Epigenetic inactivation of *SFRP* genes in oral squamous cell carcinoma

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Received December 27, 2007; Accepted February 20, 2008

Abstract. Although mutations of *APC*, *CTNNB1* (ß-catenin) and *AXIN1* are rare in oral squamous cell carcinoma (OSCC), activation of the Wnt signaling pathway is thought to play an important role in oral carcinogenesis. In the present study, we examined the relationship between Wnt signaling and epigenetic alteration of the secreted frizzled-related protein (*SFRP*) genes in OSCC. We frequently detected loss of membrane localization of ß-catenin and its cytoplasmic or nuclear accumulation in OSCC cell lines, although these cell lines showed no *APC* or *CTNNB1* (ß-catenin) mutations and no methylation of *CDH1* (E-cadherin). By contrast, we frequently detected methylation of *SFRP1* (7/17, 41%) *SFRP2* (16/17, 94%) and *SFRP5* (14/17, 82%) in a panel of OSCC cell lines, as well as in specimens of primary tumors collected from 44 OSCC patients (*SFRP1*, 10/42, 24%; *SFRP2*, 16/44, 36%; *SFRP5*, 7/43, 16%). We also observed that OSCC cell lines express various Wnt ligands, and that ectopic expression of SFRPs inhibited cancer cell proliferation. Our results confirm the frequent methylation and silencing of SFRP genes in OSCC, and suggest that their loss of function contributes to activation of Wnt signaling that leads to cell proliferation during oral carcinogenesis.

Introduction

Epidemiological studies have shown that oral squamous cell carcinoma (OSCC) is one of the most commonly occurring cancers of the head and neck (1), and that alcohol and tobacco play key roles in its development (2,3). Although it has been suggested that OSCC arises through the accumulation of multiple genetic changes (4-6), the precise molecular mechanism underlying its pathogenesis remains unclear. In addition to genetic changes, recent studies indicate that epigenetic silencing of cancer-related genes via DNA methylation also plays an important role in OSCC tumorigenesis (7,8). Genes involved in cell cycle regulation, apoptosis, DNA repair and cellular differentiation are all reportedly involved (9-12), though the precise role of DNA methylation in oral carcinogenesis is not fully understood.

Wnt ligands are secreted proteins that bind to transmembrane receptors in the Frizzled (Fz) family. During normal developmental processes, the resultant Wnt signaling plays essential roles in the regulation of cell proliferation, patterning and fate determination (13). The binding of Wnt to Fz leads to dephosphorylation and stabilization of ß-catenin, which enables its translocation into the nucleus, where it interacts with members of the T-cell factor/lymphocyte enhancer factor (TCF/LEF) family of transcription factors to stimulate the expression of target genes. This signaling pathway is strongly implicated in tumorigenesis; indeed, the first mammalian Wnt isoform was identified based on its ability to promote mouse mammary tumorigenesis (14). Moreover, activation of the canonical Wnt pathway caused by mutation of *APC*, *AXIN1* and *CTNNB1* (ß-catenin) has been observed in various human cancers (14,15). In OSCC, loss of heterozygosity at the *APC* locus is reported at frequencies ranging from 13% to 72.7% (16-20), though mutations in *APC*, *AXIN1* and *CTNNB1* are rare (21,22). On the other hand, reduced levels of membrane-bound E-cadherin and/or ß-catenin and cytoplasmic accumulation of ß-catenin is often observed in OSCC, which indicates that Wnt signaling is activated in this disease (20-25) and suggests that the activation is via an alternative pathway in OSCC.

There are several known classes of secreted Wnt antagonists, including the Cerberus, Wnt inhibitory factor 1 (WIF1), secreted frizzled-related protein (SFRP) and the Dickkopf (DKK) families (26). The SFRP family is comprised of five glycoproteins identified as putative inhibitors of the Wnt signaling pathway (27). SFRP proteins contain an N-terminal domain homologous to the cysteine-rich domain

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Key words: Wnt, ß-catenin, secreted frizzled-related protein, methylation, oral squamous cell carcinoma

(CRD) of Fz and a C-terminal domain with some homology to netrin. This enables SFRPs to inhibit Wnt signaling by competing with Fz for Wnt binding via its CRD domain or by binding directly to Fz (27).

We previously showed that *SFRP1*, *-2* and *-5* are frequently inactivated in colorectal cancer (CRC) and gastric cancer (GC), and that SFRPs suppress constitutive Wnt signaling when overexpressed in CRC and GC cells (28-30). To date, methylation of *SFRP* genes and downregulation of their expression have been identified in bladder cancer (31), prostate cancer (32), lung cancer (33) breast cancer (34), and chronic lymphocytic leukemia (35), which strongly suggests *SFRPs* function as tumor suppressor genes. Our aim in the present study was to characterize the epigenetic abnormalities of *SFRP* genes in OSCC and determine whether *SFRPs* function as tumor suppressors in OSCC.

Materials and methods

Cell lines and tissue specimens. DNA was prepared from 17 OSCC cell lines (HSC-2, HSC-3, HSC-4, Ca9-22, OSC-19, OSC-20, OSC-30, OSC-70, SAS, KOSC-3, Ho-1-u-1, Ho-1-N-1, HOC119, HOC621, MoN2, MoT and OM1) as described previously (8,36). To analyze restoration of *SFRP* gene expression, cells were treated with a DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC; 2.0 μM) (Sigma, St. Louis, MO, USA) for 72 h, renewing the drug and medium every 24 h. In addition, 44 primary oral squamous cell carcinoma specimens from 44 patients were obtained from the Department of Oral Surgery, Sapporo Medical University Hospital. Adjacent non-tumorous oral tissue was also obtained from 33 of the 44 patients. Informed consent was obtained from all patients before the collection of the specimens. Genomic DNA was extracted using the standard phenol-chloroform procedure. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then treated with a DNA-free kit (Ambion, Austin, TX, USA). Total RNA from normal oral mucosa from a healthy individual was purchased from BioChain (Hayward, CA, USA).

Mutational analysis. Analysis of *APC* and *CTNNB1* gene mutation was performed as described previously (37). Briefly, codons 1140-1545 in exon 16 of *APC* were examined for mutations. Genomic DNA corresponding to this region was amplified by PCR using primers APC-F1 and APC-R1, after which the gel-purified PCR products were directly sequenced using an ABI3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in both the sense and antisense directions with the primers used for PCR and two pairs of internal primers (APC-F2, APC-F3, APC-R2 and APC-R3). For analysis of *CTNNB1* mutation, exon 3 of the gene was amplified by PCR, after which the products were directly sequenced in both the sense and antisense directions with the primers used for PCR. PCR was run in a 50-μl volume containing 50 ng of genomic DNA, 1X Ex Taq Buffer (Takara, Otsu, Japan), 0.3 mM dNTP, 0.25 μM each primer and 1 U of Takara Ex Taq Hot Start Version (Takara). The PCR protocol entailed 5 min at 95˚C; 35 cycles of 1 min at 95˚C, 1 min at 55˚C and 1 min at 72˚C; and a 7-min final extension at 72˚C. Primer sequences are listed in Table I.

