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Plasma membrane Ca^{2+} ATPase isoform 1 down-regulated in human oral cancer

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Abstract. The plasma membrane Ca^{2+} ATPase (*PMCA*) is an essential regulator of free intracellular calcium. Recent studies have reported aberrant expression of the *PMCA1* gene, a member of the PMCA family, in several cancer cell types. To elucidate the contribution of *PMCA1* to oral carcinogenesis, we analyzed genetic and epigenetic changes and mRNA and protein expression in primary oral squamous cell carcinomas (OSCCs), oral premalignant lesions (OPLs), and OSCC-derived cell lines. The *PMCA1* gene was epigenetically inactivated, but not mutated in the eight OSCC-derived cell lines tested. In clinical samples, frequent down-regulation of PMCA1 protein expression was found not only in primary OSCCs (43%), but also in OPLs (40%). Real-time quantitative reverse transcriptase-polymerase chain reaction data were consistent with the protein expression status. These results suggest that inactivation of the *PMCA1* gene is a frequent and early event during oral carcinogenesis, and gene expression may be regulated by an epigenetic mechanism.

Introduction

Intracellular Ca^{2+} has a variety of important cellular functions. Altered or abnormal free Ca^{2+} homeostasis might be involved in excessive cellular proliferation, a hallmark of tumorigenesis (1-6). Studies have suggested that proteins involved in Ca^{2+} transport and Ca^{2+} pumps might be altered in tumorigenetic cells. One such study showed that Ca^{2+}-activated chloride channel expression was reduced in human mammary gland and tumorigenic mammary gland cell lines. Moreover, aberrant expression of recoverin, a Ca^{2+} binding protein specific to the retina, has been demonstrated in a variety of cancer cell lines (4). The eukaryotic cells maintain Ca^{2+} gradients across the sarco-endoplasmic reticulum membrane and the plasma membrane. This is primarily achieved by two ion transport systems: the sarco-endoplasmic reticulum Ca^{2+} ATPase (*SERCA*) and plasma membrane Ca^{2+} ATPase (*PMCA*). Liu *et al* demonstrated that aged mice with a single functional Atp2a2 allele ($Atp2a2^{+/-}$) have a high prevalence of spontaneously developing squamous cell carcinomas (SCCs) and premalignant lesions in keratinized epithelial cells of the upper digestive organs, including the oral cavity (7). We previously reported that SERCA2, a member of the *SERCA* family, was associated with oral carcinogenesis (8). On the other hand, *PMCA* extrudes Ca^{2+} from cells and is an essential regulator of the maintenance of Ca^{2+} homeostasis. At present, four PMCA isoforms (PMCA1, 2, 3, and 4) have been identified (9). Additional isoform diversity can be generated by alternative splicing of primary *PMCA* transcripts (9). *PMCA1* and *PMCA4* are ubiquitously expressed, whereas *PMCA2* and *PMCA3* have a limited distribution in tissue. Quantitative analyses have shown that, in general, the human *PMCA1* product is more abundant than *PMCA4* both at the mRNA and protein levels (10,11).

Previous studies reported alterations in *PMCA* activity and expression in several diseases, such as hypertension (12), non-insulin-dependent diabetes mellitus (13), rhabdomyosarcoma (14), a neuroblastoma cell line (15), and breast cancer cell lines (16). Regarding oral squamous cell carcinomas (OSCC), it is unknown whether PMCA1 is associated with oral carcinogenesis. The goals of this study were to analyze both the mRNA and protein level of the *PMCA1* gene in a series of primary OSCCs, including oral premalignant lesions (OPLs) and OSCC-derived cell lines, and correlate the expression levels with clinical classifications.

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Table I. Correlation between the expression of PMCA1 and clinical classification in OSCCs.

