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Copy number changes of *CRISP3* in oral squamous cell carcinoma

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Abstract. The aim of this study was to identify tumor suppressor genes (TSGs) in oral squamous cell carcinoma (OSCC) using whole-genome analysis of microarray technology and real-time quantitative polymerase chain reaction (QPCR). We applied whole-genome analysis of TSGs in the specimens from 3 patients of OSCC by microarray technology. A total of 11 genes, *CRISP3*, *SCGB3A1*, *AGR2*, *PIP*, *C20orf114*, *TFF1*, *STATH*, *AZGP1*, *MUC7*, *DMBT1* and *LOC389429,* were found to be down-regulated, and 2, matrix metallopeptidase (*MMP)* 1 and *MMP3,* were found to be up-regulated in the 3 OSCC patients using microarray technology. In this study, we selected the *CRISP3* gene. *CRISP3* belongs to the cystein-rich secretary protein gene family in chromosome 6p12.3. *CRISP3* has been found in the salivary gland, spleen and prostate gland and is a prominent biomarker in the gene expression of prostate cancer. Down-regulation of this gene was previously observed in OSCC. No studies examining the DNA copy number of *CRISP3* in detail exist. We analyzed the DNA copy number of *CRISP3* in 5 OSCC-derived cell lines (SAS, Ca9-22, KON, HSC2 and HSC4) and 60 OSCC tissues by real-time QPCR. The DNA copy number loss of *CRISP3* was observed in 2 of the 5 OSCC-derived cell lines (SAS, HSC2) and in 24 of 60 patients (40.0%) using real-time QPCR. A significant statistical correlation between the copy number loss and gender and T classification was observed. These results indicate that the inactivation of *CRISP3* is an early event in OSCC, since the T1/T2 classification is correlated with DNA copy number loss of *CRISP3,* whereas T3/T4 classification is not. We conclude that *CRISP3* may be involved in the carcinogenesis of OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) is a common malignancy that affects 300,000 individuals per year worldwide (1). A number of etiologic factors have been implicated in the development of OSCCs, such as the use of tobacco, alcohol or betel nut chewing, human papillomavirus infection, and the presence of incompatible prosthetic materials. Tobacco is a major risk factor for the development of OSCC and overall risk of OSCC among smokers is 7-10 times higher than that of non-smokers (2-4).

The primary treatment for management of OSCC is surgical intervention. Despite considerable advances in the treatment of OSCC over the past two decades, overall disease outcomes have only modestly improved (5). Local tumor recurrence affects approximately 60% of patients and metastasis develops in 15-25% (6). The prevention and management of this disease is likely to greatly benefit from the identification of molecular markers and targets (7,8).

However, little is known about the molecular basis of OSCC compared with other malignancies. Molecular alterations in a number of oncogenes and tumor suppressor genes (TSGs) associated with the development of OSCC may be significant clues with which to address these problems (9,10).

Inactivation of TSG is considered to be associated with carcinogenesis, and alterations in TSGs are accepted to be critical events in the multi-step process leading toward the development of cancer. Loss of chromosome 3p22-24 is a common early genetic event in OSCC (11). Specific regions of arm harbor candidate tumor suppressor genes including *FHIT* and *RSSF1A*. It is generally accepted that the transformation of normal tissue into malignant tissue follows an accumulation of genetic changes in the TSGs and oncogenes (9). High throughput investigation into the molecular characteristics of OSCC has mainly utilized microarray technology to search for gene expression profiles associated with disease and disease outcome.

In the present study, microarray technology was applied to screen novel TSGs in OSCC patients, and we selected candidate gene *CRISP3*. *CRISP3* DNA copy number was evaluated by real-time QPCR in 5 OSCC-derived cell lines and 60 primary OSCC samples. Human *CRISP3* is also likely to be involved in the pathogenesis of prostate cancer, where *CRISP3* expression is significantly upregulated (12).

