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Contribution of *NF-κB*, *TRAF1*, and *TRAF2* promoter methylation in Iranian patients with oral squamous cell carcinoma

Abstract

Many genetic modifications were seen to influence the growth of oral squamous cell carcinoma (OSCC). The nuclear factor kappa B (*NF-κB*) is a transcription factor that participates in inflammation, apoptosis, and cell survival and also causes invasion and metastasis. *TRAF1* and *TRAF2* have been recognized as genetic targets for *NF-κB* transcriptional activity. The current study investigated *NF-κB*, *TRAF1*, and *TRAF2* gene promoter methylation patterns by employing a methylation-specific polymerase chain reaction (MS-PCR) in 86 paraffin wax embedded samples of OSCC patients and compared the results with 68 normal control tissues. Analysis of data showed that no significant relationship was found for promoter methylation of *NF-κB* gene between case and control groups (*p*-value = 0.999); besides, promoter methylation of *TRAF1* and *TRAF2* genes was not considerably different between these two groups (*p*-value = 0.411 and *p*-value = 0.866, respectively). The findings of this study suggest that there is no significant correlation between promoter methylation of all three genes and the risk of establishing OSCC.

Keywords: *Squamous Cell Carcinoma of Head and Neck, TNF Receptor-Associated Factor 2, NF-kappa B, TNF Receptor-Associated Factor 1, DNA Methylation*

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Introduction

The eighth most known cancer in the world is oral squamous cell carcinoma (OSCC). Modifications like mutations or deletion in TSG genes, or silencing by hypermethylation, may lead to cancer (Scully & Bagan, 2009). Oral cancer portrays an important problem because of its high prevalence globally and the severe functional defects associated with its management. It is believed that synthetic or natural chemical compounds are in the environment; smoking and drinking are among the most significant environmental factor related to oral cancer (Katoh et al., 1999).

The epigenetic mechanism is necessary for the conservation of particular gene expression profiles in vertebrates and normal development. Alterations in this process can cause impaired gene function and the transformation of malignant cells. Alteration of the epigenetic landscape is an identifying characteristic of cancer (Sharma, Kelly, & Jones, 2010).

DNA methylation is one of the key epigenetic modifications which provide hereditary information without being encoded in the nucleotide sequence. DNA in vertebrates can only be covalently modified by 5-Methylcytosine (Jeltsch, 2002). In cancers, DNA methylation abnormalities are associated with the onset and progression of the disease (Feinberg & Vogelstein, 1983; Riggs & Jones, 1983). The epigenome of cancer can be validated by measuring hypomethylation across the genome and hypermethylation of CpG islands within its promoters (Jones & Baylin, 2002).

As a transcription factor, the nuclear factor kappa B (*NF-κB*) participates in a variety of biological procedures such as cell survival, inflammation, regulating apoptosis, proliferation, and differentiation (Sughra, Birbach, de Martin, & Schmid, 2010). Tumor necrosis factor-alpha (TNF-α) bound to the TNF receptor (TNFR) activates the cell death pathway and stimulates the transcription factor *NF-κB*, which blocks cell death. Activation of *NF-κB* blocks the activation of caspase 8 (C.-Y. Wang, Mayo, Korneluk, Goeddel, & Baldwin, 1998). Tumor necrosis factor-alpha (TNF-α) receptor-associated factors (TRAF) are the main mediator of transducing TNF signaling to various targets (Shi & Sun, 2018). Heterodimers form of *TRAF2* and *TRAF1* are *NF-κB* inducible proteins that modulate the expression of *TRAF2* and *TRAF1* (Park, Lee, Lim, & Kim, 2019). *NF-κB* stimulates a collection of proteins that cooperate effectively during the initial checkpoint to inhibit TNF-alpha-mediated cell death, thereby suppressing apoptosis induced by genotoxic agents (C.-Y. Wang et al., 1998).

In this study, we evaluated promoter hypermethylation of *NF-κB*, *TRAF1*, and *TRAF2* genes in patients with OSCC in Iran.

2. Materials and Methods

2.1. Subjects

Eighty-six paraffin wax embedded tissues with the informed consent of patients diagnosed with OSCC (mean age ± S.D.: 54.14 ± 12.6) along with 68 control oral mucosae (mean age ±

S.D.: 37.07 ± 11.07) were collected from individuals that did not have OSCC and were referred to the Periodontics Department, Dental School, Zahedan University of Medical Sciences, Iran (Table 1).

