Jpn. J. Cancer Res. **91**, 658–663, June 2000

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

Satoru Matsuyama,[1,3] Yuri Goto,[1] Naoko Sueoka,[1] Yasuo Ohkura,[2] Yoichi Tanaka,[2] Kei Nakachi[1] and Eisaburo Sueoka[1]

*[1]Saitama Cancer Center Research Institute and [2]Hospital, 818 Komuro, Ina, Kitaadachi-gun, Saitama 362-0806*

**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-$\mu$m 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

[3]To whom request for reprints should be addressed.
E-mail: matuyama@qb3.so-net.ne.jp

noncancerous 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.[4] 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.[4]

**Immunohistochemistry** Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex method as reported previously.[11] 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 cancer used as a positive control showed clear reactivity under a microscope. Counter-staining was performed with hematoxylin. For histological assessment, hematoxylin and eosin (HE) staining of other consecutive sections was performed conventionally. The expression of hnRNP B1 in cancerous regions was expressed as “enhanced,” “equal” or “decreased” compared with that of noncancerous regions.

**Cell lines** Human esophageal SCC cell lines, TE-2, TE-5, TE-8, TE-10 and TE-11[12] 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% $CO_2$.

**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 m*M* phenylmethanesulfonyl fluoride (PMSF), 1 $\mu$g/ml aprotinin and 1 $\mu$g/ml leupeptin. Following the addition of 250 $\mu$g/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 15 000*g* 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 $\mu$g of tissue lysate was heated in 4× lithium dodecyl sulfate (LDS) sample buffer (NOVEX) for 10 min at 70°C and centrifuged at 12 000*g* 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 1300*g* 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 $\chi^2$ 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.[4] 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,

