Screening of candidate tumor-suppressor genes in 3p21.3 and investigation of the methylation of gene promoters in oral squamous cell carcinoma

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Abstract. Oral squamous cell carcinoma (OSCC) is the most common type of head and neck malignant tumor. However, its pathological mechanisms have not yet been elucidated. In the present study, we screened for candidate tumor-suppressor genes (TSGs) related to OSCC among 10 candidate genes located in 3p21.3, a region abundant with TSGs based on previous studies, using semi-quantitative reverse transcription PCR (RT-PCR). Three genes, *GNAT1*, *SEMA3B* and *AXUD1*, with low or no expression in OSCC tissues and the cell line TCA8113 were selected, and the promoter methylation status was further analyzed by methylation-specific PCR (MS-PCR). Hypermethylation in the promoter regions of *SEMA3B* was found in OSCC tissues, and a significant difference in the frequency of methylation of *SEMA3B* was observed between OSCC and non-cancerous tissues. Furthermore, TCA8113 cells treated with 5-Aza-Cdc started to re-express *SEMA3B* at a concentration of 5 μM or higher. Our study confirmed that three candidate TSGs with low expression may be involved in OSCC and that hypermethylation in promoter regions may contribute to the low expression of *SEMA3B*. These findings offer novel insights for clarifying the molecular mechanisms of tumorigenesis of OSCC as well as for aiding in its clinical diagnosis and therapeutic strategy.

Introduction

Head and neck tumors are the common malignant tumors worldwide, while oral squamous cell carcinoma (OSCC) is the most prevalent type of head and neck tumor accounting for 2-3% of malignant tumors and 80% of oral and maxillofacial malignant tumors (1,2). Although the techniques of surgical treatment, radiotherapy and chemotherapy for OSCC have progressed in the past few decades, the 5-year survival rate of OSCC patients has shown no noticeable improvement (3,4). Thus, it is crucial to explore the pathological mechanisms of OSCC, and new biomarkers must be identified and used in the detection of precancerous lesions, early diagnosis and therapy of OSCC.

Numerous factors, such as genetic alterations, epigenetic changes and environmental influence, are involved in the development of OSCC. Inactivation and deletion of tumor-suppressor genes (TSGs) play important roles in the development of carcinomas (5). Previous studies have suggested that analyzing the LOH (loss of heterozygosity) in tumor tissues can facilitate the identification of relevant TSGs. For example, *RB* (6), p53 (7), Wilms tumor (*WT)* (8), cyclin-dependent kinase number 2 (*CDKN2)* (9), adenomatous polyposis coli (*APC*) (10), deleted in pancreatic carcinoma locus 4 (*DPC4*) (11), fragile histidine triad (*FHIT*) (12) and putative protein tyrosine phosphatase gene (*PTEN*) (13,14) have all been identified using LOH analysis. As for OSCC, a high frequency of LOH has been observed on chromosomes 3p, 4q, 5q, 7q, 8p, 9p, 11q, 13q, 18q, 20q, 21q and 22q regions using comparative genomic hybridization and LOH analysis (15-18). Thus, these chromosome segments appear to be target regions where TSGs of OSCC may exist.

Roz *et al* (19) found LOH at one or more loci of chromosome 3p by investigating 30 oral dysplastic lesions, presenting clinically as either erythroplakias or leukoplakias with histopathological features of either severe epithelial dysplasia or carcinoma *in situ*. Furthermore, three discontinuous deletion regions including -3p13-21.1, -3p21.3-5 and -3p25, were detected in patients with precancerous lesions and OSCC, which suggest that alterations of these three regions may be early events in the development of OSCC. Yamamoto *et al* (20) found different degrees of LOH at 3p21.3 in OSCC patients from Japan and India. At the same time, LOH at 3p21-22 was present in other common malignant tumors such as renal carcinoma (21), lung cancer (22), squamous cell carcinoma of the head and neck (23), cervical carcinoma (24) and

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Table I. Clinicopathological features of the oral squamous cell carcinoma patients.