2.2. DNA isolation and modification

DNA from control and OSCC tissue was extracted by the use of the QIAamp DNA extraction kit (Cat. No. 56404, Qiagen Co., Hilden, Germany) according to the manufacturer's guidelines; subsequently, the quality was projected by nanodrop. 1–2 mg of extracted genomic DNA diluted in 20 mL of water and was used for DNA modification considering a recently published study (Kordi-Tamandani & Birjandian, 2010). Wizard® DNA Clean-Up System (Cat. No. A7280, Promega Co., Madison, WI) was also employed for purifying the bisulfite-treated DNA concerning the guidelines. Bisulfite-treated DNA was stored at -20 °C.

2.3. Methylation-specific PCR (MSP)

The online ensemble database was where the promoter of genes was recognized. The methylation patterns in the promoter areas of *NF-κB*, *TRAF1*, and *TRAF2* genes were obtained; then, utilizing MethPrime online software, unmethylated and methylated specific primers were designed at CpG sites of the promoter areas. Finally, through the Methylation-Specific PCR (MSP) methylation patterns were examined. The PCR reaction in each tube consisted of 1 µL of bisulfite-modified DNA in a final volume of 20 µL, 0.5 µL each of primer (10 mmol/L) were loaded to each AccuPower® HotStart PCR PreMix tube (Cat. No. k-5050, Bioneer Co., Daejeon, South Korea); and in order to yield a total volume of 20 µL to the reaction mixture, double distilled water (nuclease-free) was supplied to the mix.

The MSP reaction was set as first denaturation of 94 °C for 10 min, then 40 cycles (94 °C for 40 s, the annealing temperature for *NF-κB*: M = 60 °C, U = 62 °C. *TRAF1*: M = 55 °C, U = 57 °C, *TRAF2*: M = 61 °C, U = 52 °C, for 30 s and extension at 72 °C for 1 min). The last extension is terminated at 72 °C for 10 min. Note that in all reactions a positive control and a negative control were incorporated. Ethidium bromide staining was performed on the PCR products after loading them onto a 4% agarose gel. The sequences and annealing temperature of methylated and unmethylated specific primers are shown in Table 2.

3. Statistical analysis

Statistical analysis was conducted through SPSS version 26 (SPSS, Chicago, Illinois). The threshold tests were set at $p < 0.05$ for all statistics.

4. Results

Results of the genes methylation condition in individuals who have OSCC and the healthy group are shown in Table 3. Data analysis revealed that promoter methylation at *NF-κB* was not considerably altered in patients compared to healthy individuals (OR = 0.0, CI = 0.0, and $p = 0.999$); in addition, there was an absence of any variation in the *TRAF1* and *TRAF2* genes (OR = 0.333, CI = 0.024-4.567, and $p = 0.411$ for *TRAF1*; OR = 0.850, CI = 0.128-5.633, and $p = 0.866$ for *TRAF2*). Also, the relationship between the methylation status of *NF-κB*, *TRAF1*, and *TRAF2* genes and clinicopathological characteristics are mentioned in Tables 4, 5, and 6, respectively.

5. Discussion

Alteration in epigenetic regulation and consequently changing gene expression dictate Cancer-cell biology (Bates, 2020). Epigenetic alterations can be seen in nearly every cancer of humans. All these changes consist of DNA methylation, histone modifiers and readers, chromatin remodelers, micro RNAs, and related components of chromatin (Baylin & Jones, 2016).

Rearrangement, overexpression, and chromosomal amplification of genes coding for *Rel*/*NFκB* factors were seen in human hematopoietic and solid tumors (Rayet & Gélinas, 1999). TRAF proteins come together as signal transducers. *TRAF1* and *TRAF2* communicate together to form a complex with *TNFR2*, a common mediator for *NF-κB* transcriptional stimulation (C.-Y. Wang et al., 1998).

