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Co-Stimulatory Markers OX40 and OX40L in Blood and Saliva of Oral Squamous Cell Carcinoma: A Comparative Study

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SU designed the study and provided facilitated in experiments. AS performed the experiments, statistical analysis, wrote the protocol and manuscript. ZR critically reviewed the manuscript and finalized it. SA and SI helped in sample collection and manuscript writing. ZA helped in lab work and statistical analysis. All authors read and approved the final manuscript.

Key words
OX40, OX40L, Immunotherapy, Oral squamous cell carcinoma, Costimulatory molecules

ABSTRACT

The immunotherapy has emerged as a treatment modality to treat recurrent and advanced tumors. Many immune markers are being evaluated in Oral squamous cell carcinoma (OSCC) including costimulatory molecules OX40 (Tumor necrosis factor receptor superfamily, member 4, TNFSRF4) and OX40L (tumor necrosis factor superfamily, member 4; TNFSF4). They function to potentiate T cells function to accentuate anti-tumor activity. The results of OX40 and OX40L in cancer immunotherapy are still limited and most of the studies have evaluated their expression in tumor biopsies. In this study we aimed to find out gene expression of costimulatory molecules in naïve oral squamous cell carcinoma patients in saliva and blood as noninvasive biological samples by qPCR. The results revealed significant difference between the cycle threshold (Ct) values in the saliva and blood of OX40 (p value=<0.001) and OX40L (p value=<0.001) in OSCC patients. The fold change overexpression in blood and saliva is also compared in relation to histological gradings and clinical staging. Based on our observations we suggest these particular markers can be evaluated in blood and saliva less invasively compared to tumor biopsies.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is one of the most common cancer, due to excessive smokeless tobacco usage and inadequate access to health care prevalent in developing countries (Rao *et al*., 2013). Males have a greater risk of occurrence and mortality ascribable to OSCC in comparison to females (Anwar *et al*., 2020). Overconsumption of nicotine, human papilloma virus infection and alcohol are the primary risk factors for majority of the oral cancers (Warnakulasuriya *et al*., 2010; Mehanna *et al*., 2013). Regardless of the fact, that oral cavity can be easily examined, oral malignancies are often detected late and therefore, the key cause of dire prognosis (Jafari *et al*., 2013).

The commonly practiced treatment modalities of oral cancer are surgery, radiotherapy, and chemotherapy (Bessell *et al*., 2011). The tumor microenvironment of the OSCC is reported to be immunosuppressive (Jewett *et al*., 2006). Recently, agents that affect immune system are being investigated and are already approved by FDA for treatment purposes in melanoma and non-small cell lung carcinoma (Esposito *et al*., 2020; Raedler, 2015). Immune markers are receiving more focus in the management of head and neck cancerous lesions, especially for diagnostic and prognostic purposes. The fundamental principal of immunotherapy is to manipulate biomarkers expressed on immune cells and the tumor microenvironment (TME), which interacts closely with cancer cells and immune cells (Chakraborty *et al*., 2018). Mediators such as OX40 (Tumor necrosis factor receptor superfamily, member 4; TNFRSF4) a member of the tumor necrosis factor receptor superfamily and its ligand, OX40L (tumor necrosis factor superfamily, member 4; TNFSF4) play a crucial role in potentiating T cell responses via T cell receptor followed by ligation of OX40L with OX40. OX40L is expressed on antigen-presenting cells, including dendritic cells and B cells (Redmond *et al*., 2009).

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These markers are mostly being evaluated in the tumor biopsies as they reflect the true immune status at tumor site but it is difficult to evaluate biopsies repetitively. On the other hands, there are fewer studies which evaluated expression of these molecules in blood and to best of our search no study has evaluated expression of OX40 and OX40L in saliva which can reflect the oral milieu. Apart from detecting biomarkers in blood, using saliva for identification of biomarkers for diagnostic and prognostic purposes is a promising line of research as well, especially because saliva collection is easy and non-invasive (Roblegg *et al*., 2019). This study aimed to assess the expression levels of OX40 and OX40L in the saliva and blood of patients with OSCC as well as healthy controls.

MATERIALS AND METHODS

This study investigated 20 healthy participants and 20 newly diagnosed OSCC patients. The OSCC patients that were enrolled in the study were without any prior treatment and were recruited from Dental Department of Ziauddin University. The study protocol approval was granted by the Ethics Committee of Ziauddin University (ERC#2410720ASBC). After written informed consent, blood and unstimulated saliva samples were obtained from the participants. 5mL of blood was drawn by undertaking all aseptic measures and centrifuged at 2000 rpm for 10 min to separate the buffy coat. For saliva samples pellet was separated after centrifuging the sample at 2000 rpm for 10 min.

