

Expression and promoter methylation of Wnt inhibitory factor-1 in the development of oral submucous fibrosis

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Abstract. Oral squamous cell carcinoma (OSCC) is a type of head and neck malignancy with a high mortality rate. Oral submucous fibrosis (OSF) is the pre-cancerous lesion of OSCC, whose molecular mechanisms in OSCC tumorigenesis remain largely unclear. Activation of the Wnt/ β -catenin signaling pathway plays an important role in oral mucous carcinogenesis, although rare mutations of Wnt signaling molecules are found in OSCC, suggesting an epigenetic mechanism mediating aberrant Wnt/ β -catenin signaling in OSCC. Wnt inhibitory factor-1 (WIF1) is a Wnt antagonist, and its downregulation and methylation have been reported in a number of malignancies. However, the expression and methylation of WIF1 in the development of OSF have yet to be reported. In the present study, we investigated the WIF1 expression level by immunohistochemical staining and semi-quantitative RT-PCR in normal oral, OSF and OSCC tissues, as well as the methylation status by methylation-specific PCR and bisulfite genomic sequencing. The results showed that WIF1 was readily expressed in normal oral mucous tissues, but decreased gradually in OSF early, moderately advanced and advanced tissues, and was less expressed in OSCC tissues. Moreover, WIF1 was able to translocate from the nuclear to cytoplasm in OSF and OSCC tissues. Furthermore, *WIF1* was frequently methylated in OSCC cases with betel quid chewing habit, but not in normal

oral mucous and different stages of OSF tissues, suggesting *WIF1* methylation is tumor-specific in the development of OSF. Thus, the results demonstrated that *WIF1* is frequently downregulated or silenced by promoter methylation in the carcinogenesis of OSF, which serves as a potential epigenetic biomarker for the early detection of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is a common type of cancer of the head and neck region, with a high mortality rate and rising incidence (1,2). Oral carcinogenesis is a complex multi-step process, undergoing sequential histopathological stages including hyperplasia, oral epithelial dysplasia and final malignant phenotypes (3,4). Despite significant advances in the diagnosis and treatment of OSCC in recent years, 5-year survival rate remains low due to late-stage diagnosis.

Oral submucous fibrosis (OSF), as a pre-cancerous condition (5), is a chronic and potentially malignant disorder, first described in the early 1950s by Pindborg and Sirsat (6). OSF is predominantly prevalent in Indian, mainland China (such as Hunan and Hainan), Taiwan, Bangladesh and Pakistan (7). It is characterized by submucosal fibrosis initially occurring in the oral cavity and then progressively infringing the pharynx and upper esophagus (8). The malignant transformation rate of OSF has been reported in the range of 7-30% based on the different population (9). The etiology of OSF is multifactorial and remains vague. Chewing of betel quid containing areca nut is the strongest risk factor of OSF, and other factors include tobacco use, alcohol drinking, as well as genetic and immunologic predisposition (10-12). Thus, elucidating the molecular events of OSF malignant progression may be useful in identifying potential biomarkers for the early diagnosis and prevention of OSCC.

Epigenetic modifications including the promoter CpG methylation and histone modification have been recognized as being crucial events in cancer development, such as genetic

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alterations (13,14). Aberrant promoter methylation leads to the transcriptional silencing of tumor-suppressor genes (TSGs), involved in cell malignant transformation and tumorigenesis. Detection of promoter CpG methylation in human DNA isolated from primary tumor tissues and bodily fluids has become a promising approach for non-invasive screening and early diagnosis of cancer (15). Several TSGs have been identified methylated in OSCC (16-19), such as *p16*, *p14arf*, *E-cadherin*, O-6-methylguanine-DNA methyltransferase (*MGMT*), death-associated protein kinase (*DAPK*), runt-related transcription factor 3 (*RUNX3*), as well as certain Wnt antagonists.

