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S100A8 Is Identified as a Biomarker of HPV18-Infected Oral Squamous Cell Carcinomas by Suppression Subtraction Hybridization, Clinical Proteomics Analysis, and Immunohistochemistry Staining

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The purpose of this work is to differentiate between the Human papillomaviruses 18 positive (HPV18+) and negative (HPV18−) oral squamous cell carcinomas (OSCC) in oral cancer patients with cancer-associated oral habits (betel quid chewing, cigarette smoking, and alcohol drinking). Both gene and protein expression profiles of HPV18+ and HPV18− OSCC were compared: we then further explored the biological effect of HPV in oral cancer. Suppression subtraction hybridization (SSH), clinical proteomics analysis, and immunohistochemistry (IHC) staining were carried out in the HPV18+ and HPV18− OSCC groups. HPV typing detection revealed that 11 OSCC tissues from 82 patients were positive for HPV18. The SSH experiment showed that 4 cancer-associated genes were highly transcribed within 11 cDNA libraries of HPV18+ OSCC, including poly(ADP-ribose)polymerase I (PARP1), replication protein A2 (RPA2), S100A8, and S100A2. Clinical proteomics analysis indicated that there was over 10-fold overexpression of Stratifin, F-actin capping protein alpha-1 subunit (CapZ alpha-1), Apolipoprotein A-1 (ApoA-1), Heat-shock protein 27 (HSP27), Arginase-1, $p16^{INK4A}$, and S100 calcium-binding protein A8 (S100A8) in HPV18+ OSCC. Interestingly, the results from SSH and protemics analysis showed that S100A8 was overexpressed in HPV18+ OSCC. Moreover, IHC staining demonstrated that S100A8 was up-regulated in HPV18+ OSCC tissues. Our results suggest that S100A8 plays an important role in oral carcinogenesis following HPV18 infection; therefore, S100A8 may be a powerful biomarker of HPV18 as well as a potential therapeutic target for HPV18+ OSCC patients. The study is the first to identify S100A8 as a biomarker in HPV-associated cancer. Furthermore, this is also the first study to discover a biomarker by combining SSH, clinical proteomics, and IHC stain analysis in oral cancer-associated research.

Keywords: proteomics • oral squamous cell carcinomas • human papilomaviruses 18 • suppression subtraction hybridization • S100 calcium-binding protein A8

Introduction

Cancer of the oral cavity is the sixth most common malignancy in developed countries, representing nearly 3% of malignant tumors.[1] Oral malignancies most commonly originate from the nonkeratinizing stratified mucosal epithelium (oral squamous cell carcinomas, OSCC) and show morphological similarity to squamous cell carcinomas of other body regions, like those of cervix, anus, and bronchi. The overall survival rate hasn't changed in recent years despite extensive research on the biological and molecular aspects of OSCC in oral cancer. The location and treatment of OSCC in the mouth/face/neck result in a relatively high rate of related morbidity, as the treatment frequently results in significant mutilation of the affected site and compromised functions. More than 1000 OSCC patients die every year in Taiwan and about 90% of patients are men. Survivors often suffer the disfiguring effects and inconvenient functional loss associated with surgical treatment.[2] Betel quid chewing, cigarette smoking, and alcohol

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drinking are the three major risk factors responsible for the development of OSCC in Taiwan.[3] However, there is growing evidence that OSCC can develop in young adults of both genders without such cancer-associated oral habits (OH) or risk factors, especially in developed countries. These observations suggest that mutagenesis by environmental chemical carcinogens is not the sole cause of OSCC.[4]

Recently, some studies have shown that the human papillomavirus (HPV) may be involved in the carcinogenesis of OSCC.[5−7] HPV is a small, epitheliotropic, nonenveloped DNA virus. The HPV genome comprises 7000−8000 base pairs (bp) of double-stranded closed-circular DNA. More than 100 different HPV types have been identified in humans. Some of these HPV types, especially 16, 18, 31, 33, and 58, seem to play specific roles in the development of cervical cancer and are therefore named "high-risk" types. Squamous cell carcinomas of the cervix are infected mainly by high-risk HPV types,[5] whereas other mucosa, such as the larynx, is infected by a wide range of HPV types with varying tumorgenic potential.[6] HPV has been implicated in many intraepithelial neoplasias and invasive squamous cell carcinomas.[7−10] The worldwide prevalence of HPV in cervical carcinomas has been determined to be as high as 99.7%, with HPV types 16 and 18 implicated most frequently.[11,12] Recent studies indicate that of these two high risk types of HPV, HPV16 and 18 are the most prevalent types found in oral cancers, most notably in OSCC. These studies have also provided evidence that oral infection with HPV is a significant independent risk factor for OSCC, determining that HPV is detected in 40−55% of OSCC, compared with its detection in 5−10% of normal oral mucosa.[13−16] To investigate the exact relationship between of HPV and OSCC, Miller et al.[17] performed a meta-analysis and concluded that oral infection with HPV, particularly with high-risk genotypes, was considered to be a significant independent risk factor for OSCC. In addition, previous molecular studies have suggested that infection of HPV may promote head and neck carcinogenesis.[18,19] A few studies have also shown that high-risk HPV and HPV-related E6 and E7 oncoproteins can immortalize and transform oral keratinocytes *in vitro*.[20−23] This cumulated evidence suggests that HPV might play a role in oral carcinogenesis.

