

Disruption of Transforming Growth Factor- β Signaling by Five Frequently Methylated Genes Leads to Head and Neck Squamous Cell Carcinoma Pathogenesis

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is an aggressive cancer with low survival rates in advanced stages. To facilitate timely diagnosis and improve outcome, early detection markers (e.g., DNA methylation) are crucial for timely cancer diagnosis. In a recent publication, an epigenome-wide screen revealed a set of genes that are commonly methylated and downregulated in head and neck cancers (*SEPT9*, *SLC5A8*, *FUSSEL18*, *EBF3*, and *IRX1*). Interestingly, these candidates are potentially involved in the transforming growth factor- β (TGF- β) signaling pathway, which is often disrupted in HNSCC. Therefore, we sought to determine coordinated epigenetic silencing of these candidate genes in HNSCC as potential key disruptors of TGF- β signaling, which could ultimately result in HNSCC progression. Through immunoprecipitation studies, all five of the investigated candidate genes were found to interact with components of the TGF- β pathway. Overexpression of *SLC5A8*, *EBF3*, and *IRX1* resulted in decreased mitotic activity and increased apoptosis. In addition, *EBF3* was found to increase *p21* promoter activity, and *SMAD2* significantly increased *IRX1* promoter activity. These findings are significant because they reveal a set of genes that interact with components of the TGF- β pathway, and their silencing via methylation in HNSCC results in coordinated decrease in apoptosis, increased proliferation, and decreased differentiation. [Cancer Res 2009;69(24):9301–5]

Introduction

The mortality from head and neck squamous cell carcinoma (HNSCC) has not changed significantly in the last 30 years (1). Thus, HNSCC is considered to be a very aggressive cancer because it is often diagnosed at a very late stage. More than 50% of patients present with locally advanced and metastatic disease for which effective treatments are not available (2).

DNA methylation is an epigenetic mark that can frequently occur in the initial stages of tumorigenesis, and it therefore serves as a good early indicator for cancer. Furthermore, aberrant methylation is treatable through effective demethylating drugs that are currently used clinically (3). Therefore, targets of DNA methylation may serve as the first indicators for HNSCC progression and perhaps provide innovative targets for preventing this. To facilitate

timely diagnosis and improve treatment, elucidation of novel detection markers is crucial (4). Although several genes have previously been shown to be methylated near the beginning of HNSCC onset, none have been established clinically as biomarkers (5–7).

We recently performed an epigenome-wide screen using restriction landmark genomic scanning that revealed a set of genes that are commonly methylated and downregulated in head and neck cancers: *SEPT9*, *SLC5A8*, *FUSSEL18*, *EBF3*, and *IRX1* (8). Furthermore, overexpression of the three most frequently downregulated candidates, *SLC5A8*, *IRX1*, and *EBF3*, revealed tumor suppressor potential by growth curve analysis and colony formation assay (8).

Interestingly, most of the candidates identified in this previous study are potentially involved in the transforming growth factor- β (TGF- β)/hypoxia pathway, which is often disrupted in HNSCC. *FUSSEL18* has been shown to bind SMAD2/3 and inhibit TGF- β signaling (9). Also, *SEPT9* interacts with hypoxia-inducible factor-1 α (HIF-1 α), stabilizing its expression through inhibition of its degradation (10, 11). *IRX1* is a downstream target of SMAD2-mediated transcriptional activation (12), and *SLC5A8* downregulates survivin (HIF-1 α target) to induce apoptosis (13). Also, *EBF3* activates *p21* (14), which is also a target of SMAD3 (15) and HIF-1 α (16).

Therefore, in the present study, we investigated whether the previously observed silencing of this gene panel by methylation is critical to the pathogenesis of HNSCC. If the five genes indeed interact with components of the TGF- β pathway, disruption of this signaling could result in decreased apoptosis, increased cellular proliferation, and decreased differentiation (all of which are potential outcomes of TGF- β signaling).