		Gender				Lymph node status	
Tumor subsite	Cases	Male	Female	Age (years)	Median age (years)	Positive	Negative
Tongue	28	24	4	17-80	42.1	13	15
Gum	3	2	1	40-61	50.3	1	2
Soft plate	1	1	0	73	73.0	0	1
Floor of the mouth	2	2	0	53	53.0	1	0
Jaw bones	2	1	1	48-59	53.5	2	0

breast cancer (25). Several groups have studied the relevant TSGs for nasopharyngeal carcinoma (NPC) and lung cancer at region 3p21.3 and found that *BLU*, *RASSF1A*, *SEMA3B* and *SEMA3F* play vital roles in the development and progression of NPC or lung cancer (26-30). Similar studies performed in OSCCs revealed that the expression levels of the *LTF, LIMD1*, *CACNA2D2*, *RASSF1A*, *CDC25A* and *SCOTIN* genes located at 3p21.3 were decreased by 67.6, 53.2, 23.7, 15.1, 5.3 and 0.58%, respectively. LOH and DNA methylation alterations may account for the abnormal expression of *LTF*, *LIMD1*, *CACNA2D2* and *RASSF1A*.

In the present study, based on the above-mentioned research, bioinformatics analysis was carried out to screen candidate TSGs located at 3p21.3, which to date have not been or have been inadequately studied in OSCC. The methylation state in the promoters of TSGs was also analyzed using methylation-specific PCR (MSP) to explore the role of DNA methylation in the downregulation of TSGs in OSCCs.

Materials and methods

Selecting target genes located at 3p21.3. On the basis of a database search (PubMed, Gene Card, OMIM and Gene Map), using '3p21.3' and 'tumor-suppressor genes' as search terms, 10 genes were selected for analysis based on the following inclusion criteria: i) they were previously reported to exhibit low expression or no expression in other tumor types; and ii) the functions of these genes were not previously studied or inadequately studied in OSCCs.

Cell line and tissues. TCA8113, a tongue cancer cell line, was provided by the Cancer Research Institute of Central South University. TCA8113 cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum at 37˚C in a humidified atmosphere with 5% CO_2. Thirty-six pairs of normal and tumor tissues from OSCC patients were obtained from the Hunan Cancer Hospital (Changsha, Hunan, China) and the Second Xiangya Hospital of Central South University (Changsha, Hunan, China) with informed patient consent prior to treatment. All fresh tissues were snap-frozen in liquid nitrogen and stored until required. Clinicopathological information of the patients is presented in Table I.

DNA and RNA extraction. gDNA from all specimens as well as from $1x10^6$ cells was extracted using an improved method for extracting high-molecular-weight DNA with phenol/chloroform according to previously reported procedures (31). Total RNA was isolated from a 100 mg sample from each specimen and a total number of 10^7 cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen, USA).

Reverse transcription-PCR (RT-PCR). Total RNA was treated with DNAse I (Roche Diagnostics, USA) and purified in accordance with the manufacturer's instructions. Reverse transcription was performed with 1 μg of treated total RNA using AMV reverse transcriptase (Promega, Madison, WI, USA). An equal amount of cDNA from each sample was amplified using specific primers for each gene (Table II). The conditions for PCR reactions were as follows: 95˚C for 5 min, followed by 30-34 cycles of 94˚C for 35 sec, 55-58˚C for 35 sec, 72˚C for 35 sec, and 72˚C for 10 min for the extension. The PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining.

Gray scale scanning and data analysis. The intensity of each electrophoretic band was measured using ImageMaster VDS (Pharmacia Biotech Inc., Piscataway, NJ), and analyzed by VDS software version 3.0 for band quantification with *GAPDH* as an internal control. The expression levels of target genes in the tumors and normal tissues were investigated after they were normalized by transforming them into two groups according to the ratios of the band intensity of the target genes over that of *GAPDH* for the same sample as previously described (32). A ratio >2 indicated that the corresponding gene was upregulated in OSCC and a ratio <1/2 indicated that the corresponding gene was downregulated in OSCC and all other ratio values indicated that the corresponding genes were expressed at a normal level. Each RT-PCR reaction was carried out in triplicate.

