Implications of CLEC3B as a Prognostic Marker in Oral Squamous Cell Carcinoma

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Abstract

Objective: To examine the prognostic significance of C-Type Lectin Domain Family 3 Member B (CLEC3B) and Tetranectin in Oral squamous cell carcinoma (OSCC).

Methods: 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. Regarding the inclusion criteria, OSCC patients diagnosed based on histopathological examination were included in the OSCC group. Patients diagnosed with oral premalignant lesions and conditions on the basis of histopathological examination were included in the Oculopharyngeal Muscular Dystrophy (OPMD) group. In the Persistent Suspicious Oral Mucosal lesions (PSOML) group, patients with any suspicious oral lesion based on clinical assessment were included. Controls were healthy individuals and none of the patients received any surgery other than biopsy. Regarding the exclusion criteria, patients having malignancies other than OSCC were excluded. Patients having a history of autoimmune diseases or HIV infection were also excluded from the study. 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.

Result: CLEC3B was Significantly expressed in OSCC, postoperative patients, and healthy individuals (p-value 0.019). It was significantly downregulated in OSCC compared with controls (p-value 0.014) whereas no significance was found in mRNA levels of OSCC and Postoperative OSCC cases. Tetranectin expression was significantly downregulated in OSCC compared with postoperative OSCC and controls (p-value 0.03).

Conclusion: These results indicated that CLEC3B and Tetranectin have the potential to serve as prognostic biomarkers for OSCC as it is significantly decreased in OSCC. Decreased expression of CLEC3B and Tetranectin with the progression of cancer staging and grading, suggests its tumor suppressive function. Moreover, the utilization of saliva as a noninvasive method for detecting biomarkers adds to the feasibility and relevance of these findings.

Key words: Prognostic marker, oral squamous cell carcinoma, gene expression.

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Introduction

Oral squamous cell carcinoma (OSCC) is still the eighth most deadly cancer in the world, with an estimated annual incidence rate of over 600,000 cases. Tobacco and alcohol usage are two main risk factors for OSCC in developing countries. q7q 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 tumors confined to the primary site is approximately 85.1 percent, and it drops to 40.1 per cent when regional lymph nodes and distant metastasis are involved, respectively[1].

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

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and protein modification, hence changing many signaling pathways[2]. 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 [3,4]. 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[5,6]. Tumors, on the other hand, have complicated regulatory mechanisms that limit the prognostic outcomes of patients due to their specificity, efficiency, and consistency[7].

Oral cancer stands as a prominent contributor to global mortality. The identification of reliable biomarkers for timely detection and prognosis is crucial in mitigating mortality rates and enhancing the efficacy of treatment for individuals with oral squamous cell carcinoma (OSCC).

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), but none have been demonstrated to be useful in predicting OSCC prognosis[8,9]. C-Type Lectin Domain Family 3 Member B (CLEC3B) and its translational product, Tetranectin, have been implicated in tumour invasion and metastasis across different cancer types, yet their correlation with OSCC remains unclear.

CLEC3B, a member of the C-type lectin superfamily responsible for encoding tetragonal proteins within cells, is recognized as a transmembrane calcium-binding protein. Its presence is observed in various cellular compartments, including the cell plasma, extracellular matrix, and exosomes[8,9]. Beyond its structural role, CLEC3B is implicated in the mineralization process during osteogenesis and contributes to neuroprotection. Additionally, its involvement in tumour invasion and metastasis is linked to the activation of plasminogen, resulting in extracellular effects. Furthermore, CLEC3B has been identified in a range of oncological pathologies, spanning breast, bladder, cervical, and ovarian cancers, melanoma, and gastric adenocarcinoma[9-11].

Despite reports indicating a decrease in CLEC3B levels in OSCC patients, its specific role and the mechanisms associated with tumorigenesis in this context remain ambiguous. Further research is essential to unravel the precise functions of CLEC3B, both in normal physiological processes and its involvement in disease states, particularly in Oral squamous cell carcinoma.