RT-PCR. Single-stranded cDNA was prepared using Super-Script III reverse transcriptase (Invitrogen). The integrity of the cDNA was confirmed by also amplifying glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*). PCR was run in a 50-μl volume containing 100 ng of cDNA, 1X Ex Taq Buffer (Takara), 0.3 mM dNTP, 0.25 μM each primer and 1 U of Takara Ex Taq Hot Start Version (Takara). The PCR protocol entailed 5 min at 95˚C; 35 cycles of 1 min at 95˚C, 1 min at 55˚C and 1 min at 72˚C; and a 7-min final extension at 72˚C. The primer sequences for RT-PCR analysis and PCR product sizes are listed in Table I.

Methylation analysis. Bisulfite treatment of genomic DNA was performed as described previously (30). Methylation was analyzed using methylation-specific PCR (MSP) and bisulfite-sequencing, as described previously. PCR was run in a 25-μl volume containing 50 ng of bisulfite treated DNA, 1X MSP buffer [67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$ and 10 mM 2-mercaptoethanol], 1.25 mM dNTP, 0.4 μM each primer and 0.5 U of JumpStart RedTaq DNA Polymerase (Sigma). The PCR protocol for MSP entailed 5 min at 95˚C; 35 cycles of 30 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C; and a 7-min final extension at 72˚C. The PCR protocol for bisulfite-sequencing entailed 5 min at 95˚C; 35 cycles of 1 min at 95˚C, 1 min at 60˚C and 1 min at 72˚C; and a 7-min final extension at 72˚C. Amplified bisulfite-sequencing PCR products were cloned into pCR2.1-TOPO vector (Invitrogen), and 10 clones from each sample were sequenced. The primer sequences and PCR product sizes are listed in Table I.

Western blotting. Western analysis was carried out as described previously (30). Briefly, samples (20 μg) of cell lysate were subjected to 10% SDS-PAGE, after which the resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were then blocked with 5% non-fat milk and 0.1% Tween-20 in TBS and probed with anti-ß-catenin (BD Biosciences, San Jose, CA, USA) or anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), after which the blots were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA).

Immunofluorescence microscopy. Cells cultured on chamber slides were washed with PBS and fixed with 4% paraformaldehyde, after which they were incubated with anti-ß-catenin monoclonal antibody (BD Biosciences), stained with anti-mouse IgG Alexa Fluor 488 (Invitrogen), and examined under an FV300-IX71 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Colony formation assays. Cells ($1x10^5$) were plated in 6-well plates 24 h before transfection, and then transfected with 4 μg of one of the pcDNA3.1His-SFRP vectors or with empty vector using a Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The SFRP vectors were prepared as described previously (29). The cells were

Table I. Sequences of the primers used in this study and the expected product sizes.

	Sense	Antisense	Product size (bp)
Mutation analysis			
APC	F1: 5'-GCCTACCAATTATAGTGAACGT-3'	R1: 5'-TGATTCTTTAGGCTGCTCTGAT-3'	1219
	F2: 5'-GCCACTTGCAAAGTTTCTTC-3'	R2: 5'-GAAGATCACTGGGGCTTATA-3'	
	F3: 5'-TTGAGAGTCGTTCGATTGCC-3'	R3: 5'-TCAGCTGATGACAAAGATGA-3'	
CTNNB1	5'-CCAATCTACTAATGCTAATACTG-3'	5'-CTGCATTCTGACTTTCAGTAAGG-3'	310
Methylation analysis			
CDH1 MSP-U	5'-TTTTTAGTTAATTAGTGGTATGGGGGGT-3'	5'-AATACAATCAAATCAAACCAAACTAAAACA-3'	160
CDH1 MSP-M	5'-TTAGTTAATTAGCGGTACGGGGGGC-3'	5'-CGATCGAATCGAACCGAACTAAAACG-3'	153
SFRP1 MSP-U	5'-GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT-3'	5'-CTCAACCTACAATCAAAAACAACACAAACA-3'	135
SFRP1 MSP-M	5'-TGTAGTTTTCGGAGTTAGTGTCGCGC-3'	5'-CCTACGATCGAAAACGACGCGAACG-3'	126
SFRP2 MSP-U	5'-TTTTGGGTTGGAGTTTTTTGGAGTTGTGT-3'	5'-AACCCACTCTCTTCACTAAATACAACTCA-3'	145
SFRP2 MSP-M	5'-GGGTCGGAGTTTTTCGGAGTTGCGC-3'	5'-CCGCTCTCTTCGCTAAATACGACTCG-3'	138
SFRP5 MSP-U	5'-GTAAGATTTGGTGTTGGGTGGGATGTTT-3'	5'-AAAACTCCAACCCAAACCTCACCATACA-3'	136
SFRP5 MSP-M	5'-AAGATTTGGCGTTGGGCGGGACGTTC-3'	5'-ACTCCAACCCGAACCTCGCCGTACG-3'	141
SFRP1 bis-seq	5'-GTTTTGTTTTTTAAGGGGTGTTGAG-3'	5'-ACACTAACTCCRAAAACTACAAAAC-3'	209
SFRP2 bis-seq	5'-TAAGAAAATTTTGGTTGTGTTTTAGTAA-3'	5'-CAACRAACCAAAACCCTACAACAT-3'	290
SFRP5 bis-seq	5'-TTAAATGTTTAGGGAGGTAGGGAGT-3'	5'-AATCGCCCAAATAAATAACAACCTAC-3'	293
RT-PCR			
CDH1	5'-GATCCATTTCTTGGTCTACGCCT-3'	5'-TCACCTTCAGCCATCCTGTTTCT-3'	365
SFRP1	5'-CCAGCGAGTACGACTACGTGAGCTT-3'	5'-CTCAGATTTCAACTCGTTGTCACAGG-3'	497
SFRP2	5'-ATGATGATGACAACGACATAATG-3'	5'-ATGCGCTTGAACTCTCTCTGC-3'	322
SFRP5	5'-CAGATGTGCTCCAGTGACTTTG-3'	5'-AGAAGAAAGGGTAGTAGAGGGAG-3'	346
WNT1	5'-GTCTGATACGCCAAAATCCGG-3'	5'-CTCGTTGTTGTGAAGGTTCATG-3'	404
WNT2	5'-TTGAAACAAGAGTGCAAGTGCC-3'	5'-ACTTACACCCACACTTGGTCAT-3'	379
WNT2B	5'-GGACTGATCTTGTCTACTTTGAC-3'	5'-TTGAGTTGAGAGGCTTGAATTGG-3'	338
WNT3	5'-ATGACAGCCTGGCCATCTTTG-3'	5'-AGCCCGTGGCACTTGCATTTG-3'	349
WNT3A	5'-GGCATCAAGATTGGCATCCAG-3'	5'-CACTTGAGGTGCATGTGGCTG-3'	404
WNT4	5'-ATGCTCTGACAACATCGCCTA-3'	5'-TGCGGCTTGAACTGTGCGTTG-3'	333
WNT5A	5'-TGGAAGTGCAATGTCTTCCAAG-3'	5'-AGGTGTTATCCACAGTGCTGCA-3'	314
WNT5B	5'-GAAGCTGTGCCAATTGTACCA-3'	5'-ATCCACAAACTCCTTGGCGAA-3'	355
WNT6	5'-TCACGCAGGCCTGTTCTATGG-3'	5'-AATGGAGGCAGCTTCTGCCAG-3'	370
WNT7A	5'-GCAAGCATCATCTGTAACAAGA-3'	5'-TCTCTTTGTCGCAGCCACAGT-3'	310
WNT7B	5'-CATCAACGAGTGCCAGTACCA-3'	5'-CCTCATTGTTATGCAGGTTCAT-3'	353
WNT11	5'-GAACTGCTCCTCCATTGAGCT-3'	5'-CTTACACTTCATTTCCAGAGAG-3'	364
GAPDH	5'-CGGAGTCAACGGATTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	307

