

Original Article

Effects of *EZH2* promoter polymorphisms and methylation status on oral squamous cell carcinoma susceptibility and pathology

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Abstract: Oral squamous cell carcinoma (OSCC), which is malignant tumors in oral cavity, is the fourth most common male cancer in Taiwan. *EZH2* plays a key role in transcriptional repression through chromatin remodeling and in cancer development. Although the *EZH2* expression in OSCC is highly correlated with tumorigenesis, it has not been determined if specific *EZH2* genetic variants are associated with OSCC risk. The aim of this study was to investigate the relationship between genetic polymorphisms of *EZH2* and susceptibility to OSCC in Taiwan. Here, four SNPs of *EZH2* (rs6950683, rs2302427, rs3757441, and rs41277434) were analyzed by a real-time PCR genotyping in 576 patients with oral cancer and 552 cancer-free controls. After adjusting for other co-variants, we found that carrying CC genotype at *EZH2* rs6950683 and rs3757441 had a lower risk of developing OSCC than did wild-type carriers. The CCGA or CCTA haplotype among the four *EZH2* sites was also associated with a reduced risk of OSCC. Furthermore, OSCC patients who carried CC genotype at *EZH2* rs6950683 had a higher methylation than TC genotype. Our results suggest that the two SNPs of *EZH2* (rs6950683 and rs3757441) might contribute to the prediction of OSCC susceptibility. Moreover, rs6950683 CC genotype exhibits hypermethylation in *EZH2* promoter. This is the first study to provide insight into risk factors associated with *EZH2* variants and epigenetic changes in carcinogenesis of OSCC in Taiwan.

Keywords: *EZH2*, oral squamous cell carcinoma, single-nucleotide polymorphism (SNP), bisulfite sequencing, methylation

Introduction

Oral squamous cell carcinoma (OSCC) represents the fourth common male cancer of cancer-related deaths per year in Taiwan [1]. The development of OSCC is a multistep process modulated by the accumulation of endogenous and environmental factors including alcohol drinking, tobacco smoking, betel nut chewing, and chronic inflammation [2-4]. The current treatment for OSCC is surgery combined with radiotherapy and chemotherapy [5]. However, the prognosis of OSCC is poor due to aggressive local metastasis and invasion. The per-

centage of OSCC recurrence rate was 32.7% and 5-year survival for patients was 54.5% [6].

Polycomb group (PcG) proteins are important epigenetic regulators of embryonic development, cell-fate determination, proliferation, stem cell pluripotency and self-renewal [7]. *EZH2* is a subunit of the multi-enzyme complex polycomb repressive complex 2 and functions as a histone H3 Lys27 (H3K27) trimethyltransferase [8]. Recent findings implicate that *EZH2* is overexpressed in a wide range of cancer types, including brain [9], breast [10], and liver cancer [11]. Overexpression of *EZH2* has been

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correlated with advanced stages of human cancer progression and poor prognosis [12]. Moreover, EZH2 promotes epithelial-to-mesenchymal transition (EMT) and enhanced cell migration and invasion [13]. Although EZH2 contributes to the formation of many types of cancer, the association between *EZH2* variants and OSCC risk and prognosis has been poorly investigated.

Recently, several studies suggested that genetic variation will affect cancer susceptibility and response to environmental carcinogens [14]. Among these genetic factors, SNPs are one of the most common types of genetic variation and have a significant impact on the diagnosis of the causes, treatment and prevention of human genetic diseases [15-17]. We therefore performed a case-control study of four SNPs of *EZH2* to clarify the associations between these SNPs and OSCC susceptibility and clinicopathologic features.

Epigenetic regulation is an important genetic modification in silencing of tumor suppressor genes and activation of oncogenes [18]. It is commonly known that hypermethylation within the gene promoter regions cause inactivation of certain tumor-suppressor genes and numerous studies have demonstrated DNA methylation is associated with gene silencing in different cancer types [19]. In this study, we analyzed associations among SNPs of *EZH2*, epigenetic changes, and OSCC susceptibility. To our knowledge, this is the first study that has used a bisulfite DNA sequencing approach to examine methylation status with *EZH2* gene SNPs in oral carcinogenesis.

Materials and methods

Ethics statement

Study protocols were approved by the institutional review boards of the Taichung Chung Shan Medical University Hospital. All methods were carried out in accordance with the approved guidelines. All subjects provided written informed consent to participate in the study.

