Heterogeneous Nuclear Ribonucleoprotein B1 Expressed in Esophageal Squamous Cell Carcinomas as a New Biomarker for Diagnosis

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We recently reported that heterogeneous nuclear ribonucleoprotein (hnRNP) B1 was overexpressed in most human lung cancers, especially squamous cell carcinoma (SCC), as well as human oral SCC. To find the significance of hnRNP B1 in cancer diagnosis, we studied hnRNP B1 expression in 16 paraffinized sections of esophageal SCC, using immunohistochemical staining with anti-hnRNP B1 polyclonal antibody, raised in a rabbit. We compared the expression of hnRNP B1 in cancerous and noncancerous regions of the same specimen: enhanced expression was observed in 63% of cancerous regions (10/16), whereas none of the noncancerous regions showed enhanced expression. The enhanced expression of hnRNP B1 in cancerous regions was compared with that in noncancerous tissue in relation to histopathological grade: 83% for well differentiated (5/6), 83% for moderately differentiated (5/6) and 0% for poorly differentiated (0/4). Histologically, enhanced expression of hnRNP B1 was observed around cancer pearls, as well as in the cells of nests lacking keratinization in well and moderately differentiated SCC. Western blotting analysis revealed enhanced expression in three frozen specimens of moderately differentiated SCC. Using esophageal cancer cell lines, we further confirmed the decreased expression in poorly differentiated SCC cells, compared with other differentiation types. All our results support the significance of hnRNP B1 expression in esophageal SCC as a unique diagnostic marker with regard to association between expression level and histopathological grading.

Key words: Esophageal cancer — Squamous cell carcinoma — hnRNP

Heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, which is an RNA binding protein and a major component of the hnRNP core complex, is involved in RNA splicing in nuclei and in RNA shuttling between nucleus and cytoplasm.1,2) HnRNP B1 of 37 kDa is identical to hnRNP A2 with the addition of 12 amino acids at the N-terminus, and is probably derived from an alternative splicing of hnRNP A2 mRNA.3) We recently demonstrated the significance of hnRNP B1 as a biomarker for detection of human lung cancer, especially squamous cell carcinoma (SCC), by immunohistochemical staining with polyclonal antibody, which was raised in a rabbit using 19-mer synthetic peptides based on sequences within hnRNP B1 protein.4) All examined lung SCC specimens (15/15) showed intensive staining of hnRNP B1 protein in their nuclei, but not in nuclei of normal adjacent tissue. Thus, the overexpression of hnRNP B1 is associated with the rapid cell growth of human lung cancer cell lines.4) Moreover, it has been reported elsewhere that hnRNP A2/B1 protein binds to a telomeric DNA sequence,5,6) suggesting that hnRNP plays an important role in the development of cancer. This is consistent with our recent evidence that overexpression of hnRNP B1 was found in SCC of the lungs, and also in leukoplakia as a precancerous lesion of oral SCC.7) In the light of this, we investigated the expression of hnRNP B1 in SCC of the esophagus by immunohistochemical staining and western blotting. In Japan, SCC of the esophagus accounts for over 90% of esophageal cancer.8)

MATERIALS AND METHODS

Tissue samples Specimens of esophageal SCC were collected, regardless of clinical stage, from paraffinized sections of sixteen esophageal cancer patients (6 well, 6 moderately, 4 poorly differentiated SCC) who had undergone esophagectomy without preoperative treatment using irradiation or anti-cancer agents at our Cancer Center Hospital, from 1993 to 1999. The pathological characteristics of the cancer, such as longitudinal diameter of the tumor, depth of tumor invasion, regional lymph node metastasis, distant metastasis and stage grouping, were determined according to the TNM Classification of Malignant Tumours (Fifth Edition).9) Differentiation grading was performed according to the criteria of the World Health Organization.10) Surgically resected tissues were fixed in 10% buffered neutral formalin solution and embedded in paraffin; 4-µm consecutive sections were cut and mounted on glass slides. Fresh cancerous specimens were frozen in liquid nitrogen and stored at −80°C until use, along with

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synthesize specimens cut as far as possible from cancer regions in the resected tissues.

**Anti-hnRNP B1 antibody** Preparation of anti-hnRNP B1 antibody was reported previously. Briefly, the antibody was raised in a rabbit using 19-mer synthetic peptides (amino acid residues 3–20+cysteine), which included 12 residues that differ between hnRNP A2 and B1. The immunized sera were affinity-purified by use of the antigen and then purified by Mono-Q column chromatography.