replated on 60-mm culture dishes 24 h after transfection and then selected for 14 days with 0.6 mg/ml G418, after which colonies were stained with Giemsa and counted using National Institute of Health IMAGE software.

Statistical analysis. Statistical analyses were carried out using SPSS software (version 11.0; SPSS Inc., Chicago, IL). Mann-Whitney U test and Fisher's exact test (2-sided) were used to evaluate the association between methylation of *SFRP* genes and clinicopathological features. Values of $P<0.05$ were considered significant.

Results

Analysis of ß-catenin expression in OSCC cell lines. To investigate Wnt signaling in OSCC, we initially analyzed expression of ß-catenin in a set of OSCC cell lines. Western analysis using an anti-ß-catenin antibody revealed detectable levels of endogenous ß-catenin in all of the cell lines tested (Fig. 1A). Immunocytochemical analysis of the cellular distribution of endogenous ß-catenin revealed cytoplasmic or nuclear accumulation of the protein in 14 of the 15 (93%) OSCC cell lines studied (Table II; representative

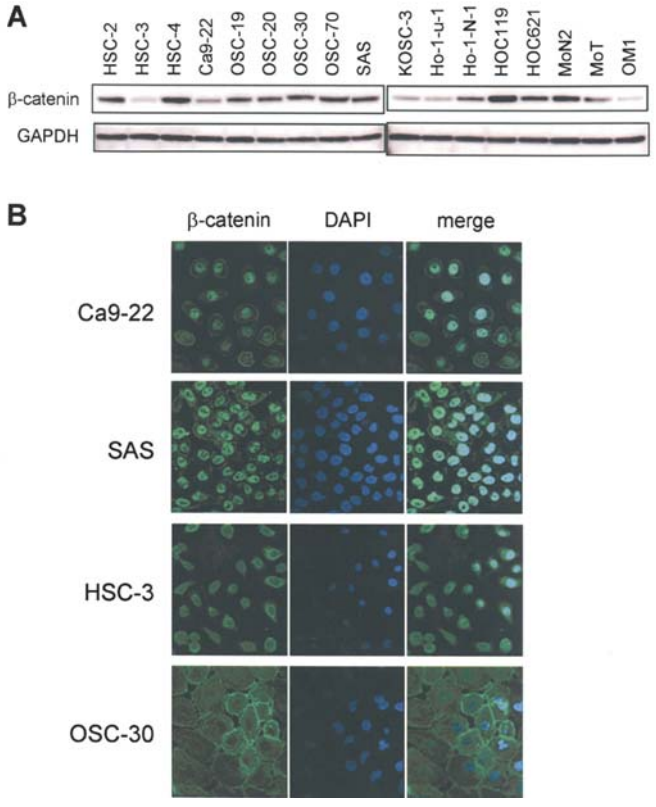

Figure 1. Analysis of ß-catenin expression in OSCC. (A) Western analysis of ß-catenin in a set of OSCC cell lines. Levels of GAPDH protein were used as a loading control. (B) Fluorescence immunocytochemical analysis of the intracellular distribution of ß-catenin in OSCC cell lines. Cells were stained with anti-ß-catenin antibody (green); nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI; blue).

results in Fig. 1B). Seven cell lines (47%) showed the presence of membrane-bound ß-catenin, but in many of those cells, nuclear or cytoplasmic localization was also observed (Table II; representative results in Fig. 1B). These results confirmed activation of the Wnt signaling pathway in all but one of the OSCC cell lines tested, though the apparent degree of activation varied.

Analysis of APC, CTNNB1 and CDH1 in OSCC cells. To clarify the mechanism by which Wnt signaling is activated in OSCC cells, we next looked for mutations of *APC* and *CTNNB1*. This entailed examination of 17 OSCC cell lines for mutations in codons 1140-1545 of *APC*, which include the mutation cluster region (37). It has been well documented that the majority of somatic *APC* mutations seen in CRC occur within this region; nonetheless, we detected no *APC* mutations in any of the OSCC cell lines studied (Table II). We also looked for *CTNNB1* mutations in these OSCC cell lines. Exon 3 was selected for sequencing because most mutations found in human cancers occur at the serine/threonine residues in this exon. As with *APC*, however, we detected no *CTNNB1* mutations in any of the OSCC cell lines. In addition, because loss of E-cadherin could be responsible for the reduced membrane localization of ß-catenin, we tested whether *CDH1* was epigenetically silenced in the OSCC cell lines. MSP analysis revealed that *CDH1* was unmethylated in all of the OSCC cell lines tested, and RT-PCR analysis confirmed that they expressed CDH1 mRNA (data not shown). Thus, we found no genetic or epigenetic alterations of *APC*, *CTNNB1* or *CDH1* in our panel of OSCC cell lines.