Clinical classification	Total	Immunostaining results - no. of patients (%)		P-value
		PMCA1 (-)	PMCA1 (+)	
Age at surgery (years) (%)				
<60	32	14 (44)	18 (56)	0.184039
60-69	34	18 (53)	16 (47)	
≥70	24	11 (46)	23 (54)	
Gender (%)				
Male	50	22 (44)	28 (56)	0.121335
Female	50	21 (42)	29 (58)	
T-primary tumor (%)				
T1	17	6 (35)	11 (65)	0.465124
T2	46	20 (43)	26 (57)	
T3	16	6 (38)	10 (62)	
T4	20	11 (55)	9 (45)	
N-regional lymph node (%)				
N (-)	34	15 (44)	19 (56)	0.438578
N (+)	66	28 (42)	38 (58)	
Stage (%)				
I	13	4 (31)	9 (69)	0.561363
II	16	6 (38)	10 (62)	
III	21	12 (57)	9 (43)	
IV	47	18 (38)	29 (62)	
Histopathologic type (%)				
Well-differentiated	92	41 (45)	51 (55)	0.375825
Moderately differentiated	5	1 (20)	4 (80)	
Poorly differentiated	3	1 (33)	2 (67)	
Tumor site (%)				
Gingiva	38	15 (39)	23 (61)	0.573155
Tongue	41	19 (46)	22 (54)	
Buccal mucosa	12	6 (50)	6 (50)	
Oral floor	6	3 (50)	3 (50)	
Oropharyngeal	2	0 (0)	2 (100)	
Others	1	0 (0)	1 (100)	
Leukoplakias	32	13 (41)	19 (59)	

Materials and methods

Cell line and cell culture. We used the following OSCC-derived cell lines in this study: SAS, HSC-2, HSC-3, HSC-4, Ca9-22, Ho-1-u-1, Ho-1-N-1 (Human Science Research Resources Bank, Osaka, Japan), and OK-92 (established from carcinoma of the tongue in our department). All OSCC-derived cell lines were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 50 units/ml of penicillin and streptomycin.

Tissue samples and nucleic acid isolation. Tumors or premalignant lesions (leukoplakias) with patient-matched normal epithelium (when available) were obtained at the time of surgical resection at Chiba University Hospital after informed consent was obtained from the patients according to a protocol that was reviewed and approved by the Institutional Review Board of Chiba University. The resected tissues were divided into two parts; one was frozen immediately after removal of the surrounding normal tissue and stored at -80˚C until DNA or RNA extraction, and the other portion was fixed in 10% buffered formaldehyde solution for pathologic diagnosis and immunohistochemical staining. Histopathologic diagnosis of each cancerous tissue was performed according to the International Histological Classification (IHC) (17) of Tumors by the Department of Pathology, Chiba University Hospital. The clinicopathologic staging was determined by the TNM classification of the

