

# Association between Tissue Expression of Toll-Like Receptor and Some Clinicopathological Indices in Oral Squamous Cell Carcinoma

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## Abstract

**Background:** The oral squamous cell carcinoma (OSCC) composes about 90% of all head and neck cancers. The toll-like receptor (TLR)<sup>+</sup> immune cells have potential of invasion and malignancy transformation. The aim of this study was assessment of possible associations between clinicopathological indices and TLR2 and TLR9 gene expression in OSCC.

**Methods:** Forty-two OSCC samples with related healthy margins including 25 early and 17 advanced stages were gathered. The samples were classified histologically from grade I to II. The expression of TLR2 and TLR9 was evaluated by Real-time PCR. The patient's disease-free survival (DFS) and overall survival (OS) were analyzed using SPSS V.23 software.

**Results:** The expression of TLR2 and TLR9 genes in tumor tissues (especially in grade I and II) were higher than healthy surgical margin tissue ( $p < 0.001$ ). TLR9 expression in grade II was statistically significant than grade I in tumor tissue ( $p < 0.001$ ). TLR9 expression in advanced stage was statistically significant in compare to early stage ( $p = 0.012$ ). In advanced stage both overall survival ( $p = 0.029$ ) and disease-free survival ( $p = 0.012$ ) were statistically lower than early stage. The follow-up time to recurrence in advanced stage was statistically lower than early stage ( $p = 0.007$ ).

**Conclusions:** Overexpression of TLRs 2, 9 play role in the pathogenesis and tumor development of OSCC and can be applied as biomarker in prognostic approaches.

**Keywords:** Disease-free survival, Oral squamous cell carcinoma, Overall survival, TLR2, TLR9.

## Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the 7th most common malignancy with high mortality rates that constitutes approximately 10% of all the cancers. The oral squamous cell carcinoma (OSCC) composes about 90% of all head and neck cancers (1). Smoking, radiation, and viral infection are known as the main risk factors for OSCC

pathogenesis. In addition to main causes involved in etiopathogenesis of OSCC that mentioned above, recent studies highlighted the pivotal role of tumor microenvironment in cancer development or tumor cell apoptosis. It is demonstrated surrounded tissue matrix and immune cells that infiltrate to tumor microenvironment (TME) affect tumor

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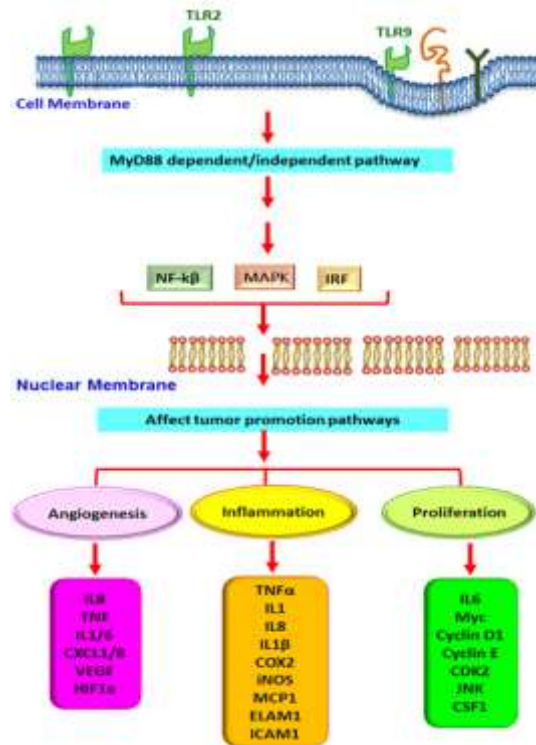
progression, stages of carcinogenesis and survival rate (2, 3).