Study subjects and specimen collection

This hospital-based case-control study recruited 576 OSCC patients between 2010 and 2014

from the Chung Shan Medical University Hospital in Taichung and the Changhua Christian Hospital and Show Chwan Memorial Hospital in Changhua, Taiwan, to serve as the case group. The diagnosis of OSCC was made according to the criteria specified in the national guidelines for OSCC. An additional 552 race- and ethnic group-matched non-cancer patients who entered the hospital for health check-ups were enrolled as the control group. OSCC patients were clinically staged at the time of diagnosis according to the tumor/node/metastasis staging system of the American Joint Committee on Cancer. The patients' clinicopathological characteristics, including clinical staging, lymph node metastasis, and histopathologic grading levels, were verified by chart review. Whole-blood specimens collected from the controls and OSCC patients were placed in tubes containing EDTA, immediately centrifuged, and stored at -80°C.

Selection of EZH2 polymorphisms

A total of four SNPs in *EZH2* (NM_004456) were selected from the International HapMap Project data for this study. We included the non-synonymous SNP rs2302427 (D185H in exon 6) in the coding sequence of the gene. The other SNPs (rs6950683, rs3757441, and rs41277434) were selected in this study because they have been found in cancer patients.

Genomic DNA extraction

Genomic DNA was extracted using QIAamp DNA blood mini kit reagents (Qiagen, Valencia, CA). DNA was dissolved in buffer containing 10 mM Tris (pH 7.8) and 1 mM EDTA and then quantified by measurement of the optical density at 260 nm. Final DNA preparations were stored at -20°C and used as templates for PCR.

Real-time PCR

Allelic discrimination of the *EZH2* polymorphisms rs6950683, rs2302427, rs3757441, and rs41277434 was assessed using an ABI StepOne™ Real-Time PCR System (Applied Biosystems), SDS v3.0 software (Applied Biosystems), and the TaqMan assay. The final volume for each reaction was 5 µL, containing 2.5 µL TaqMan Genotyping Master Mix, 0.125 µL TaqMan probes mix, and 10 ng genomic

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Table 1. The distributions of demographical characteristics in 552 healthy controls and 576 patients with oral cancer

Variable	Controls (N=552)	Patients (N=576)	p value
Age (yrs)	Mean ± S.D. 51.65 ± 14.62	Mean ± S.D. 54.37 ± 11.28	$P < 0.001^*$
Gender	n (%)	n (%)	
Male	449 (81.3%)	554 (96.2%)	
Female	103 (18.7%)	22 (3.8%)	$P < 0.001^*$
Betel nut chewing			
No	460 (83.3%)	136 (23.6%)	
Yes	92 (16.7%)	440 (76.4%)	$P < 0.001^*$
Alcohol consumption			
No	345 (62.5%)	235 (40.8%)	
Yes	207 (37.5%)	341 (59.2%)	$P < 0.001^*$
Tobacco consumption			
No	336 (60.9%)	87 (15.1%)	
Yes	216 (39.1%)	489 (84.9%)	$P < 0.001^*$

Mann-Whitney U test or Fisher's exact test was used between healthy controls and patients with oral cancer. *P value < 0.05 as statistically significant.

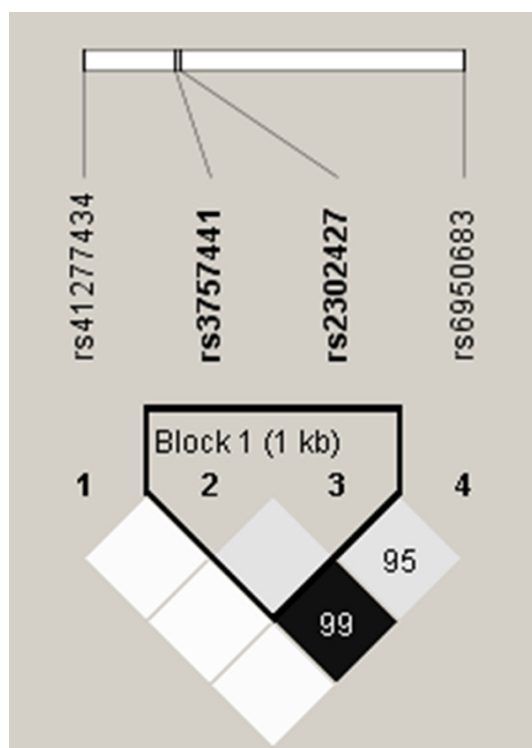


Figure 1. EZH2 pairwise linkage disequilibrium (LD) patterns. Schematic presentation of the EZH2, indicating the locations of the SNP polymorphism and the pairwise linkage disequilibrium measures D' . The measure of D' of SNP is shown graphically according to a grey scale, where white represents low D' and dark represents high D' .