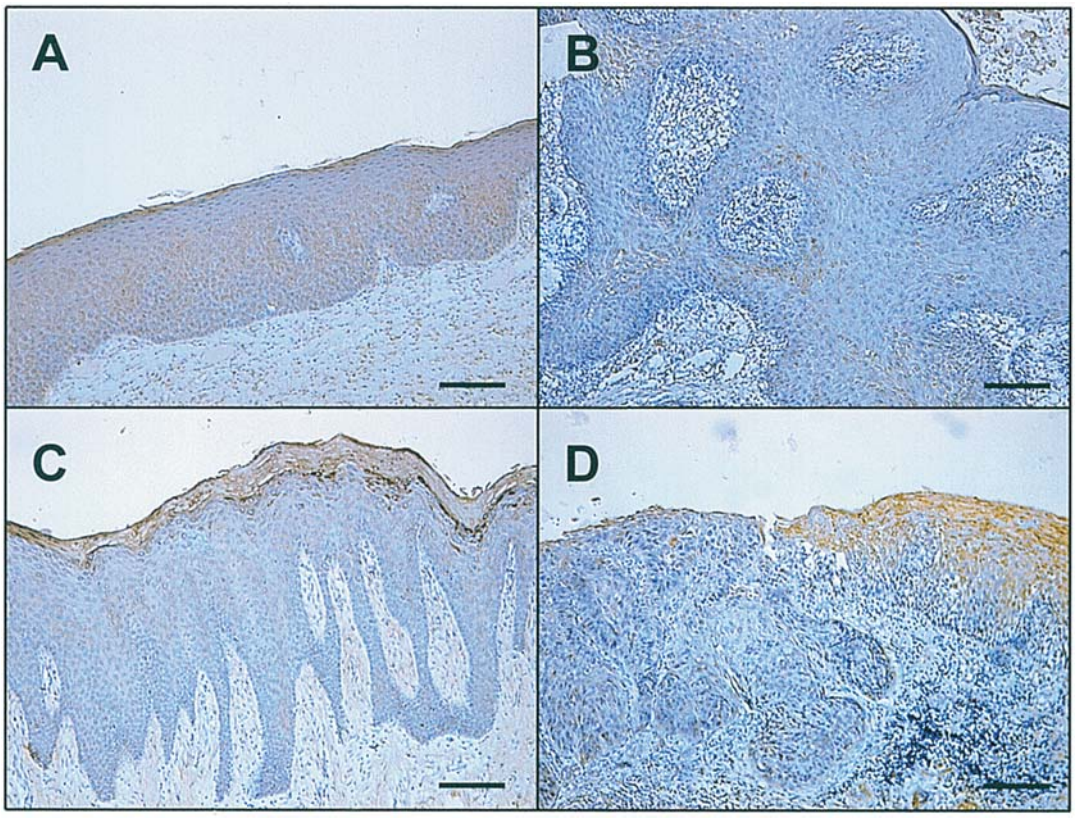

Figure 1. Immunohistochemical staining of PMCA1 in normal and tumorous oral tissue. (A) Normal oral tissue shows positive PMCA1 protein expression (original magnification, x100). (B) Most of the tumor cells of primary OSCC revealed negative staining for PMCA1 (original magnification, x100). (C) Representative result of a patient with OPL (leukoplakia). While positive PMCA immunoreaction is evident in the horny layer, cells in the spinous layer reveal a significant down-regulation of PMCA1 expression (original magnification, x100). (D) The border between normal epithelium (right side) and the dysplastic lesion (left side) is seen. While no PMCA1 expression is detected in the lesion, PMCA1 protein expression is evident in the normal epithelial cellular cytoplasm (original magnification, x100). Bar, 100 μm.

International Union against Cancer (18). All patients had SCC that was histologically confirmed, and the tumor samples were checked to ensure the presence of tumor tissue in more than 80% of the specimens. Genomic DNA was extracted by a proteinase K digestion procedure as described previously (19), and total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols.

Immunohistochemistry. Immunohistochemical staining was performed on the 4-μm paraffin-embedded specimens. Briefly, after deparaffinization and hydration, the slides were treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min, and the sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in phosphate-buffered saline (PBS) before reacting with goat anti-human PMCA1 polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:20. Sections were then incubated with primary antibody for 30 min at room temperature in a moist chamber, washed 3 times in PBS buffer and treated with biotinylated secondary antibody and avidin-biotin enzyme reagent (Santa Cruz Biotechnology), followed by color development in 3,3'-diaminobenzidine tetrahydrochloride (Dako, Kyoto, Japan). Finally, the slides were lightly counterstained with hematoxylin and mounted. As a negative control, duplicate sections were immunostained without exposure to primary antibodies. To quantitate the state of PMCA1 protein expression, a scoring method was used in which the mean percentage of positive tumor cells was determined in at least five random fields at x400 magnification in each section. The intensity of the PMCA1 immunoreaction was scored as: 1+, weak; 2+, moderate; and 3+, intense. The percentage of positive tumor cells and staining intensity were then multiplied to produce a PMCA1 IHC staining (PMCA1-IHC) score for each case. Cases with a PMCA1-IHC score <30 were considered negative. Two independent pathologists, neither of whom had knowledge of or information pertaining to the clinical status of the patients, scored the cases.

