

## PTEN expression and methylation status in oral squamous cell carcinoma

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**Abstract.** *PTEN* is a tumor suppressor gene located on chromosome 10q23.3. In addition to genetic mutations and deletions, the down-regulation of *PTEN* has been found in various malignant tumors. However, little is known about the profile of *PTEN* gene in oral carcinomas. In this study, the expression profiles and genetic alterations of *PTEN* were examined in 113 oral squamous cell carcinoma (OSCC) cases and 9 OSCC-derived cell lines. An immunohistochemical analysis showed statistically significant differences in the immunohistochemical (IHC) scores for *PTEN* protein in normal tissues and in cancerous regions ( $P=0.0104$ ), suggesting that *PTEN* protein expression is down-regulated in OSCC. No significant correlations existed between the down-regulation of *PTEN* protein expression and the clinicopathological features of the tumor. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed a lower *PTEN* mRNA expression in the 9 OSCC-derived cell lines examined, as compared to the normal oral epithelium cells. However, treatment with a demethylating reagent restored *PTEN* mRNA expression in 4 cell lines. No genetic mutations were detected in these cell lines by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. The results suggest that epigenetic changes may be related to the down-regulation of *PTEN* expression. We therefore conclude that *PTEN* is a crucial molecule in the tumorigenesis of OSCC.

### Introduction

The *PTEN* gene was recently identified as a tumor-suppressor gene located on chromosome 10q23.3 (1,2). *PTEN* consists of 9 exons and encodes a 403-amino acid protein with the signature motif HCXXGXXR in the protein tyrosine phosphatase active sites, and has homology with the cytoskeletal proteins, tensin and auxilin (3). Tensin is an actin-binding protein localized in focal adhesion complexes. *PTEN* functions as a protein phosphatase and has been implicated in the inhibition of cell migration and invasion (4), and in the inhibition of cell cycle progression (5). Moreover, *PTEN* exhibits phosphatase activity with lipid substrates, and it is generally believed that the major substrate of *PTEN* is phosphatidylinositol (3,4,5)-triphosphate (PIP-3), a product of PI3-kinase (6). The loss of *PTEN* function results in increased concentrations of PIP-3, leading to Akt hyperactivation. Thus, *PTEN* is thought to act as a tumor suppressor gene through negative regulation of the PI3-kinase/Akt cell survival pathway (7,8).

Deletions or somatic mutations of *PTEN* have been detected in various malignant tumors, including glioma (9), breast (1), small-cell lung (10) and prostate cancer (1), endometrial carcinoma (11) and malignant melanoma (12). However, little is known about the *PTEN* gene in oral squamous cell carcinoma (OSCC). To clarify the role of *PTEN* in OSCC, 113 tissue samples of OSCC were analyzed by immunohistochemical staining, and the structural changes and methylation status of *PTEN* were examined in 9 cell lines derived from OSCC cells.

### Materials and methods

**Tissue samples and cell lines.** A total of 113 pairs of tumor and corresponding normal oral mucosa samples were obtained from 113 unrelated Japanese patients with OSCC at the time of surgical resection at the Department of Oral Surgery at Chiba University Hospital. Informed consent was obtained from all patients and their families. The OSCC cases are summarized in Table I. Resected tissues were divided into two parts, one of which was frozen immediately after careful removal of the surrounding normal tissues, and was then

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**Abbreviations:** OSCC, oral squamous cell carcinoma; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism

**Key words:** oral squamous cell carcinoma, *PTEN*, methylation

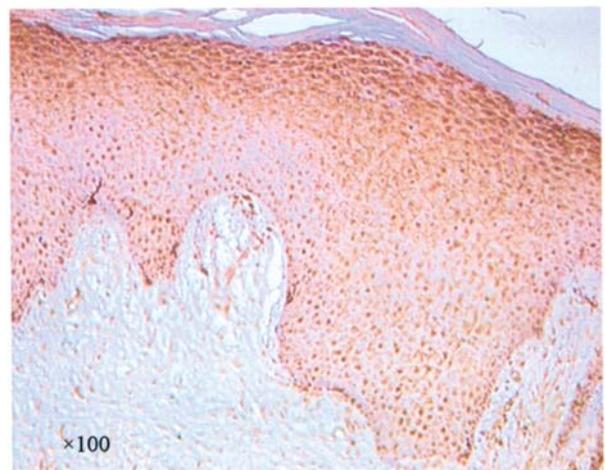
Table I. Profiles of the OSCC cases examined in the study.