There is a well-established association among oncogenic HPV, uterine cervical carcinoma, and anogenital carcinoma.[7,24] To clarify the exact relationship between intratumor HPV status and carcinogenesis of OSCC patients, we investigated the differentially expressed genes and proteins associated with HPV18 infections in OSCC patients. We then screened for potential biomarkers of HPV18+ OSCC. Suppression subtraction hybridization (SSH), clinical proteomics analysis, and immunohistochemistry (IHC) staining demonstrated that only S100A8 was overexpressed in HPV18+ OSCC tissues. These experiments will serve as a model for searching for new biomarkers of oral cancer carcinogenesis in patients infected with HPV.

Experimental Procedures

Patient Characterization and Sample Collection. Eighty-two patients with OSCC were identified from the 1990−2004 surgical pathology archives of the Department of Otorhinolaryngology, China Medical University Hospital. Formalin-fixed paraffin-embedded blocks of tumor specimens that had been obtained from cancer patients who had undergone surgery for oral cancer were used in our study. Lip and tonsillar malignancies were excluded in this investigation. Clinical data were reviewed to identify tumor stage. Haematoxylin and eosin−stained slides were reexamined to confirm the original diagnosis. The histology of specimens from all patients was confirmed by two independent histopathologists following fixation, embedding, sectioning, and H&E staining. Overall, the tumor specimens all contained over 90% tumor cells. No tumor cells were detected in the surrounding mucosal tissue. Information on past or current tobacco smoking, alcohol drinking, and betel quid chewing was recorded. The cutoff value for (self- or heteroanamnestically reported) alcohol drinking, tobacco smoking, and betel quid chewing was 25 g alcohol per day, 20 cigarettes per day, and 20 betel quid per day, respectively. Tumor size, nodal metastases, and distant metastases (TNM) staging of tumors were determined according to criteria for pathologic stages set forth by the American Joint Committee on Cancer (AJCC); histological grading was done according to the World Health Organization classification. The study was approved by the Ethical Committee of the China Medical University Hospital (Taichung, Taiwan). All OSCC samples and adjacent normal tissues that had been collected during surgery were kept frozen at −80 °C; the freezing chain was not interrupted until DNA, RNA, and protein extraction.

DNA Extraction and HPV Detection. The OSCC cells were suspended in 50 mM Tris-HCl (pH 8.0) with 10 mM EDTA containing 200 μg/mL proteinase K and incubated overnight at 37 °C for cell lysis. DNA was extracted from this lysis solution by the phenol-chloroform-isoamylalcohol method. To avoid contamination, we used disposable utensils and discarded them immediately after a single use. A reaction mixture without template DNA was included in every set of PCR runs as a negative control. To assess the quality of the isolated DNA for PCR, a 156 bp segment of the β-actin gene was amplified by PCR using the primers β-actin (F) and β-actin (R) as an internal control to monitor the genomic DNA extraction procedure.

Primary detection of HPV DNA was performed using the PCR Human Papillomavirus Typing Kit (TaKaRa Biomedicals Japan) according to the manufacturer's instructions with some modifications of the PCR reaction. In brief, the E6 and E7 genes of the HPV genome were amplified by two pairs of consensus sequence primers. Malignant HPV types (HPV-16, 18, 31, 33, 52b, and 58) were amplified using the pU-1M/pU-2R primer pair, whereas benign HPV types (HPV-6 and 11) were amplified using the pU-31B/pU-2R primer pair. Besides, the all HPV18-postive samples from PCR Human Papillomavirus Typing Kit were conformed by a HPV18 specific PCR for E6 oncogene (forward: 5′-ATGGC GCGCT TTGAG GATCC-3′; reverse: 5′-TTATA CTTGT GTTTC TCTGC GTC-3′). The positive control cell line was HeLa (HPV18). For each of the 82 samples, 200 ng of OSCC tumor DNA was tested for the presence of HPV DNA. PCR samples, which showed amplification products indicating the presence of HPV, were purified using PCR purification columns (Qiagen, Valencia, CA) and subjected to bidirectional sequence analysis.

Total RNA and mRNA Extraction. Total RNA was isolated from HPV18+ and HPV− OSCC tissues with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Poly (A+) mRNA was isolated from the total RNA using Oligotex mRNA spin columns (Qiagen, Valencia, CA) following the manufacturer's instructions to collect a tester group of mRNA from 11 HPV18+ OSCC and a driver group of mRNA from 70 HPV−OSCC for the SSH.

Suppression Subtraction Hybridization (SSH). SSH was performed with the PCR-Select cDNA Subtraction Kit according