Table II. Summary of ß-catenin staining and genetic/epigenetic analysis of Wnt-related genes in OSCC cell lines.

	ß-catenin staining	*APC*	*CTNNB1*	*CDH1*	*SFRP1*	*SFRP2*	*SFRP5*
HSC-2	C	WT	WT	U	M	M	M
HSC-3	C	WT	WT	U	M	M	M
HSC-4	C/M	WT	WT	U	M	M	M
Ca9-22	N	WT	WT	U	M	M	M
OSC-19	C	WT	WT	U	U	M	M
OSC-20	C	WT	WT	U	U	M	M
OSC-30	C/M	WT	WT	U	U	M	M
OSC-70	C	WT	WT	U	U	M	M
SAS	N	WT	WT	U	M	M	M
KOSC-3	C	WT	WT	U	M	M	M
Ho-1-u-1	ND	WT	WT	U	U	M	M
Ho-1-N-1	N/C/M	WT	WT	U	U	M	U
HOC119	M	WT	WT	U	M	M	U
HOC621	C/M	WT	WT	U	U	U	U
MoN2	C/M	WT	WT	U	U	M	M
MoT	ND	WT	WT	U	U	M	M
OM-1	C/M	WT	WT	U	U	M	M

ß-catenin: C, cytoplasmic; M, membrane-bound; N, nuclear; ND, not done. Genetic/epigenetic analysis: WT, wild-type; M, methylated; U, unmethylated.

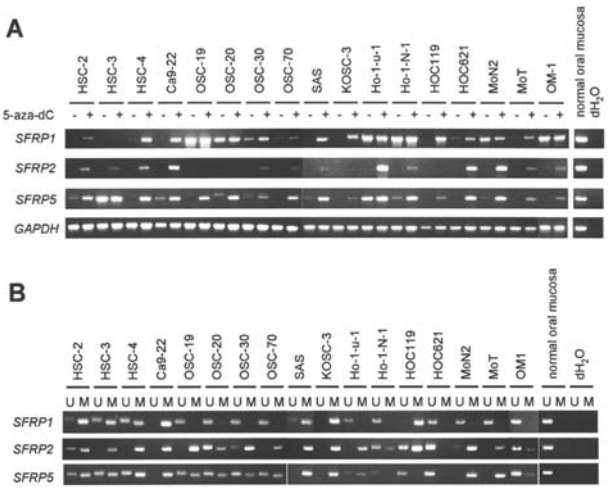

Figure 2. Analysis of the expression and methylation of *SFRP* genes in OSCC cell lines. (A) RT-PCR analysis of *SFRP1*, *-2* *-5* expression in the indicated OSCC cell lines, with and without 5-aza-dC treatment, and in normal oral mucosa. Expression of *GAPDH* was assessed in all samples to ensure the cDNA quality; dH_2O indicates no RNA added. (B) MSP analysis of the indicated OSCC cell lines and normal oral mucosa. Bands in the 'M' lanes are PCR products obtained with methylation-specific primers; those in the 'U' lanes are products obtained with unmethylated-specific primers; dH_2O indicates no DNA added.

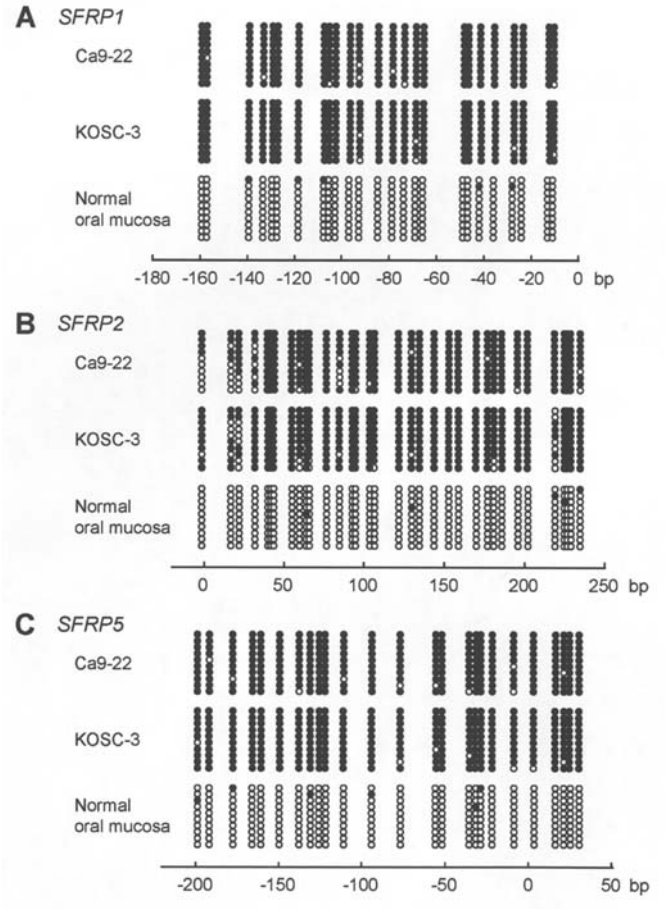

Figure 3. Bisulfite sequencing analysis of *SFRP* gene methylation. (A) Bisulfite sequencing of *SFRP1* in the indicated OSCC cell lines and normal oral mucosa. Open and filled circles represent unmethylated and methylated CpG sites, respectively. The location of each CpG site relative to the transcription start site is shown below. (B and C) Bisulfite sequencing of *SFRP2* (B) and *SFRP5* (C). CpG sites are represented as in (A).

Analysis of SFRP expression in OSCC cell lines. We previously reported that three of the five *SFRP* genes *(SFRP1*, *-2* and *-5*) were often methylated and silenced in CRC and GC cells (28,30). Therefore, in the present study we initially evaluated the expression status of *SFRP1*, *-2* and *-5* in our panel of OSCC cell lines. We found that SFRP1 mRNA was completely absent or its levels were significantly reduced in 7 of the 17 cell lines tested (HSC-2, HSC-3, HSC-4, Ca9-22, SAS, KOSC-3 and HOC119), and that treating the affected cells with the DNA methyltransferase inhibitor 5-aza-dC rapidly restored its expression (Fig. 2A). In addition, four cell lines showed some basal expression of SFRP1 mRNA that was also upregulated by 5-aza-dC (OSC-30, OSC-70, HOC621 and MoT) (Fig. 2A). SFRP2 mRNA was not seen or was barely detectable in all of the OSCC cell lines, and 5-aza-dC treatment restored its expression in many of these cells (Fig. 2A). SFRP5 mRNA was absent or its levels were reduced in 15 cell lines (HSC-2, HSC-4, Ca9-22, OSC-19, OSC-20, OSC-30, OSC-70, SAS, KOSC-3, Ho-1-N-1, HOC119, HOC621, MoN2, MoT and OM-1), and again 5-aza-dC enhanced expression in the affected cell lines (Fig. 2A). In contrast to the cancer cells, all three *SFRP* genes were expressed in normal oral mucosa from a healthy individual (Fig. 2A). Taken together, these results strongly suggest that, in OSCC, *SFRP* genes are frequent targets of epigenetic silencing through methylation.