CRISP3 is secreted and can be detected in human tissue fluids including saliva, sweat, blood and seminal plasma, rendering it an ideal candidate biomarker for pathophysiological conditions, including ectopic pregnancy (12-14). Ye *et al* reported the expression of *CRISP3* in OSCC using microarray technology (15). Therefore, the purpose of the present study was to assess the role of *CRISP3* in OSCC.

Materials and methods

Cells. The 5 human OSCC-derived cell lines used in this study were SAS, Ca9-22, KON, HSC2, and HSC4 (Human Science Research Resources Bank, Osaka, Japan). SAS was from male tongue, Ca9-22 from male gingiva, KON from male oral floor, HSC2 from male mouth and HSC4 from male tongue. The cell lines were maintained at 37˚C (humidified atmosphere 5% CO_2/95% air) in 150x200 mm tissue culture dishes (Nunc, Roskilde, Denmark) and cultured in Dulbecco's modified Eagle's medium F-12 HAM (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (Sigma) plus 50 U/ml penicillin and streptomycin.

Normal oral keratinocyte (NOKs) strains from two patients who had undergone dental surgery served as the controls, and the patients provided written informed consent prior to 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 was 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 were then seeded into Collagen I Cellware 60-mm dish biocoat cell environments (Becton Dickinson Labware, Bedford, MA, USA) and cultured in Keratinocyte Basal Medium-2 (Cambrex, Walkersville, MD, USA) 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) (16).

Patient characteristics. A total of 60 patients with OSCC were included in the present study. Surgical resection of primary tumors and marginal normal tissues from all patients was performed at the Hospital of Chimei, Tainan, Taiwan, and the Hospital of Tokyo Dental College, Chiba, Japan, between July 1999 and September 2009. Written informed consent was obtained from all patients and the study approved by the ethics committees of the Hospital of Chimei and the Tokyo Dental College, ethical clearance number 205. Informed consent was obtained from each patient prior to surgical resection.

Staging of tumors was performed according to the International Union Against Cancer TNM staging system (17). Cervical LNM during the 12-month follow-up period was evaluated by computed tomography and magnetic resonance imaging. In case of a positive signal, metastasis was further confirmed by histopathological examination of the resected tissues. Patients not exhibiting any cervical LNM for 12 months after surgery were considered metastasis-free. Patients with distant metastasis at the time of clinical examination or those receiving preventive radiotherapy or chemotherapy were excluded from this study. Detailed patient characteristics are shown in Table I.

Primary tumor samples. Resected primary tumor tissues were divided into two sections. Of these, one section was frozen immediately in liquid nitrogen and stored at -80˚C. The other was fixed in 10% formalin for histopathological examination. The resected marginal normal tissues were frozen immediately in liquid nitrogen and stored at -80˚C.

Microarray gene expression profiling. A total of 3 OSCC patients (2 tongue and 1 oral floor patient) were subjected to whole-genome analysis using microarray technology to determine the TSGs.

Total RNA was extracted from 3 OSCC patients using the Qiagen RNeasy mini kit and the SuperScript double-stranded cDNA synthesis kit (Invitrogen) was used to generate cDNA according to the manufacturer's instructions. Cy3 labeling of ds-cDNA was performed overnight using the NimbleGen One-Color DNA labeling kit.

Cy3-labeled ds-cDNA (4 μg) was hybridized to the *Homo sapiens* 4x72 K gene expression array (Roche NimbleGen) representing 24,000 protein-coding genes, according to the manufacturer's instructions. The mRNA expression data were analyzed using NimbleScan software version 2.4, which applied quintile normalization (18), and expression values were obtained using the Robust Multi-Chip Average algorithm as described by Irizarry *et al* (19). Expressional alterations of 2-fold across the two biological repeats were considered significant.

