Decreased expression of S100A6 in oral squamous cell carcinoma

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Abstract. We previously established an *in vitro* cellular carcinogenesis model of oral squamous cell carcinoma (OSCC) with a line of human immortalized oral epithelia cells (HIOECs), a line of cancerous HB96 cells, and other cells (HB56 cells) at the early stage of carcinogenesis. In this study, comparative proteomic analysis identified a panel of differentially expressed proteins among these cells, and S100A6 was shown as one of the significantly down-regulated proteins accompanying cellular transformation. S100A6 was further validated for its expression in the three cell lines and in the clinical samples of cancerous and paracancerous tissues from 30 primary OSCC patients. Western blot analysis and real-time PCR revealed the decreased S100A6 protein and mRNA levels in the cancerous HB56 and HB96 cells over HIOECs. Immunohistochemistry and real-time PCR also showed decreased S100A6 protein and mRNA levels in the cancerous tissues compared to the paracancerous tissues from OSCC patients. The results presented here suggest that the expression of S100A6 decreases along with the cancerization in OSCC both *in vitro* and *in vivo*.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the oral and maxillofacial region. Although efforts has been made to improve the diagnosis and treatment of OSCC patients, the prognosis is still poor with a 5-year survival rate of approximately 50-60% (1,2). The mechanism of carcinogenesis of OSCC is still not very clear.

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In vitro cellular model is an important object in understanding cellular events related to pathological or physiological conditions in humans. It is an indispensable study tool in investigation of molecular mechanisms, because it has many advantages such as homogeneity of cell population, accessibility, reproducibility, controllable growth rate, and hence enough amount of material for analysis (3). Thus, *in vitro* cellular carcinogenesis model is very important, especially for cancer research, not only on the aspect of molecular biomarkers, but also on the aspect of molecular mechanisms. Previously, we have successfully established a human immortalized oral epithelia cell line (HIOEC) by transfecting HPV16 E6/E7 gene to normal human oral epithelia cells (4), and a cancerous cell line (HB96) by treating the HIOEC with benzo-[a]pyerene for 6 months, which can develop well to moderately differentiated squamous cell carcinoma in nude mice (5). An *in vitro* cellular carcinogenesis model of OSCC has also been established including the HIOECs, HB56 cells (at the early stage of cancerization), and HB96 cells (5). Based on this cellular carcinogenesis model, further comparative proteomic analysis is applied to identify the differentially expressed proteins, and the protein of S100A6 is one of them.

S100A6, an EF-hand calcium binding protein of the S100 protein family, is found in epithelial cells and fibroblasts (6) as well as in some neurons (7), Schwann cells and subpopulations of astrocytes (8). Most of previous studies on S100A6 have reported the increased expression of S100A6 in many malignancies, such as colorectal carcinoma/adenocarcinoma, gastric cancer, pancreatic ductal adenocarcinoma, cutaneous melanoma, thyroid papillary carcinoma, astrocytoma, choleteatoma, osteosarcoma, acute myeloid leukemia, and craniopharyngioma (9-18). However, decreased expression of S100A6 has also been reported in prostate cancer, thyroid anaplastic carcinoma, breast cancer, and hepatocellular carcinoma (10,11,14,19-21). In this study, we described the significantly decreased expression of S100A6 in the HB96 cells compared with the HIOECs, as well as the S100A6 expression in OSCC cell lines and tissue samples.

Materials and methods

Cell cultures. The HIOECs, HB56 cells, and HB96 cells in the *in vitro* cellular carcinogenesis model of OSCC (4,5) were

used in this study. The HIOECs were cultured in the Defined keratinocyte-SFM (Gibco, USA). The HB56 and HB96 cells were cultured in the DMEM (Dulbecco's modified Eagle's medium, Gibco) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37˚C.

Tissue samples. From February 2007 to July 2007, 30 patients with primary OSCC were involved in this study. Informed consent was obtained from each patient. None of them had received radiotherapy or chemotherapy before surgery. Surgical tissue samples of cancerous and paracancerous tissues were collected during surgery as previously described (22,23). There were 21 males and 9 females, the age ranged from 31 to 84 years with a mean of 53.8 years. The sites of primary carcinoma were tongue (n=17), buccal mucosa (n=4), retromolar region (n=3), floor of mouth (n=3), gingiva (n=2), and palatoglossal arch (n=1). The stage of disease was determined according to the tumor-node-metastasis (TNM) staging system of the International Union Against Cancer (24). The histological grade was determined according to the WHO histological criteria (25).