Analysis of SFRP methylation in OSCC cell lines. We next used MSP to analyze the methylation status of *SFRP* genes. We observed S*FRP1* methylation in 7 of the 17 (41%) OSCC cell lines tested (Fig. 2B; Table II). *SFRP2* methylation was detected in 16 (94%) cell lines, and *SFRP5* methylation was detected in 14 (82%) (Fig. 2B; Table II). In general, we observed a significant inverse correlation between methylation and expression of *SFRP* genes, but there were some exceptions. Although some methylation of *SFRP5* was detected in HSC-3 and Ho-1-u-1 cells (Fig. 1B), they showed a substantial basal expression of SFRP5 mRNA (Fig. 1A), which was probably transcribed from the unmethylated allele. Conversely, RT-PCR analysis revealed that *SFRP1* transcription was downregulated in four cell lines (OSC-30, OSC-70, HOC621 and MoT), although no methylation was observed in these cells (Fig. 2). Likewise, HOC621 cells showed no methylation of *SFRP2* or *-5*, but neither gene was transcribed (Fig. 2). Despite the apparent absence of methylation, 5-aza-dC restored expression of both genes, suggesting some other epigenetic mechanism is responsible for their silencing in these cells (Fig. 2A). In contrast to OSCC cell lines, normal oral mucosal tissue did not show methylation of *SFRP* genes (Fig. 2B).

To assess methylation status in more detail, we carried out bisulfite sequencing in selected cell lines and a sample of normal oral mucosa. Sequencing analysis revealed that the CpG islands of *SFRP1*, *-2* and *-5* are extensively methylated in Ca9-22 and KOSC-3 cells, whereas nearly all of the CpG sites were unmethylated in normal oral mucosa (Fig. 3).

Table III. SFRP methylation and clinicopathological features in primary OSCC.

		SFRP1[a]		*SFRP2*		*SFRP5*[b]		All *SFRP*	At least one
	No.	Unmethylated	Methylated	Ummethylated	Methylated	Unmethylated	Methylated	Unmethylated	Methylated
Total	44	32	10	28	16	36	7	22	22
		76.20%	23.80%	63.60%	36.40%	83.70%	16.30%	50.00%	50.00%
Sex									
Male	25	16	9	15	10	21	3	12	13
Female	19	16	1	13	6	15	4	10	9
		p=0.031		p=0.753		p=0.680		p=1.000	
Age (years, mean ± SD)	64.32±14.11	64.50±14.17	65.00±10.52	63.44±15.01	64.82±13.82	63.56±14.38	67.14±14.05	62.41±14.00	66.23±14.28
		p=0.919		p=0.758		p=0.548		p=0.376	
pT category									
pT1	8	7	0	5	3	7	1	4	4
pT2	26	20	6	18	8	22	3	16	10
pT3	4	3	1	2	2	3	1	1	3
pT4	6	2	3	3	3	4	2	1	5
		p=0.060		p=0.526		p=0.339		p=0.145	
pN category									
pN0	27	20	5	17	10	22	5	14	13
pN1	9	6	3	7	2	7	1	5	4
pN2	7	5	2	3	4	6	1	2	5
pN3	1	1	0	1	0	1	0	1	0
		p=0.652		p=0.911		p=0.664		p=0.686	
pM category									
pM0	44	32	10	28	16	36	7	22	22
pM1	0	0	0	0	0	0	0	0	0
		p=1.000		p=1.000		p=1.000		p=1.000	
Stage									
1	8	7	0	5	3	7	1	4	4
2	15	11	4	10	5	12	3	9	6
3	10	8	2	8	2	8	1	6	4
4	11	6	4	5	6	9	2	3	8
		p=0.146		p=0.535		p=0.936		p=0.273	
Differentiation									
Moderate	18	14	4	14	4	16	1	13	5
Well	22	16	5	13	9	17	5	9	13
		p=1.000		p=0.312		p=0.206		p=0.062	
Mode of invation									
2	6	5	1	4	2	4	2	4	2
3	17	13	3	13	4	14	3	9	8
4C	9	5	4	5	4	8	1	4	5
4D	2	2	0	1	1	1	0	1	1
		p=0.378		p=0.684		p=0.693		p=0.868	
Smoking									
+	23	15	8	13	10	19	3	11	12
-	20	16	2	15	5	16	4	11	9
		p=0.142		p=0.336		p=0.691		p=0.763	
Alcohol									
+	26	19	6	17	9	22	3	15	11
-	17	12	4	11	6	13	4	7	10
		p=1.000		p=1.000		p=0.413		p=0.358	

[a]Total sample number is 42 for *SFRP1*. [b]Total sample number is 43 for *SFRP5*.

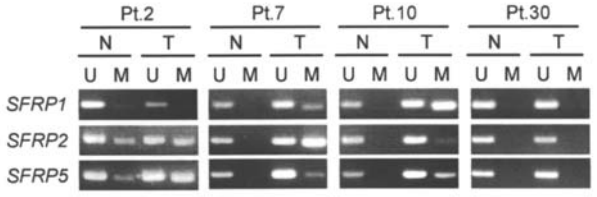

Figure 4. Analysis of S*FRPs* methylation in primary OSCC. Shown are representative results of MSP analysis of *SFRP1*, *-2*, *-5* and in primary OSCC tissues (T) and adjacent normal oral mucosa from the same patients (N).