mRNA expression analysis. Among the OSCC cases studied by immunohistochemistry, the expression levels of PMCA1 mRNA were examined in 40 patients with OSCC, including 25 PMCA1-negative and 15 PMCA1-positive cases from whom RNA was available from primary tumors and paired specimens of normal oral tissue. Control reactions were prepared in parallel without reverse transcriptase. Before cDNA synthesis, residual genomic DNA was removed from total RNA with a DNase I treatment (DNA-free; Ambion, Austin, TX). The primer sequences used for analysis of *PMCA1* mRNA expression were 5'-GCATGCTCCTCCTTC AGAAC-3' (nucleotides 3073-3092) and 5'-GGTTTTCCA CCAAACTGCAC-3' (nucleotides 3270-3251). The sequence of specific primers was checked before use to avoid amplification of genomic DNA or pseudogenes using the Primer3 program (available at http://www.genome.wi.mit.

edu/cgi-bin/primer/primer3_www.cgi). The amplified products were analyzed by 3% agarose gel electrophoresis to ascertain the size and purity of the products. To confirm the identity of the polymerase chain reaction (PCR) products, they were cloned into a pCR 2.1 vector (Invitrogen) and sequenced as described previously (20).

Real-time quantitative reverse transcriptase (RT)-PCR was performed with a single method using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). To prepare the standard curve, 1.5 μg of total RNA from normal oral tissue was reverse transcribed with Superscript™ reverse transcriptase (Life Technologies, Grand Island, NY) and oligod$(T)_{12\text{-}18}$ primer, after which serial dilutions were made corresponding to cDNA transcribed from 300, 30, 3.0, and 0.3 ng of total RNA. The PCR reactions using a LightCycler (Roche Diagnostics GmbH) apparatus were carried out in a final volume of 20 μl of reaction mixture consisting of 2 μl of FirstStart DNA Master SYBR Green I mix (Roche), 3 mM $MgCl_2$, 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, 59˚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 *PMCA1* gene was estimated from the respective standard curves and normalized to a glyseraldehyde-3-phosphate dehydrogenase transcript amount determined in corresponding samples. Statistical significance of the gene expression levels between PMCA1-positive and -negative cases was calculated with the Mann-Whitney U-test. P<0.05 was considered significant.

Mutational analyses. To screen the sequence variations of the *PMCA1* gene, PCR-single strand confirmation polymorphism (SSCP) analysis was performed as described previously (21). Sets of oligonucleotide primers (n=22) were used to amplify the entire coding region (exons from 1 to 22) of the *PMCA1* gene. The sequence of each primer and each PCR condition was based on those reported previously (22). After amplification, the PCR products were electrophoresed on 10% polyacrylamide gels under several different conditions at 4˚C, 15˚C, and room temperature. After electrophoresis, the gel was silver-stained using a DNA Silver Staining kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Methylation analyses. To determine if methylation of a CpG island of the PMCA1 promoter region could contribute to the mRNA expression of PMCA1, DNA samples obtained from eight OSCC-derived cell lines were applied for the MSP assay. Bisulfite modification and DNA purification were carried out on 1 μg of *EcoR*I-digested DNA using the CpGenome™ DNA Modification kit (Intergen Discovery Products, Purchase, NY). PCR amplification for both methylated and unmethylated DNA was carried out using specific primers: sense 5'-TCCTTCTGATATATTATACTT GTAATGGGC-3' and antisense 5'-GACCTGAGCTCCATG AGAGCCCG-3' for methylated sequences and sense 5'-TTTTTTGATATATTATATTTGTAATGGGTG-3' and antisense 5'-TCCATAAAAACCCACAACTCTACA-3' for unmethylated sequences. The PCR reactions were performed in a final volume of 25 μl containing 1 μl of digested DNA, 2.5 pmol of each specific primer, 50 μM of dNTPs, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 unit of AmpliTaq (Applied Biosystems, Foster City, CA). PCR amplification was carried out in a 9700 Perking-Elmer Thermal Cycler at 95˚C for 30 sec, 35 cycles at 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 30 sec, followed by an extension step at 72˚C for 5 min. The amplified PCR products were separated on 3% agarose gel and visualized by ethidium

