

Genome-wide analysis of DNA methylation in tongue squamous cell carcinoma

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Abstract. Tongue squamous cell carcinoma (TSCC) is one of the most common types of oral cancer; however, its molecular mechanisms remain unclear. In this study, methylated DNA immunoprecipitation (MeDIP) coupled with methylation microarray analysis was performed to screen for aberrantly methylated genes in adjacent normal control and TSCC tissues from 9 patients. Roche NimbleGen Human DNA Methylation 385K Promoter Plus CpG Island Arrays were used to detect 28,226 CpG sites. A total of 1,269 hypermethylated CpG sites covering 330 genes and 1,385 hypomethylated CpG sites covering 321 genes were found in TSCC tissue, compared to the adjacent normal tissue. Furthermore, we chose three candidate genes (FBLN1, ITIH5 and RUNX3) and validated the DNA methylation status by methylation-specific PCR (MS-PCR) and the mRNA expression levels by reverse transcription PCR (RT-PCR). In TSCC tissue, FBLN1 and ITIH5 were shown to be hypermethylated and their expression was found to be decreased, and RUNX3 was shown to be hypomethylated, however, its mRNA expression was found to be increased. In addition, another three genes (BCL2L14, CDCP1 and DIRAS3) were tested by RT-PCR. In TSCC tissue, BCL2L14 and CDCP1 expressions were markedly upregulated, and DIRAS3 expression was significantly downregulated. Our data demonstrated that aberrant DNA methylation is observed in TSCC tissue and plays an important role in the tumorigenesis, development and progression of TSCC.

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Introduction

Oral cancer accounts for 2-4% of all cancer cases worldwide (1). An estimated 263,900 new cases and 128,000 deaths from oral cancer occurred globally in 2008 (2). More than 90% of all oral cancer cases are oral squamous cell carcinomas (OSCCs), which are a result of multistep processes that develop from the combined effects of a patient's genetic predisposition and exposure to environmental influences, including tobacco, alcohol, betel quid, chronic inflammation and viral infection (3). Tongue squamous cell carcinoma (TSCC) is a common OSCC. Imaging systems and treatment strategies in TSCC are improving substantially, however, 5-year survival statistics remain low. The molecular mechanisms underlying its clinical characteristics including carcinogenesis, development, progression, invasion, and metastasis have yet to be fully elucidated. Epigenetics studies recently showed that aberrant DNA methylation is involved in the progress of TSCC (4). DNA methylation is an important regulator of gene transcription and its role in carcinogenesis and development has been a topic of considerable interest in recent years. DNA methylation, which represses transcription of the promoter region of tumor suppressor genes leading to gene silencing, has been studied extensively (5).

DNA methylation is a very stable epigenetic mark and next generation sequencing studies have shown that several genes are aberrantly methylated in various types of cancer (6,7). Tissue specific DNA methylation patterns are stabilized during embryonic development, and are maintained through cell divisions. Aberrant DNA methylation patterns have been associated with a large number of human malignancies and are found in two distinct forms: hypermethylation and hypomethylation (8). The majority of DNA methylation studies focus on the analysis of CpG islands located in the promoter areas of candidate genes. However, differentially methylated areas may be located within genes and at large distances from the nearest neighboring genes (9). In the past decade, researchers focused only on a few candidate genes of DNA methylation in TSCC-related genes (4,5,10). Nevertheless, systematic studies on the genome-wide distribution of methylated loci in TSCC

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Gene	Primer	Sequence
FBLN1	M primer	F: 5'-ATTAGGAGATTCGCGGTTTC-3'
		R: 5'-GCTCCATAAACGACGAACG-3'
	U primer	F: 5'-GATTAGGAGATTTGTGGTTTTG-3'
		R: 5'-CACACTCCATAAACAACAACA-3'
ITIH5	M primer	F: 5'-TTGGCGATAGAAATTAAGTAAGTTC-3'
		R: 5'-AACCACCTATATTAACCCACG-3'
	U primer	F: 5'-TTGGTGATAGAAATTAAGTAAGTTTGT-3'
		R: 5'-AAAACCACCTATATTAACCCCACA-3'
RUNX3	M primer	F: 5'-TTACGAGGGGCGGTCGTACGCGGG-3'
		R: 5'-AAAACGACCGACGCGAACGCCTCC-3'
	U primer	F: 5'-TTATGAGGGGTGGTTGTATGTGGG-3'
		R: 5'-AAAACAACCAACAACAACACCTCC-3'

Table I. List of p	primers of methylated	(M) and unmeth	ylated (U) sequences	for MS-PCR analysis.
1	2			2

remain scarce. With the advent of next generation sequencing, genome-wide screening has become an attractive and useful tool for profiling TSCC.

