Down-regulation of 1D-myo-inositol 1,4,5-trisphosphate 3-kinase A protein expression in oral squamous cell carcinoma

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Abstract. Functional proteomics is a useful method to explore changes in protein expression in human diseases, including carcinomas. To identify tumor-associated proteins as biomarkers or molecular targets of human oral squamous cell carcinomas (OSCCs), we performed two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Comparison of the protein expression profiles of OSCC cell lines and normal oral keratinocytes identified six proteins with markedly different expression levels. Of the six proteins, we found a 1D-myo-inositol 1,4,5-trisphosphate 3-kinase A (ITPKA) protein that was down-regulated in OSCC cell lines. ITPKA phosphorylates inositol 1,4,5-trisphosphate, which regulates the calcium (Ca^{2+}) level within the cell by releasing Ca^{2+} from intracellular stores, and is responsible for regulating the levels of a large number of inositol polyphosphates that are important in cellular signaling. Western blots revealed dramatically down-regulated ITPKA expression in all OSCC cell lines examined. Real-time quantitative reverse transcriptase-polymerase chain reaction showed down-regulated *ITPKA* mRNA expression in nine of 12 (75%) OSCC cell lines. Immunohistochemistry analysis showed that 40 of 100 OSCC clinical samples had a significant decrease in ITPKA. Poorly differentiated tumors showed significantly lower immunoreactivity of the protein compared to well- and moderately-differentiated tumors. These data suggest that ITPKA may be related to carcinogenesis by the modulation of inositol polyphosphates and Ca^{2+} homeostasis and that ITPKA may be a potential novel molecular target, biomarker, parameter, or all of these of cellular differentiation and of intracellular Ca^{2+} homeostatic characteristics in clinical medicine.

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Introduction

Biological responses consist of protein components resulting from transcriptional control, post-transcriptional regulation, and post-translational modifications. Thus, proteome analysis can provide important information in the study of the biological sciences. To identify the proteins associated with the development of human oral squamous cell carcinoma (OSCC), we compared the protein expression of OSCC cell lines with normal oral keratinocytes (NOKs), using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-mass). We found that 1D-myo-inositol 1,4,5-trisphosphate 3-kinase A protein (ITPKA) has markedly decreased expression in OSCC cell lines. To our knowledge, ITPKA has not been reported in oncogenesis. ITPKA phosphorylates inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which regulates intracellular calcium (Ca^{2+}) homeostasis (1-3).

Ca^{2+} is a ubiquitous intracellular signal responsible for controlling numerous cellular processes, including fertilization, cell growth, transformation, secretion, smooth muscle contraction, sensory perception, and neuronal signaling (4,5). Intracellular Ca^{2+} levels are buffered to a low concentration (100 nM) and rapidly rise to 1 μM or more in response to incoming signals (6). A second messenger, $Ins(1,4,5)P_3$, is part of various internal Ca^{2+} signals that regulate many different cellular functions (7). When the extracellular agonists, such as hormones and neurotransmitters, occupy a cell surface receptor, phosphatidylinositol 4,5-bis-phosphate in plasma membranes is hydrolyzed to $Ins(1,4,5)P_3$ and 1,2-diacylglycerol (DAG) by phospholipase C activity that is enhanced by guanosine triphosphate (GTP). The primary function of $Ins(1,4,5)P_3$ is to mobilize Ca^{2+} from intracellular stores, probably in the endoplasmic reticulum, by binding to $Ins(1,4,5)P_3$ receptors, whereas DAG is a direct activator of protein kinase C (1-3,8-12). This bifurcating messenger system operates throughout the life of a typical cell in a variety of specialized cells in animals and plants (7). The levels of $Ins(1,4,5)P_3$ are tightly regulated and rapidly metabolized by two mechanisms: dephosphorylation via an $Ins(1,4,5)P_3$ 5-phosphatase to inositol 1,4-bisphosphate $(Ins(1,4)P_2)$ or phosphorylation via an inositol 1,4,5-trisphosphate 3-kinase (IPK3) to inositol 1,3,4,5-tetrakisphosphate $(Ins(1,3,4,5)P_4)$ (3). Human IPK3 exists as three isoforms, ITPKA, B, and C, of which ITPKA is the most highly characterized isoform and is expressed intensely in rat brain and testes (3,13-15). ITPKA protein encoded by the gene located on chromosome 15q14-q21 is approximately 51 kDa and consists of 461 amino acids. IPK3 activity is stimulated in the presence of Ca^{2+}/calmodulin via phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, or Ca^{2+}/calmodulin-dependent protein kinase II (3,14,16-18).