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

This case-control study involved the collection of a total of 60 saliva samples from 60 participants. These samples were categorized into three groups: 20 samples from patients with Oral Squamous Cell Carcinoma (OSCC), 20 samples from post-operative OSCC patients, and 20 samples from healthy individuals serving as controls. The sample size was calculated to be 18 by open epi at the confidence interval of 95% and error at 0.05%. A convenient sampling technique was used. Regarding the inclusion criteria, OSCC patients diagnosed based on histopathological examination were included in the OSCC group. Patients diagnosed with oral premalignant lesions and conditions based on histopathological examination were included in the Oral Potentially Malignant Disorder (OPMD) group. In the Persistent suspicious oral mucosal lesions (PSOML) group, patients with any suspicious oral lesion based on clinical assessment were included. Controls were healthy individuals and none of the

patients received any surgery other than biopsy. Regarding the exclusion criteria, patients having malignancies other than OSCC were excluded. Patients having a history of autoimmune diseases or HIV infection were also excluded from the study. The samples were obtained from Abbasi Shaheed Hospital and Ziauddin University Hospital. The duration of this study was 10 months. Prior to saliva collection, each participant provided written informed consent. Additionally, the demographic characteristics, including age, and gender were recorded for each participant. OSCC patients were determined based on histopathological reports. This study was approved by the Ziauddin Ethics Review Committee ref no: 2941220ZAPAT

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 controls. Individuals with malignancies other than OSCC and autoimmune diseases and patients who underwent radiotherapy or chemotherapy after surgical excision were among the exclusion criteria.

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.

RNA extraction for gene expression was conducted using the Trizol technique. Phase separation was facilitated by introducing 200µl of chloroform to the mixture, and RNA precipitation was achieved by combining 2ml of isopropanol. Following centrifugation, the supernatant was removed, and the resulting pellets were air-dried before being suspended in 20 µl of Nuclease-free water at -80°C. The RNA concentration and purity were assessed using the Multi Scan Sky Spectrophotometer. For cDNA synthesis, the “Revert Aid First Strand cDNA Synthesis Kit” was employed according to the provided manual. Primers for subsequent analysis were designed using Primer 3 software from Penicon. GAPDH was utilized as an internal control.

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to analyze CLEC3B expression. In a final volume of 20 µl, a mixture of 10 µl of cDNA and primer mix was combined with 10 µl of SYBR green master mix. The reaction underwent 40 cycles, including denaturation at 92°C, annealing, and extension at 72°C. Subsequently, CT values were acquired, and the relative fold change was calculated for expression analysis.

The data analysis was conducted using SPSS version 22. For numerical variables, the mean and standard deviation were calculated. To discern differences among groups, analysis of variance (ANOVA) was performed, followed by post hoc Tukey’s test. Categorical variables were expressed in frequencies and percentages. Quantitative variables (CLEC3B and Tetranectin) were expressed in mean and standard deviations. The data was computed at a 95% confidence interval, and a significance level of p-value less than 0.05 was considered statistically significant.

Results

Table 1. Demographics characteristics of participants

Variables	OSCC n(%)	Post-Operative OSCC n(%)	Healthy individuals n(%)
Gender	20	20	20
Male	9 (45)	10(50)	8 (40)
Female	11 (55)	10 (50)	12 (60)
Age			
<45	13 (65)	14 (70)	10 (50)
>45	7 (35)	6 (30)	10 (50)
Habits			
Smoking	10 (50)	4 (20)	9 (45)
Betel quid	4 (20)	-	4 (20)
Pan	3 (15)	-	2 (10)
Pan&Betelquid	3 (15)	-	-
None	-	16 (80)	5 (25)

To determine the prognostic significance of CLEC3B, OSCC cases were compared with controls and postoperative cases. Our results depicted a significant difference in the quantitative analysis between the control samples, post-operative OSCC samples, and the OSCC samples (Table. 2). The mean Ct values were significantly higher in the control samples than in post-operative OSCC and OSCC samples (P-value 0.019). On comparison within

the groups, a significant difference was observed in OSCC and controls.

Table 2. Prognostic potential of CLEC3B

Samples	Mean ± Std. Dev	P-value
OSCC	37.7 ± 1.15	0.019*
Post Operative	36.4 ± 3.69	
Control	34.7 ± 4.21	
	Mean Difference, Std. Error	
OSCC Post Operative	1.35,1.02	0.38
OSCC Control	3.04,1.04	0.014*

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

Relative quantification was employed to assess the expression levels of CLEC3B, using reference standards for comparison. The analysis involved a comparison among control samples, postoperative samples, and OSCC samples to elucidate the expression levels, as indicated in Table 3. The expression was notably higher in the control samples, and it was found to be under-expressed in OSCC samples compared to postoperative samples (P-value 0.001) from OSCC patients, as detailed in Table 3. Furthermore, within-group comparisons revealed significantly distinct expression values.

