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ORIGINAL ARTICLE

Expression of CLEC3B in Oral Squamous Cell Carcinoma and its Association with Clinicopathological Features

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ABSTRACT

Background: Oral squamous cell carcinoma is one of the leading causes of cancer related morbidity worldwide. The socioeconomic status and clinicopathological parameters have predictive importance specifically for head and neck squamous cell carcinoma. Literature has reported various studies on CLEC3B, and its correlation with OSCC has yet to be explored.
Aim: To examine the association of CLEC3B expression with the clinicopathological parameters in the blood and saliva of OSCC patients and healthy individuals.
Methodology: This case-control study was conducted on 40 blood and 40 saliva samples recruited from Ziauddin University Hospital and the Abbasi Shaheed Hospital. Blood and saliva samples were taken from 20 OSCC patients as well as 20 healthy individuals as controls. Demographic details including age, gender, and habits were collected directly from participants on a proforma. RT-qPCR was carried out to determine the association of CLEC3B expression in the blood and saliva of OSCC patients and healthy individuals with clinicpathological parameters.
Result: Significant expression was observed in blood and saliva samples of OSCC (p-value 0.001*). CLEC3B was significantly expressed in saliva samples with respect to pathological grading and staging of OSCC. Moreover the foldchange analysis was considerably underexpressed in both samples (saliva p-value 0.042, blood p-value 0.014).Whereas no significant association was found with respect to the habits, age and gender of patients. The significant expression of CLEC3B in saliva may improve the ability of clinicians to detect OSCC at an early stage and may help to reduce the mortality rate of patients.
Conclusion: A significant association of salivary CLEC3B was observed with the staging and grading of OSCC and it was found that CLEC3B may have the potential to be used as a suitable biomarker for the detection and diagnosis of OSCC. This study could provide insight into ongoing evidence on the importance of CLEC3B in early detection of oral cancer. Furthermore, the salivary gene expression can be used to characterize tumor grading and staging.
Keywords: CLEC3B, Biomarkers, Gene expression, Oral cancer, RT-qPCR, molecular diagnostics

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the eighth most prevalent carcinoma around the globe. It is one of the leading causes of cancer-related morbidity worldwide[1]. Its incidence shows variation with increased prevalence and is estimated to double in developing countries by 2030[2]. In Asia, Pakistan is the second most affected country with a prevalence of 18.2%[3]. Tobacco, alcohol, areca nut, and human papilloma viruses are known risk factors (HPV) for OSCC. Other than these contributory factors genetic predisposition play a major role in the pathogenesis of OSCC development[4]. Moreover, socioeconomic status and clinicopathological parameters have predictive importance specifically for head and neck squamous cell carcinoma[5].

It is quite challenging to diagnose oral lesions, representing different etiology and various prognoses. Although tissue biopsy has been widely used as a gold standard, its usefulness in detecting OSCC remains debatable, especially in patients with multiple oral lesions, such as taking tissue from a single site and omitting another cancer site. Despite intensive research, the prognosis of OSCC remained poor, with 50% 5-year survival rate, which has not changed for the past 30 years.[(6)]Currently, researches have been undergoing to reduce the mortality rate of OSCC patients and researchers are working on various genetic indicators to validate their role as potential biomarkers for OSCC[7,8].

During the last few years, salivary biomarkers have gained much attention because of their potential to diagnose various diseases[9]. Moreover, saliva has direct contact with the oral lesion, it can be used to diagnose and track the evolution of oral cancer. It is a complex oral fluid made up of numerous salivary gland secretions and gingival crevicular fluid. It may be acquired using noninvasive procedures and can aid in monitoring and identifying a wide range of biomarkers, making it an important diagnostic and prognostic tool[10]. C-type lectin domain family 3 member B (CLEC3B), a C-type lectin that encodes tetranectin intracellularly, has been isolated from human plasma[11]. It regulates proteolytic activity by binding to the Kringle 4 domain of plasminogen in a lysine-dependent manner and activating plasminogen[12]**.** It has been reported as diagnostic and prognostic marker in various tumors such as hepatocellular, renal, ovarian and breast carcinoma[13-16]. However, its potential in diagnosing OSCC in saliva has not been evaluated. Despite ongoing progress in oral cancer therapy, OSCC cases are still rising, as the current clinical and histopathological methods are not enough to predict OSCC early . Therefore, this study was aimed to explore the association of CLEC3B expression with the clinicopathological parameters in blood and saliva of OSCC patients and healthy individuals to detect OSCC at an early stage.

