Overexpression of Septin1: Possible contribution to the development of oral cancer

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Abstract. Our previous study using proteomic profiling demonstrated significant up-regulation of Septin1, a conserved family of GTPase proteins, in oral squamous-cell carcinoma (OSCC)-derived cell lines. In the current study, to determine the potential involvement of Septin1 in oral carcinogenesis, we evaluated the state of septin1 protein/mRNA expression in OSCC-derived cell lines, oral premalignant lesions (OPLs), and primary OSCCs. A significant ($P<0.05$) increase in Septin1 expression was evident in all OSCC-derived cell lines examined compared to human normal oral keratinocytes (HNOKs) and OPLs. In immunohistochemistry, while the vast majority of the OSCCs (89%) were positive for Septin1, no immunoreaction was observed in corresponding normal tissues and OPLs. In addition, real-time quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) data were consistent with the protein expression status. These results suggest that Septin1 expression could contribute to cancer progression, proliferation, or both, and that Septin1 may be a potential diagnostic marker of highly active cancer and a therapeutic target for OSCCs.

Introduction

Oral squamous-cell carcinomas (OSCCs), the most common cancer of the head and neck, accounts for over 300,000 new cancer cases worldwide annually (1). With the currently available clinical assessment and treatment methods, patients are often diagnosed in the late stage of the disease, and the survival rate has not improved substantially. This highlights the need for continued efforts to discover suitable biomarkers for early diagnosis of the disease and to understand the disease pathogenesis as a first step toward improving treatment. Elucidation of the genetic changes leading to the development of OSCCs will probably result in improved molecular assays for the early diagnosis of, therapy for, and improved prognosis of this cancer. The availability of biomarkers of malignancy would also be a key factor for monitoring cancer recurrence and evaluating the efficacy of novel treatment.

The proteomic study includes post-translational modifications such as acetylation, ubiquitination, phosphorylation, or glycosylation (2,3). Proteomic methods detect the functioning units of expressed genes using protein fingerprinting (4,5). In addition, many cancer biomarkers are a manifestation of differences in post-transcriptional splicing, post-translational modifications, or both. Thus, proteomic tools are used increasingly in the post-genomic era to discover new cancer biomarkers. This information will likely prove to be crucial in cancer prognosis, diagnosis, prevention, and therapy, with the ultimate goals being therapeutic target discovery, rational drug design, and identification of early-detection surrogate biomarkers (5,6).

In our previous study, using a fluorescent two-dimensional differential in-gel electrophoresis (2-D-DIGE) system and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), we compared protein expression profiles in human normal oral keratinocytes (HNOKs) and OSCC-derived cell lines and identified several OSCC-associated proteins (7). Among them, the protein, Septin1, was found to be a putative OSCC-related protein. The current study focused on the state of septin1 expression at protein/mRNA level in OSCC-derived cell lines, human primary OSCCs, and oral premalignant lesions (OPLs).

Materials and methods

Tissue specimens and cell lines. Tissue samples were obtained during surgical resection from 85 unrelated Japanese patients

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Table I. Clinical of pathological characteristics of patients and their tumor.

Clinical classification	Total	Immunostaining results [no. of patients (%)]		P-value
		septin1 (+)	septin1 (-)	
Age at surgery				0.665114
<60	24	21 (88)	3 (12)	
60-70	25	23 (92)	2 (8)	
≥70	36	33 (92)	3 (8)	
Gender				0.891534
Male	55	50 (91)	5 (9)	
Female	30	27 (90)	3 (10)	
T-primary tumor				0.0156749
T1	6	3 (50)	3 (50)	
T2	37	34 (92)	3 (8)	
T3	18	16 (89)	2 (11)	
T4	24	24 (100)	0	
N-regional lymph node(s)				0.70461
N (-)	54	48 (89)	6 (11)	
N (+)	31	29 (94)	2 (6)	
Stage				0.155804
I	5	2 (50)	3 (50)	
II	17	17 (100)	0	
III	16	14 (88)	2 (12)	
IV	47	44 (94)	3 (6)	
Histopathological type				0.0168169
Well differentiated	55	53 (97)	2 (3)	
Moderately differentiated	24	19 (80)	5 (20)	
Poorly differentiated	6	5 (83)	1 (17)	
Tumor site				0.164478
Gingiva	25	25 (100)	0	
Tongue	39	33 (85)	6 (15)	
Buccal mucosa	8	8 (100)	0	
Oral floor	8	7 (88)	1 (12)	
Oropharyngeal isthmus	4	3 (75)	1 (25)	
Lower lip	1	1 (100)	0	

with primary SCC of the oral cavity who were treated at Chiba University Hospital between 1998 and 2005 (Table I) and from 33 unrelated Japanese with leukoplakia of the oral cavity who were treated at the Tokyo Dental College Hospital between 2005 and 2006 (Table II). Tumors and patient-matched normal epithelium were obtained intraoperatively at Chiba University Hospital after the patients provided informed consent for a protocol approved by the institutional review board of Chiba University.