The Wnt/ β -catenin signaling pathway plays an important role in human malignancies including OSCC (20,21). Wnt inhibitory factor-1 (*WIF1*) as a secreted WNT antagonist inhibits Wnt/ β -catenin signaling by directly binding to Wnt proteins (22). However, few studies have focused on the epigenetic disruption of *WIF1* in OSF progression. In the present study, we investigated the expression level and methylation status of *WIF1*, a key antagonist in WNT signaling, in the carcinogenesis of OSF.

Materials and methods

Tissue specimens. OSCC (n=55), OSF (n=45) and normal oral mucosa (n=15) tissue specimens were obtained at the time of surgical resection at the Xiangya Second Hospital and Xiangya Hospital, Central South University (Changsha, China) and Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China) from January 2013 to June 2014. Informed consent from the patients was obtained under a protocol reviewed and approved by the Institutional Review Boards of the Xiangya School of Medicine or Shanghai Jiaotong University School of Medicine. The clinical diagnosis and pathological stage of OSF was determined in terms of the Pindborg criteria (23). OSCC was diagnosed according to the World Health Organization criteria of 2005. Fifteen normal specimens were obtained from healthy oral mucosa. Forty-five cases of OSF were incident, newly diagnosed without OSCC or neoplastic disease. OSF was classified into 3 grades: early stage (E, n=15), moderately advanced stage (M, n=15) and advanced stage (A, n=15). The collected tissues were divided into two groups. Tissues in the first group were frozen immediately after careful removal of the tumor mass, OSF tissue in epithelium layer, and grossly normal-looking epithelium, all at -80°C. Tissues in the second group were fixed in 4% buffered formalin solution for pathological diagnosis and immunohistochemical staining. Clinicopathological staging of OSCC was determined by the TNM classification of the International Union Against Cancer in 2009.

Immunohistochemistry. Immunohistochemical staining was performed on 4- μ m serial sections from formalin-fixed paraffin-embedded specimens. Following deparaffinization and hydration, the slides were treated with endogenous peroxidase in 3% H₂O₂ for 20 min, after which the sections were blocked for 30 min at 37°C with 1.5% blocking serum in phosphate-buffered saline (PBS) prior to reacting with *WIF1* antibody (1:100 dilution, AP2723a; Abgent, San Diego, CA, USA) at 4°C in a moist chamber overnight. Negative control slides were duplicate sections in the absence of primary

antibodies. To evaluate *WIF1* expression, a scoring method was used. A mean percentage of positive tumor cells was determined by the examination of 500 cells in at least 5 areas at x400 magnification. The cells were assigned to 1 of the 5 following categories according to percentage of positive cells (PP): 0) <5%, 1) 5-24%, 2) 25-49%, 3) 50-75%, and 4) >75%. The intensity of the *WIF1* staining (SI) then was scored as follows: 0) no, -; 1) weak, +; 2) moderate, ++; and 3) intense, +++. The final immunoreactive score (IRS=SI + PP) was as follows: -) 0, 1; +) 2, 3; ++) 4, 5; and +++)) 6, 7. The stained tissues were scored blindly with regard to clinical patient data. Statistical analyses were performed with the SPSS 17.0 software. Statistical significance was evaluated by the Chi-square test (χ^2).

Reverse transcription-polymerase chain reaction. Total RNAs were extracted using TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed with the use of a kit from Promega (Madison, WI, USA). Quantitative PCR was performed to detect *WIF1* expression, according to the manufacturer's instructions (HT7500 system; Applied Biosystems, Foster City, CA, USA). Primers for the amplification of *WIF1* mRNA sequences (accession no. U75285) were synthesized as previously described (24,25). The 257-bp mRNA of *WIF1* was amplified by PCR using the primers: forward, 5'-TATGGA TCGATGCTCACCAG and reverse, 5'-CAGAGGGACATT GACGGTTG. *GAPDH* was used as an internal control. Primers used for *GAPDH* were: forward, 5'-ATCTCTGCC CCCTCTGCTGA-3' and the reverse, 5'-GATGACCTTGCC CACAGCCT-3'. PCR amplification was performed as follows: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec in 32 cycles. The PCR products were visualized on 2% agarose gels under ultraviolet transillumination.