DNA copy number analysis by real-time QPCR. DNA from the frozen 60 tumor and marginal normal tissues was extracted using the QIAamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real-time QPCR was performed using 60 tumor and marginal normal tissue samples with SYBR-Green I fluorescence detection on a LightCycler (Roche Diagnostics, Basel, Switzerland). Oligonucleotide primers for real-time QPCR were designed using Primer 3 software (Whitehead Institute for Biomedical Research), and uniqueness in the human genome was confirmed using a BLAST search. The primer set specific for *CRISP3* was forward: 5'-ATCAGGCTGCATC CCAATAC-3', and reverse: 5'-AACACCAAATCCCCACA GAA-3'. The 20-μl reaction mixture consisted of 10 μl 2X iQ SYBR-Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 10 ng genomic DNA, and 800 nM of each PCR primer. The reaction mixture was loaded into glass capillary tubes and submitted to an initial denaturation at 95˚C for 10 min, followed by 45 cycles of amplification at 95˚C for 10 sec for denaturation, 58˚C for 10 sec for annealing, and 72˚C for 15 sec for extension, with a temperature slope of 20˚C/sec, performed in the LightCycler. The crossing point for each amplification curve was determined by the second derivative maximum method. The copy numbers are presented as the log ratio of each target locus in tumor normalization to internal reference loci (*GAPDH*) and relative to the normal DNA. The primer set specific to *GAPDH* was forward: 5'-CCACTAGGCGCTCACTGTTCT-3', and reverse: 5'-GCG AACTCACCCGTTGACT-3'. DNA copy number loss was

Table I. Clinical characteristics of 60 OSCC patients.

Case	Gender	Age	Ethnic group	Tumor site	T	N	Stage	pN	Tobacco	Alcohol
1	M	43	Taiwanese	Buccal mucosa	3	0	III	-	+	+
2	M	66	Taiwanese	Lower gingiva	2	0	II	-	+	+
3	M	31	Taiwanese	Tongue	2	0	II	-	+	+
4	M	55	Taiwanese	Buccal mucosa	1	0	I	-	+	+
5	M	37	Taiwanese	Tongue	3	0	III	-	+	+
6	M	58	Taiwanese	Buccal mucosa	1	0	I	-	+	+
7	F	71	Taiwanese	Upper gingiva	2	0	II	-	-	-
8	M	43	Taiwanese	Oral floor	2	0	II	-	+	+
9	M	39	Taiwanese	Buccal mucosa	2	0	II	-	+	+
10	M	56	Taiwanese	Tongue	1	0	I	-	+	+
11	M	49	Taiwanese	Buccal mucosa	3	0	III	-	+	+
12	M	72	Taiwanese	Tongue	1	1	III	+	+	+
13	M	51	Taiwanese	Lower gingiva	4	0	IV	-	+	+
14	M	55	Taiwanese	Tongue	2	2	IV	+	-	-
15	M	59	Taiwanese	Buccal mucosa	3	1	III	+	-	-
16	M	54	Taiwanese	Buccal mucosa	1	0	I	-	+	+
17	M	68	Taiwanese	Buccal mucosa	2	0	II	-	+	-
18	M	33	Japanese	Tongue	2	1	III	+	-	+
19	M	55	Japanese	Oral floor	3	2c	IV	+	+	+
20	M	74	Japanese	Tongue	2	1	III	-	+	+
21	M	54	Japanese	Buccal mucosa	4	2c	IV	+	-	+
22	M	64	Japanese	Lower gingiva	4	2a	IV	+	-	+
23	M	53	Japanese	Tongue	2	1	III	+	+	-
24	F	70	Japanese	Buccal mucosa	3	1	III	+	-	-
25	M	44	Japanese	Tongue	1	0	I	-	-	+
26	F	50	Japanese	Upper gingiva	1	0	I	-	-	+
27	M	36	Japanese	Upper gingiva	2	0	II	-	-	+
28	F	37	Japanese	Tongue	2	2c	IV	-	-	-
29	M	47	Japanese	Tongue	2	0	II	+	-	+
30	M	43	Japanese	Tongue	1	0	I	-	-	+
31	M	57	Japanese	Tongue	2	2b	IV	-	+	+
32	M	52	Japanese	Tongue	1	0	I	-	+	+
33	F	58	Japanese	Tongue	2	0	II	-	-	-
34	M	59	Japanese	Upper gingiva	3	1	III	-	-	+
35	M	62	Japanese	Tongue	1	0	I	-	+	+
36	F	70	Japanese	Upper gingiva	2	1	III	-	-	-
37	M	82	Japanese	Upper gingiva	3	0	III	-	-	+
38	F	69	Japanese	Buccal mucosa	2	0	II	-	-	-
39	M	80	Japanese	Lower gingiva	2	2b	IV	+	-	-
40	M	48	Japanese	Tongue	1	0	I	-	-	+
41	M	70	Japanese	Lower gingiva	2	1	III	-	+	+
42	M	49	Japanese	Lower gingiva	2	2b	IV	-	-	-
43	M	85	Japanese	Tongue	2	1	III	-	-	-
44	M	71	Japanese	Lower gingiva	4	1	IV	-	-	+
45	F	73	Japanese	Tongue	1	0	I	-	-	-
46	F	58	Japanese	Lower gingiva	3	0	III	-	-	-
47	M	41	Japanese	Tongue	3	2b	IV	-	+	+
48	M	58	Japanese	Tongue	1	1	III	+	+	+
49	F	66	Japanese	Buccal mucosa	2	1	III	+	-	-
50	M	63	Japanese	Tongue	1	0	I	-	+	+
51	F	60	Japanese	Lower gingiva	2	0	II	-	-	-
52	M	52	Japanese	Tongue	2	0	II	-	+	+
53	F	72	Japanese	Upper gingiva	2	2a	IV	-	-	-
54	F	63	Japanese	Lower gingiva	4	2b	IV	-	-	-
55	F	66	Japanese	Buccal mucosa	1	0	I	-	-	-
56	M	66	Japanese	Oral floor	4	2c	IV	+	+	+
57	M	66	Japanese	Lower gingiva	4	2b	IV	-	-	+
58	M	76	Japanese	Oral floor	2	1	III	+	-	+
59	M	77	Japanese	Oral floor	2	1	III	-	-	+
60	F	69	Japanese	Lower gingiva	4	2b	IV	+	-	-