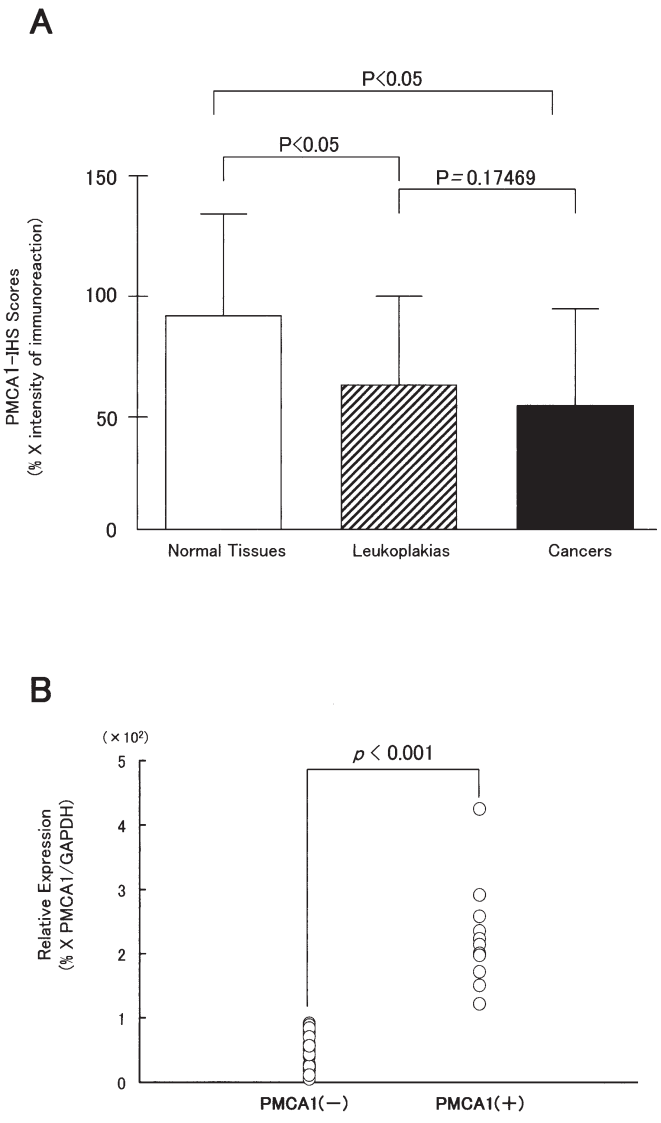

Figure 2. (A) State of PMCA1 protein expression in normal oral tissues (n=100), OPLs (n=32), and OSCCs (n=100). PMCA1-IHC scores were calculated as: PMCA1-IHC score = (the percentage of positive tumor cells) x staining intensity. PMCA1 protein expression in leukoplakias and OSCCs is significantly lower than in normal oral tissues (P<0.05; Mann-Whitney U test), whereas no statistical difference in protein expression is observed between leukoplakias and OSCCs (P=0.17469; Mann-Whitney U test). Results are expressed as means ± SD. (B) *PMCA1* mRNA expression status in primary OSCCs and OSCC-derived cell lines. Comparison of *PMCA1* mRNA expression levels between PMCA1-positive and PMCA1-negative cases classified by immunohistochemical analysis. The relative mRNA expression level in the negative and positive cases ranged from 4 to 91 (mean, 55.5), and from 150 to 2791 (mean, 578.9), respectively. There is a significant difference in the *PMCA1* mRNA expression levels between the negative and positive cases (P<0.001; Mann-Whitney U test).