In this study, methylated DNA immunoprecipitation (MeDIP) in combination with microarray-based hybridization was performed in adjacent normal control and tumor tissues from 9 patients with TSCC (11). Extensive DNA methylation distribution information from human TSCC and its adjacent normal tissues provided a preliminary genomic DNA methylation profile. Selected differentially expressed genes were further validated using methylation-specific PCR (MS-PCR) and reverse transcription PCR (RT-PCR).

Materials and methods

Patients and samples. Samples were obtained from 20 patients diagnosed with TSCC who underwent surgery at the Second Xiangya Hospital of Central South University and the Hunan Provincial Tumor Hospital. The study protocol was approved by the Ethics Committees of Central South University and written informed consent was obtained from all patients. None of the patients were given adjuvant chemotherapy or radiation prior to the operation. For each TSCC case, fresh samples were obtained from the primary tumor tissue and the adjacent normal tongue mucosa with a clear surgical margin to tumor. Surgical margins were considered as tumor-free or negative at least 5 mm of histologically normal tissue according to the pathological examination. The samples were snap frozen in liquid nitrogen and stored at -80°C until subsequent analyses.

MeDIP and methylation microarray hybridization. Genomic DNA from TSCC and adjacent normal control tissue (n=9) were extracted and purified according to the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD, USA). Then, DNA samples were pooled to one pair and sheared by sonication to obtain fragments between 400 and 500 bp and were immuno-precipitated by anti-5-methylcytidine antibody according to the MagMeDIP kit (Diagenode, Liège, Belgium). Fully

methylated DNA was obtained by whole-genome amplification using the GenomePlex[®] Whole Genome Amplification kit (Sigma-Aldrich, St. Louis, MO, USA). MeDIP DNA and input DNA were labeled with Cy5 and Cy3, respectively, and then analyzed using Human DNA Methylation 385K Promoter Plus CpG Island Arrays (Roche NimbleGen, Madison, WI, USA), which were used to detect 28,226 CpG sites. Gene chips were scanned by GenePix 4000B microarray scanner and hybridization signals were analyzed using GenePix Pro 6.0 hybridization analysis software. Probe-rich value setting was ≥ 2.0 .

MS-PCR analysis. To determine the methylation status of the three selected genes (*FBLN1*, *ITIH5* and *RUNX3*), we used MS-PCR in 20 pairs of samples from adjacent normal control and TSCC tissues. Genomic DNA was isolated by using Wizard[®] Genomic DNA Purification kit (Promega, Madison, WI, USA). Complete bisulfite conversion and purification of DNA were performed according to the EpiTect[®] Bisulfite kit (Qiagen). The fully methylated and fully unmethylated DNA samples were used as controls, and a water blank reaction was used as control for contamination. Following amplification by PCR, products were resolved on 1% agarose gels containing 0.5 μ g/ml ethidium bromide and visualized under UV transillumination. Primers for MS-PCR analysis are shown in Table I.

RT-PCR analysis. To determine the mRNA expression level of the six selected genes (*FBLN1*, *ITIH5*, *RUNX3*, *BCL2L14*, *CDCP1* and *DIRAS3*), we used RT-PCR in 20 pairs of samples from adjacent normal control and TSCC tissues. Total RNA was isolated from the sample tissues using TRIzol[®] reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. RNA was efficiently reverse transcribed into cDNA using the Reverse Transcription System (Promega). Primers for RT-PCR analysis and the PCR conditions of the six selected genes are shown in Table II. The expression level of each gene was confirmed by comparison to the expression.



Table II. List of primers for RT-PCR analysis and the condition of PCR.