Two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass chromatography (LC-MS/MS). When the HIOECs and HB cells grew to 80% confluency, they were lysed in a 300-μl ice-cold lysis buffer containing 8 M Urea, 65 mM DTT, 4% (w/v) CHAPS, 40 mM Tris-HCl, and 1 mM PMSF. The protein concentration of the supernatant was determined using the Bradford dye-based protein assay reagent (Bio-Rad, USA). The first-dimensional isoelectric focusing (IEF) was performed with an IPGphor Isoelectric Focusing System (Amersham Biosciences, Sweden), 17-cm prefabricated immobilized pH gradient (IPG) strips with a linear pH range of 3-10 (Bio-Rad Catalog No. 163-2009). Total proteins (400 μg) were mixed into a 500 μl of rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, and 0.001% bromophenol blue. After rehydration for 12 h with mineral oil covering the fluid, IEF was carried out at 17˚C with a maximum current setting of 70 μA/strip using the following conditions: 250 V for 30 min in linear mode, 1000 V for 1 h in rapid mode, 10000 V for 5 h in linear mode, 10000 V for 6 h in rapid mode, and then at 500 V with the temperature maintaining at 17˚C. The gel trips were equilibrated for 2x15 min with equilibration buffer. Then, the second-dimensional SDS-PAGE was performed with a Hofer SE 600 Ruby System (Amersham Biosciences). The constant current was first 5 mA/gel and then 30 mA/gel until the bromophenol blue frontier reached the bottom of the gels. Two-dimensional standards were added to the protein samples as internal markers to determine the isoelectric point (pI) and molecular weight (M_r). After 2-DE, the analytic gels were stained with ammoniacal silver nitrate for analysis. The preparative gel was stained with Coomassie brilliant blue. The silver stained gels were scanned using a GS710 imaging densitometer (Bio-Rad) in transmissive mode. Spot detection and matching were performed using PDQuest software version 7.3.0 (Bio-Rad). Expression intensity >5.0-fold or <0.2-fold was set as a threshold indicating significant changes. In-gel tryptic digest was performed using a digestion buffer comprising 0.1 μg/μl trypsin in 25-mM ammonium bicarbonate. The peptide solutions were dried by vacuum centrifugation, desalted and cleaned using a C18 Ziptip (Millipore, USA). The peptide mixtures were separated and identified by a Finnigan LTQ mass spectrometercoupled with a Surveyor HPLC system (ThermoQuest, USA). Microcore RP column (C18 0.15x150 mm, ThermoHypersil, USA) was used to separate the protein digests, the trap column was Zorbax 300SB-C18 peptide traps (Agilent Technologies, USA). Solvent A was 0.1% formic acid, and solvent B was 0.1% formic acid in 100% acetonitrile. The gradient was increased linearly from 3 to 38% solvent B in 50 min. The peptides were electrosprayed directly into the mass spectrometer with the application of spray voltage of 3.2 kV and with the capillary temperature 170˚C. The full scan ranged from m/z 400 to 2000. Protein identification using MS/MS raw data was performed with SEQUEST program in the BioWorks 3.1 software suite (University of Washington, licensed to Thermo Finnigan) based on the IPI Human database version 3.15.1. Trypsin was selected as protein cleavage specificity. Both b ions and y ions were also included in the database search. Protein identification results were filtered with the Xcorr (≥1.9 for a 1+ tryptic peptide, ≥2.2 for a 2+ tryptic peptide, ≥3.75 for a 3+ tryptic peptide) and ΔCn (≥0.1).

Western blot analysis. Total protein was prepared from the cultured cells grown to 80% confluency, lysed in ice-cold 2X lysis buffer containing 125 mM Tris-HCl (pH 6.8), 5% w/v SDS, and 24.75% glycerol as we previously described (4,5). Each sample (2 μl) was used for protein concentration assessment using the Bradford assay (Bio-Rad). Extracted proteins (50 μg/lane) were separated using 12% SDS-PAGE. Proteins were then electrophoretically transferred onto the PVDF membranes using a wet transfer system (Invitrogen, USA). The membranes were blocked with blocking buffer containing 5% dry milk in PBS with 0.1% Tween-20 and incubated overnight with the primary antibodies of monoclonal anti-mouse S100A6 antibody (clone CACY-100, Abcam, UK) at 1:200 dilution, then incubated with fluorescent-based secondary antibodies at 1:1000 dilution for 1 h. Finally, the immunoreactive bands were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA). ß-actin was used as internal control protein.

Real-time RT-PCR. Total RNA of cultured cells was isolated from cells grown to 80% confluency using TRIzol reagent according to the manufacturer's instructions. The procedure of total RNA extraction and cDNA synthesis was performed as we previously described (22,23). The M-MLV (Promage, USA) and random primers (Amersham Biosciences, USA) were used to perform reverse transcription with 2 μg total RNA. The alterations of S00A6 was confirmed using fluorescent-based real-time PCR quantification (SYBR Premix Ex Taq, Takara, Japan) using the Thermal Cycler Dice™ Real-Time System (Takara). The primer sequences of S100A6 (NM_014624) were designed by primer premier 5.0 (Premier Biosoft International, CA), forward primer 5'-GGGAGGGTGACAAGCACAC-3', reverse primer 5'-AGCTTCGAGCCAATGGTGAG-3', the length of PCR product was

Table I. The differentially expressed proteins identified in the HIOECs, HB56 and HB96 cells.

