

Significance of Heterogeneous Nuclear Ribonucleoprotein B1 as a New Early Detection Marker for Oral Squamous Cell Carcinoma

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The development of an early tumor detection marker for oral cancer is an obvious need due to the high recurrence rate and poor survival rate. Based on our previous report that overexpression of heterogeneous nuclear ribonucleoprotein (hnRNP) B1 protein was found in 100% of squamous cell carcinomas of human lung, we applied the same immunohistochemical method, using anti-hnRNP B1 antibody, to human oral squamous cell carcinoma (OSCC). Seven human tissue sections of OSCC showed strong staining with anti-hnRNP B1 antibody, and hnRNP B1 protein of 37 kDa was identified in protein fractions isolated from six of the cancerous tissue sections, while it was not found in adjacent noncancerous tissue. Moreover, three non-homogeneous (nodular) leukoplakia sections showed significant anti-hnRNP B1 staining. The results suggest that this antibody detects precancerous lesions as well as advanced lesions (stages I to IV) of OSCC. We also present positive results of cytodiagnosis for two smear specimens. All of the above results indicate that hnRNP B1 is a new and useful marker for early detection of OSCC.

Key words: Oral precancerous lesion — Oral leukoplakia — Cytodiagnosis — Biomarker — Chemoprevention

Recently we reported that overexpression of heterogeneous nuclear ribonucleoprotein (hnRNP) B1 of 37 kDa—and not hnRNP A2, as had previously been believed—is a good biomarker for the detection of human lung cancer.¹⁾ hnRNP B1 is identical to hnRNP A2 with the addition of 12 amino acids at the N-terminus, a difference probably deriving from alternative splicing.²⁾ hnRNP A2/B1 protein is a major component of the hnRNP core complex in mammalian cell nuclei, and is involved in RNA splicing in nuclei, as well as in mRNA transport from nucleus to cytoplasm.³⁾ In a study on the expression of hnRNP B1 in both human lung cancer cell lines and human lung cancer tissue, we found that cancer cells showed significant positive staining of hnRNP B1 with anti-hnRNP B1 antibody, and that squamous cell carcinomas showed 100% positive staining.¹⁾ These results encouraged us to see whether molecular diagnosis of human lung cancer with anti-hnRNP B1 could be extended to human oral squamous cell carcinoma (OSCC).

Success in early detection of oral cancer is of great value, since human oral cancer has a high recurrence rate and is associated with cosmetic disruption in the advanced stage. Although various tumor markers, such as carcinoembryonic antigen (CEA),⁴⁾ squamous cell carcinoma-antigen (SCC),⁵⁾ glutathione *S*-transferase- π (GST- π),^{6,7)} and cytokeratins,^{8,9)} are currently being used as diagnostic aids for OSCC, all these markers have proved to be insuffi-

ciently sensitive and reliable for the early detection of OSCC and prediction of its recurrence. Thus, the establishment of a new tumor marker with increased specificity and sensitivity for early lesion of OSCC is a high priority.

We first found that anti-hnRNP B1 antibody stained cancer cells of seven human tissue sections of OSCC. Next, by western blotting we detected a protein band (37 kDa) of hnRNP B1 in six samples of cancerous tissue obtained from OSCC patients, a band not detected in adjacent noncancerous tissue. These results all indicate the potential of the anti-hnRNP B1 antibody for cytodiagnosis of smear specimens. Furthermore, although the results are preliminary, this represents the first evidence that hnRNP B1 is, in addition to its previously demonstrated effectiveness in lung cancer, also a useful biomarker for early detection of OSCC.

MATERIALS AND METHODS

Patients and tissue preparations The oral tissues from 12 OSCC and three leukoplakia patients were taken by surgical resection during the eleven months from December 1997 to October 1998 at the Department of Oral and Maxillofacial Surgery, Tokyo Medical University. The resected tissues obtained from seven OSCC patients listed in Table I and tissues obtained from three leukoplakia patients listed in Table III were fixed in 10% formalin and paraffin-embedded. Five-micrometer sections were cut and subjected to immunohistochemical analysis. Similarly resected cancerous and noncancerous tissues from the six

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Table I. Characteristics of Seven OSCC Patients, Histological and Immunohistochemical Results for hnRNP B1 Protein

Patient No.	Sex	Age	Stage	Site	Histology ^{a)}	Anti-hnRNP B1 antibody ^{b)} Positive staining
1	F	56	I	Gingiva	M/D	+
2	F	76	I	Tongue	M/D	+++
3	M	59	II	Tongue	W/D	+
4	M	61	II	Buccal mucosa	P/D	++
5	F	49	III	Tongue	W/D	++
6	M	62	IV	Gingiva	P/D	+++
7	M	25	IV	Tongue	P/D	+

a) W/D: well differentiated, M/D: moderately differentiated, and P/D: poorly differentiated.

b) Data represent relative intensity of staining, (+) mild, (++) medium, (+++) strong.