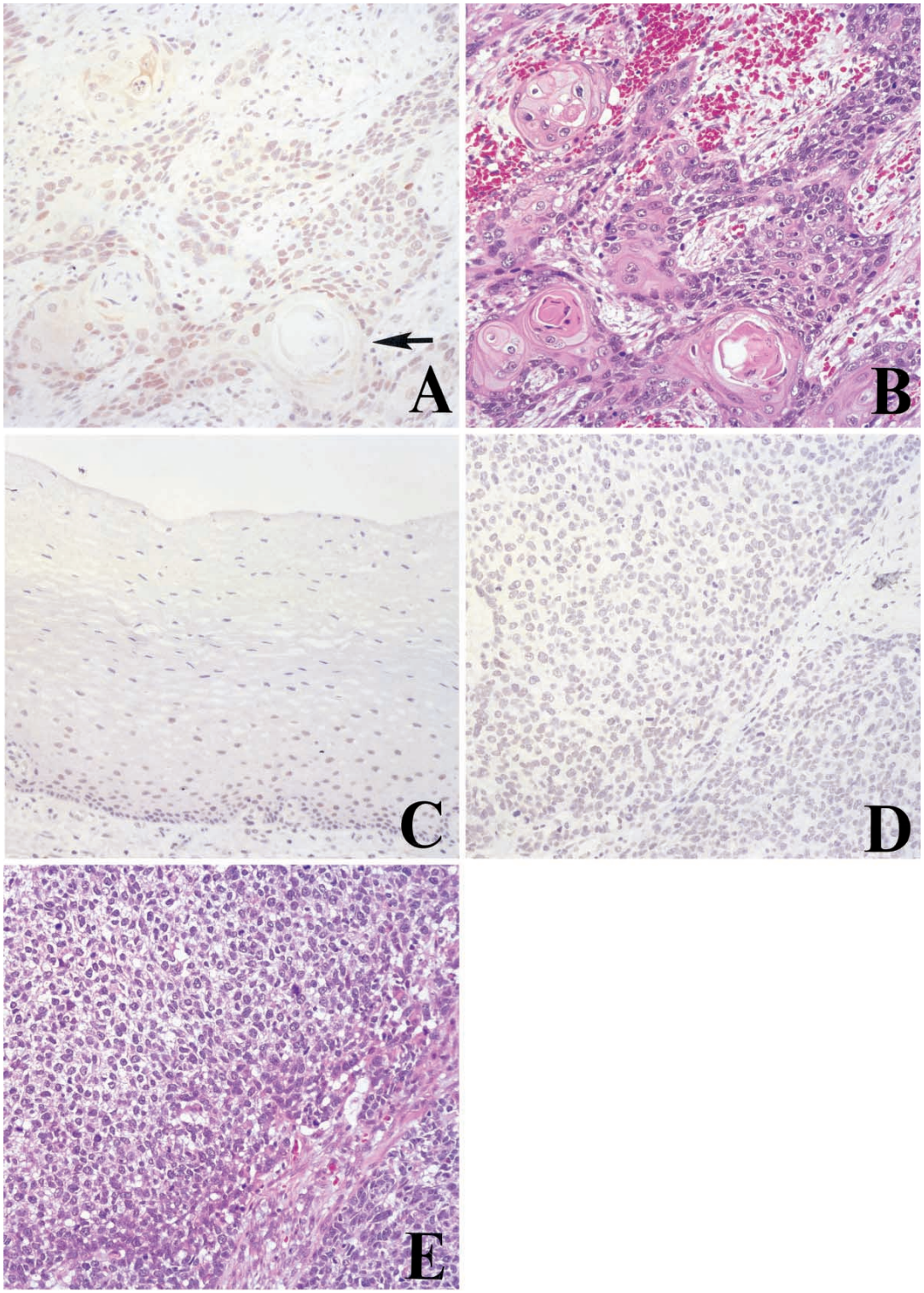


Fig. 1. Immunohistochemical staining with anti-hnRNP B1 antibody (A, C, and D) and corresponding HE staining (B and E). A and B: Moderately differentiated SCC, where peripheral cells of cancer pearls (arrows) and cancer nests without keratinization were strongly stained. C: Cells in parabasal layers 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)

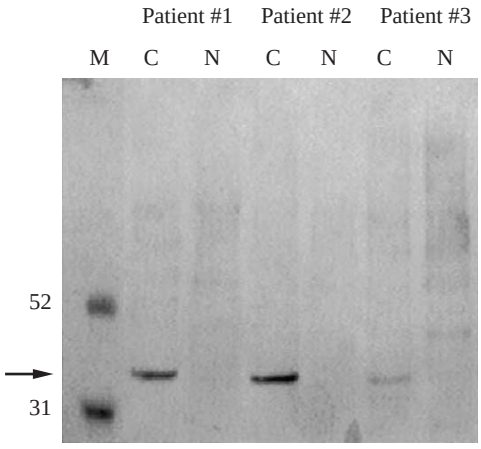


Fig. 2. Western blot analysis of hnRNP B1 protein in cancerous and noncancerous tissue of moderately differentiated SCC patients. M, molecular weight markers; C, cancerous; and N, noncancerous tissue. The arrow indicates a band corresponding to hnRNP B1 (37 kDa).

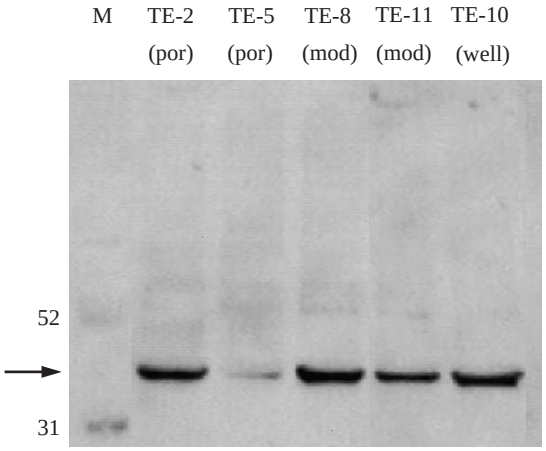


Fig. 3. Western blot analysis of hnRNP B1 protein in esophageal SCC cell lines. Por, established from poorly differentiated SCC; mod, established from moderately differentiated SCC; well, established from well-differentiated SCC; M, molecular weight markers. The arrow indicates a band corresponding to hnRNP B1 (37 kDa).

among the lung, oral and esophageal SCC cells we examined, TE-5 was unique in its low expression of hnRNP B1, having an expression level lower than normal human epidermal keratinocytes (data not shown).

**Expression of hnRNP B1 and clinicopathological characteristics** Table I summarizes the results of immunohistochemical staining, showing histopathological grade and

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

| Intensity of staining[a)] | 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 IIB | 0 | 0 | 1 |
| Stage III | 2 | 2 | 0 |
| Stage IVB | 4 | 1 | 0 |

*a*) C, cancer; N, noncancerous region.

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

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.[4, 7] 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.[13] 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.

The next aim of our study was to clarify the clinical characteristics of both enhanced and decreased expression of hnRNP B1 in esophageal SCC. Various biomarkers, such as p53, MIB-1 (Ki-67), epidermal growth factor receptor (EGFR), cyclin D1, and constituents of the intercellular adherens junction, have been examined in an effort to characterize SCC. Overexpression of p53[14] and MIB-1[15] labeling index were shown to be associated with poor prognosis, and MIB-1 labeling index was also found to have a correlation with lymph node metastasis, tumor size and proliferation pattern of primary tumor; *EGFR* gene amplification has an impact on lymph node metastasis[16]; and overexpression of cyclin D1 might have some prognostic significance related to lymph node metastasis and retinoblastoma (RB) protein expression.[17] However, except for that between constituents of the intercellular adherens junction and SCC differentiation type, we found no relation between any of these activities. Although reduced expression of E-cadherin, β-catenin and plakoglobin was also found in moderately and poorly differentiated SCC of the esophagus, these SCC did not show any significant differences in expression levels.[18] However, since the expression of hnRNP B1 was specifically decreased in poorly, not moderately differentiated SCC, we think that hnRNP B1 is distinct from other biomarkers.

Further studies are needed to clarify the precise function of hnRNP B1 in squamous cell carcinogenesis, particularly its relation to differentiation in esophageal cancer and its expression in precancerous lesions, i.e., dysplasia.