The resected tissues were divided into two parts, one of which was frozen immediately after careful removal of the surrounding normal tissues and stored at -80˚C until protein isolation; the second part was fixed in 10% buffered formaldehyde solution for pathologic diagnosis and immuno-histochemical staining. Histopathologic diagnosis of each neoplastic tissue was performed according to the World Health Organization criteria by the Department of Pathology, Chiba University Hospital. Clinicopathological staging was determined by the TNM classification of the International Union against Cancer. All patients had SCC that was confirmed histologically, and tumor samples were checked to ensure that tumor tissue was present in >80% of the specimens. The

Table II. Patients and their OPLs.

Clinical classification	No. of patients (%)
Age at surgery	
<60	16 (49)
60-70	10 (30)
≥70	7 (21)
Gender	
Male	18 (55)
Female	15 (45)
Leukoplakia site	
Gingiva	13 (40)
Tongue	10 (30)
Buccal mucosa	2 (6)
Plate	7 (21)
Lips	1 (3)

OSCC-derived cell lines used in this study were HSC-2, HSC-3, HSC-4, HO-1-N-1, KON (Human Science Research Resources Bank, Osaka, Japan), and OK92 (established from carcinoma of the tongue in our department) (8). All OSCC-derived cell lines were grown in Dulbecco's modified Eagle's medium/F-12 HAM (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) and 50 U/ml penicillin and streptomycin (Sigma). Healthy oral gingival specimens were collected from patients aged 22-35 years at Chiba University Hospital. Five independent HNOK cell lines were cultured and maintained in defined keratinocyte-SFM (Gibco BRL, Gaithusberg, Germany) (7).

Protein and mRNA extraction. Protein was extracted from the cells when they reached 80-90% confluence; they were washed twice with phosphate-buffered saline (PBS), scraped into a tube, and centrifuged briefly. The cell pellets were incubated for 30 min in a lysis buffer (LB) containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, and 10 mM Tris pH 8.0, and lysed by sonication (3x10 sec pulses on ice). After the sample was centrifuged at 13,000 rpm for 20 min, the supernatant containing the cell proteins was recovered, and the protein concentration was measured with a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 1 mg/ml with LB. The pH of the protein sample was adjusted to 8.5 with 30 mM Tris-HCl. Total RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Each specimen of extracted RNA was stored separately at -80˚C until use.

Immunofluorescence. Cells were plated on chamber slides at 50% confluency, washed with ice-cold PBS, and fixed with 1% paraformaldehyde-PBS for 20 min, then permeabilized in PBS containing 0.2% Triton X-100 as previously described (9). The following primary antibodies were used: polyclonal anti-Septin1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution for 2 h, washed with PBS, and incubated with donkey anti-goat IgG secondary antibody labeled with Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands) for 2 h. Coverslips were examined by fluorescence microscopy. The microscope used was a Leica DMIRBE inverted stand equipped with a Leica TCS2-MP confocal system (Leica Laserteknik, Mannheim, Germany) and Coherent Mira tunable pulsed titanium sapphire laser (Coherent Laser Group, Santa Clara, CA, USA).

Western blot analysis. Protein extracts were electrophoresed on 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad), and blocked for 1 h at room temperature in 5% skim milk. Immunoblot PVDF membranes were washed five times with 0.1% Tween-20 in TBS (TBS-T), and 2 μg/ml affinity-purified rabbit antihuman Septin1 polyclonal antibody (Abcam) was added directly to the TBS-T solution for 2 h at room temperature. PVDF membranes were washed again and incubated with a 1:700 of horseradish peroxidase-conjugated antigoat IgG Envision$^+$ (Dako Japan Inc., Kyoto, Japan) as a secondary antibody for 90 min at room temperature. Finally, the membranes were incubated with ECL$^+$-horseradish peroxidase substrate solution included in the ECL$^+$ kit (Amersham Biosciences UK Ltd., UK), and immunoblotting was visualized by exposing the membrane to Hyperfilm (Amersham) (10,11).