Materials and Methods

Cell lines, antibodies, and plasmids. The established human HNSCC cell lines used in the study (SCC22B, SCC11B, and SCC17as) were kindly provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). The cell line was maintained in DMEM with 10% fetal bovine serum and 1% streptomycin/penicillin antibiotics. The antibodies used in the study were as follows: IL-6 (Abcam, ab6672), ARTS (Sigma-Aldrich, A4471), *SLC5A8* (Santa Cruz, sc-34189), *SEPT9* (aka MSF; Abgent, AP6215a), *EBF3* (Aviva Systems Biology, ARP31837_T100), survivin (Santa Cruz, sc-17779), α -tubulin (Cell Signaling Technologies), TGF- β 1 (Santa Cruz, sc-146), caspase-3 (Cell Signaling, 9662), actin (Santa Cruz, sc-8432), HIF-1 α (Cell Signaling, 3716), and involucrin (Aviva Systems Biology, ARP48675_P050). The *IRX1* and *p21* promoter constructs contained 0 to -1.2 kb with respect to the transcription start site for each gene. These sequences were amplified via PCR and cloned into the pGL3 basic vector (Promega). The *SLC5A8* overexpression vector was provided by Dr. Joe Costello (University of California, San Francisco, CA). The *EBF3* and *IRX1* cDNAs were cloned into the *Bam*HI and *Sal*I sites of pBABE. The *SEPT9* cDNA was cloned into the *Nhe*I and *Xho*I sites of pBABE, and FLAG-tagged *FUSSEL18* cDNA was cloned into the *Bam*HI and *Xho*I sites. The 1.66-kb *EBF3* and 1.45-kb *IRX1* cDNAs were each cloned into the *Bam*HI and *Sal*I sites of pBABE.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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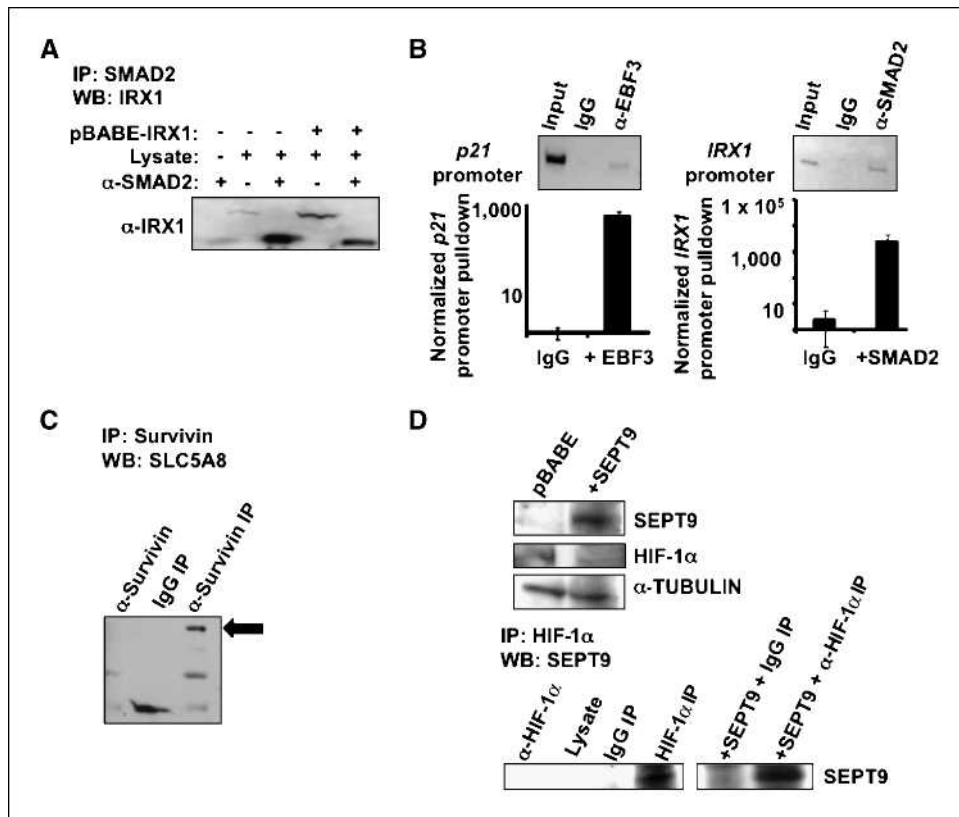


Figure 1. Interaction between frequently methylated genes and TGF-β pathway components in HNSCC. *A*, immunoprecipitation was done with SMAD2 antibody using lysates from WT SCC22B and SCC22B + IRX1 overexpression. This revealed no IRX1 association with SMAD2. *B*, chromatin immunoprecipitation analysis using EBF3 and SMAD2 antibodies in SCC22B cells. *p21* and *IRX1* promoter pull-down was assessed through quantitative PCR, which revealed increased pull-down of both promoters with their respective antibody compared with the negative IgG control. *C*, immunoprecipitation analysis of SLC5A8 pull-down by survivin antibody. *Arrow*, SLC5A8, which is only pulled down by survivin and not by the IgG control. *D*, overexpression of SEPT9 in SCC22B and immunoprecipitation analysis of SEPT9 pull-down by HIF-1α antibody. HIF-1α associates with SEPT9, and this binding is increased in SEPT9-overexpressing cells (+SEPT9). Furthermore, SEPT9 expression correlates with decreased HIF-1α expression.