Methylation-specific PCR and bisulfite genomic sequencing. Bisulfite modification of DNA, methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) were performed as previously described (24-27). Bisulfite-treated DNA was amplified with the methylation-specific primer set *WIF1*-m3: 5'-TGTCGTTTTTATTTTCGTTTCGC, *WIF1*-m4: 5'-CGTTTAAACGACTAAACGCG or the unmethylation specific primer set *WIF1*-u3: 5'-TTTTTGTTGTTTTTATTT TTGTTTGT, *WIF1*-u4: 5'-TCCCATTAAACAACACTAAAC ACA. MSP was performed using AmpliTaq Gold (methylation-specific primer: annealing temperature 60°C, 40 cycles, unmethylation-specific primer: annealing temperature 58°C, 40 cycles). For BGS, bisulfite-treated DNA was amplified using primers BGS1: 5'-GTTTTAGGGGTTTTTGAGTGTT and BGS2: 5'-CAACTCCCTCAACCAAACTA. The 463-bp PCR products were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA), with 8-10 colonies being randomly selected and sequenced.

Results

Downregulation of *WIF1* expression at the protein level in the carcinogenesis of OSF. To evaluate *WIF1* protein expres-

Table I. WIF1 expression in the carcinogenesis of oral submucous fibrosis (OSF).

Group	n	WIF1				WIF1 expression	Mean WIF1 score
		-	+	++	+++		
Normal	15	1	0	5	9	93.3%	5.73
OSF	45	12	10	17	6	73.3%	3.29
E	15	2	2	8	3	86.7%	4.13
M	15	3	3	7	2	80.0%	3.60
A	15	7	5	2	1	53.3%	2.13
OSCC	55	35	18	2	0	36.4%	1.27

E, early stage of OSF; M, moderately advanced stage of OSF; A, advanced stage of OSF; OSCC, oral squamous cell carcinoma.

