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ORIGINAL ARTICLE

May High MMP-2 and TIMP-2 Expressions Increase or Decrease the Aggressivity of Oral Cancer?

Bijayatha Shrestha[1] · **Dipshikha Bajracharya**[2] · **Aditi Amit Byatnal**[3] · **Asha Kamath**[4] · **Raghu Radhakrishnan**[5]

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Abstract Matrix metalloproteinases-2 (MMP-2) and the tissue inhibitor of metalloproteinase-2 (TIMP-2), may presumably have an important role on the invasion and metastatic spread of malignancies attributed to an uncontrolled degradation of the extracellular matrix (ECM). A retrospective chart analysis was carried out to study the expression of MMP-2 and TIMP-2 on the archival samples of oral squamous cell carcinoma (OSCC) (n = 30) and normal mucosa (n = 10) by immunohistochemistry and compared with the clinicopathologic parameters of cases. Both MMP-2 and TIMP-2 expressions showed a positive correlation with the grades, stages and metastatic capacities of tumors (Spearman's correlation, $p < 0.05$). Concomitant increase in the expression of TIMP-2 and MMP-2 suggested that the rate of MMP-2/TIMP-2 expression is a better marker for characterization of MMP-2 concentration. High expression and/or activity of MMP-2 were linked with poorer survival in OSCC cases, while TIMPs have been shown to apparently act as either growth-stimulating or suppressor factors for tumors. It was also revealed that MMP-2 and TIMP-2 were secreted by both tumor cells and stromal cells. A new concept, supposing the dynamic, anticancer partnership between the residual genome stabilizer machinery of tumor cells and defensive cells adjacent to tumors, may illuminate the controversial results. In conclusion, the stronger the infiltrative and metastatic capacity of cancers, the higher is the rate of MMP-2/TIMP-2 expression helping the arrival of humoral and cellular anticancer forces.

Keywords Oral squamous cell carcinoma · Invasion · Matrix metalloproteinases · Metastasis · MMP-2 · TIMP-2

Introduction

Oral cancer is the most common head and neck cancer constituting nearly one third of all cancer cases in India. Local invasiveness and high rate of metastasis to cervical lymph nodes in oral cancer is attributed to various adhesion molecules and/or matrix degrading enzymes. Matrix metalloproteinases (MMPs), a family of zinc dependent endopeptidases, degrades most of the components of extracellular matrix (ECM) including the basement membrane, which is a prerequisite for cancer cells to invade and metastasize [1]. Together with the degradation of the basement membrane, cancer dissemination requires the induction of angiogenesis where the roles of MMPs have been implicated [2, 3]. Additionally the proteolytic activities of MMPs are controlled by appropriate activation and inhibition by tissue inhibitors of metalloproteinases (TIMPs). Besides being implicated in anti-angiogenic, anti-mitogenic and anti-apoptotic, it is not clear whether TIMPs have a protective role in cell growth or are they contributory to metastasis [4]. In this study, expression of MMP-2 and TIMP-2 in oral cancer cases is correlated with the clinicopathologic parameters. Deciphering its expression in oral cancer cases may offer newer insight in understanding oral cancer progression.

✉ Raghu Radhakrishnan
raghu.radhakrishnan@gmail.com

[1] Department of Oral Pathology, Chitwan Medical College, Bharatpur, Chitwan, Nepal

[2] Department of Oral Pathology, Kantipur Dental College, Hospital and Research Center, Kathmandu, Nepal

[3] Oral Pathology & Microbiology, Hubli, Karnataka, India

[4] Department of Community Medicine, Kasturba Medical College, Manipal University, Manipal, India

[5] Department of Oral Pathology, Manipal College of Dental Sciences, Manipal University, Manipal 576104, India

Material and Methods

The material for the present study included 30 cases of carcinoma of the bucco-alveolar mucosal complex from the year 2009 to 2012, whose clinical details were retrieved from medical records. Prior to carrying out the study, University Ethics Committee approval was obtained (UEC/58/2009) from Manipal University. A retrospective chart analysis was carried out to study the expression of MMP-2 and TIMP-2 in formalin fixed paraffin embedded (FFPE) tissue blocks in all the cases by immunohistochemistry (IHC) correlating with the tumor staging, histological grading and lymph node metastasis. Ten healthy mucosal tissues, obtained at the time of extraction of impacted teeth were used as controls. Only those patients with tobacco habits who were histologically the confirmed cases of oral squamous cell carcinoma (OSCC) and in whom the radiotherapy or chemotherapy had not begun at the time of biopsy were included.

Immunohistochemical Staining

Two identical sections, 4 μm thick obtained from FFPE tissue blocks were taken on APES (3-aminopropyl triethoxysilane, Sigma Aldrich Co. St. Louis, USA) coated glass slides for immunohistochemical staining by polymer chain two step indirect technique. The anti MMP-2 monoclonal antibody (MMP-2 NCL-MMP2–507 clone 17B11) and anti TIMP-2 monoclonal antibody (TIMP-2 NCL-TIMP2–487 clone 46E5) obtained from Novocastra Leica Biosystems, Newcastle Ltd. were used. Tissue sections of inflammatory bowel disease constituted the positive control for MMP-2 while sections from placenta constituted the positive control for TIMP-2.