Western blot analysis. Whole-cell lysates were suspended in Laemlli buffer and boiled for 15 min, followed by centrifugation. Lysates were loaded onto acrylamide gels (varying between 7.5% and 20%) and run for 2 h at 110 V followed by semidry transfer of the proteins to nitrocellulose membranes. The membranes were then blocked in 5% milk for 1 h. The primary antibodies (1:1,000 dilution) were incubated with the blot with

overnight shaking at 4°C. One-hour incubation with the secondary antibodies (1:5,000 dilution) followed. The blots were then washed in 1× TBS-Tween for 1 h and exposed using enhanced chemiluminescence solutions.

Chromatin immunoprecipitation analysis. Chromatin immunoprecipitation analysis was done as previously described (17), according to the Upstate Cell Signaling Solutions protocol. Sequences of the primers

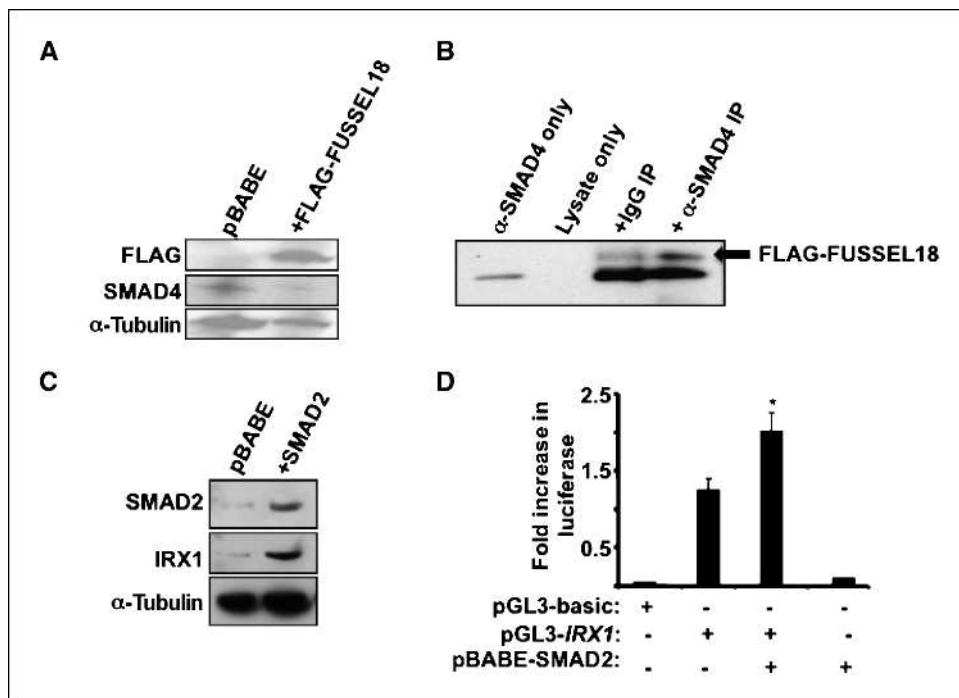


Figure 2. Impact of interactions on target gene expression. *A*, FLAG-tagged FUSSEL18 overexpression in SCC22B. SMAD4 expression inversely correlates with FUSSEL18 expression. *B*, immunoprecipitation analysis showing increased FUSSEL18 pull-down by the SMAD4 antibody compared with the IgG control in SCC22B cells. *C*, SMAD2 overexpression in SCC22B correlates with increased IRX1 expression. *D*, luciferase analysis of *IRX1* promoter activity in SCC22B cells with and without SMAD2 overexpression. Overexpression of SMAD2 significantly increases *IRX1* promoter activity. *, $P < 0.045$.

used for the quantitative chromatin immunoprecipitation PCRs are provided on request.

