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Expression of MMP-7 and MT1-MMP in oral squamous cell carcinoma as predictive indicator for tumor invasion and prognosis

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BACKGROUND: Squamous cell carcinoma of the oral cavity is a highly invasive neoplasm that spreads locally and metastasizes to regional lymph nodes. This process involves multiple proteolytic enzymes including matrilysin (MMP-7) and membrane type I-matrix metalloproteinase (MT1-MMP). This study was designed to explore the association between MMP-7 and MT1-MMP in the invasiveness and prognosis of oral squamous cell carcinoma (OSCC).

METHODS: About 4-μM, formalin-fixed, paraffin-embedded tissue sections from 69 patients with OSCC were immunohistochemically studied using specific antibodies against MMP-7 and MT1-MMP proteins. Immunostaining was semiquantitatively scored, and results were correlated with histologic and clinical variables including clinical behavior and survival.

RESULTS: MMP-7 was observed only in cancer cells, and MT1-MMP in both tumoral tissue and stroma. MMP-7 expression was significantly correlated with lymph node metastasis ($P = 0.03$; RR = 3.2). MT1-MMP showed a significant association with TIMP-2 (in N+ cases) and p53 expression ($P = 0.01$). MMP-7 and MT1-MMP displayed a survival relevance, and in multivariate analysis they were independent prognostic indicators, particularly in neck node-positive cases.

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Keywords: immunohistochemistry; MMP-7; membrane type I-matrix metalloproteinase; oral squamous cell carcinoma; prognosis

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Introduction

Oral squamous cell carcinoma (OSCC), a common cancer in the head and neck region, is characterized by an aggressive growth pattern, a high degree of local invasiveness and cervical lymph node spread (1). Over the last two decades, diagnosis and treatment of this disease has improved, but long-term survival rates have increased only marginally. Currently, the only specific prognostic factors that are commonly used in OSCC are: site where the primary tumor arises, tumor size, neck node status and presence of distant metastasis. However, in their biologic behavior OSCC are characterized by a significant heterogeneity, and tumors of the same clinical stage often show differences in clinical course and treatment response. Thus, identification of factors affecting invasion and metastasis, and establishment of biomarkers to predict malignant potential and identify different risk groups are of a paramount importance. Among these biomarkers, matrix metalloproteinases (MMPs) have been implicated in tumor invasiveness and several of them have shown prognostic significance in several human cancers (2). MMP-7 (matrilysin) has the capacity to start an activation cascade of MMPs, and is able to degrade a variety of extracellular matrix (ECM) substrates, including elastin, laminin, type IV collagen, and others (3). But matrilysin not only degrades ECM macromolecules, but also acts on other substrates that may modulate cell behavior (4, 5). Thus, MMP-7 seems to play a central role in tumor invasion and metastasis (6). Other important characteristic of MMP-7 is that, unlike the other MMPs which are also synthesized by stromal cells, matrilysin is produced exclusively by cancer cells (7).

There are other MMPs that are believed to play a major role in tumor invasion and metastasis, such as MMP-2, MMP-9, and MMP-14. MMP-2 and MMP-9 cleave type IV collagen, the main component of the basement membrane (8, 9), and MT1-MMP (MMP-14),

that has been originally identified as an activator of proMMP-2 (10), was lately demonstrated that it also degrades other components of the ECM such as proMMP-13, types I, II, III collagen, gelatin, fibronectin, laminin, fibrin, and proteoglycans (11). Hence, it has been shown that this membrane anchored MMP serves as the dominant purveyor of the tissue-invasive activities necessary to support the trafficking of normal and neoplastic cells through the ECM (12).

Expression of MMP activity can be controlled at different levels such as gene transcription, proenzyme activation, and specific inhibitors (13). A family of tissue inhibitors of matrix metalloproteinases (TIMPs) is capable of inhibiting fully activated MMPs, and the local balance between MMPs and TIMPs seems to be crucial in the control of tumor invasion and metastasis. The most studied of these inhibitors are TIMP-1 and TIMP-2. Expression of MMPs, specifically MMP-7 and MT1-MMP in OSCC has already been reported (14–17); however, the correlation between expression and clinical parameters or prognosis in oral carcinoma is still controversial.