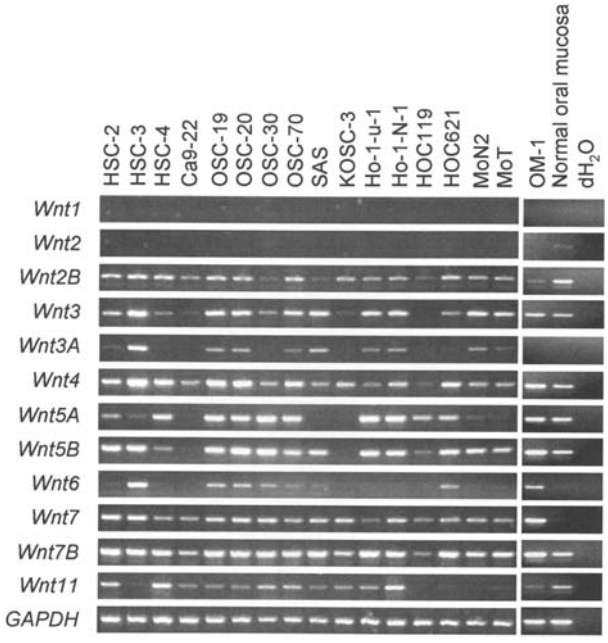

Figure 5. RT-PCR analysis of Wnt ligand genes expression in the indicated OSCC cell lines. *GAPDH* expression was assessed in all samples to ensure the cDNA quality; dH_2O indicates no RNA added.

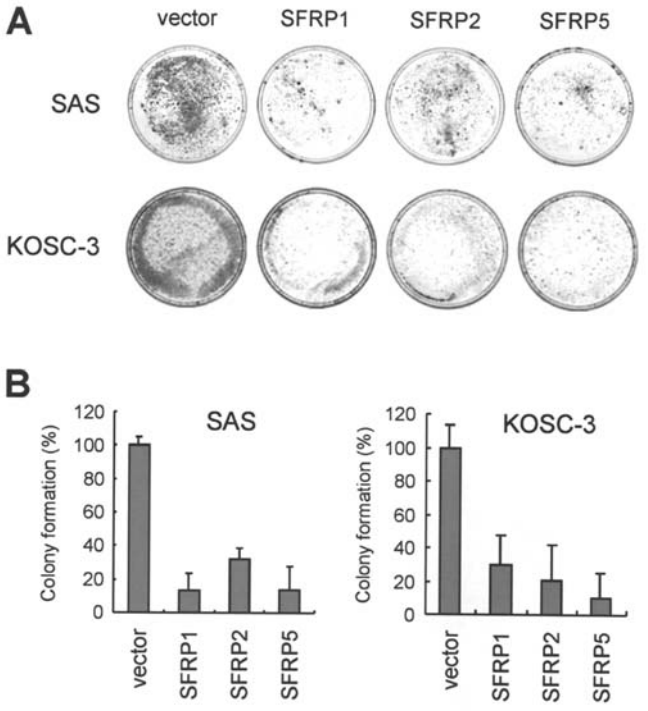

Figure 6. Growth suppression of OSCC cells by ectopic SFRP expression. (A) Representative results from a colony formation assay carried out using the indicated OSCC cell lines transfected with SFRP or control plasmid (vector). (B) Relative colony formation efficiencies of OSCC cells transfected with SFRP or control plasmid (vector). Shown are means of three replications; error bars represent standard deviations.

Methylation of SFRP genes in primary OSCC. Given that *SFRP* genes were frequently methylated in cultured OSCC cell lines, we next attempted to determine the extent to which they are aberrantly methylated in primary OSCC tumors. When we used MSP to examine *SFRP* methylation in 44 OSCC specimens, we detected methylation of *SFRP1* in 10 of 42 specimens (24%), *SFRP2* in 16 of 44 (36%) and *SFRP5* in 7 of 43 (16%) (Table III; representative results in Fig. 4). Two specimens were not informative for *SFRP1* methylation and one was not informative for *SFRP5* methylation due to insufficient PCR amplification. Among the 44 specimens tested, 22 (50%) showed methylation of at least one *SFRP* gene (Table III). In general, methylation of *SFRPs* was tumor-specific or tumor-predominant, but weak methylation of *SFRP* genes was observed in some non-tumorous oral mucosa from the OSCC patients (representative results in Fig. 4). This may have been caused by field defects.

We next correlated the methylation status of each *SFRP* gene and the clinicopathological features of primary OSCCs (Table III). We found no statistically significant correlations between methylation status and age, pT status, pN status, pM status, differentiation and tumor stage, smoking and alcohol consumption. We did, however, find that *SFRP1* was methylated more frequently in male patients.

Suppression of OSCC cell proliferation by ectopic expression of SFRPs. Our data suggest that loss of *SFRP* expression may be the primary cause of Wnt signal activation in OSCC cells. To investigate the consequences of silencing *SFRP* genes, we used RT-PCR to assess expression of Wnt ligands in OSCC cell lines. Although Wnt1 and -2 were not expressed in any of the OSCC cell lines, we found that all the cells expressed at least 6 of the 12 Wnt ligands tested (Fig. 5). Notably, expression of Wnt3A, -6 and -7 was detected in many of the OSCC cell lines, whereas these genes were not expressed in normal oral mucosa (Fig. 5). These results indicate that loss of SFRPs may enhance the effect of Wnt ligands in OSCC cells. We then investigated whether SFRPs could function as tumor suppressors by transfecting SFRP1, -2 or -5 into SAS and KOSC-3 cells, which otherwise do not express an SFRP, and carrying out colony formation assays (Fig. 6). Notably, induction of any one of these SFRPs markedly suppressed cell proliferation, suggesting SFRPs act as tumor suppressors by negatively regulating Wnt signaling in OSCC.

Discussion

Compelling evidence suggests that activation of Wnt signaling plays an important role in carcinogenesis. ß-catenin is

the center of the Wnt pathway, and its accumulation in the cytoplasm and nucleus is frequently observed in various cancers including CRC, GC, liver cancer and pancreatic cancer (14,15). Previous studies have also shown that *APC*, *CTNNB1* and *AXIN* are frequently mutated in these cancers; for instance, *APC* is mutated in the germ line of individuals with familial adenomatous polyposis coli (FAP), and acquired mutations in *APC*, *CTNNB1* or *AXIN2* initiate the majority of sporadic CRCs (14,15).

Several groups have reported that the distribution of ß-catenin is different in OSCC cells than in normal mucosal cells. Whereas ß-catenin is localized exclusively to the membrane in normal oral mucosal epithelium (23), such membrane-bound ß-catenin is absent or diminished in OSCC, and there is a corresponding increase in cytoplasmic localization (20-25). We also observed that membrane-bound ß-catenin is reduced or absent in more than half of the OSCC cell lines we tested, and the majority of those cell lines showed cytoplasmic and/or nuclear accumulation of ß-catenin. Previous reports also demonstrated that aberrant expression of ß-catenin is inversely correlated with differentiation and is significantly associated with invasion and poor prognosis (23-25). Thus Wnt signaling appears to play a key role in oral carcinogenesis.