Table II. List of aberrantly expressed genes.

	Gene symbol	Gene name	Location
Upregulated genes			
	MMP1	Matrix metallopeptidase 1	11q22
	MMP3	Matrix metallopeptidase 3	11q22
Downregulated genes			
	STATH	Statherin	4q11-q13
	MUC7	Mucin 7	4q13-q21
	SCGB3A1	Secretoglobin, family 3A, member 1	5q35-qter
	CRISP3	Cysteine-rich secretory protein 3	6p12.3
	LOC389429	Hypothetical LOC389429	6q21
	AGR2	Anterior gradient homolog 2	7p21.3
	AZGP1	α-2-glycoprotein 1, zinc-binding	7q22.1
	PIP	Prolactin-induced protein	7q34
	DMBT1	Deleted in malignant brain tumors 1	10q26.13
	C20orf114	Chr. 20 open reading frame 114	20q11.21
	TFF1	Trefoil factor 1	21q22.3

determined as <1.2 (20). The data were analyzed as the mean ± SD of three independent experiments with samples in triplicate.

Statistical analysis. The association between copy number loss and any clinical findings were assessed by the Fisher's exact test. The association between the DNA copy number of tumor and normal tissues was assessed by the unpaired U test (SAS Institute, Cary, NC, USA). P-<0.05 was considered to indicate statistical significance.

Results

Clinicopathological findings. This study included 60 patients who had undergone surgical resection of primary OSCC. The patients, 45 males and 15 females, had average ages of 56.7 years for the males (range 31-85) and 63.1 years for the females (range 37-73). Regarding patient ethnicity,17 patients of Taiwanese and 43 of Japanese ethnicity were included in this study. The tumor sites were as follows: 23 patients, tongue; 13, buccal mucosa; 12, lower gingiva; 7, upper gingiva; and 5, oral floor. The T classifications, which indicate the sizes of the primary clinical tumors, were as follows: 15 patients with T1, 27 with T2, 10 with T3 and 8 with T4. The classifications by TNM stage were: 13 patients with Stage I, 12 with Stage II, 19 with Stage III, and 16 with Stage IV. A total of 18 of the 60 patients had histopathologically-confirmed cervical lymph node metastasis (LNM) at the time of diagnosis or during the 12-month follow-up period (LNM present).