Name of cells	Abbreviations of gene names according to the identified differentially expressed proteins
HIOECs	ACP1, ANXA1, ECH1, ETHE1, GLO1, GRB2, HCC1, ISG15, KRT7, NDUFV2, PCBP2, PFN2, PRDX1, PSMB3,Q8N849, RANBP1, RPS12, SOD2, S100A6, S100A8, TPD52L2, TPI1, TXNL4A, VBP1, ZYX, 44 kDa protein-ENSP00000319797
HB56 cells	ANXA2, CFL, KRT17
HB96 cells	ANXA2, CAPZA1, CTSB, EEF2, ERH, GAPDH, GNB1, LASP1, LGALS1, M6PRBP1, PDHB, PFN2, RANBP1, RPP2, SERPINB5, STMN1, TUBB2C, TUFM, UCHL1

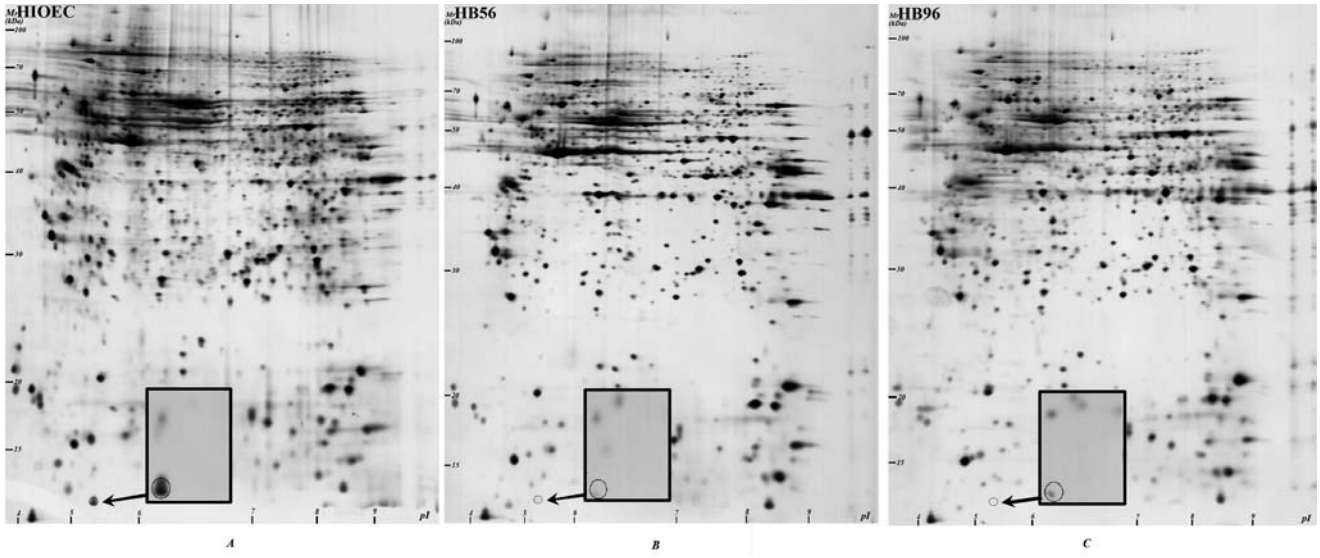


Figure 1. (A) 2-DE electrophotogram of the protein profile in the HIOECs. (B) 2-DE electrophotogram of the protein profile in the HB56 cells. (C) 2-DE electrophotograms of the protein profile in the HB96 cells. The IEF was pH 3-10. The differentially expressed protein spot of S100A6 was marked with a circle.