Methylation-specific PCR (MSP). MSP was performed to analyze the methylation state in the promoter regions of the target genes both in OSCC tissues and TCA8113 cells. One microgram gDNA from OSCC tissues and TCA8113 cells was treated with bisulfite, similar to previous methods (33,34). Each sample was amplified with two sets of primers, one set for methylated DNA and one set for unmethylated DNA. When gDNA was treated with bisulphite, the unmethylated cytosine was tranformed to uracil while methylated cytosine was unaffected. Thus, the methylated and unmethylated promoters were distinguished by MSP. The primers used for the methylated and unmethylated target gene promoter regions are listed in

Table II. Summary of primer sequences, annealing temperatures and product sizes.

	Squence (5'-3')	Product size (bp)	Annealing temperature (˚C)	Cycles	Ref.
Primer for RT-PCR					
GAPDH	F: GAGATCCCTCCAAAATCAAGTG R: GAGTCCTTCCACGATACCAAAG	282	58	28	
AXUD1	F: TGCGTAACAGTCTCCCACTG R: CCTGATTTCAGCCCTGTCTC	301	58	30	
BAP1	F: TACGCTACAACCGTGCTGTC R: TCAGCCTCCACACACTTCAG	295	58	30	
FUS1	F: GGATCTGGCTCACGAGTTCT R: GAAGGTTATGGGCCAACAGA	296	55	30	
GNAT1	F: CATCTGCAACCACCGCTAC R: CGCACGTCATGTGGGAATA	221	62	32	
LARS2	F: TTACTGGATGCCTGTGGATT R: CCCGTTGTGTTTGGACTTAC R: GCCTGCGACATACTGTGGTC	295	56	30	
NPRL2	F: CGCATTGCTATCCAGAACCT R: CTTCATAAGCCCGAACTGGA	300	60	30	
RASSF1A	F: ACACGTGGTGCGACCTCT R: GATGAAGCCTGTGTAAGAACCGTCCT	308	58	32	
SEMA3B	F: GTCCTCTTCATTGGCACAGAC R: GTCGCCATTCCTTACGTCTT	345	56	30	
SEMA3F	F: AGTGTCCGTACGATCCCAAG R: ATGACAGGGTTCCTCACGTC	466	58	32	
ZNF35	F: GCGCACATAGGCAGTACTCA R: TGTCTGAAGACCCTGCACTG	296	58	30	
Primer for MSP					
GNAT1	MF: GTAAAAGATATATTTATGGTCGGC MR: CCTCCCAAATAACTAAAATTACGAA	198	59		
	UF: AAAAGATATATTTATGGTTGGGTGA UR: CCTCCCAAATAACTAAAATTACAAA	195	59		
SEMA3B	MF: TGGTTAGGCGGGGTATTTTC MR: TCAACAATAAAAACGAAAACG	133	54		(52)
	UF: GTGGTTAGGTGGGGTATTTTT UR: ATCAACAATAAAAACAAAAACA	135	54		

F, forward primer; R, reverse primer; M, methylation; U, unmethylation.

Table II. The PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining.

Sequencing. DNA sequencing was carried out to identify the methylated and unmethylated sequences. MSP products were electrophoresed on a 1% agarose gel, and the products were recovered by using the QIAquick Gel Extraction kit (Qiagen, USA). The purified DNA was then cloned into the pMD18-T (Takara, Japan) vector and transformed into *Escherichia coli* strain Top 10 according to the manufacturer's instructions. The recombinant plasmid DNA extracted from the 5 randomly selected positive clones was used for sequencing.