to the manufacturer's protocol (BD Biosciences Clontech, USA). First- and second-strand cDNA synthesis and blunt-ending of DNA ends by T4 DNA polymerase were carried out according to the manufacturer's protocol. After PCR amplification, PCR products from all groups of cDNAs (specific for tester cDNA fragments) were ligated to pGEM-T easy vector (Promega) by incubating 300 ng of each of the PCR products with 50 ng of vector and 1 μL of T4 DNA ligase (Biolabs) in the appropriate buffer for 48 h at 4 °C, in a total volume of 20 μL. The ligation mixtures were diluted 5-fold and used to transform *Escherichia coli* DH5α. The transformation titer was evaluated on 10-cm LB-agar plates prepared with 100 μg/mL ampicillin and isopropylthiogalactoside /X-Gal. The following day, approximately 275 colonies from each subtraction were plated on 22 × 22 cm^2 LB-agar trays. After an overnight growth period, colonies were manually picked and grown individually in 96 well megaplates containing 1 mL LB·100 μg^{-1}·mL ampicillin and 10% glycerol. The plates were covered with porous covers and grown overnight at 37 °C under rocking at 200 rpm. Two hundred microliters of mixtures were then kept in 96 well microtiter plates at −80 °C. Sequencing was carried out by transferring a frozen aliquot of the bacteria to 3 mL LB·100 μg^{-1}·mL ampicillin. After an overnight growth period, the plasmid DNA was isolated by classical miniprep and sequenced using an ABI PRISM 3100 Genetic Analyzer. The sequences obtained were blasted against the National Center for Biotechnology Information (NCBI) library (http://www.ncbi.nlm.nih.gov/).

Protein Extractions. All tissues were minced into pieces of about 1−2 mm^3 and then homogenized with 0.5 mL of lysis buffer (8M urea, 4% CHAPS) containing protease inhibitor cocktail (Sigma, USA) by a homogenizer (MagNA Lyser, Roach) for 60 s, 6500 rpm. The lysates were centrifuged at 8000 rpm for 20 min at 4 °C and, supernatants were collected for protein purification. The protein pellet was denatured with sample buffer (8 M urea, 4% CHAPS, 65 mM DTE, 0.5% ampholytes), and the denatured proteins were incubated at 4 °C for 2−3 h before centrifuging 13 000 rpm for 15 min. The protein concentrations of the resulting supernatants were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) according to the NenoDrop instructions.

Two-Dimensional Gel Electrophoresis (2-DE). We used 200 μg of extracted protein per sample for 2D-gel separation. The extracted sample was diluted with rehydration buffer consisting of a 8 M urea, 4% CHAPS, 65 mM DTE, 0.5% ampholytes, and a trace of bromophenol blue; the final volume was 350 μL. The rehydration solution (containing sample) was then placed into a 17 cm immobilized pH gradient pH 3−10 IPG strip (ReadyStrip IPG strip, Bio-Rad) overnight. Electrophoresis in the first dimension was focused for a total of 60 kVhr (PROTEAN IEF cell, Bio-Rad) at 20 °C and then stored at −20 °C until SDS-PAGE electrophoresis. Before SDS-PAGE electrophoresis, IPG strips were equilibrated with 3 mL of a equilibrium solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue, and DTE (1% w/v) for 20 min, followed by a second equilibration for 20 min in the same equilibrium solution containing iodoacetamide (2.5% w/v) instead of DTE. Finally, the strips were transferred to the top of 12% polyacrylamide gels and held in position with molten 0.5% agarose in running buffer containing 25 mM Tris, 0.192 M glycine, 0.1% SDS. Gels were run at 16 mA/gel for 30 min followed by 50 mA/gel for 4−5 h.

Detection of Protein Spots and Data Analysis. Gels were routinely stained with silver nitrate and then scanned by a GS-800 imaging Densitometer with PDQuest software version 7.1.1 (Bio-Rad). The images were processed using Photoshop (Adobe) and PowerPoint (Microsoft) software. Protein spots were quantified and compared using the PDQuest software. To compare the HPV18+ OSCC (n = 11) and HPV− OSCC (n = 71) specimens, we screened for proteins in HPV18+ OSCC that were overexpressed (>10 fold) for the advanced analysis. Normalized volume differences were statistically calculated for all specimens. A total of 7 proteins were consistently and significantly overexpressed (>10 fold) and were selected for analysis by nanoelectrospray mass spectrometry (nanoLC−MS/MS).

Enzyme Digestion. The in-gel digestion method used in this study was a modification of that reported by Gharahdaghi et al.[25] and Terry et al.[26] Each protein spot (1−2 mm diameter) that represented >10-fold overexpression was cut with a pipet tip and transferred into a microcentrifuge tube (0.6 mL). The gel pieces were washed twice with 50 μL of 50% acetonitrile (ACN): 50% 200 mM ammonium bicarbonate for 5 min and shrunk with 100% acetonitrile until the gels turned white; the gels were then dried for 5 min in a speed vac. The gel pieces were rehydrated at room temperature in 15 μL of 50 mM ammonium bicarbonate (37 °C, 4 min). An equivalent volume (15 μL) of trypsin (Promega, Madison, WI) solution (20 ng/μL in 50 mM ammonium bicarbonate) was then added, and the gel pieces were incubated at 37 °C for 4 h or 30 °C for at least 16 h. After digestion, the gel pieces were vortexed and spun down; the resulting supernatant representing the peptide solution was stored at −80 °C until mass analysis.

