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ORIGINAL ARTICLE

Immunohistochemical expression and significance of MMP1 in oral squamous cell carcinoma in relation to tumour depth

Immunohistochemiczna ekspresja i znaczenie MMP1 w raku płaskonabłonkowym jamy ustnej w odniesieniu do głębokości guza

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Abstract

Introduction. *Oral squamous cell carcinoma (OSCC) is a malignancy of stratified squamous epithelium, beginning as an epithelial dysplasia and progressing until the dysplastic epithelial cells break the basement membranes (BM) and invade the underlying connective tissue. It is estimated that over 90% of all oral neoplasms are OSCCs. Matrix metalloproteinase-1 (MMP1) is an enzyme that in humans is encoded by the MMP1 gene which causes degradation of the extracellular matrix (ECM) and BM, and thus may play a key role in development and local invasion of OSCC. It can serve as a potential biomarker molecule for diagnosis, treatment and prognostic evaluation. Tumour depth (TD) is considered to be a more reliable feature, as many studies have shown that the risk of metastasis and spread to cervical lymph node (LN) increases with an increase in TD.* ***Materials and Methods.*** *Forty-five formalin-fixed, paraffin-embedded blocks of totally excised OSCC collected pro- and retrospectively were included in this study. Hematoxylin & Eosin stain was performed for each block for reassessment of histopathological examination. An immunohistochemical (IHC) staining was performed using anti-MMP1 monoclonal antibodies.* ***Results.*** *The majority of the OSCC sample revealed TD of more than 7 mm (57.78%), with a maximum registered depth of 18 mm. Furthermore, the data demonstrated a significant correlation between TD and cervical lymph node metastasis. Immunohistochemically, the tumour*

Streszczenie

Wstęp. *Rak płaskonabłonkowy jamy ustnej (OSCC) to nowotwór złośliwy wielowarstwowego nabłonka płaskiego, zaczynający się jako dysplazja nabłonka do momentu naruszenia ciągłości błony podstawnej przez komórki dysplastyczne i zaatakowaniu leżącej u podstaw tkanki łącznej. Szacuje się, że ponad 90% wszystkich nowotworów jamy ustnej to OSCC. Metaloproteinaza macierzy pozakomórkowej-1 (MMP1) to enzym u ludzi kodowany przez gen MMP1, powodujący degradację macierzy pozakomórkowej i błony podstawnej i w ten sposób może odgrywać kluczową rolę w rozwoju raka płaskonabłonkowego. Może również służyć jako potencjalny biomarker dla diagnozy, leczenia i oceny rokowania. Głębokość nacieku guza jest uważana za bardziej wiarygodny wskaźnik gdyż wiele badań wykazało, że ryzyko przerzutu do szyjnych węzłów chłonnych wzrasta wraz ze wzrostem głębokości naciekania.* ***Materiał i metody.*** *Prospektywnie i retrospektywnie zgromadzono czterdzieści pięć utrwalonych w formalinie i zatopionych w parafinie bloczków całościowo wyciętych guzów płaskonabłonkowych, które włączono do obecnego badania. Każdy bloczek barwiono hematoxyliną i eozyną w celu powtórnej oceny badania histopatologicznego. Immunohistochemiczne (IHC) barwienie przeprowadzono przy użyciu anty-MMP1 monoklonalnych przeciwciał.* ***Wyniki.*** *Większość próbek raka płaskonabłonkowego ujawniło głębokość guza większą niż 7 mm (57,78%), a*

KEYWORDS:
OSCC, MMP1, Immunohistochemistry, tumour depth

HASŁA INDEKSOWE:
OSCC, MMP1, immunohistochemia, głębokość nacieku guza

cells mostly showed MMP1 overexpression in score 4 (55.56%). Statistically, the MMP1 showed significant correlation with TD. ***Conclusion.*** *A significant correlation was seen regarding the expression of MMP1with TD suggesting that degradation and collagenolytic activity against collagens in carcinoma tissue extract was associated with deeper invasion.*

maksymalna głębokość wyniosła 18 mm. Ponadto dane pokazały istotną zależność między głębokością guza a przerzutem do szyjnych węzłów chłonnych. Immunohistochemicznie, komórki guza przeważnie wykazywały nadekspresję MMP1 (score 4 – 55,56%). Statystycznie, MMP1 wykazało istotną korelację z głębokością guza. ***Wniosek.*** *Wykazano istotną korelację pomędzy ekspresją MMP1 a głębokością nacieku guza sugerując, że degradacja i rozkład kolagenu w tkankach nowotworowych ma miejsce w przypadkach pogłębionej inwazji.*