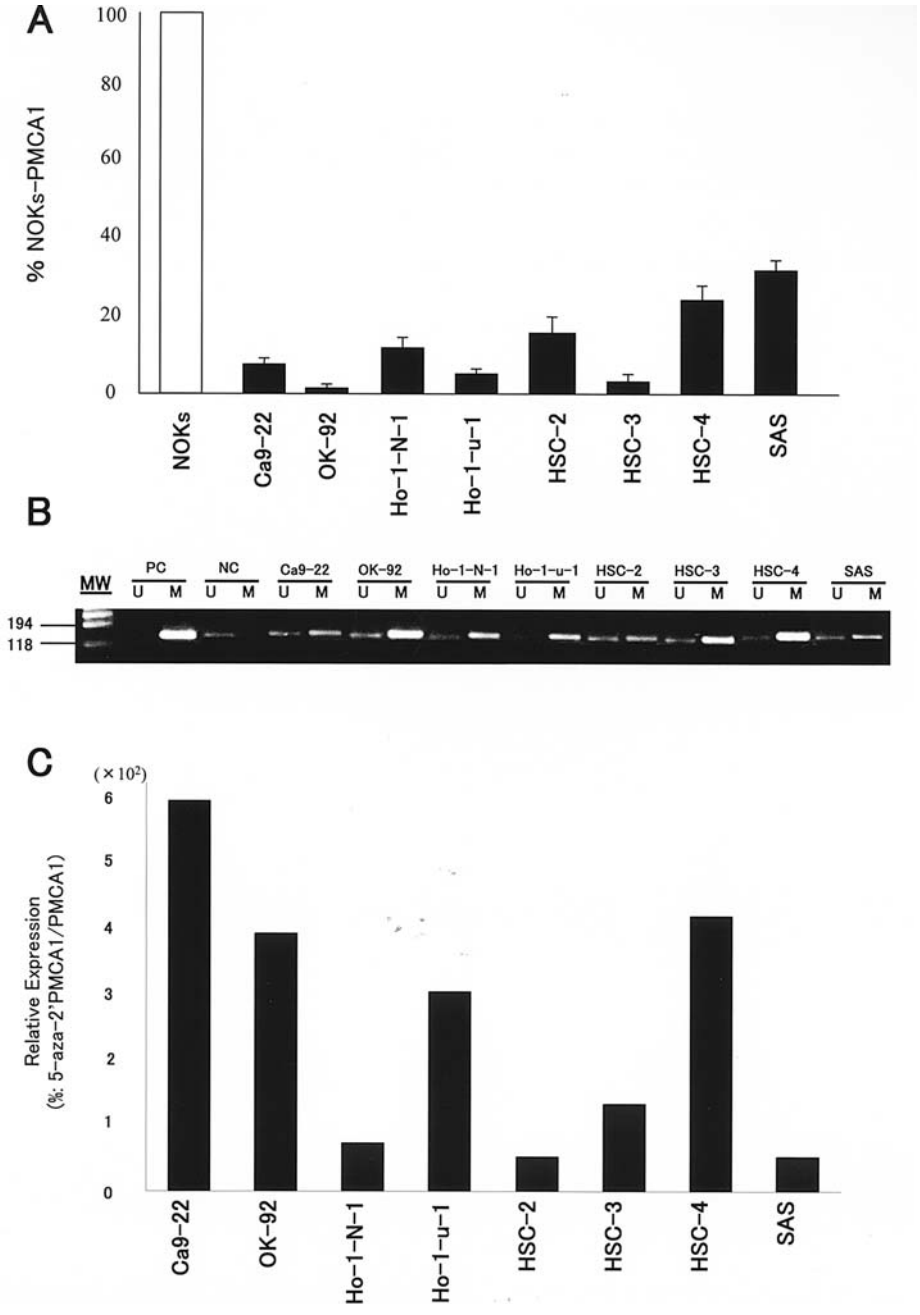

Figure 3. (A) Quantification of mRNA levels in OSCC-derived cell lines by real-time RT-PCR analysis. Significant down-regulation of the *PMCA1* gene is seen in all cell lines examined compared to *PMCA1* mRNA expression in normal oral keratinocytes (NOKs). Data are expressed as means ± SD. (B) Methylation-specific PCR analysis using specific primers for the *PMCA1* gene shows that *PMCA1* methylation is detected in all cell lines. Primer sets used for amplification are designated as unmethylated (U) or methylated (M). Peripheral blood DNA treated *in vitro* with *Sss*I methylase is used as a positive control (lane PC) for methylated alleles. The *Sss*I-untreated DNA is used as a negative control (lane NC) for methylated genes. MW, molecular weight marker (φX174-*Hae*III). (C) Real-time quantitative RT-PCR for demethylation assay in *PMCA1*-methylated OSCC cell lines. Up-regulation of *PMCA1* mRNA expression is detected in 5 of 8 OSCC-derived cell lines (Ca9-22, OK-92, Ho-1-u-1, HSC-3, and HSC-4) after 5-aza-2'-dC treatment.

bromide after the run. Peripheral blood DNA treated *in vitro* with *Sss*I methylase (New England Biolabs, Beverly, MA) was used as a positive control for methylated alleles. The *Sss*I-untreated DNA was used as a negative control for methylated genes.