It is well known that impaired intracellular Ca^{2+} homeostasis, which ITPKA partly regulates, causes epithelial disorders. For instance, mutations in ATP2C1 (SPCA1), encoding a Ca^{2+} pump (Ca^{2+}-transport ATPase), cause Hailey-Hailey disease, which is an autosomal dominant disorder characterized by persistent blisters and erosions of the skin with impaired adhesion of keratinocytes (19,20). In addition, mutations in ATP2A2 (SERCA2), encoding a Ca^{2+} pump (Ca^{2+}-transport ATPase) located in the scarcoendoplasmic reticulum (ER) and sequestering Ca^{2+} from the cytosol into the ER, cause Darier-White disease, an autosomal dominant disorder characterized by loss of adhesion in epidermal cells and abnormal keratinization (21-23). Mice with a SERCA2 heterozygous mutant have a high rate of spontaneously developing SCCs and premalignant lesions in keratinized epithelial cells of the upper digestive organs, including the oral cavity (24,25). Endo *et al* showed that ATP2A2 is downregulated in human OSSCs (26). Furthermore, IgG from pemphigus, characterized by blister formation on the skin, causes a transient increase in the intracellular Ca^{2+} by $Ins(1,4,5)P_3$ (27). Based on this evidence, we can presume that ITPKA is related to intracellular Ca^{2+} homeostasis and also causes epithelial disorders, including OSCCs.

To verify the result of proteome analysis using 2D-PAGE and MALDI-TOF-mass and to investigate the contribution of changes in ITPKA to carcinogenesis in the oral squamous epithelium, we also analyzed the mRNA and protein levels in OSCC cell lines and NOKs and expression levels in immunohistochemistry (IHC) of OSCC clinical samples.

Materials and methods

Cell cultures. We used the human OSCC cell lines HSC-2, HSC-3, HSC-4, Ho-1-N-1, Ca9-22, SKN3, Kosc2, KON, SAT, SAS (Human Science Research Resources Bank, Osaka, Japan), OK92 (established from carcinoma of the tongue in our department), and Sa3 and H1 (kindly provided by Dr Fujita at Wakayama Medical University, Wakayama, Japan). All cell lines were maintained at 37˚C (humidified atmosphere 5% CO_2/95% air) on 150x20-mm tissue culture dishes (Nunc, Roskilde, Denmark) and cultured in Dulbecco's modified Eagle's medium F-12 HAM (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Sigma) and 50 units/ml penicillin and streptomycin.

Five NOK strains from five patients who had undergone dental surgery served as the controls in the experimental procedures. All patients provided written informed consent before the start of the study. The normal oral specimens were washed in Dulbecco's phosphate-buffered saline (PBS) (Sigma) and then placed overnight in 0.25% trypsin-EDTA solution (Sigma) at 4˚C. After the epithelial tissue separated from the connective tissue, it was disaggregated by incubation in 0.25% trypsin-EDTA solution for 15 min with gentle pipetting at 37˚C. Isolated epithelial cells then were seeded into Collagen I Cellware 60-mm dish biocoat cell environments (Becton-Dickinson Labware, Bedford, MA) and cultured in Keratinocyte Basal Medium-2 (Cambrex, Walkersville, MD) with 0.4% bovine pituitary extract, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% hydrocortisone, 0.1% transferrin, 0.1% epinephrine, and 0.1% GA-1000 (Cambrex).

Tissue samples for IHC. Tumors or oral leukoplakias with patient-matched normal oral tissue were obtained at the time of surgery 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 tissue was fixed in 10% buffered formaldehyde solution for pathological diagnosis and IHC staining. Histopathological diagnosis of each cancerous tissue was performed according to the International Histological Classification of Tumours 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 histologically-confirmed SCC.

2D-PAGE. We used seven OSCC cell lines (Ca9-22, OK92, Ho-1-N-1, H1, HSC-4, Sa3, SAS) and five NOK strains. When the cultured cells grew into a full monolayer, they were washed with cold PBS (Sigma) three times and treated with lysis buffer containing 30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail to generate protein lysates for 10 min at 4˚C. The cells were collected using a scraper and centrifuged at 15000 x g for 10 min at 4˚C. Protein concentrations were determined by

the Bradford method. Total protein concentrations were normalized to 1 μg/μl for all samples, and samples were stored at -20˚C until use.