Treatment of TCA8113 cells with 5-aza-2'-deoxycytidine (5-Aza-Cdc). TCA8113 cells were plated in 6-well plates at a density of $1x10^5$ cells/well and cultured overnight. On the morning of the next day, the TCA8113 cells were treated with 5-Aza-Cdc, a type of demethylation reagent, at final concentrations (as control) of 0 1, 5, 10, 50 and 100 μM, respectively. After changing the medium and treating cells with 5-Aza-Cdc

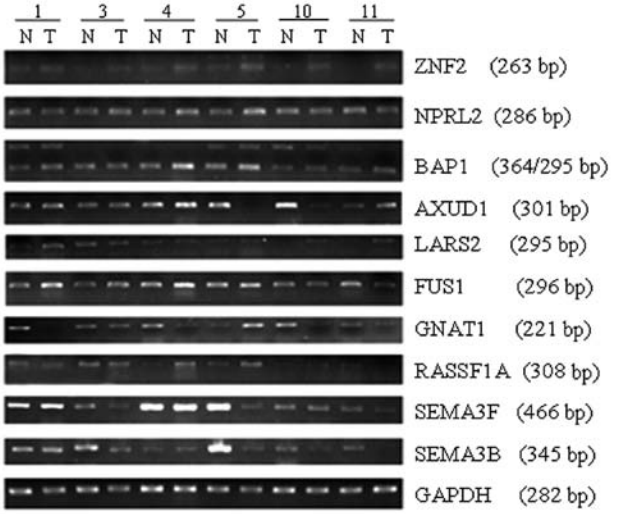

Figure 1. Expression of genes located at region 3p12.3 in OSCC tissues by RT-PCR. N, normal tissues as control; T, OSCC tissues.

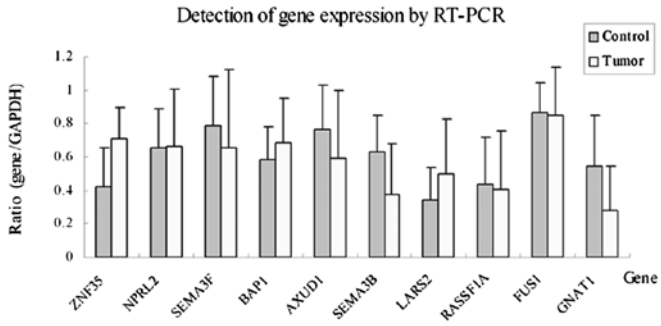

Figure 2. Relative expression of genes located at region 3p12.3 in OSCC tissues.

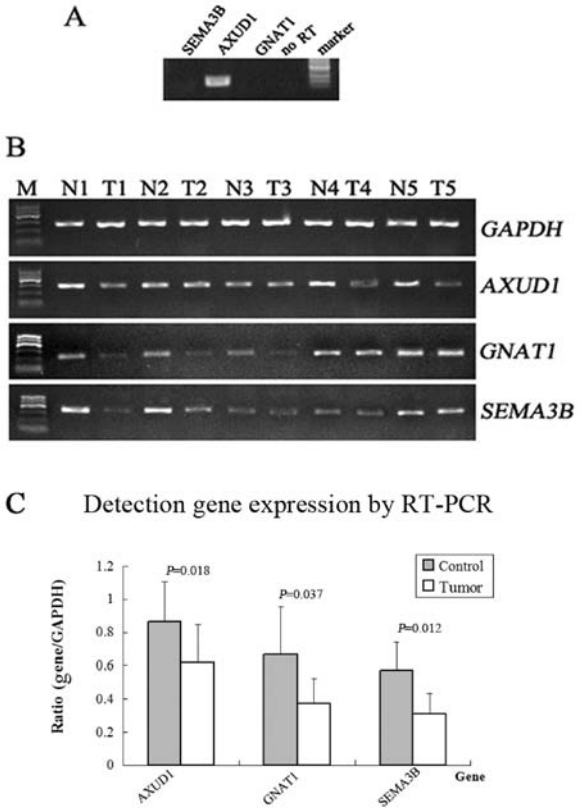

Figure 3. Expression of *GNAT1*, *SEMA3B* and *AXUD1* in OSCC tissues and the TCA8113 cell line. (A) Expression of *GNAT1*, *SEMA3B* and *AXUD1* in TCA8113 cells. (B) Verification of the expression of *GNAT1*, *SEMA3B* and *AXUD1* in additional OSCC tissue samples. (C) Verification of relative expression of *GNAT1*, *SEMA3B* and *AXUD1* located at region 3p12.3 in additional OSCC tissues.

once per 24 h for 4 consecutive days, TCA8113 cells were collected for RNA and DNA extraction to perform RT-PCR and MSP analysis.

