CLEC3B, A Prognostic marker of Oral squamous cell carcinoma

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CLEC3B, A Prognostic marker of Oral squamous cell carcinoma

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ABSTRACT

Oral cancer is one of the leading cause of mortality worldwide. Effective biomarkers for early diagnosis and prognosis would be beneficial to reduce the mortality and ensure effective treatment of OSCC patients. C-Type Lectin Domain Family 3 Member B(CLEC3B) and its translatory protein, Tetranectin has been involved in tumor invasion and metastasis in various cancers, but its

association with OSCC remains unclear. Therefore, we aimed to investigate the prognostic importance of CLEC3B and Tetranectin in OSCC.

A case control study was conducted on saliva samples of 60 participants including 20 OSCC, 20 Postoperative OSCC and 20 healthy individuals, to determine the salivary expression of CLEC3B in OSCC. All samples were collected from Abbasi Shaheed hospital and ziauddin university hospital. Written informed consent was obtained before taking samples. mRNA levels were detected through RT-qPCR and Tetranectin levels were identified by ELISA.

CLEC3B is Significantly expressed in OSCC, postoperative patients and healthy individuals (p-value0.019). It is significantly downregulated in OSCC compared with controls (p-value 0.014) whereas no significance found in mRNA levels of OSCC and Postoperative OSCC cases. Protein expression is significantly downregulated in OSCC compared with postoperative OSCC and with controls ($P < 0.01$). These findings suggest that CLEC3B and Tetranectin could serve as a potentially prognostic biomarker for OSCC.

KEY WORDS

Prognostic marker, oral squamous cell carcinoma, gene expression

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INTRODUCTION

Oral squamous cell carcinoma is still the eighth most deadly cancer in the world, with an estimated annual incidence rate of over 600,000 cases(Qadir, Lalli et al. 2019).Tobacco and alcohol usage are two main risk factors for OSCCC in developing countries (Abulaizi, Tomonaga et al. 2011). OSCC's clinical result is inextricably linked to the moment of diagnosis. According to the SEER(surveillance,epidemiology,end result) programme, the estimated 5-year relative survival rate of patients with tumor confined to the primary site is approximately 85.1 percent, and it drops to 40.1 percent when regional lymph nodes and distant metastasis are involved, respectively(Nagy, Lánczky et al. 2018).

OSCC's evolution is still a source of contention. Most researchers thought it was a multistep process involving the accumulation of genetic and epigenetic alterations that affect gene expression and protein modification, hence changing many signaling pathways(Li, Severson et al. 2016). However, no molecular technique has yet been found to aid in the diagnosis and prognosis of OSCC. Despite recent breakthroughs in OSCC treatment methods that have greatly improved

patients' quality of life and life expectancy, overall clinical results of patients have remained poor, particularly in those who are diagnosed at an advanced stage(Arellano-Garcia, Li et al. 2010, Lim, Tay et al. 2014)As a result, effective prognostic models can forecast the overall survival rate of patients, which may benefit clinicians in the therapy process. However, contemporary prognostic models place a premium on numerous clinicopathological characteristics of OSCC, such as age, gender, smoking habits, advanced clinical stage, and grading at the time of diagnosis(Lee, Nagano et al. 2010, Boxberg, Steiger et al. 2018). Tumors, on the other hand, have complicated regulatory mechanisms that limit the prognostic outcomes of patients due to their specificity, efficiency, and consistency(Shen, Bai et al. 2017).

For the diagnosis and prognosis of OSCC, molecular biomarkers have recently received a lot of attention in oncology. However, a few biomarkers have been found (LDOC1, IL8, S100P), none have been demonstrated to be useful in predicting OSCC prognosis(Liu, Chen et al. 2019, Sivadasan, Gupta et al. 2020).

As a result, an in-depth understanding of the molecular process behind OSCC is urgently needed, as this could lead to the development of novel therapeutic ways to improve long-term survival. CLEC3B has not been linked to OSCC in comparison to post-operative OSCC, so far. As a result, this study was carried out to examine CLEC3B expression and establish its prognostic value in OSCC. Furthermore, improving the use of currently existing therapy outcomes for better prognosis will benefit from the classification of OSCC patients.