Immunohistochemistry. Immunohistochemical staining was performed on 4-μm sections of paraffin-embedded specimens with the use of goat anti-human Septin1 polyclonal antibody (Abcam). Briefly, after deparaffinization and hydration, the slides were treated with endogenous peroxidase in 0.3% hydrogen peroxide for 30 min, after which the sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz Biotechnology) in PBS before reacting with anti-Septin1 antibody (1:200 dilution) at room temperature in a moist chamber for 2 h. Upon incubation with the primary antibody, the specimens were washed three times in PBS and treated with Envision reagent (Dako) followed by color development in 3,3'-diaminobenzidine tetrahydrochloride (Dako). Finally, the slides were lightly counterstained with hematoxylin, dehydrated with ethanol, cleaned with xylene, and mounted. As a negative control, duplicate sections were immunostained without exposure to primary antibodies. To quantitate the state of Septin1 expression, the mean percentage of positive tumor cells was determined in at least five random fields at magnification x400 in each section. The intensity of a Septin1-immunoreaction was scored as follows: 1+, weak; 2+, moderate; and 3+, intense. The percentage of positive tumor cells and the staining intensity were then multiplied to produce a Septin1-immunohistochemical staining score (12,13). A Septin1-immunohistochemistry scores are calculated as follows: the Septin1-immunohistochemistry score = (% of positive tumor cells) X the staining intensity. Cases with a Septin1 score >130.25 (the highest score for normal tissue) were defined as positive. These judgments were made by two independent pathologists, neither of whom had knowledge of the patients' clinical status. Statistical significance was evaluated by the Fisher's exact test or the Mann-Whitney U test. $P<0.05$ was considered significant.