Toll-like receptors (TLRs) are cell surface or intracellular transmembrane proteins that represent on both immune cells including macrophages and DCs and also non-immune cells like keratinocytes of skin and oral mucosa. The TLRs detected exogenous agents by pathogen-associated molecular patterns (PAMPs). They able to regulate innate and adaptive immune responses by activation of NF- $\kappa$ B transcription factor that released inflammatory and anti-inflammatory cytokine including tumor necrosis factor (TNF)- $\alpha$ , IL-6, and IL-1. Moreover, TLRs detected endogenous agents such as damaged and dying cells by damage/danger-associated molecular patterns (DAMPs) (4). It was demonstrated TME TLRs<sup>+</sup> immune cells interact with DAMPs -as ligand- and stimulated cytokine production and T-cell activation. The tumor cells that liberated DAMPs have potential of invasion and malignancy transformation (5).

Recent studies reported that TLR 2 and TLR9 increase expression of Cyclin D1 and IL6 and tumor proliferation in gastric, colon, prostate and breast cancers (6). So, they can

boost the migration and invasion of tumor cells by upregulating of MMP enzymes such as MMP2 and 9 (Fig. 1) (7, 8). On the other hand, some other studies demonstrated TLRs can restrict tumorigenesis process. They enhance the antitumor response by triggering the maturation of APCs and prohibiting the tumor growth by apoptosis induction (9). It is found that lack of TLR9 expression can be associated with higher stages, poor differentiation, and poor prognosis in patients with mucoepidermoid carcinoma. In this way, TLRs act as double-edged sword (10).

Conventional therapeutic approaches of OSCC patients showed low five-year survival rate. Moreover, one of the main causes of 50% failure in OSCC patient's therapy is late diagnosis that appears local recurrence or metastasis to the lymph nodes. Therefore, early diagnosis of the patients in the early stages is recommended and it can provide by reliable biomarkers (11). The aim of this study was evaluation of the role of TLR2 and TLR9 in biological behavior and pathogenesis of OSCC patients in compare to healthy margin for assessment of possible associations between TLR2 and TLR9 gene expression and survival rate.



**Fig.1.** The activation of TLRs 2, 9 affect some tumor promotion pathways such as angiogenesis, inflammation, and proliferation.

## Materials and Methods

### Study population

The Mashhad University Ethics committee confirming approval of the research (IR.MUMS.DENTISTRY.REC.1396.046). The informed consent form was obtained from all OSCC patients before biopsy. Finally, 42 OSCC tissue samples selected for molecular assessment in this study.

Forty-two OSCC tissue samples embed in paraffin-blocks that collected from Omid, Imam Reza, and Ghaem Hospitals and Department of Oral and Maxillofacial Pathology, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran. Radical surgery of target tissues in OSCC patients applied to consider surgical healthy margins as normal specimens (12). The inclusion criteria included whose OSCC patients without any medical history of previous malignancy, autoimmune disease, anti-tumoral drugs consumption, radiotherapy, or chemotherapy, and also samples with sufficient amounts of tissues for molecular tests with no necrosis, and the patients with a complete demographic recording profile regards the tumor staging.

### Sample collection

All demographic information of participants was registered and physical examination was done. The tissue biopsies fixed in 10% formalin were embedded in paraffin. The clinical stage, histological grade, age, sex, and the location of the lesions were recorded according to the patients' profiles analysis of survival rate.

### Real time PCR

The total RNA was extracted from paraffin blocks. After deparaffinization by Xylene and alcohol, the total RNA was extracted by High Pure RNA Paraffin Kit, FFPET RNA Tissue (Roche, Germany). The quantification of extracted RNA was assessed by Nanodrop system (Thermo Scientific 2000, USA) following absorbance ratio of 260 nm/280nm wavelengths optical density. The RNA

qualification was observed by electrophoresis of 2% agarose gel. The complementary DNA (cDNA) was immediately made by Revert Aid First Strand cDNA (Thermos Scientific, Germany) kit as manufacture instruction: 65 °C for 5 minutes using Oligo dT in total 20 µl volume reactions. Quantitative real-time PCR was applied by SYBR Green master mix (Thermos Scientific, Germany) on ABI system (One Step, USA). The total volume of real-time PCR reaction was 20 µl including 10 µl SYBR Green, 0.5 µl of each primer (10 pM), 2 µl of cDNA template, 0.5 µl ROX stain, and 6.5 µl distilled sterile water. The PCR condition was 10 min at 95 °C pre-incubation, and then 15 sec at 95 °C, keeping 61 °C for 30 sec, and then maintaining 72 °C for 30 sec for 40 cycles in thermo cycler. The sequence of target genes (TLR2 and TLR9) and house-keeping gene ( $\beta$ -actin) included were as following:

TLR2	Forward:	5'
	GGCCAGCAAATTACCTGTGT 3',	
TLR2	Reverse:	5'
	TTCTCCACCCAGTAGGCATC 3',	
TLR9	Forward:	5'
	CACCCTCAACTTCACCTTGGA 3',	
TLR9	Reverse:	5'
	TGCACGGTCACCAGGTTGT 3',	
$\beta$ -act	Forward:	5' AGCGGGAAATCGTGCGTG 3' and
$\beta$ -act	Reverse:	5' GGGTACATGGTGGTGCCG 3'.

The quantification of gene expression was evaluated by  $\Delta\Delta CT$  method (13). The differential expression of TLR2 and TLR9 were assessed in compare to  $\beta$ -act as reference gene. More therefore, the melting curve was analysis to confirm the specificity of PCR products.

### Statistical analysis

Data analysis was performed by SPSS software (version 23). The association between TLR2 and TLR9 gene expressions were determined by Chi-squared, Spearman's, and Fisher's exact test. The P-value less than 0.05 was considered statistically significant for all statistical analysis.

## Results

In this study 42 OSCC patients participated including 26 men (61.9%) and 16 women (38.1%) with 57.3±15.6 years mean age (±standard deviation) between the age ranges of 27-82 year. The pathologic stage classification was based on previous studies (12, 14). The grade (I or II), stage (early or advanced), the mean time of recurrence and overall survival (OS) were mentioned in Table 1. The OS refers to long term of diagnosis date to up to the time of death and always is stated as a five-year survival rate. The disease-free survival (DFS) is defined as length of time between primary treatment to tumor recurrence or death. In advanced stage, both OS and DFS were statistically lower than early stage ( $p=0.029$  and  $p=0.012$ , respectively)

(Table 2). Both OS and DFS were lower in well differentiated grade in compare to moderate differentiated grade, but there was not statistically significant difference. The DFS in no TLR2 expression group in compare to expression group demonstrated statistically significant difference ( $p=0.044$ ). There was not statistically significant difference for the other parameters.

The median time of follow up was 24 months with range of 6 to 126 months. Thirty-one patients (73.8%) were alive until the end of this study. The median time of follow up to recurrence was 17 months with range of 2 to 126 months. It was not happened recurrence for 35 (73.8%) patients until the end of the study.

**Table 1.** Clinical characteristics of OSCC patients participated in this study.

Variable	Mean±SD or n (%)	
Age (year)	57.3±15.6	
Gender	Male	26 (61.9)
	female	16 (38.1)
Clinical Stage	Early	25 (59.5)
	Advanced	17 (40.5)
Histological grade	Well-differentiated	21 (50)
	Moderate differentiated	21 (50)
Number of recurrences	7 (16.7)	
Number of deaths	11 (26.2)	
Time of recurrence (year)	31.1±29.3	
Time of survival (year)	35.4±29.1	

**Table 2.** Prognostic factors for DFS and OS in OSCC patients.

Variable	Number of patients	OS N (%)	p-value	DFS N (%)	p-value	
Gender	Male	26	76.9	0.720	79.9	0.222
	female	16	68.8		93.8	
Clinical Stage	Early	25	88.0	0.029	96.0	0.012
	Advanced	17	52.9		64.7	
Histological grade	Well-differentiated	21	66.7	0.292	81.0	>0.99
	Moderate differentiated	21	81.0		85.7	
TLR2	No expression	4	50.0	0.079	50.0	0.044
	expression	38	76.3		86.8	
TLR9	No expression	21	71.4	0.712	81.0	0.707
	expression	21	76.2		85.7	

Oral squamous cell carcinoma; OSCC, Disease-free survival; DFS, Overall survival; OS, Toll-like receptor 2; TLR2, TLR9; Toll-like receptor 9.