Microarray analysis. Three OSCC patients were subjected to microarray analysis to screen for TSGs in this population. As in previous studies, we know that there are 17 upregulated genes in OSCC, comprising matrix metallopeptidase 1 *MMP1*, *MMP10*, *MMP3*, *MMP12*, *PTHLH*, *INHBA*, *LAMC2*, *IL8*, *KRT17*, *COL1A2*, *IF16*, *ISG15*, *PLAU*, *GREM1*, *MMP9*, *IFI44* and *CXCL1,* and that there are 18 downregulated genes in OSCC, comprising *KRT4*, *MAL*, *CRNN*, *SCEL*, *CRISP3*, *SPINK5*, *CLLA4*, *ADH1B*, *P11*, *TGM3*, *RHCG*, *PPP1R3C*, *CEACAM7*, *HPGD*, *CFD*, *ABCA8*, *CLU* and *CYP3A5* (15,21). In this study, we found 2 upregulated genes, *MMP1* and *MMP3,* and 11 downregulated genes, *CRISP3*, *SCGB3A1*, *AGR2*, *PIP*, *C20orf114*, *TFF1*, *STATH*, *AZGP1*, *MUC7*, *DMBT1* and *LOC389429,* in all three patients. The detected genes of up- and down-regulation are shown in Table II. *CRISP3* was selected as it is currently unknown as to whether this gene is associated with OSCC.

Assessment of CRISP3 by real-time QPCR. To evaluate microarray data at *CRISP3*, real-time QPCR was performed using non-amplified genomic DNA as a template. Initially, PCR primer sets located in *CRISP3* were successfully designed to meet the criteria for reliable quantification. One PCR primer set was designed in a housekeeping gene, *GAPDH,* located on chromosome 12p, as a reference for normalization. Real-time QPCR analysis was performed with the above-mentioned primer sets using genomic DNA of 5 OSCC cell lines and 60 OSCC patients. DNA copy number loss of *CRISP3* was observed in 2 of the 5 OSCC-derived cell lines (SAS and HSC2). The copy number of *CRISP3* was significantly reduced in OSCC-derived cell lines compared with NOKs (P=0.024, Unpaired U-test: Fig. 1). The copy number loss of *CRISP3* was observed in 24 (40%) of the 60 patient specimens. We evaluated statistically significant differences in all clinical characteristics pertaining to the *CRISP3* copy number between OSCCs and normal tissues. No statistically significant differences were noted. Moreover, no statistically significant differences were observed in the *CRISP3* copy number between OSCC tumor tissues and normal tissues in the 60 OSCC patients (P=0.401, Mann-Whitney's U-test; Fig. 2).

Clinicopathological findings and statistical analysis. We compared our results with the clinicopathological findings for each tumor. The copy number loss of *CRISP3* was observed

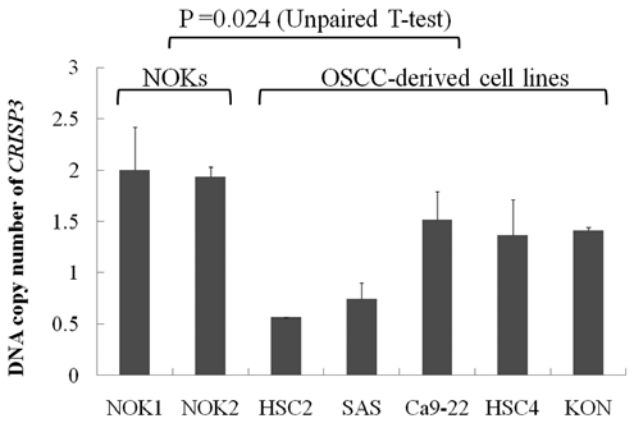

Figure 1. Quantification of *CRISP3* DNA copy numbers in OSCC-derived cell lines and NOKs by real-time QPCR. Two OSCC cell lines decreased *CRISP3* copy numbers compared with NOKs. A statistically significant difference was found in the *CRISP3* copy numbers between OSCC-derived cell lines and NOKs (P=0.024, unpaired U test).

