RESEARCH PAPER

Quantification of TIMP-1 and MMP-2 Proteins in Saliva from Oral Squamous Cell Carcinoma (OSCC) patients with and without Diabetes Mellitus

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ABSTRACT

Introduction: Squamous cell carcinoma (SCC) is the most prevalent malignant neoplasm in the maxillofacial region, known for its local invasiveness. Matrix metalloproteinases (MMPs) play crucial roles in the alterations associated with carcinogenesis, while tissue inhibitors of metalloproteinases (TIMPs) are peptidases involved in extracellular matrix degradation. This study aimed to quantify TIMP1 and MMP2 proteins in the saliva of patients with oral squamous cell carcinoma (OSCC), with or without diabetes mellitus.

Materials and Methods: Saliva samples were collected from three groups: OSCC patients with diabetes mellitus, OSCC patients without diabetes mellitus, and normal patients. The levels of TIMP1 and MMP2 were determined using an ELISA kit, and statistical analysis was performed using SPSS software version 23.0.

Results: Elevated levels of TIMP1 and MMP2 were observed in oral squamous cell carcinoma (OSCC) patients with coexisting Diabetes Mellitus compared to those without diabetes. MMP-2 and TIMP-1 concentrations were notably higher in OSCC patients with diabetes mellitus, indicating potential prognostic implications.

Conclusion: In conclusion, our study highlights TIMP1 and MMP2 as potent prognostic markers for OSCC patients with diabetes mellitus. These findings contribute to a deeper understanding of the disease's pathophysiology and could aid in the development of targeted therapies for improved patient outcomes.

KEYWORDS: Oral Squamous Cell Carcinoma, Diabetes Mellitus, Tissue Inhibitor of Metalloproteinase-1 (TIMP-1), Matrix Metalloproteinase-2 (MMP-2), Saliva Analysis.

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INTRODUCTION

Over 657,000 new cases of oral cancer cases have been reported annually, with the incidence rising steadily each year. By 2035, the total number of cases will reach approximately 856,000 per year. A significant proportion of these cases—more than half—occur in Asia, where the mortality rate is alarmingly high at 68%. The average 5-year survival rate for oral cancer patients remains around 50%. Among oral cancers, oral squamous cell carcinoma (OSCC) is the most common, accounting for more than 90% of cases. OSCC often arises from premalignant mucosal lesions, which are recognized as oral potentially malignant disorders (OPMD) due to their higher risk of progressing to invasive cancer. (1,2)

The process of carcinogenesis is complex, involving multiple steps and factors that disrupt the balance between activated proto-oncogenes and tumor suppressor genes. This disruption triggers cellular transformation, leading to uncontrolled growth and the development of cancer. (3)These changes result from exposure to carcinogens and host-related factors over time, leading to alterations in multiple genes. The loss of regulatory control over these genes underpins the phenotypic changes associated with cancer, including cellular immortality, tissue invasion, metastasis, and angiogenesis. (4)The progression of oral cancer typically begins with a precursor lesion, such as leukoplakia, which is well-documented in the literature. (5,6)

Early detection is crucial for the effective management of oral cancer. Saliva, a biological fluid that is easy to collect, cost-effective, and non-invasive, offers a promising medium for diagnostic purposes. Recent studies have highlighted the potential of using validated salivary biomarkers for the early detection of OSCC, with a focus on the analysis of RNA and proteins.

Matrix metalloproteinase-2 (MMP-2), a 72-kD type IV collagenase, is a key enzyme that degrades type IV collagen, the primary structural component of basement membranes. MMP-2 plays significant roles in various physiological processes, including endometrial breakdown during menstruation, regulation of vascularization, and the inflammatory

response. It has also been implicated in cancer progression. For instance, Kenny et al. (2008) reported that MMP-2 expression was induced when ovarian cancer cells attached to the mesothelium. TIMP metallopeptidase inhibitor 1 (TIMP-1), a member of the TIMP family, is a natural inhibitor of MMPs, which are enzymes involved in the degradation of the extracellular matrix (ECM). TIMP-1 plays a crucial role in regulating MMP activity, which is essential for maintaining ECM integrity. (7)