The median time of OS was 42 months with range of 6 to 126 months in early stage and 12 months with 6 to 71 months range in advanced stage. Twenty-two (88%) patients with early stage and 9 (52.9%) patients with advanced stage were alive until the end of the study. The follow-up time to recurrence was 42 months with 2 to 126 months range in early stage and 12 months with 2 to 61 months range in advanced stage. There was not happened recurrence for 24 (96%) early-stage patients and 11 (64.7%) advanced stage cases until the end of the study. The follow-up time to recurrence in advanced stage was statistically lower than early stage ( $p=0.007$ ).

The median time of OS in no TLR2 expression group was 11.5 months with 6 to 71 months range and in TLR2 expression group was 33 months with 6 to 126 months range. Both groups were not demonstrated statistically significant difference to each other in OS ( $p=0.079$ ). The median follow-up time to recurrence was 9 months with 3 to 17

months range in no TLR2 expression group and also 24 months with 2 to 126 months range in TLR2 expression group. Both groups showed statistically significant difference each other in follow-up time to recurrence ( $p=0.044$ ).

The median time of OS in no TLR9 expression group was 24 months with 6 to 126 months range and in TLR9 expression group was 24 months with 6 to 97 months range. Both groups were not demonstrated statistically significant difference to each other in OS ( $p=0.712$ ). The median follow-up time to recurrence was 17 months with 2 to 126 months range in no TLR9 expression group and also 17 months with 2 to 97 months range in TLR9 expression group. Both groups were not statistically significant difference each other in follow-up time to recurrence ( $p=0.707$ ).

The expression of TLR2 and TLR9 genes in tumor tissue were statistically significant than healthy margin tissue ( $p<0.001$  for each one) (Table 3).

**Table 3.** The relation of TLR2 and TLR9 gene expressions between and within grade and two groups.

Gene	Grade	N	Tumor	Healthy margin	p-value	Tumor	Healthy margin
			Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)
TLR2	I	21	5.89 (4.80)	0.78 (1.16)	<0.001	6.10 (4.75)	0.58 (1.12)
	II	21	6.55 (5.81)	0.45 (1.19)	<0.001		
<b>p-value</b>			p=0.497	p=0.801		<0.001	
TLR9	I	21	1.78 (2.65)	0.51 (0.62)	0.021	2.49 (2.96)	0.29 (0.55)
	II	21	3.73 (2.48)	0.28 (0.47)	<0.001		
<b>p-value</b>			p<0.001	p=0.606		<0.001	

Toll-like receptor 2; TLR2, TLR9; Toll-like receptor 9, Interquartile range; IQR.

As mentioned in Table 3, the overexpression of TLR2 in grade I and II tumor-tissue were statistically significant than healthy margin tissue ( $p<0.001$  for each one). The overexpression of TLR9 in grade I and II tumor-tissue were statistically significant than healthy margin tissue ( $p<0.021$  and  $p<0.001$ , respectively). The expression of TLR9 in grade II was statistically significant than grade I in tumor tissue ( $p<0.001$ ). The expression of TLR2 in both healthy margins and tumor tissues was not statistically significant between

stage 1 and 2 ( $p=0.317$  and  $p=0.990$ , respectively). The expression of TLR9 in stage 2 was statistically significant in compare to stage 1 in tumor tissue ( $p=0.012$ ) (Table 4). There was not statistically significant association between TLR2 and TLR9 expressions in tumor tissue ( $r=0.188$ ,  $p=0.232$ ,  $N=42$ ), (Fig. 2).