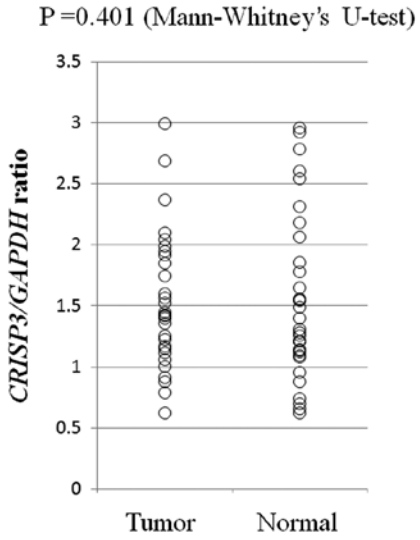

Figure 2. DNA copy numbers of *CRISP3* in 60 OSCC patients. The manifestation amount of DNA copy numbers of *CRISP3* among tumor and normal tissues. The statistically significant difference could not be confirmed.

Table III. Correlation between the DNA copy numbers of *CRISP3* and clinical characteristics in OSCCs.

	Loss	Normal	P-value
Gender			
Male	13	32	0.005
Female	11	4	
Age			
<65	17	21	0.104
≥65	15	7	
Ethnic group			
Taiwanese	6	11	0.773
Japanese	18	25	
Tumor site			
Tongue	6	17	0.491
Buccal mucosa	6	7	
Lower gingiva	6	6	
Upper gingiva	4	3	
Oral floor	2	3	
T classification			
T1/T2	21	21	0.021
T3/T4	3	15	
LNM			
Present	6	12	0.573
Absent	18	24	
Stage			
I	5	8	0.111
II	8	4	
III	6	13	
IV	5	11	
Tobacco			
Yes	7	19	0.11
No	17	17	
Alcohol			
Yes	12	27	0.058
No	12	9	

LNM, lymph node metastasis confirmed by histopathological examination within the 12-month follow-up period.

in 24 (40%) of the 60 patients. The Fisher's exact test was performed to evaluate the significance of correlations between copy number loss of *CRISP3* and clinicopathological findings (Table III). A significant statistical correlation between the copy number loss and gender and T classification was observed. The copy number loss of *CRISP3* was detected in early stage lesions; and the copy number loss of *CRISP3* tended to be higher in early clinical stages. Moreover, no statistically significant correlation was found between the copy number loss of *CRISP3* and other clinicopathological findings such as age, ethnicity, lymph node metastasis, tobacco and alcohol associated with the tumor samples.

Discussion

OSCC is often associated with loss of eating and speech function, disfigurement and psychological distress. The development of OSCC is strongly associated with smoking and excessive alcohol consumption (22). The prevention and management of this disease is likely to benefit from the identification of molecular markers and targets (7,8).

Recently, the development of tools for measuring gene expression and copy numbers across the entire genome has revolutionized our ability to characterize cancers at the molecular level. Cytogenetic analyses in conjunction with molecular genetics analyses by a number of research groups have shown an accumulation of genetic abnormalities during the development and/or progression of OSCC (23). In this study, we identified 11 downregulated genes, *CRISP3*, *SCGB3A1*, *AGR2*, *PIP*, *C20orf114*, *TFF1*, *STATH*, *AZGP1*, *MUC7*, *DMBT1* and *LOC389429*, and 2 up-regulation genes,

MMP1 and *MMP3,* using microarray technology. *CRISP3* was selected due to the fact that it is unknown as to whether *CRISP3* is associated with OSCC. However, to the best of our knowledge no previous OSCC studies have identified *CRISP3* as we observed in the current study. Our findings indicate that DNA copy number loss of *CRISP3* is involved in carcinogenesis in OSCC patients.