For 2D-PAGE, each protein sample (100 μg) was mixed with sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonic acid, and 1% (w/v) dithiothreiol. Isoelectric focusing for the first dimension of protein separation was performed using a Multiphore II electrophoresis system (Amersham Biosciences, Piscataway, NJ). Immobiline Dry IPG strips (7 cm, pH 3.0-10.0 nonlinear IPG strips; Amersham Biosciences) were rehydrated overnight with sample/rehydration buffer mixture. The strips were subjected to electrophoresis using a ramping IPG strip (200-5000 V) focusing algorithm. After isoelectric focusing, the gel strips were equilibrated with sodium dodecyl sulfate (SDS) equilibration buffer and electrophoresed in vertical SDS-PAGE slab gels containing 12.5% acrylamide. Gels of the microdissected samples then were fixed and silver-stained using Silver Quest silver staining kit (Invitrogen Japan, K.K., Tokyo, Japan), according to the manufacturer's instructions.

2D gel analysis and MALDI-TOF-mass. The silver-stained gels were scanned into Adobe Photoshop 4.0J (Adobe, San Jose, CA) using a Umax PowerLook II scanner (Umax, Dallas, TX) and printed. Differences in protein levels were defined as clear visual differences in size, density, or both of the protein spot on the gel.

Phoretix 2D Advanced software (version 5.01; Nonlinear Dynamic, Ltd., Newcastle, UK) was used to estimate the relative differences in spot intensity for a candidate protein. Spots that were consistently and significantly different were selected for analysis by MALDI-TOF-mass. The protein spots were excised from the gels, and in-gel digestion was performed with an enzyme solution containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 12.5 ng/μl trypsin. Aliquots of the purified samples were spotted on matrix crystals of α-cyano-4-hydroxyl-cinnamic acid on a stainless-steel target and air-dried. Mass determinations were performed on the AXIMA-CFR mass spectrometer (Shimadzu Co. Ltd., Kyoto, Japan). The proteins were identified by the peptide-mass fingerprinting (PMF) method using Mascot Search on the Web (Matrix Science, Ltd., London, UK).

Reverse transcription-PCR (RT-PCR). Total RNA from 12 OSCC cell lines (HSC-2, HSC-3, HSC-4, Ho-1-N-1, OK92, Ca9-22, SKN3, Kosc2, KON, SAT, SAS, Sa3) and the NOK strains was isolated using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's protocol. Total RNA (1.0 μg) was reverse transcribed with Superscript II Rnase H⁻ reverse transcriptase (Invitrogen Corp.) and oligo(dT) primer (Sigma Genosys, Ishikari, Japan), according to the manufacturer's protocol. The primer sequences used for analysis of *ITPKA* mRNA expression were 5'-GGCGTCA GGACTTACCTAGA-3' (nucleotides 850-869) and 5'-AGC ACTTCCTCATCTCCTTG-3' (nucleotides 1163-1144). The sequence of specific primers was checked before use using the Primer3 program (http:www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) to avoid amplification of genomic DNA or pseudogenes. Real-time quantitative RT-PCR was performed using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). PCR reactions using LightCycler (Roche) 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 $MgCl_2$, 1 μM of the primers, and 50 ng of template cDNA, according to the manufacturer's instructions. The reaction mixture then was loaded into glass capillary tubes and submitted to initial denaturation at 95˚C for 10 min, followed by 45 rounds of amplification at 95˚C (10 sec) for denaturation, 68˚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 *ITPKA* gene was estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript amount determined in corresponding samples.

Western blotting. The protein samples from the 12 OSCC cell lines (HSC-2, HSC-3, HSC-4, Ho-1-N-1, OK92, Ca9-22, SKN3, Kosc2, KON, SAT, SAS, Sa3), the NOK strains, and rat brain were prepared as previously described. Protein extracts from the latter two were used as positive controls. Samples containing 30 μg of protein were electrophoresed on 12.5% SDS-polyacrylamide electrophoresis gels and transferred to Immunoblot PVDF membranes (Bio-Rad, Hercules, CA). Equal loading of protein was confirmed by silver-staining. Membranes were blocked for 1 h with 5% non-fat skim milk in 50 mM Tris (pH 8.0), 150 mM NaCl, and 1% Tween-20 (TBS-T) at room temperature. After the membranes were washed three times with TBS-T, the blots were incubated for 2 h with 0.8 μg/ml affinity-purified goat antihuman ITPKA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T containing 5% non-fat skim milk at room temperature. The membranes were washed briefly in TBS-T and incubated with a 1:5000 of antigoat IgG as secondary antibody for 20 min at room temperature. After several washings, the blots were developed using ECL and Western blotting detection reagents (Amersham), and immunoblotting was visualized by exposing the membrane to X-ray film.