Table 3. Relative gene expression of CLEC3B

Samples	Mean ± Std. Dev	P-value
OSCC	1.76 ± 0.05	0.001*
Post Operative	1.56 ± 0.20	
Control	1.50 ± 0.20	
	Mean Difference, Std. Error	
OSCC Post Operative	0.20,0.55	0.001*
OSCC Control	0.25,0.55	0.001*

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

To validate the mRNA expression, tetranectin concentration was determined in OSCC, Postoperative OSCC, and healthy individuals through ELISA as shown in Fig 1. The saliva levels in OSCC were significantly decreased compared with healthy controls ($P < 0.01$). Moreover, a significant difference was shown in postoperative OSCC compared with controls (P-value 0.04). Whereas no significance was shown in postoperative OSCC cases in comparison with OSCC (p-value-0.48)

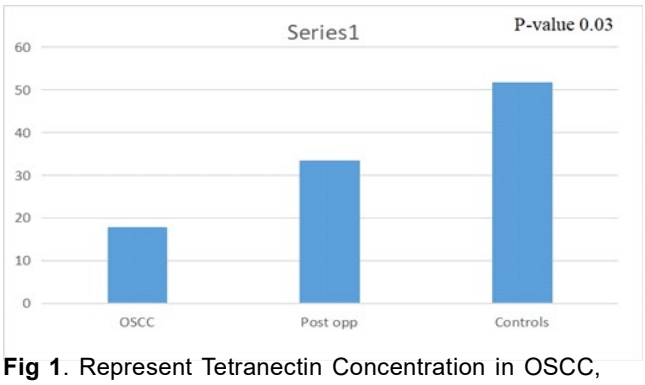

Fig 1. Represent Tetranectin Concentration in OSCC, Postoperative OSCC and Controls (P-value 0.03)

Discussion

Flexible malignancies are presently addressed through surgical resection and subsequent repair, followed by supplementary radiotherapy. Meanwhile, different tumours are managed using a combination of chemotherapy and radiotherapy. In contrast to patients with breast and lung cancer, individuals with oral squamous cell carcinoma (OSCC) undergo identical therapeutic combinations, irrespective of the genetic characteristics of their tumours[12]. 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 tumour[13,14]. With this approach this study was aimed to explore the significance of CLEC3B in the prognosis of OSCC.

Previous studies reported that downregulation of CLEC3B is associated with the aggressiveness of OSCC[3,13]. 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. A study on head and neck squamous cell carcinoma reported that CLEC3B was significantly decreased in the HNSCC tissue compared to the control group[12]. The mRNA levels of CLEC3B, along with C6 and CLCN1, have been demonstrated to accurately predict the survival of

patients with oral squamous cell carcinoma (OSCC). A reduced expression of CLEC3B was associated with a poor prognosis[15]. Furthermore, a substantial down-regulation of CLEC3B was observed in metastatic OSCC, suggesting a potential role for CLEC3B in suppressing the progression of OSCC. Moreover, the Patients with a high expression level of CLEC3B exhibited a greater survival benefit.[3] Another study showed a positive correlation between CLEC3B and proliferation inhibitors, indicating its tumour-suppressive role[15]. The tumour 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[14-18]. The positive expression of CLEC3B in both ovarian cancer tumour tissues and sera has been reported to correlate with a more favourable outcome for patients. Furthermore, there is documentation indicating a reduction of CLEC3B in hepatocellular carcinoma (HCC)[9,19]. However there has been no report detecting CLEC3B in saliva of postoperative OSCC patients.

Furthermore, the analysis of relative gene expression revealed significantly altered values in our study. Specifically, the relative expression of CLEC3B in OSCC patients was markedly decreased compared to postoperative OSCC patients and controls. Meanwhile, the mean Ct of postoperative OSCC patients was slightly higher than that of controls.