DNA. The reaction conditions included an initial denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

Bisulfite modification

Genomic DNA (0.5 µg) was treated with sodium bisulfite via the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). Bisulfite treatments changed unmethylated cytosines into uracils while leaving methylated cytosines unmodified. Bisulfite-treated genomic DNA was used for further analysis of methylation status of CpG sites via bisulfite DNA sequencing.

Bisulfite DNA sequencing

Bisulfite-modified DNA was amplified by PCR with primer designed to amplify a 412-bp-long promoter region of the EZH2 gene. Bisulfite primer sequences were: 5'-AGTTTTGAATTGGTTTAAATTTGGT-3' (forward) and 5'-CCTCCAATCACAAAACCC-3' (reverse). Purified PCR products were cloned into the pGEM vector (GeneMark, Taichung, Taiwan) by using the manufacturer's standard protocol. Seven clones were submitted for DNA sequencing at the Genomics BioSci & Tech, Ltd. (New Taipei City, Taiwan).

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD), and categorical variables were expressed as number (%). Hardy-Weinberg equilibrium was assessed using a χ^2 goodness-of-fit test for biallelic markers. A Mann-Whitney U-test and a Fisher's exact test were used to compare differences of age and demographic characteristics distributions between controls and OSCC patients. The odds ratios (ORs) with 95% confidence intervals (CIs) were estimated by logistic regression models. The adjusted odds ratios (AORs) with 95% CIs of the association between genotype frequencies and OSCC risk as well as clinical pathological characteristics were estimated by multiple logistic regression models after controlling for other covariates. The haplotype-based analysis was carried out using the Phase program.

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Table 2. Distribution frequency of *EZH2* genotypes in 552 controls and 576 oral cancer patients

Variable	Controls (N=552) n (%)	Patients (N=576) n (%)	OR (95% CI)	AOR (95% CI)
rs6950683				
TT	264 (47.8%)	308 (53.5%)	1.00	1.00
TC	220 (39.9%)	221 (38.4%)	0.861 (0.671-1.104)	0.893 (0.622-1.280)
CC	68 (12.3%)	47 (8.2%)	0.592 (0.395-0.890)*	0.492 (0.274-0.884)*
TC+CC	288 (52.2%)	268 (46.5%)	0.798 (0.631-1.008)	0.790 (0.563-1.108)
rs2302427				
CC	346 (62.7%)	356 (61.8%)	1.00	1.00
CG	171 (31.0%)	200 (34.7%)	1.137 (0.883-1.463)	0.930 (0.646-1.405)
GG	35 (6.3%)	20 (3.5%)	0.555 (0.314-1.002)	0.611 (0.265-1.405)
CG+GG	206 (37.3%)	220 (38.2%)	1.038 (0.816-1.321)	0.883 (0.623-1.250)
rs3757441				
TT	271 (49.1%)	312 (54.2%)	1.00	1.00
TC	223 (40.4%)	221 (38.4%)	0.861 (0.672-1.102)	0.887 (0.620-1.269)
CC	58 (10.5%)	43 (7.5%)	0.644 (0.420-0.987)*	0.524 (0.284-0.968)*
TC+CC	281 (50.9%)	264 (45.8%)	0.816 (0.646-1.031)	0.804 (0.573-1.127)
rs41277434				
AA	517 (93.6%)	540 (93.7%)	1.00	1.00
AC	34 (6.2%)	35 (6.1%)	0.986 (0.606-1.604)	1.546 (0.784-3.048)
CC	1 (0.2%)	1 (0.2%)	0.957 (0.060-15.347)	3.341 (0.157-70.898)
AC+CC	35 (6.4%)	36 (6.3%)	0.985 (0.609-1.592)	1.590 (0.814-3.104)

The odds ratios (ORs) and with their 95% confidence intervals (CIs) were estimated by logistic regression models. The adjusted odds ratios (AORs) with their 95% confidence intervals (CIs) were estimated by multiple logistic regression models after controlling for age, gender, betel nut chewing, tobacco and alcohol consumption.

