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

SHANHUI ZHOU1, LING CHEN2, MUBARAK MASHRAH1, YUN ZHU1, ZHUJING HE3, YUHUA HU4, TINGXIU XIANG2, ZHIGANG YAO2, FENG GUO6 and CHENPING ZHANG1

1Department of Oral and Maxillofacial-Head and Neck Oncology, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200011; 2Molecular Oncology and Epigenetics Laboratory, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016; 3Department of Oral and Maxillofacial Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410008; 4Department of Oral Pathology, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 20001; 5Department of Oral Pathology, Xiangya Stomatological Hospital, Central South University, Changsha, Hunan 410078; 6Department of Oral and Maxillofacial Surgery, Xiangya Hospital, Central South University, Changsha, Hunan 410008, P.R. China

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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 an 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 nucleus 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...
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, 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 surgical intervention, and grossly normal-looking epithelium, all at -80˚C. Tissues in the second group were fixed in 4% buffered formalin layer, and transillumination. The collected tissues were scored blindly with regard to clinical patient data. Statistical analyses were performed using the SPSS 17.0 software. Statistical significance was evaluated by the Chi-square test ($\chi^2$).

**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 CCCCTCTGCTGA-3' and the reverse, 5'-GATGACCTTGCC CAACACGCT-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'-TGTGCTTATTTATTTGTTGTCWC, WIF1-m4: 5'-CGTTTAAACAGACTAAACCGC and the unmethylation specific primer set WIF1-u3: 5'-TTTTGGTGTGTGTTATTTT TTGTGGTGTGG, WIF1-u4: 5'-TCCCCATTTAACAACTAACC 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'-GTTTTTGGGTTTTGTTGTTGTTT and BGS2: 5'-CAACTCCCCTCAACAAAACACTA. 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-
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

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

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

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).
Zhou et al.: WIF1 Methylation in OSF and OSCC

In OSF and OSCC tissues, WIF1 expression is reduced 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 OSCC 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 CGI (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 or OSF tissues (Fig. 4C-E).

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

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>Total (n)</th>
<th>Results of immunostaining, (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WIF1 (+)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>43</td>
<td>18</td>
</tr>
<tr>
<td>≥50</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Gender</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
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<td>0</td>
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<tr>
<td>Tumor site</td>
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<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>40</td>
<td>17</td>
</tr>
<tr>
<td>Others</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Primary tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1+T2</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>T3+T4</td>
<td>43</td>
<td>18</td>
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<tr>
<td>TNM stage</td>
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<td>I+II</td>
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<td>7</td>
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<tr>
<td>III+IV</td>
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<td>Well</td>
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</tr>
<tr>
<td>Moderately-poorly</td>
<td>35</td>
<td>15</td>
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</table>

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

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.

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 OSCC 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.
These results suggested that the promoter methylation of \textit{WIF1} is a tumor-specific event in the carcinogenesis of OSF.

\textit{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 \textit{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 mucous tissues, and OSF early stage and moderately advanced stage tissues, while
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.

WIF1, 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, WIF1 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 WIF1 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, WIF1 is frequently methylated in OSCC tissues, but not in normal oral mucous tissues, and their paired adjacent normal or OSF tissues. We also detected WIF1 methylation in a panel of OSCC patient tissues with betel quid chewing habit, and observed WIF1 methylation. These results are consistent with those of other studies on WIF1 methylation in OSCC in Western populations (18). WIF1 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 WIF1 methylation as an epigenetic biomarker in OSCC in the Chinese population.

In summary, we provide evidence that WIF1 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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