Gene	Primer	Size of product (bp)	Annealing temperature (°C)	Cycles
FBLN1	F: 5'-TCAATACAGTTGCCTAGAGCA-3' R: 5'-GAGGAACAAGAGGACCCAT-3'	409	60	28
ITIH5	F: 5'-CACAGTTCTCCAGCGACA-3' R: 5'-CAGGACCGTTTCAGTATCAT-3'	386	60	32
RUNX3	F: 5'-CAGAAGCTGGAGGACCAGAC-3' R: 5'-TCGGAGAATGGGTTCAGTTC-3'	180	62	32
BCL2L14	F: 5'-TCCAGGTGTCTTTCTAACG-3' R: 5'-TTGACCTCTGATTCTCCC-3'	407	58	34
CDCP1	F: 5'-CATAAGAGCATCGGTTTAGAG-3' R: 5'-GGGTAGTTGGCAGACATCA-3'	329	58	28
DIRAS3	F: 5'-TCTCTCCGAGCAGCGCA-3' R: 5'-CGTCGCCACTCTTGCTGTCG-3'	410	62	34
β -actin	F: 5'-TAAGGAGAAGCTGTGCTACG-3' R: 5'-GACTCGTCATACTCCTGCTT-3'	459	58	28



Figure 1. Genome-wide DNA methylation was analyzed from adjacent normal and TSCC tissues, detecting 28,226 CpG sites. The hybridization signals of DNA methylation microarray from nine paired adjacent normal (A) and TSCC (B) tissues are shown.

sion amounts of β -actin which served as an internal control. Optical density of positive band was subsequently measured using Quantity One 4.62 (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data are expressed as the means \pm standard deviation (SD). Data were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA). The results of MS-PCR analysis were analyzed using a two-tailed Fisher exact test. The data of RT-PCR analysis were analyzed using a Student's t test. P<0.05 was considered to indicate statistically significant differences.

Results

Global DNA methylation changes in TSCC tissues. In order to investigate the aberrant genomic DNA methylation in TSCC, we performed methylated DNA amplification coupled with CpG island microarray analysis using a set of TSCC tissue specimens (adjacent normal control tissue vs. tumor tissue) (Fig. 1). Methylation status of the individual 28,226 CpG sites was compared between the 9 pairs of samples. There were 2,654 DNA hypermethylated sites that significantly differed in methy-

		FBLN1		ITIH5		RUNX3	
Group	Count	U n (%)	M n (%)	U n (%)	M n (%)	U n (%)	M n (%)
N	20	16 (80%)	4 (20%)	15 (75%)	5 (25%)	9 (45%)	11 (55%)
Т	20	9 (45%)	11 (55%)	6 (30%)	14 (70%)	17 (85%)	3 (15%)
		P=0	.048ª	P=0	.004 ^b	P=0	.019ª

Table III. Frequency of DNA methylation status of three candidate genes in paired adjacent normal and TSCC tissues.

Statistical significance of the difference between the adjacent normal (N) and TSCC (T) tissues was calculated using a two-tailed Fisher exact test. ^aP<0.05, ^bP<0.01. U, unmethylated; M, methylated.

lation level between adjacent normal control and TSCC tissue. A total of 1,269 CpG sites covering 330 genes were found to be significantly hypermethylated in TSCC tissues. In our analysis, these genes were located in different chromosomes. Among these hypermethylated genes, 28 genes (8.48%) and 27 genes (8.18%) were located on chromosome 1 and 19, respectively. Furthermore, chromosome 17, 16, 7 and 2 had 26 (7.88%), 24 (7.27%), 23 (6.97%) and 22 (6.67%) hypermethylated genes, respectively. Chromosome 18 had only 4 hypermethylated genes, accounting for 1.21%. In addition, chromosome 15 or Y had 5 hypermethylated genes (1.52%). Of the 330 methylated genes in TSCC, the hypermethylated sites of 218 genes (66.1%) were located in the promoter region, 77 (23.3%) in the coding region, and 45 (13.6%) in the downstream of gene, respectively.

In adjacent normal tissue, there were 1,385 hypermethylated CpG sites, covering 321 genes. Among these genes, up to 40 genes were located on chromosome 19, accounting for 12.46%. Chromosome X and 1 had 32 and 23 genes, accounting for 9.97 and 7.17%, respectively. Moreover, chromosome 14 had only 2 genes (0.62%). Among the 321 genes, 210 genes (65.4%) were located in the promoter region. Familial aggregation was found in these genes of differential methylation. For example, four genes of keratin (KRT) family and three keratin-related proteins (KRTAP) were shown to be hypermethylated in tumor tissue, including KRT 14 and 31 on chromosome 17, KRT 72 and 75 on chromosome 12, KRTAP10-3 and 10-7 on chomosome 21 and KRTAP2-4 on chromosome 17. Seven members of MAGE (melanoma antigen-encoding gene) family, MAGEA2, MAGEA3, MAGEA4, MAGEA9, MAGEA9B, MAGEA10 and MAGEA11, were found to be hypermethylated in adjacent normal tissue and located on chromosome X.