In this research, MS-PCR is used to describe the patterns of *NF-κB*, *TRAF1*, and *TRAF2* gene methylation in OSCC in the Southeastern Iranian community. Our result shows no considerable variation in methylation pattern within OSCC patients and healthy individuals for *NF-κB*, *TRAF1*, and *TRAF2* genes; similarly, the methylation status of these genes did not differ with clinicopathological conditions.

OSCC is the most common type of all oral cancers, and numerous oncogenes are associated with this disease. *NF-κB*, *TRAF1*, and *TRAF2* genes can be activated in several cancers; however, this study did not significantly alter the methylation patterns of the genes mentioned above.

In line with our results, Van Laere et al. revealed overexpression of the *NF-κB* gene in Inflammatory breast cancer (Van Laere et al., 2006). Also, Tang et al. have suggested overexpression of *NF-κB* p65 nuclear is an occurrence in the pathogenesis of lung cancer (Tang et al., 2006). Trauzold et al. revealed that *TRAF2* overexpression blocks the apoptosis induced by CD95 and invasiveness is also mediated by the death receptor (Trauzold et al., 2005). Zhang et al. proved that *TRAF1* expression did not have a

considerable prognostic value for glioblastoma (GBM). Conversely, increased expression of *TRAF2* may propose a poorer prognosis of GBM and be seen as an individual biomarker in glioblastoma prognosis (Zhang, Sun, Liu, & Li, 2017). Likewise, it demonstrated that gastric cancer cells strongly express *TRAF1* and *TRAF2* (Rossi, Contiero, Manoel-Caetano, Severino, & Silva, 2019; Wan et al., 2016). Another study suggested a novel role for *TRAF1* in BRAF/MEK/ERK signaling pathway in non-small cell lung cancer and suggested it could be a promising therapeutic strategy (Q. Wang et al., 2018).

6. Conclusion

Despite the pivotal contribution of *NF-κB* methylation in the advancement of various cancers, our study has shown that CpG sites found at the promoter of this gene in healthy and a individuals might have exhibited similar methylation patterns. *TRAF1* and *TRAF2* genes, which are involved in *NF-κB* signaling, have also displayed the same patterns. We sincerely recommend that more studies should be carried out to recognize the precise molecular process involved in OSCC through the employment of more advanced molecular techniques like microarray and methyl-seq in various and greater populations.

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Ethical Statement:

Informed Consent Forms were signed by all participants at the Zahedan University of Medical Sciences before they were permitted to participate in this study.

Conflict of interests

The authors declare that there is no conflict of interest.

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Tables

Table 1

Detailed demographic information about the patients and healthy groups.

Variables	Patient (n = 86)	Healthy (n = 68)	*p*-value [a]
Sex			0.205
Male	41 (47.67%)	27 (39.71%)	
Female	45 (52.33%)	41 (60.29%)	
Age groups			0.001
50>	27 (31.4%)	49 (56.98%)	
50<	59 (68.6%)	19 (43.02%)	
Cancer stage			-
I	19 (22.1%)		
II	17 (19.77%)		
Well-differentiated	37 (43.02%)		
Moderately	8 (9.30%)		
Metastasis	5 (8.81%)		

[a] Chi-square test
n: number

Table 2

Methylation and unmethylation primer sequences.

Primers	Sequences (5′–3′)	Annealing temperatures (°C)	Product size (bp)
***NF-κB* M**	F: GAAGTTAGAGTTTCGTAGGGGTC R: AACGAAAACGAAAAATAAAATCG	60	187
***NF-κB* U**	F: AGGAAGTTAGAGTTTTGTAGGGGTT R: ACAAAAACAAAAAATAAAATCACT	62	188
***TRAF1* M**	F: TTTTATATTTTATCGGAAGTTTCGG R: AAATAAACCCCTACGAAACCG	55	272
***TRAF1* U**	F: TTTTATATTTTATTGGAAGTTTTGG R: AAAAAATAAACCCCTACAAAACCA	57	275
***TRAF2* M**	F: TAAAAATATAAATATTAGTCGGGCGT R: TTTAAAACGAAAACTCACTCTATCG	61	166
***TRAF2* U**	F: ATTAAAAATATAAATATTAGTTGGGTGT R: TAAAACAAAAACTCACTCTATCACC	52	166

U: unmethylated, M: methylated, bp: base pair, R: reverse, F: forward

Table 3

Assessing the risk of OSCC based on gene promoter methylation status.