Statistical analysis. Statistical analysis was performed using the Chi-square test and the Student's t-test. In all analyses, SPSS 11.5 statistical software (SPSS, Chicago, IL) was used, and a P-value <0.05 was considered to indicate a statistically significant result.

Results

Differential expression of the selected genes in OSCC and normal tissues. We selected 10 genes using '3p21.3' and 'tumor-suppressor genes' as the search terms in several databases (PubMed, Gene Card, OMIM and Gene Map). The selected genes are listed in Table II.

Differential expression of the target genes was assessed using semi-quantitative RT-PCR combined with Gray Scale Scanning analysis in 12 pairs of OSCC tissues and contralateral normal tissues. The results revealed that the expression of *GNAT1*, *SEMA3B* and *AXUD1* was downregulated or deficient in the OSCC tissues with percentages of 58.3 (7/12), 41.7 (5/12) and 41.7% (5/12), respectively (P<0.05). The expression of *ZNF35* was upregulated in 33.3% (4/12) of OSCC tissues. No significant difference in expression for *NPRL2*, *BAP1*, *FUS1*, *LARS2*, *RASSF1A* and *SEMA3F* was observed between the OSCC and normal tissues (P>0.05) (Figs. 1 and 2). We selected three downregulated genes, *GNAT1*, *SEMA3B* and *AXUD1*, for further study.

Expression of GNAT1, SEMA3B and AXUD1 in OSCC tissues and the TCA8113 cell line. RT-PCR was performed to verify the low expression of *GNAT1*, *SEMA3B* and *AXUD1* in 36 OSCC tissues, corresponding adjacent normal tissues and TCA8113 cells. The results confirmed that the expression of *GNAT1*, *SEMA3B* and *AXUD1* was downregulated or undetectable in 44.4 (16/36) 50 (18/36) and 47.2% (17/36) of the OSCC tissues, respectively, compared with the normal expression of these genes in adjacent normal tissues (P<0.05) (Fig. 3B and C). No expression of *GNAT1* and *SEMA3B* was detected and *AXUD1* was expressed at a normal level in the TCA8113 cells (Fig. 4A) as compared with the expression in OSCC tissues. No significant correlation was observed between the downregulated genes and OSCC metastasis and patient gender when compared with the clinical data (Table III).

Hypermethylation of GNAT1 and SEMA3B in OSCC tissues. We analyzed the methylation status of CpG islands in the promoter region of *GNAT1* and *SEMA3B* in several

Table III. Statistical analysis of the correlation between the downregulated genes and tumor metastasis.

		Genes					
		AXUD1		*GNAT1*		*SEMA3B*	
Groups	Cases	N (%)	U (%)	N (%)	U (%)	N (%)	U (%)
Metastasis	17	10 (27.8)	7 (19.4)	10 (27.8)	7 (19.4)	10 (27.8)	7 (19.4)
Non-metastasis	19	9 (25.0)	10 (27.8)	9 (25.0)	10 (27.8)	8 (22.2)	11 (30.1)
χ^2		0.472		0.139		1.003	
P-value		0.492		0.709		0.317	

N, normal expression; U, underexpression.