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METHODOLOGY

This case-control study comprised of 40 blood and 40 saliva samples that were recruited from patients with OSCC admitted in Ziauddin University Hospital and the Abassi Shaheed Hospital. Ethical approval was taken from Ziauddin Ethics Review Committee ref no: 2941220ZAPAT. All subjects gave their informed consent before providing samples. Detailed pathology reports were studied to obtain information regarding tumor grade, stage and site. Demographic details including age, gender and habits were collected directly from participants on a proforma.

Blood and saliva sample were taken from 20 OSCC patients similarly for controls both blood and saliva sample were taken from 20 healthy individuals. Patients with OSCC diagnosed on histopathological bases were included as cases whereas healthy people were taken as controls. Individuals with cancers other than OSCC were excluded. All of the obtained saliva pellets and blood samples were centrifuged and kept at -80 C.

The Trizole reagent was employed to extract the RNA. For phase separation, 200ul of chloroform was utilized. After that, centrifugation was used. Isopropanol precipitation was used to retrieve the RNA. It was centrifuged and air dried. At -80°C, the pallets were suspended in 20ul Nuclease-free water. The extraction of RNA was quantified using a Multi Scan Sky Spectrophotometer. The manufacturer's protocol was followed while synthesizing cDNA with the "Revert Aid First Strand cDNA synthesis Kit." Penicon's primer 3 software was used to design the primer sequence[8]. GAPDH was taken as a housekeeping gene. The primer sequence was as follows:

GAPDH; forward 5′-CCAGAACATCATCCCTGCCT-3′
Reverse; 5′CCTGCTTCACCACCTTCTTG- 3′
CLEC3B; forward 5′- TGGTGTAACCTCAGAAGTG-3′
Reverse; 5′- GTCAACTCCAGGCTTGTA- 3′

RT-qPCR was used to investigate CLEC3B expression. To make a 20ul volume, CDNA and primer combination were mixed to SYBR green master solution. Denaturation, annealing, and extension cycles were performed at 40 cycles in each reaction. For expression analysis, CT values were obtained[8].

Statistical analysis: Independent sample t test was used to calculate the difference between CT value of saliva and blood and Kruskal walis and Post Hoc analysis was applied to identify the intra group comparison among cases and controls. p- value less than 0.05 was considered as significant at 95% confidence interval.

RESULTS

Demographics and clinicopathological characteristics of subjects: Table 1 represents the distribution of subjects with respect to age, gender and clinicopathological parameters. The 20 patients with OSCC includes 10 males and 10 females out of which 9(22.5%) were above 40 years of age whereas in controls there were 8 males and 12 females and 7(17.5%) were above 40 years of age. There was no significant difference in the gene expression with respect to age and gender of cases and controls. Association of CLEC3B expression with clinicopathological parameters of OSCC

Table 2 shows the analysis of expression levels of CLEC3B in OSCC samples by RT-qPCR showed significant difference in blood and saliva samples. The mean CT values was higher in saliva samples than in blood, indicating delayed expression of CLEC3B in saliva. Moreover, the comparison of CT values with pathological grading and clinical staging was statistically significant in saliva than in blood.

Table1. Demographics and clinicopathological characteristics of subjects

Parameters	Cases (%)	P-value Blood	P-value Saliva	Control(%)	P-value Blood	P-value Saliva
Gender						
Male	10 (25%)	0.3	1	8 (20%)	0.6	1
Female	10 (25%)			12 (30%)		
Age						
≥40	9 (22.5%)			7 (17.5%)		
≤40	11 (27.5%)	0.5	0.7	13 (32.5%)	0.76	0.61
Risk Factors						
Smoking	10 (25%)			12 (30%)		
betel quid	6 (15%)	0.6	0.5	7 (12.5%)	0.8	0.9
Pan	4 (10%)			1 (2.5%)		

P < 0.05 was considered statistically significant.Kruskal walis and Post Hoc analysis was applied.