Linkage disequilibrium (LD) coefficients, $D' = D / D_{max}$ (or D / D_{min} if the D' value was negative), were assessed for pairs of alleles between the two sites of *EZH2* polymorphisms. All P values < 0.05 were considered significant. The data were analyzed using SAS statistical software (Version 9.1, 2005; SAS Institute Inc., Cary, NC).

Results

Population characteristics

A total of 1128 subjects were enrolled in this case-control study, which containing 576 OSCC patients and 552 healthy controls (**Table 1**). The healthy controls were comparable with the OSCC patients in regard to significantly different distributions of age ($P < 0.001$), gender ($P < 0.001$), betel nut chewing ($P < 0.001$), alcohol consumption ($P < 0.001$) and tobacco consumption ($P < 0.001$) between healthy controls and OSCC patients. To reduce possible interference of confounding variables, AORs with 95% CIs were estimated by multiple logistic regression models after controlling for age, gender,

betel nut chewing, tobacco and alcohol consumption in each comparison.

Association between *EZH2* SNPs and OSCC risk

In our recruited control group, the frequencies of *EZH2* genes were in Hardy-Weinberg equilibrium. The reconstructed linkage disequilibrium plot for the four SNPs was shown in **Figure 1**. We found that rs6950683 and rs3757441 show a high degree of D' in our study. **Table 2** shows the genotype distributions and the association between OSCC and *EZH2* polymorphisms. The alleles with the highest distribution frequency at *EZH2* rs6950683, rs2302427, rs3757441, and rs41277434 in both OSCC patients and controls were homozygous T/T, homozygous C/C, homozygous T/T, and homozygous A/A, respectively. After adjusting variables, people with rs6950683 CC showed a 0.492-fold (95% CI: 0.274-0.884), and rs3757441 CC showed a 0.524-fold (95% CI: 0.284-0.968) lower risk of OSCC, respectively, compared with individuals carrying the wild-type allele. Individuals with polymorphisms at

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Table 3. Distribution of the allele frequencies of EZH2 genotypes in controls and oral cancer patients

Variable	Controls (N=1104) n (%)	Patients (N=1152) n (%)	OR (95% CI)	p value
rs6950683				
T	748 (67.8%)	837 (72.7%)	1.00	
C	356 (32.2%)	315 (27.3%)	0.791 (0.660-0.948)	P=0.011*
rs2302427				
C	863 (78.2%)	912 (79.2%)	1.00	
G	241 (21.8%)	240 (20.8%)	0.942 (0.770-1.153)	P=0.564
rs3757441				
T	765 (69.3%)	845 (73.4%)	1.00	
C	339 (30.7%)	307 (26.6%)	0.820 (0.683-0.984)	P=0.033*
rs41277434				
A	1068 (96.7%)	1115 (96.8%)	1.00	
C	36 (3.3%)	37 (3.2%)	0.984 (0.617-1.570)	P=0.948

The odds ratios (ORs) and with their 95% confidence intervals (CIs) were estimated by logistic regression models. *Statistically significant at $P < 0.05$.

rs2302427 and rs41277434 showed no reduction in OSCC risk compared with wild-type individuals.

The distribution of clinical status/*EZH2* genotypes in OSCC patients was estimated to clarify the role of *EZH2* polymorphisms in the clinicopathologic state of OSCC patients. Clinical status assessments included TNM clinical stage, primary tumor size, lymph node involvement, distant metastasis. Compared with control subjects having the wild-type genotype, patients with at least one polymorphic C allele at *EZH2* rs6950683 showed significant differences in *EZH2* genotypic frequencies (Table 3), clinical stage and tumor size (Table 4); however, patients with at least one polymorphic C allele at *EZH2* rs3757441 showed no significant differences in the clinicopathologic state.

Haplotypes analysis of EZH2 SNPs with OSCC risk

We further explored the haplotypes to evaluate the combined effect of the four polymorphisms on oral-cancer susceptibility. The haplotype distributions of *EZH2* rs6950683, rs2302427, rs3757441, and rs41277434 were further evaluated, and seven haplotypes were derived from these four SNPs in our recruited individuals. The most common haplotype in the control group was TCTA (42.4%), and it was, therefore, chosen as the reference. Compared with this

reference, two minor haplotypes, CCCA and CCTA, significantly reduced the risk of OSCC by 0.763-fold (95% CI: 0.626-0.930) and 0.250-fold (95% CI: 0.100-0.627), respectively (Table 5).