Validation of aberrantly methylated genes by MS-PCR. To verify the results obtained from our previous DNA methylation microarray study, we selected three candidate genes (FBLN1, ITIH5 and RUNX3), which were based on reports of tumorspecific methylation genes, for further analysis by MS-PCR. Twenty cases of TSCC and paired adjacent tissues were detected by MS-PCR for these three genes. Of these three genes, FBLN1 and ITIH5 were hypermethylated in TSCC tissue, and RUNX3 was hypermethylated in adjacent normal tissue. FBLN1 and ITIH5 showed a significantly higher incidence of DNA methylation in TSCC, while RUNX3 had markedly lower incidence of DNA methylation in TSCC than in adjacent normal



Figure 2. Three candidate genes (*FBLN1*, *IT1H5* and *RUNX3*) were validated by MS-PCR. Representative bands of MS-PCR using primers of methylated (M) and unmethylated (U) sequences are shown in paired adjacent normal (N) and TSCC (T) tissues.

tissue. As shown in Fig. 2 and Table III, *FBLN1* showed DNA methylation in 11/20 cases (55%) in TSCC and in 4/20 cases (20%) in adjacent normal tissue (P=0.048 <0.05). Moreover, methylation of *ITIH5* was detected in 14/20 cases in TSCC and in 5/20 cases in adjacent control tissues, of which the incidence was 70 and 25%, respectively (P=0.004 <0.01). In addition, we observed that 11/20 cases (55%) in adjacent normal tissues and only 3/20 cases (15%) in TSCC showed DNA methylation of *RUNX3* (P=0.019 <0.05). Differences in DNA methylation status of these three genes were statistically significant between adjacent normal and TSCC tissue. These findings were consistent with the microarray study.

Analysis of mRNA expression level of candidate genes by RT-PCR. According to our microarray analysis data, we selected six genes, which were highly related to tumorigenesis and development of TSCC, to detect their mRNA expression in the 20 paired samples. These genes included FBLN1, ITIH5, RUNX3, BCL2L14, CDCP1 and DIRAS3. As compared with adjacent normal tissue, TSCC tissue showed markedly upregulated expression levels of RUNX3 (P=0.006 <0.01), BCL2L14 (P=0.001 <0.01) and CDCP1 (P=0.032 <0.05). By contrast, expression levels of FBLN1 (P=0.042 <0.05), ITIH5 (P=0.021 <0.05) and DIRAS3 (P=0.037 <0.05) were significantly down-regulated in TSCC tissue as compared with adjacent normal



Figure 3. Gene expressions of six selected genes (*FBLN1*, *ITIH5*, *RUNX3*, *BCL2L14*, *CDCP1* and *DIRAS3*) were detected by RT-PCR. (A) Representative bands of these six genes and β -actin are shown in paired adjacent normal (N) and TSCC (T) tissues. (B) Relative mRNA expression levels of these six genes were assessed. All results were normalized to that of β -actin. Data are expressed as the means \pm SD (n=20). *P<0.05 vs. the adjacent normal control tissue; **P<0.01 vs. the adjacent normal control tissue.

tissue (Fig. 3). The gene expression levels of *FBLN1*, *ITIH5* and *RUNX3* in TSCC tissue were inversely correlated with the DNA methylation status.

Discussion

In the present study, a total of 9 paired samples from adjacent normal control and TSCC tissues were screened at the individual 28,226 CpG sites. The results demonstrated that 2,654 CpG sites were significantly different in methylation level: 1,269 sites, covering 330 genes, were hypermethylated in TSCC tissue and 1,385 sites, covering 321 genes, were hypermethylated in adjacent normal tissue. The microarray data could provide evidence and insights to further clarify the molecular mechanism in TSCC.