Introduction

OSCC is a malignancy of stratified squamous epithelium, beginning as an epithelial dysplasia, and progressing until the dysplastic epithelial cells break the BM and invade the underlying connective tissue.[1,2] Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However, it is estimated that more than 90% of all oral neoplasms are OSCC.[3]

MMP1 is an enzyme that in humans is encoded by the MMP1 gene. The matrix metalloproteinases (MMPs) cause degradation of the ECM and BM, and thus may play a key role in cancer development.[4] The relative expression level of MMP1 mRNA was higher in histological grade II/III tissues than in grade I, higher in OSCC in advanced stages (III/IV) than in tumours in early stages (I/II). MMP1 gene may play a role in local invasion of OSCC, and can serve as a potential biomarker molecule for diagnosis, treatment and prognostic evaluation of OSCC.[5] MMPs can be inactivated by specific tissue inhibitors of matrix metalloproteinases [TIMPs]. Thus far, four different TIMPs [TIMP-1, -2, -3, -4] have been identified.[6] Recent studies have associated increased MMPs expression and decreased TIMP expression with tumour aggressiveness; however, other studies have shown overexpression of TIMPs in some patients with advanced tumours.[7]

TD has not been uniformly measured to date. Most authors used an optical micrometer to measure thickness.[8] Some of them measured the distance from the deepest point of tumour invasion to the most protruding part of the lesion (tip of the papilla) in exophytic type, and to the ulcer base in ulcerated lesions,[8,9] whereas others performed measurements from the deepest point of the tumour to an imaginary line that reconstructed the healthy mucosa.[9,10] TD is considered to be a reliable feature. Since many studies have shown that the risk of metastasis and spread to cervical LN increases with an increase in TD, it is reasonable to think that the most aggressive tumours are those with the greatest capacity to grow downwards vertically.[11]

The purpose of the present study is to assess the correlation of expression of the MMP1 in OSCC and TD.

Materials and methods

A total of forty-five retrospective formalin-fixed, paraffin-embedded blocks of totally excised OSCC were collected pro- and retrospectively from the archives of Oral and Maxillofacial Pathology Department, College of Dentistry, University of Baghdad, Al-Shaheed Ghazi hospital, Al-Yarmok hospital. Four µm thick sections were cut and hematoxylin and eosin slides were prepared for histopathological reassessment. Another 4 µm thick sections were cut for IHC staining with anti-MMP1monoclonal antibodies (Abcam, UK). Negative and positive controls were included in each IHC run. Tissue blocks of cervical carcinoma were used for MMP1 (according to antibodies' manufacturer).

Tumour depth measurement

An optical micrometer was used to measure the distance (to the nearest mm) from an granular

cell layer to the deepest point of tumour invasion – in ulcerated lesions the thickness was measured from the base of the ulcer instead of granular cell layer, disregarding any superficial keratin layer or inflammatory infiltrate that may exist in all cases. For each section, the power field (4X) was used. The patients were classified into three groups according to their TD: 1: ≤3mm, 2: 4–7 mm, 3: >7 mm.

Evaluation of IHC results

The immunoreactions evaluation was analyzed according to the presence or absence of brown immunostaining in the ECM and cytoplasm. The percentage of positive cells was scored as follows: 0: <10% positive cells, 1: <25% positive cells, 2: 25-50% positive cells, 3: 50-75% positive cells and 4: >75% positive cells.

