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Investigation of the *NF-κB*, *TRAF1*, and *TRAF2* Relative Gene Expressions in a Population of Iranian Patients with Oral Squamous Cell Carcinoma: A Case-Control Study

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A B S T R A C T

Objective: Oral squamous cell carcinoma (OSCC) is a frequently prevalent subtype of oral cancer with late detection and diagnosis and an elevated mortality rate. It can be triggered via genetic and environmental factors, creating several patient difficulties. The main concern is to comprehend the interaction between variables to explain the regulation of oral cancer, as well as the process of its occurrence. Nuclear factor kappa B (*NF-κB*) belongs to a class of induced transcription factors, controlling various genes responsible for several aspects of inflammation and immunological processes. It is regulated through diverse genes, such as TNF receptor-associated factor 1 (*TRAF1*) and TNF receptor-associated factor 2 (*TRAF2*). The disruption of this pathway may contribute to various health problems. Materials and Methods: Eighteen paraffin-embedded oral cancer tissues and 18 oral mucosae tissues were applied to evaluate gene expression profiles via quantitative real-time PCR (qRT-PCR) in a population in Southeast Iran. Results: *NF-κB* (95% CI = -0.804 to -0.222, *p*-value = 0.001), *TRAF1* (95% CI = -3.201 to -1.018, *p*-value < 0.0001), and *TRAF2* (95% CI = -1.802 to -0.86, *p*-value = 0.019) gene expressions were revealed a strong correlation with OSCC. Conclusion: Our findings revealed that the increased gene expression levels of *NF-κB*, *TRAF1*, and *TRAF2* are closely linked to clinical manifestations in OSCC.

Introduction

There are nearly 50% of fatalities from oral cancer worldwide, making it one of the top cancers (Li et al., 2020). GLOBOCAN estimated that there were approximately 378 thousand new cases of lip and oral cavity cancer in 2020, with 178 thousand new deaths (Sung et al., 2021). On a global scale, the highest rates of oral cancer are found in countries

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that are located in South Asia (Warnakulasuriya, 2010). It is estimated that roughly 75% of all oral cancers originate from the consumption of smoking and alcohol (Ogden, 2005). In oral cancer, the male-to-female ratio (m/f) varies from 1.45 to 10.5 depending on geographical area and age group; however, the impact is more noticeable in men than women. For example, the m/f ratio of oral cancer in Iran and Taiwan was 1.9 and 10.5, respectively. This ratio was reversed in Thailand (1:1.56). About half of oral cancers manifest on the tongue in Asian, North American, and European populations (Kujan, 2017). There is also evidence that the tongue is the most commonly encountered site of oral cancer in Iran (Maleki et al., 2015). In Iran, oral cancer has a high economic impact (Rezapour et al., 2018). In 2018, oral cancer accounted for $64,245,173, the majority of which was due to loss of productivity (50%) due to the disease (Rezapour et al., 2018).

Various studies have demonstrated that ncRNAs (e.g., lncRNAs, snoRNAs, circRNAs, and miRNAs) can serve as prospective biomarkers for the preliminary diagnosis and tracking of oral cancer as well as promising therapeutic purposes for current and future treatments. As a result of large heterogeneity among tumors, as well as the omnipresent nature of ncRNA expression, it may not always be sufficient to determine cancer status with a single ncRNA (Balakittnen et al., 2022).

In principle, epigenetic modifications are heritable and can be reversed changes in gene expression that are not attributed directly to changes in the sequence of DNA bases but instead results from hereditary processes. Therefore, epigenetic mechanisms change the phenotype without interference in DNA sequences. Among epigenetic processes, methylation of DNA is considered one, histones are considered another, and non-coding RNAs are considered to influence chromatin structure. Other factors, including diet, alcohol, tobacco, toxins, or pharmaceuticals, can influence epigenetic patterns. Evidence suggests that disruptions in epigenetic mechanisms play a crucial role in developing many types of neoplasms (Li et al., 2020).

In order for OSCC to progress in a multistep manner, there must be an accumulation of mutations that occur both genetically and epigenetically in various genes, either oncogenes or tumor suppressor genes. These lead to dysregulation of the cell cycle and the inhibition of growth suppressors (Li et al., 2020).

A screening test does not serve as a diagnostic tool. However, this helps identify cases containing anomalous oral lesions and facilitates consultation with a specialist to determine specific diagnostic approaches (including follow-up and, if required, histopathological diagnostic testing and tissue biopsy). Choosing the best screening program that suits a particular population is critical based on disease frequency, funding, and the country's health care system (Warnakulasuriya & Kerr, 2021). Dental professionals must actively participate in prospective screening, especially in developed countries such as the United States (Psoter et al., 2019; Warnakulasuriya & Kerr, 2021). Primary healthcare employees are often employed in widespread oral cancer screening programs in developing countries because of the comparatively insufficient number of healthcare professionals (Warnakulasuriya & Kerr, 2021).