For antigen retrieval, slides were immersed completely in the antigen retrieval solution (Tris EDTA, pH 9.0) at 800 W in a microwave oven for 10 min and eventually allowed to cool at room temperature. The slides were washed with TBS, pH 7.6 twice for 5 min. For endogenous peroxidase blocking, sections were incubated in 3 % hydrogen peroxide for 20 min following which the slides were agitated in TBS, pH 7.6 for 5 min. Non-specific binding sites were blocked by protein block for 10 min provided by Novacastra Leica Biosystems, Newcastle Ltd. The slides were drained of the reagent and the sections were washed twice in TBS for 5 min. Incubation with primary antibodies were carried out by covering the sections completely with Anti MMP-2 and Anti TIMP-2 monoclonal antibodies diluted with Tris buffer, pH 7.6 at 1:20 dilution each and incubated for 3 h at 37 °C in a moist chamber. Post primary block was done by a polymer penetration enhancer containing 10 % *v*/v animal serum in TBS and thereafter the sections were washed twice in TBS for 5 min. Incubation with secondary antibody was carried out at room temperature for 30 min by applying anti-mouse IgG-Poly-HRP (8 μg/ml) containing 10 % *v*/v animal serum in TBS. Following a thorough wash with TBS for 5 min, visualization of this reaction was done with the addition of chromogen containing 1.74 % *w*/*v* 3, 3′ – diaminobenzidine (DAB), Novacastra Leica Biosystems, Newcastle Ltd. in a stabilizer solution for 6 min. The sections were washed under running tap water and counterstained with Mayer's hematoxylin for 5 min.

Staining Interpretation

To quantify the expression of MMP-2 and TIMP-2, we evaluated the number of MMP-2 and TIMP-2 positive cells. A positive cell demonstrated a diffuse brown signal in the cytoplasm of cells, independent of its intensity [5]. Tissue sections were scored based on the proportion of cells expressing MMP-2 and TIMP-2 positivity. Tissue sections were considered positive even if 1 % of tumor cells showed positive staining. Expression was graded as low (+) when 1–25 % of the tumor cells and/or stromal cells stained positively, moderate (++), when 26–50 % of the tumor cells and or stromal cells stained positively and strong (+++) when more than 50 % of the tumor cells and or stromal cells were positive for MMP-2 and TIMP-2. The expression was assessed in the tumor cells at the invasive front, tumor cells within the tumor islands, stromal cells adjacent to the invasive tumor front and stromal cells between the tumor islands. The evaluation of positive cells was assessed in five different fields at × 40 magnifications and a total of 500 cells were examined.

Statistics

Two observers independently assessed the proportion of positive cells. Statistical analysis was carried out using SPSS (Statistical package for social service) version 16.0 for Windows. Kendall's tau-b statistics was applied to assess the measure of agreement between two observers. Frequency analysis was done to calculate the median measure of expression of MMP-2 and TIMP-2 observable in tumor cells and stromal cells in different areas with regard to grading and staging of oral cancer. Mann Whitney U test was used to compare the median expression of MMP-2 and TIMP-2 in normal and all the cases of oral cancer. Kruskal-Wallis test was done to calculate the difference in expression of MMP-2 or TIMP-2 in tumor cells in different areas with regard to grading and staging of oral cancer. Mann Whitney U test was used to study the relationship with regional lymph node status. Correlation of expression of MMP-2 with TIMP-2 in different areas, grades, stages and lymph node status of the patient was carried out by Spearman's correlation. $p < 0.05$ was considered significant for all statistical analysis.

Results

The clinicopathological details of the patients included in the study are presented in Table 1. The cases were selected by convenience sampling and were categorized into well differentiated, moderately differentiated and poorly differentiated squamous cell carcinoma. The immunostaining of inflammatory bowel disease (Fig. 1a) and human placenta (Fig. 1b) were used as positive control for MMP-2 and TIMP-2 respectively. The sections of normal oral mucosal tissue did not show immunopositivity for MMP-2 (Fig. 1c) or TIMP-2 (Fig. 1d) except in the endothelial cells lining the blood vessels.

MMP-2 and TIMP-2 expression was positive in all except four of OSCC cases included in our study. The expression of MMP-2 and TIMP-2 was observed within the tumor cells at the invasive front, the stromal cells adjacent to the invasive tumor front (Fig. 1e and Fig. 1f) as well as within the tumor islands and stromal cells between the tumor islands (Fig. 1g and Fig. 1h). The representative images of the expression of MMP-2 and TIMP-2 in positive cases are shown. A high level of agreement was achieved for the expression of both MMP-2 (0.977, $p < 0.001$) and TIMP-2 (0.979, $p < 0.001$) by Kendall tau-b test. The expression scores of MMP-2 ($p = 0.008$) and TIMP-2 ($p = 0.002$) in 4 different areas examined is present as a bar diagram (Fig. 2).

The expression score of MMP-2 and TIMP-2 at the invasive front of the tumor, the stromal cells adjacent to invasive tumor front, the tumor cells within the tumor islands and the stromal cells between the tumor islands in all the clinical stages (Table 2), the lymph node status when it was first diagnosed (Table 3), and histological grades (Table 4) were found to be statistically significant (Fig. 3).