As mentioned in Table 5, there was not statistically significant difference in expression of both TLR2 and TLR9 genes between men and women in tumor tissue ( $p=$

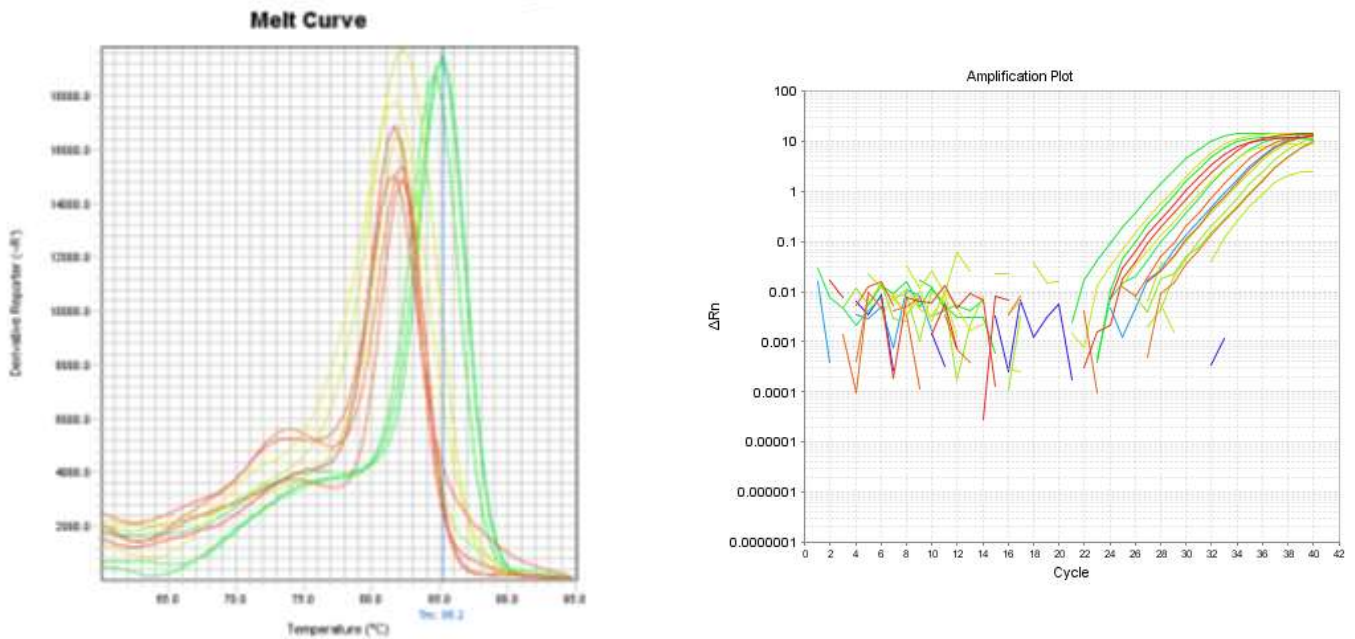
0.126 and  $p= 0.445$  respectively). On the other hand, the expression of both TLR2 and TLR9 genes showed statistically significant

difference in tumor tissue in compare to healthy margin in men and women ( $p< 0.001$  for each one).

**Table 4.** The relation of TLR2 and TLR9 gene expressions between stages.

Gene	Stage	N	Tumor
			Median (IQR)
TLR2	1	25	5.69 (4.68)
	2	17	6.55 (6.90)
p-value			0.990
TLR9	1	25	2.12 (2.86)
	2	17	3.79 (3.44)
p-value			0.012

Toll-like receptor 2; TLR2, TLR9; Toll-like receptor 9, Interquartile range; IQR.



**Fig. 2.** The melting curve and amplification plot of TLR2 (Red), TLR9 (Green) and GAPDH (Yellow) gene expressions in real-time PCR.

Only TLR2 gene expression was directly correlated to age in both tumor and healthy

margin tissues ( $p= 0.041$  and  $p= 0.028$ , respectively).