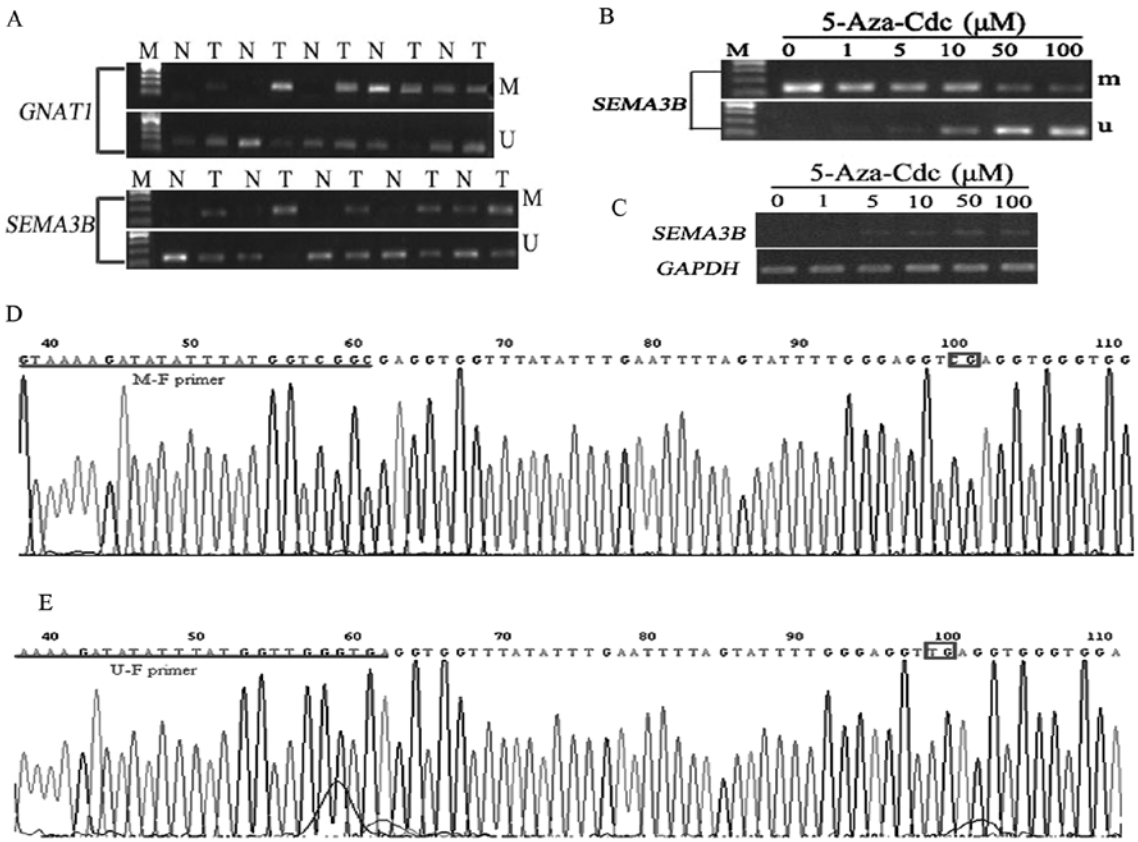

Figure 4. The hypermethylation status of *GNAT1* and *SEMA3B* was detected in OSCC tissues by MSP. 5-Aza-Cdc partially rescued the expression of *SEMA3B* in TCA8113 cells. (A) Analysis of the methylation of *GNAT1* and *SEMA3B* in OSCC tissues by MSP. N, normal control tissues; T, OSCC tissues; M, methylated; U, unmethylated. (B and C) Expression of *SEMA3B* in TCA8113 cells treated with different concentrations of 5-Aza-Cdc. m, methylated; u, unmethylated. (D and E) Confirmation of the outcome of MSP by sequencing.

OSSC tissue samples and adjacent non-cancerous tissues. Hypermethylation of *GNAT1* and *SEMA3B* was found in 75 (12/16) and 77.8% (14/18) of OSSC tissues, respectively. In the non-cancerous tissues, different levels of methylation were also detected with a percentage of 56.25% (9/16) for *GNAT1* and 38.9% (8/18) for *SEMA3B* (Fig. 4A). The results were also confirmed by the sequencing of the MSP products (Fig. 4D and E). Statistical analysis indicated that there was a significant difference in the frequency of methylation of *SEMA3B* between the OSCC and non-cancerous tissues (χ^2 test, P=0.018 <0.05) (Table IV), while no statistical significance was found in the frequency of methylation of *GNAT1* between the OSCC and non-cancerous tissues (χ^2 test, P=0.709 >0.05), suggesting that methylation of the promoter plays an important role in the downregulation of the *SEMA3B* gene in OSSC tissues.