Nanoelectrospray Mass Spectrometry (nanoLC−MS/MS) Analysis. nanoscale capillary LC−MS/MS was used to analyze the meaningful proteins involved in the reaction. All analyses were performed using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTARXL quadrupole-time-of-flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA). The nanoscale capillary LC separation was performed on a RP C18 column (15 cm × 75 μm i.d.) with a flow rate of 200 nL/min and a 60 min linear gradient of 5−50% buffer B. Buffer A contained 0.1% formic acid in 5% aqueous ACN; buffer B contained 0.1% formic acid in 95% aqueous ACN. The nanoLC tip used for on-line LC−MS used was a PicoTip (FS360−20-10-D-20; New Objective, Cambridge, MA). Data acquisition was performed by Automatic Information Dependent Acquisition (IDA; Applied Biosystem /MDS Sciex). The IDA automatically finds the most intense ions in a TOF MS spectrum, and then performs an optimized MS/MS analysis on the selected ions. The product ion spectra generated by nanoLC−MS/MS were compared against NCBI databases for exact matches using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT search program (http://www.matrixscience.com).[27] A homo sapiens taxonomy restriction was used and the mass tolerance of both precursor ion and fragment ions was set to ± 0.3 Da. Carbamidomethyl cysteine was set as a fixed modification, whereas serine, threonine, tyrosine phosphorylation, and other modifications were set as variable modifications. All phosphopeptides identified were confirmed by manual interpretation of the spectra.

Immunohistochemistry (IHC). We selected rabbit anti-human S100A8 polyclonal antibody (sc-20174; Santa Cruz Biotechnology) at 1:500 in the experiment. Briefly, paraffin-embedded tissues were sectioned (5 μm) and collected in serial sections on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma). The sections were deparaffinized by immersion in xylene, followed by immersion in alcohol and then

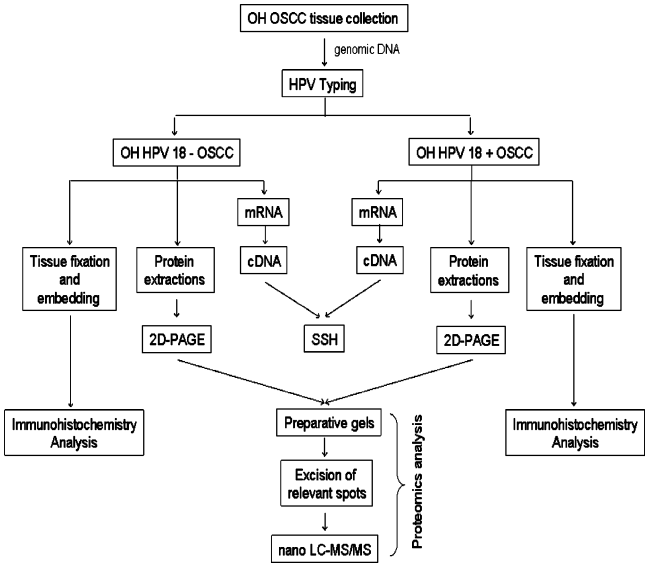

Figure 1. Schematic representation of the procedures used for screening potential biomarkers in HPV18+ OSCC tissues.

incubation with 3% hydrogen peroxide diluted in Tris buffered saline (TBS) (pH 7.4) for 40 min. Next, the sections were immersed in citrate buffer (pH 6.0; P4809; SIGMA) for 20 min at 95 °C for antigen retrieval. Soon afterward, the sections were blocked by incubation with 5% normal goat serum diluted in distilled water, at room temperature, for 30 min. The slides were then incubated with the primary antibodies at 4 °C overnight in a humidified chamber. After washing in TBS, the sections were treated with the labeled streptavidin−biotin (LSAB) kits (DAKO). The sections were then incubated in a 3,3′-diaminobenzidine (DAB) in a chromogen solution (DAKO) for 5−10 min at room temperature. Finally the sections were stained with Mayer's hematoxylin and covered. Negative controls were obtained by the omission of primary antibodies, which were substituted by 1% PBS-BSA and by non-immune rabbit (DAKO) serum. Finally, the sections were dehydrated, made transparent with graded ethanol and xylene, and mounted in GEL/ MOUNT (Biømeda Corp, Foster City, CA). All photographs were taken using a video camera (Sony, Japan) with a microscope adapter on a Zeiss Universal microscope (Carl Zeiss, Germany). The brown signals used to determine the density of each tissue were recorded as faintly positive, moderately positive, or very strongly positive.

Statistical Analysis. The data were stored and analyzed by SPSS.11 software (SPSS Inc, Chicago, IL). Chi-square test was used for univariate analysis of categorical data whereas the *t*-test was used for continuous data. Tests were considered significant when their *P* values were less than 0.05.

Results

In this study, our cDNA subtraction studies were designed to identify which genes were up-regulated by HPV18 infection. Furthermore, we used OSCC as a model system to investigate the reliability of protein detection by coupling an improved extraction method and a 2-DE method previously optimized for soluble proteins. A flow chart of the methods used in this work is depicted in Figure 1.

HPV Detection and Patient Characteristics. Amplication of the human β-actin gene in all DNA samples indicated that sufficient DNA was present in the 82 OSCC tissues used for the amplification of the HPV E6 gene. In the PCR detection, to rule out the possibility of contamination and PCR artifacts, at least 3 negative control samples containing all reaction components except template DNA were included for each round of PCR reaction. The Human Papillomavirus Typing Kit and classical PCR detection revealed that a total of 11 OSCC tumor specimens were positive for HPV18 (patient No.1028T, 1073T, 1105T, 1129T, 1132T, 1151T, 1154T, 1167T, 1184T, 1188T, and 1213T). The study population included 62 men and 20 women with OSCC (median age of 53 years). Table 1 summarizes the clinical and behavioral characteristics of the patients. The mean age at diagnosis was 53 years (range 40−70 years). The specimens from the 82 OSCC patients were classified histologically into 16 (20%) well differentiated, 34 (41%) moderately differentiated, and 32 (39%) poorly differentiated tumors. Fifteen (18%) patients had lymph node involvement. HPV positivity was not found to correlate with tumor stage, histological differentiation, or other factors examined.