**Table 5.** The relation of TLR2 and TLR9 gene expressions between and within stage and gender.

Gene		N	Healthy margin tissue	Tumor tissue	p-value
TLR2	male	26	0.39 (0.90)	5.85 (5.21)	<0.001
	female	16	1.16 (1.55)	6.83 (9.23)	<0.001
	p-value		0.085	0.126	
TLR9	male	26	0.24 (0.39)	2.34 (4.10)	<0.001
	female	16	0.65 (1.13)	3.38 (2.33)	<0.001
	p-value		0.004	0.445	

Toll-like receptor 2; TLR2, TLR9; Toll-like receptor 9.

## Discussion

Our results demonstrated both TLR2 and TLR9 genes overexpressed in tumor tissues in compared to healthy margins. Both genes were more expressed in grade I, II and also stage 1, 2 than healthy margins. Our data approved overexpression of TLR2 and 9 can promote tumorigenic process and development of OSCC. Outcome of present research highlighted the effect of clinical stage on overall survival. Besides, analysis of DFS illustrated advanced stage increase potential of recurrence and rate of patient's expiration, so the follow up time were reduced.

Recent studies demonstrated endogenous factors like some molecular pathways involved in development and progression of OSCC (12, 15). The pathways that mediated by TLR play role in progression and suppression of OSCC. For instance, TLRs showed pro-apoptotic activity that present anti-tumor effects. Moreover, they can also stimulate tumor development, metastasis and progression following inflammation, proliferation and angiogenesis. In this way, genetically changes and alternation in expression of TLRs influence OSCC pathogenesis (6). The TLRs active innate immune response and they act as key regulator during inflammation process (16). The result of microarray analysis illustrated TLR9 expression impact cell cycle by regulating p16 protein, this post translational modification induces carcinogenesis (17). Moreover, some polymorphism in TLR 2 gene that affect

inflammation process following bacterial infection can increase risk of OSCC (18). It was reported a SNP on TLR9 (rs5743836) promoter area led to TLR9 overexpression that associated with HBeAg seroconversion (19, 20). In addition, some polymorphism in TLR genotype is proposed to apply as potential prognostic biomarker (21), like the other previous studies that proposed alternation in some gene expression can consider as diagnostic or prognostic biomarker (22, 23). The lack of TLR9 expression provide poor immune response in viral related cancers, for instance Epstein Barr virus (EBV) allow to a reduce TLR9 mRNA levels that correlated to cellular transformation (24).

Previous investigations demonstrated association between TLR2 and TLR9 expressions and invasion, metastases and recurrence. In addition, it was approved the strong connection between TLR2 and TLR9 expression and increased depth of invasion, tumor size and lower tumor grade (25). It was reported a significant association between the activation of TLR9 pathway, higher stages and lower survival rate (26).

The expression of TLR9 was simultaneously increased with Treg cells in HNSCC patients that confirm the antitumor effect of TLR9 that modulated tumor-immune suppression (27). The significant increasing of co-expression of both FoxP3<sup>+</sup>TLR2<sup>+</sup> cell in compare to FoxP3<sup>+</sup> and TLR2<sup>+</sup> cells demonstrated its role in improvement of antitumor response (28). Some

previous studies suggested TLRs can trigger in therapeutic approaches to inhibit tumor growth (29). Altogether, although TLRs play antitumor role, but as mentioned above they can improve carcinogenesis and tumor development. These results demonstrated TLRs such as TLRs 2, 9 have multiple performance. Their function as anti-tumorigenic factor or tumor promotion point may rely on nature of tumor and its loco regional.

Previous experiments demonstrated local delivery of TLR2, 9 agonists can active innate immune responses and repressed primary tumor growth and metastasis in HNSCC models (30).

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As mentioned above, previous studies reveal that activation of TLRs can improve tumorigenic process. Overexpression of TLRs such as TLR2, 9 affect OS and DFS in HNSCC patients. In this way, our study approved that TLRs 2, 9 are valuable to apply as prognostic factors.

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