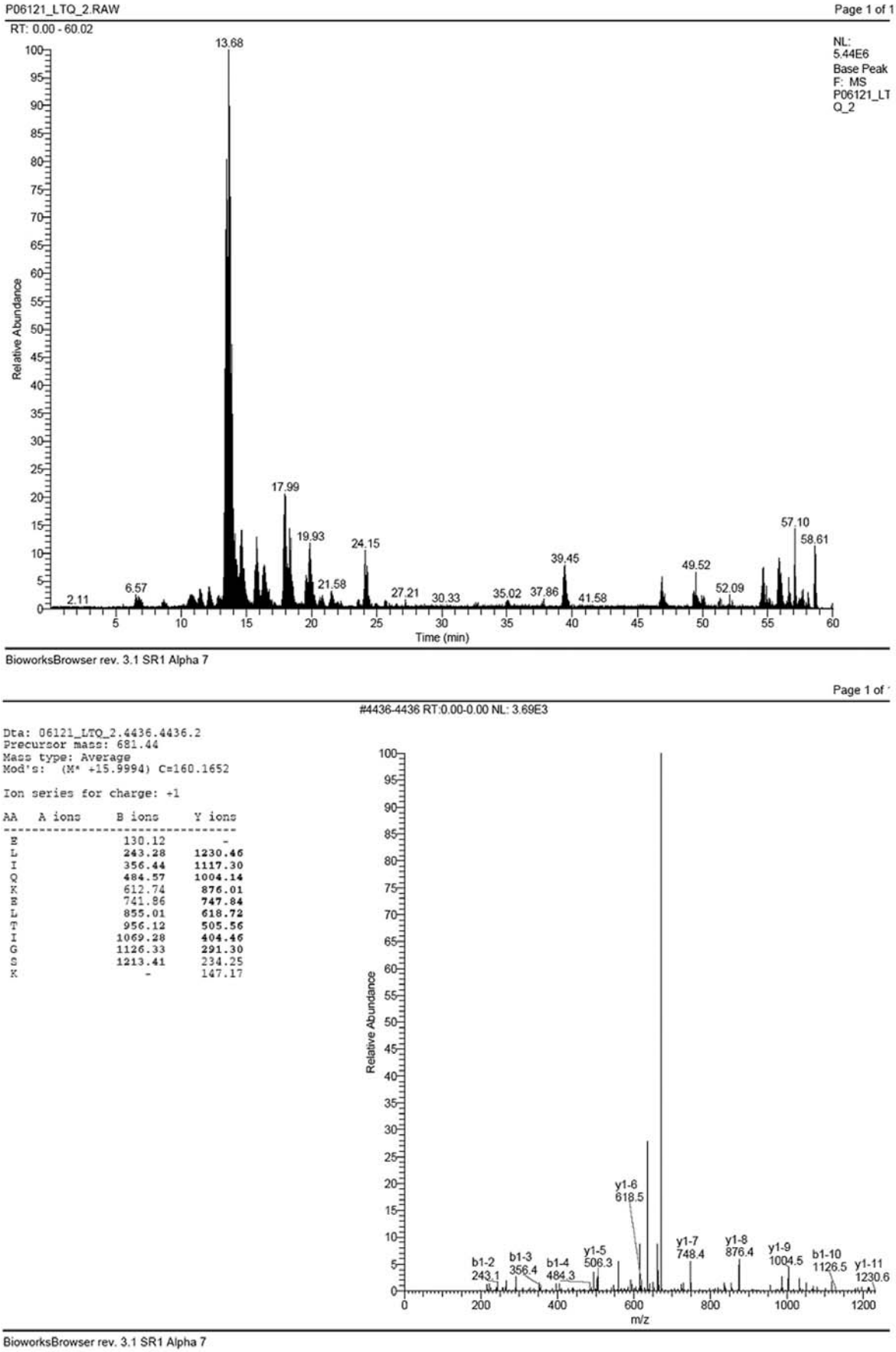
79 bp. The primer sequences of ß-actin were forward primer 5'-TCACCCACACTGTGCCCATCTACGA-3', reverse primer 5'-CAGCGGAACCGCTCATTGCCAATGG-3', the length of PCR product was 100 bp. The conditions for PCR reactions were: 95˚C for 10 sec followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing elongation at 60˚C for 30 sec. The relative quantification of S100A6 mRNA level compared with internal control gene ß-actin was calculated according to the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (26). $-\Delta\Delta Ct = [Ct_{(\text{cancerous tissue ß-actin})}-Ct_{(\text{cancerous tissue S100A6})}]/[C_{t(\text{paracancerous tissue ß-actin})}-Ct_{(\text{paracancerous tissue S100A6})}]$. All the samples were run in duplicates and the relative quantification of each target gene expression was done twice.

Immunohistochemistry. The procedure of immunohistochemistry was performed as we previously described (22,27). Briefly, after deparaffinization and endogenous peroxidase blocked, the sections were heated by water bath at 98˚C with 0.01 M citrate buffer solution (pH 6.0) for 20 min, then incubated with the monoclonal anti-mouse S100A6 antibody (clone CACY-100, Abcam, UK) at 1:100 dilution overnight at 4˚C, and visualized using DAB detection kit (Dako Cytomation, Denmark). Negative control was prepared using PBS instead of primary antibody. Microscopic examination was performed by two pathologists and all samples were blinded. The immunoreactive positive score was determined based on the proportion of stained cells on a scale of negative to strong as follows: negative, 0% of stained cells with score of 0; weak, 1-25% of stained cells with score 1; moderate, 26-50% of stained cells with score 2; and strong, >50% of stained cells with score 3.

Statistical analysis. The data were analyzed using the statistical software of SPSS10.0 for Windows (SPSS Inc., USA). The statistical difference of the initial data was analyzed using the non-parametric tests. When the P-value was <0.05, the difference was regarded as statistically significant.

Results

2-DE and LC-MS/MS. Using the two-dimensional gel electrophoresis, we compared the protein profiles between the HIOECs, HB56, and HB96 cells. There were >50 protein spots up- or down-regulated in the protein profiles between



A

Figure 2. (A) Peptide fingerprints of S100A6 identified by LC/MS-MS.