Luciferase assays. Luciferase analyses were done according to the Promega protocol. Briefly, ~20,000 SCC22B cells per well were plated in a 24-well plate in triplicates for each test plasmid (including a negative control, pGL3-basic only). Twenty-four hours after plating, the cells were transfected with 1- μ g luciferase plasmid, 60-ng renilla control plasmid, and 6- μ L Fugene transfection reagent (Roche). Forty-eight hours after transfection, the cells were lysed in the Promega passive lysis buffer, and 30 μ L were aliquoted into the 96-well opaque plate. Analysis was done using a luminometer. Luciferase substrate (100 μ L), followed by renilla substrate (100 μ L), was injected into the plate, and the fluorescence was read. The luciferase values were normalized to the renilla values, and the normalized values were presented in terms of fold increase over the pGL3-basic control vector.

Immunoprecipitation. Immunoprecipitation analyses were done according to the Santa Cruz protocol. Briefly, 500 μ g of cell lysate were precleared with 1 μ L of normal IgG and 20 μ L of protein A/G PLUS-agarose (Santa Cruz, sc-2003), rotating for 1 h at 4°C. After centrifugation at 4,800 rpm, the supernatant was transferred into a new Eppendorf tube. Thirty microliters were removed for lysate control loading for the Western blot analysis. Two micrograms of antibody (either normal IgG or the test antibody) were incubated with the lysate for 2 h, rotating at 4°C. Protein A/G PLUS-agarose (60 μ L) was added, and the lysate rotated at 4°C overnight. After centrifugation, the pellet was washed three times with Mammalian Protein Extraction Reagent (Pierce) and suspended in 30 μ L of Laemmli buffer.

RT-PCR. RT-PCRs were done as previously described (17).

Cell cycle analysis. Cells were suspended in cold PBS and centrifuged, and PBS was removed. This step was repeated, and the cells were then suspended in 500 μ L of PBS. The cells were mixed with 3 mL of cold 100% ethanol, vortexing constantly while adding the cells. After 30 min, the cells were subjected to flow cytometry via core services.

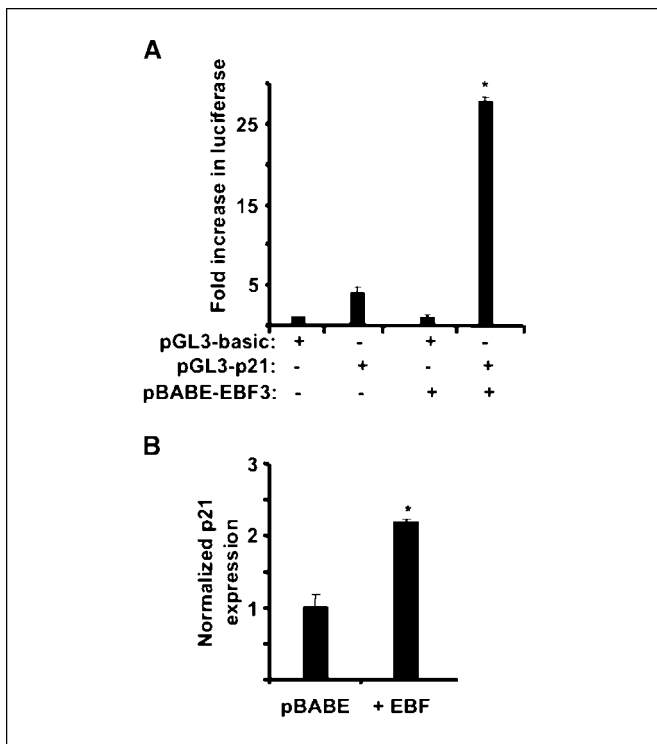


Figure 3. EBF3 regulates *p21* gene expression. **A**, luciferase analysis of *p21* promoter activity in SCC22B cells with and without EBF3 overexpression. Overexpression of EBF3 significantly increases *p21* promoter activity. *, $P < 0.0006$. **B**, quantitative RT-PCR analysis of *p21* mRNA expression in SCC22B cells with and without EBF3 overexpression. EBF3 overexpression results in a significant increase in *p21* transcription. *, $P < 0.05$.