Little is known about the function of the mammalian CRISPs; however, *CRISP1* and *CRISP2* are known to be involved in various steps in reproduction (24). The C-terminal domain of *CRISP2* has been shown to interact with calcium channels (25) and to bind a kinase present in the acrosome of mouse sperm (26). Less is known about human *CRISP3*. *CRISP3*, which is also known as specific granule protein of 28 kDa (SGP28), belongs to a family of CRISPs characterized by their size (220-230 amino acids), their secretory properties and a content of 16 highly conserved cysteine residues, which form an intra-molecular disulphide bond (27). Apart from its ability to bind A1BG in serum, Udby *et al* have shown that *CRISP3* forms similar complexes with one of the three major proteins secreted from the prostate in seminal plasma, β-microseminoprotein (28). Human *CRISP3* is also likely to be involved in the pathogenesis of prostate cancer, where *CRISP3* expression is significantly upregulated (12). However, to the best of our knowledge no previous reports have identified the *CRISP3* gene in OSCC.

Table II lists other candidate genes that may be associated with carcinogenesis in OSCC. These genes exhibit correlations with various types of carcinoma excluding *C20orf114*, *STATH*, and *LOC389429*. *SCGB3A1* (*HIN1*) is a TSG that is highly expressed in a number of epithelial tissues, including the breast, lung, trachea, pancreas, prostrate and salivary gland. Inactivation of SCGB3A1 expression by promoter methylation is frequent in many types of epithelial carcinoma and carcinoma *in situ*, including breast, lung and nasopharyngeal carcinoma (29,30). *AGR2* is a putative member of the protein disulfide isomerase family and was first identified as a homolog of the *Xenopus laevis* gene *XAG-2*. *AGR2* was down-regulated in gastric tumor tissue compared to the control (31). *AGR2* has previously been found to be one of several genes that encode secreted proteins showing an increased expression in prostate cancer cells compared to normal prostatic epithelium (32). We observed that the *MMP1* gene is up-regulated in OSCC. *MMP1* is located on chromosome 11q22.3 and belongs to the MMP family, which is responsible for the degradation of extracellular matrix components. There is clear evidence indicating that *MMP1* is involved in various cell and tumour events, including cancer-cell development, growth, proliferation, apoptosis, invasion and metastasis, as well as angiogenesis and immune surveillance (33-36).

Our results suggest that the *CRISP3* gene is a novel TSG particular to OSCC, and inactivation of the *CRISP3* gene may play one or more roles in the carcinogenesis of OSCCs. In the present study, we applied whole-genome analysis of TSGs in specimens from 3 patients with OSCC by microarray techno-logy. A significant statistical correlation was observed between the DNA copy number of *CRISP3* and T-classification and gender. No significant correlation was found between DNA copy number loss of *CRISP3* and age, ethnic group, tumor site, lymph node metastasis, tumor stage, tobacco or alcohol. In this population, 15 patients were habitual consumers of tobacco, alcohol and/or betel nuts. We compared these 15 patients with 22 patients who did not consume tobacco, alcohol or betel nuts. No significant correlation was found between the DNA copy number loss of *CRISP3* and tobacco, alcohol and/or betel nut consumption. These results indicate that inactivation of the *CRISP3* is an early event in OSCC, since T1/T2 classification is correlated with DNA copy number loss of *CRISP3* rather than T3/T4 classification. We were not able to apply the functional analysis of the *CRISP3* gene. Further studies employing techniques including immunoblotting, immunofluorescence, and immunohistochemistry are required to clarify the function of this gene in the development and progression of OSCC. Furthermore, a study with a larger patient series is requred to validate these results, in order that more appropriate treatment modalities can be offered to OSCC patients in Taiwan, Japan, and worldwide.

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