Heterogeneous Nuclear Ribonucleoprotein B1 as a New Marker of Early Detection for Human Lung Cancers

Eisaburo Sueoka, Yuri Goto, Naoko Sueoka, Yasuko Kai, Tomoko Kozu, and Hirotu Fujiki

Saitama Cancer Center Research Institute, Ina, Kitadachi-gun, Saitama 362-0806, Japan [E. S., Y. G., Y. K., T. K., H. F.], and University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [N. S.]

Abstract

Heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 is an RNA binding protein that is required for maturation of mRNA precursor. Tockman et al. previously reported that hnRNP A2/B1 with a Mr of 31,000 is overexpressed from the early clinical stage of human lung cancer (M. S. Tockman et al., J. Clin. Oncol., 6: 1685–1693, 1988). However, when hnRNP A2/B1 mRNA and hnRNP B1 mRNA were separately studied, we found unique evidence that hnRNP B1 mRNA, which is a splicing variant of hnRNPA2 mRNA, was more significantly elevated in lung cancer tissues than hnRNP A2/B1 mRNA. Our hnRNP B1-specific polyclonal antibody specifically recognized hnRNP B1 protein as a Mr of 37,000, not hnRNP A2 protein. Immunohistochemical staining with the hnRNP B1 antibody revealed that hnRNP B1 protein was specifically stained in the nuclei of human cancer cells, and in squamous cell carcinomas in particular, but not in those of normal adjacent lung epithelial cells. We think that hnRNP B1 protein of Mr 37,000, not hnRNP A2, is well qualified as a biomarker for the detection of human lung cancer.

Introduction

In Japan, lung cancer is the leading cause of death by cancer among males (20.9%) and the second leading cause among females (11.9%) (1). The overall survival rate is only 40% at 5 years, mainly because of the recurrence of the disease or the occurrence of second-primary cancers, even among patients with completely resected cancers (2). For early clinical diagnosis of lung cancer, various tumor markers have been investigated, such as pro-gastrin-releasing peptide and neuron specific enolase for small cell lung cancer, and carci-noembryonic antigen, SCC (3), antigen, for non-small cell lung cancer (3–6). However, a biomarker that can reliably detect lung cancer in its very early stage has not yet been confirmed.

In 1988, Tockman et al. first reported that a lung cancer-specific monoclonal antibody, 703 D, recognized human hnRNP A2/B1 protein with a Mr of 31,000, and that hnRNP A2/B1 protein was frequently overexpressed in the early clinical stage of primary non-small cell lung cancer (7). In addition, they reported that hnRNP A2/B1 was strongly expressed in bronchial epithelial cells by sputum cytology before x-ray imaging diagnosis of lung cancer (7–10). These results indicated that hnRNP A2/B1 protein could be a useful tool for the early detection of human lung cancer.

hnRNP A2/B1 protein is a major component of the hnRNP core complex in mammalian cell nuclei. Although the function of hnRNP A2/B1 has not yet been fully elucidated, recent studies have revealed that hnRNP A2/B1 is involved in RNA splicing in nuclei as well as in mRNA transport from nucleus to cytoplasm (11, 12). One of our authors (T. K.) previously elucidated (13) a complete sequence of human hnRNP A2/B1 gene. She found that hnRNP B1 mRNA is a splicing variant of hnRNP A2 mRNA and that hnRNP B1 protein contains an additional 12 amino acids that hnRNP A2 protein does not, with predicted Mr of 37,000 for hnRNP B1 protein and 34,000 for hnRNP A2 protein. On the basis of evidence that hnRNP B1 mRNA constitutes 2–5% of hnRNP A2/B1 mRNA (13), we studied expression of hnRNP A2/B1 mRNA and hnRNP B1 mRNA separately. We found that hnRNP B1 mRNA is more specifically elevated in human lung cancer cells than hnRNP A2/B1 mRNA. We further raised a hnRNP B1-specific antibody and used it to develop a specific and sensitive immunohistochemical method for the detection of human lung cancer. This is the first report that hnRNP B1 is well qualified as a new tumor marker for human lung cancer, which suggests that our anti-hnRNP B1-specific antibody will be useful for the early detection of lung cancer.