Results

***IRX1*, *EBF3*, *SLC5A8*, *SEPT9*, and *FUSSEL18* interact with TGF- β pathway components.** To address the hypothesis that key hypermethylated genes in HNSCC progression interact within the TGF- β pathway, we performed immunoprecipitation studies of the five frequently methylated genes (*IRX1*, *EBF3*, *SLC5A8*, *SEPT9*, and *FUSSEL18*) and their respective targets in the TGF- β pathway. To assess whether *IRX1* and *SMAD2* interact, coimmunoprecipitation was done, which revealed no protein-protein interaction (Fig. 1A). However, chromatin immunoprecipitation showed *SMAD2* binding to the *IRX1* promoter in three HNSCC cell lines (Fig. 1B; Supplementary Fig. S1A). Because *EBF3* has previously been shown to activate the *p21* promoter via promoter assays in Saos2 cells (14), chromatin immunoprecipitation analysis was also done to assess the potential for *EBF3* to bind to *p21* in HNSCC. Similar to *SMAD2* and *IRX1*, we observed a protein-DNA interaction between *EBF3* and *p21*, but not a protein-protein interaction (Fig. 1B; Supplementary Fig. S1A). Coimmunoprecipitation studies also revealed that *SLC5A8* binds with survivin (Fig. 1C; Supplementary Fig. S1B), and *SEPT9* binds to HIF-1 α (Fig. 1D; Supplementary Fig. S1C). Furthermore, *SEPT9* overexpression seemed to increase this interaction with HIF-1 α (Fig. 1D). FLAG-tagged *FUSSEL18*-overexpressing cells were used to analyze protein interaction with *SMAD4* (because there is not an available antibody against *FUSSEL18*), which has previously been shown. This coimmunoprecipitation revealed pull-down of *FUSSEL18* with the *SMAD4* antibody (Fig. 2B; Supplementary Fig. S1D). It is important to note that these coimmunoprecipitation studies do not necessarily suggest direct protein-protein interactions. However, even indirect interactions are still interesting in the context of relevance to the TGF- β signaling pathway.

***IRX1*, *EBF3*, *FUSSEL18*, *SEPT9*, and *SLC5A8* regulate or are regulated by the expression of their TGF- β targets.** To assess the functional relevance of the five candidate genes to TGF- β signaling, each candidate was overexpressed in two HNSCC cell lines (SCC22B and SCC17as). *SEPT9*-overexpressing cells correlated with decreased HIF-1 α expression (Fig. 1D; Supplementary Fig. S2A). Similarly, *FUSSEL18*-overexpressing cells revealed a decrease in target *SMAD4* gene expression (Fig. 2A; Supplementary Fig. S2A). *SMAD2* overexpression resulted in an increase in *IRX1* at both the mRNA (Supplementary Fig. S2B) and protein (Fig. 2C; Supplementary Fig. S2A) levels, likely from increased promoter activity (Fig. 2D; $P = 0.045$). Similarly, *EBF3* overexpression in HNSCC cells resulted in a significant increase in *p21* promoter activity (Fig. 3A; $P = 0.0006$), leading to increased *p21* mRNA expression (Fig. 3B; $P = 0.05$) and protein expression (Supplementary Fig. S2A).

Functional relevance of *IRX1*, *EBF3*, *SLC5A8*, *SEPT9*, and *FUSSEL18* to TGF- β signaling in the context of HNSCC progression. To validate the relevance these candidate genes may have in the context of TGF- β signaling in HNSCC cells, their effects on apoptosis, cellular proliferation, differentiation, and inflammatory response were investigated as downstream outcomes of TGF- β signaling. With the exception of *FUSSEL18*, overexpression of all of the other candidates showed increased caspase-3 cleavage in a HNSCC cell line (Fig. 4A). Also, overexpression of *IRX1*, *FUSSEL18*, and *SEPT9* resulted in increased ARTS expression (the protein specifically related to apoptosis in the TGF- β pathway; Fig. 4A). Cell lines that exhibited increased TGF- β expression were analyzed for effects on differentiation using a differentiation marker, involucrin. Interestingly, *IRX1*-overexpressing cells exhibited increased involucrin expression compared with the wild-type (WT) cells (Fig. 4A).