Statistical analysis

Numerical values were used in this study for describing the variables which included: Number, mean, SD for age, MMP1. Categorical variable which included: sites, grade, gender and clinical presentation was described using number and percentage. Chi-square test for the relationship between categorical variables. Statistical analysis was done using SPSS (statistical package for social sciences) V16. The <0.05 level was considered significant, while the <0.001 level was considered highly significant for the interpretation of P values.

Results

Clinicopathological findings of OSCC cases were designed as follows: most of the cases (29 – 64.44%) were older than 50 years with an age range from 22 to 82 years (mean ± SD = 55.67+15.45), and the majority of the cases were males (27 – 60 %). The most common site was the tongue (22 cases – 48.89%) and most of the cases were presented as mass 24 cases (53.33%).

Hematoxylin and eosin pathological analysis revealed that out of 45 OSCC cases, more than a half were well differentiated OSCC 23 cases (51.11%), 18 cases (40%) were moderately differentiated OSCCs, and the remaining 4 cases (8.89%) were poorly differentiated OSCCs (Table 1).

Table 1. Clinico-pathological characteristics of 45 OSCC cases

	Frequency	Percentage %
Age		
22-50	16	35.56
>50	29	64.44
Gender		
Male	27	60
Female	18	40
Clinical Presentation		
Ulcer	21	46.67
Mass	24	53.33
Site		
Buccal mucosa	6	13.33
Tongue	22	48.89
Lip	8	17.78
Gingiva	3	6.67
Palate	2	4.44
Floor of mouth	1	2.22
Maxilla	3	6.67
Histological Grading		
Well	23	51.11
Moderate	18	40.00
Poor	4	8.89

Table 2. Frequency of tumour depth in 45 OSCC

Tumour depth	Frequency	Percentage
≤3 mm	6	13.33
4-7 mm	13	28.89
>7 mm	26	57.78
Total	45	100

Table 3. Correlation of tumour depth and lymph node

LN	Tumour depth					
	<7mm		≥7mm		Total	
	No.	%	No.	%	No.	%
Absent (0)	17	37.78	16	35.56	33	73.33
Present (1)	2	4.44	10	22.22	12	26.67
Total	19	42.22	26	57.78	45	100