Table 1. Clinicopathological Parameters of HPV18+ and HPV18− OSCC Patients

factor	HPV+ *n* (%)	HPV− *n* (%)	total (*n* = 82)	*P* value[a]
age (median, range)	46(40−61)	56(44−70)	53(40−70)	0.547
sex				0.201
male	8(73%)	54(76%)	62(76%)	
female	3(27%)	17(24)%	20(24%)	
tobacco use				
yes	11(100%)	71(100%)	82(100%)	
no	0(0%)	0(0%)	0(0%)	
alcohol use				
yes	10(91%)	71(100%)	81(99%)	
no	1(9%)	0(0%)	1(1%)	
betel quid chewing				
yes	11(100%)	71(100%)	82(100%)	
no	0(0%)	0(0%)	0(0%)	
clinical stage				0.775
I−II	3(27%)	30(42%)	30(37%)	
III−IV	8(73%)	41(58%)	52(63%)	
tumor size				0.334
T1, T2	2(18)	16(23%)	18(22%)	
T3, T4	9(82)	55(77%)	64(78%)	
lymph node metastasis				0.506
N0	9(82%)	58(82%)	67 (82%)	
N1−3	2(12%)	13(18%)	15 (18%)	
tumor differentiation				0.119
well	2(18%)	14(20%)	16 (20%)	
moderate	5(45%)	29(41%)	34 (41%)	
poor	4(37%)	28(39%)	32 (39%)	
clinical outcome				0.232
alive	10(91%)	64(90%)	74(90%)	
dead	1(9%)	7(10%)	8(10%)	

[a] *P* values were obtained from χ2-test.

cDNA SSH Studies. Our cDNA subtraction studies were designed to identify genes that were up-regulated by HPV18+ in OSCC. Among the cDNA sequences from the 275 colonies per plate that were picked, a total of 14 high-transcript genes were identified from each of 11 subtracted libraries in this study. The 14 genes were classified into four groups based on the predicted function of the encoded proteins: protein processing molecules (n = 7), nuclear protein gene (n = 1), cancer associated genes (n = 4), and genes of yet unknown function (n = 2) (data not show). The cDNA subtraction study identified 4 cancer relative genes: PARP1, RPA2, S100A8, and S100A2. Other information (chromosome location and biofunction) associated with those genes was obtained from the NCBI website and is listed in Table 2.

Table 2. Gene Transcripts in HPV18+ OSCC from cDNA Subtraction Studies

accession no.	gene[a]	chromosome location	description
NM_001618	PARP1	1q41	involved in the base excision repair pathway
NM_009246	RPA2	1p35	involved in DNA recombination, repair and replication
NM_002964	S100A8	1q21	calcium binding protein and cancer-related protein
NM_005978	S100A2	1q21	a role in suppressing tumor cell growth

[a] PARP1, poly (ADP-ribose) polymerase family, member 1; RPA2, replication protein A2; S100A8, S100 calcium binding protein A8; S100A2, S100 calcium binding protein A2.

2-DE Analysis. We used 2-DE technology to monitor changes in the abundance of proteins to identify the overexpressed proteins in HPV18+ OSCC tissues. Total protein extraction rates didn't differ significantly between HPV18+ and HPV18− OSCC tissues. The proteins were quite evenly distributed in the gel. The pH ranged from 3 to 10 and the molecular masses ranged from 10 to 100 kDa. Figure 2 shows representative images of the 2-DE master gels for HPV18+ OSCC (1132T, (A)) and HPV18− OSCC (1288T, (B)). The two gels were randomly selected from 11 HPV18+ and 71 HPV− specimens, respectively. Some proteins were present in train spots, indicating that modified proteins or isoforms were well separated by 2-DE.

Fully automated spot detection and quantification were also performed by PDQuest software, image pairs from each case, followed by automated image to image matching and statistical analysis in the experiment. Averages of 600−900 spots were detected across all gels. The abundance ratios between HPV18+ OSCC and HPV18− OSCC specimens from gels were calculated. The ratio values are expressed as fold changes, i.e., a 10-fold increase in HPV18+ OSCC is expressed as 10.0. The 7 protein spots that consistently showed more than 10-fold overexpression in HPV18+ OSCC tissues in all specimens analyzed are highlighted in circles. Statistical data for these proteins are summarized in Table 3 with fold differences in HPV18+ OSCC tissues relative to fold differences in HPV− OSCC (for an average of each HPV18+OSCC indicated). PDQuest analysis showed that the expression of heat-shock protein beta-1(HSP27) increased 17.3 fold in HPV18+OSCC, representing the highest-level of overexpression among the 7 proteins. Arginase-1 was the protein with the lowest level of overexpression (10.9 fold). The other proteins were overexpressed as follows: Stratifin (16.8-fold), F-actin (15.5-fold), capping protein alpha-1 subunit (CapZ alpha-1)(16.2-fold), ApolipoproteinA-1 (ApoA-1)(17.3-fold), $p16^{INK4A}$ (10.9-fold), and S100 calcium-binding protein A8 (S100A8) (13.7-fold).