Reference	PepCount	UniquePep Count	Cover Percent	MW	PI	Identified Name							
File, Scan(s)	Sequence	MH+	Diff(MH+)	Charge	Rank	XC	DeltaCn	Sp	RSp	Ions	PI	Group Count	Protein Count
	14	6	36.67%	10179.74	5.32	IPI:IPI00027463.1|SWISS-PROT:P06703|TREMBL:Q5RHS4|ENSEMBL: ENSP00000349740|REFSEQ:NP_055439|H-INV:HIT000030408;HIT000034143| VEGA:OTTHUMP00000015472;OTTHUMP00000015473 Tax_Id=9606 Calcyclin							
P06121_LTQ_2,4436	K.ELIQKELTIGSK.L	1359.5939	-2.2701	2	1	4.0014	0.446	518.7	1	17|22	6.24	1	1
P06121_LTQ_2,4457	K.ELIQKELTIGSK.L	1359.5939	1.1019	2	1	2.9989	0.4417	949.5	1	19|22	6.24	1	1
P06121_LTQ_2,2763	K.ELTIGSK.L	747.8602	-1.6058	1	1	1.9799	0.1184	166.4	1	8|12	6.1	1	1
P06121_LTQ_2,3174	K.KELKELIQK.E	1129.3751	0.4621	2	1	2.5695	0.1241	1188.6	1	14|16	8.5	1	1
P06121_LTQ_2,2598	K.LQDAEIAR.L	916.0142	-1.4148	1	1	2.1236	0.1418	453.1	1	10|14	4.37	1	1
P06121_LTQ_2,2785	K.LQDAEIAR.L	916.0142	0.0852	2	1	2.5083	0.3172	829.9	1	13|14	4.37	1	1
P06121_LTQ_2,2796	K.LQDAEIAR.L	916.0142	-0.8498	2	1	2.6562	0.3331	1059.2	1	13|14	4.37	1	1
P06121_LTQ_2,3104	K.LQDAEIAR.L	916.0142	0.4872	2	1	2.5288	0.2624	871.5	1	13|14	4.37	1	1
P06121_LTQ_2,3709	K.LQDAEIAR.L	916.0142	0.2292	2	1	2.4984	0.3235	589.1	3	12|14	4.37	1	1
P06121_LTQ_2,9880	K.LQDAEIAR.L	916.0142	-0.8468	2	1	2.2996	0.2471	873.5	1	13|14	4.37	1	1
P06121_LTQ_2,10507	K.LQDAEIAR.L	916.0142	-1.6258	2	1	2.3119	0.2677	819	1	13|14	4.37	1	1
P06121_LTQ_2,2147	R.LM*EDLDRNK.D	1150.2883	-0.7547	2	1	2.5105	0.1242	324.4	1	12|16	4.56	1	1
P06121_LTQ_2,3017	R.LMEDLDRNK.D	1134.2889	0.1969	2	1	2.7315	0.1776	1202.1	1	14|16	4.56	1	1
P06121_LTQ_2,3027	R.LMEDLDRNK.D	1134.2889	0.9299	2	1	2.5598	0.2732	1374	1	14|16	4.56	1	1

MACPLDQAIGLLVAIFHKYSGREGDKHTLSK**KELKELIQKELTIGSKLQDAEIARLMED**
LDRNKDQEVNFQEYVTFLGALALIYNEALKG

B

Figure 2. (B) Protein identification using MS/MS raw data was performed with Sequest program in the BioWorks 3.1 software suite based on the IPI Human database version 3.15.1. Matched peptides are shown in bold and underlined.

the HIOECs, HB56, and HB96 cells (Table I). Among these protein spots, one significantly down-regulated protein in the HB cells compared with the HIOECs (marked with a circle in Fig. 1), was identified as S100A6 protein using LC-MS/MS. The peptides fingerprint and the matched peptides are shown in Fig. 2. These results were obtained by searching the IPI Human database version 3.15.1 with SEQUEST program in the BioWorks 3.1 software suite (University of Washington, licensed to Thermo Finnigan).

Protein expression and mRNA level of S100A6 in the HIOECs, HB56 cells, and HB96 cells. Using Western blot analysis, the protein expression of S100A6 in the HB56 and HB96 cells was found decreased compared with the HIOECs normalized against ß-actin (Fig. 3A and B). The protein expression of S100A6 in the cellular carcinogenesis model was identical to the comparative proteomic analysis result. The relative mRNA level of S100A6 was also found decreased in the HB56 and HB96 cells compared with the HIOECs normalized against ß-actin using real-time PCR detection (Fig. 3C).

Protein expression and mRNA level of S100A6 in tissue samples. Using immunohistochemistry, the paracancerous tissues were found strongly immunoreactive for S100A6 in the cellular membrane and cytoplasm. The positive rate of S100A6 protein in the paracancerous tissues was 96.7% (29/30); the positive rate of S100A6 in the cancerous tissues was 70.0% (21/30). The positive score of S100A6 in the cancerous tissues (1.17±0.91) was significantly lower than that in the paired paracancerous tissues (2.10±0.92) (Wilcoxon Signed Ranks test, Z=-4.365, P<0.001) (Table II). Although decreased expression of S100A6 was found in the cancerous tissues of different pathological differentiation