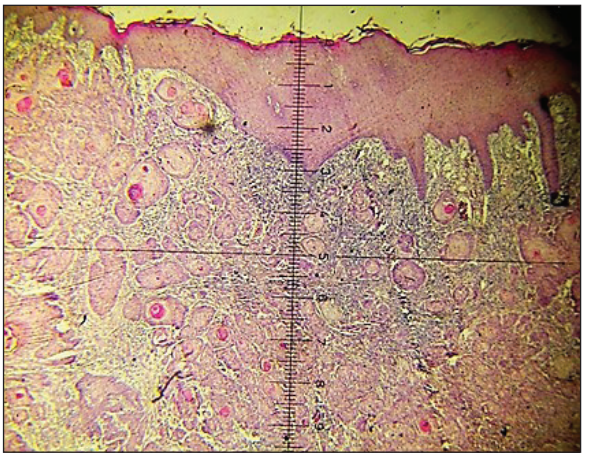

Fig. 1. *Measuring tumour depth (40X).*

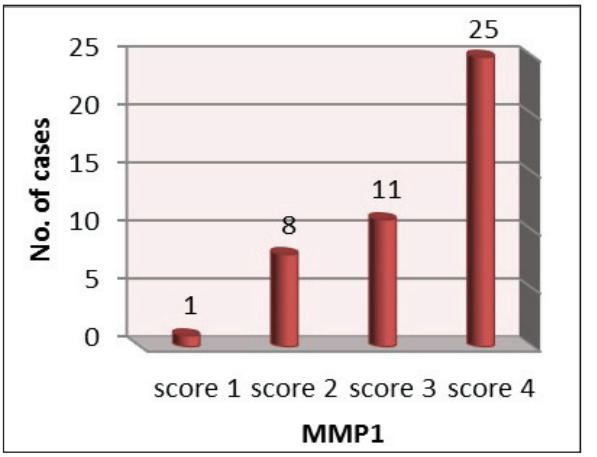

Fig. 2. *Frequency of MMP1 expression in 45 cases of OSCC.*

Concerning the TD, 26 cases (57.78%) revealed TD from the granular cell layer (or the surface if the lesion is ulcerated) to the deepest point of tumour cells invasion of more than 7 mm, 13 cases (28.89%) showed 4-7 mm depth, and 6 cases (13.33%) were less than 3 mm in depth. The mean depth of tumour invasion was 6.98 mm (SD+2.67) with a maximum registered depth of 17 mm (Table 2 and Fig. 1). Moreover, the present study revealed a statistically significant association between TD and lymph node involvement (P=0.036) (Table 3).

Evaluation of MMP-1 IHC

MMP1 expression was detected as a brown staining in the cytoplasm and ECM, all 45 cases showed positive expression (100%). The majority of the cases strongly expressed MMP1 in score 4 (25 cases – 55.56%), followed by score 3 reported in 11 cases (24.44%), score 2 in 8 cases (17.78%), while score 1 had the lowest percentage which occurred in only one case (2.22%) (Fig. 2).

According to the Chi-Square test, the present study revealed a statistically significant correlation regarding MMP1 expression in relation to the age (P-value = 0.012), while gender (P-value = 0.263),

Table 4. Correlation of MMP1 with Tumour depth

MMP1 scoring	Tumour thickness		
	≤3 mm	4-7 mm	>7 mm
1	0	0	1
2	1	4	3
3	1	2	8
4	4	7	14
Total	6	13	26

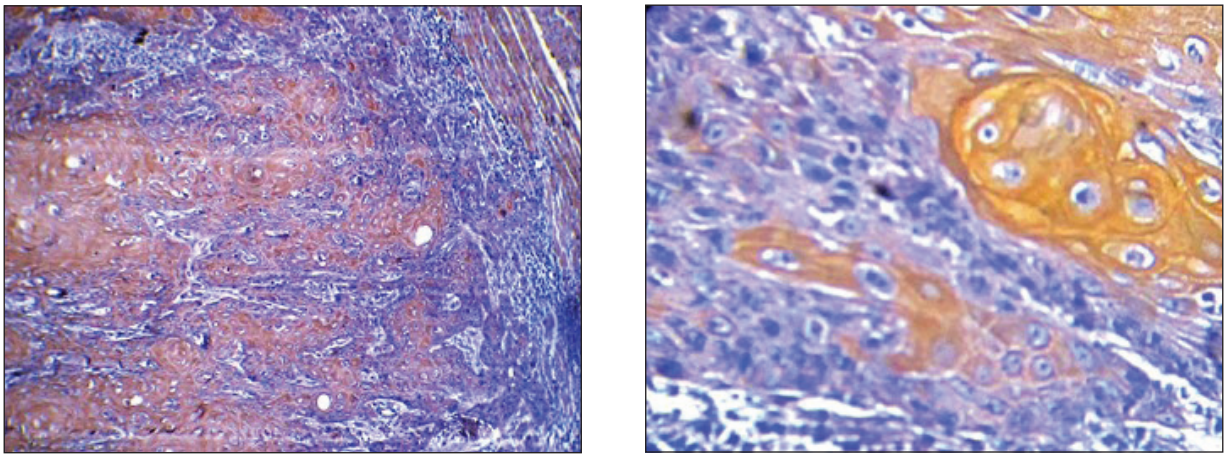

Fig. 3. *Positive immunostaining expression of MMP1 in OSCC.*

site (P-value = 0.879), and clinical presentation (P-value = 0.262) showed statistically insignificant correlation . Furthermore, the present study showed statistically insignificant association between MMP1 and the stage of the tumour (P-value = 0.847), while significant association was found with tumour grade (P-value = 0.047).

Concerning the TD, the present study revealed a statistically significant correlation between MMP1 and TD (P-value = 0.037) (Table 4).

Discussion

This study is not a large epidemiological one that expressed the incidence and prevalence of different clinical-pathological features of OSCC, therefore the limited number and the random selection of the available cases precludes definitive clinical findings.