To assess reactivation of PMCA1 gene expression, the cells were treated with different concentrations (0 and 2 μM) of the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-2'-dC) (Sigma Chemical Co., St. Louis, MO) as described previously (23). The following OSCC-derived cell lines were analyzed: Ho-1-u-1, Ho-1-N-1, Ca9-22, HSC-2, HSC-3, HSC-4, SAS, and OK-92. On day 5, the cells were washed with PBS and grown for another 10 days without the demethylating chemical, after which the cells were harvested. To evaluate expression of the *PMCA1* gene, RNA was extracted and a real-time quantitative RT-PCR assay was performed as described previously.

Results

Immunohistochemistry. Among 100 OSCCs analyzed by immunohistochemical staining, 43% (43 of 100) showed significantly decreased expression (P<0.001) of PMCA1 (IHC score <30) and 41% of OPLs (13 of 32 cases). In contrast,

all normal tissues showed strong membrane immunoreaction for PMCA1. There was no statistically significant difference between the state of PMCA1 expression in OSCCs and clinicopathologic features (Table I). Fig. 1 shows representative results for PMCA1 protein expression in normal oral tissue, OPLs, and primary OSCCs. The PMCA1-IHC scores for normal tissues, OPLs, and OSCCs ranged from 30 to 174 (mean, 91), 13 to 32 (mean, 61), and 0 to 152 (mean, 52), respectively. The PMCA1 expression levels in primary OSCCs and leukoplakias were significantly lower than those in normal oral tissues ($P<0.05$; Fig. 2A). In contrast, we found no significant difference in PMCA1-IHC scores between OSCCs and OPLs ($P=0.17469$; Fig. 2A).

mRNA expression. To analyze mRNA levels of the *PMCA1* gene, real-time quantitative RT-PCR was carried out. We confirmed the presence of PMCA1 mRNA by cDNA sequence analysis of a 200-bp PCR product in human oral normal tissues. The expression levels of mRNA were examined in primary OSCCs and matched adjacent normal oral tissues and eight OSCC-derived cell lines. Some 25 of 40 OSCCs (62.5%) and all cell lines showed reduced *PMCA1* gene expression (Fig. 3A).

The real-time quantitative RT-PCR analysis data were matched to those protein expression states studied by immunohistochemistry. The mRNA expression of *PMCA1* was significantly reduced in primary tumors of randomly selected PMCA1-negative cases (n=25) compared with the selected PMCA1-positive cases (n=15) ($P<0.001$; Fig. 2B). The relative mRNA expression level in the negative and positive cases ranged from 0 to 158 (mean, 53.2) and 39 to 200 (mean, 115.0), respectively.

Mutational analyses. We screened DNA samples obtained from the eight OSCC-derived cell lines by PCR-SSCP. No band shifting was detected in any exons of the *PMCA1* gene.

Methylation analyses. To investigate the mechanisms responsible for down-regulation of PMCA1 expression, we analyzed the methylation status of the PMCA1 promoter in the eight OSCC-derived cell lines. PMCA1 methylation was detected in all eight cell lines. Fig. 3B shows the result of methylation status in the cell lines.

To further study the consequences of loss of expression of PMCA1 in association with promoter hypermethylation, the eight OSCC-derived cell lines, which showed methylation and transcriptional inactivation of *PMCA1,* were subjected to 5-aza-2'-dC treatment. Significant ($P=0.02534$) up-regulated expression of the *PMCA1* gene was observed after 5-aza-2'-dC treatment in 5 of the 8 cell lines (Ca9-22, OK-92, Ho-1-u-1, HSC-3, and HSC-4) (Fig. 3C).