Comparison of the difference in median expression of MMP-2 and TIMP-2 in normal and all the cases of OSCC combined was statistically highly significant (Mann-Whitney-U test, $p < 0.001$). No statistically significant difference was noted in median expression of either MMP-2 ($p = 0.95$) or TIMP-2 ($p = 0.76$) (Kruskal-Wallis test) between the four areas studied. The median expression score of MMP-2 ($p = 0.054$) and different stages of oral cancer was not statistically significant. However, a significant relationship was associated between TIMP-2 ($p = 0.013$) and different stages of oral cancer (Kruskal-Wallis analysis). A statistically significant association of MMP-2 ($p = 0.01$) and TIMP-2 ($p = 0.049$) could be elicited (Mann-Whitney U test) with the lymph node status of the cases. With regard to different grades of carcinoma, no statistically significant relationship could be elicited between the expression of MMP-2 ($p = 0.221$) or TIMP-2 ($p = 0.661$) (Kruskal-Wallis analysis).

Spearman's correlation coefficient to study the correlation between the expression of MMP-2 and TIMP-2 in different areas of OSCC examined showed a significant positive correlation with each of the clinicopathologic parameters studied (Table 5).

Discussion

The in vivo activity of MMPs is controlled by transcriptional regulation, zymogen activation, and specific tissue inhibitors of metalloproteinases (TIMPs) [6]. The activation of proMMP-2 is mediated by the membrane type – matrix metalloproteinase (MT-MMPs) and the effective activation of proMMP-2 on the cell surface requires TIMP-2. This occurs by the formation of the ternary proMMP-2/MT1-MMP/TIMP-2 complex. This process takes place at lower TIMP-2 concentrations relative to MT1-MMP to permit availability of enough inhibitor-free MT1-MMP to initiate pro-MMP-2 activation [7]. On the other hand, high levels of TIMP-2 inhibit MMP-2 activation by blocking all free MT1-MMP molecules [8]. Any imbalance in MMPs and TIMPs may lead to an uncontrolled degradation of the ECM and is most likely the cause of invasion [9].

The negative expression of MMP-2 and TIMP-2 in normal oral mucosa observed in our study has been described earlier [10]. It is attributable to the absence of Nuclear factor kappa B (NF-kB) isoform (p65) immunoreactivity in normal oral tissue. Contrary to which, its presence has shown to enhance the expression of MMPs in oral cancer, and its inactivation inhibit the malignant phenotypic features of OSCC [11].

The distribution and the proportion of cells expressing MMP-2 and TIMP-2 in the form of diffuse intra cytoplasmic staining observed in our study is in agreement with several studies [12–17]. It was assumed for long that cancer cells were responsible for producing the MMPs in human tumors until this concept was challenged and the role of stromal cells as the principal source of MMPs were implicated [18]. This was first demonstrated in breast carcinoma where the myofibroblasts that were in close contact with pre-invasive and invasive tumor clusters expressed both the MMP-2 and its activator MT1-MMP [19]. Similarly, the degradative enzymes in the skin tissue were not necessarily produced by malignant cells but by induction or recruitment of non-malignant stromal cells [20]. Expression of MMPs thus, appears to be the property of the whole tumor and not just the reaction of advancing tumor to the host tissue [21].

Increased expression of MMP-2 and TIMP-2 in the four areas studied is in agreement with Charous et al. [21]. Alternatively, Ondruschka et al. [22] found marked expression of MMP-2 and TIMP-2 at the invasive front compared to the central tumor islands. Our observation of MMPs expressing not only in the tumor cells but also in the adjacent stromal cells and inflammatory cells has been reported by Franchi et al., [23] suggesting that the entire tumor may non selectively

Table 1 Clinicopathological details of the patients included in the study

Patient characteristics		Patients (30)	Male (26)	Female (4)
Age in years	40–50	9	8	1
	51–60	16	14	2
	> 60	5	5	1
Tobacco habit	Smoking	30	26	4
	Chewing	30	26	4
Anatomical sites	Buccal mucosa	28	25	3
	Alveolar mucosa	2	1	1
Stage	Stage I	1	1	–
	Stage II	3	2	1
	Stage III	9	8	1
	Stage IV	17	15	2
Regional	Involved	26	23	3
Lymph node	Not Involved	4	3	1
Histological grading of OSCC	Well differentiated	10	8	2
	Moderately differentiated	10	9	1
	Poorly differentiated	10	9	1

cause secretion of these factors, than only the tumor cells at the invasive front.

It is conceived that MMP-2 is localized principally in cancer cells, [10, 23–26] possessing the docking sites for MMPs secreted by stromal cells thus functioning as a receptacle for stromal MMPs [27]. Tumor cells interact with fibroblasts via extracellular matrix metalloproteinase inducer (EMMPRIN) and lead to fibroblast-induced local degradation of basement membrane and ECM components, thus facilitating tumor cell invasion [28]. In addition to the stimulation of stromal cells, EMMPRIN may also facilitate tumor cell dissemination by stimulating MMP production by both tumor cells and endothelial cells [29].