Materials and Methods

Human Lung Cancers and Cell Lines. All lung cancers used for this study were taken from patients who were subjected to surgery during the year 1997 at Saitama Cancer Center Hospital. Resected cancerous and noncancerous tissues were separately frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed. Formalin-fixed and paraffin-embedded tissues were used for subsequent immunohistochemical analysis. The following human lung cancer cell lines were used: (a) A549 (adenocarcinoma) from RIKEN Cell Bank, Japan; and (b) H226B (SCC), SK-MES (SCC), H596 (adenosquamous cell carcinoma) and ChaGo K-1 (SCC) from Dr. Jonathan Kurie at University of Texas M. D. Anderson Cancer Center, Houston, TX.

RT-PCR. Total RNA was isolated from cancerous and noncancerous tissues by the acid guanidinium/phenol/chloroform extraction method as reported previously (14). One μg total RNA was applied to RT with MuLV reverse transcriptase (Roche Molecular Systems, New Jersey) at 37°C for 60 min. Obtained cDNAs (1 μg) were amplified using hnRNP A2/B1 and hnRNP B1 specific primers in the following conditions: (a) initial denaturation at 95°C for 5 min; and (b) 25-cycle amplification for hnRNP A2/B1 or 28-cycle for hnRNP B1. The PCR products were subjected to 5% PAGE in 0.5X Tris-boric acid-EDTA buffer. Radioactivity of PCR products was determined by BAS 2000 Bioimage analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan). After normalizing by an amount of β-actin mRNA as a control, the expression of hnRNP A2/B1 mRNA and hnRNP B1 mRNA in cancerous tissue was compared with that in adjacent noncancerous tissue. The results were obtained in duplicate assays.

Preparation of Anti-hnRNP A2/B1 and Anti-hnRNP B1 Antibodies. Anti-hnRNP A2/B1 and anti-hnRNP B1 antibodies were produced in rabbit using 18-mer synthetic peptide (amino acid residues 194–210 + cysteine) for hnRNP A2/B1 protein and 19-mer synthetic peptide (amino acid residues 1404-1407, April 1, 1999)
3–20 + cysteine) for hnRNP B1 protein. The immunized sera were affinity-purified by the antigen and then purified by Mono-Q column chromatography.

Western Blot Analysis and Immunohistochemistry. Human lung cancer cell lines were maintained in RPMI 1640 supplemented with 10% FCS. For the preparation of cytosolic and nuclear fractions, cells were lysed in 2-(N-morpholino)-ethanesulfonic acid buffer (pH 7.0) containing 17 mM 2-(N-morpholino)-ethanesulfonic acid, 20 mM EDTA, 250 mM sucrose, 50 μg/ml leupeptin, 300 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride. The supernatant was obtained as cytosolic fraction by centrifugation at 100,000 g for 60 min at 4°C. Nuclei were sonicated in buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Tween 20, 50 μg/ml leupeptin, 300 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride and centrifuged at 15,000 g for 20 min at 4°C. The supernatant was used as nuclear fraction. The cytosolic and nuclear fractions were subjected to SDS-PAGE. Western blotting was performed using anti-hnRNP A2/B1 and anti-hnRNP B1 antibodies, and hnRNP A2/B1 protein was visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Immunohistochemical staining was performed by the standard method as reported previously (15). In brief, a deparaffinized 5-μm-thin tissue section was heated by microwave for 5 min, twice, and then treated with anti-hnRNP A2/B1 and anti-hnRNP B1 antibodies overnight at 10°C after visualization with DAKO ENVISSION system (DAKO Co. Carpinteria, CA). Immunohistochemical examination was conducted by two independent investigators (E. S. and Y. G.).