Additionally, the analysis of relative gene expression in our study demonstrated significant differences. Specifically, the relative expression of CLEC3B in OSCC patients was markedly decreased compared to postoperative OSCC patients and controls. Meanwhile, the mean Ct values of postoperative OSCC patients and controls were nearly equal, suggesting that in the absence of tumour cells, CLEC3B levels were high. Furthermore, significant differences were observed in relation to tumour staging and grading, underscoring the importance of CLEC3B in tumour characterization. In a study conducted on lung cancer patients, the expression of CLEC3B showed an inverse correlation with the TNM stage, indicating that individuals with lower CLEC3B levels were more prone to manifest late-stage TNM disease. This suggests that CLEC3B functions as a tumour suppressor gene in lung cancer[20]. Liu et al. identified that CLEC3B functions within the mitogen-activated protein kinase pathway and demonstrates a positive correlation with proliferation inhibitors. As a result, the reduction in CLEC3B expression in tumour tissue contributes to carcinogenesis by promoting the uncontrolled proliferation of tumour cells[15]. An additional study proposed that CLEC3B may serve as a tumor suppressor in the context of tumor-immune interactions[20].

Moreover, the validation results of its translatory protein Tetranectin (TN) indicated a significant decrease in OSCC compared to postoperative cases and healthy individuals in our study. TN has been suggested as a serum marker for malignant growth and is believed to play a role in cancer cell progression and metastasis through extracellular matrix (ECM) remodelling or cell proliferation. The impact of TN on cell proliferation remains unclear, as conflicting outcomes have been reported[17,21]. Decreased plasma tetranectin levels were found to be a strong predictor for poor prognosis in ovarian carcinoma[22]. In breast cancer, low tetranectin levels were associated with a poor treatment response[23]. However, in renal cell carcinoma, it was reported that tetranectin inhibited cell proliferation[15].

In our study, we observed decreased TN levels with the progression of oral cancer. This decrease in TN levels may be attributed to the fact that oral cancer cells are immersed in the salivary milieu, and in the tumour microenvironment, tetranectin may be consumed in proteolytic activity for invasion and metastasis. Therefore, a lower concentration of tetranectin in saliva was observed in OSCC. In humans, it forms a homotrimer characterized by a triple alpha-helical coiled coil. Each monomer within this homotrimer comprises a C-type lectin domain, also known as a carbohydrate recognition domain, connected to an extended alpha helix. The activation of plasminogen is proposed to play a crucial role in the invasion and metastasis of tumours. can-

cer cells release plasminogen activators, initiating the transformation of inactive plasminogen into the active protease known as plasmin. Plasmin, in its active state, facilitates the breakdown of proteins in basement membranes and the extracellular matrix (ECM), thereby promoting the invasion of cancer cells into the surrounding tissues[8].

Apart from its specific binding to plasminogen, tetranectin engages in a calcium-dependent interaction with plasminogen-like hepatocyte growth factor and tissue-type plasminogen activator (tPA), but not with urokinase-type plasminogen activator (uPA). The ability of tetranectin to bind and accumulate tPA in an active conformation is postulated to enhance the activation process. In the realm of the serine protease inhibitor superfamily, plasminogen activator inhibitors PAI-1 and PAI-2 assume a regulatory role by impeding the binding between uPA and its receptor. Notably, PAI-2 has been identified as a potential biomarker for invasive head and neck squamous cell carcinoma (HNSCC). This assertion is supported by a noteworthy reduction in its expression, both at the mRNA and protein levels, observed in cultured HNSCC cells and biopsy specimens during invasion into the underlying connective stroma[24]. The study's findings underscore the prognostic significance of CLEC3B and tetranectin in oral squamous cell carcinoma (OSCC). Nevertheless, further exploration is needed to elucidate the precise roles of tetranectin and CLEC3B in tumour progression. Limitations of this study include a limited sample size of oral potentially malignant cases.

Conclusion

These results suggested that CLEC3B and Tetranectin have the potential to be the prognostic biomarkers for OSCC. This is evident from their significantly lower expression in OSCC samples compared to healthy ones, as well as their increased expression, approaching healthy levels, in post-operative OSCC samples. Additionally, the use of saliva as a noninvasive tool for biomarker detection further enhances the practicality and applicability of these findings.

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