Among the other factors involved in tumor-stromal cell communications is transforming growth factor (TGF), which plays a major role in the transformation of fibroblasts into myofibroblasts and in the stimulation of their production of ECM macromolecules and proteolytic enzymes such as MMP-2 and MT1-MMP [30, 31]. In addition to effecting MMP expression, TGF-1 can also regulate MMP activity by inducing the synthesis of TIMP-2 [32].

It is apparent that stromal cells have the ability to synthesize proforms of MMPs and provide them to the neoplastic epithelial cells [13]. Thus it seems possible that the neoplastic cells receiving proenzymes from surrounding stromal cells activate them by their own MT1-MMP suggesting that MMPs produced by tumor cells and stromal cells are both involved in tumor progression and metastasis [33].

Our study also revealed the expression of TIMP-2 in both the tumor cells and the peritumor stromal cells, as has been demonstrated earlier [34]. The varied expression of MMP-2 and TIMP-2 in our study suggests that OSCC is heterogeneous in its potential to produce MMP-2 and TIMP-2. Absence of expression may be attributed to the decrease in antigenicity of MMP-2 during the process of preparation of paraffin sections after removal of these materials or to smaller amount of MMP being produced in these patients compared with that produced in patient who had MMP expression [25]. It may also suggest that in cases with absent MMP-2/TIMP-2 expression, the tumor invasion and progression of OSCC may be influenced by other independent factors such as MMP-9 and growth factors [23, 25, 35] and an array of cytokines and chemokines that induce leukocyte infiltration to the tumor [36].

The significance of MMP-2 expression with presence of lymph node metastasis has been shown by Danilewitch et al. [37]. Studies have shown that high expression or activity of MMP-2 is linked with poorer survival in HNSCC indicating that with higher expression of MMP-2, tumor cells have increased propensity to invade blood and lymphatic vessels [3, 22, 37–39].

The TIMPs have a critical role in the homeostasis of ECM by regulating the activity of MMPs. In addition to the inherent property of inhibiting MMP collagenolytic activity, a large body of evidence suggests that TIMPs are multifunctional proteins that regulate cell proliferation, apoptosis, angiogenesis and pro-MMP activation [40]. Therefore, the immunoreactivity of TIMP-2 is likely to be useful for monitoring MMP-2 activation. This indicates that TIMPs may have paradoxical roles in tumorigenesis in that it is both inhibitory [41] and stimulatory [22, 39, 41]. Because of the inhibitory and regulatory function of TIMPs a general down-regulation of TIMPs with up regulation of MMPs in malignant tumors might be expected.

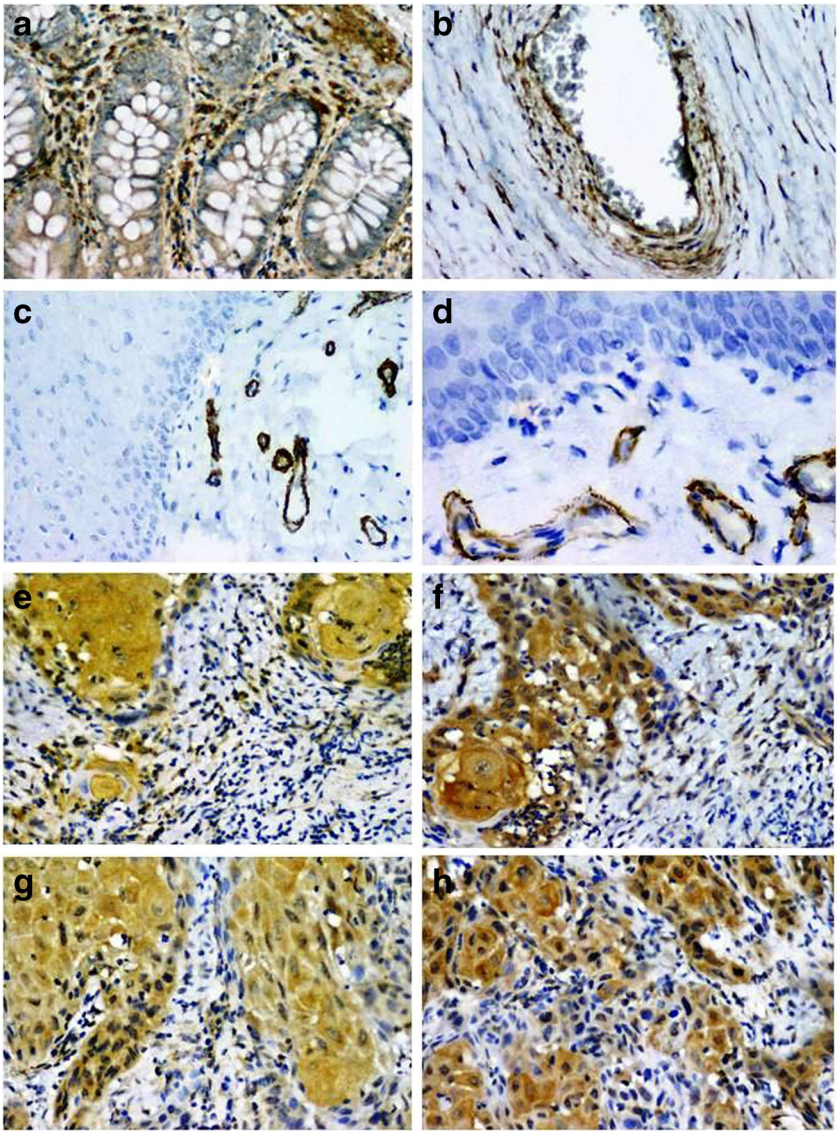
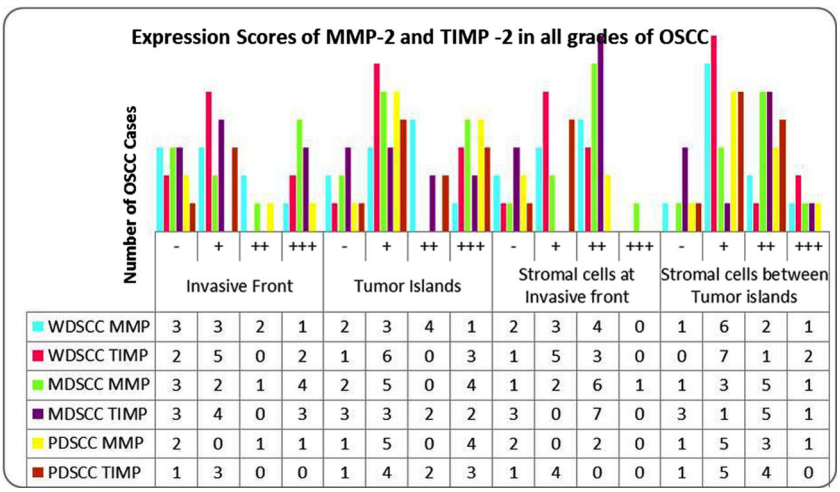