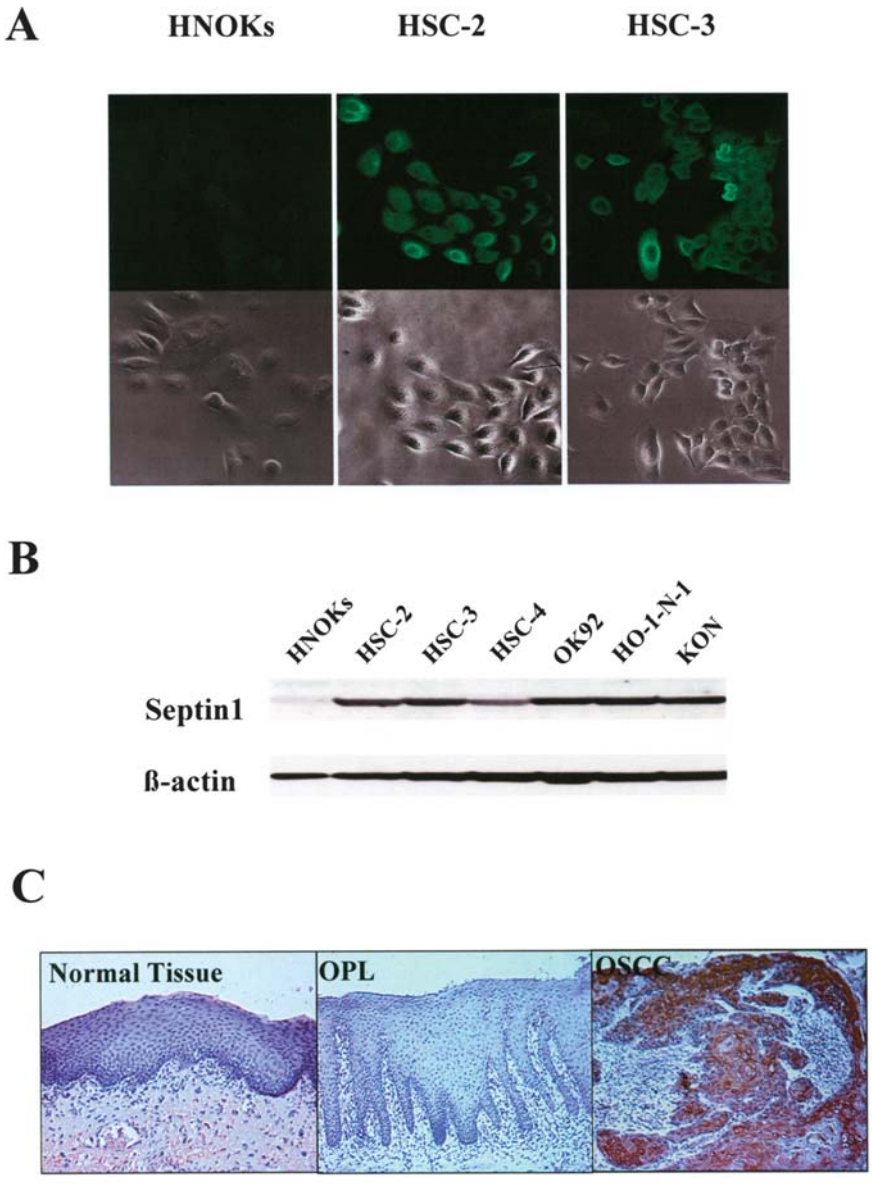

Figure 1. Septin1 expression in OSCC-derived cell lines. (A) Protein expression and subcellular localization of Septin1 in HNOks and OSCC-derived cell lines are identified by immunofluorescent staing. Septin1 is visualized with the antibody against Septin1 and Alexa Fluor 488 fragment of donkey anti-goat IgG. Immunocytochemical analysis shows strong immunoreactivity of Septin1 in OSCC-derived cell lines (HSC-2 and HSC-3) compared with the HNOKs. (B) Western blot analysis of Septin1 in OSCC-derived cell lines and HNOKs. HNOK extracts do not significantly express Septin1. OSCC-derived cell line extracts exhibiting Septin1 expression. (C) Immunohistochemical staining of Septin1 in normal and primary OSCCs. Normal oral tissue shows no Septin1 expression. The border between normal epithelium and the dysplastic lesion is seen. While no Septin1 expression is detected in normal epithelial and OPL cellular cytoplasms, strong Septin1 expression is evident in the lesion. Septin1-positive case of OSCCs. Strong positive immunoreaction for Septin1 is detected in the cytoplasm. Original magnification x200.

mRNA expression analysis. Among the OSCC cases studied by immunohistochemistry, expression levels of *septin1* mRNA were examined in 52 patients with OSCC from whom mRNA was available from primary tumors and from paired specimens of normal oral tissue. Control reactions were prepared in parallel without reverse transcriptase. In addition, expression levels of *septin1* mRNA were examined in OSCC-derived cell lines (HSC-2, HSC-3, HSC-4, OK92, HO-1-N-1, and KON) and in HNOKs. Real-time quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was performed with a single method using a LightCycler FastStart DNA Master SYBR Green 1 Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the procedure provided by the manufacturer. The primer sequences used to analyze *septin1* mRNA expression were forward 5'-AGAACCCACATCACTGCGA TTT-3' and reverse 5'-TAGCCCTCGTAGAGCAGAGTCT-3'. The sequence of specific primers was checked before use to avoid amplification of genomic DNA or pseudogenes using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The PCR reactions using LightCycler (Roche) apparatus were carried out in a final volume of 20 μl of a reaction mixture consisting of 2 μl of FirstStart DNA Master SYBR Green I mix (Roche), 3 mM $MgCl_2$, and 0.2 μl of the primers, according to the manufacturer's instructions. The reaction mixture then was loaded into glass capillary tubes and subjected to an initial denaturation

at 95˚C for 10 min, followed by 45 rounds of amplification at 95˚C (10 sec) for denaturation, 63˚C (10 sec) for annealing, and 72˚C for extension, with a temperature slope of 20˚C/sec, performed in the LightCycler. The transcript amount for the *septin1* gene was estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-CATCTCTGCCCCCT CTGCTGA-3' and reverse 5'-GGATGACCTTGCCCACAG CCT-3') transcript amount determined in corresponding samples.

Statistical analysis. The statistical significance of the gene expression levels between *septin1*-positive and *septin1*-negative cases was calculated with the Mann-Whitney U test. P<0.05 was considered significant. Data are expressed as the means (± SD) of two independent experiments with triplicate samples. The relation of protein expression and histological types of oral cancer was evaluated using the Mann-Whitney U test performed in SPSS 13.0 for Windows. Differences were considered significant at P<0.05. The disease-free survival and overall survival probabilities were calculated using the Kaplan-Meier method. The long-rank statistic was used to test the difference in survival times between the groups.