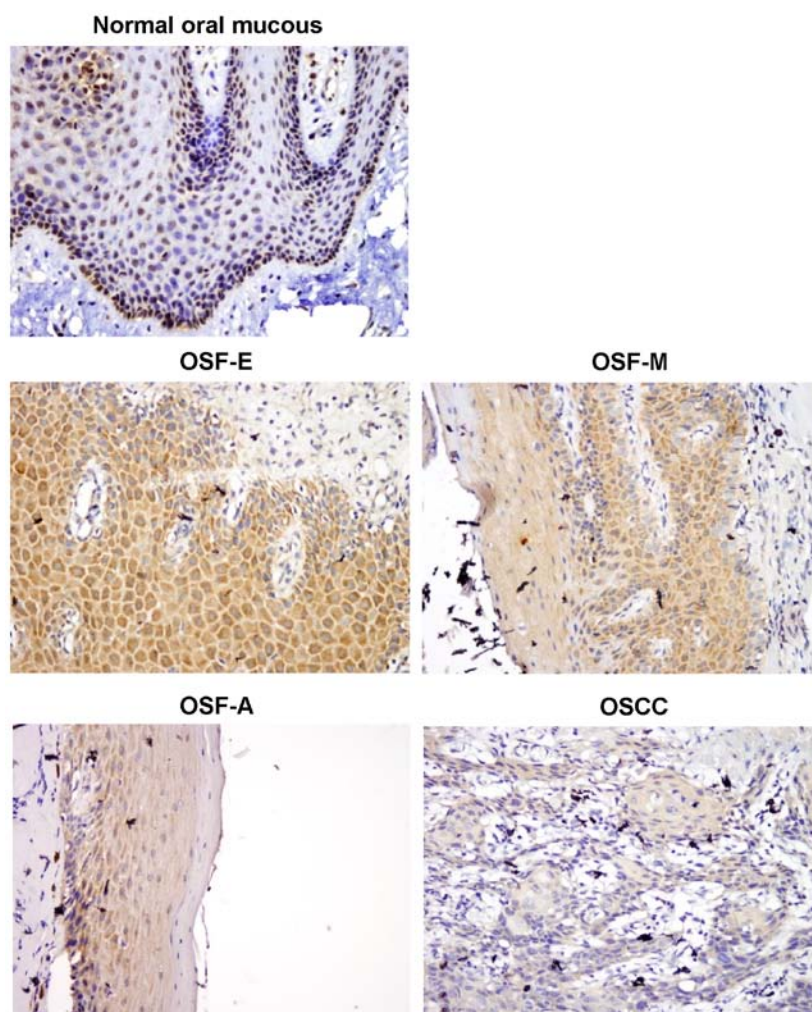


Figure 1. Immunohistochemical staining of WIF1 in normal oral mucosa, OSF and OSCC tissues. Normal oral tissues exhibited strongly positive WIF1 protein expression in the nucleus. OSF early stage and moderately advanced stage tissues showed WIF1-positive expression in the cytoplasm and nucleus. OSF advanced stage tissues showed a weak WIF1 expression in cytoplasmic parts. Primary OSCC showed very weak cytoplasmic immunoreaction for WIF1 (original magnification, x400).

sion, we initially performed immunohistochemical staining using WIF1-specific antibody in normal oral mucous tissues, OSF and OSCC tissues. Fourteen of 15 (93.3%) normal oral mucous cases showed nuclear WIF1 positivity, while 33 of 45 (73.3%) OSF tissues showed cytoplasmic WIF1 expression, including tissues from 13 of 15 (86.7%) early stage, 12

of 15 (80%) moderately advanced stage and 8 of 15 (53.3%) advanced stage, as well as 20 of 55 (36.4%) OSCC (Fig. 1). The average values of WIF1 expression varied in different tissue samples, including mean score of 5.37 in normal oral mucous tissues, 3.29 in OSF tissues, and 1.27 in OSCC tissues (Table I). WIF1 expression was gradually reduced in normal