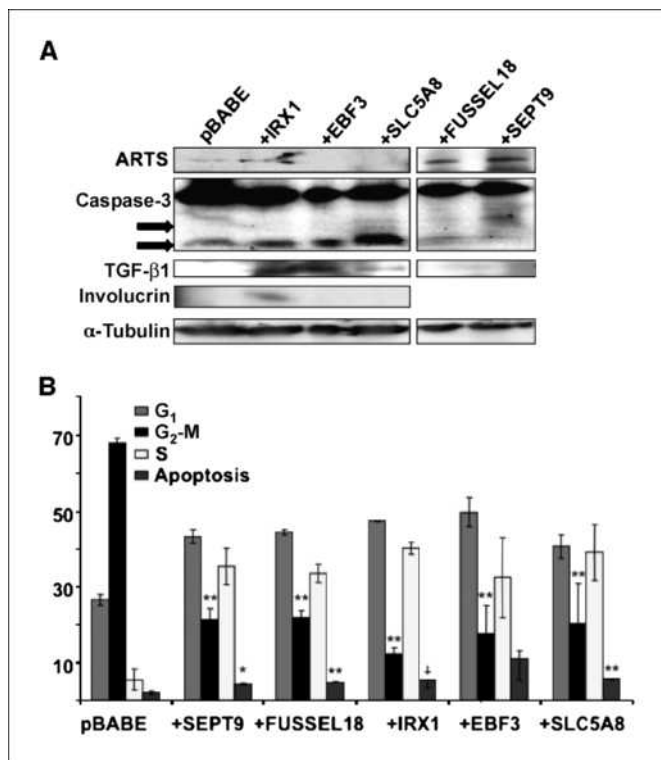


Figure 4. Functional relevance of the interactions between the methylated candidates and components of the TGF- β pathway. *A*, Western blot analysis of ARTS, caspase-3, TGF- β , and involucrin expression in the five overexpressing cell lines (tubulin serves as a loading control). *B*, cell cycle analysis of the five overexpressing cell lines revealed a significant decrease in mitosis in IRX1-, EBF3-, and SLC5A8-overexpressing cells. *, $P < 0.05$; **, $P < 0.01$.

Cell cycle analysis of HNSCC cells overexpressing SLC5A8, FUSSEL18, or SEPT9 revealed a significant increase in apoptotic cells (Fig. 4B; *, $P < 0.05$ to $P = 0.0008$). Additionally, overexpression of each of the five genes resulted in a concomitant significant decrease in mitotic cells (Fig. 4B; **, $P < 0.01$). As a readout of inflammatory response in the context of TGF- β signaling, we interrogated IL-6 and IL-1 β protein expression after overexpression of each of the five candidate genes. We observed that EBF3 was capable of decreasing IL-6 expression, and IRX1, SLC5A8, and FUSSEL18 increased IL-1 β expression (Supplementary Fig. S3).

Discussion

In the present study, five frequently methylated genes in HNSCC (*EBF3*, *IRX1*, *SLC5A8*, *FUSSEL18*, and *SEPT9*; ref. 8) were found

to interact with components of the TGF- β and hypoxia pathways. The interactions were found to have functional relevance to TGF- β signaling (i.e., apoptosis, proliferation, and inflammatory response). For example, inhibition of IL-6 by EBF3 overexpression reveals its tumor suppressor abilities because IL-6 has previously been shown to increase tumor cell survival and proliferation via signal transducer and activator of transcription-3 activation (18).

The TGF- β pathway has been shown to be commonly disrupted in HNSCC progression and can be targeted in therapy (19). Aberrant expression of hypoxia-induced proteins has been shown to have relevance to HNSCC progression and prognosis prediction (20). Furthermore, evidence supports the synergistic cooperation between the hypoxia and TGF- β signaling pathways (21). Therefore, epigenetic downregulation of these candidate genes may be critical to disrupt their roles in TGF- β signaling, allowing uncontrolled proliferation and apoptosis resistance for HNSCC progression.

The findings of this study are novel because they not only show for the first time the relevance of these genes to disruption of TGF- β signaling but they also show their association with the pathogenesis of HNSCC. Perhaps more interestingly, however, is the phenomenon of epigenetic silencing of a panel of genes that are all involved in the same pathway relevant to HNSCC pathogenesis. Therefore, identifying this set of targets relevant to a larger spectrum of HNSCC patients may be crucial in preventing HNSCC progression through improved diagnoses and treatment of HNSCC. Furthermore, methylation analysis via methylation-specific PCR on saliva samples has already been established as a noninvasive and highly sensitive means of screening for HNSCC (22). Therefore, methylation of the genes described in this study deserves further attention to discern their potential worth for HNSCC diagnosis and prognosis prediction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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