Results and Discussion

Elevation of hnRNP B1 mRNA in Human Lung Cancer Tissue.

We first determined the level of both hnRNP A2/B1 mRNA and hnRNP B1 mRNA in the total RNA isolated from cancerous (T) and 3-20 + cysteine) for hnRNP B1 protein. The immunized sera were affinity-purified by the antigen and then purified by Mono-Q column chromatography.

Western Blot Analysis and Immunohistochemistry. Human lung cancer cell lines were maintained in RPMI 1640 supplemented with 10% FCS. For the preparation of cytosolic and nuclear fractions, cells were lysed in 2-(N-morpholino)-ethanesulfonic acid buffer (pH 7.0) containing 17 mM 2-(N-morpholino)-ethanesulfonic acid, 20 mM EDTA, 250 mM sucrose, 50 μg/ml leupeptin, 300 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride. The supernatant was obtained as cytosolic fraction by centrifugation at 100,000 g for 60 min at 4°C. Nuclei were sonicated in buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Tween 20, 50 μg/ml leupeptin, 300 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride and centrifuged at 15,000 g for 20 min at 4°C. The supernatant was used as nuclear fraction. The cytosolic and nuclear fractions were subjected to SDS-PAGE. Western blotting was performed using anti-hnRNP A2/B1 and anti-hnRNP B1 antibodies, and hnRNP A2/B1 protein was visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Immunohistochemical staining was performed by the standard method as reported previously (15). In brief, a deparaffinized 5-μm-thin tissue section was heated by microwave for 5 min, twice, and then treated with anti-hnRNP A2/B1 and anti-hnRNP B1 antibodies overnight at 10°C after visualization with DAKO ENVISSION system (DAKO Co. Carpinteria, CA). Immunohistochemical examination was conducted by two independent investigators (E. S. and Y. G.).

Results and Discussion

Elevation of hnRNP B1 mRNA in Human Lung Cancer Tissue.

We first determined the level of both hnRNP A2/B1 mRNA and hnRNP B1 mRNA in the total RNA isolated from cancerous (T) and
noncancerous tissue (N) of six patients by RT-PCR using their specific primers. As Fig. 1A shows, hnRNP A2/B1 mRNA was equally expressed in the cancerous and noncancerous tissues of the six patients. However, we found that the expression of hnRNP B1 mRNA was specifically elevated in cancerous tissues of all six of the patients compared with the levels in noncancerous tissues. The enhancements were from 1.5- to 4-fold (Fig. 1B). When the hnRNP mRNA levels were normalized by β-actin mRNA, hnRNP B1 mRNA was elevated 0.5–2.5 times that of hnRNP A2/B1 mRNA in cancerous tissues of the six patients.

Zhou et al. (9) had also reported that hnRNP A2/B1 mRNA was overexpressed in human lung cancer cells. However, we demonstrate here the first evidence that hnRNP B1 mRNA, not hnRNP A2/B1 mRNA, is specifically elevated in human lung cancer compared with its levels in noncancerous adjacent tissue. Because hnRNP B1 mRNA makes up 2–5% of hnRNP A2/B1 mRNA, our discovery of the elevated expression of hnRNP B1 mRNA is an essential confirmation of the finding of Tockman’s group.

**Immunohistochemical Detection of hnRNP A2/B1 and hnRNP B1 Proteins.** To detect the expression of both hnRNP A2/B1 and hnRNP B1 proteins in human lung cancer cells, we used anti-hnRNP A2/B1 and anti-hnRNP B1 polyclonal antibodies. Anti-hnRNP A2/B1 antibody recognized two protein bands with Mr of 37,000 and 34,000 in the nuclear fraction of adenocarcinoma cell line A549 cells but not in the cytosolic fraction. The molar ratio of Mr 37,000 and 34,000 proteins is comparable to that of hnRNP B1 mRNA to hnRNP A2/B1 mRNA.
mRNA. Because anti-hnRNP B1 antibody significantly detected a single Mr 37,000 protein in the nuclear fraction (Fig. 2A), we concluded that the Mr 37,000 protein is hnRNP B1 and the Mr 34,000 protein is hnRNP A2. Preimmune normal rabbit serum did not react with these two proteins.