IHC. Formalin-fixed, paraffin-embedded serial tissue sections were cut at 4 microns, deparaffinized in xylene, and rehydrated in a series of graded ethanol. Antigen retrieval for ITPKA was performed using citrate buffer with a microwave method. Hydrogen peroxide (0.3%) was used to quench endogenous peroxidase activity. The sections were incubated first with 1.5% blocking serum (Santa Cruz Biotechnology) for 1 h at room temperature to block non-specific staining and then with affinity-purified goat antihuman ITPKA polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:40 overnight at 4˚C in a moist chamber. Immunodetection was performed using the avidin-biotin-peroxidase complex method (Santa Cruz Biotechnology), according to the manufacturer's instruction. Finally, the slides were counterstained with hematoxylin, dehydrated, and cover-slipped with permanent mounting medium. As a negative control, duplicate sections were immunostained without exposure to primary antibodies. To quantitate ITPKA protein expression, a scoring method was used in which the mean percentage of positive tumor cells
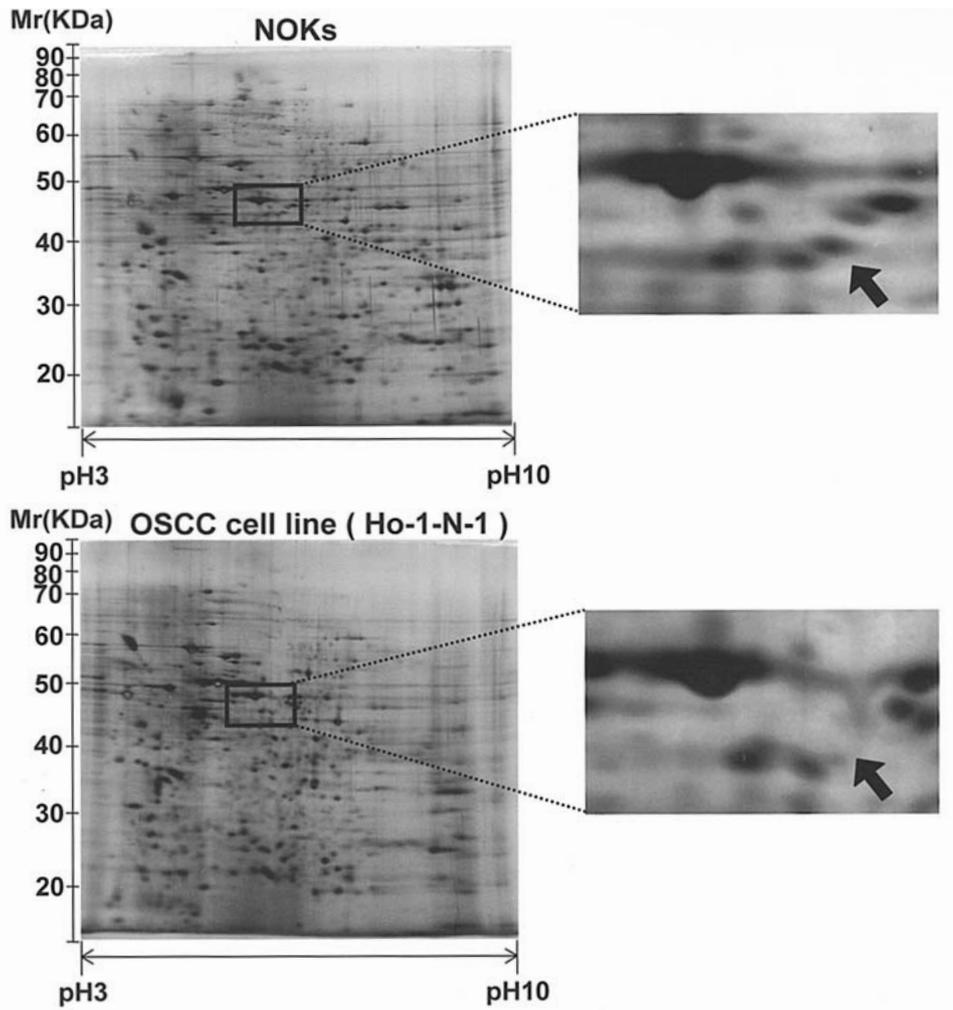
Figure 1. Representative master 2D-gel images and detailed alteration patterns of ITPKA protein in NOK strains and OSCC cell line samples.

Table I. Results of MALDI-TOF MS spectra and database analysis for protein identification.