Results

Analyses of protein expression of Septin1 in OSCC-derived cell lines. We previously showed up-regulation of Septin1 in OSCC-derived cell lines (HSC-3 and HSC-2) (7). We evaluated the state of Septin1 expression in four OSCC-derived cell lines (HSC-4, OK92, HO-1-N-1, and KON) by Western blot analysis. Representative results of the analysis are shown in Fig. 1B. As expected, the size of the band was detected at 42 kDa, and a significant increase in Septin1 expression was observed in all OSCC-derived cell lines compared with the HNOKs. Western blot experiments confirmed the results from 2-D-DIGE analysis and protein identification by the mass spectrometry performed in our previous study (7).

Immunofluorescence. We examined the expression level of Septin1 based on the results of proteomic analyses by immunofluorescence analysis. Two OSCCs-derived cell lines and HNOKs were examined. Septin1 in the OSCC-derived cell lines were surrounded by obvious green fluorescence; no fluorescence was seen in any HNOKs, indicating that the data matched the proteomic study. Fig. 1A shows a representative result of the immunofluorescence analysis.

Immunohistochemistry. A total of 85 patients with OSCC were identified for whom there was adequate histologic material available for immunohistochemical analysis. The correlation between the clinicopathologic characteristics of the patients with OSCCs and the status of Septin1 expression is summarized in Table I. Normal oral mucosa specimens had no significant down-regulation of Septin1 expression and were considered Septin1-negative. Among the tumors examined, 77 of 85 cases (89%) had Septin1 immunoreactivity in the cytoplasm of the tumor cells. Representative results for Septin1 expression in normal oral tissue and primary OSCCs are shown in Fig. 1C. The Septin1 immunohistochemistry scores for normal tissues, OPLs and OSCCs ranged from 2.21 to 137.35 (mean 37.25), 19.29 to 91.27 (mean 43.57) and 50.36 to 275.0 (mean 203.8), respectively. Septin1 expression levels in primary OSCCs were significantly higher than in normal oral tissues (Mann-Whitney U test, P<0.019) (Fig. 2).

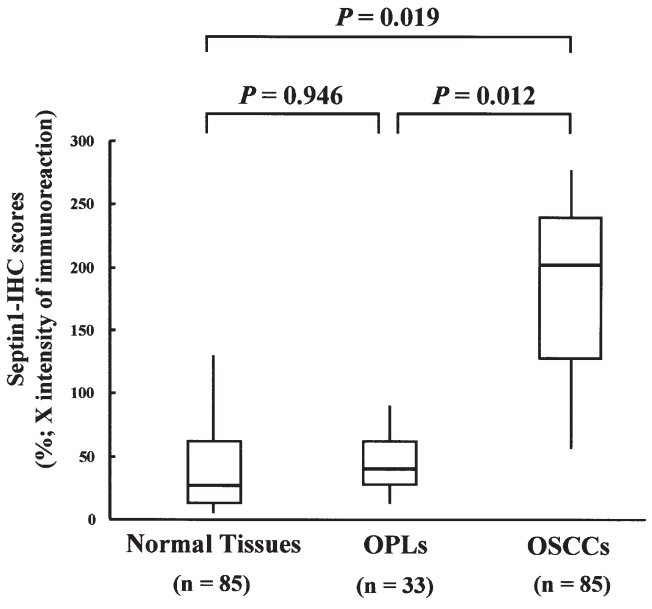

Figure 2. State of Septin1 expression in normal tissues (n =85), OPLs (n=33) and primary OSCCs (n=85). Septin1 expression in OSCCs is significantly higher than in normal oral tissues and OPLs (Mann-Whitney U test). The results represent the mean ± SD. IHC, immunohistochemistry.

mRNA expression analysis. *Septin1* mRNA expression levels were significantly increased in primary OSCCs (n=52) compared with matched non-cacerous squamous samples (n=52) (Mann-Whitney U test, P<0.010) (Fig. 3A). Relative mRNA expression levels in normal tissues, OPLs, and OSCCs ranged from 1.12 to 11.3 (mean 4.76), 1.24 to 5.26 (mean 2.57), and 3.18 to 39.1 (mean 15.74), respectively. A significant increase in the expression of *septin1* occurred in all OSCC-derived cell lines examined compared with the HNOKs (Fig. 3B). *Septin1* mRNA expression levels for the HNOKs and OSCC-derived cell lines ranged from 0.18 to 2.7 (mean 1.13) and 4.89 to 14.8 (mean 7.35), respectively. Therefore, *septin1* mRNA expression levels were consistent with the protein expression status.