It was reported that hnRNP A2/B1 protein was overexpressed in human lung cancer cells (7–10). We analyzed the expression of hnRNP B1 protein in various human lung cancer cell lines, and the results with five human lung cancer cell lines examined (one adenocarcinoma, one adenosquamous cell carcinoma, and three squamous cell carcinomas) showed that the expression levels of hnRNP B1 protein were varied (Fig. 2B). Table 1 shows that expression of hnRNP B1 mRNA and that of the protein are relatively well correlated among all of the cell lines except SK-MES. To determine the consequence of the expression of hnRNP B1, we studied the doubling time of these cell lines (cell growth rate). As Table 1 shows, rapid growth cell lines, such as A549 and H226B, expressed high amounts of hnRNP B1 protein, whereas slow growth cell lines, such as H596 or ChaGo K-1, expressed smaller amounts. hnRNP A2/B1 protein was originally assumed to be involved in RNA splicing, mRNA transport, and transcriptional regulation (10,11,16). Furthermore, recent study has revealed that hnRNP A2/B1 protein binds to a telomeric DNA sequence (17,18). The question of how overexpression of hnRNP B1 protein in cancer cells influence dysregulation of cellular functions remains to be further investigated.

**Overexpression of hnRNP B1 Protein in Cancer Cells but not in Normal Epithelial Cells of Human Lung.** Primary cancer and non-cancerous adjacent tissues obtained from 43 lung cancer patients were subjected to immunohistochemical examination with anti-hnRNP A2/B1 and anti-hnRNP B1 antibodies. Fig. 3 shows that hnRNP B1 expression was detected in nuclei of the cancer cells only and not in nuclei of normal adjacent tissue, such as normal bronchial epithelial cells and alveolar epithelial cells (Fig. 3, A and C).

Next, we analyzed the specificity and sensitivity of anti-hnRNP B1 antibody for detection of lung cancer cells, compared with the results using the anti-hnRNP A2/B1 antibody. The anti-hnRNP A2/B1 antibody showed positive tissue response in 22 (55%) of 43 total lung cancer patients and 8 (53%) of 15 SCC patients. Significantly, overexpression of hnRNP B1 protein stained with anti-hnRNP B1 antibody was observed in 32 (74%) of the 43 cancer patients, including in a unique finding, all of the SCC patients. Furthermore, 12 of 43 were stage I cancer patients and in every single case showed the cancer tissue-positive staining with hnRNP B1 antibody.

Considered together, our results indicate that hnRNP B1 protein is overexpressed in the early stage of human lung cancer development and is, therefore, a useful marker for early detection of human lung cancer, especially for SCC. Thus, we think that hnRNP B1 protein is a more specific and sensitive marker for lung cancer than hnRNP A2/B1 protein. It is still unclear how hnRNP B1 protein was recognized by our assay system to be overexpressed compared with hnRNP A2 protein, inasmuch as hnRNP B1 protein is identical to hnRNP A2 protein except for the 12 additional amino acids in hnRNP B1 (13). It was reported previously (19) that hnRNP is a complex of about 30 major proteins with Mr ranging from 30,000 to 120,000, and that these proteins are phosphorylated by casein kinase 2 and bind to calmodulin (20). hnRNP B1 protein is more basic than hnRNP A2 protein (20) because its NH2-terminal additional peptide contains a cluster of basic amino acids. For this reason, we think that hnRNP B1 protein can be more easily recognized by its antibody than hnRNP A2 protein. It is now anticipated that anti-hnRNP B1 antibody will be used in the examination of sputum specimens during the screening of the general population for lung cancer.

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**References**


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