Protein name	Peptides matched	Sequence coverage (%)	MOWSE score[a]	Change[b]
Creatine kinase, mitochondrial	8	20	97	Decrease
1D-myo-inositol 1,4,5-trisphosphate 3-kinase A	6	19	69	Decrease
Acetyl-coenzyme A acyltransferase	9	28	92	Decrease
Aldolase C, fructose-bisphosphate	9	23	86	Increase
Similar to hydroxyacyl-coenzyme A dehydrogenase	8	37	106	Increase
Dystrophin-related protein 3	8	19	70	Increase

[a]MOWSE scores over 67 were significant ($P<0.05$). [b]In OSSC cells compared to NOKs.

was determined in at least five random fields at magnification x400 in each section. The intensity of the ITPKA immuno-reaction was scored as follows: 1+, weak; 2+, moderate; and 3+, intense. The percentage of positive tumor cells and the staining intensity then were multiplied to produce an ITPKA-IHC staining score for each case. Cases with an ITPKA-IHC score of less than 38, the minimal score in normal tissue, were defined as ITPKA-negative. Two independent pathologists with no knowledge of the patients' clinical status scored the cases. Statistical differences between the ITPKA-IHC scores and the clinicopathological features were determined by the χ^2 for independence test or the Mann-Whitney U test. In

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( B )    MTLPGHPTGM ARPRGAGPCS PGLERAPRRS VGELRLLFEA RCAAVAAAAA
         AGEPRARGAK RRGGQVPNGL PRAAPAPVIP QLTVTSEEDV APASPGPPDR
         EGNWLPAAGS HLQQPRRLST SSLSSTGSSS LLEDSEDDLL SDSESRSRGN
         VQLETSEDVG QKSHWQKIRT MVNLPVMSPF KKRYSWVQLA GHTGSFKAAG
         TSGLILKRSS EPEHYCLVRL MADVLRGCVP AFHGVVERDG ESYLQLQDLL
         DGFDGPCVLD CKMGVRTYLE EELTKARERP KLRKDMYKKM LAVDPEAPTE
         EEHAQRAVTK PRYMQWREGI SSSTTLGFRI EGIKKADGSC STDFKTTRSR
         EQVTRVFEEF MQGDAEVLKR YLNRLQQIRD TLEISDFFRR HEVIGSSLLF
         VHDHCHRAGV WLIDFGKTTP LPDGQILDHR RPWEEGNRED GYLLGLDNLI
         GILANLAER
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Figure 2. (A), Mass spectometry of ITPKA by MALDI-TOF-mass. (B), The amino acid sequences of the ITPKA protein analyzed by the PMF method are underlined in the full-length sequence of the protein.

addition, statistical analysis of the ITPKA-IHC scores of histopathological type was performed using the Mann-Whitney U test. p<0.05 was considered significant.

Results

Protein separation by 2D-PAGE. Fig. 1 shows typical master gel images of the NOK strains and OSCC cell line samples. About 750 protein spots in each gel were detected using the Phoretix Two-Dimensional Advanced v5.01 analysis program. When we compared the protein spots from seven OSCC cell lines (Ca9-22, OK92, Ho-1-N-1, H1, HSC-4, Sa3, SAS) with five NOK strains, we found that three protein spots common to both showed increases and 27 protein spots common to both showed decreases.

Protein identification by PMF. We selected three protein spots that increased in the OSCC cell lines and three of the 27 that decreased in the OSCC cell lines because their expression levels were more intense than the other 24 spots in the NOK strains. Each protein spot was excised and subjected to in-gel tryptic digestion, MALDI-TOF-mass, and database matching. Isoforms with an identical primary structure in the protein matching were classified as one protein. Table I shows the six proteins identified; most of the matched proteins had high sequence coverage, mass accuracy, and MOWSE scores. Fig. 1 shows the changes in expression levels of the identified ITPKA protein in the OSCC cell lines and NOK strains. The ITPKA protein levels dramatically decreased in the OSCC cell lines.

Figure 3. *ITPKA* mRNA expression status in OSCC cell lines by real-time quantitative RT-PCR analysis. Nine of 12 (75%) OSCC cell lines have significantly down-regulated *ITPKA* mRNA expression compared to the NOK strains. Data are expressed as the means ± SD.

Fig. 2 shows the results of mass spectometry and the matched sequence of ITPKA.