Many researchers have investigated genetic alteration of Wnt signal-related genes in OSCC. Loss of heterozygosity at the *APC* locus has been observed in OSCC at frequencies ranging from 13% (4/31) (20) to 72.7% (8/11) (17). Largey *et al* (16) and Huang *et al* (18) observed loss of *APC* heterozygosity in 25% (3/12) and 53.8% (14/26) of primary OSCCs, respectively. In addition, Chang *et al* (19) analyzed 34 OSCC cases and found loss of *APC* heterozygosity in 29% of the cases, but detected no *APC* mutations in the OSCC specimens. Iwai *et al* (22) also failed to detect APC mutations in 20 primary OSCCs, and found *AXIN1* mutation in only one case, though cytoplasmic accumulation of ß-catenin was observed in 19 of 20 cases studied. Yeh *et al* (21) analyzed *CTNNB1* and *AXIN1* mutations in 30 primary OSCCs, but found no mutations in any of the samples. In the present study, we analyzed exon 3 of *CTNNB1* and the mutation cluster region of *APC* in 17 OSCC cell lines, but found no mutations of either gene. Because aberrant expression of ß-catenin might be associated with the loss of E-cadherin protein, and epigenetic inactivation of *CDH1* is frequently observed in various cancers including OSCC (38,39), we also analyzed the methylation and expression status of *CDH1* in our panel of OSCC cell lines. However, *CDH1* was neither methylated nor silenced in any of the OSCC lines studied. Taken together, these observations strongly suggest that activation of Wnt signaling is a common event in OSCC, one that is observed more often than genetic or epigenetic alterations of the key component genes.

We previously showed that epigenetic silencing of *SFRP* genes is one of the mechanisms by which Wnt signaling is activated in CRC and GC (29,30). Four *SFRP* family genes (*SFRP1*, *-2*, *-4* and *-5*) are presently known to be targets of epigenetic silencing in human tumors (28). Among them, *SFRP1*, *-2* and *-5* are methylated at particularly high frequencies in CRC and GC, and ectopic expression of these SFRPs suppresses both Wnt signaling and cell proliferation (28,30). Consistent with those findings, we observed that *SFRP1*, *-2* and *-5* were respectively methylated in 41, 94 and 82% of the OSCC cell lines tested; that expression of all three genes was lost or downregulated in the methylated cell lines; and that treatment with a DNA methyltransferase inhibitor, 5-aza-dC, restored their expression. Moreover, similar results were obtained with primary OSCCs.

To examine the effects of Wnt signaling in OSCC, we evaluated the expression status of the Wnt ligands in the OSCC cell lines. We previously found that at least 3 of the 11 known Wnt ligands are expressed CRC cell lines (29), and even more were expressed in OSCC cell lines. As described above, we observed cytoplasmic/nuclear accumulation of ß-catenin in 14 of 15 OSCC cell lines in which multiple *SFRP* genes were methylated and silenced. Moreover, ectopic expression of SFRP suppressed OSCC cell proliferation in colony formation assays. Thus loss of SFRP expression appears to contribute to activation of Wnt signaling leading to cell proliferation in OSCC.

On the other hand, our findings obtained with a TOPFLASH reporter assay system indicate that TCF/LEF transcriptional activity is not upregulated in any of the OSCC cell lines tested, even when they show a loss of Wnt antagonist genes and cytoplasmic accumulation of ß-catenin (data not shown), which is consistent with our earlier findings in GC cells (30). Although it is well known that mutation in *APC*, *CTNNB1* and *AXIN* is rare in GC, we found that the majority of GC cells showed activation and nuclear accumulation of ß-catenin and methylation of multiple *SFRP* genes (30). Among those GC cells, only a few cell lines in which *APC* or *CTNNB1* was mutated showed significant upregulation of TCF/LEF transcriptional activity (30). It may be that the TOPFLASH reporter assay is not sufficiently sensitive to detect weak or moderate activation of Wnt signaling.

In summary, we have shown that epigenetic silencing of multiple *SFRP* genes is a common event in OSCC. Methylation of *SFRP* genes is more frequently observed than genetic or epigenetic alterations of any other Wnt signal-related gene in OSCC. Our data support the hypothesis that epigenetic silencing of SFRP genes is one of the major causes of Wnt signal activation in OSCC cells. In addition, our results not only shed light on a mechanism contributing to OSCC tumorigenesis, but also suggest that *SFRP* methylation may be useful molecular target for the diagnosis and treatment of OSCC.

Acknowledgements

The authors thank M. Ashida for technical assistance, and Dr W.F. Goldman for editing the manuscript. This study was supported in part by Grants-in-Aid by Ministry of Education, Culture, Sports, Science and Technology (M.T. and T.T.).

References

1. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 55: 74-108, 2005.
2. Gangane N, Chawla S, Subodh A, Gupta S and Sharma SM: Reassessment of risk factors for oral cancer. Asian Pac J Cancer Prev 8: 243-248, 2007.
3. Petti S and Scully C: Oral cancer: the association between nation-based alcohol-drinking profiles and oral cancer mortality. Oral Oncol 41: 828-834, 2005.