Fig. 1 Photomicrographs showing the immunohistochemical expression of MMP-2 and TIMP-2 in tissue sections (**a**), Inflammatory bowel used as positive control for MMP-2, [IHC (×20)]; (**b**), Placenta used as positive control for TIMP-2, [IHC (×20)]; (**c**), Normal buccal mucosa showing negative expression for MMP-2, [IHC (×20)]; (**d**), Normal buccal mucosa showing negative expression for TIMP-2, [IHC (×20)]; (**e**), Oral squamous cell carcinoma showing MMP-2 positive areas within the tumor cells and at the invasive front, [IHC (×20)]; (**f**), Oral squamous cell carcinoma showing MMP-2 positive areas within tumor cells at the invasive front and in the tumor islands, [IHC (×20)]; (**g**) Oral squamous cell carcinoma showing the expression of MMP-2 positive areas within the tumor cells and in stromal cells between tumor islands [IHC (×40)]; (**h**) Oral squamous cell carcinoma showing the expression of TIMP-2 positive areas within the tumor cells and in stromal cells between tumor islands [IHC (×40)]



	Invasive Front				Tumor Islands				Stromal cells at Invasive front				Stromal cells between Tumor islands			
	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
WDSCC MMP	3	3	2	1	2	3	4	1	2	3	4	0	1	6	2	1
WDSCC TIMP	2	5	0	2	1	6	0	3	1	5	3	0	0	7	1	2
MDSCC MMP	3	2	1	4	2	5	0	4	1	2	6	1	1	3	5	1
MDSCC TIMP	3	4	0	3	3	3	2	2	3	0	7	0	3	1	5	1
PDSCC MMP	2	0	1	1	1	5	0	4	2	0	2	0	1	5	3	1
PDSCC TIMP	1	3	0	0	1	4	2	3	1	4	0	0	1	5	4	0

Fig. 2 Bar diagram showing the expression of MMP-2 and TIMP-2 in all the grades of squamous cell carcinoma [WDSCC- Well differentiated Squamous cell carcinoma; MDSCC- Moderately differentiated Squamous cell carcinoma; PDSCC-Poorly differentiated Squamous cell carcinoma]

Table 2 Expression score of MMP-2 and TIMP-2 in 4 designated areas in all the stages of oral cancer

Area Studied	Stage of OSCC	MMP-2 Score				Total	Chi-square	*P* value	TIMP-2 Score				Total	Chi-square	*P* value
		−	+	++	+++				−	+	++	+++			
Tumor Cell (Invasive Front)	Normal	10	0	0	0	10	18.013	$p = 0.022$ Significant	10	0		0	10	21.334	$p = 0.006$ Significant
	Stage I	1	0	0	0	1			1	0		0	1		
	Stage II	1	2	0	0	3			1	2		0	3		
	Stage III	2	0	2	3	7			1	3		3	7		
	Stage IV	4	3	2	3	12			3	7		2	12		
	Total	18	5	4	6	33			16	12		5	33		
Tumor Cell (Island)	Normal	10	0	0	0	10	27.369	$p < 0.001$ Significant	10	0	0	0	10	29.530	$p = 0.003$ Significant
	Stage I	0	0	1	0	1			0	0	0	1	1		
	Stage II	1	2	0	0	3			1	2	0	0	3		
	Stage III	2	3	1	3	9			1	3	2	3	9		
	Stage IV	1	8	2	6	17			3	8	2	4	17		
	Total	14	13	4	9	40			15	13	4	8	40		
Stromal cell (Invasive front)	Normal	10	0	0	0	10	31.984	$p = 0.006$ Significant	10	0	0	−	10	21.280	$p = 0.006$ Significant
	Stage I	1	0	0	0	1			1	0	0	−	1		
	Stage II	1	1	1	0	3			1	1	1	−	3		
	Stage III	1	0	5	1	7			0	3	4	−	7		
	Stage IV	2	4	6	0	12			3	4	5	−	12		
	Total	15	5	12	1	33			15	8	10	−	33		
Stromal cell (Island)	Normal	10	0	0	0	10	30.892	$p = 0.001$ Significant	10	0	0	0	10	30.837	$p = 0.002$ Significant
	Stage I	0	1	0	0	1			0	1	0	0	1		
	Stage II	1	1	1	0	3			1	1	1	0	3		
	Stage III	1	3	3	2	9			0	3	4	2	0		
	Stage IV	1	9	6	1	17			3	8	5	1	17		
	Total	13	14	10	3	40			14	13	10	3	40		