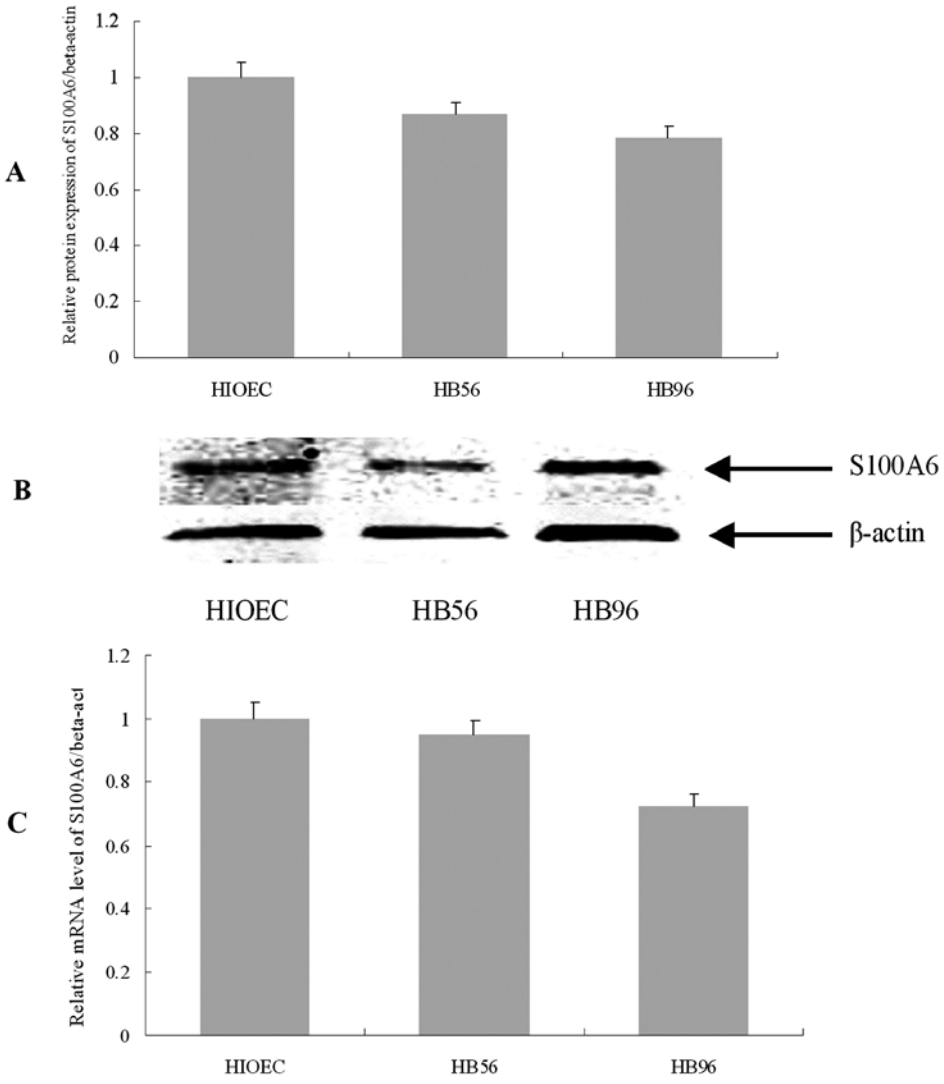


Figure 3. (A and B) Western blot analysis of S100A6 protein expression in the HIOECs, HB56 cells, and HB96 cells. The S100A6 protein expression for each sample was normalized against ß-actin. Decreased S100A6 protein expression was found in both the HB56 and HB96 cells. (C) S100A6 mRNA level in the HIOECs, HB56 cells, and HB96 cells, which was normalized against ß-actin. Decreased S100A6 mRNA level was also found in both of the HB56 and HB96 cells.

Table II. The immunohistochemical S100A6 positive scores in the different types of tissues from oral squamous cell carcinoma patients.

		S100A6 positive score			
Type of tissue	Total cases	0	1	2	3
No. of paracancerous tissue	30	1	8	8	13
No. of cancerous tissue	30	9	8	12	1

Wilcoxon Signed Ranks Test, the difference of S100A6 positive scores between the cancerous and paracancerous tissues was significant with Z= -4.365, P<0.001.

grade, the correlation between the positive score of S100A6 protein expression and pathological differentiation grade was not significant (P=0.850) (Fig. 4). Furthermore, no significant relationship was found between the S100A6 positive score with T stage (P=0.529), N stage (P=0.930), clinical stage (P=0.4892), smoking (P=0.412) and drinking (P=0.946) exposure (Table III).

The good efficacy and specificity of real-time PCR was confirmed by melting curve and 3% agarose gel electrophoresis. The distribution of relative quantification data