Influence of Septin1 expression on patient's survival. The overall survival and disease-free survival rates for the patients with positive Septin1 expression were 84.9 and 79.8%, respectively, and negative Septin1 expression was 81.4 and 68.3% respectively (log-rank test, P>0.05) (Fig. 4). Survival analysis using the Kaplan-Meier method showed that in the group of high Septin1 expression the overall survival and disease-free survival were not significant compared with that of the group with low Septin1 expression.

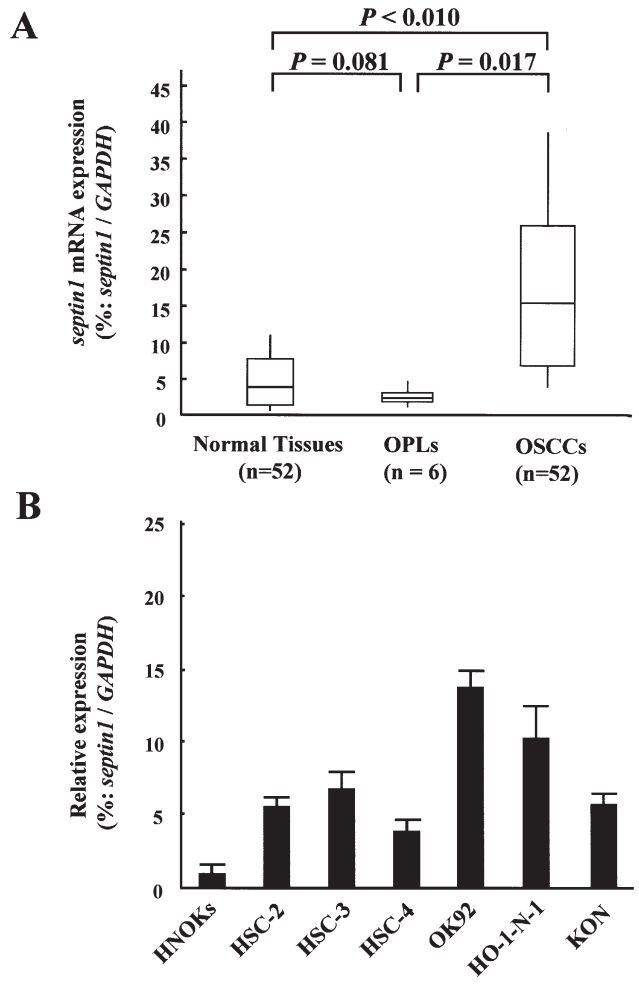

Figure 3. (A) State of *septin1* mRNA expression in normal tissues (n=52), OPLs (n=6) and primary OSCCs (n=52). *Septin1* mRNA expression in OSCCs is significantly higher than in normal tissues and OPLs (Mann-Whitney U test). The results represent the mean ± SD. (B) *Septin1* mRNA expression status in primary OSCCs and OSCC-derived cell lines. Quantification of mRNA levels in OSCC-derived cell lines by real-time QRT-PCR analysis. Significant up-regulation of *septin1* mRNA is seen in all OSCC-derived cell lines compared to *septin1* mRNA expression in HNOKs. Data are expressed as means ± SD.