In this study we found a positive correlation of MMP-2 with TIMP-2 in different stages, grades and lymph node status. This process may occur only at low TIMP-2 concentrations relative to MT1-MMP to permit availability of enough inhibitor-free MT1-MMP to initiate pro-MMP-2 activation. On the other hand, high levels of TIMP-2 inhibit activation by blocking all free MT1-MMP molecules [24].

In previous immunohistochemical studies, [22, 39, 41–43] elevated TIMP-2 levels had been identified as indicators of aggressive behavior and poor prognosis among patients with

Table 3 Expression of MMP-2 and TIMP-2 in different areas correlating with lymph node status

Area Studied	Lymphnode Metastasis	MMP-2 Score				Total	Chi-square	*P* value	TIMP-2 Score				Total	Chi-square	*P* value
		−	+	++	+++				−	+	++	+++			
Tumor Cell (Invasive Front)	No	12	2	0	0	14	11.711	$p = 0.008$ Significant	12	2	0	0	14	11.711	$p = 0.008$ Significant
	Yes	6	3	4	6	19			6	3	4	6	19		
	Total	18	5	4	6	33			18	5	4	6	33		
Tumor Cell (Island)	No	11	2	1	0	14	18.904	$p < 0.001$ Significant	11	2	1	0	14	18.904	$p < 0.001$ Significant
	Yes	3	11	3	9	26			3	11	3	9	26		
	Total	14	13	4	9	40			14	13	4	9	40		
Stromal cell (Invasive front)	No	12	1	1	0	14	16.146	$p = 0.001$ Significant	12	1	1	0	14	16.146	$p = 0.001$ Significant
	Yes	3	4	11	1	19			3	4	11	1	19		
	Total	15	5	12	1	33			15	5	12	1	33		
Stromal cell (Island)	No	11	2	1	0	14	21.070	$p < 0.001$ Significant	11	2	1	0	14	21.070	$P < 0.001$ Significant
	Yes	2	12	9	3	26			2	12	9	3	26		
	Total	13	14	10	3	40			13	14	10	3	40		

Table 4 Expression score of MMP-2 and TIMP-2 in 4 designated areas in all the grades of oral cancer

Area Studied	Grades of OSCC	MMP-2 Score				Total	Chi-square	*P* value	TIMP-2 Score				Total	Chi-square	*P* value
		−	+	++	+++				−	+	++	+++			
Tumor Cell (Invasive Front)	Normal	10	0	0	0	10	17.230	$p = 0.045$ Significant	10	0	0	0	10	18.013	$p = 0.006$ Significant
	WDSCC*	3	3	2	1	09			2	5	0	2	09		
	MDSCC**	3	2	1	4	10			3	4	0	3	10		
	PDSCC***	2	0	1	1	04			1	3	0	0	04		
	Total	18	5	4	6	33			16	12	0	5	33		
Tumor Cell (Island)	Normal	10	0	0	0	10	39.106	$p < 0.001$ Significant	10	0	0	0	10	27.369	$p = 0.001$ Significant
	WDSCC	2	3	4	1	10			1	6	0	3	10		
	MDSCC	1	5	0	4	10			3	3	2	2	10		
	PDSCC	1	5	0	4	10			1	4	2	3	10		
	Total	14	13	4	9	40			15	13	4	8	40		
Stromal cell (Invasive front)	Normal	10	0	0	0	10	22.477	$p = 0.007$ Significant	10	0	0	−	10	31.984	$p < 0.001$ Significant
	WDSCC	2	3	4	0	09			1	5	3	−	09		
	MDSCC	1	2	6	1	10			3	0	7	−	10		
	PDSCC	2	0	2	0	4			1	3	0	−	04		
	Total	15	5	12	1	33			15	8	10	−	33		
Stromal cell (Island)	Normal	10	0	0	0	10	30.892	$p < 0.001$ Significant	10	0	0	−	10	30.892	$p < 0.001$ Significant
	WDSCC	1	6	2	1	10			0	7	1	2	10		
	MDSCC	1	3	5	1	10			3	1	5	1	10		
	PDSCC	1	5	3	1	10			1	5	4	0	10		
	Total	13	14	10	3	40			14	13	10	3	40		