METHODOLOGY:

A total of 60 saliva samples were collected from 60 participants, with 20 samples taken from OSCC patients, 20 samples from post-operative OSCC patients, and 20 samples from healthy people as controls. Abbassi Shaheed Hospital and Ziauddin University Hospital provided all of the samples. Before obtaining a saliva sample, each subject gave written informed consent, and the demographic and clinicopathological characteristics of these patients, such as age, gender, tumor site, tumor differentiation, and tumor stages, were recorded. Histopathological reports were used to determine the tumor stage and differentiation of OSCC patients. This study was approved by the Ziauddin Ethics Review Committee ref no: 2941220ZAPAT

Inclusion and exclusion criteria for study subjects:

OSCC patients diagnosed by histological investigation and post-operative OSCC patients who underwent surgical excision of OSCC within two months were among the cases. Healthy people, on the other hand, were used as controls. Individuals with malignancies other than OSCC and autoimmune diseases and patients underwent radiotherapy or chemotherapy after surgical excision were among the exclusion criteria.

Sample collection:

Each patient provided 5ml of entire saliva, which was centrifuged at 2600 x g for 15 minutes at 4°C.It was then stored at -80 degrees Celsius. By qPCR, the expressions of CLEC3B in OSCC, post-operative OSCC, and healthy individuals were determined using a saliva pallet.

Gene Expression Analysis

RNA was extracted using the Trizol technique for gene expression. The phase separation was achieved by adding 200µl of chloroform to the mixture. 2ml isopropanol was combined for RNA precipitation. The supernatant was aspirated after centrifugation, and the pallets were air dried before being suspended in 20 µl of Nuclease-free water at -80C. The Multi Scan Sky Spectrophotometer was used to determine the RNA concentration and purity, and the "Revert Aid First Strand cDNA synthesis Kit" was used to synthesize cDNA according to the manual's methodology. Primer 3 software, made by Penicon, was used to design primers. As an internal control, GAPDH was used. The following was the primer sequence utilized in this study:

GAPDH; forward 5′-CCAGAACATCATCCCTGCCT-3′

Reverse; 5′- CCTGCTTCACCACCTTCTTG- 3′

CLEC3B; forward 5′- TGGTGTAACCTCAGAAGTG- 3′;

Reverse; 5′- GTCAACTCCAGGCTTGTA- 3′

qRT-PCR was used to examine CLEC3B expression. To make a total amount of 20 µl, 10 µl of cDNA and primer combination were mixed to 10 µl of SYBR green master mix. For 40 cycles of denaturation (920C), annealing, and extension, the reaction was run (720C). CT values were acquired, and relative fold change was computed for expression analysis.

STATISTICAL ANALYSIS

The data was analyzed by using spss version 23. The numerical variables were calculated by mean and standard deviation and to identify the differences in groups Anova followed by post hoc tukys test was applied and catagorical variables were calculated in frequencies and percentages and chi square test was applied to find the association. the data was calculated at 95% confidence interval and p-value less than 0.05 was considered as significant.

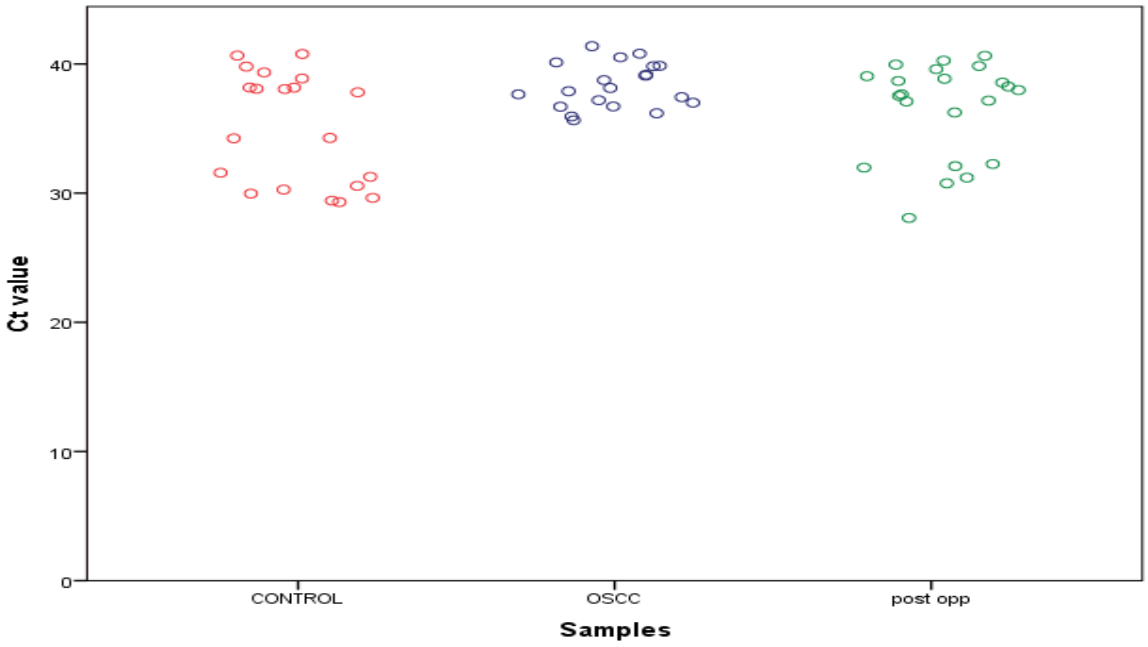
RESULTS:

CLEC3B expression in tumorous and non-tumorous sample

To identify the significance of CLEC3B in OSCC. Saliva samples from 20 healthy, 20 post-operative OSCC and 20 OSCC patients were quantitatively profiled using RT-qPCR.The post-operative group was evaluated to assess the expression levels of CLEC3B after the surgical

excision of OSCC. Fig1 Showed Ct values in all three groups representing delayed expression in OSCC group more than other groups.

Fig 1



OSCC: oral squamous cell carcinoma.

Post opp: post-operative oral squamous cell carcinoma patients.

Prognostic potential of CLEC3B

To determine the prognostic significance of CLEC3B, OSCC cases were compared with controls and postoperative cases. Our results depict significant difference in the quantitative analysis between the control samples, post-operative OSCC samples and the OSCC samples (Table. 1). The mean Ct values were significantly higher in the control samples than post-operative OSCC and OSCC samples ($P \leq 0.01$). On comparison within the groups, significant difference was observed in OSCC and controls.

Table 1

SAMPLES	MEAN ± STD. DEV	P-value
OSCC	37.7 ± 1.15	
POST OPERATIVE	36.4± 3.69	0.019*
CONTROL	34.7 ± 4.21	
	Mean Difference, Std. Error	
OSCC	1.35,1.02	0.38
POST OPERATIVE		
OSCC	3.04,1.04	0.014*
CONTROL		

OSCC: oral squamous cell carcinoma.

Post opp: post-operative oral squamous cell carcinoma patients.

Relative quantification of CLEC3B

A relative quantification was done to determine the expression level of CLEC3B with reference standards. The comparison was done between the controls samples, postoperative and OSCC samples to understand the expression levels(Table.2). The expression was higher in the control samples and was under-expressed in the OSCC more than post-operative samples ($P \leq 0.001$) of OSCC patients as shown in (Table. 2). Within the group comparison showed significantly expressed values.

Table 2

SAMPLES	MEAN ± STD. DEV	P-value
OSCC	1.76 ± 0.05	
POST OPERATIVE	1.56 ± 0.20	0.001*
CONTROL	1.50 ± 0.20	
	Mean Difference, Std. Error	
OSCC		
POST OPERATIVE	0.20,0.55	0.001*
OSCC	0.25,0.55	
CONTROL		0.001*

OSCC:oral squamous cell carcinoma.

Post opp: post-operative oral squamous cell carcinoma patients.

Correlation of CLEC3B with Clinicopathological parameters of OSCC

Table 3. showed significant difference in CLEC3B expression levels with reference to staging and grading of OSCC patients. On post Hoc analysis, significance was found in between stage I-II (P<0.001) and in stage I-III (p value 0.05). Whereas in stage II-III no significance found(Pvalue0.86).

Table 3.

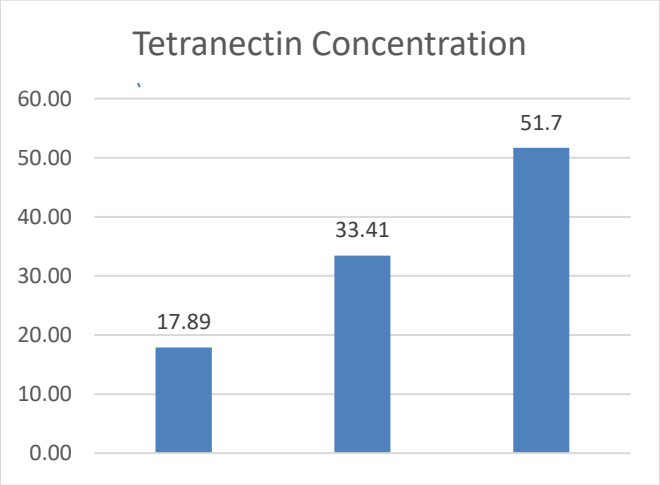
Pathological Parameters	N(%)	P-value
Stage		
I	8(40%)	0.002*
II	9(45%)	
III	3(15%)	
IV	-	
Grade		
I	8(40%)	0.001*
II	12(60%)	
III	-	