Table II. Characteristics of Western Blot Analysis of Examined OSCC Patients and Histological Features

Patient No.	Sex	Age	Stage	Site	Histology ^{a)}
1	M	25	IV	Tongue	P/D
2	M	69	IV	Tongue	M/D
3	F	77	IV	Tongue	W/D
4	F	65	re ^{b)}	Gingiva	P/D
5	F	73	re	Maxilla mucosa	W/D
6	M	77	re	Buccal mucosa	W/D

a) W/D: well differentiated, M/D: moderately differentiated, P/D: poorly differentiated.

b) Recurrence.

Patient No. 1 is the same as patient No. 7 of Table I.

Table III. Characteristics of Three Leukoplakia Patients

Patient No.	Sex	Age	Site	Anti-hnRNP B1 antibody Positive staining
1	M	61	Buccal mucosa	+
2	M	28	Tongue	++
3	M	65	Tongue	++

Table IV. Characteristics of Two Smear Specimens

Patient No.	Sex	Age	Stage	Site	Anti-hnRNP B1 antibody Positive staining
1	M	25	IV	Tongue	++
2	F	77	IV	Buccal mucosa	++

Patient No. 1 is the same as patient No. 7 of Table I and No. 1 of Table II.

OSCC patients listed in Table II were immediately frozen in liquid nitrogen and stored at -80°C until protein extraction for western blotting analysis. Tables I to IV summa-

rize patients' sex, age, stage of OSCC, site of the tumor, site of leukoplakia and histological results classified by the grade of differentiation. Smear specimens were obtained from two advanced OSCC patients listed in Table IV. Patient No. 7 of Table I is the same as patient No. 1 of Table II and No. 1 of Table IV. The samples were immediately fixed with Cytokeep II (Nippon Shoji Co., Osaka).

Immunohistochemistry Anti-hnRNP B1 antibody was raised in rabbit using a 19-mer synthetic peptide derived from hnRNP B1 protein as reported previously.¹⁾ Immunohistochemical staining was performed by a standard method as follows¹⁰⁾: a deparaffinized 5- μm tissue section was heated in 0.01 M sodium citrate buffer, pH 6.0 by microwave irradiation (750 W) twice for 5 min, after which endogenous peroxidase activity in each tissue section or smear specimen was blocked with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min at room temperature. Each tissue section was rinsed with PBS and incubated with anti-hnRNP B1 antibody at a dilution of 1:200 for 14 h at 4°C , while each the smear specimen was incubated with anti-hnRNP B1 antibody at 1:50. hnRNP B1 protein was detected with the DAKO ENVISSION system (DAKO Co., Carpinteria, CA), and counter-staining was performed with hematoxylin. Immunohistochemical examination was conducted by two independent investigators.

Western blot analysis A frozen tissue sample was crushed and homogenized in 1 ml of ISOGEN (Nippon Gene, Toyama). Protein was isolated by a standard procedure as reported previously.¹¹⁾ In brief, 0.2 ml chloroform was added to ISOGEN solution, and the mixture was centrifuged at 12,000g for 15 min at 4°C . To the supernatant, 0.3 ml of ethanol was added and centrifugation at 2,000g for 5 min at 4°C afforded supernatant, interphase and organic phases. To the supernatant, 1.5 ml of isopropanol was added, and protein was precipitated by centrifugation at 12,000g for 10 min at 4°C . The protein pellet was dissolved in 1% sodium dodecyl sulfate (SDS) solution.

