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Hypermethylation of DcR1, DcR2, DR4, DR5 gene promoters and clinical significance in tongue carcinoma

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A B S T R A C T

Objective: Tongue squamous cell carcinoma (TSCC) is one of the most common malignancies in the oral cavity, and its incidence and mortality have been constantly increasing these years. A large number of tumor suppressor genes are involved in the development of the TSCC and it has been reported that the aberrant hypermethylation of tumor suppressor genes may play a key role in the process of the TSCC. In this study, we sought to analyze the association of methylation of DcR1, DcR2, DR4 and DR5 gene promoters and clinical significance in the TSCC to evaluate association between methylation of DcR1, DcR2, DR4 and DR5 gene and Clinical Significance in tongue squamous cell carcinoma.

Methods: Methylation-specific PCR(MSP) was used to analyze the methylation of the promoters of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) receptors in 45 TSCC cases. Real-Time PCR was used to detect the expression of the DcR1, DcR2, DR4 and DR5 gene.

Results: All the four genes (DcR1, DcR2, DR4 and DR5) showed different methylation of promoters in TSCC, while methylation of these promoters in paired adjacent normal tissues were almost undetectable. Patients with high methylation index were diagnosed at younger age when compared with the ones with low methylation index. DcR1 and DR4 hypermethylation was correlated significantly with patients' TNM stage.

Conclusions: Methylation of DcR1, DcR2,DR4 and DR5 promoters are found in TSCC and may associate with its occurrence and development. Taking the reversibility of methylation into account , methylation is a potential targeted therapy of TSCC.

1. Introduction

Tongue squamous cell carcinoma (TSCC) is one of the most common malignancies in the oral cavity. There were an estimated 48,100 new cases and 22,100 deaths from TSCC in China in 2015 [1], and an estimated 12,060 new cases and 2030 deaths from TSCC in the United States in 2011 [2]. During the last decades, a notable increase of incidence and mortality of TSCC was reported in most countries [3]. These years, the TSCC patient population diagnosed with younger age is increasing worldwide, which is considered to be more aggressive, associated with early lymph node metastasis and a higher incidence of regional treatment failure [4–6]. Although great effort has been making to identify molecular interactions with clinical characteristics in order to improve the diagnosis and treatment of TSCC, the prognostication of TSCC is still on the way.

Epigenetic changes, including mutations, methylation, microRNAs expression alteration, protein coding and copy number variations, have been proved to play a crucial role in the development of the TSCC [7–10]. Among these different modes of genetic modification, DNA methylation and chromatin remodeling by histone modification is regarded as the most important one [11–13]. DNA methylation, as an important epigenetic marker, is one of the best-characterized chemical modification of mammalian DNA and provides a stable, heritable, and critical component of epigenetic regulation [11,14]. DNA methylation is a natural modification that occurs in mammalian DNA and typically affects cytosine followed by guanine (CpG) [15]. Tumor suppressor genes (p14ARF, p15INK4B, p16INK4A and p53) have various changes in the development of tongue carcinoma and these tumor suppressor genes and their protein changes are of great importance to clinical therapy [16–19]. In addition, Several tumor suppressor genes including RUNX3, RASSF1A, CDH1 and p16INK4A have been found to be inactivated by hypermethylation in promoter region in TSCC [20–22]. Therefore, determining the methylation status in TSCC has a great potential value in early detection, monitoring, and treatment.

Death receptors (DR4 and DR5) and decoy receptors (DcR1 and DcR2) genes are generally found in normal human cells and play an important role in apoptosis [23–25]. TNF-related apoptosis-inducing ligand (TRAIL) selectively induces programmed cell death (apoptosis)

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in various cancer cells but not in normal cells and TRAIL is known to bind to 4 different receptors, 2 proapoptotic (DR4 and DR5), and 2 potentially antiapoptotic receptors lacking death domains (DcR1 and DcR2) [26]. All the four TRAIL receptors work together and balance the cell apoptosis signaling. The aberrant methylation of TRAIL receptors has been reported in some tumors, however, the association of the methylation of TRAIL receptors and TSCC is still little known. Our study is trying to look into the hypermethylation of TRAIL receptors in TSCC and their possible roles.