Profile	Number
Age (years)	Range: 21-87 Mean: 62.8
Gender	
Male	65
Female	48
Differentiation	
Well	82
Moderate	23
Poor	8
Tumor size (T)	
T1	14
T2	50
T3	17
T4	32
Regional neck metastasis (N)	
N0	47
N1	43
N2a	3
N2b	11
N2c	8
N3a	1
Distant metastasis (M)	
M0	54
M1	3
MX	56
Stage classification	
I	11
II	26
III	21
IV	55

stored at  $-80^{\circ}\text{C}$  until DNA and RNA extraction. The other was fixed in 10% buffered formaldehyde solution for pathological diagnosis and immunohistochemical staining. A histopathological diagnosis of cancerous tissues was performed according to the International Histological Classification of Tumors by the Department of Pathology, Chiba University Hospital. The patients were diagnosed as having OSCC, and the tumor samples were examined to ensure that tumor tissue was present (>80% of specimens). Nine cell lines derived from human OSCC were analyzed: SAS, HSC-2, HSC-3, HSC-4, Ca9-22, Ho-1-u-1, Ho-1-N-1, SCC4 (Human Science Research Resource Bank, Osaka, Japan) and OK-92 (established in our department from tongue carcinoma).

**Immunohistochemical analysis.** Paraffin-embedded tissue sections ( $4\ \mu\text{m}$ ) from 113 paired primary OSCCs and adjacent normal oral tissue were used for the immunohistochemical

(A) Normal epithelial tissue. IHC score: 180.



(B) OSCC. IHC score: 15.

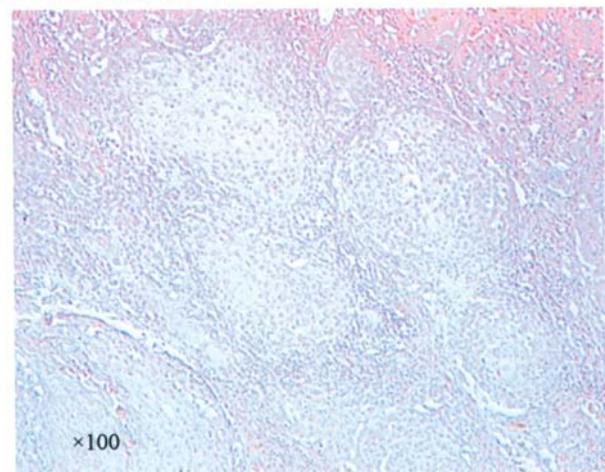


Figure 1. Immunohistochemical staining of the PTEN protein in normal and OSCC tissues. (A) Normal oral epithelial tissue. Representative image with an IHC score of 180. A strong PTEN protein expression was detected in the cytoplasm of normal oral epithelial tissue. (B) OSCC sample. Representative image showing the negative PTEN expression. Very weak immunoreactions were observed in the tumor cells. The IHC score was 15 in this case.

detection of PTEN protein. After deparaffinization and hydration, slides were heated in a microwave for 15 min in 0.01 M citrate buffer (pH 6.0), followed by rinsing three times in PBS solution. After quenching endogenous peroxidase activity in 0.3%  $\text{H}_2\text{O}_2$  for 30 min, the sections were blocked for 2 h at room temperature with 5% BSA before reacting with mouse anti-human PTEN monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:50. The sections were then incubated with primary antibody for 1 h at room temperature in a humidified chamber. The incubated sections were treated with biotinylated rabbit anti-mouse immunoglobulin (Dako Japan, Tokyo, Japan), diluted to a ratio of 1:50 in PBS for 30 min and incubated with biotin-streptavidin-peroxidase complex (Dako) for 30 min, followed by color development in 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide mixture (Dako). The slides were then lightly counterstained with hematoxylin, dehydrated