Methylation level of EZH2 rs6950683

DNA methylation plays an important role in regulating gene expression. To explore the methylation status of rs6950683 on *EZH2* promoter, we first examined the 2 OSCC cell lines (TC genotype) and 4 OSCC patient samples (CC genotype), followed by

detailed investigation of the methylation status of rs6950683 via bisulfite sequencing analysis. Therefore, the *EZH2* promoter further upstream spanning nucleotides -440 to -29 bp that include the rs6950683 was sequenced after bisulfite modification of genomic DNA from cell lines and patient samples. As shown in Figure 2, individual cell colonies derived from patients were fully methylated at rs6950683. In contrast, cell lines SAS and SCC25 were both methylated and unmethylated at rs6950683. Moreover, we also found that *EZH2* expression from total cell lysates were much lower in C/C genotype than T/C genotype. These results suggested that the rs6950683 C/C genotype was more easily methylated than the T/C genotype and associated with low *EZH2* expression in OSCC.

Discussion

The present study focused on the association of genetic polymorphisms and haplotypes of the *EZH2* gene with oral cancer risk. *EZH2* is a member of the polycomb group of genes and is important in cell cycle regulation. *EZH2* is also associated with cell proliferation, apoptosis, metastasis and invasion in OSCC development [20]. In previous studies, the results suggested that *EZH2* acts as an oncogene and correlated with malignant potential and poor prognosis in OSCC [21].

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Table 4. Clinical status and *EZH2* genotypic frequencies in 576 oral cancer patients

Variable	rs6950683			rs2302427			rs3757441			rs41277434		
	TT (N=308)	TC + CC (N=268)	<i>p</i> value	CC (N=356)	CG + GG (N=220)	<i>p</i> value	TT (N=312)	TC + CC (N=264)	<i>p</i> value	AA (N=540)	AC + CC (N=36)	<i>p</i> value
Clinical Stage												
Stage I/II	122 (39.6%)	133 (49.6%)	0.016*	159 (44.7%)	96 (43.6%)	0.810	127 (40.7%)	128 (48.5%)	0.061	241 (44.6%)	14 (38.9%)	0.502
Stage III/IV	186 (60.4%)	135 (50.4%)		197 (55.3%)	124 (56.4%)		185 (59.3%)	136 (51.5%)		299 (55.4%)	22 (61.1%)	
Tumor size												
≤T2	178 (57.8%)	177 (66.0%)	0.042*	224 (62.9%)	131 (59.5%)	0.418	183 (58.7%)	172 (65.2%)	0.110	336 (62.2%)	19 (52.8%)	0.259
>T2	130 (42.2%)	91 (34.0%)		132 (37.1%)	89 (40.5%)		129 (41.3%)	92 (34.8%)		204 (37.8%)	17 (47.2%)	
Lymph node metastasis												
No	191 (62.0%)	174 (64.9%)	0.469	228 (64.0%)	137 (62.3%)	0.668	192 (61.5%)	173 (65.5%)	0.322	344 (63.7%)	21 (58.3%)	0.517
Yes	117 (38.0%)	94 (35.1%)		128 (36.0%)	83 (37.7%)		120 (38.5%)	91 (34.5%)		196 (36.3%)	15 (41.7%)	
Distant metastasis												
No	304 (98.7%)	264 (98.5%)	0.843	353 (99.2%)	215 (97.7%)	0.154	308 (98.7%)	260 (98.5%)	0.812	533 (98.7%)	35 (97.2%)	0.462
Yes	4 (1.3%)	4 (1.5%)		3 (0.8%)	5 (2.3%)		4 (1.3%)	4 (1.5%)		7 (1.3%)	1 (2.8%)	

T2: tumor size > 2 cm in the greatest dimension. **P* value < 0.05 as statistically significant.

