cells contained in sputum samples. Until now, we have analyzed the sputum samples of 12 lung cancer cases. Three of 12 samples that were diagnosed as class V by cytology showed cancer cells stained positively, whereas the other 9 samples diagnosed class I or II were negative in immunostaining.

DISCUSSION
Early detection of lung cancer is the key to long-term survival: the 5-year survival rate of patients diagnosed at the microscopic stage of cancer is estimated at over 90%. Early diagnosis of lung cancer patients, however, has remained an elusive goal, with <1% of the cases being so diagnosed (1–3). Therefore, the development of a new diagnostic method specifically for the very early stage of lung cancer is urgently required. We recently demonstrated that hnRNP B1 is overexpressed in nuclei of lung cancer cells, but not in normal bronchial cells or alveolar cells (11). In this study, we confirmed that 100% of lung cancer tissue at clinical stage I was positively stained with anti-hnRNP B1 antibody. Here, we found that 58.1% of roentgenographically occult lung squamous cell carcinomas and 63.6% of bronchial dysplasias were positive in staining with anti-hnRNP B1 antibody. These results strongly suggested that overexpression of hnRNP B1 protein is a very early event in lung carcinogenesis, one that may be an early biomarker for detection of premalignant lesions of the lungs as well as the very early stages of various squamous cell carcinomas.

Our results suggested that overexpression of hnRNP B1 representing cell proliferation appears in very early lesions of lung cancers. Still to be determined are the mechanisms of hnRNP B1 overexpression in lung cancer tissue, or its relationship with other molecular markers that are early events in lung carcinogenesis including mutation of p53, loss of p16 expression, expression of telomerase, and loss of heterozygosity of 3p, 9p or 17p (14–18), we assumed that hnRNP B1 overexpression was partly related to cell cycle regulation, based on our previous evidence that hnRNP B1 expression is associated with rapid cell growth of lung cancer cell lines (11). The expression of hnRNP B1 is thought to be up-regulated by both transcriptional and posttranscriptional mechanisms in lung cancer tissue (11), and Michelson et al. (19) recently found that nuclear DEAF-1-related protein NUDR binds to hnRNP A2/B1 promoter and regulates its promoter activity. However, our previous results presented that the elevated expression of the *hnRNP B1* gene and protein in lung cancer cell line and cancer tissue was more apparent than that of the *hnRNP A2* gene.

Table 2 Characteristics of clinical features of roentgenographically occult cancers

<table>
<thead>
<tr>
<th>Invasive grade</th>
<th>No.</th>
<th>Pathological diagnosis</th>
<th>No. of positive staining/total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma in situ</td>
<td>11</td>
<td>Sq*</td>
<td>4/11 (36.4)</td>
</tr>
<tr>
<td>Intrabronchial invasion</td>
<td>14</td>
<td>Sq</td>
<td>10/14 (71.4)</td>
</tr>
<tr>
<td>Extrabronchial invasion</td>
<td>18</td>
<td>Sq</td>
<td>11/18 (61.1)</td>
</tr>
<tr>
<td>Total number</td>
<td>43</td>
<td>Sq</td>
<td>25/43 (58.1)</td>
</tr>
</tbody>
</table>
* Number of patients with tumor of given cell type or stage.
Sq, squamous cell carcinoma.

Table 3 Characteristics of clinical features of bronchial dysplasia patients

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Smoking index</th>
<th>Pathological diagnosis</th>
<th>Immunoreactivity of hnRNP B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>1400</td>
<td>Mild D*</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>600</td>
<td>Mild D</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>2000</td>
<td>Mild D</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>800</td>
<td>Moderate D</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>1500</td>
<td>Moderate D</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>740</td>
<td>Moderate D</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
<td>900</td>
<td>Severe D</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>1000</td>
<td>Severe D</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>63</td>
<td>600</td>
<td>Severe D</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>1200</td>
<td>Severe D</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>56</td>
<td>800</td>
<td>Severe D</td>
<td>Positive</td>
</tr>
</tbody>
</table>
* D, dysplasia.

Fig. 3. Detection of lung cancer cells in sputum by immunocytochemistry of hnRNP B1. Immunocytochemistry of sputum sample was performed using anti-hnRNP B1 antibody. Positive staining of hnRNP B1 was found only in cancer cells.
primary cancer, but also in recurrent and second primary cancers in humans.

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