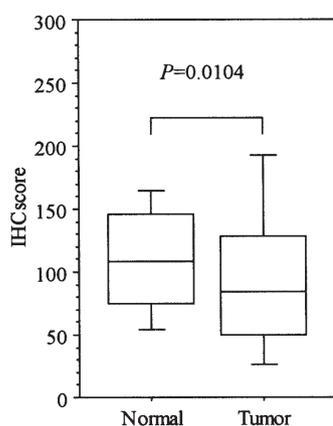


Figure 2. IHC scores in normal and tumor tissues. Median values in the normal and OSCC tissues were 109.2 and 84.0, respectively. Statistically significant differences were found between the normal and OSCC tissues ( $P=0.0104$ ).

in a graded ethanol series, cleaned in xylene and mounted. To quantitate PTEN protein expression, a scoring method was applied (13). The mean percentage values of positive tumor cells were determined on 10 fields at  $\times 400$  magnification in each section. The intensity of the PTEN immunoreactions was scored as follows: negative, 0; weak, 1; moderate, 2 and intense, 3. The percentage of positive cells and the intensity of staining were multiplied to produce a PTEN immunohistochemical staining score (IHC) for each case. These judgments were made by two independent pathologists. The IHC scores were used for analyses, and the statistical significance was evaluated by Mann-Whitney's U-test.

**RT-PCR analysis for PTEN mRNA expression.** Total RNA was isolated from 9 OSCC-derived cell lines (OSCC, SAS, HSC-2, HSC-3, HSC-4, Ca9-22, OK-92, Ho-1-u-1, Ho-1-N-1 and SCC4) using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. To synthesize first-strand cDNA, 1.5  $\mu\text{g}$  of total RNA was used for the reverse transcription reaction. The reaction was performed using a Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). To investigate the reproducible quantitative performance of the RT-PCR assay, we titrated the amount of starting cDNA and the number of amplification cycles. All subsequent assays were carried out using parameters that yielded amplification of *PTEN* and *GAPDH* genes within a linear range. cDNAs were amplified by PCR using primers specific for the *PTEN* and *GAPDH* genes (5'-CATCTCTGCCCCCTCTGCTGA-3', sense and 5'-GGATGACCTTGCCACAGCCT-3', antisense) (Table II). cDNA preparations were performed in the presence or absence of reverse transcriptase, the latter of which acted as a control for amplification of the contaminating genomic DNA. PCR analyses were performed in a 9700 Perkin-Elmer Thermal Cycler at 95°C for 10 min, 30-35 cycles of 95°C for 30-40 sec, 55°C for 30 sec and 72°C for 30 sec, followed by an extension step at 72°C for 5 min. After amplification, an aliquot of PCR products was separated on a 1.5% agarose gel, and stained with ethidium bromide. The density of the ethidium bromide-stained bands was analyzed using NIH

Image software. The results were normalized against each specific mRNA signal for *GAPDH* within the same RNA sample. cDNA derived from normal oral epithelium was used as a positive control. Reproducibility was confirmed by processing the samples at least three times.

**PCR-SSCP analysis for PTEN gene mutation.** Genomic DNA was isolated from the OSCC tissue samples and cell lines, and subjected to PCR-SSCP analyses in order to examine the mutations of the *PTEN* gene as previously described (14). Nine sets of oligonucleotide primers were used to amplify exons 1-9 of the *PTEN* gene (Table II).

**Evaluation of PTEN gene methylation status in OSCC-derived cell lines.** The chemical 5-aza-2'-deoxycytidine (5-Aza-CdR) is known to demethylate DNA and restore transcription from promoters inactivated by hypermethylation (15,16). The OSCC-derived cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum and 50 U/ml penicillin and streptomycin. Cells were treated with 2  $\mu\text{M}$  DNA methyltransferase inhibitor (5-Aza-CdR) for 5 days. As a control, cells were cultured at the same time without the 5-Aza-CdR reagent. The cells were subsequently washed with PBS and cultured for another 10 days without the demethylating reagent. Total RNA was extracted from the cells, and was subjected to cDNA synthesis and RT-PCR analysis in order to assess the restoration of the *PTEN* gene expression.