RNAase Erase spray was used for sanitization of pipettes, glassware and bench top. The buffy coat and pellet were resuspended in 1 mL of Trizol reagent and vortexed for 15 sec. Chloroform (200 µL per 1 mL of Trizol-1/5 volume) was added and was followed by vortex for 15 sec and subsequent incubation at room temperature for 15 min. Phase separation was done through centrifugation at 12000g for 10-15 min at 4°C. The aqueous phase that was separated by centrifuge was carefully shifted into another new 1.5mL Eppendorf tube. Precipitation was done by adding 1 mL chilled isopropanol succeeded by thirty min incubation at room temperature and afterwards, centrifuging it at 12000 g for 10 at 4 °C. In the next step, supernatant was removed and 1 mL of seventy percent ethanol was added and centrifuged at 12000 g for 10 min at 4 °C. Again, the supernatant was discarded and RNA pellet was left to air dry. The air-dried RNA pellet was then eluted in 30 µL of DNase and RNase free water. The concentration of isolated RNA was analyzed by using Multi Scan Sky Spectrophotometer.

The RNA was used for synthesis of cDNA according to the kit's protocol of Revert Aid First Strand cDNA synthesis kit (Thermo Scientific™).

The qPCR was performed by using Maxima SYBR green/ROX qPCR Master mix (Thermo Scientific™). For each gene, 0.4 µL of cDNA was added in 4.6 µL of 1X SYBR green Master Mix in a PCR tube. In this mixture 2.5 µL of forward and reverse primers (Table I) were added to make up total volume of 10 µL in each well.

The amplification conditions were as follows: for initiation 95°C for 4 min followed by 40 cycles of 95°C for 30 seconds then 57 °C for 30 seconds and extension phase was at 72 °C for thirty seconds. The OX40 and OX40L were normalized by GAPDH. The relative fold change expression levels of genes of interest i.e., OX40 and OX40L were calculated by the comparative Ct method presented as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

The analysis of Data was performed by using SPSS version 25. The categorical data is presented as frequencies and percentages while the descriptive data is shown as median (Interquartile range). Mann Whitney test is used to analyze expression between blood and saliva among the OSCC and healthy individuals. A p value < 0.05 was considered to be statistically significant.

RESULTS

This comparative cross-sectional study evaluated 20 OSCC patients and 20 controls. Overall, total 14 males and 6 females were recruited in OSCC group. The mean age of participants was 43 ± 6 years in the control group and 52.31± 12.75 years in the OSCC group, other characteristics of OSCC patients are given in Table II. Figure 1 depicts fold change expression of OX40 and OX40L in saliva and blood samples.

Table I. List of primers.

S. no	Gene	Primer sequence 5`→3`	Annealing temperature	Product size
1	*GAPDH*	F CCAGAACATCATCCCTGCCT R CCTGCTTCACCACCTTCTTG	58°C	185 bp
2	*OX40*	F CAAGCGTGGACTTGACTGTG R GGTCCCTGTCCTCAGATT	58°C	155 bp

3	*OX40L*	F GTCTGGGATGTGATGCTTT R GTGTTGCTTTGCCTGTCTGT	58°C	208 bp

Table II. Characteristics of OSCC patients.

Variables	OSCC patients (n=20)
Gender	
Male	14
Female	6
Clinical stage	
Stage 1	0
Stage 2	5
Stage 3	5
Stage 4	10
Grading	
Well differentiated tumor	10
Moderately differentiated tumor	8
Poorly differentiated tumor	2

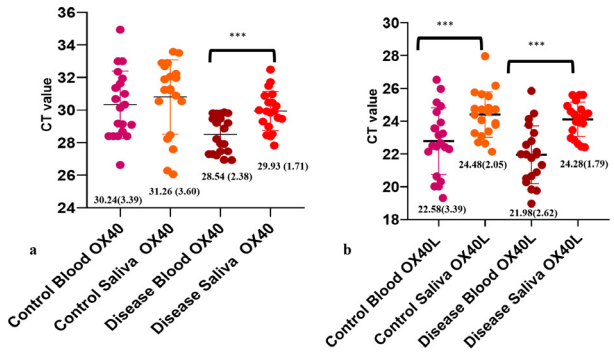

Fig. 1. Comparison of OX40 and OX40L CT values in blood and saliva of healthy individuals and OSCC patients. a, OX40 CT values in blood and saliva of healthy individuals (HI) and OSCC patients. Mann Whitney U test revealed p value of 0.398 between Blood and Saliva of HI, while p value of <0.001 between blood and saliva expression in OSCC. b, OX40L CT values in blood and saliva of healthy individuals and OSCC patients. Mann Whitney U test revealed p value of <0.001 between Blood and Saliva of HI, while p value of <0.001 between blood and saliva expression in OSCC.

The Ct value of OX40 and OX40L is depicted in Figure 1. This signifies higher the Ct (Cycle threshold) value lower is the expression and vice versa. The relative expression of OX40 and OX40L showed statistically significant difference between blood and saliva of OSCC patients with p value =<0.001 for both the markers.