4. Chiba I, Shindoh M, Yasuda M, *et al*: Mutations in the p53 gene and human papillomavirus infection as significant prognostic factors in squamous cell carcinomas of the oral cavity. Oncogene 12: 1663-1668, 1996.
5. Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM and Sidransky D: Allelotype of head and neck squamous cell carcinoma. Cancer Res 54: 1152-1155, 1994.
6. Partridge M, Emilion G, Pateromichelakis S, Phillips E and Langdon J: Location of candidate tumour suppressor gene loci at chromosomes 3p, 8p and 9p for oral squamous cell carcinomas. Int J Cancer 83: 318-325, 1999.
7. Ha PK and Califano JA: Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. Lancet Oncol 7: 77-82, 2006.
8. Ogi K, Toyota M, Ohe-Toyota M, *et al*: Aberrant methylation of multiple genes and clinicopathological features in oral squamous cell carcinoma. Clin Cancer Res 8: 3164-3171, 2002.
9. Nakayama S, Sasaki A, Mese H, *et al*: The E-cadherin gene is silenced by CpG methylation in human oral squamous cell carcinomas. Int J Cancer 93: 667-673, 2001.
10. Ogi K, Toyota M, Mita H, Satoh A, Kashima L, Sasaki Y, Suzuki H, Akino K, Nishikawa N, Noguchi M, Shinomura Y, Imai K, Hiratsuka H and Tokino T: Small interfering RNA-induced CHFR silencing sensitizes oral squamous cell cancer cells to microtubule inhibitors. Cancer Biol Ther 4: 773-780, 2005.
11. Shaw RJ, Liloglou T, Rogers SN, *et al*: Promoter methylation of P16, RARbeta, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing. Br J Cancer 94: 561-568, 2006.
12. Tran TN, Liu Y, Takagi M, Yamaguchi A and Fujii H: Frequent promoter hypermethylation of RASSF1A and p16INK4a and infrequent allelic loss other than 9p21 in betel-associated oral carcinoma in a Vietnamese non-smoking/non-drinking female population. J Oral Pathol Med 34: 150-156, 2005.
13. Cadigan KM and Nusse R: Wnt signaling: a common theme in animal development. Genes Dev 11: 3286-3305, 1997.
14. Polakis P: Wnt signaling and cancer. Genes Dev 14: 1837-1851, 2000.
15. Moon RT, Kohn AD, De Ferrari GV and Kaykas A: WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 5: 691-701, 2004.
16. Largey JS, Meltzer SJ, Sauk JJ, Hebert CA and Archibald DW: Loss of heterozygosity involving the APC gene in oral squamous cell carcinomas. Oral Surg Oral Med Oral Pathol 77: 260-263, 1994.
17. Uzawa K, Yoshikawa H, Suzuki H, Tanzawa H, Shimazaki J, Seino S and Sato K: Abnormalities of the adenomatous polyposis coli gene in human oral squamous-cell carcinoma. Int J Cancer 58: 814-817, 1994.
18. Huang JS, Chiang CP, Kok SH, Kuo YS and Kuo MYP: Loss of heterozygosity APC and MCC genes in oral squamous cell carcinoma in Taiwan. J Oral Pathol Med 26: 322-326, 1997.
19. Chang KW, Lin SC, Mangold KA, Jean MS, Yuan TC, Lin SN and Chang CS: Alteration of adenomatous polyposis coli(APC) gene in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 29: 223-226, 2000.
20. Gao S, Eiberg H, Krogdahl A, Liu CJ and Sorensen JA: Cytoplasmic expression of E-cadherin and beta-Catenin correlated with LOH and hypermethylation of the APC gene in oral squamous cell carcinomas. J Oral Pathol Med 34: 116-119, 2005.
21. Yeh KT, Chang JG, Lin TH, Wang YF, Chang JY, Shih MC and Lin CC: Correlation between protein expression and epigenetic and mutation changes of Wnt pathway-related genes in oral cancer. Int J Oncol 23: 1001-1007, 2003.
22. Iwai S, Katagiri W, Kong C, Amekawa S, Nakazawa M and Yura Y: Mutation of the APC, beta-catenin, and axin1 genes and cytoplasmic accumulation of beta-catenin in oral squamous cell carcinoma. J Cancer Res Clin Oncol 131: 773-782, 2005.
23. Williams HK, Sanders DSA, Jankowski JAZ, Landini G and Brown AMS: Expression of cadherins and catenins in oral epithelial dysplasia and squamous cell carcinoma. J oral Pathol Med 27: 308-317, 1998.
24. Lo Muzio L, Staibano S, Pannone G, Grieco M, Mignogna MD, Cerrato A, Testa NF and De Rosa G: Beta- and gamma-catenin expression in oral squamous cell carcinomas. Anticancer Res 19: 3817-3826, 1999.
25. Ueda G, Sunakawa H, Nakamori K, Shinya T, Tsuhako W, Tamura Y, Kosugi T, Sato N, Ogi K and Hiratsuka H: Aberrant expression of ß- and γ-catenin is an independent prognostic marker in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 35: 356-361, 2006.
26. Kawano Y and Kypta R: Secreted antagonists of the Wnt signalling pathway. J Cell Sci 116: 2627-2634, 2003.
27. Jones SE and Jomary C: Secreted Frizzled-related proteins: searching for relationships and patterns. Bioessays 24: 811-820, 2002.
28. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG and Baylin SB: A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 31: 141-149, 2002.
29. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG and Baylin SB: Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet 36: 417-422, 2004.
30. Nojima M, Suzuki H, Toyota M, Watanabe Y, Maruyama R, Sasaki S, Sasaki Y, Mita H, Nishikawa N, Yamaguchi K, Hirata K, Itoh F, Tokino T, Mori M, Imai K and Shinomura Y: Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. Oncogene 26: 4699-4713, 2007.
31. Stoehr R, Wissmann C, Suzuki H, Knuechel R, Krieg RC, Klopocki E, Dahl E, Wild P, Blaszyk H, Sauter G, Simon R, Schmitt R, Zaak D, Hofstaedter F, Rosenthal A, Baylin SB, Pilarsky C and Hartmann A: Deletions of chromosome 8p and loss of sFRP1 expression are progression markers of papillary bladder cancer. Lab Invest 84: 465-478, 2004.
32. Lodygin D, Epanchintsev A, Menssen A, Diebold J and Hermeking H: Functional epigenomics identifies genes frequently silenced in prostate cancer. Cancer Res 65: 4218-4227, 2005.
33. Fukui T, Kondo M, Ito G, Maeda O, Sato N, Yoshioka H, Yokoi K, Ueda Y, Shimokata K and Sekido Y: Transcriptional silencing of secreted frizzled related protein 1 (SFRP 1) by promoter hypermethylation in non-small-cell lung cancer. Oncogene 24: 6323-6327, 2005.
34. Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P and Sukumar S: Epigenetic suppression of secreted frizzled related protein 1 (SFRP1) expression in human breast cancer. Cancer Biol Ther 5: 281-286, 2006.
35. Liu TH, Raval A, Chen SS, Matkovic JJ, Byrd JC and Plass C: CpG island methylation and expression of the secreted frizzled-related protein gene family in chronic lymphocytic leukemia. Cancer Res 66: 653-658, 2006.
36. Kanaseki T, Ikeda H, Takamura Y, Toyota M, Hirohashi Y, Tokino T, Himi T and Sato N: Histone deacetylation, but not hypermethylation, modifies class II transactivator and MHC class II gene expression in squamous cell carcinomas. J Immunol 170: 4980-4985, 2003.
37. Sasaki Y, Morimoto I, Kusano M, Hosokawa M, Itoh F, Yanagihara K, Imai K and Tokino T: Mutational analysis of the beta-catenin gene in gastric carcinomas. Tumour Biol 22: 123-130, 2001.
38. Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 8: 286-298, 2007.
39. Chang HW, Chow V, Lam KY, Wei WI and Yuen A: Loss of E-cadherin expression resulting from promoter hypermethylation in oral tongue carcinoma and its prognostic significance. Cancer 94: 386-392, 2002.