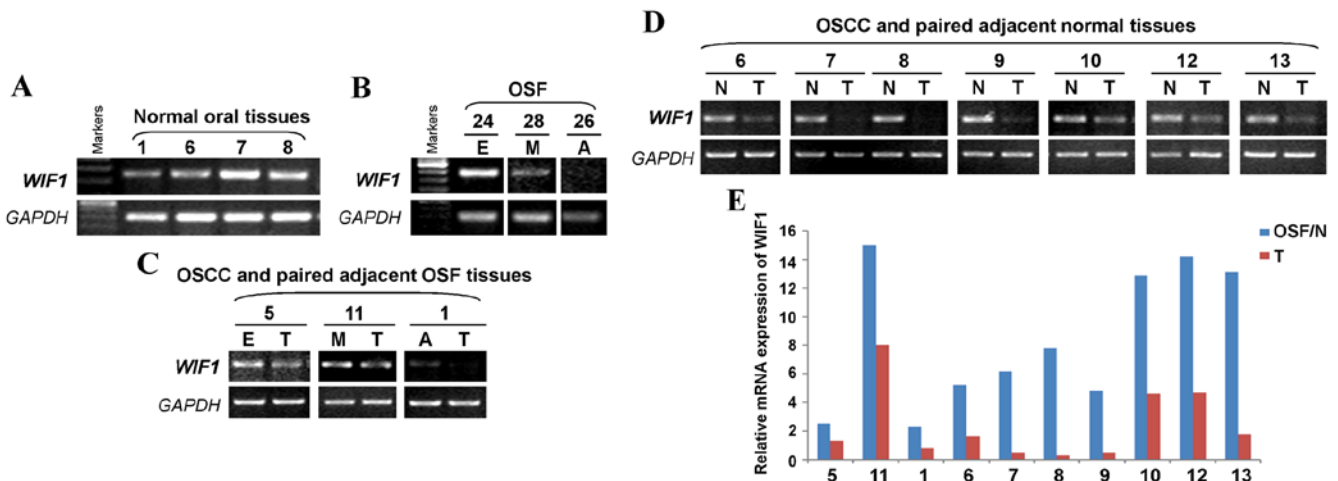


Figure 2. Detection of *WIF1* mRNA expression in normal oral mucosa, OSF and OSCC tissues. Semi-quantitative RT-PCR examined *WIF1* expression in (A) normal oral mucosa tissues, (B) OSF tissues, (C) OSCC and paired adjacent OSF tissues, as well as (D) OSCC and paired adjacent normal tissues. *GAPDH* was used as an internal control. (E) RT-qPCR was used to confirm *WIF1* expression in representative samples from OSCC and paired adjacent OSF or normal tissues. E, early stage of OSF; M, moderately advanced stage of OSF; A, advanced stage of OSF; N, normal tissue; T, OSCC.

Table II. Correlation between *WIF1* expression and clinicopathological characteristics in OSCC cases

Clinicopathological characteristics	Total (n)	Results of immunostaining, (n)		
		<i>WIF1</i> (+)	<i>WIF1</i> (-)	P-value
Age (years)				>0.05
<50	43	18	25	
≥50	12	2	10	
Gender				>0.05
Male	52	20	32	
Female	3	0	3	
Tumor site				>0.05
Tongue	40	17	23	
Others	15	3	12	
Primary tumor				>0.05
T1+T2	12	2	10	
T3+T4	43	18	25	
TNM stage				>0.05
I+II	12	7	5	
III+IV	43	13	30	
Differentiation grade				>0.05
Well	20	5	15	
Moderately-poorly	35	15	20	

oral tissues, OSF and OSCC tissues (P=0.00001). However, no statistically significant correlation was observed between *WIF1* expression and the clinicopathological characteristics of OSCC (P>0.05) (Table II). These results suggested that *WIF1* is downregulated at a protein level in the carcinogenesis of OSF.

WIF1 mRNA expression is reduced in the carcinogenesis of OSF. We also examined *WIF1* expression at the mRNA

level in normal oral mucous tissues, OSF tissues, OSCC and their paired adjacent tissues by semi-quantitative RT-PCR. The presence of the *WIF1* mRNA was shown by the 257-bp RT-PCR product. We found that *WIF1* was readily expressed in normal oral mucous tissues (Fig. 2A) and OSF early stage tissue, but decreased in OSF moderately advanced stage tissue, while rarely expressed in OSF advanced stage tissue (Fig. 2B). We also detected *WIF1* expression in OSCC and their adjacent OSF tissues. The results showed that *WIF1* was highly expressed in OSF early stage and moderately advanced stage tissues, but markedly downregulated in OSF advanced stage and OSCC tissues (Fig. 2C). We also found that *WIF1* was barely detected in OSCC tissues, compared with their paired adjacent normal tissues (Fig. 2D). RT-qPCR also confirmed that a reduced expression of *WIF1* mRNA in OSCC tissues, compared to the adjacent normal or OSF tissues (Fig. 2E). Therefore, *WIF1* mRNA expression levels are decreased in the carcinogenesis of OSF.

Promoter methylation of WIF1 in the carcinogenesis of OSF. We investigated the possible regulatory mechanism of *WIF1* reduction in the carcinogenesis of OSF. As promoter methylation mediates transcriptional repression of TSGs, we initially examined the presence of CpG island (CGI) in the *WIF1* promoter and exon 1 by bioinformatics analysis. The region spanning the *WIF1* promoter and exon 1 fulfilled the criteria of a CpG island (Gardiner-Garden and Frommer, 1987): GC content, 58.8%; observed/expected CpG ratio, 0.73; and a total of 46 CpG sites in a 2026-bp region (Fig. 3), thus as a typical CGI.