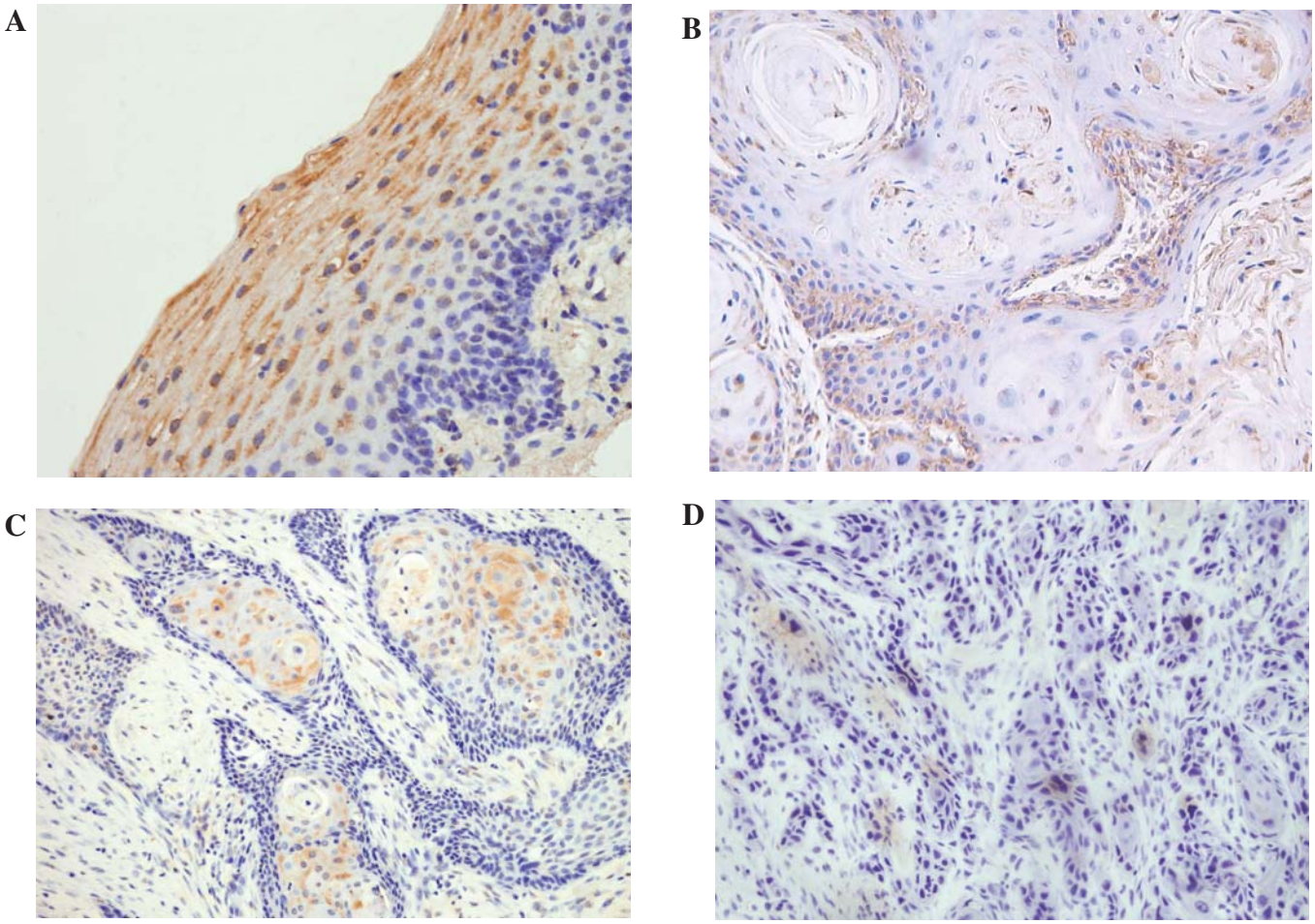


Figure 4. Immunoreactions of S100A6 protein was strong (A) in the paracancerous tissues (x200), and relatively low in the well- (B), moderately- (C), and poorly- (D) differentiated cancerous tissues (x200). The location of the protein immunoreaction was the cellular membrane and the cytoplasm.

of S100A6 mRNA level was firstly tested using one-sample Kolmogorov-Smirnov test, and the distribution was normal (P=0.258), but the CV value was 83.1%. Therefore, non-parametric analysis was performed. By descriptive statistics, S100A6 mRNA level in the cancerous tissues was 0.753-fold lower than that in the paracancerous tissues, with standard error value of 0.114, standard deviation of 0.626, and 95% of confidence interval for mean from 0.519 to 0.987. S100A6 mRNA level decreased significantly in the cancerous tissues compared with the paracancerous tissues (t=-2.163, P=0.039). The correlation between the clinicopathological characteristics and the relative S100A6 mRNA level was analyzed (Table III), and no significant difference could be found, for T stage (P=0.558), N stage (P=0.708), clinical stage (P=0.755), pathological differentiation grade (P=0.953), smoking (P=0.950), and drinking (P=0.641).

Discussion

Proteomic analysis is an accurate, sensitive and high-throughput protein identification strategy. In the research of the molecular mechanisms of diseases, comparative proteomic analysis has been used as an innovative method to investigate the protein expressions between cancerous and normal tissues/cells. Several studies on OSCC have reported the application of comparative proteomic analysis in identifying differentially expressed proteins in tissue (28-30), cell (31,32), serum (33-35), and saliva (36,37). Many differentially expressed proteins have been identified between the cancerous and normal samples. Tissue, serum and saliva samples from different subjects generally have individual variations, and their availability is limited. In contrast, cell line offers a stable supply of homogeneous samples; further *in vitro* cellular carcinogenesis model offers a very useful and important tool for cancer research, especially on the aspects of molecular biomarkers and their mechanisms. It is an indispensable study tool in investigations of molecular mechanisms, because it has many advantages such as homogeneity of cell population, accessibility, reproducibility, controllable growth rate, and hence enough amount of material for analysis (4). Park *et al* (38) has established an *in vitro* multistep carcinogenesis model for OSCC from immortalized normal human oral keratinocytes. This model has been used for the *in vitro* investigation of gene expressions (39,40). However, no systematic study of the total protein expression is reported.