Grade: well differentiated(I), moderately differentiated(II), poorly differentiated(III)

Validation of Tetranectin

The validate the mRNA expression, tetranectin concentration was determined in OSCC, Postoperative OSCC and in healthy individuals through ELISA as shown in fig 2. The saliva levels in OSCC were significantly decreased compared with healthy controls (P < 0.01). Moreover, significant difference was shown in postoperative OSCC compared with controls (P-0.04). Whereas no significance was shown in postoperative OSCC cases in comparison with OSCC (p value-0.48)

Fig 2



Association of Tetranectin concentration between the groups

Table 4 represents the significant association of tetranectin between all the groups. The mean concentration in OSCC was significantly decreased as compared to postoperative cases and controls.

Table 4.

SAMPLES	MEAN ± STD. DEV	P-value
OSCC	17.8 ± 12.3	0.03*
POST OPERATIVE	33.4 ± 33.3	
CONTROL	51.6 ± 54	

DISCUSSION:

Pliable malignancies are currently treated by surgical resection and repair followed by adjunctive radiotherapy, whilst other tumors are treated with a mix of chemotherapy and radiotherapy. Unlike breast and lung cancer patients, all OSCC patients receive the same therapy combinations, regardless of their tumor's genetic makeup. This is primarily due to a knowledge gap in molecular biomarkers that may be used to identify the most appropriate intervention based on the molecular profile of a certain tumor(Qadir, Lalli et al. 2019). With this approach we explored the significance of CLEC3B in the prognosis of OSCC.

Previous studies reported that downregulation of CLEC3B is associated with the aggressiveness of OSCC(Arellano-Garcia, Li et al. 2010, Qadir, Lalli et al. 2019) .Our results also support these studies and further observed that in post-operative OSCC patients and in healthy individuals , the expression of CLEC3B was increased than in OSCC patients, suggesting the possible role of CLEC3B in the progression of OSCC. Another study showed positive correlation between CLEC3B and proliferation inhibitors, indicating its tumor suppressive role.(Liu, Liu et al. 2018). The tumor suppression activity of CLEC3B has also been detected in various cancers and was found to be downregulated in pancreatic, ovarian, breast, renal and colorectal carcinoma. (Christensen and Clemmensen 1991, Liu, Liu et al. 2018, Zhu, Zhang et al. 2019).However there is no report detecting CLEC3B in saliva of postoperative OSCC patients.

Moreover, the comparison of relative gene expression showed significantly expressed values in our study and reported that the relative expression of CLEC3B in OSCC patient was significantly decreased in comparison to postoperative OSCC patients and controls. Whereas the mean Ct values of postoperative OSCC patients and controls were nearly equal, suggesting that in the absence of tumor cells CLEC3B levels were high. Additionally, significant difference was

observed in relation to tumor staging and grading, indicating the importance of CLEC3B in tumor characterization.

Furthermore, the validation result of its translatory protein Tetranectin was significantly decreased in OSCC as compared to postoperative case and in healthy individuals in our study. TN has been proposed as a malignant growth marker in serum, and has been regarded to be involved in cancer cell progression and metastasis through ECM remodeling or cell proliferation. The impact of TN on cell proliferation has not yet clearly comprehended, as the outcomes are conflicting. (Zhu, Zhang et al. 2019, Go, Park et al. 2021) Decreased plasma tetranectin levels were found to be a strong predictor for poor prognosis in ovarian carcinoma(Høgdall, Høgdall et al. 1993).Moreover in breast cancer, low tetranectin levels were associated with poor treatment response.(H∅ gdall, Christensen et al. 1993) . Whereas in renal cell carcinoma it was reported that cell proliferation was inhibited by TN. (Liu, Liu et al. 2018). In this study we found decreased TN levels with the progression of cancer. Decreased TN levels were may be due to the reason that cells of oral cancer are immersed in salivary milieu, and in tumor microenvironment tetranectin may consumed in the proteolytic activity for invasion and metastasis.(Hu, Arellano et al. 2008)Therefore less amount of saliva concentration of tetranectin was observed in OSCC. Our results revealed the prognostic value of CLEC3B and tetranectin in OSCC, however the precise role of tetranectin and CLEC3B in tumor progression still needs further exploration.

CONCLUSION:

Hence, these findings indicated that CLEC3B and Tetranectin could serve as a potentially prognostic biomarker for OSCC, due to its significantly lower expression in OSCC samples compared to healthy ones and the increased expression close to the healthy level in post-operative OSCC samples. Moreover, saliva can be a useful and noninvasive tool for biomarker detection.

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