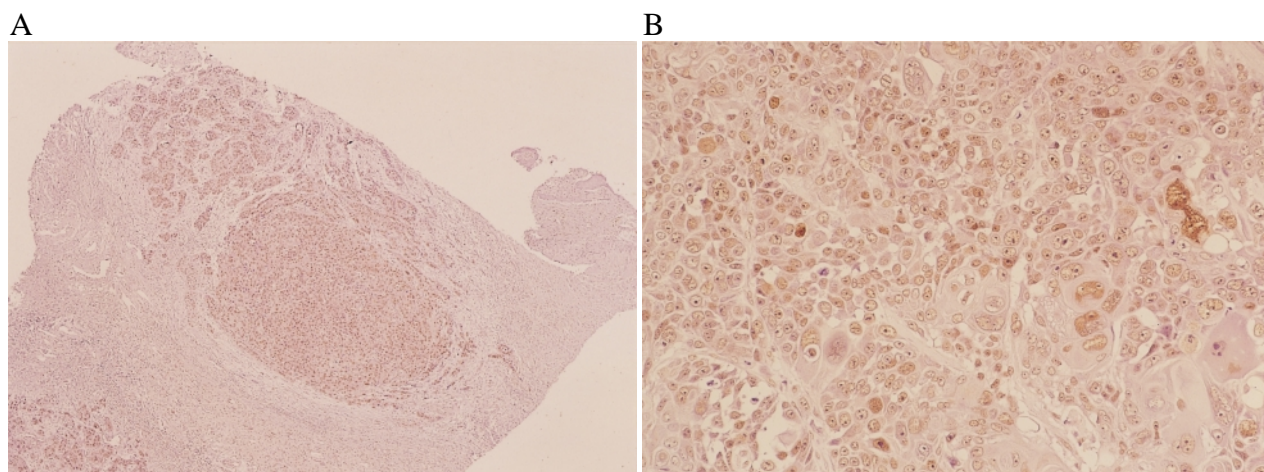


Fig. 1. Overexpression of hnRNP B1 in tissue of OSCC patient No. 6 listed in Table I. A, B: Tissue of a patient (No. 6) with stage IV OSCC was immunohistochemically stained with anti-hnRNP B1 antibody. (A) Immunohistochemical staining of OSCC section (Magnification: $\times 40$). (B) Intense immunohistochemical staining was found in the nucleus of cancer cells (Magnification: $\times 400$).

Proteins (10 μg) were size-fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), followed by western blotting using anti-hnRNP B1 antibody. The protein bands were visualized with an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK).

RESULTS AND DISCUSSION

Overexpression of hnRNP B1 protein in OSCC Tissue sections obtained from the seven OSCC patients listed in Table I were subjected to immunohistochemical staining with anti-hnRNP B1 antibody. Table I shows the characteristics of the patients along with the histological features: four males and three females, average age of 55.4, two stage I, two stage II, one stage III and two stage IV; histologically, two well, two moderately and three poorly differentiated. Anti-hnRNP B1 antibody detected cancer cells in all seven OSCC patients. Fig. 1A shows representative immunohistochemical staining: anti-hnRNP B1 antibody clearly stained cancer cells in the tissue and differentiated cancer cells from noncancerous ones, with nuclei, rather than cytoplasm, being intensively stained (Fig. 1B). Noncancerous oral epithelium of the same tissue section did not show positive staining with anti-hnRNP B1 antibody (data not shown). As shown in Table I, OSCC cancer cells even at an early stage showed strong staining with anti-hnRNP B1 antibody, and the intensity of the staining was not associated with clinical stage. Therefore, we think that

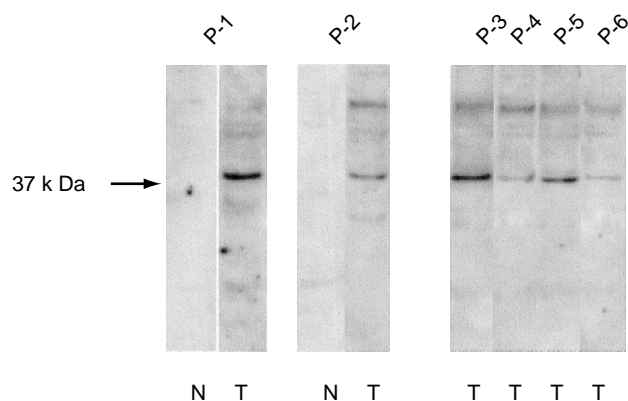


Fig. 2. Western blot analysis of hnRNP B1 in noncancerous (N) and cancerous (T) tissues of six OSCC patients listed in Table II. Noncancerous tissue was obtained from normal adjacent tissue of patients (No. 1 and 2). The arrowhead indicates hnRNP B1 protein of 37 kDa.

hnRNP B1 protein is overexpressed even in the early stage of oral cancer, just as it is in lung cancer.¹⁾

Next, we studied the expression of hnRNP B1 in cancerous and noncancerous tissue by western blotting. Proteins were isolated from cancerous (T) and noncancerous tissue (N) of the six OSCC patients listed in Table II. Characteristics of the six patients were as follows: three males and three females, average age of 64.3, three stage IV and three recurrent cases. Anti-hnRNP B1 antibody