Expression of ITPKA. Nine of 12 (HSC-2, HSC-3, Ho-1-N-1, OK92, Ca9-22, SKN3, Kosc2, KON, Sa3) OSCC cell lines (75%) had significantly down-regulated *ITPKA* mRNA expression on real-time quantitative RT-PCR compared with the NOK strains used as a control (Fig. 3). Three OSCC cell lines (HSC-4, SAT, SAS) did not show down-regulated *ITPKA*
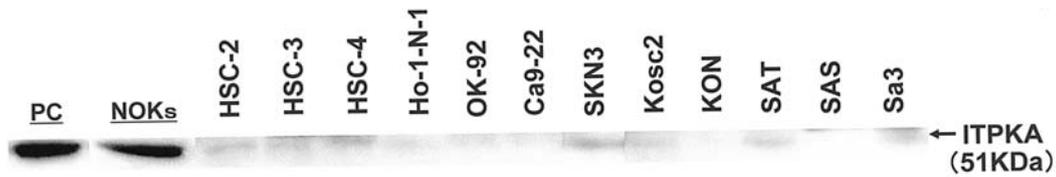
Figure 4. The ITPKA protein expression status in OSCC cell lines by Western blotting. The ITPKA protein expression of all OSCC cell lines is significantly reduced compared with that of the rat brain (lane PC) and NOK strains used as positive controls.
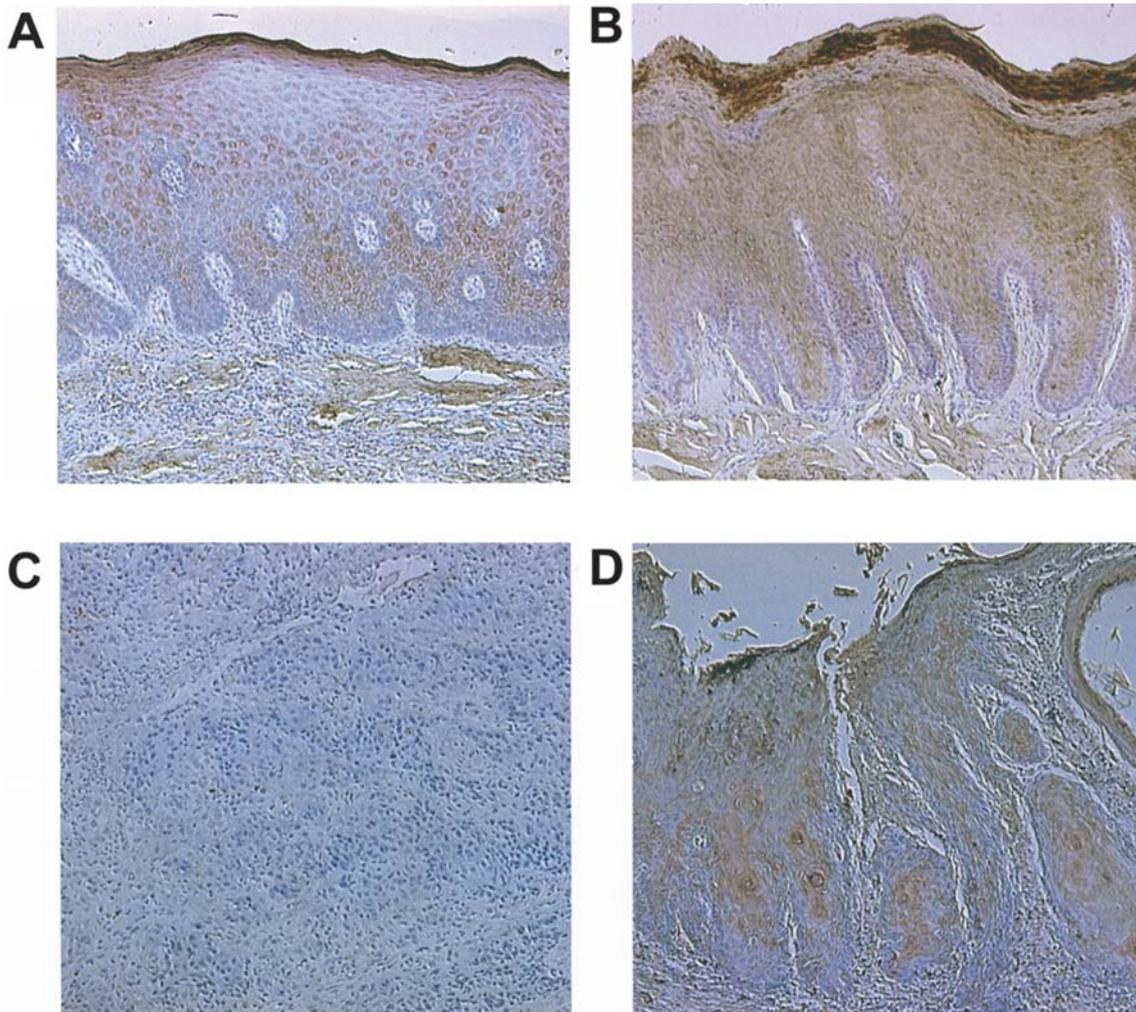

Figure 5. IHC staining of ITPKA in normal oral tissue, OPL, and primary OSCCs. (A) Normal oral tissue exhibits cytoplasmic immunoreaction of ITPKA in prickle, granular, and corneum cell layers but not in the basal cell layer. (B) OPL (leukoplakia) exhibits strong positive immunoreaction for ITPKA. (C) ITPKA-negative case of primary OSCC. (D) ITPKA-positive case of primary OSCC. Original magnification x200.

mRNA expression, although their ITPKA protein expression levels were significantly reduced. The data shown are the mean of three independent experiments with triplicate samples and are expressed as the mean ± SD.