The comparison of relative expression of OX40 in relation to histological grading showed statistically significant results between blood and saliva of OSCC patients in well differentiated (WD) and moderately differentiated tumors (MD) p value =0.001 and p=0.015, respectively. While, poorly differentiated (PD) (n=2) no statistical significance was found between blood and saliva samples. Likewise, OX40 L expression was significant different in both samples for WD and MD tumors but no significance was found in poorly differentiated samples between the two biological samples. The comparative analysis of relative fold change expression of OX40 and OX40L in relation to grading is demonstrated in Table III.

Table III. Comparison of relative expression of OX40 and OX40L in blood and saliva of OSCC patients according to the histological grading

Grade	Blood	Saliva	p value
OX40 (Median; IQR)			
Well differentiated	20.1(17.24)	3.08 (3.11)	0.001**
Moderately differentiated	24.07(24.96)	2.03 (1.63)	0.015*
Poorly differentiated	23.23	2.74	0.333
OX40L (Median; IQR)			
Well differentiated	9.26 (20.2)	2.38(2.68)	0.005**
Moderately differentiated	7.25(11.41)	2.22(2.63)	0.021*
Poorly differentiated	20.09	1.8	0.333

Table IV. Comparison of relative expression of OX40 and OX40L in blood and saliva of OSCC patients according to the clinical stages.

Stage	Blood	Saliva	p value
OX40 (Median; IQR)			
Stage II	24.07 (17.73)	2.36(32.97)	0.421
Stage III	24.07 (27.60)	3.53(4.51)	0.056
Stage IV	17.45(22.29)	2.1(2.07)	0.000*
OX40L (Median; IQR)			
Stage II	9.14 (15.91)	3.47(2.68)	0.016*
Stage III	16.47(37.31)	3.09 (2.99)	0.151
Stage IV	9.98 (14.23)	1.42(1.63)	0.002*

The comparative analysis of relative expression of OX40 revealed significant difference of expression of OX40 between blood and saliva of Stage IV (p value = <0.001). On the other hand, no significant difference was observed in relative expression of OX40 between blood

and saliva in relation to Stage II and III. The OX40L relative expression in blood and Saliva for different stages is shown in Table IV.

DISCUSSION

The molecular components of innate and adaptive immune system can be found in the blood and saliva, making them promising biomarker candidates (Lim *et al*., 2016). Immune biomarkers are impacted by local immunity processes in the mouth cavity, which is a distinct environmental niche (Williamson *et al*., 2012). Immune components are used as biomarkers in many clinical investigations to represent patient well-being or intervention results. According to the current results, the expression of OX40 and OX40L in blood were significantly higher compared to salivary samples. Moreover, this study was the first to measure the OX40 and OX40L expression in OSCC patients and compared their expression levels in biological samples of blood and saliva.

Most of the studies have evaluated expression of co-stimulatory molecules in tumor microenvironment (TME). The expressions of OX40 and OX40L were seen higher in blood sample in comparison to saliva and could be due to higher number of leukocytes in the blood as compared to saliva as OX40 and OX40L are majorly expressed on immune cells and for that reason it is expressed significantly higher in the blood samples as compared to the saliva sample (Ostheim *et al*., 2020). Our results also reveal that the saliva had late expression of OX40 and OX40L as compared to blood samples may be due to the same reason.

Multiple studies have evaluated OX40 and OX40L in head and neck cancer in tumor biopsies. A study by Lecerf *et al*. (2019) analyzed tumor biopsies and reported OX40L overexpression was associated with poor prognosis. Higher serum levels of OX40 were observed in late-staged oral cancer (Sani *et al*., 2021). Montler *et al*. (2016) reported higher percentage of OX40 positive T regulatory cells (Treg) in tumor infiltrating leukocytes (TILs) as compared to peripheral blood mononuclear cells (PMBCs) in head and cancer patients (Montler *et al*., 2016). The significant results were observed for well differentiated and moderately differentiated tumors, however, no significant difference was observed in poorly differentiated due to the lesser number of cases i.e., only two. Similarly, the stage III cases did not show significant results for OX40 and OX40L gene expression between the 2 biological samples.

Potential research may be able to employ saliva collection instead of blood to measure OX40 and OX40L in order to ascertain future OX40 and OX40 treatment drugs. However, the appropriateness of substitution varies per analyte, and the salivary sample in our investigation however, adequately reflect the costimulatory immunological markers. Therefore, we suggest costimulatory molecules OX40 and OX40L expression in saliva can be employed and larger sample size is able to establish the cutoff expression levels or to validate through other techniques such as ELISA or western blot or more sensitive techniques. This is the first study of its kind which have investigated OX40 and OX40L in saliva samples of newly diagnosed OSCC patients without undergoing any treatment. The limitation of our study is sample size plus tumor tissue was not evaluated to collate the expression among these samples.

CONCLUSION

The expression of OX40 and OX40L is seen comparatively higher in blood samples as compared to the saliva samples. Based on our observations we suggest these particular markers can be evaluated be in blood and saliva and can be utilized as an alternative to tumour biopsies if repeated analysis is required.

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IRB approval and ethical statement

The approval of the study was granted by Ethical Research Committee of Ziauddin Medical University, Karachi.

Statement of conflict of interest

The authors have declared no conflict of interest.

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