OSCC Oral Squamous Cell Carcinoma, *WDSCC** Well differentiated squamous cell carcinoma, *MDSCC*** Moderately differentiated squamous cell carcinoma, *PDSCC**** Poorly differentiated squamous cell carcinoma

HNSCC. Katayama et al. [42] showed that the marked expression of TIMP-2 strongly correlated with lymph metastasis as well as with poor prognosis in early-stage OSCC, and was the only independent factor for poor prognosis. Likewise, Yoshizaki et al. [39] and Ondruschka et al. [22] have demonstrated high expression of TIMP-2 as the only independent factor for poor prognosis in OSCC. In contrast these findings, Imanishi et al. [13] and Danilewicz et al. [37] found no difference in expression of TIMP-2 in cases with or without regional lymph node metastasis.

The role of TIMPs in the process of tumor growth, invasion and metastasis formation is somewhat unclear. TIMPs have

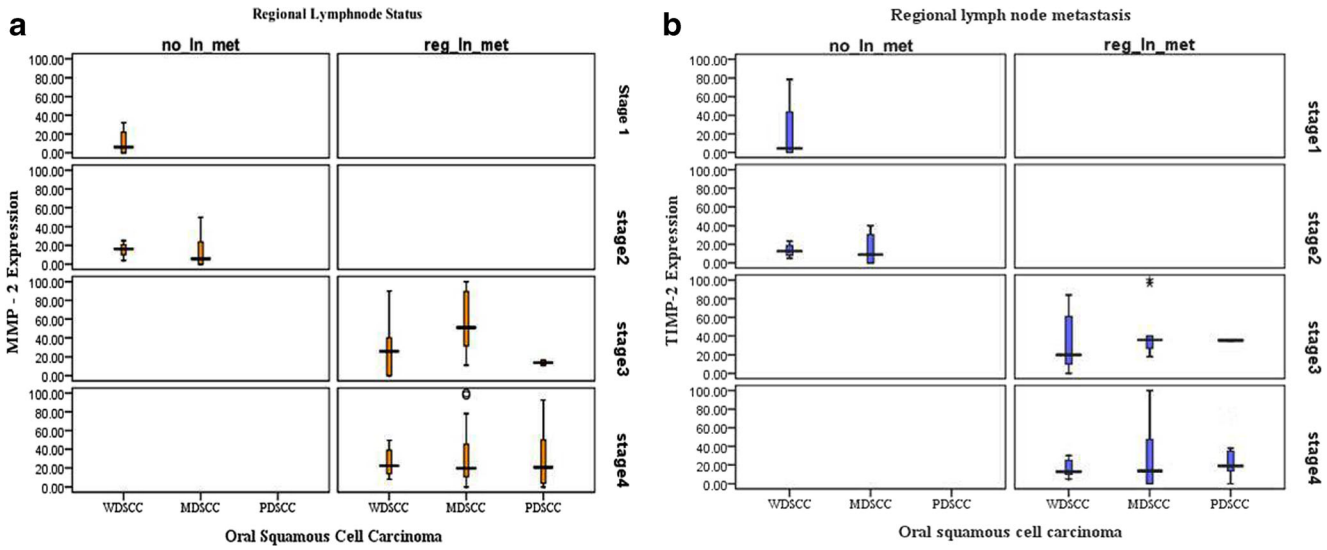


Fig. 3 Box plot showing the median scores of (**a**) MMP-2 and (**b**) TIMP-2 expression level of TIMP-2 with regard to different histological grades, clinical stages and lymph node status respectively

Table 5 Spearman's correlation between expression of MMP-2 and TIMP-2 in different areas in different grades, stage and patients' with different lymph node status

Variables	Categories	Markers	Spearman's rho Correlation Coefficient		*P* value
			MMP-2	TIMP-2	
Tumor Areas	Tumor Cells – Invasive Front	MMP-2	1.000	0.891	<0.001
		TIMP-2	0.891	1.000	
	Tumor Cells – Tumor Islands	MMP-2	1.000	0.828	<0.001
		TIMP-2	0.828	1.000	
	Stromal Cells – Invasive Front	MMP-2	1.000	0.822	<0.001
		TIMP-2	0.822	1.000	
	Stromal Cells – Tumor Islands	MMP-2	1.000	0.810	<0.001
		TIMP-2	0.810	1.000	
Grading of OSCC	Well differentiated SCC	MMP-2	1.000	0.448	=0.005
		TIMP-2	0.448	1.000	
	Moderately differentiated SCC	MMP-2	1.000	0.873	<0.001
		TIMP-2	0.873	1.000	
	Poorly differentiated SCC	MMP-2	1.000	0.521	=0.004
		TIMP-2	0.521	1.000	
Staging of Oral Cancer	Stage I	MMP-2	1.000	1.000	<0.001
		TIMP-2	1.000	1.000	
	Stage II	MMP-2	1.000	0.848	<0.001
		TIMP-2	0.848	1.000	
	Stage III	MMP-2	1.000	0.701	<0.001
		TIMP-2	0.701	1.000	
	Stage IV	MMP-2	1.000	0.616	<0.001
		TIMP-2	0.616	1.000	
Regional Lymph node metastasis	Without Lymph node metastasis	MMP-2	1.000	0.995	<0.001
		TIMP-2	0.995	1.000	
	With lymph node metastasis	MMP-2	1.000	0.642	<0.001
		TIMP-2	0.642	1.000	