Discussion

Although lifestyle considerations such as the use of tobacco and alcohol are important factors associated with the development of OSCCs, some patients develop OSCC in the absence of such habits. This suggests that host susceptibility may play a role. Molecular alterations in a number of oncogenes and tumor suppressor genes associated with OSCC development could be an important clue for addressing these problems. Considerable evidence regarding mutation of the *p53* gene, hypermethylation of the *p16* gene, cyclin D1 overexpression, and loss of heterozygosity in several chromosomal locations may explain the possibility of host susceptibility (24-27). However, to date, we have little information on a useful or specific molecular marker for human OSCC.

It has been generally accepted that calcium is a ubiquitous second messenger controlling a broad range of cellular functions including growth and proliferation. Several studies showed that the increased influx of Ca^{2+} or an increase in intracellular Ca^{2+} load was associated with excessive proliferation and tumorigenesis (14-16). Lipskaia and Lompre reported that proliferation is associated with a sustained increase in cytosolic calcium due to decreased Ca^{2+} removal resulting from the inhibition of *PMCA* and *SERCA* (28). Liu *et al* first reported that most aged heterozygous mutant *SERCA2* mice developed SCCs of the forestomach, esophagus, skin, and oral cavity (7). Moreover, our previous study showed frequent down-regulation of *SERCA2* gene expression and the mutational and methylation state of SERCA2 in OSCC (8). Thus, we hypothesized that inactivation of the *PMCA1* gene also could contribute to human oral carcinogenesis, and this gene is one of the major targets of OSCC.

We found that *PMCA1* expression is decreased or lost in OSCCs and OSCC-derived cell lines, but expressed abundantly in normal oral epithelial cells. Moreover, down-regulation of protein expression was observed even in the premalignant state, and we failed to find a significant correlation between *PMCA1* expression status and any clinicopathologic features, suggesting that the marked down-regulation of *PMCA1* may play a role and can be considered a precipitous event in oral carcinogenesis.

Several studies have reported *PMCA1* gene overexpression in common cancer types, such as breast cancer (16), rhabdomyosarcoma (14), and hepatocarcinoma (29). Our results differ from those reports. At present, it is unclear why these cancers with *PMCA1* expression exist. A possible explanation is that overexpression of *PMCA* may reduce apoptosis because an excessive increase in cytosolic calcium causes cell death (30).

The current study evaluated the state of the *PMCA1* gene mutation and its mRNA/protein expression in primary OSCCs, OPLs, and OSCC-derived cell lines. When DNA from OSCC-derived cell lines was analyzed, we did not detect any mutation in the entire coding region of the *PMCA1* gene by PCR-SSCP. In contrast to mutational analyses, real-time quantitative RT-PCR analysis of *PMCA1* mRNA showed frequent down-regulation of the gene (8/8, 100%), indicating that other mechanisms, such as post-transcriptional modification and up-regulated degradation, may be involved in *PMCA1* gene silencing. In this context, with accumulating knowledge of the mechanism of inactivation of tumor suppressor genes, abnormal methylation at the promoters of tumor suppressor genes is another mechanism that suppresses gene activity. In cases of oral tumors, the promoters of several tumor-suppressor genes, such as *p16*, *p15*, *p14*, and *E-cadherin*, are highly methylated in addition to the presence of a rare gene mutation in human OSCCs (25,31,32). In the current study, we found an association between methylation

ression of *PMCA1* gene expression. Furthermore, t of OSCC-derived cell lines showing *PMCA1* methylation with a demethylating agent was effective in restoring or significantly up-regulating *PMCA1* mRNA expression. Thus, these results suggest that the *PMCA1* gene may act as a class II tumor suppressor gene that is structurally intact in sequence, but underexpressed or unexpressed due to down-regulation or silencing in transcription or translation (33).

In conclusion, our results suggest that down-regulation of *PMCA1* is an early event during OSCC development and DNA methylation may play a role in gene inactivation. Further studies with a greater number of clinical samples will improve our ability to diagnose, prevent, and treat this neoplasm.

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