2. Materials and methods

2.1. Patients and tissue specimens

Tissue specimens were obtained from TSCC patients who underwent tumor resection at Guangzhou HuaDu affiliated hospital of Guangzhou medical university between January 2013 and December 2017. The study protocol was approved by the Ethics Committees of Guangzhou medical University and written informed consent was obtained from all patients. None of the patients were given a neoadjuvant therapy 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 analysis.

2.2. RNA extraction and RT-PCR analysis

RNA extraction from tumor tissues and adjacent normal control tissue were done using TRIzol reagent (Takara, Otsu, Japan). First-strand cDNA was synthesized from total RNA using the PrimeScript RT Reagent kit (TaKaRa, Shiga, Japan). PCR amplification was performed using SYBR® Premix Ex Taq™ (Takara). Amplification and qPCR measurements were carried out using the CFX96 Real-Time PCR Detection System (Bio-Rad). All quantities were expressed as number of folds relative to the expression of GAPDH. The primer sequences were as follows:

DR4 forward, CCAACAAGACCTAGCTCCCCAGC,
and reverse, AAGACTACGGCTGCAACTGTGACTCC;
DR5forward, GTCCTGCTGCAGGTCGTACC,
and reverse, GATGTCACTCCAGGGCGTAC;
DcR1 forward, CCCAAAGACCCTAAAGTTCGTC,
and reverse, GCAAGAAGGTTCATTGTTGGA;
DcR2 forward, ACCCCAAGATCCTTAAGTTCG,
reverse, CAAGAAGGCAAATTGTTGGAA.

2.3. MSP

Genomic DNA was isolated by using Wizard® Genomic DNA Purification kit (Promega, Madison,WI, USA). Bisulfite modification of genomic DNA from tumor and normal tissues was performed using the DNA Bisulfite Conversion Kit (Tiangen Biotech, Shanghai). The bisulfite-modified DNA (20 μg) was used in each MSP system (Tiangen Biotech, Shanghai) with primers specific for methylated DNA and primers specific for unmethylated DNA. The primer sequences are (5- to −3) are shown in the Table .1.

3. Results

3.1. Methylation of the TRAIL Receptors in TSCC tissues

To identify the methylation of the genes of TRAIL receptors, tumor tissues and paired adjacent tissues of forty-five diagnosed with TSCC were eligible for evaluation by MS-PCR in this study. All of the four genes were hypermethylated in TSCC while in adjacent normal tissues the four genes showed almost unmethylated. As shown in Table 2 and Fig. 1, the methylation level of DcR1 in TSCC (40%) was much higher than that of adjacent normal tissues (2%). In addition, the methylation of DcR2 and DR5 in 45 TSCC cases were detected in 21 (47%) and 16(36%) respectively. Interestingly, methylation level of DcR2 and DR5 in 45 adjacent normal tissues was the same (5%). Moreover, DR4 was observed that 35/45 cases (78%) in TSCC and 1/45 case (2%) in adjacent normal tissues were methylated. These results showed the methylation of the TRAIL receptors genes in TSCC tissues may be involved in epigenetic down-regulation of gene expression.

Table 1
List of primers of methylated (M) and unmethylated (U) sequences for MS-PCR analysis.

Gene	Primer	Sequence
DcR1	M primer	F:5′-TTACGCGTACGAATTTAGTTAAC-3′ R: 5′-ATCAACGACCGACCGAAACG-3′
	U primer	F: 5′-GAATTTTTTTATGTGTATGAATTTAGTTAAT-3′ R: 5′-CCA TCAAACAACCAAAACA-3′
DcR2	M primer	F: 5′-GGGATAAAGCGTTTCGATC-3′ R: 5′-CGACAACAAAACCGCG-3′
	U primer	F: 5′-TTGGGGATAAAGTGTTTTGATT-3′ R: 5′-AAACCAACAACAAAACCACA-3′
DR4	M primer	F: 5′-TTCGAATTTCGGGAGCGTAGC-3′ R: 5′-GTAATTCAATCCTCCCCGCGA-3′
	U primer	F: 5′-GTAGTGATTTTGAATTTTGGGAGTGTAGT-3′ R: 5′-CTCATAATTCAATCCCCACAA-3′
DR5	M primer	F: 5′-GAGGTAGTGAAAGTATAGTCGCGTC-3′ R: 5′-CCCTTAAAATAAATCGAACATCGT-3′
	U primer	F: 5′-GGTAGTGAAAGTATAGTTGTGTTGT-3′ R: 5′-ACCCTTAAAATAAATCAAACATCATC-3′