## Results

**Immunohistochemical analysis.** An immunohistochemical analysis using anti-human PTEN monoclonal antibody was performed in order to clarify the changes in PTEN protein expression in the OSCC samples. The PTEN immunoreactions were successfully detected, primarily in the cytoplasm (Fig. 1A), and weaker positive signals were seen in the OSCC samples when compared to the normal epithelium (Fig. 1B). The IHC scores for PTEN in normal and OSCC specimens ranged from 48 to 280 and 0 to 234, respectively. The mean IHC scores (standard deviation) for PTEN in normal and OSCC samples were 117.2 (52.5) and 93.6 (56.7), respectively, and the median values were 109.2 and 84.0, respectively (Fig. 2). Statistically significant differences were found between PTEN IHC scores for normal and OSCC specimens ( $P=0.0104$ ). These results indicate that the PTEN protein expression is down-regulated in OSCC samples. However, although the relationship between the immunohistochemical data and clinicopathological features was analyzed, no significant associations were found (Table III).

**Examination of the PTEN gene in OSCC cell lines.** To investigate the genetic alterations in *PTEN*, a characterization of OSCC-derived cell lines was performed. First, mRNA expression in OSCC-derived cell lines was examined by RT-PCR analysis. The density of the ethidium bromide-stained bands was analyzed using NIH Image software (Fig. 3A). Relative values versus the normal oral epithelium were calculated. A lower expression of *PTEN* mRNA was seen in the 9 cell lines examined. In HSC-3, HSC-4, Ca9-22, OK-92, Ho-1-u-1, Ho-1-N-1 and SCC4, *PTEN* mRNA expression was

Table II. Specific primer sequences for amplification of the *PTEN* gene.

	Forward	Reverse
(A) RT-PCR	5'-ATGACAGCCATCATCAAAGAG-3'	5'-AGGATATTGTGCAACTCTGCA-3'
(B) PCR-SSCP		
Ex.1	5'-AGAGCCCCGCCACCAG-3'	5'-AAAGAGGAGCCGCAGAA-3'
Ex.2	5'-TGACCACCTTTTATTACTCCA-3'	5'-AGTATCTTTTTCTCGTGGCTTA-3'
Ex.3	5'-ATAGAAGGGGTATTTGTTGGA-3'	5'-CTTCACTCTAACAAGCAGATA-3'
Ex.4	5'-TTCAGGCAATGTTTGTGA-3'	5'-TGATAATCTGGATGACTCA-3'
Ex.5	5'-CTTATTCTGAGGTTATCTTTTTTACC-3'	5'-CTCAGAATCCAGGAAGAGGA-3'
Ex.6	5'-ACATTTTTTTTCAATTTGGATTCTC-3'	5'-TAGATATGGTTAAGAAAAGTGTCC-3'
Ex.7	5'-ATCGTTTTTGACAGTTTG-3'	5'-TCCCAATGAAAGTAAAGTACA-3'
Ex.8	5'-AGGACAAAATGTTTCACTTTTGGG-3'	5'-ACATACATACAAGTCACCAACCC-3'
Ex.9	5'-CTTTCTCRTAGGTGAAGCTCTACTT-3'	5'-TTCATGGTGTTTTATCCCTCTTGA-3'