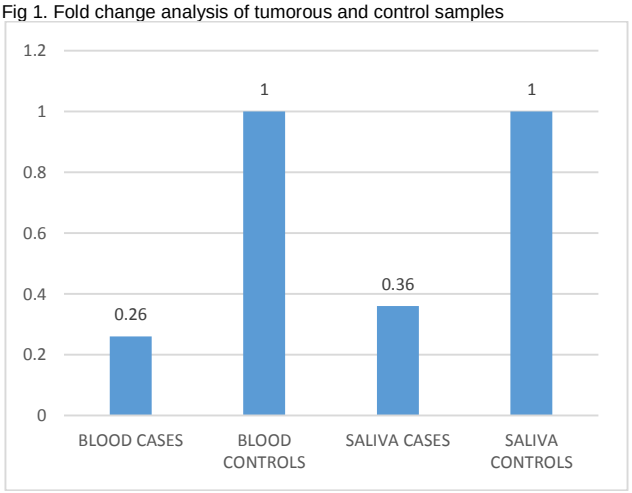
Table 2. Association of CLEC3B expression with clinicopathological parameters of OSCC

Variables	Frequency	Blood Mean ± SD	Saliva Mean ± SD
Cases	N(%)		
OSCC	20(50%)	32.9 ± 2.16	37.8 ± 1.056
p-value		**0.001***	
Comparison of CLEC3B expression with pathological grades			
Well differentiated	8(20%)	33.36± 1.1	36.9±1
Moderately differentiated	12(30%)	32.65±2.66	38.4±0.95
p-value		**0.48**	**0.001***
Comparison of CLEC3B expression with Clinical stages			
I	7(17.5%)	32.5±2.49	37.3±0.7
II	10(25%)	32.9±2.2	37.8±1.08
III	3(7.5%)	33.8±1.34	39.06±0.812
p-value		**0.699**	**0.049**
Comparison of CLEC3B expression with habits			
Buccal mucosa	8(20%)	33.1± 1.1	37.5±1.2
Tongue	4(10%)	31.6± 3.01	38.4±1.2
Soft palate	3(7.5%)	34.1± 1.01	37.5±0.9
Hard palate	5(12.5%)	32.01±4.02	38.4±0.2
p-value		**0.35**	**0.45**

OSCC: oral squamous cell carcinoma. P < 0.05 was considered statistically significant.Kruskal walis and Post Hoc analysis was applied.

Fold change analysis of samples: The significance of CLEC3B expression in OSCC was assessed by calculating fold change. The gene expression was significantly under expressed in both saliva (p-value 0.042) and blood (P-value 0.014) samples of OSCC than in controls showing delayed expression of CLEC3B in cancer patients as shown in fig 1.

Fig 1. Fold change analysis of tumorous and control samples



The bar graph shows Foldchange analysis of CLEC3B expression in blood and saliva of OSCC patients and controls. Control fold change is taken as 1.

DISCUSSION

In order to reduce the risk of mortality in OSCC, it is crucial to diagnose it at its initial stage. Moreover, the clinical characteristics including age, gender, habit, staging and grading of tumor are important for selecting the appropriate treatment strategy[17].Therefore the present study aimed to analyze the expression of CLEC3B in clinical and pathological parameters of OSCC in both saliva and blood samples.

The salivary RNA transcriptome was analyzed and compare with blood and gene expression were compared between healthy and cancer subjects in both samples, in order to detect the significance of a salivary biomarker in early detection of OSCC. The current study revealed significant expression of CLEC3B gene in both blood and saliva samples taken from OSCC patients and healthy individuals. Whereas the downregulation of gene in both samples of OSCC depicts its tumor suppressive role and suggests that it may have cancer specific functions[18]. Similarly downregulation of CLEC3B was observed in a comparative study conducted on OSCC saliva samples but they did not compare it with blood samples and clinicopathological parameter's[8]. The expression of CLEC3B in various malignancies is still poorly understood and controversial. It has been found to be downregulated in blood samples from patients with ovarian cancer, myeloma, breast cancer, colon cancer, B-chronic lymphocytic leukemia, oral cancer and pancreatic cancer[13,16,19]. However, patients with endometrial adenocarcinoma had contradictory results[20]. Except for serum, CLEC3B expression was found in the stroma and cancer cells of tumor tissues, as well as saliva with different expression states and different distribution patterns[21].