Protein expression of ITPKA. The ITPKA protein expression levels of all OSCC cell lines were significantly reduced compared with the rat brain and NOK strains used as positive controls (Fig. 4). Western blot analysis data from six OSCC cell lines (Ca9-22, OK92, Ho-1-N-1, HSC-4, Sa3, SAS) were

matched to their respective protein expression states studied using 2D-PAGE analysis.

IHC. Among 100 OSCCs analyzed by IHC staining, 40 had significantly decreased expression of ITPKA (IHC score <38). In contrast, all normal tissue revealed a strong cytoplasmic immunoreaction of ITPKA in the prickle, granular, and corneum cell layers but not in the basal cell layer. In oral premalignant lesions (OPLs), all 33 were ITPKA-positive. The representative results for ITPKA protein expression in

Table II. Correlation between expression of ITPKA and clinical classification in OSCC.

Clinical classification	Results of immunostaining No. of patients (%)			
	Total	ITPKA (-)	ITPKA (+)	P-value
Age at surgery (years)				
<60	34	15 (44)	19 (56)	0.260
60-69	29	14 (48)	15 (52)	
≥ 70	37	11 (30)	26 (70)	
Gender				
Male	53	21 (40)	32 (60)	0.935
Female	47	19 (40)	28 (60)	
T-primary tumor				
T1	9	6 (67)	3 (33)	0.655
T2	54	25 (46)	29 (54)	
T3	9	3 (33)	6 (67)	
T4	28	6 (21)	22 (79)	
N-regional lymph node				
N (-)	68	30 (44)	38 (56)	0.220
N (+)	32	10 (31)	22 (69)	
Stage				
I	8	5 (62)	3 (38)	0.661
II	38	20 (53)	18 (47)	
III	15	5 (33)	10 (67)	
IV	39	10 (26)	29 (74)	
Histopathological type				
Well differentiated	67	22 (33)	45 (67)	0.032
Moderately differentiated	27	13 (48)	14 (52)	
Poorly differentiated	6	5 (80)	1 (20)	
Tumor site				
Gingiva	38	8 (21)	30 (79)	0.936
Tongue	37	17 (46)	20 (54)	
Buccal mucosa	11	5 (45)	6 (55)	
Oral floor	7	6 (86)	1 (14)	
Oropharyngeal isthmus	6	4 (67)	2 (33)	
Lip	1	0 (0)	1 (100)	

normal oral tissue, OPLs, and primary OSCCs are shown in Fig. 5. There were statistically significant differences in clinicopathological features between ITPKA-negative and ITPKA-positive cases by histopathological type (Table II). The ITPKA-IHC scores for normal tissue, OPLs, and primary OSCCs that are well, moderately, and poorly differentiated respectively ranged from 38-155 (mean, 52±17.2 SD), 40-162 (mean, 67±21.8 SD), 18-149 (mean, 57±34.1 SD), 14-190 (mean, 53±43.4 SD), and 14-55 (mean, 26±15.1 SD). We found some significant differences in ITPKA-IHC scores by histopathological type (Fig. 6).
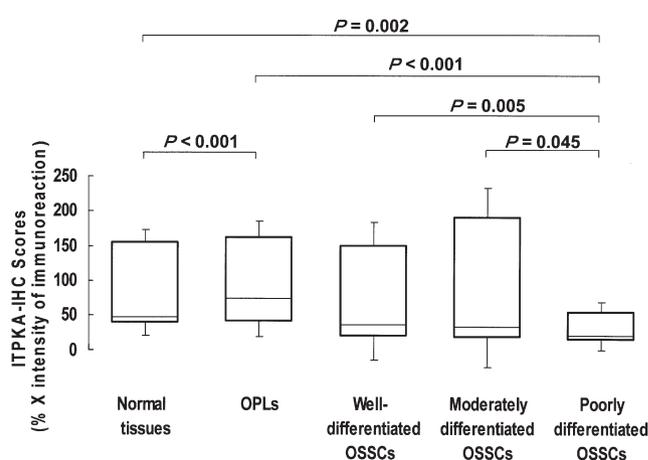

Figure 6. State of ITPKA protein expression in normal oral tissue (n=100), OPLs (n=33), primary OSCCs that are well (n=67), moderately (n=27), and poorly differentiated (n=6). There were statistically significant differences between normal oral tissue, OPLs, and primary OSCCs that are poorly differentiated. In addition, there were statistically significant differences between primary OSCCs that are well, moderately, and poorly differentiated. Results are expressed as the ranges and the means ± SD.