We detected the promoter methylation of *WIF1* in normal oral mucous and OSF tissues, OSCC and their paired adjacent OSF or normal tissues. We found that *WIF1* methylation was not detected in 10 normal oral tissues, 10 OSF tissues from early stage, moderately advanced stage and advanced stage (Fig. 4A and B). We also found that *WIF1* was frequently methylated in 16 of 20 (80%) OSCC tumor tissues, but not any in their paired adjacent normal and OSF tissues (Fig. 4C-E).

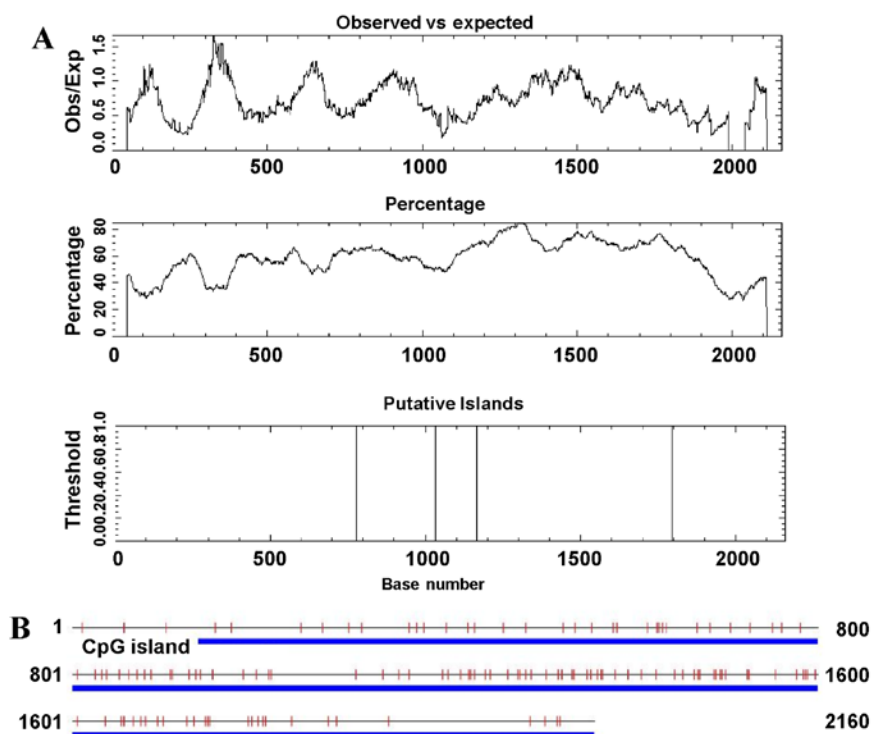


Figure 3. Promoter CpG island analysis of the *WIF1* promoter and exon 1 regions by (A) the EMBOSS Cpgplot (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) and (B) CpG island searcher (<http://cpgislands.usc.edu/>).