IHC of S100A8 on OSCC Sections. In our study, transcriptional and translational regulation correlated positively only for S100A8. The comparative approach also demonstrated that some proteins were either not differentially regulated on the transcription level or were regulated by reverse transcription. To test whether HPV18+ OSCC did show an increase in S100A8 protein expression, the IHC stain was performed on a total of 82 OSCC specimens. There was a significantly higher prevalence of high level S100A8 expression in HPV18+ OSCC tissues (Figure 3A) than in HPV18- OSCC tissues (Figure 3B). The brown, positive signal was observed in nearly all HPV18+ OSCC tissues, especially in tumor nests and nuclei. The overall frequency of S100A8 expression in 11 HPV18+ and 71 HPV18− OSCC specimens are listed in Table 4.

Discussion

New data on the effects of HPV infection as a possible etiologic agent in head and neck squamous cell carcinoma (HNSCC) has emerged in recent years, indicating that HPV infected tumors may represent a distinct subset that can be identified by specific molecular features.[28,29] Recently, many interesting reports have been published that focused on biomarkers of HPV-associated HNSCC.[30] However, a comprehensive profile of both gene and protein expression between HPV+ and HPV− OSCC has not been reported until now. In this article, we organized global gene and protein expression in 11 HPV18+ and 71 HPV18− OSCC tissues and compared the two groups in expressed patterns by SSH and clinical proteomics analysis. In our patient population, all of the 11 HPV18+ and 71 HPV18− OSCC patients had cancer-associated oral habits (betel quid chewing, cigarette smoking, and alcohol drinking). Thus, we support the findings by Chang et al.[31] that HPV infection is not associated with the abovementioned oral habits. Highly transcripted genes were identified in the HPV18+ OSCC tissue by subtractive hybridization technique. There are a number of techniques available to identify and isolate differentially expressed transcripts. Some of the most widely used ones are differential display (DD), representative differential analysis (RDA), and suppression subtractive hybridization (SSH). One advantage of the SSH technique over the others is the equalization of high- and low-abundance messages, which allows for the identification of differentially expressed low-abundant mRNAs. It is also reported that there is a lower incidence of false positives for SSH than for DD, especially when SSH is used for the identification of genes in tissues with many differentially expressed transcripts.[32]

We focused on the cancer-related genes, including PARP1, RPA2, S100A8, and S100A2, when performing the SSH technique. PARP1 is the principal member of a family of enzymes possessing poly(ADP-ribosylation) catalytic capacity. It is a conserved nuclear protein that binds rapidly and directly to both single- and double-strand breaks.[33] The understanding of the role and involvement of PARP-1 in many biological mechanisms and diseases as well as its role in carcinogenesis has steadily increased in recent years.[34] Recently, some studies have demonstrated that inhibition of PARP can control tumor growth and that PARP inhibition or genetic deletion of PARP1 prevents tumor promotion by inhibiting their cooperate with the activation AP-1, NF−KB, and HIF-1A.[35] Generally, it is believed the PARP1 is a biomarker of carcinogenesis, but no reports have provided evidence that PARP1 is associated with HPV infection, until now. We are the fist to demonstrate that the PARP1 gene was up-regulated in HPV-infected cancer cells. RPA2, a number of replication protein A (RPA), was shown by DNA microarray analysis to be increased in HPV-infected HNSCC.[30] RPA is a tetrameric protein complex that binds single strain DNA and facilitates DNA damage sensing.[36] The HPV E1 protein, an ATP-dependent viral DNA helicase, was reported to bind and recruit RPA to sites of DNA replication through binding of the 70-kDa subunit of RPA1.[37] S100A8 and S100A2 are members of the S100 protein family, a calcium-binding protein family. They have recently been shown to be associated with many cancer types, including lung cancer, prostate cancer, and SCC.[38−41] Moreover, S100A2 has been reported to play an antitumor role in SCC, partly by reducing the expression of cyclooxygenase-2 in recent