Gene	Methylation status	Patient (n = 86)	Healthy (n = 68)	Unadjusted			Adjusted [b]		
				OR	95% CI	*p*-value	OR	95% CI	*p*-value [a]
NF-κB	U (ref)	0	2	0.000	0.000	0.999	0.000	0.000	0.999
	M	86	66						
TRAF1	U (ref)	4	2	0.716	0.119-4.296	0.715	0.333	0.024-4.567	0.411
	M	82	66						
TRAF2	U (ref)	5	1	0.563	0.054-5.837	0.630	0.850	0.128-5.633	0.866
	M	81	67						

[a] Binary logistic regression analysis.
[b] Adjusted by age and sex status.
U: unmethylated, n: number, M: methylated, ref: reference, CI: confidence interval, OR: odds ratio

Table 4

The relationship between *NF-κB* gene methylation status and clinicopathological features in patient and healthy groups.

Characteristics	Patient (n = 86)			Healthy (n = 68)		
	Methylation status			Methylation status		
	M n (%)	U n (%)	*p*-value [a]	M n (%)	U n (%)	*p*-value
Age (year)			-			0.48
<50	27 (31.40%)	0 (0%)		48 (55.81%)	1 (1.47%)	
>50	59 (68.60%)	0 (0%)		18 (41.23%)	1 (1.47%)	
Sex			-			0.763
Male	41 (47.67%)	0 (0%)		26 (38.42%)	1 (1.47%)	
Female	45 (52.33%)	0 (0%)		40 (58.62%)	1 (1.47%)	
Cancer stage			-			
I	19 (22.1%)	0 (0%)				
II	17 (19.77%)	0 (0%)				
Well-differentiated	37 (43.02%)	0 (0%)				
Moderately	8 (9.30%)	0 (0%)				
Metastatic	5 (5.81%)	0 (0%)				

[a] Chi-square test

U: unmethylated, n: number, M: methylated

Table 5

The relationship between *TRAF1* gene methylation status and clinicopathological features in patient and healthy groups.

Characteristics	Patient (n = 86)			Healthy (n = 68)		
	Methylation status			Methylation status		
	M n (%)	U n (%)	*p*-value [a]	M n (%)	U n (%)	*p*-value
Age (year)			0.778			0.48
<50	26 (30.23%)	1 (1.17%)		48 (70.59%)	1 (1.47%)	
>50	56 (65.11%)	3 (3.49%)		18 (26.47%)	1 (1.47%)	
Sex			0.924			0.763
Male	39 (45.34%)	2 (2.33%)		26 (38.24%)	1 (1.47%)	
Female	43 (0.5%)	2 (2.33%)		40 (58.82%)	1 (1.47%)	
Cancer stage			0.471			
I	19 (22.1%)	0 (0%)				
II	15 (17.43%)	2 (2.33%)				
Well-differentiated	35 (40.70%)	2 (2.33%)				
Moderately	8 (9.30%)	0 (0%)				
Metastatic	5 (5.81%)	0 (0%)				

[a] Chi-square test

U: unmethylated, n: number, M: methylated

Table 6

The relationship between *TRAF2* gene methylation status and clinicopathological features in case and control groups.

Characteristics	Patient (n = 86)			Healthy (n = 68)		
	Methylation status			Methylation status		
	M n (%)	U n (%)	*p*-value [a]	M n (%)	U n (%)	*p*-value
Age (year)			0.572			0.530
<50	26 (30.23%)	1 (1.17%)		48 (70.59%)	1 (1.47%)	
>50	55 (63.95%)	4 (4.65%)		19 (27.94)	0 (0%)	
Sex			0.723			0.214
Male	39 (45.34%)	2 (2.33%)		26 (38.24%)	1 (1.47%)	
Female	42 (48.84%)	3 (3.49%)		39 (57.35%)	2 (2.94%)	
Cancer stage			0.454			
I	19 (22.1%)	0 (0%)				
II	16 (18.60%)	1 (1.17%)				
Well-differentiated	33 (38.37%)	4 (4.65%)				
Moderately	8 (9.30%)	0 (0%)				
Metastatic	5 (5.81%)	0 (0%)				

[a] Chi-square test

U: unmethylated, n: number, M: methylated