## ACKNOWLEDGMENTS

We thank Dr. Hirota Fujiki (Saitama Cancer Center Research Institute) for helpful discussions. This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture and from the Ministry of Health and Welfare, Japan, and for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control and for Comprehensive Research on Aging and Health from the Ministry of Health and Welfare, Japan, as well as a grant from the Smoking Research Foundation of Japan.

*(Received February 2, 2000/Revised March 15, 2000/Accepted March 23, 2000)*

## REFERENCES

1) Dreyfuss, G., Matunis, M., Pinol-Roma, S. and Burd, C. G. HnRNP proteins and biogenesis of mRNA. *Annu. Rev. Biochem.*, **62**, 289–321 (1993).
2) Mayeda, A., Munroe, S. H., Caceres, J. F. and Krainer, A. R. Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. *EMBO J.*, **13**, 5483–5495 (1994).
3) Kozu, T., Henrich, B. and Schäffer, K. P. Structure and expression of the gene (*HNRPA2B1*) encoding the human hnRNP protein A2/B1. *Genomics*, **25**, 365–371 (1995).
4) Sueoka, E., Goto, Y., Sueoka, N., Kai, Y., Kozu, T. and Fujiki, H. Heterogeneous nuclear ribonucleoprotein B1 as a new marker of early detection for human lung cancers. *Cancer Res.*, **59**, 1404–1407 (1999).
5) Ishikawa, F., Matunis, M. J., Dreyfuss, G. and Cech, T. R. Nuclear proteins that bind the pre-mRNA 3′ splice site sequence r(UUAG/G) and the human telomeric DNA se-

quence d(TTAGGG)n. *Mol. Cell. Biol.*, **13**, 4301–4310 (1993).
6) McKay, S. J. and Cooke, H. hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeat TTAGGGn. *Nucleic Acids Res.*, **20**, 6461–6464 (1992).
7) Goto, Y., Sueoka, E., Chiba, H. and Fujiki, H. Significance of heterogeneous nuclear ribonucleoprotein B1 as a new early detection marker for oral squamous cell carcinoma. *Jpn. J. Cancer Res.*, **90**, 1358–1363 (1999).
8) Japanese Research Society for Esophageal Diseases, National Cancer Center, Tokyo. "The Report of Treatment Results of Esophageal Carcinoma in Japan (1983·1984·1985·1986·1987), No.16" (1997). Mitamura Printing Co., Tokyo.
9) UICC. "TNM Classification of Malignant Tumours, Fifth Ed.," International Union Against Cancer (1997). John Wiley & Sons, Inc., New York.
10) World Health Organization. "International Histological Classification of Tumours: Histological Typing of Oesophageal and Gastric Tumours, Second Ed." (1990). Springer-Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo and Hong Kong.
11) Vitarelli, E., Sippelli, G., Tuccari, G. and Barresi, G. The use of microwave irradiation for immunohistochemistry: a new methodological proposal. *Histopathology*, **10**, 35–38 (1995).
12) Nishihira, T., Hashimoto, Y., Katayama, M., Mori, S. and Kuroki, T. Molecular and cellular features of esophageal cancer cells. *J. Cancer Res. Clin. Oncol.*, **119**, 441–449 (1993).
13) Hoek, K. D., Kidd, G. J., Carson, J. H. and Smith, R. HnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. *Biochemistry*, **37**, 7021–7029 (1998).
14) Shimaya, K., Shiozaki, H., Inoue, M., Tahara, H., Monden, T., Shimano, T. and Mori, T. Significance of p53 expression as a prognostic factor in oesophageal squamous cell carcinoma. *Virchows Arch. A Pathol. Anat. Histol.*, **422**, 271–276 (1993).
15) Youssef, E. M., Matsuda, T., Takada, N., Osugi, H., Higashino, M., Kinoshita, H., Watanabe, T., Katsura, Y., Wanibuchi, H. and Fukushima, S. Prognostic significance of the MIB-1 proliferation index for patients with squamous cell carcinoma of the esophagus. *Cancer*, **76**, 358–366 (1995).
16) Kitagawa, Y., Ueda, M., Ando, N., Ozawa, S., Shimizu, N. and Kitajima, M. Further evidence for prognostic significance of epidermal growth factor receptor gene amplification in patients with esophageal squamous cell carcinoma. *Clin. Cancer Res.*, **2**, 909–914 (1996).
17) Ishikawa, T., Furihata, M., Ohtsuki, Y., Murakami, H., Inoue, A. and Ogoshi, S. Cyclin D1 overexpression related to retinoblastoma protein expression as a prognostic marker in human oesophageal squamous cell carcinoma. *Br. J. Cancer*, **77**, 92–97 (1998).
18) Nakanishi, Y., Ochiai, A., Akimoto, S., Kato, H., Watanabe, H., Tachimori, Y., Yamamoto, S. and Hirohashi, S. Expression of E-cadherin, α-catenin, β-catenin and plakoglobin in esophageal carcinomas and its prognostic significance. *Oncology*, **54**, 158–165 (1997).