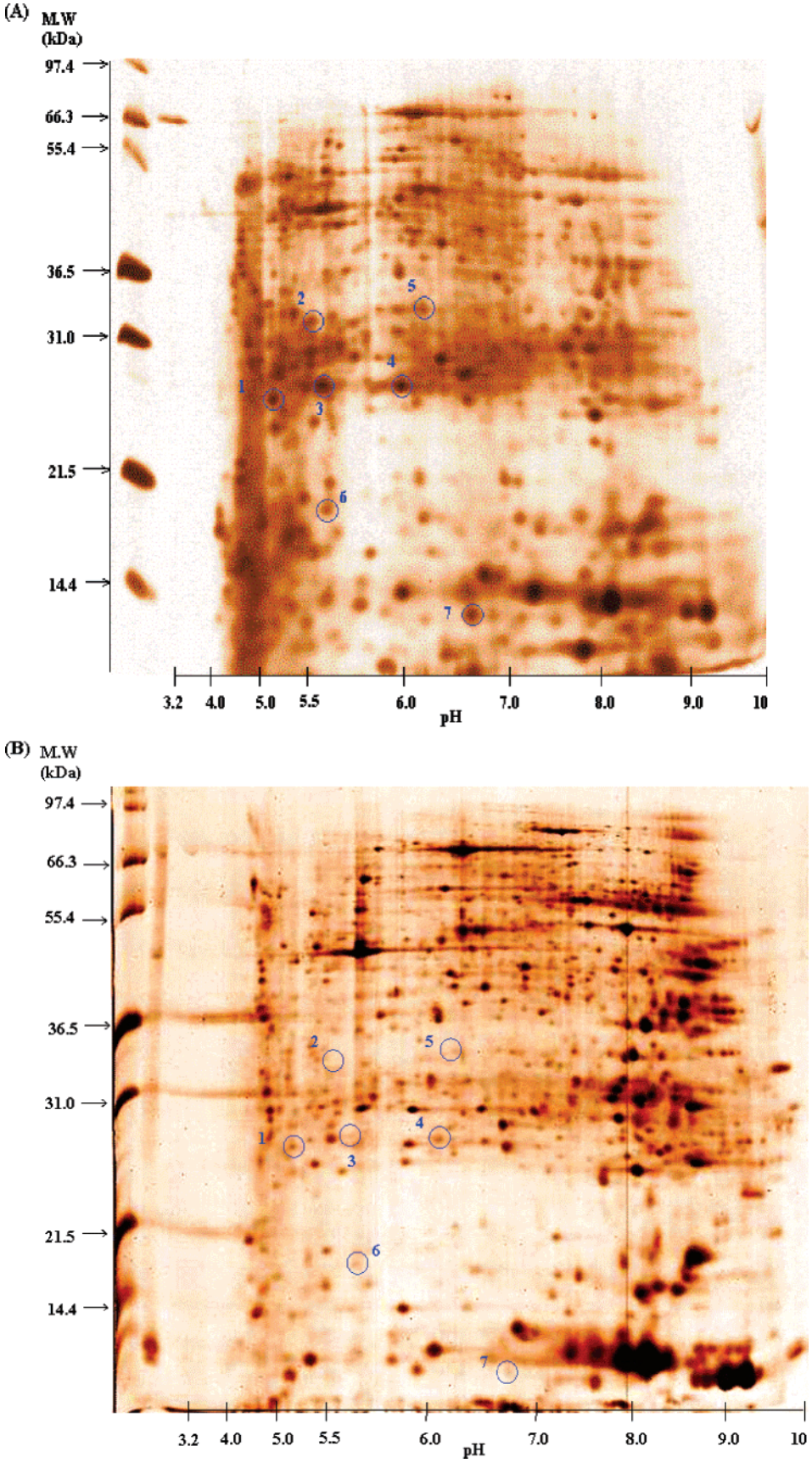

Figure 2. 2-DE protein patterns from HPV18+ and HPV18− OSCC tissue extracts. Two hundred micrograms of protein were separated on 17 cm gels followed by silver staining. Protein expression profiles of (A) HPV18+ OSCC tissue (1132T) and (B) HPV18− OSCC tissue (1288T). The seven proteins were overexpressed (>10 fold) in HPV18+ OSCC tissues. The identified proteins were numbered, and the protein IDs are provided in Table 1.

Table 3. Identification of Overexpressed Proteins (>10 Fold) in HPV18+ OSCC by Q-TOF Mass Spectra and Database Searching

spot no.	accession no.	protein ID[a]	theoretical M_r (kDa)/p*I*	sequence coverage	chromosome location	average fold change
1	P31947	Stratifin	27.8/4.68	35%	11q23	+16.8
2	P52907	CapZ alpha-1	32.9/5.45	22%	6q23.2	+15.5
3	P02647	ApoA-1	30.8/5.56	39%	9p21	+16.2
4	P04792	HSP27	22.7/5.98	38%	1p13.2	+17.3
5	P05089	Arginase-1	34.7/6.72	51%	7q11.23	+10.9
6	P42771	$p16^{INK4A}$	16.5/5.52	16%	1q21	+13.7
7	P05109	S100A8	10.8/6.51	49%	1p36.11	+17.0

[a] CapZ alpha-1, F-actin capping protein alpha-1 subunit; ApoA-1, Apolipoprotein A-1; HSP27, Heat-shock protein 27; S100A8, S100 calcium-binding protein A8.

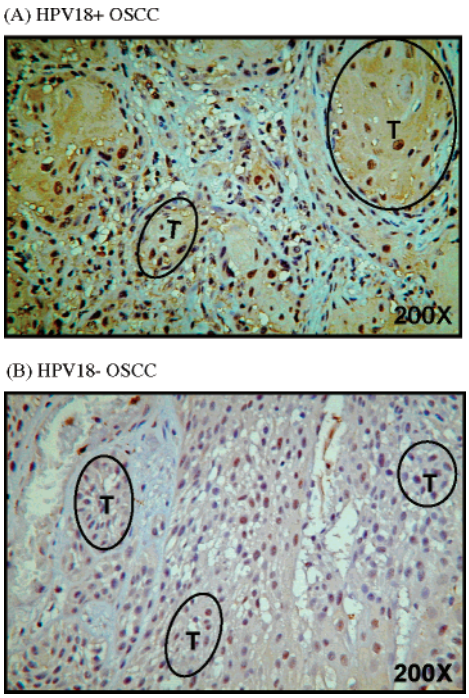

Figure 3. Immunohistochemical validation of S100A8 by immunohistochemistry. IHC of (A) normal HPV18+ OSCC and (B) HPV18− OSCC tissues are shown. The antibody used was against S100A8. (A) and (B) are shown at 200×. No. 1129T and 1080T were taken randomly from HPV18+ and HPV18− groups, respectively. **N**, normal epithelium; **T**, tumor nest.