The sub sites of oral cancer represent a variety of trends in type and onset of cancers. In developing countries like Pakistan and India, tobacco chewing leading repetitive insult to oral mucosa results in the cancer affecting buccal mucosa[6,22,23] which is consistent with our finding showing buccal mucosa as the most frequently affected site. Whereas in western world due to alcohol consumption and excessive smoking, tongue is the most affected site[24]. This geographical variation depends on different habits of smoking, tobacco chewing and consumption of alcohol in these areas[24]. A study on 3010 OSCC cases reported that in majority of cases tumor was found on buccal mucosa followed by tongue and alveolar ridges[25]. However our study showed no significant association of CLEC3B expression with tumor sub sites.

OSCC being the leading cause of cancer associated mortality, its survival largely depends on the stage at diagnosis. The TNM classification has been widely accepted for cancer staging. Our study showed no significant association of CLEC3B expression in blood with stages. However, it showed significance in clinical staging in saliva samples, indicating that saliva has the potential to aid in determining cancer stages. Tumor differentiation has been reported to have an impact on the treatment outcomes of OSCC patients[26]. It has been reported that CLEC3B expression and TNM staging system may improve prognostic accuracy and overall survival of HCC patients. Moreover, in HCC decreased expression was seen with the advance stages[27]. Similar results found in clear cell renal cell carcinoma showed downregulated expression was associated with advance clinical stage and lower differentiation[16]. Further more in lung cancer CLEC3B was downregulated in stage IA patients and revealed its diagnostic potential in differentiating lung cancer patients from healthy individuals[16]. In addition to it, our findings showed significance in tumor differentiation in saliva samples. This implies that salivary expression of CLEC3B may has a role in detecting OSCC. whereas in blood samples, there were no significant difference regarding differentiation. The reason for this could be limited sample size and lack of poorly differentiated cases of OSCC in our sample.

Although age or gender are not specific for OSCC, yet most of the patients present with tumor in later life. However males are more affected than females .A study conducted on 276 OSCC patients reported majority of patients above 60 years of age with male predominance . [(28)]This may be due to the fact that males consumes more tobacco containing products than females[29]. However, it is inconsistent with the findings of our study, showed no association between age and gender with OSCC.

Moreover, this study has taken numerous patients characteristics into consideration in order to identify important risk factors that might contribute in the progression of tumor. The association of smoking and risk of tumor progression has been reported in previous studies[18]. In our region high consumption of chewable tobacco such as pan gutka etc. together with any other form of intoxication such as smoking, and alcohol increases the changes of OSCC development .However we did not find any significant association between the risk factors and the expression of CLEC3B in both blood and saliva samples. Whereas CLEC3B was reported to be associated with smoking in lung cancer[16]. In future ,studies must be carried out to validate the utility of CLEC3B with larger sample size and different techniques.

CONCLUSION

A significant association of CLEC3B was observed in salivary expression of gene with staging and grading of OSCC and it was found that CLEC3B may have the potential to be used as a suitable biomarker for the detection and diagnosis of OSCC. This study could provide an insight into ongoing evidence on importance of CLEC3B in early detection of oral cancer. Furthermore, the salivary gene expression can be used to characterize tumor grading and staging.

Conflict of Interest: There is no conflict of interest.

Ethical approval: This study was approved by the Ziauddin Ethics Review Committee ref no: 2941220ZAPAT

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Authors' Contributions: Z.A and **S.U**: conception of idea and study design. Author Z.A collected the data, prepare the manuscript and performed the bench work. **F.S** supervise the project. **Z.A and S.U** wrote the protocol and finalized the manuscript. **A.A** performed the statistical analysis. **S.K and J.A** did the literature search.

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