Discussion

We identified six proteins using 2D-PAGE analysis, MALDI-TOF-mass, and the PMF method: mitochondrial creatine kinase is responsible for the transfer of high-energy phosphate from mitochondria to the cytosolic carrier, creatine (28); ITPKA phosphorylate Ins(1,4,5)P$_3$ regulates intracellular Ca^{2+} homeostasis (1-3); acetyl-coenzyme A acyltransferase is an enzyme in the ß-oxidation system of the peroxisomes (29); idolase C, fructose-bisphosphate is a glycolytic enzyme in mammalian brain (30); hydroxyacyl-coenzyme A de-hydrogenase is an enzyme associated with fatty acid ß-oxidation (31); and dystrophin-related protein 3 is a peripheral membrane protein associated with dystrophin and dystrophin-related proteins (32). Of these, we selected ITPKA because it has not been reported in oncogenesis.

When we compared the OSCC cell lines with the NOK strains, the expression level of ITPKA protein was dramatically decreased in Western blotting, as indicated by proteomics analysis, whereas, *ITPKA* mRNA expression was down-regulated in nine of 12 (75%) OSCC cell lines. Thus, the results suggest that post-transcriptional regulation and post-translational modifications may be related to ITPKA down-expression. On the other hand, high frequent loss of hetero-zygosity (LOH) was found at chromosome 15q14-21 near the genomic region containing ITPKA, which has been reported to be the region involving the tumor suppressor gene in human tumors, including head and neck SCCs (33-39). Therefore, LOH also may be related to ITPKA down-expression.

We found statistically significant differences by histo-pathological type. As the differentiation became less pronounced, the mean IHC scores became increasingly smaller. In SCC, the differentiated degree means the difference in the keratinizing state. ITPKA expression may be related to the process of keratinization for two reasons: first, ITPKA expression was seen in the prickle, granular, and corneum cell layer but not in the basal cell layer; and second, there was

intense immunoreaction of ITPKA in the parts that were well keratinized, such as cancer pearl and OPLs. Furthermore, Ca^{2+} is a known regulator of epithelial differentiation. The increase in the intracellular Ca^{2+} level, which ITPKA partly regulates, stimulates the terminal differentiation of NOKs in culture. The inability of SCC lines to differentiate is related to a defect in achieving adequate levels of Ca^{2+} (40-44). Based on this evidence, we speculate that ITPKA expression, which is thought to control a critical level of cytosolic Ca^{2+}, may trigger the mechanisms required for initiation of SCC differentiation.

ITPKA may not only be related to differentiation of SCC by Ca^{2+} regulation but also may influence various intracellular biological responses by controlling inositol polyphosphate metabolism because ITPKA produces $Ins(1,3,4,5)P_4$ and various inositol polyphosphates that are involved in several aspects of cell regulation. For example, $Ins(1,3,4,5)P_4$ is thought to control Ca^{2+} entry from the extracellular fluid across the plasma membrane together with $Ins(1,4,5)P_3$ (2,45-50). As a result, down-regulation of ITPKA may lead to reduced Ca^{2+} influx into the cell. In addition, $Ins(1,3,4,5)P_4$ also may play a role in regulating cross-talk between Ca^{2+} and other signaling pathways, because an $Ins(1,3,4,5)P_4$ binding protein has been identified that can stimulate the GTPase activity of the *ras* and *rap* small GTP binding proteins (51,52). Moreover, $Ins(1,3,4,5)P_4$ activates K^+ channels in the plasma membrane in cooperation with the internal Ca^{2+} (53). In the same way, ITPKA down-expression may impair these functions. The physiological roles of other inositol polyphosphates, formed via the dephosphorylation or phosphorylation pathways downstream of $Ins(1,3,4,5)P_4$, have not yet been established (1,54-60). However, the function of inositol 3,4,5,6-tetrakis-phosphate regulates Ca^{2+}-dependent chloride conductance (61). Based on these reports, we presumed that decreased production of $Ins(1,3,4,5)P_4$ and inositol polyphosphates by ITPKA down-expression alters the intracellular biological responses including oncogenesis.

Although we initially hypothesized that ITPKA may be a tumor-suppressor gene for down-expression compared to NOKs in OSCC cell lines, we did not find histopathological evidence of this. Alternatively, we propose that down-regulation of ITPKA might contribute to several aspects of acquisition, progression, and maintenance of the malignant phenotype via irregular Ca^{2+} homeostasis and inositol polyphosphate metabolism. Histopathological differentiation of carcinoma frequently correlates with the prognosis of cancer. Therefore, ITPKA may be a potential novel prognostic marker, a therapeutic molecular target of cancer, or both in future studies of the complexity of cytosolic Ca^{2+} regulation using a greater number of clinical samples.

Acknowledgements

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