The activity of MMPs is regulated at several levels, including transcription, proteolytic activation, and inhibition by natural enzyme inhibitors such as TIMPs. (8,9) The balance between active MMPs and TIMPs is critical in controlling ECM degradation. While TIMPs are known to inhibit MMP activity, which is crucial for preventing tumorigenesis, there is evidence that TIMPs can paradoxically promote cancer progression by influencing cell proliferation, apoptosis, and angiogenesis. (10)

TIMPs are considered multifunctional molecules in the context of tumorigenesis. They regulate various processes, including cell proliferation, apoptosis, MMP-2 activity, and (10,11)angiogenesis. Although TIMPs generally inhibit tumor growth and metastasis, overexpression of TIMP-1 in neoplastic cells has been shown to paradoxically stimulate tumor growth and inhibit apoptosis, thereby potentially facilitating disease progression. (12) The aim of the present study is to quantify the levels of TIMP-1 and MMP-2 proteins in the saliva of OSCC patients with and without diabetes mellitus.

MATERIALS AND METHODS

Study Setting
This research was conducted at Saveetha Dental College and Hospitals in Chennai. Saliva samples were collected in sterilized containers from three distinct groups: 10 OSCC patients with diabetes mellitus, 10 OSCC patients without diabetes mellitus, and 10 healthy individuals serving as controls. The study included pre-operative OSCC patients, with or without diabetes mellitus, while exclusion was based on age and sex criteria.

Sample Analysis
TIMP-1 and MMP-2 levels in the saliva samples were determined using specific ELISA kits. An automatic microplate reader was used for measurement, and the data were recorded in an Excel sheet for statistical analysis using SPSS software version 23.0. The results were presented as mean ± standard deviation.

Principle of the MMP-2 Assay
The MMP-2 ELISA kit is based on a sandwich enzyme-linked immunosorbent assay. Anti-MMP-2 polyclonal antibodies pre-coated onto 96-well plates capture the antigen from the sample. Detection was performed using a biotin-conjugated anti-MMP-2 antibody, followed by the addition of an Avidin-Biotin-Peroxidase Complex. The HRP reaction was visualized using TMB substrate, resulting in a color change measured at 450 nm, proportional to the MMP-2 concentration.

MMP-2 Assay Procedure
All reagents were equilibrated to room temperature (37°C) before use. A standard curve was prepared for each assay. Standards, samples, and controls were added in duplicate to pre-coated wells. After incubation at 37°C for 90 minutes, the plates were washed to remove unbound substances. Biotin-conjugated anti-MMP-2 antibody was then added, followed by another incubation and wash. The Avidin-Biotin-Peroxidase Complex was then added, followed by TMB substrate. After a final incubation in the dark for 25-30 minutes, the reaction was stopped with an acidic solution, and the absorbance was read at 450 nm. The concentration of MMP-2 in each sample was determined using the standard curve.

Principle of the TIMP-1 Assay
The TIMP-1 ELISA kit employs a double antibody sandwich method to quantify TIMP-1. A specific antibody for TIMP-1 was pre-coated onto a microplate. Samples and standards were added, and any TIMP-1 present was bound by the immobilized antibody. Detection was carried out using an HRP-conjugated antibody, with color development measured at 450 nm.

TIMP-1 Assay Procedure
Diluted standards and samples were added in duplicate to the pre-coated wells. After a 2-hour incubation at room temperature, the wells were washed to remove unbound material. HRP-conjugated antibodies were then added, followed by additional washing and incubation. TMB substrate was added, and after color development, the reaction was stopped, and absorbance was read at 450 nm. The TIMP-1 concentration was calculated based on the standard curve.

RESULTS

Our study revealed that both TIMP-1 and MMP-2 levels were significantly elevated in patients with oral squamous cell carcinoma (OSCC) coexisting with diabetes mellitus.

MMP-2 Levels
As illustrated in Graph 1, the concentration and

optical density (OD) of MMP-2 in saliva were compared among three groups: healthy controls, OSCC patients without diabetes mellitus, and OSCC patients with diabetes mellitus. The data presented in Table 1 demonstrate that the OD value in the healthy control group was 1.012, with a corresponding concentration of 80.7984 pg/mL. In OSCC patients without diabetes mellitus, the OD value was lower at 0.763, with a concentration of 60.91816 pg/mL. Conversely, in OSCC patients with diabetes mellitus, both the OD value and concentration were markedly higher at 1.65 and 131.7365 pg/mL, respectively. These findings indicate that MMP-2 levels are significantly elevated in OSCC patients with diabetes mellitus compared to the other two groups.