**Immunohistochemistry** Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex method as reported previously. In brief, after deparaffinization, tissue sections were heated twice in 10% sodium citrate buffer (pH 6.0) by microwave irradiation for 5 min. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min at room temperature. Following incubation with anti-hnRNP B1 antibody in a humidified chamber at 4°C overnight, the bound antibody was detected with a peroxidase-labeled polymer-conjugated anti-rabbit antibody (ENVISION System; DAKO Co., Carpinteria, CA). The reaction with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) was stopped when the specimen of lung tissue samples subjected to western blotting were equal to that of noncancerous specimens.

**Cell lines** Human esophageal SCC cell lines, TE-2, TE-5, TE-8, TE-10 and TE-11 were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. The cells were cultured in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified incubator with 5% CO2.

**Western blot analysis of tissue and cultured cells** Frozen tissue samples subjected to western blotting were crushed and homogenized in strong lysis solution (CytoSignal, Irvine, CA), to which were added 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 µg/ml aprotinin and 1 µg/ml leupeptin. Following the addition of 250 µg/ml of ribonuclease A (Sigma Chemical Co., St. Louis, MO), samples were stored on ice for 1 h and then sonicated for 5 min. The whole lysate was centrifuged at 15 000g for 10 min at 4°C; its protein content was determined using a protein assay kit (Bio-Rad Lab., Hercules, CA). Western blotting was conducted by use of the NuPAGE electrophoresis system (NOVEX, San Diego, CA) according to the manufacturer’s instructions. Briefly, 50 µg of tissue lysate was heated in 4X lithium dodecyl sulfate (LDS) sample buffer (NOVEX) for 10 min at 70°C and centrifuged at 12 000g for 10 min at 4°C. After having been fractionated by 10% Bis-Tris pre-cast gel (NOVEX) electrophoresis, the proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Lab.). The detection of protein was performed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK), using anti-hnRNP B1 as a primary antibody, and peroxidase-linked species-specific whole antibody (Amersham Pharmacia Biotech) as a secondary antibody.

Cultured cells were centrifuged at 1300g for 5 min at 4°C after having been rinsed and scraped in PBS. Immediately after centrifugation, the pellets were pipetted a few times in strong lysis solution and afterward treated in the same manner as tissue.

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

**RESULTS**

**Expression of hnRNP B1 in esophageal SCC tissue** The results of immunohistochemical staining show that five out of six well differentiated SCC were strongly stained in nuclei with anti-hnRNP B1 antibody compared with adjacent noncancerous squamous epithelium, and the staining was similar in five out of six moderately differentiated SCC (Fig. 1, A and B). The remaining two specimens of well and moderately differentiated SCC showed inconspicuous intensity, equivalent to that of noncancerous epithelium. The enhanced expression was found in the peripheral cells of cancer pearls (Fig. 1A, arrow), which were differentiating to keratinization, and in the cells of nests without cancer pearls. Noncancerous squamous epithelial cells of some layers above the basal cell layers, known as parabasal layers, were immunohistochemically stained (Fig. 1C), whereas in lung cancer, normal epithelial cells were not stained. Two poorly differentiated SCC showed decreased staining (Fig. 1, D and E) compared with that in noncancerous regions, while in the other two poorly differentiated SCC the intensity of immunohistochemical staining in cancerous regions was equivalent to that in noncancerous ones.