DNA methylation is the most common gene modification in eukaryotic cells. This epigenetic event has been studied extensively in the field of cancer research. In the past decade, several studies focused on the aberrant DNA methylation in various types of cancer, suggesting that the CpG island hypermethylation in gene promoter was closely related to inactivation of tumor suppressor gene or candidate tumor suppressor gene expression (12). Sardi *et al* (13) demonstrated that promoter hypomethylation resulted in the activation of oncogene expression in human bladder cancer and local hypermethylation may be considered a potential mechanism for increasing genetic alterations in bladder cancer formation. Furthermore, aberrant DNA methylation can be detected before tumors are clinically evident. Kresty et al (14) showed that hypermethylation of the cell cycle-regulatory genes $p16^{INK4a}$ and $p14^{ARF}$ were detected in 57.7 and 3.8% of patients, respectively, and the highest rates of $p16^{INK4a}$ hypermethylation occurred in lesions of the tongue and the floor of the mouth. The alterations of $p16^{INK4a}$ and $p14^{ARF}$ locus are frequent events preceding the development of oral cancer. As described by Palmisano et al (15), aberrant methylation of the *p16* and/or O⁶-methylguanine-DNA methyltransferase (MGMT) promoters can be detected in DNA from sputum in 100% of patients with lung squamous cell carcinoma up to three years before clinical diagnosis. In addition, Rosas et al (10) reported that aberrant methylation of at least one of these genes, p16^{CDKN2A}, MGMT and death-associated protein kinase (DAP-K), were detected in 17 (56%) of 30 head and neck primary tumors; 14 (47%) of 30 at p16, 10 (33%) of 30 at Dap-K and 7 (23%) of 30 at MGMT. In 11 (65%) of 17 methylated primary tumors, abnormally methylated DNA was detected in the matched saliva samples.

In order to verify our microarray data, we selected three genes (*ITIH5*, *FBLN1* and *RUNX3*), which have previously been reported in studies on aberrant DNA methylation in various types of cancer, and tested their levels of DNA methylation and mRNA expression in adjacent normal and TSCC tissues.

FBLN1, fibulin 1, was identified as a tumor suppressor gene whose inactivation may contribute to carcinogenesis. FBLN1 plays a tumor suppressive role in gastric cancer (16) as well as in other types of cancer such as breast (17) and prostate cancer (18). Cheng et al (16) demonstrated that aberrant promoter hypermethylation resulted in downregulated expression of FBLN1 in all gastric cancer cell lines and the primary gastric carcinoma tissues and was significantly restored after pharmacological demethylation. Kanda et al (19) reported that promoter hypermethylation of FBLN1 was significantly associated with advanced stage hepatocellular carcinoma, multiple tumors and increased tumor size. The present study showed that FBLN1 was hypermethylated and its mRNA level was significantly decreased in TSCC tissue compared with those in adjacent normal tissue. The exact molecular mechanism of how FBLN1 suppresses tumorigenesis remains unclear. Its expression could suppress cell motility, inhibit the phosphorylation of extracellular signal-regulated kinase and myosin light chain, and reduce the intracellular calcium level (20).

The inter- α -trypsin inhibitors (ITIs) family constitutes a group of plasma proteins consisting of one light (bikunin) and two heavy chains (ITIHs). *ITIH5* is a member of the *ITIH* gene family (21) and is a tumor suppressor gene. ITI plays a role in extracellular matrix stabilization and in the prevention of tumor metastasis (22). The studies of Veeck *et al* (21) and Himmelfarb *et al* (22) revealed that promoter methylation-mediated downregulation of *ITIH5* expression was associated with unfavorable outcome in breast cancer patients. Hamm *et al* (23) reported that *ITIH2*, *ITIH3*, *ITIH4* and *ITIH5* were strongly downregulated in a variety of human solid tumors (breast, endometrium, ovary, cervix, stomach, small intestine, colon, rectum, lung, thyroid, prostate, kidney and pancreas). *ITIH5* could be involved in the progression, invasion and metastasis of breast cancer, as its absence is associated with increased proliferation rates and a prognostic value indicating poor clinical outcome (21,22). Data from our study indicated that aberrant *ITIH5* promoter hypermethylation occurred in 14 out of 20 cases (70%) in TSCC tissue. There were statistically significant differences in *ITIH5* expression between TSCC and adjacent normal tissues.