been shown to act as an antiapoptotic agent and growth-stimulating factor [44] on one hand and on other hand, there is some evidence showing that increased TIMP expression might suppress tumor progression [8, 26, 45]. There is evidence that TIMPs bind to the cell surface with high affinity, indicating that they may work as a ligand similar to some cytokines and growth factors [44, 46, 47]. ProMMP-2 forms a tight complex with TIMP-2 through their C-terminal domains, therefore permitting the N-terminal inhibitory domain of TIMP-2 in the complex to bind to MT1-MMP on the cell surface. Alternatively, MT1-MMP inhibited by TIMP-2 can act as a "receptor" of proMMP-2. This MT1-MMP/TIMP-2/proMMP-2 complex is then presented to an adjacent free MT1-MMP for activation. Clustering of MT1-MMP on the cell surface through interactions of the hemopexin domain facilitates the activation process [46, 48]. By this model it could be demonstrated that when the molar ratio of MT1-MMP to TIMP-2 is in the range 3:1 to 3:2, activation of proMMP-2 will be enhanced compared to the absence of exogenous TIMP-2 where no increase in proMMP-2 activation has been noted. Also when the molar ratio is 7:6 and excess, TIMP-2 resulted in the inhibition of activation [47].

The role of TIMPs in cancer cell growth and progression is extremely complex. The net effect of TIMPs on tumorigenesis may depend on the bioavailability of the local amount of TIMPs in the tumor environment. Higher levels of TIMPs may have a tumor suppressing effect due to their dominant anti-MMP effect, while lower levels of TIMPs may favor tumor growth, due to their anti-apoptotic role [3, 48].

In the present study, a positive correlation could be established between the expressions of TIMP-2 with the stage of tumor. These data although are contradictory to the report of Kugler et al. [49] and Kallakury et al., [50], are similar to those of Liu et al. [38] and Katayama et al. [42]. These findings support our hypothesis that the expression of MMP-2 could be an intrinsic, biological feature of individual tumors that may indicate aggressive behavior of the tumor regardless of the disease stage whereas TIMP-2 shows relationship with disease stage.

Some studies demonstrate that the expressions of MMP-2 and TIMP-2 correlate with histological grade [17, 23, 50, 51]. Our study showed no significant relationship between degree of expression of MMP-2 and TIMP-2 and histologic differentiation of OSCCs, a finding observed by several others [3, 25, 38]. With increase in grade, cancer cells may lose their ability to

produce MMP on their own because of dedifferentiation [9]. Baker et al. [52] on the other hand found a positive MMP-2 and negative TIMP-2 correlation between the grades of OSCC.

It remains unclear whether the overexpression of a particular enzyme reflects its functional role in the malignant process or whether such overexpression is a sign of the host response to the tumor itself as MMP produced by inflammatory cells may make a significant contribution to squamous cell carcinogenesis [23].

In summary, our observations support and strengthen that MMP-2 and TIMP-2 are secreted by both the tumor cells and stromal cells, and their activity regulate the concentration of MMP-2 adjacent to the tumor. Expressions of MMPs and their inhibitors may not be separately evaluated as both of them are the physiologic players of regulation. A rate of MMP-2/TIMP-2 expression is better for characterization of MMP-2 activity. In our study, an increased MMP-2 enzyme activity adjacent to the invasive front of tumors exhibited apparently direct correlation with infiltrative and metastatic capacities of tumors. These results deceivingly support the erroneously presumed partnership between tumor cells and their environment for the promotion of intense tumor propagation and dissemination.

According to recent data, tumor cells should not be regarded as wicked enemies, rather as integrative but defective parts of the whole body of patients. Nevertheless, tumor cells are embarrassed by the partial or near total loss of the highest intranuclear control machinery, which earlier safeguarded their somatic and reproductive controls [53]. Consequently, cancer cell invasion does not depend on the mechanical resistance of dense connective tissue. In the vicinity of tumors, the degradation of local connective tissue and the induction of angiogenesis by increased MMP expression may be evaluated as a possibility for the recruitment of cellular and humoral factors for hormonal and immunologic defense against the proliferation of malignant cells. A new insight suggests that in case of infiltrative and even metastatic cancers, the stronger MMP-2 secretion produced by both tumor cells and stromal components may serve as local cellular defense against cancer cell proliferation.

In conclusion; there is no controversial “dual role” of TIMP-2 expression. In mammalians, the local tissue reactions against cancers are always protective and even malignant tumor cells may have some preserved regulatory activities helping their own apoptotic death [54]. At the advanced stage of tumors, the intense defensive partnership between tumor cells and their environment may become insufficient in spite of the increased concentration of MMPs.Earlier studies predominantly support the role of MMP-2 and TIMP-2 synthesis in determining the aggressiveness of oral cancer. A new concept, supporting the dynamic, anticancer partnership between tumor cells and stromal cells may illuminate the source of controversial results [-55] concerning the correlations between the regulation of MMP-2/TIMP-2 expression and the aggressiveness of oral cancer.

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