TIMP-1 Levels

Graph 2 depicts the concentration and optical density of TIMP-1 in the saliva of the same groups. As shown in Table 2, the OD value for TIMP-1 in the healthy control group was 0.769, with a concentration of 120.1563 pg/mL. In OSCC patients without diabetes mellitus, the OD value was 0.49, with a concentration of 76.5625 pg/mL. In OSCC patients with diabetes mellitus, the OD value and concentration were significantly higher at 1.206 and 188.4375 pg/mL, respectively. These results clearly demonstrate that TIMP-1 levels are also elevated in OSCC patients with diabetes mellitus when compared to the other groups.

Overall, our data suggest a strong association between elevated TIMP-1 and MMP-2 levels and the presence of both OSCC and diabetes mellitus, underscoring the potential role of these biomarkers in the pathophysiology of OSCC in diabetic patients.

GRAPH 1: In graph 1, X axis depicts the concentration of MMP2 in the saliva and Y axis depicts the Optical density

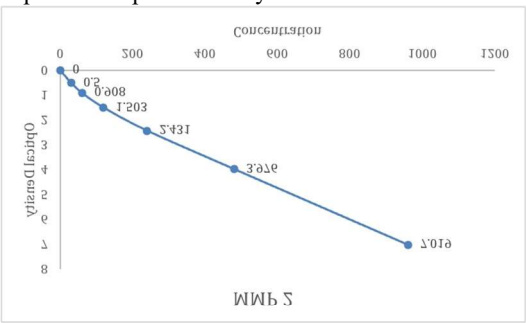

TABLE 1: From table 1 it is seen that, the value of normal patients OD is 1.012 and the concentration is 80.7984. The OD value of OSCC patients without diabetes mellitus is 0.763 and the concentration is 60.91816. The OD value OSCC patients with diabetes mellitus 1.65 and the concentration is 131.7365. From this table it can be inferred that the OD and concentration value of MMP2 is greater in OSCC patients with diabetes mellitus when compared to other two groups

Sample details	OD	ng/ml
Normal	1.012	80.7984
1	0.763	60.91816
2	1.65	131.7365

Graph 2: In graph 2, X axis depicts the concentration of TIMP1 in saliva and the Y axis depicts the Optical density of TIMP1.

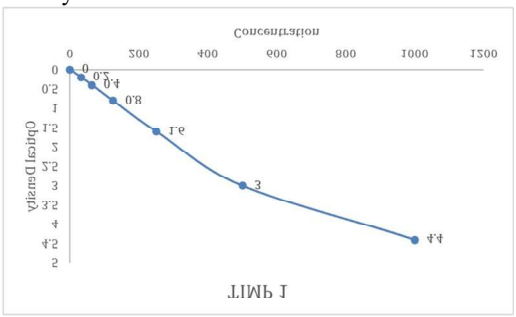

TABLE 2: From table 2 it is seen that, the value of normal patients OD is 0.769 and the concentration is 120.1563. The OD value of OSCC patients without diabetes mellitus is 0.49 and the concentration is 76.5625. The OD value of OSCC patients with diabetes mellitus 1.206 and the concentration is 188.4375. From this table it can be inferred that the OD and concentration value of TIMP1 is greater in OSCC patients with diabetes mellitus when compared to other two groups

Sample details	OD	pg/ml
Normal	0.769	120.1563
1 (OSCC without Diabetes Mellitus)	0.49	76.5625

2 (OSCC with Diabetes Mellitus)	1.206	188.4375

DISCUSSION:

Tumor invasion is a complex biological process involving the detachment of tumor cells from the primary mass and their subsequent infiltration into surrounding tissues. This process requires the disruption of cell-to-cell connections, active cell migration, adherence to the extracellular matrix (ECM), and proteolytic degradation of the ECM. Various molecules, including cadherins, integrins, proteases, and growth factors, play a crucial role in regulating cancer invasiveness at the molecular level. Matrix metalloproteinases (MMPs) are particularly important in tumor invasion and metastasis, often expressed in both invasive tumor cells and the surrounding stroma. Interestingly, the majority of MMPs are produced by neighboring stromal cells rather than the tumor cells themselves, as demonstrated by in situ hybridization techniques. It is hypothesized that tumor cells secrete extracellular matrix metalloproteinase inducer (EMMPRIN), which stimulates stromal fibroblasts to produce MMPs. (13,14)

The activities of MMPs are tightly regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs), which bind to MMPs and inhibit their proteolytic functions. An imbalance favoring MMP activity can lead to excessive ECM degradation, contributing to tumor cell invasion and angiogenesis, key processes in cancer progression. (15) Several MMPs, including MMP-1, -2, -8, -13, and MT1-MMP, are involved in degrading interstitial collagen in the ECM. (16) These observations suggest that the degradation of ECM is not solely dependent on a single MMP but rather requires the coordinated activity of multiple MMPs and TIMPs for effective disruption of the basement membrane and interstitial stroma.