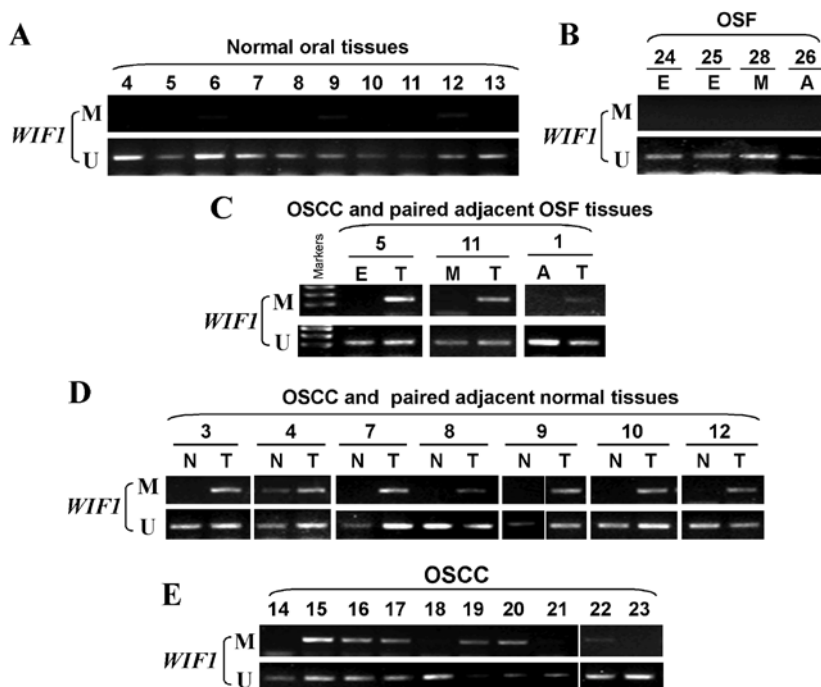


Figure 4. Promoter methylation of *WIF1* in normal oral mucosa, OSF and OSCC tissues. MSP was used to detect *WIF1* methylation in (A) normal oral mucosa tissues, (B) OSF tissues, (C) OSCC and paired adjacent OSF tissues, (D) OSCC and paired adjacent normal tissues, as well as (E) OSCC tissues. M, methylated; U, unmethylated; E, early stage of OSF; M, moderately advanced stage of OSF; A, advanced stage of OSF; N, normal tissue; T, OSCC.

These results suggested that the promoter methylation of *WIF1* is a tumor-specific event in the carcinogenesis of OSF.

Bisulfite genomic sequencing of the WIF1 promoter in OSF and OSCC tumor tissues. To confirm MSP data, high resolution

methylation analysis on every CpG site in the *WIF1* promoter was carried out by using bisulfite genomic sequencing (BGS). We found that no or very few methylated CpG sites were detected in representative normal oral mucosa tissues, and OSF early stage and moderately advanced stage tissues, while