Our previous work established an *in vitro* cellular carcinogenesis model of OSCC, including three kinds of HIOEC, HB56, and HB96, whose biological characteristics have been previously described including cell morphology, cell ultrastructure, cell growth, cell circle analysis, immunocytochemistry, *in vitro* invasion ability, and tumorigenicity. In mice injected subcutaneously with HIOECs, no neoplasm

Table III. The correlation between the status of S100A6 expression and the clinicopathological characteristics of oral squamous cell carcinoma patients.

Classification	Case no.	Relative mRNA level	Non-parametric test value	P-value	Cancerous protein positive score	Non-parametric test value	P-value
Smoking							
Yes	13	0.723±0.505	Z=-0.063	0.950	1.00±0.91	Z=-0.820	0.412
No	17	0.776±0.720			1.29±0.92		
Drinking							
Yes	12	0.754±0.477	Z=-0.466	0.641	1.17±1.03	Z=-0.067	0.946
No	18	0.752±0.722			1.17±0.86		
T stage							
T1	6	1.052±1.153		0.558	1.17±0.98		0.529
T2	13	0.702±0.515	χ^2=2.072		1.07±0.86	χ^2=2.216	
T3	4	0.469±0.294	df=3		1.75±0.50	df=3	
T4	7	0.752±0.259			1.00±1.15		
N stage							
N0	16	0.821±0.817	Z=-0.374	0.708	1.19±0.98	Z=-0.088	0.930
N1-2	14	0.674±0.305			1.14±0.86		
Clinical stage							
I	5	1.151±1.260			1.40±0.89		
II	8	0.663±0.612	χ^2=3.511	0.319	0.75±0.89	χ^2=2.424	0.489
III	2	0.341±0.074	df=3		1.50±0.71	df=3	
IV	15	0.723±0.292			1.27±0.96		
I + II	13	0.850±0.899	Z=-0.523	0.601	1.00±0.91	Z=-0.820	0.412
III + IV	17	0.680±0.302			1.29±0.92		
Pathological differentiation grade							
Well	12	0.652±0.301			1.08±0.90		
Moderately	15	0.720±0.473	χ^2=0.097	0.953	1.27±0.96	χ^2=0.326	0.850
Poorly	3	1.319±1.753	df=2		1.00±1.00	df=2	

formed. In mice injected with HB56 cells, the neoplasms formed had an intact envelope with 2-3 layers of epithelial cells under the envelope, and massive keratinocytes under the epithelial cells, parakeratosis could also be found in some keratinocytes with few atypical hyperplasia. In the mice injected with HB96 cells, the neoplasm formed was typical squamous cell carcinoma; the differentiation of tumor cells was good to moderate with obvious atypical hyperplasia and pathological mitosis (4,5). Using this *in vitro* cellular carcinogenesis model, more than 40 differentially expressed proteins were identified by comparative proteomic analysis during the cancerization process. S100A6, being one of the differentially expressed proteins, was identified in the HIOECs, indicating the decreased expression of S100A6 along with the cancerization process from the HIOECs to the HB96 cells. Further validations in OSCC cellular and tissue levels confirm the decreased protein expression of S100A6 in the cancerous cell lines and cancerous tissues. These results suggest the decreased expression of S100A6 in OSCC, not only *in vitro*, but also *in vivo*.

Most of previous studies have reported the increased expression of S100A6 in many malignancies, such as colorectal carcinoma/adenocarcinoma, gastric cancer, pancreatic ductal adenocarcinoma, cutaneous melanoma, thyroid papillary carcinoma, astrocytoma, choleteatoma, osteosarcoma, acute myeloid leukemia, and craniopharyngioma (9-18). However, decreased expression of S100A6 has also been reported in prostate cancer, thyroid anaplastic carcinoma, breast cancer, hepatocellular carcinoma (10,11,14,19-21). In OSCC, increased S100A6 mRNA level has been reported by semi-quantitative reverse transcription-polymerase chain reaction (41), and decreased S100A6 mRNA level has also been reported by semi-quantitative reverse transcription-PCR and quantitative real-time-PCR (42). No studies on protein expression of S100A6 in OSCC have been reported. In the present study, we found decreased expression of S100A6 in OSCC in both protein level and mRNA level, and both in cancerous cell line (compared with immortalized human oral epithelial cell line) and cancerous tissue (compared with paracancerous

tissue from OSCC patients). Even though, large sample investigation is suggested in future studies.

Although, the precise mechanism is still not clear, S100A6 is involved in the cellular proliferation, regulation of actin cytoskeleton and ß-catenin, membrane dynamics, p53 and heat-shock response. The targets of S100A6 include glyceraldehyde-3-phosphate dehydrogenase, p53, annexins (II, VI, XI), tropomyosin, caldesmon, calponin, lysozyme, calcyclin-binding protein/Siah-1-interacting protein, hop, sgt1, melusin, and kinesin light chain. However, the expression of S100A6 is regulated by DNA methylation, p53, nuclear transcription factor κB, platelet-derived growth factor, epidermal growth factor, tumor necrosis factor α, retinoic acid, and some hormones (43). Further studies on the S100A6 detailed mechanism are clearly warranted.

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