The NF-κB family of transcription regulatory proteins comprises five subfamily members: *NF-κB1* (p50), *NF-κB2* (p52), *RelA* (p65), *RelB*, and *c-Rel*, which all contain an N-terminal REL homology domain critical to nuclear localization. *NF-κB* (*NF-κB1*) gene acts homodimerically or heterodimerically using at least one component that constitutes the *NF-κB* family, and a series of chromatin binding, nuclear translocation, post-translational modification, and stimulus-dependent inhibitor degradation activate it (Ernst, Vayttaden, & Fraser, 2018). According to the NCBI database, the nuclear factor kappa B subunit 1 *(NFKB1)* gene, known as *NF-κB,* contains 27 exons and is located on 4q24; its DNA sequence consists of 115944 base pairs, 4055 base pairs make up its mRNA, and 969 amino acids make up its protein, TNF receptor-associated factor 1 (*TRAF1*) is mapped on 9q33.2 and has ten exons; TNF receptor-associated factor 2 (*TRAF2*) is located on 9q34.3 and contains 17 exons (Sayers et al., 2021). *NF-κB* is present exclusively in cells capable of transcribing immunoglobulin light chain genes, interacting at a particular location containing the enhancer of immunoglobulin kappa (Sen & Baltimore, 1986). Many triggers, including infections, therapy, and environmental factors, initiate inflammation, resulting in tumor proliferation, angiogenesis, and invasion. Bacterial infection promotes the initiation and progression of

cancerous processes, and *NF-κB* activation contributes to this process (Chattopadhyay, Verma, & Panda, 2019).

TRAF2 is expressed most frequently in head and neck squamous cell carcinoma (HNSCC), accompanied by cervical cancer out of all the cancer types that were sampled (17 cancer types), based on The Human Protein Atlas (THPA). There is a direct interaction between *TRAF2* and TNF receptors as well as a complex formed with another member of the TRAF family, *TRAF1*, which is similarly overexpressed in human papillomavirus positive (HPVP) HNSCC. These factors are required for *TNFα*-mediated activation of *NF-κB* and *MAPK8/JNK*, both of which play a role in cell survival (Hinić et al., 2022).

Our study was designed to assess how *NF-κB*, *TRAF1*, and *TRAF2* gene expression status affect OSCC risk.

Material and Methods

Sampling

Eighteen formalin-fixed paraffin-embedded (FFPE) samples of OSCC (as the case group) and 18 biopsy samples of the healthy oral mucosa (as the control group) from the patients assigned to Zahedan University of Medical Sciences were obtained after diagnostic and pathological evaluations. Sampling and experimental testing were performed between 2013 and 2014. These groups were characterized according to their demographic and clinicopathological characteristics, as shown in **Table 1**.

RNA extraction and cDNA synthesis

Total RNA extracting was done on 18 paraffin-embedded OSCC tissues using High Pure FFPE RNA Micro Kit (Cat no .04823125001, Roche, Germany) and 18 normal mucosae tissues using Cinna Pure RNA Purification Kit (Cat no . PR891620, Sinaclon, Iran), according to their manufacturers' instruction. An amount of 0.3 uL of total RNA samples was loaded into the chipcuvette® of ScanDrop® 250 (Analytik Jena, Germany) device to evaluate their quantity and their purity; their integrity was also verified through horizontal gel electrophoresis containing 1% agarose. Afterward, RevertAid First Strand cDNA Synthesis Kit (Cat no .K1621, Fermentas, USA) was employed to reverse-transcribe 1 µg of RNA in a final volume of 20 µL.

Table 1 - The demographic characteristics and clinicopathological features of the cases and controls.

Criteria	Cases (N=18)	Controls (N=18)	*p*-value [a]
Sex			0.738
Male	8 (44.44%)	9 (50%)	
Female	10 (55.56%)	9 (50%)	
Age (year)	52.89 ± 9.43	40.50 ± 8.52	0.424
Age groups			0.008*
<45	5	13	
≥45	13	5	
Histological grade			-
Grade I	5	-	
Grade II	3	-	
Well-differentiated	6	-	
Moderately	2	-	
Metastatic	2	-	

[a]. Chi-Square test, N. Number, *. statistically significant at $p < 0.05$

quantitative Real-Time PCR

RNA 18S gene (Saberi et al., 2014) was used as an internal control for the normalization of the expression of all genes. The following conditions were applied to all qRT-PCR experiments: the initial denaturation at 95 °C for 10 min; a series of 40 cycles at 95 °C for 15 s, the annealing temperature of primers (mentioned in **Table 2**) for 30 s; and in the end, the melting curve was measured over a range of 58 to 95 °C. *NF-κB*, *TRAF1*, and *TRAF2* qPCR primers were designed using the Primer3 web tool (https://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Koressaar & Remm, 2007).