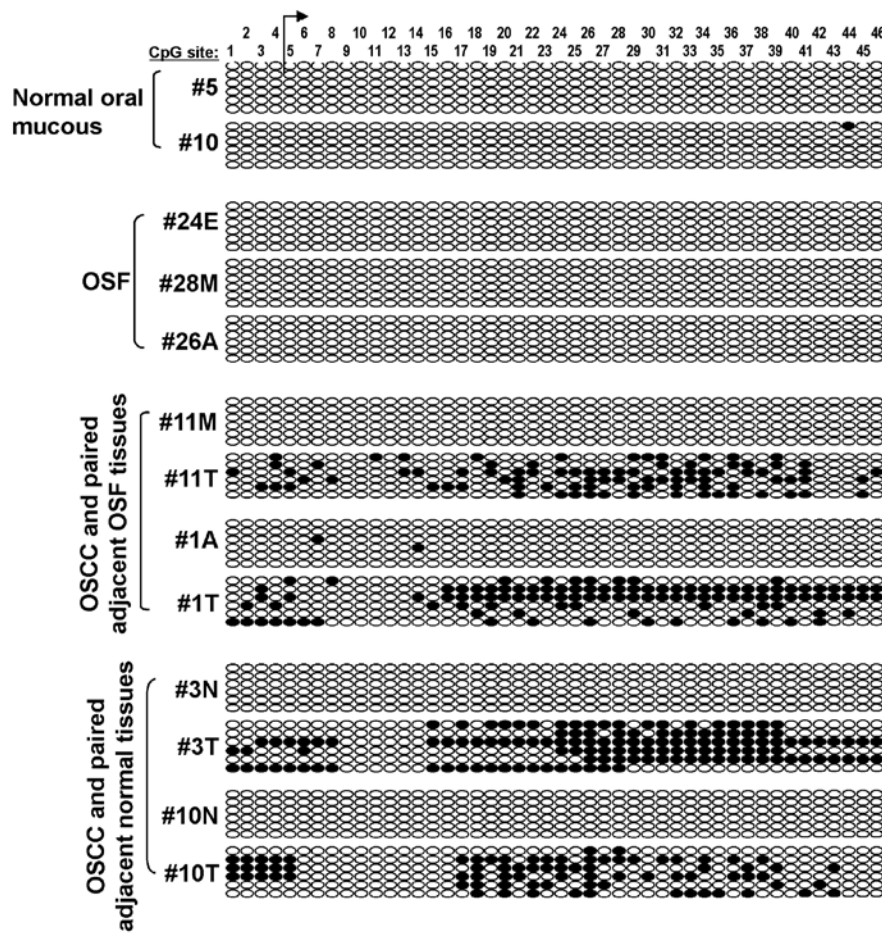


Figure 5. High-resolution mapping of individual CpG sites in the *WIFI* CGI by BGS in normal oral mucosa, OSF and OSCC tissues. Circles indicate CpG site. Filled circles indicate methylated sites. Open circles indicate unmethylated sites. Rows indicate individual alleles analyzed.

methylated alleles were detected in OSCC tissues, but not in their paired normal and OSF tissues. The results confirmed the MSP analysis (Fig. 5).

Discussion

Emerging evidence has shown that the epigenetic silencing of cancer genes via promoter CpG methylation plays an important role in OSCC pathogenesis. Several genes have been identified to be aberrantly methylated in OSCC, some of which exhibited a potential as biomarkers, for example, *E-cadherin* promoter methylation is associated with the poor survival in advanced OSCC (16), thus is an ideal epigenetic biomarker for OSCC. Thus, identifying more methylated genes may be useful to develop epigenetic biomarker for the early detection of OSCC.

Wnt signaling pathways include the canonical Wnt, the non-canonical planar cell polarity and the non-canonical Wnt/calcium pathways (28). The canonical Wnt pathway, also known as Wnt/ β -catenin pathway, accumulates cytoplasmic β -catenin in and eventual translocates into the nucleus, thus acting as a transcriptional coactivator to activate multiple oncogenic genes, leading to tumorigenesis (29). The Wnt/ β -catenin pathway is deregulated in various common human cancers (30). Frequent mutations of the signaling molecules in the Wnt/ β -catenin pathway have been identified in human cancers, including colon, hepatocellular, breast

and prostate carcinomas as well as glioblastoma. However, infrequent mutations of APC, Axin1 and β -catenin genes were reported in OSCC (31), indicating that mechanisms such as epigenetic modulation mediate inactivation of the Wnt/ β -catenin pathway in OSCC.

WIFI, a secreted Wnt inhibitor, is downregulated and methylated in various carcinomas including hepatocellular (32), nasopharyngeal (24,33), esophageal (5), prostate (34), breast (25), lung (35) and gastrointestinal (36) malignancies, and promotes tumor development and progression by activating β -catenin. Notably, *WIFI* silencing may be an early event in tumorigenesis. Although OSF is the precancerous lesion of OSCC, its molecular mechanisms remain to be elucidated. In the present study, we found that *WIFI* either at the protein or mRNA level is highly expressed in normal oral mucous tissues, and is gradually decreased in the different stages of OSF and OSCC tissues. Additionally, *WIFI* is frequently methylated in OSCC tissues, but not in normal oral mucous tissues, and their paired adjacent normal or OSF tissues. We also detected *WIFI* methylation in a panel of OSCC patient tissues with betel quid chewing habit, and observed *WIFI* methylation. These results are consistent with those of other studies on *WIFI* methylation in OSCC in Western populations (18). *WIFI* methylation was also reported to be correlated with shorter survival in oral cancer patients (37). Thus, it may become a tumor marker for the early detection of OSCC. Large-scale studies focusing on

OSF and OSCC samples should be conducted to confirm the potential value of *WIFI* methylation as an epigenetic biomarker in OSCC in the Chinese population.

In summary, we provide evidence that *WIFI* is frequently methylated in OSCC in a Chinese population, but not in normal oral mucous and OSF tissues, which is associated with its reduced expression. The present study reveals a novel epigenetic event in the carcinogenesis of OSF, which shed light on the development of a valuable epigenetic biomarker that may be useful for the early detection of OSCC.

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