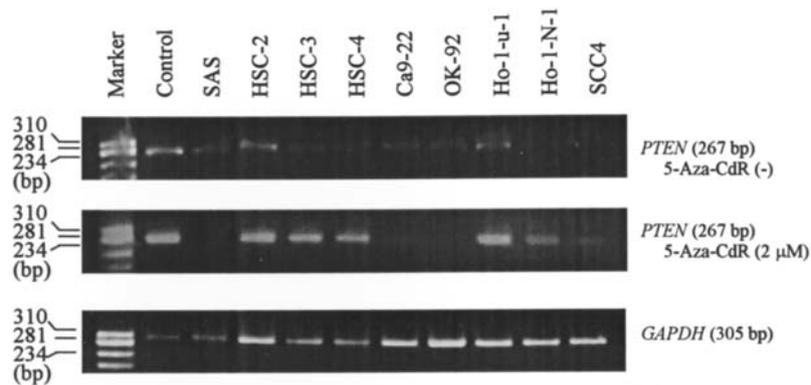
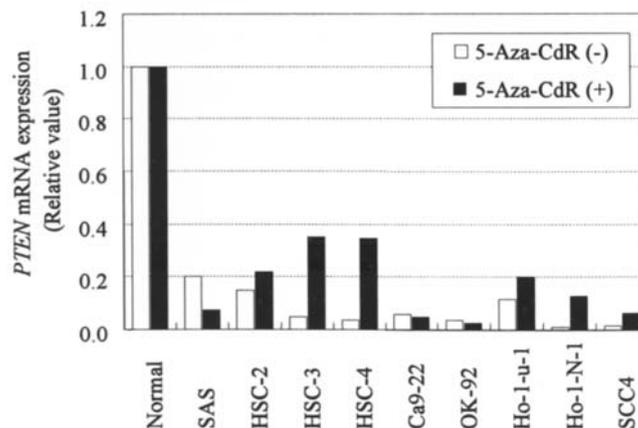
(A) RT-PCR analysis for mRNA expression of *PTEN* gene.(B) Relative mRNA expression of *PTEN* gene against that in normal oral epithelium.

Figure 3. RT-PCR analysis for mRNA expression of the *PTEN* gene in OSCC-derived cell lines. (A) RT-PCR was performed with *PTEN*- and *GAPDH*-specific primers, and PCR products were subjected to electrophoresis. The panel shows the representative electrophoresis data for PCR products running on a 1.5% agarose gel. PCR products derived from normal epithelial tissue are shown as a control. (B) Band density was quantified and expressed as relative values normalized against *GAPDH* signals. The white columns indicate *PTEN* mRNA expression without the demethylating treatment. A lower expression of *PTEN* mRNA was observed in the 9 cell lines examined. *PTEN* mRNA expression of HSC-3, HSC-4, Ca9-22, OK-92, Ho-1-u-1, Ho-1-N-1 and SCC4 was below one-tenth, indicating the apparent down-regulation of *PTEN* mRNA. The black columns indicate *PTEN* mRNA expression after the demethylating treatment with 5-Aza-CdR. The graph shows the significant down-regulation of *PTEN* mRNA in 8 out of the 9 OSCC-derived cell lines examined.

Table III. PTEN protein expression and clinicopathological profiles.

Clinicopathological profiles	Number	IHC score		P-value <sup>a</sup>
		Average	(SD)	
Tumor size (T)				
T1+T2	64	93.1	(57.2)	P=0.869
T3+T4	49	94.2	(56.5)	
Regional neck metastasis (N)				
Negative	47	81.2	(49.4)	P=0.096
Positive	66	102.4	(60.1)	
Stage classification				
I+II	37	102.7	(57.1)	P=0.276
III+IV	76	89.1	(56.3)	
Differentiation				
Well	82	95.8	(57.8)	P=0.373
Moderate + poor	31	87.6	(54.0)	

<sup>a</sup>P-value is calculated by Mann-Whitney's U-test.

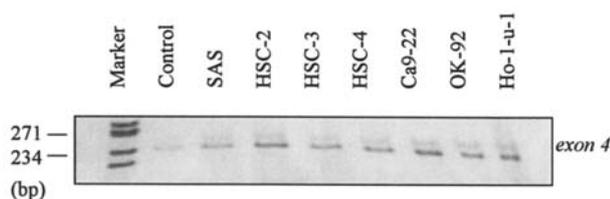


Figure 4. PCR-SSCP analysis of OSCC-derived cell lines. Representative results using *PTEN* exon 4-specific primers are shown. No mobility shift was observed in any of the exons (1 to 9).

below one-tenth, indicating the apparent down-regulation of *PTEN* mRNA (Fig. 3B). To elucidate whether the methylation status of the *PTEN* promoter is associated with the inactivation of *PTEN*, the restoration of *PTEN* mRNA expression by treatment with the demethylating reagent (5-Aza-CdR) was attempted. After treatment, a distinct recovery of *PTEN* mRNA expression was observed in HSC-3, HSC-4, Ho-1-N-1 and SCC4. These results suggest that the inactivation of *PTEN* may be due to hypermethylation of the *PTEN* gene promoter. PCR-SSCP analysis was performed to detect structural changes in the *PTEN* gene. No mobility shift was observed in SSCP patterns in exons 1-9 in any of the OSCC-derived cell lines examined. Representative PCR-SSCP data for exon 4 are shown in Fig. 4.