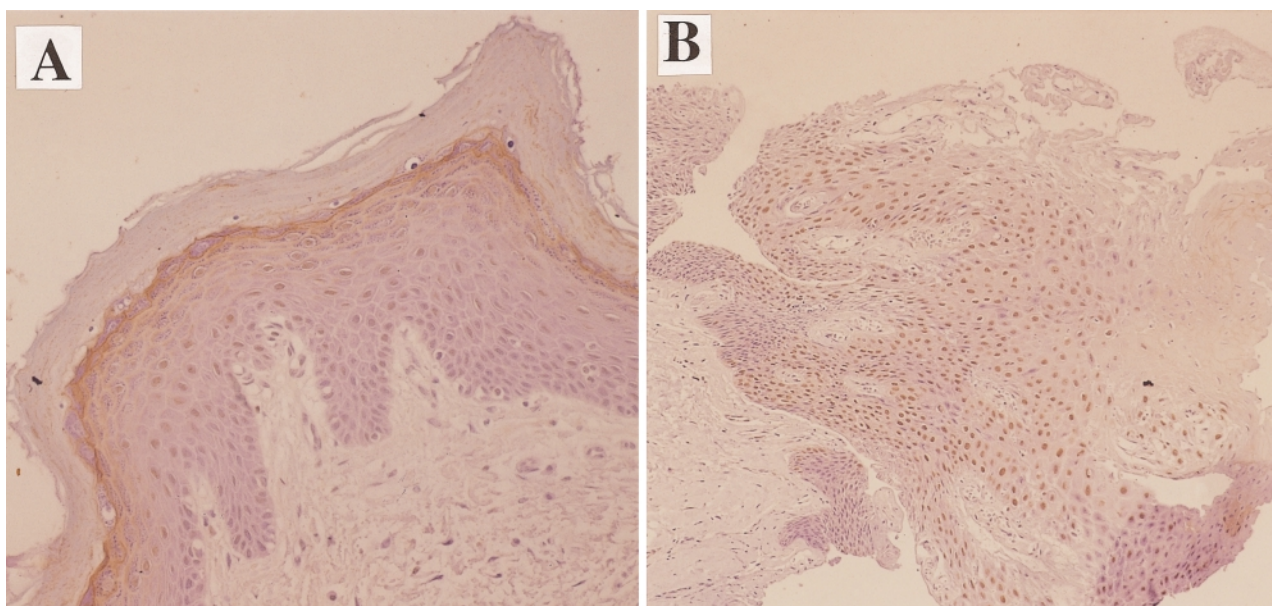


Fig. 3. Expression of hnRNP B1 in two oral leukoplakias listed in Table III. A: leukoplakia of buccal mucosa (patient No. 1) stained with anti-hnRNP B1 antibody. B: leukoplakia of tongue (patient No. 3) stained with anti-hnRNP B1 antibody (Magnification: $\times 100$).