Table 2 - The primers were used for gene expression.

Gene	Sequences (5′-3′)	Annealing (°C)	Amplicon (bp)
NF-κB	F: AATTAACGGCGACAATCTGGAA	58	205
	R: ACTTCACAAGCATAGCCATCAG		
TRAF1	F: CCGGAACAAGGTCACCTTCATGC	62	133
	R: TGGGCATCCACTGGCCACG		
TRAF2	F: GGCCCTTCAACCAGAAGGTGACC	62	126
	R: CGATGTTCATGTCGTTGACTGGC		
RNA 18S	F: GTAACCCGTTGAACCCCATT	60	112
	R: CCATCCAATCGGTAGTAGCG		

bp. base pair, R. Reverse, F. Forward.

Statistical analysis

The analysis of the demographic data was carried out using the Chi-Squared test. One-Sample Kolmogorov-Smirnov test was used to determine the status of normal distribution for each gene. The Independents-Samples T-test or the Mann-Whitney U-test was conducted to evaluate the relative gene expression between OSCC and healthy groups. Pearson's or Spearman's correlation coefficient tests assessed the correlation between genes and clinicopathological characteristics. SPSS version 26.0 (SPSS Inc, USA) software was utilized for all statistical analysis; a statistical significance threshold of $p < 0.05$ was applied to examine significant values. Furthermore, GraphPad Prism version 9.5.0 software (GraphPad Software, USA) was employed for illustrating **Fig. 1**.

Results

Table 3 shows a comparison of gene expression levels between case and control groups. Correlations of gene and clinicodemographic characteristics between OSCC and healthy individuals are shown for each gene in **Table 4**.

The relative gene expression is shown in **Fig. 1** for patients and healthy individuals (panel 'A' for *NF-κB*, panel 'B' for *TRAF1*, and panel 'C' for *TRAF2*).

Discussion

The present study applied the qRT-PCR assay to assess the *NF-κB*, *TRAF1*, and *TRAF2* relative gene expression profiles in patients affected by OSCC and the healthy group. The results in **Table 3** indicated a significant difference in the expression status of these three genes between cases and healthy controls. According to **Table 4**, most differences are due to the advanced histological grade of patients, which indicates that these genes might potentially impact the aggressive aspect of OSCC (except for *NF-κB*). The correlation between age and age groups with the genes was seen only in *TRAF1*. Furthermore, there appears to be no correlation between the effect of sex in genes.

Several scholarly papers have been published in recent years concerning the regulation of *NF-κB* and its molecular effects. A recent study by Hou *et al.* (2022) showed that the sphingolipid metabolic pathway was profoundly activated in OSCC. Its vital enzyme, *SPHK1*, was dramatically overexpressed in OSCC tissues. This overexpression was positively associated with advanced neoplasms and poor OSCC outcomes. This gene targets *NF-κB* by stimulating p65 expression to control the progression of OSCC tumors and may provide novel therapeutic approaches to identify and combat oral malignancies (Hou et al., 2022). According to the findings of Quintana *et al.* (2013) study, the expression of the *NF-κB* gene is considered to serve as a radiosensitivity biomarker in head and neck squamous cell carcinoma (HNSCC) patients. Increased expression of the *NF-κB* correlates significantly with a decrease in local control (Balermpas et al., 2013). The study conducted by Wen *et al.* (2022) demonstrated that orally administered sesamin significantly prevented the growth of tumors or esophageal squamous cell carcinoma (ESCCs) in nude mice.

Table 3 - Relative gene expression status in case and control groups.

Gene	Groups	N	Expression mean ± SD	Expression range	95% CI	*p*-value
NF-κB	Cases	18	1.545 ± 0.549	0.639-2.503	-0.804 to -0.222 [a]	0.001[a, *]
	Controls	18	1.032 ± 0.260	0.613-1.389		
TRAF1	Cases	18	3.563 ± 2.250	0.859-8.701	-3.201 to -1.018 [c]	< 0.0001[b, *]
	Controls	18	1.040 ± 0.260	0.387-1.465		
TRAF2	Cases	18	2.490 ± 2.168	0.519-9.347	-1.802 to -0.86 [c]	0.019 [b, *]
	Controls	18	1.090 ± 0.478	0.592-2.165		

N. Number, SD. Standard Deviation, [a]. Independents-Samples T-test, [b]. Mann–Whitney U-test, [c]. obtained using Hodges-Lehmann estimate, *. statistically significant at $p < 0.05$

Table 4 - Gene-clinicodemographic characteristics correlation between OSCC and healthy subjects.