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Table 5. Distribution frequency of *EZH2* haplotype in controls and oral cancer patients

Variable				Controls	Patients	OR (95% CI)	p value
rs6950683 T/C	rs2302427 C/G	rs3757441 T/C	rs41277434 A/C	(N=1104) n (%)	(N=1152) n (%)		
T	C	T	A	468 (42.4%)	562 (48.8%)	Reference	
C	C	C	A	335 (30.3%)	307 (26.6%)	0.763 (0.626-0.930)	0.007*
T	G	T	A	240 (21.7%)	238 (20.7%)	0.826 (0.665-1.026)	0.084
T	C	T	C	36 (3.3%)	37 (3.2%)	0.856 (0.532-1.376)	0.520
C	C	T	A	20 (1.8%)	6 (0.5%)	0.250 (0.100-0.627)	0.001*
T	C	C	A	4 (0.4%)	0 (0.0%)	---	
C	G	T	A	1 (0.1%)	2 (0.2%)	1.665 (0.151-18.425)	0.674

*P value < 0.05 as statistically significant.

Epidemiological studies have provided evidences that genetic variation of humans can affect how humans develop diseases [14]. Analyzing these DNA polymorphisms may help in identifying progression and susceptibility of malignancy. Therefore, *EZH2* polymorphisms may be associated with OSCC development. However, the predictive value of *EZH2* for susceptibility to OSCC has not previously been investigated. In this study, we provide novel information of SNPs of *EZH2* on the oral cancer susceptibility and clinicopathologic statuses association.

In our hospital-based case-control study, 4 polymorphisms of *EZH2* were examined in 552 healthy controls and 576 patients with oral cancer. Patients recruited among the oral cancer at our hospital showed a higher interference of environmental factors than did the control cases, such as alcohol drinking, tobacco smoking and betel-nut chewing (Table 1), which indicates environmental factors are highly associated to the increased risk of oral cancer. Exposure to environmental carcinogen might involve the process of tumorigenesis, but more evidence indicates that genetic variants might more helpful in predicting cancer risk [22, 23]. Therefore, the association between the SNPs and the risk of oral cancer was analyzed by controlling for environmental influences.

Data in Table 2 show that individuals with the *EZH2* polymorphisms rs6950683 CC and rs3757441 CC genotype have lower risks for OSCC compared to the TT genotype. Although the functional importance of these two SNPs has not been tested experimentally, clinical research from previous studies also shows that these two SNPs carrying CC genotype have a protective effect of risk than those carrying TT wild type in lung cancer and colorectal cancer

[24, 25]. We also observed in the *EZH2* gene haplotypes “CCCA” and “CCTA” that were associated with a decreased the risk of developing OSCC. These results suggest that *EZH2* gene polymorphisms rs6950683 and rs3757441 have a strong impact on oral cancer susceptibility.

Epigenetic regulations including DNA methylation and histone modification, which can lead to genomic instability, control gene expression and play important roles during cancer development [19, 26]. DNA methylation typically occurs in the promoter CpG-rich regions of tumor suppressor genes, which mediates gene silencing. SNP rs6950683 is located in a tissue-specific CpG island within the *EZH2* promoter region upstream of the *EZH2* coding sequence, several studies have shown promoter might play an important role in regulating the process of transcription and protein expression. One of the intriguing findings of our study is that rs6950683 remains hypermethylation status when patient sample carrying CC genotype. This result raises the possibility that methylation of rs6950683 most likely impact *EZH2* expression by affecting promoter function, and consequently reduce the risk of OSCC. Further functional studies are needed to confirm the specific mechanisms by epigenetic regulation of *EZH2* polymorphisms influence the development of OSCC. However, the number of case subjects in this study was relatively small and only considers a Taiwanese population, which may limit the application of these findings. Therefore, additional studies with larger sample sizes are needed to validate the epigenetic regulation of *EZH2* polymorphisms to OSCC. Further investigations should focus on epigenetic changes of *EZH2* polymorphisms and their biological function in OSCC patients.