**Western blot analysis of esophageal SCC tissue and cell lines** Western blotting of three moderately differentiated SCC revealed the enhanced expression of hnRNP B1 in cancerous tissue compared with noncancerous regions (Fig. 2). Fig. 3 shows the results of western blotting of cell lines of esophageal SCC (poorly, moderately and well differentiated). As for the lack of enhanced expression of hnRNP B1 in poorly differentiated SCC, the poorly differentiated SCC cell line (TE-5) showed a more reduced expression of hnRNP B1 than the other cell lines. In fact,
Fig. 1. Immunohistochemical staining with anti-hnRNP B1 antibody (A, C, and D) and corresponding HE staining (B and E). A and B: Moderately differentiated SCC, where peripheral cells of cancer pearls (arrows) and cancer nests without keratinization were strongly stained. C: Cells in parabasal layers above basal cells in noncancerous regions of D and E sections. These cells showed apparent staining. D and E: Poorly differentiated SCC, where strong staining was not displayed. (Magnification of A, B, D and E, ×200; C, ×100)
the clinicopathological characteristics of patients. Overall, 63% (10/16) of esophageal SCC showed enhanced expression of hnRNP B1 protein. Specifically, the well and moderately differentiated SCC exhibited a significantly high percentage (83%, 5/6 for each type) of enhanced expression, while no enhancement in hnRNP B1 expression was observed in any of the poorly differentiated SCC (P = 0.03 in 2×3 tables after combining well and moderately differentiated SCC). Furthermore, only in poorly differentiated SCC was decreased expression demonstrated. The longitudinal diameter of tumors displaying enhanced expression ranged from 23 to 80 mm; that of tumors with inconspicuous expression (C=N), from 54 to 90 mm; that of tumors with decreased expression (C<N), from 16 to 65 mm. In short, every degree of staining was found in various sizes of tumor. Enhanced expression was demonstrated in tumors invading both shallowly and deeply (pT), and in tumors with or without metastasis to regional lymph nodes (pN) and to distant organs (pM). Although the number of tumors showing inconspicuous or decreased intensity of staining was small, there is no statistically significant correlation between intensity and clinicopathological characteristics except differentiation grade. Consequently, there

Table I. Immunohistochemical Staining with Anti-hnRNP B1 Antibody and Clinicopathological Characteristics

| Intensity of staining<sup>a</sup> | C>>N | C=N | C<N |
|----------------------------------|------|-----|-----|
| Differentiation                  |      |     |     |
| Well-differentiated              | 5    | 1   | 0   |
| Moderately                       | 5    | 1   | 0   |
| Poorly                           | 0    | 2   | 2   |
| Longitudinal diameter (Mean±SE, mm) | 48.4±6.8 | 73.5±9.6 | 37.5±27.5 |
| Primary tumor                    |      |     |     |
| pT1                              | 2    | 0   | 1   |
| pT2                              | 1    | 0   | 0   |
| pT3                              | 7    | 2   | 1   |
| pT4                              | 0    | 2   | 0   |
| Regional lymph nodes             |      |     |     |
| pN0                              | 5    | 1   | 1   |
| pN1                              | 5    | 3   | 1   |
| Distant metastasis               |      |     |     |
| pM0                              | 6    | 3   | 2   |
| pM1b                             | 4    | 1   | 0   |
| Stage grouping                   |      |     |     |
| Stage I                          | 2    | 0   | 0   |
| Stage IIA                        | 2    | 1   | 1   |
| Stage IIIB                       | 0    | 0   | 1   |
| Stage III                        | 2    | 2   | 0   |
| Stage IVB                        | 4    | 1   | 0   |

<sup>a</sup> C, cancer; N, noncancerous region.
is no association between staining status and clinicopathological parameters, including TNM classification. Nor did other clinical features, such as intraepithelial spread, blood vessel invasion or lymphatic invasion, show any association with intensity of staining (data not shown).

**DISCUSSION**

HnRNP B1 is now established as a new biomarker for early detection of lung cancer and oral SCC, in both of which it is overexpressed. In this paper, we confirmed the elevated expression of hnRNP B1 in esophageal SCC by immunohistochemical staining and western blotting. One of the new findings reported here is that the expression was increased in well and moderately differentiated SCC, but decreased in poorly differentiated SCC. In addition, while intensively stained cells were more numerous around cancer pearls, cells of parabasal layers were also stained, suggesting that hnRNP B1 is involved in differentiation of esophageal SCC and epithelial cells. The interpretation of the staining of parabasal layers is uncertain at present: the expression of hnRNP B1 may not be related to cell proliferation, since the expression levels of hnRNP B1 in cell lines used in this study remained unchanged regardless of different proliferation rates induced by different medium conditions, 5% FBS or 10% FBS (data not shown). Although quantitative analysis of hnRNP B1 expression levels, looking at each histological differentiation grade and using a large population, has not yet been performed, results so far indicate that hnRNP B1 may be a new objective differentiation marker of SCC.

Hoek et al. previously reported that hnRNP A2 binds to myelin basic protein (MBP) mRNA and regulates its transport from nucleus to cytoplasm in neurogenic cells. We found a clear difference between the intracellular localization of hnRNP B1 in basal layer cells and in keratinized cells on the surface of squamous epithelium (data not shown), suggesting that hnRNP B1 binds to a specific mRNA in squamous cells and regulates its transport. Thus, altered expression and intracellular localization of hnRNP B1 might have an effect on squamous differentiation. The target molecule of hnRNP B1 in squamous cells, and its role, are now under investigation.

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