RUNX3, runt-related transcription factor 3, part of the runtrelated (RUNX) family, appears to be an important component of the transforming growth factor- β (TGF- β)-induced tumor suppression pathway. RUNX3 has a critical role in the regulation of cell proliferation and cell death by apoptosis, as well as in angiogenesis, cell adhesion and invasion (24). RUNX3 acts as a tumor suppressor gene in gastric cancer (25,26). Aside from gastric cancer, it has been reported that downregulated expression of RUNX3 is observed in bladder (27), gastric (28), liver (29-31), colorectal (32,33) and lung (34) cancer. In these tumors, reduced expression of RUNX3 was frequently caused by CpG island hypermethylation. It was reported that RUNX3 expression levels were upregulated in head and neck squamous cell carcinoma (35), basal cell carcinoma of skin (36) and ovarian cancer (37). RUNX3 may have an oncogenic role in HNSCC as well as in basal cell carcinoma of skin (38,39). In addition, RUNX3 expression was observed in the tongue and palate epithelium of mouse embryos and disappears in newborn and adult mice (24,40). The results of this study are in accordance with these previous findings. RUNX3 was hypomethylated and its expression was upregulated in TSCC tissue. DNA hypermethylation of RUNX3 was observed in normal epithelial cells of the tongue, suggesting that RUNX3 may be silenced by methylation in normal oral mucosa. The exact molecular mechanism of RUNX3 regulation in normal adult oral epithelium and TSCC remains unclear and requires further investigation.

Of the other three candidate genes, *BCL2L14* and *CDCP1* were hypermethylated in adjacent normal tissue and *DIRAS3* was hypermethylated in TSCC tissue. In the RT-PCR analysis, the expressions of *BCL2L14* and *CDCP1* were significantly upregulated in TSCC tissue compared to adjacent normal tissue, whereas the expression of *DIRAS3* was markedly downregulated. Therefore, these findings indicate that the expressions of the three genes in TSCC tissues may be caused by the aberrant DNA methylation during the development of TSCC.

Apoptosis facilitator Bcl-2-like protein 14 (BCL2L14, also known as Bcl-G), is a protein encoded by the BCL2L14gene, and belongs to the Bcl-2 family. Bcl-2 family members act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities (41). The human Bcl-G gene encodes two proteins through alternative mRNA splicing, Bcl-G(L) (long) and Bcl-G(S) (short). Bcl-G(L) mRNA is widely expressed in adult human tissues, whereas Bcl-G(S)mRNA is found only in testis. Overexpression of this gene has been shown to induce apoptosis in cells. Bcl-G was downregulated in prostate (42) and breast cancer (43). However, in this study, BCL2L14 was observed to be hypomethylated with upregulated mRNA expression in TSCC tissue. CUB domain containing protein (CDCP1), a transmembrane protein with intracellular tyrosine residues which are phosphorylated upon activation, is assumed to be involved in proliferative activities and resistance to apoptosis of cancer cells (44). CDCP1 is a novel stem cell marker that is expressed in several types of cancer. *CDCP1* mRNA highly overexpressed in human colon cancer (45) and lung adenocarcinoma (44). In the breast cancer samples of Ikeda *et al* (46), tumors with high-level *CDCP1* expression showed higher levels of proliferation. *CDCP1* may be involved in regulating the adhesion and motility of cancer cells (47).

In our study, CDCP1 was shown to be hypermethylated in CpG site using microarray analysis but its expression was found to be upregulated using RT-PCR in TSCC tissue. DIRAS3 (also known as ARHI and NOEY2), GTP-binding protein Di-Ras3, is a novel imprinted tumor suppressor gene that encodes a small GTPase with 60% homology to Ras and Rap (48). Only the paternal allele of DIRAS3 is expressed due to maternal imprinting. DIRAS3 was absent or expressed at lower levels with allelic loss and promoter hypermethylation in ovarian, breast, and liver cancer as well as in other tumor tissues (49-51). DIRAS3 may play an important role physiologically in regulating cell growth through regulating expression of the cyclins and cyclin dependent kinase inhibitors (49). Our results showed that DIRAS3 gene expression was downregulated in TSCC tissue with hypomethylation status. The increased expression of BCL2L14 may be regulated by hypomethylation of its promoter region in TSCC tissue. However, the mechanism by which CDCP1 expression was upregulated with DNA hypermethylation and DIRAS3 expression was downregulated with DNA hypomethylation in TSCC tissue compared with that in adjacent normal tissue, remains unclear.

In summary, genome-wide DNA methylation microarray was used to identify the methylated genes in TSCC. Distinctly different DNA methylation profiles were obtained from adjacent normal and TSCC tissues. Furthermore, we validated the methylation status of three candidate genes by MS-PCR and the mRNA expression level of six candidate genes by RT-PCR. These results are consistent with the microarray data. Our study may provide useful data for further investigation of TSCC biomarkers for diagnosis, treatment and prognosis. Future gene-specific investigations are required to explore the molecular mechanism of the tumorigenesis, development and progression of TSCC.

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