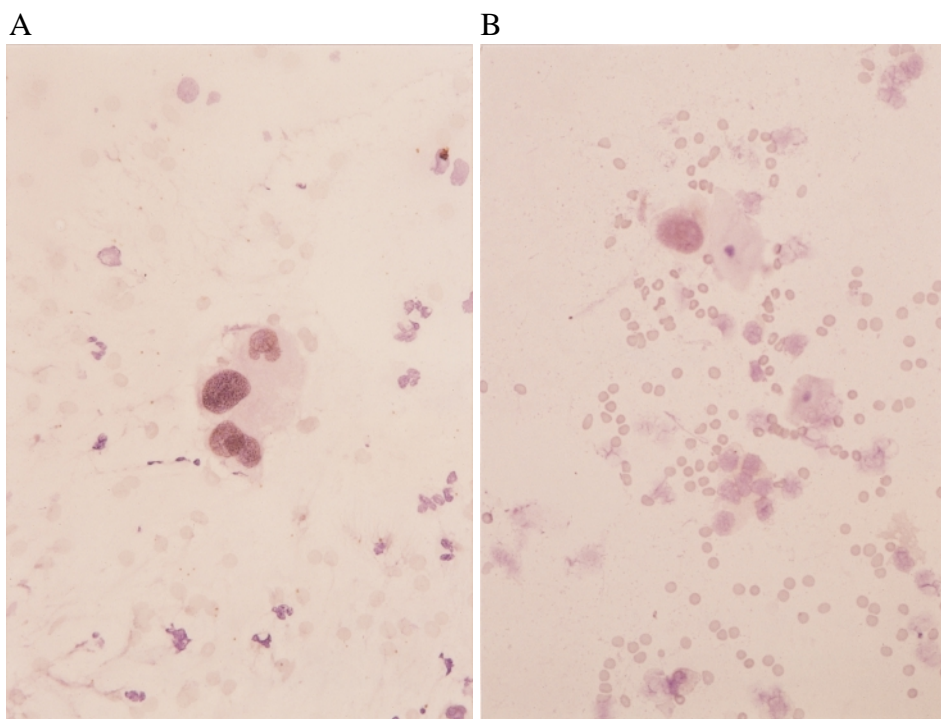


Fig. 4. Staining of cancer cells in smear specimens by immunocytochemistry with anti-hnRNP B1 antibody. Positive staining of cancer cells in smear specimens from OSCC patients listed in Table IV. A: smear specimen of patient No. 1, B: that of patient No. 2 (Magnification: $\times 400$).

identified a protein of 37 kDa as hnRNP B1 in all samples from six OSCC tissues (Fig. 2). Noncancerous tissue did not express the protein, indicating that intense staining of the 37 kDa protein in cancer cells by anti-hnRNP B1 antibody was due to overexpression of hnRNP B1 protein. All the results clearly showed that hnRNP B1 is overexpressed specifically in cancer cells of OSCC patients, even at an early clinical stage, suggesting that hnRNP B1 can be used as a new marker for the early stage of OSCC.

Elevated expression of hnRNP B1 protein in oral leukoplakia Oral leukoplakia is a well defined precancerous lesion.^{12,13} About ten percent of oral leukoplakias are thought to develop into malignant oral cancer, and when leukoplakia is clinically classified into homogeneous and non-homogeneous (nodular) leukoplakia,¹³ it is generally considered that 15 to 40% of non-homogeneous (nodular) leukoplakias progress to malignant transformation. However, in a standard clinical examination, it is difficult to determine which non-homogeneous (nodular) leukoplakia constitutes the high-risk group for OSCC. Since we recently found that hnRNP B1 is expressed in the severe grade of squamous metaplasia in human lung (data not shown), we applied the same method to oral leukoplakia: anti-hnRNP B1 antibody clearly stained three non-homogeneous (nodular) leukoplakia sections (Table III). The results for two non-homogeneous leukoplakias are shown in Fig. 3. Although anti-hnRNP B1 antibody-positive cells were found in all layers of oral epithelium, the basal cell layers showed stronger staining than the other layers (Fig. 3). From this, we concluded that anti-hnRNP B1 antibody detects precancerous lesion of OSCC, as well as the later stages. The precise molecular relationship between the high expression of hnRNP B1 and development of OSCC is now under investigation.

Application of anti-hnRNP B1 antibody to cytodagnosis Because it is simple and practical, cytodagnosis is an important clinical tool. In order to utilize anti-hnRNP B1 antibody in cytodagnosis, smear specimens were next subjected to immunocytochemistry. As Fig. 4 shows, cancer cells in smear specimens from two OSCC patients (Table IV) showed positive staining, whereas normal oral epithelial cells did not (data not shown), confirming, we

think, that anti-hnRNP B1 antibody is indeed applicable in cytodagnosis of OSCC.

OSCC is one of the important targets for chemoprevention, since there are high risks of recurrence and second primary cancers, and oral leukoplakia as a precancerous lesion is also a primary target for chemoprevention.^{14,15} We report here the first evidence that hnRNP B1 is apparently overexpressed in the cells of oral leukoplakia from the early stage of OSCC to recurrence, results which strongly indicate that hnRNP B1 is a useful new biomarker for early detection of OSCC, as well as human lung cancer.

A note should be added concerning the overall significance of hnRNP B1 in cancer research. Tockman *et al.* in 1988 reported that hnRNP A2/B1 was frequently observed in the early stage of primary non-small cell lung cancer.¹⁶ hnRNP A2/B1 is a major component of the hnRNP core complex consisting of hnRNP A1 to hnRNP U in mammalian cell nuclei, and it is involved in both RNA splicing in nuclei and mRNA transport from nucleus to cytoplasm.^{3,17} Building on these results, we previously reported that overexpression of hnRNP B1 is associated with rapid cell growth of human lung cancer cell lines.¹ The precise functions of hnRNP proteins are tantalizingly complex, but the overexpression of hnRNP B1, derived from alternative splicing of hnRNP A2 gene, should command immediate attention, aimed at its further elucidation.

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