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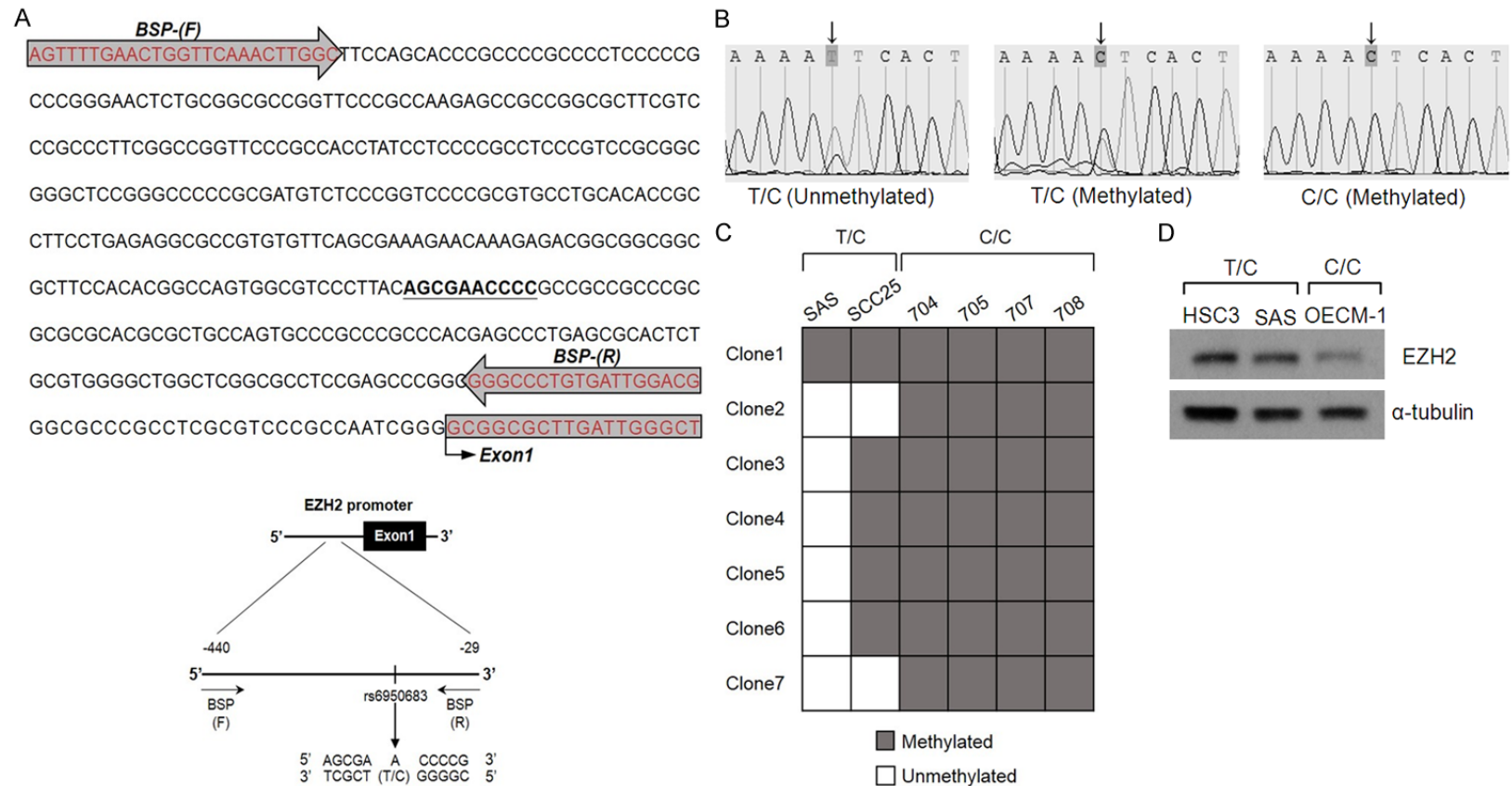


Figure 2. Bisulfite sequencing of the rs6950683. A. Positions of bisulfite sequencing PCR (BSP) primers. BSP primers (-440 to -29) were designed to include the rs6950683. B. Bisulfite sequence analyses of the EZH2 promoter reveal TC (two signal) or CC genotype (one signal) at the rs6950683 location at which the arrow points. C. Bisulfite sequencing results from oral cancer patient samples (704, 705, 707 and 708) and oral cancer cell lines (SAS and SCC25). Open squares, un-methylated status; gray squares, methylated status. Each square represents an individual sequenced DNA strand. D. Western blot analysis of EZH2 in oral cancer cell lines. α -tubulin was used as a loading control.

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In conclusion, this is the first study to associate *EZH2* polymorphisms and epigenetic change with risk of OSCC. Results showed genotypes of "CC" of rs6950683 and rs3757441 in the *EZH2* gene that decreased OSCC susceptibility. Moreover, OSCC patients with the rs6950683 CC genotype have a hypermethylation status in *EZH2* promoter. These results suggest that *EZH2* variants might play important roles in the susceptibility to OSCC.

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Disclosure of conflict of interest

None.

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