5-Aza-Cdc partially recovers the expression of SEMA3B in TCA8113 cells. RNA and DNA were extracted from TCA8113 cells treated with different concentrations of 5-Aza-Cdc for 4 days for expression and methylation analysis of *SEMA3B* by RT-PCR and MSP. The results demonstrated that *SEMA3B* began to be expressed in the TCA8113 cells when treated

Table IV. Statistical analysis of the gene promoter methylation status in OSCC tissues.

	Genes					
	GNAT1			*SEMA3B*		
Groups	n	M (%)	U (%)	n	M (%)	U (%)
Control	16	9 (56.25)	7 (43.75)	18	7 (38.9)	11 (61.1)
Tumor	16	12 (75.0)	4 (25.0)	18	14 (77.8)	4 (22.2)
χ^2		1.247			5.60	
P-value		0.264			0.018	

Tumor, OSCC tissues; Control, non-cancerous tissues; M, methylated product; U, unmethylated product.

with a concentration of 5-Aza-Cdc at 5 μM (Fig. 4C). More unmethylated products were amplified from the DNA samples of TCA8113 cells when the higher concentration of 5-Aza-Cdc was administered (Fig. 4B). This suggests that methylation of the promoter plays a critical role in the downregulation of this gene in TCA8113 cells.

Discussion

Inactivation of TSGs and overexpression of oncogenes are the main causes for tumorigenesis. LOH and hypermethylation in promoter regions are the common reasons accounting for the inactivation of TSGs. The short arm of human chromosome 3, a region with a high frequency of LOH, is considered to be rich in TSG-containing sites as indicated by previous studies on lung cancer, breast cancer, ovarian cancer and other malignant tumors (35). Screening tumor-related TSG candidates is an available method based on loss of a chromosome region. Numerous candidate TSGs have been identified in high frequency chromosome-deleted regions such as *DLC-1*, a potential TSG at 8p21, a region frequently deleted in various types of cancers (33,36) and *DMRT1*, *DMRT3* and *DOCK8* located at 9p24.3, a deleted region in squamous cell lung carcinoma (37). In this study, we selected 10 genes including *AXUD1*, *BAP1*, *FUS1*, *GNAT1*, *LARS2*, *NPRL2*, *RASSF1A*, *SEMA3F*, *SEMA3B* and *ZNF35* which were located on region 3p21.3 and analyzed the expression of these genes using RT-PCR to find candidate TSGs in OSCC. Three downregulated genes, *SEMA3B*, *AXUD1* and *GNAT1*, were identified and both *SEMA3B* and *GNAT1* were downregulated in TCA8113 cells.

AXUD1, also termed cysteine-serine-rich nuclear protein 1 gene (*CSRNP1*), was cloned by Ishiguro *et al* (38) while studying the functions of AXIN1 which encodes one of the apoptotic proteins induced by TGF-β in hepatoma carcinoma cells. The full length *AXUD1* cDNA is 3188 bp and encodes a 64-kDa protein containing 596 amino acids and is located mainly in the nucleus. AXUD1, a downstream responsive protein for AXIN1, negatively regulates the Wnt pathway which plays an important role in early embryonic development. Studies have shown that the abnormal regulation of the Wnt pathway is related to tumorigenesis. The expression of *AXUD1*, which is high in normal tissues such as lung, placenta, skeletal muscle, pancreas and leukocytes, was found to be downregulated in lung cancer hepatoma and colorectal carcinoma, suggesting that *AXUD1* may be a candidate TSG (38). Simultaneously, knockdown of the expression of *DAXUD1*, a orthologue of the *AXUD1/CSRNP* family in *Drosophila*, was found to induce imaginal cells to proliferate. Conversely, overexpression of *DAXUD1* retarded cell cycle progression at mitosis behaving similar to a TSG (39). Our study showed that the expression of *AXUD1* was downregualted in 47.2% (17/36) of the OSCC tissues. However, this downregulation had no correlation with patient gender and tumor metastasis, indicating that *AXUD1* may be involed in the early malignant transformation of the oral mucous membrane. The function of this gene in OSCC warrants further study.