TIMPs' anti-MMP activity is especially crucial later in the course of a tumor, when it has the potential to significantly reduce metastasis. On the other hand, because of their growth-promoting and anti-apoptotic properties, TIMPs may encourage cell proliferation and survival during the early phases of lesion formation. TIMPs consist of four homologous components, the most extensive studies are on TIMP-1 and TIMP-2. (17) In addition to suppressing MMPs, these multifunctional proteins also decrease cell invasion in vitro and suppress angiogenesis, tumorigenesis, tumour invasion, and metastasis in vivo.(18) TIMP-1 and TIMP-2, which have molecular weights of 28.5 kDa and 21.0 kDa, respectively, bind to gelatinases B and A as well as other MMPs to create 1:1 stoichiometric complexes that suppress MMP activity. (19)Although TIMPs were originally believed to have only anti-invasive properties, new research indicates that they may have a more intricate, multidimensional role in cancer, possibly with consequences for prognosis. Serine proteinases that cleave the amino-terminal domains of MMPs activate them extracellularly after they are released as inactive zymogens. Membrane-type MMPs (MT-MMPs) and MMP-11 (stromelysin-3) are two examples of this not being the case.

The most well-known member of the TIMP family, TIMP-1, is created in response to external stimuli like cytokines (like interleukin 6, IL-6) and growth factors (like platelet-derived growth factor, PDGF), such as basic fibroblast growth factor, b-FGF. Numerous cell types, including fibroblasts, chondrocytes, smooth muscle cells, endothelium and epithelial cells, osteoblasts, and various tumour cell types, express it. (12) Although TIMP-1's function in erythroid activity was initially identified, other cell types such as keratinocytes, fibroblasts, lung adenocarcinoma cells, and melanoma cells are also affected by its ability to promote growth. (10) Inversely, poor prognosis has been linked to elevated TIMP-1 expression in a number of solid tumours, such as lung, colorectal, stomach, and breast malignancies. Progression of the cancer and metastasis have also been linked to elevated plasma levels of TIMP-1 in these patients. Consequently, researching TIMP-1 expression and regulation, especially in OSCC (oral squamous cell carcinoma), may yield important information on the pathways underlying tumor invasion as well as possible prognostic indicators (20). In our study, we observed increased levels of TIMP-1 in the saliva of OSCC patients with coexisting diabetes mellitus. This finding is consistent with previous research, such as the study by J Carlos et al., which reported TIMP-1 expression in 66.2% of cases, with expression noted both in tumor tissue and in the stroma. (21) The pattern of TIMP-1 expression observed in cancer tissues varied, appearing as homogenous, central, or irregular distributions. These results further support the role of TIMP-1 as a critical factor in the progression and prognosis of OSCC, particularly in patients with comorbid diabetes mellitus.

The integration of molecular biology techniques in the study of oral carcinogenesis provides a deeper understanding of the processes involved in tumor initiation, growth, invasion, and metastasis. The expression, regulation, and localization of TIMP-1 could serve as potential indicators for assessing the invasiveness and prognosis of epithelial tumors, such as OSCC.

CONCLUSION:

Our study demonstrates that the levels of TIMP-1 and MMP-2 are significantly elevated in patients with oral squamous cell carcinoma (OSCC) coexisting with diabetes mellitus, suggesting a potential role for these biomarkers in the progression of OSCC in this patient population. These findings highlight the importance of considering comorbid conditions like diabetes mellitus when studying the molecular mechanisms underlying OSCC.

Future research should focus on expanding the sample size to validate these findings further and explore the association of additional proteins and biomarkers with OSCC. Such studies could provide deeper insights into the pathophysiology of OSCC and aid in the development of more targeted therapeutic strategies.

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