## Discussion

*PTEN* was previously identified as a tumor suppressor gene located in human chromosome 10q23, and its association with various carcinomas has been confirmed. In the present study, *PTEN* protein expression in OSCC samples and *PTEN* mRNA expression in OSCC-derived cell lines were examined. Immunohistochemical analysis showed that *PTEN* protein

expression was significantly lower in the OSCC regions when compared to the normal regions in OSCC samples ( $P=0.0104$ ), while RT-PCR analysis showed a distinctly lower expression of *PTEN* mRNA in 7 out of 9 OSCC-derived cell lines (77.8%). These data suggest that the down-regulated expression of *PTEN* may be associated with some types of oral squamous cell carcinoma. The role of *PTEN* in oral carcinomas remains uncertain. Squarize *et al* evaluated the expression of *PTEN* by immunohistochemical analysis in OSCC and concluded that aggressive tumors with a high malignancy did not express *PTEN*, and that *PTEN* is related to the histology and behavior of OSCC and may therefore be used as a prognostic marker (17). Shin *et al* demonstrated that the inactivation of *PTEN* by either mutation or loss of transcription is related to the pathogenesis of some oral cancers (18). These findings are comparable to our results. However, other investigators have suggested that no relationship between *PTEN* and oral carcinomas exists (19-22). Taken together, these reports suggest that *PTEN* plays a crucial but limited role in certain types of oral carcinoma.

Although no mutations were observed in *PTEN* on PCR-SSCP analysis, the down-regulated expression was recovered after treatment using the demethylation reagent in 4 out of the 6 OSCC-derived cell lines that showed a lower expression of *PTEN* mRNA, which suggests that the down-regulation of *PTEN* is due to hypermethylation. Thus, we speculate that changes in epigenetic regulation are more significant than structural changes in the *PTEN* gene in OSCC. Similar findings were reported after an analysis of the uterine cervix SCC samples, in which no mutations and few losses of heterozygosity were observed, although *PTEN* promoter methylation was detected in 58% (23). Murao *et al*, however, suggested that *PTEN* methylation was not found in cutaneous SCCs (24). Further study is necessary to determine whether this discrepancy may be based on tissue specificity.

The physiological function of PTEN in tumors has been widely discussed, and it is generally believed that the main activity is counteracting phosphatidylinositol (3,4,5)-phosphate (25). The down-regulation of PTEN results in the accumulation of phosphatidylinositol (3,4,5)-phosphate, which leads to an increased Akt phosphorylation. Furthermore, previous studies have demonstrated that PTEN dephosphorylates focal adhesion kinase (FAK) and negatively regulates the integrin function, which is closely related to cell migration, cell growth, invasion and focal adhesion (26). We believe that similar mechanisms are involved in OSCC tumorigenesis, although no studies have been performed using an OSCC system. *Pten*<sup>+/-</sup> mice showed spontaneous tumor development in the intestines, lymphoid, breast, thyroid, endometrial and adrenal glands (27-30), indicating that the role of PTEN in tumorigenesis is tissue-specific. The investigation of OSCC-specific roles for PTEN inactivation is therefore important for further therapeutic approaches.

In conclusion, the present study demonstrated that the down-regulation of PTEN protein expression was found in OSCC samples, and that a significantly decreased mRNA expression of *PTEN* was observed in 7 out of 9 OSCC-derived cell lines (77.8%). Although no mutational alterations were detected, the demethylation reagent-induced restoration of *PTEN* mRNA expression was observed in 4 out of the 7 OSCC-derived cell lines, suggesting that epigenetic changes are related to the down-regulation of PTEN expression. We thus conclude that PTEN probably plays a crucial role in the tumorigenesis of OSCC.

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