3.2. Expression of the TRAIL Receptors in TSCC tissues

According to the results of methylation of the TRAIL receptors in TSCC tissues, we determined to detect the expression of the four genes, DcR1, DcR2, DR4 and DR5, and verify whether the methylation worked for epigenetic down-regulation. RT-PCR was used to detect expression of the TRAIL receptors in all of the 45 TSCC tissues and paired normal samples. As shown in Fig. 2, expression of the all four genes in methylated group of TSCC tissues was reduced compared with non-methylated group of TSCC tissues. Moreover, expression of DR4 genes is found to be dramatically downregulated in TSCC tissues compared with the other three genes. This may indicate that the higher the TRAIL receptors genes were methylated, the lower the TRAIL receptors genes were expressed.

3.3. Methylation of the TRAIL Receptors in TSCC tissues may be involved in tumor metastasis

Having revealed the methylation of the TRAIL receptors was epigenetic down-regulation, we further detected the association between methylation of the TRAIL receptors and clinicopathological characteristics in TSCC. The results are summarized in Table 3. There were statistically significant association between the three studied genes, DcR1, DR4 and DR5, with demographic and clinicopathologic characteristic of patients' age. In addition, DcR1 and DR4 hypermethylation was correlated significantly with patients' TNM stage.

4. Discussion

Series of studies reported that TRAIL receptors functioned as a suppressor tumor gene through inducing apoptosis in cancer cells and their down regulation could promote the initiation and process of carcinomas [27–29]. It is reported that methylation of promoters in TRAIL receptors may lead to epigenetic down-regulation of expression

Table 2
Frequency of DNA methylation status of four candidate genes in paired adjacent normal and TSCC tissues.

Group	Number	DcR1		DcR2		DR4		DR5	
		U	M	U	M	U	M	U	M
		n(%)	n(%)	n(%)	n(%)	n(%)	n(%)	n(%)	n(%)
N	45	44(98%)	1(2%)	43(96%)	2(4%)	44(98%)	1(2%)	43(95%)	2(4%)
T	45	27(60%)	18(40%)	24(53%)	21(47%)	10(22%)	35(78%)	29(64%)	16(36%)
		P**		P**		P***		P**	

Statistical significance of the difference between the adjacent normal (N) and TSCC (T) tissues was calculated using a two-tailed Fisher exact test. P** < 0.01, P*** < 0.001. U, unmethylated; M, methylated.

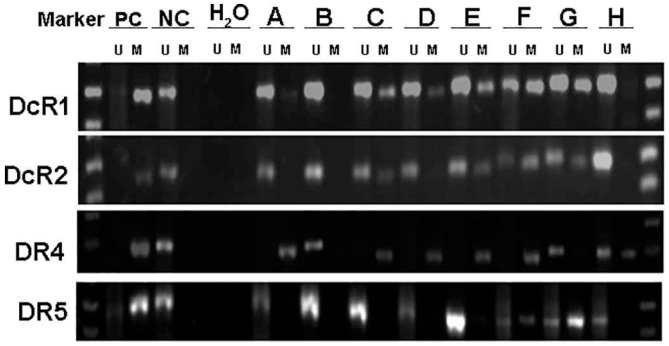

Fig. 1. Four candidate genes (DcR1, DcR2, DR4 and DR5) were validated by MS-PCR. Representative bands of MS-PCR using primers of methylated (M) and unmethylated (U) sequences are shown in paired adjacent normal (N) and TSCC (T) tissues. The methylated/unmethylated tissues DNA were used as positive controls (PC) and negative controls (NC), respectively.