Table 4. Differential Levels of Significant *S100A8* Protein Expression between HPV18+ and HPV18− OSCC Tissues among 82 Patients

signal intensity	HPV18+ OSCC	HPV18− OSCC
faintly positive	0	67
moderately positive	1[a]	2[b]
very strongly positive	10	2[c]
total	11	71

[a] 1105T of 11 HPV18+ OSCC cases. [b] 1121T and 1274T of 71 HPV18- OSCC cases. [c] 1066T and 1215T of 71 HPV18- OSCC cases.

study.[42] We have developed techniques to obtain and analyze frozen OSCC tissues and have so far presented data at the RNA level using SSH. Alterations at the RNA level may, but cannot in general, be expected to correlate with alterations at the protein level.[43,44] In this investigation, we identified differentially expressed proteins from silver stained 2-DE and relate it to the original data derived from SSH. We hypothesize that proteomic data are more relevant than transcripted data with regard to the biological function. Proteomics is a promising approach in the identification of proteins with changed levels which may be useful as diagnostic markers for the early detection of cancer.[45] Proteomics has been successfully employed in studies of lung,[46] breast,[47] and prostate[48] and in studies of OSCC.[49]

Seven proteins were overexpressed in HPV18+ OSCC, including Stratifin, CapZ alpha-1, ApoA-1, HSP27, Arginase-1, $p16^{INK4A}$, and S100A8. In our previous study, Stratifin and HSP27 were also identified as overexpressed proteins in OSCC tissues; in fact, they were expressed 3.6- and 4.3-fold higher, respectively, than their paired normal tissues.[49] Here, stratifin and HSP27 were overexpressed by 16.8- and 17.3-fold, respectively, in HPV18+ OSCC. The significant difference between the results suggests that Stratifin and HSP27 are not the only indicators of tumorgenesis in OSCC. They may also be associated with the interference of HPV in OSCC or other types of SCC. More research is necessary. According to the proteomics data, six differential proteins (Stratifin, CapZ alpha-1, ApoA-1, HSP27, Arginase-1, and $p16^{INK4A}$) were not regulated at the RNA level, suggesting that the differential expression of these proteins could be due to the stability or differences in secretion or accumulation of these proteins. Interestingly, we found that only the S100A8 gene and protein were both up-regulated in HPV18+ OSCC. To confirm our findings, we used paraffin series sections obtained from the Department of Pathology and Otorhinolaryngology to carry out the IHC. The result demonstrated that S100A8 was overexpressed in HPV18+ OSCC tissues.

S100A8 is a member of the S100 protein family, which comprises 23 different members. Proteins in the family are characterized by high homology, low-molecular weight, two calcium-binding EF-hands, and by tissue-specific expression. Although most S100 proteins form homodimers, some members, like S100A4, S100A6, S100A8, and S100A9, form heterodimers with another member of the S100 family. S100A8/A9, also known as calprotectin, is the most abundant naturally occurring S100 heterodimer and is found in high levels in neutrophils, macrophages, and endothelial cells.[50,51] Typically, S100A8 and S100A9 are differentially expressed at sites of acute and chronic inflammation.[52] Moreover, at least 16 genes of the multigenic S100 family, including the genes coding for S100A8 and S100A9, are clustered on human chromosome 1q21,[53] a region that is a frequent target for chromosomal rearrangements that occur during tumor development.[54,55] A recent study found that S100A8 and S100A9 were implicated in tumor development and progression.[55] Moreover, there is much evidence from gene expression and proteomic studies that S100A8 is overexpressed in breast,[56] gastric,[57] colorectal,[68] pancreatic,[69] and prostate cancer[60] but down-regulated in tumors derived from squamous cells, such as squamous

oesophageal carcinoma (ESCC).[61] However, very little information is known regarding the regulation of S100A8 and/or S100A9 in several human common malignancies, and little is known about the possibility of abnormal expression of S100A8 in ESCC. Following the characterization, we will involve the S100A9 into our future investigation. In 2004, a study demonstrated that S100A8 and S100A9 were preferentially expressed in normal esophageal epithelia and lowly expressed in ESCC.[61] According to the proteomics data from our previous study, the expression of S100 family proteins did not differ significantly between OSCC tissue and the paired normal tissue.[49] It is interesting to note that S100A8 may be expressed differently between ESCC and OSCC. Whether S100A8 expression is an important predictor for histological grade needs to be confirmed by clinical follow-up studies. In a virology study, the S100A8/A9 protein complex was shown to play an important role in the regulation of CKII-mediated oncogene E7 phosphorylation in HPV-transfected epithelial cells.[62] However, no clinical studies have demonstrated that S100A8, S100A9, or S100A8/A9 is involved in oral cancer caused by viral infection. The biofunctions of S100A8 in virus-associated cancer development and progression require more detailed examination.

Conclusion

S100A8 was identified as a biomarker of HPV18+ OSCC by SSH, proteomics, and IHC analysis. We found that either S100A8 or the S100A8/A9 complex may be involved in the viral oncogene-associated molecular pathway in OSCC carcinogenesis. The mechanisms of S100A8 overexpression in HPV18+ OSCC tissues are not well understood but may involve the multistep cell transformation process. Briefly, S100A8 may be providing useful diagnostic information about HPV18− infected SCC.

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Supporting Information Available: Graph showing overexpression of HPV18+ patients. This material is available free of charge via the Internet at http://pubs.acs.org.

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