Guanine nucleotide-binding protein G (G protein) subunit α-1a encoded by *GNAT1* is an intracellular protein involved in light signal transduction in the retina (40). G protein, as an energy regulator, participates in the regulation of different transmembrane signal transduction pathways (40). Yi *et al* (41) found that expression of *GNAT1* was downregulated in 72.7% (24/33) of nasopharyngeal carcinoma tissues, which was markedly lower than that in chronic nasopharyngitis tissues (100%, 15/15). The study also demonstrated that downregulation of *GNAT1* was associated with LOH, but not with abnormal methylation of the promoter region. In our study, in comparison with the expression in normal control tissues, the expression of *GNAT1* was significantly downregulated in 44.4% of the OSCC tissues (P=0.037 <0.05) and in TCA8113 cells. However, the downregulation of *GNAT1* had no correlation with patient gender and tumor metastasis, suggesting that *GNAT1* may be involved in the early development of OSCC.

SEMA3B, a member of the human semaphoring family is involved in apoptosis (42,43) and in antitumor pathways regulated by p53 (44). *SEMA3B* was cloned by Sekido *et al* (45). *SEMA3B* cDNA encodes an 83-kDa protein of 749 amino acids. Using Northern blot analysis, Lerman and Minna (46) detected wide expression of a 3.4-kb *SEMA3B* transcript that was strongest in placenta, weaker in other tissues including lung and testis and not detectable in small-cell lung cancer cell lines. Missense mutations were found in non-small cell lung cancer cell lines. The authors also showed that SEMA3B is likely to be an extracellular secreted protein. Lerman and Minna concluded that *SEMA3B* is an attractive candidate tumor-suppressor gene for methylation and functional analysis. In the present study, we detected the expression of *SEMA3B* mRNA in OSCC tissues and adjacent control tissues. The results showed that the expression level of *SEMA3B* was significantly lower in OSCC tissues than that in control tissues, which was confirmed by undetectable expression of *SEMA3B* in TCA8113 cells. The χ^2 test confirmed that no significant correlation existed between the downregulated expression of *SEMA3B* and patient gender and tumor metastasis.

DNA hypermethylation, one of the most common epigenetic alterations, is involved in inactivation of the expression of many TSGs (47-49). MSP is a technique that has facilitated the detection of promoter hypermethylation at CpG islands in cell lines and clinical samples, including fresh and frozen tissues (50). Riquelme *et al* (51) found that the methylation ratio of the promoter region of *SEMA3B* is 92% (46/50) in

gallbladder carcinoma, suggesting that the methylation of *SEMA3B* may participate in the carcinogenesis and progression of gallbladder carcinoma. The hypermethylation status of *SEMA3B* was also recorded in non-small cell lung cancer decreasing the expression of *SEMA3B* (52,53) and in nasopharyngeal carcinoma for *GNAT1,* however, it did not account for the low expression of *GNAT1* (41).

Thus, MSP was performed with DNA samples from OSCC tissues, control tissues and 5-Aza-Cdc-treated TCA8113 cells. Hypermethylation status of *SEMA3B* in promoter regions was assessed in OSCC tissues, and the expression of *SEMA3B* was partly recovered in TCA8113 cells treated with 5-Aza-Cdc, demonstrating that the hypermethylation status in the promoter region led to, at least partially, low expression of *SEMA3B* in OSCC in accordance with studies in other cancer types (51-53). As for *GNAT1*, although hypermethylation in the promoter regions was observed in OSSC tissues, there was no significant difference in expression between OSSC tissues and control samples. Futher studies should be conducted to explore the reasons for the low expression of *GNAT1*.

In conclusion, our study demonstrated that *AXUD1*, *GNAT1* and *SEMA3B*, three candidate TSGs located at 3p21.3, may be involved in the development of OSCC, and methylation in the promoter region plays a critical role in the low expression of *SEMA3B*. These findings may lead to new avenues for the early diagnosis and therapy of OSCC.

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