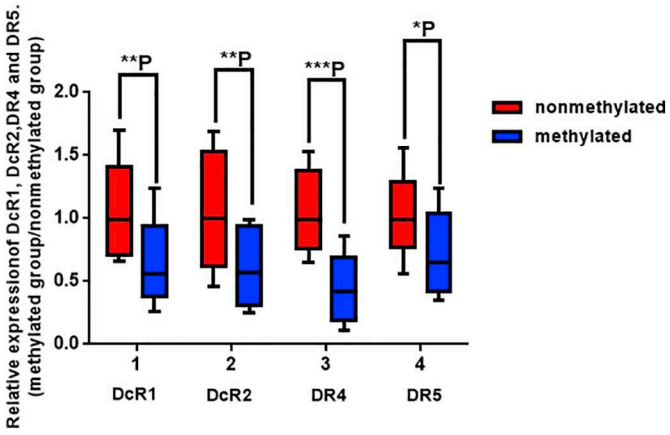

Fig. 2. Comparisons of expression of four candidate genes between the methylated group and nonmethylated group.

[30,31]. In the present study, methylation level of the four TRAIL receptor genes were determined by MSP in TSCC and paired adjacent normal tissues. Here, as shown in Table 2, it was indicated that the four TRAIL receptor genes were differently hypermethylated in TSCC tissues, while hypermethylation was almost undetectable in paired normal samples. It is noteworthy that the DR4 hypermethylated level in TSCC is higher than the other three suppressor tumor genes, and this indicates that DR4 may act as a potential biomarker in TSCC diagnosis and treatment.

Aberrant methylation and subsequent down-expression of DcRs and DRs genes are frequent in multiple tumor types including breast cancers, lung cancers and mesotheliomas [32]. The low expression of DcRs and DRs resulting from aberrant methylation may be strongly associated with the processing of TSCC. In our study, the four TRAIL

Table 3
Demographic and clinicopathological characteristics.

Clinical characteristics		Frequency (Percentage)	P value of methylated genes			
			DcR1	DcR2	DR4	DR5
Age (yr)	≤44	11(24%)	0.038*	0.248	0.011*	0.023*
	> 44	34(76%)				
Sex	Male	30(67%)	0.568	0.568	0.128	0.335
	Female	15(33%)				
Multicentricity	No	36(80%)	0.866	0.578	0.487	0.787
	Yes	9(20%)				
Cervical LN metastasis	No	40(89%)	0.325	0.124	0.575	0.689
	Yes	5(11%)				
TNM	I/II	12(27%)	0.014*	0.367	0.001***	0.398
	III/IV	33(73%)				

* Significant difference for promoter hypermethylation in DcR1, DcR2, DR4 and DR5 with tumor site by Fisher's Exact test and patients' age by independent-samples *t*-test.
*** p < 0.005.

receptor genes (DcRs and DRs genes) in TSCC is downregulated compared with their paired normal samples. The expression of DR4 in TSCC is dramatically less than the other three TRAIL receptors (DR5,DcR1 and DcR2) and the methylation of DR4 is much higher than that of the DR5,DcR1 and DcR2,which demonstrated the expression of TRAIL receptors may be subject to the degree of methylation in response genes. Our results indicated that methylation of promoter may be responsible for the down-regulation of the TRAIL receptors in the TSCC. To the best of our knowledge, this is the first reported hypermethylation of DcRs and DRs promoter in TSCC. Moreover, our results suggested that clinicopathologic stage may be associated with methylation level of the DcR1 and DR4. We have performed a sequence of analysis of the correlation between methylation of DcRs and DRs and clinicopathologic of the 45 TSCC. As shown in the Table 3, the hypermethylation level of DcR1 in stage III/IV is much higher than that of stage I/II in our TSCC samples. Likewise, we found that methylation level of DR4 stage I/II is lower than that of stage III/IV. Shivapurkar et al. have inferred that DcRs may function as oncogenes because of their postulated anti-apoptotic effect, and represent pro-apoptotic effect in cancer [32]. However, the degree of methylation of DR4 in our study is higher than that of DcR1, and this demonstrates that methylation of DR4 acts as a main role in the development of TSCC.

In summary, our results showed that the four TRAIL receptor genes were differently hypermethylated and the methylation of DR4 and DcR1 are more frequently seen in stage III/IV. Hypermethylation of DR4 and DcR1 are potential biomarkers for clinical detection of TSCC for diagnosis, treatment and prognosis.

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