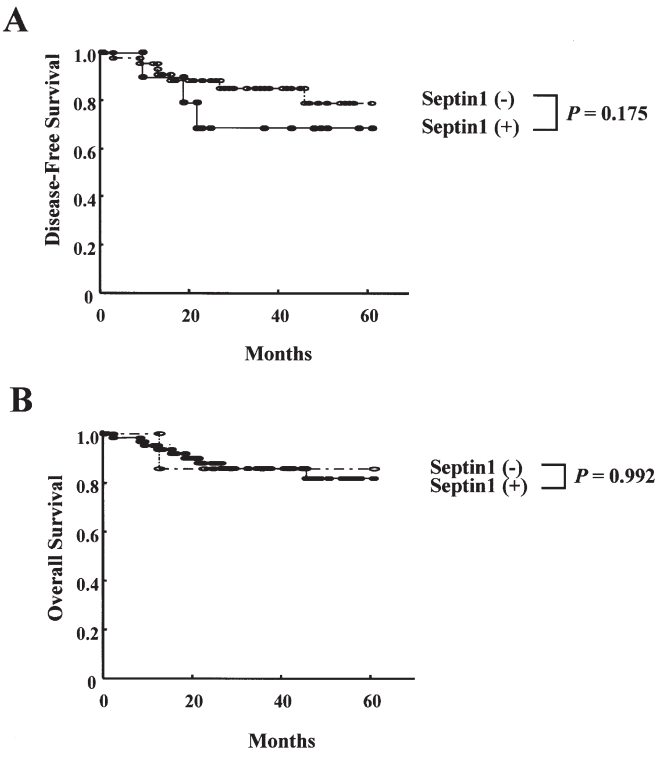

Figure 4. Kaplan-Meier survival curve for disease-free survival and overall survival rate of patients with OSCCs according to the levels of Septin1 expression. High levels of Septin1 expression are not significantly associated with lower disease-free survival (A) and overall survival (B) rates (A, P=0.175; B, P=0.992, respectively). The log-rank statistic was used to test the difference in survival times between the groups. There is no significance difference between survival and disease rate when Septin1 overexpression was observed.

Discussion

Because the functional molecules in cells are protein, proteome analysis based on 2-dimensional gel electrophoresis is believed to have several advantages over cDNA/oligonucleotide microarray systems for clinical use. Proteomic studies of clinical tumor samples identified cancer-specific protein markers, which provide a basis for developing new methods for early diagnosis and early detection and clues to understanding the molecular characterization of cancer progression (14-18). We recently reported that a significant increase in Septin1 expression was observed in OSCC-derived cell lines, using a 2-D-DIGE system and MALDI-TOF/MS (7).

Human Septins have been newly identified as a conserved family of GTPase proteins (19-28). Septin1 remains at the centrosomes and the nearby spindle region throughout mitosis and only appears at the cleavage furrow or the midbody in telophase and cytokinesis, when it partially co-localizes with Aurora-B. Septin1's role in the regulation of cytokinesis is related to its phosphorylation by Aurora-B (29). Aurora-B is a 'chromosomal passenger' protein that localizes to centromeres from prophase to metaphase, to the midzone of the mitotic spindle in anaphase, and to the midbody in telophase (30). Aurora-B plays a crucial role in chromosome segregation and cytokinesis (31,32). Overexpression of Aurora-B has been reported in several human cancer cell lines and in primary tumors including colorectal cancer (33), seminomas (34), thyroid anaplastic carcinoma (35) and non-small cell lung carcinoma (36). Based on the above evidence (29,33-36), we have hypothesized that overexpression of Septin1 is correlated with the activator of Aurora B members of chromosomal passenger protein and contributed to the development of carcinogenesis. We suggested that overexpression of Septin1 might contribute to the development of OSCCs. At present, status of Septin1 in OSCCs remains unclear and for this reason we selected Septin1 for further investigation.

To clarify the relative status of Septin1 in OSCCs, we investigated septin1 protein/mRNA expression in a series of OSCC-derived cell lines and human primary OSCCs using immunofluorescence, Western blot analysis, real-time QRT-PCR, and immunohistochemistry. Significant increases in septin1 protein/mRNA expression levels were observed in the OSCC-derived cell lines examined compared with the

HNOKs. We also found that the vast majority of the OSCCs (77 of 85 samples) showed strong tumor cell-localized cytoplasmic Septin1-immunoreaction. Moreover, by evaluating the Septin1 immunohistochemistry scores using the Mann-Whitney U test, a significant up-regulation of Septin1 was observed in primary OSCCs (P=0.019) compared with normal tissues and OPLs, and real-time QRT-PCR analysis data were matched to protein expression levels. In contrast, we could not find any significant correlation between Septin1 overexpression and clinicopathologic features with the exception of tumor differentiation (P=0.0168). Thus, we speculate that Septin1 overexpression is not a prognostic marker but is a key event in the development of OSCCs. Further studies with a greater number of clinical material will be needed to address more detailed status of Septin1 in oral carcinogenesis.

Based on our data, we conclude that Septin1 is frequently overexpressed in OSCCs, but, not in OPLs, and thus Septin1 may also play an important role in the course of oral tumorigenesis. Follow-up studies are required to determine whether this reflects a non-causative role of Septin1 expression changes or a more complex involvement in the etiology of OSCCs and may lead to the development of new approaches for effective diagnosis and therapy.

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