Genes	Correlation	Sex	Age	Age groups	Groups	Histological grades
NF-κB	Pearson's Correlation Coefficient	-0.128	0.198	0.208	0.524**	0.438
	p-value	0.457	0.246	0.224	0.001	0.069
	N	36	36	36	36	18
TRAF1	Spearman's Correlation Coefficient	0.019	0.394*	0.342*	0.781**	0.616**
	p-value	0.914	0.017	0.041	< 0.0001	0.006
	N	36	36	36	36	18
TRAF2	Spearman's Correlation Coefficient	0.099	0.154	0.235	0.396*	0.483*
	p-value	0.565	0.370	0.167	0.017	0.042
	N	36	36	36	36	18

N. number, *. Correlation is significant at the 0.05 level (2-tailed), **. Correlation is significant at the 0.01 level (2-tailed).

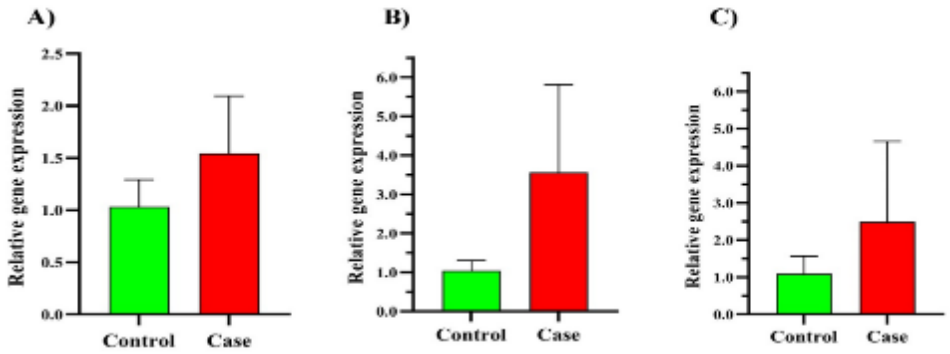

Fig 1 - These bar plots illustrate the mean of expressions and their standard deviation for *NF-κB* (panel A), *TRAF1* (panel B), and *TRAF2* (panel C) genes in case and control groups.

According to their study, sesamin provides anti-tumor activity in ESCC by blocking the *NF-κB* pathway, suggesting its promising application for treating ESCC (Wen et al., 2022). A study by Han *et al.* (2022) aimed to characterize the role of the *NF-κB* gene on mir488 in pancreatic cancer, which showed that *NF-κB* transcriptionally restricts mir-488 expression by binding to its promoter region. Consequently, mir-488 cannot bind to the 3′-UTR of the *ERBB2* gene and interfere with it; therefore, it affects the downstream genes of *ERBB2*, impacting apoptosis and cell cycle progression. A decrease in inhibitory effects was observed after the application of anti-*NF-κB* agents (Han et al., 2022). A study by Xiao *et al.* (2020) on chronic lymphocytic leukemia (CLL) showed that the expression of *TRAF1* and HuR (an RNA-binding protein) was considerably higher in LCLs than it was in B lymphocytes. Upon inhibition of HuR, the expression of *NF-κB*-inducing kinase (*NIK*), inhibitor NF-κB kinase α (*IKK-α*), and *TRAF1* is considerably elevated in both B lymphocytes and LCLs. Furthermore, suppression of HuR function can enhance CLL cells' drug resistance and regulate the inflammatory response and apoptosis (Xiao et al., 2020). *NF-κB* participates in addiction by inducing the expression of a broad spectrum of target genes along with various inflammatory factors potentially involved with the development of addictions, particularly neuropeptides and opioid receptors (Nennig & Schank, 2017). Sodium salicylate and aspirin have been demonstrated to interfere with *NF-κB* through the inhibition of the degradation of IκBα and consequently neutralizing *NF-κB* (Yu et al., 2020). An increased expression of *TRAF2* reducing and its suppression elevating the homeodomain interacting protein kinase 2 (*HIPK2*) protein level. Protease inhibitors prevented *TRAF2*-mediated reductions in *HIPK2* protein expression. A further effect of *TRAF2* was a shortening in *HIPK2*'s protein half-life (Lee et al., 2022).

Conclusion

Collectively, our findings indicate that gene expression of *NF-κB* and its related genes (*TRAF1* and *TRAF2*) participate in OSCC pathogenesis, as reported in previous studies (mentioned in the discussion section). Future studies should apply alternative inclusion criteria and enlarge the population size to improve their quality. It will also be necessary to investigate other genes and signaling pathways that may be related directly/indirectly to OSCC.

Ethical consideration

All participants signed consent forms indicating their agreement to participate in the study.

Conflict of interest

The authors declare no conflict of interest.

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