The tumour size usually affects the choice and outcome of treatment.[12] It also affects the surgeon's ability to achieve complete resection, especially in deep invading tumours. Increased tumour size has been linked to cervical involvement, high recurrence rate,[13] and poor prognosis.[14] The association of TD with LN metastasis is believed to reflect the aggressiveness of tumour growth.[15]

In the present study, the majority of the OSCC sample revealed TD of more than 7 mm (57.78%), the mean TD was 6.98 mm + 2.67, with a maximum registered depth of 18 mm. Furthermore, the data demonstrated a significant correlation between TD and cervical LN metastasis (P=0.036). Similar results were recorded in previous studies.[16,17] Moreover, *Haksever* et al. concluded that the critical TD is when the tumour is invading the musculature and that is highly associated with subclinical nodal metastases.[17] The relationship between thickness of the primary tumour and occurrence of contralateral cervical metastasis were reported to increase by 5% in T1/ T2 SCC of the tongue.[18] However, recent studies suggested that tumour size did not predict nodal disease, and it is now widely accepted that TD is a more accurate predictor of sub-clinical nodal metastasis, local recurrence and survival than tumour size.[13,19]

The MMPs are a family of zinc-dependent endopeptidases, proteolytic enzymes that can decompose ECM components, like collagen, gelatin, elastin, fibronectin, and the proteoglycans. They also break down BM around transformed keratinocytes and vessel or lymphatic duct epithelium, and thus assisst local tumour invasion and metastasis.[20] The most abundant member of this family is MMP1, which has been reported to be strongly associated with tumour development, invasion, and metastasis as well as angiogenesis and thrombosis.[21] *Yang* et al. concluded that overexpression of MMP1 induces epithelialmesenchymal transition and results in the acquisition of cancer stem cells-like properties in SCC cells, with increased expression of mesenchymal markers (vimentin and fibronectin).[22] *George* et al. found that 100%

of OSCCs showed cytoplasmic immune reactivity for MMP1 in the epithelial and connective tissue cells.[23]

In our study sample, the tumour cells mostly showed MMP1 overexpression in score 4 (55.56%), while the lowest expression was noted in score 1 (2.22%). This is in agreement with the findings of *Zhu* et al.,[24] *George* et al.,[23] and *Yang* et al.[22] However, the nature of their roles in each head and neck primary site remains unresolved due to methodological differences between studies in terms of MMP1 detection and the relatively small sample sizes.[20]

Concerning the tumour stage and grade, the result of the present study showed insignificant association between expression of MMP1 and the tumour stage. This was also observed in the study by *Gomes* et al.[25] By contrast, *Chiu* et al. found MMP1to increase the risk of HNSCC progression for advanced stages (III-IV).[26] Regarding the grade, our series revealed a significant association with tumor grade (P=0.047), which is consistent with *George* et al.[23] who found that in OSCC the epithelial and connective tissue cells MMP1 expression was elevated as the histopathological grade varied from well to poorly differentiated, and concluded that elevated MMP1 protein expression is associated with higher histopathological grade of OSCC. In accordance with these figures, Chiu et al. suggest that MMP1 most likely contributes to tumour development and cell differentiation of OC patients in Taiwan.[26] But also opposite results on MMP1 immunohistochemical expression and tumour grade exist.[25,27]

Regarding the TD, *Shiozawa* et al. found that MMP1 immunoreactivity was significantly correlated with the depth grading of tumour invasion, and that the degree of MMP1 expression was higher in cases showing an infiltrative growth pattern than in cases showing an expansive pattern.[28] *Van der Stappen* et al. have shown that increased collagenolytic activity against collagens of types I and III in carcinoma tissue extract was associated with deeper invasion, and that such degradation is effected mainly by MMP1.[29] Consistently, *O-Charoenrat* et al. found the similar result in European population and concluded that the 2G allele was associated with larger tumour size.[30] Our results support this contention and indicate that MMP1 expression by tumour cells is closely involved in the facilitation of the degree of invasion depth in the OSCC.

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