Other factors related to cancer biology, and with potential clinical relevance are the p53 gene and the proliferative cell index. The p53 tumor suppressor gene is inactivated in 40–50% of OSCC (18) and it has been suggested that this abnormality plays a critical role in the onset or progression of many cancers. The proliferative capacity showed by the carcinoma cells is believed to have prognostic significance. Ki-67 nuclear antigen is associated with cell proliferation and is a reliable marker to evaluate tumor growth rate (19).

The aim of the current study was to investigate immunohistochemically the expression of MMP-7 and MT1-MMP as well as the relation of these proteases with MMP-2, MMP-9, TIMP-1 and TIMP-2, p53, and cell proliferation. Using this data, we then determined whether MMP-7 and/or MT1-MMP may be associated with progression of OSCC and also whether they may be used as prognostic indicators.

Materials and methods

Patients

This study is based on a retrospective cohort of 69 patients with primary OSCC diagnosed at the Department of Oral and Maxillofacial Surgery, Asturias University Hospital, Oviedo, Spain, between January of 1990 and December of 1992. Inclusion criteria were surgical treatment performed according to standard procedures and consisting of the resection of the primary tumor and a radical or selective ipsilateral or bilateral neck dissection, complete clinicopathologic data, and availability of sufficient paraffin-embedded tumor material. Clinicopathologic information on each case, including age, gender, smoking and alcohol intake history, tumor size, nodal status, location, histologic grade, treatment, and presence or absence of tumor recurrence was obtained from patient files.

There were 15 (22%) women and 54 (78%) men, ranging in age from 24 to 87 years (mean, 59.3 years).

Table 1 Clinical features in 69 patients with oral squamous cell carcinoma

Characteristic	*Number of cases (%)*
Gender	
Male	54 (78)
Female	15 (22)
Age (yearsl mean, 59.28, range: 24–87)	
< 65	44 (64)
≥65	25 (36)
Primary sites	
Lip	4 (6)
Tongue	29 (42)
Floor of the mouth	18 (26)
Gum	8 (11)
Palate	4 (6)
Buccal	6 (9)
T	
T1	19 (27)
T2	28 (41)
T3	6 (9)
T4	16 (23)
N	
N0	44 (64)
N1	13 (19)
N2	12 (17)
N3	0 (0)
Clinical stage	
I	14 (20)
II	19 (28)
III	12 (17)
IV	24 (35)
Tumor differentiation	
Well	45 (65)
Moderate	18 (26)
Poor	6 (9)
Initial therapy	
Surgery alone	38 (55)
Surgery and radiotherapy combined	31 (45)
Recurrence	
No	40 (58)
Yes	29 (42)
Clinical status at the end of the follow up	
Live and without recurrence	38 (55)
Dead or with non-treatable recurrence	28 (41)
Lost	3 (4)

The main clinical characteristics of the 69 patients selected for this study are detailed in Table 1. All patients were staged according to the 1997 UICC TNM Classification of Malignant Tumors (20). The follow-up period ranged from 4 to 128 months (average: 56.4 months). All patients had been treated surgically with curative intention, and 31 (45%) underwent postoperative radiotherapy, receiving 40–70 Gy, according to the following indications: T4, poorly differentiated carcinomas, tumor-positive resection margins, and metastases in neck nodes. Surgical margins of resection were free of tumor infiltration in 66 of 69 cases (96%). The recurrence-free survival and the survival of the patients were quantified. Clinical outcome was measured by two end points: death caused by disease recurrence and non-treatable disease presence at the end of the follow-up time. At the end of this period 28 patients (40.6%) had died of tumoral recurrence, and 38 cases (55.1%) were alive and free of recurrence. Three cases were lost during the follow up.

Table 2 Details of the antibodies used

Antibodies	*Supplied by*	*Code*	*Dilution*	*Control*
MMP-7	Chemicon®, Temecula, CA, USA	AB19135	1:400	Skeletal muscle
MT1-MMP	Chemicon®, Temecula, CA, USA	MAB3317	1:700	Smooth muscle
MMP-2	Zymed®, San Francisco, CA, USA	18-0218	1:20	Colon cancer
MMP-9	Novocastra®, Newcastle-upon-Tyne, UK	NCL-MMP9-439	1:20	Liver cancer
TIMP-1	RD Systems®, Wiesbaden-Nordenstadt, Germany	MAB 970	1:40	Breast cancer
TIMP-2	RD Systems®, Wiesbaden-Nordenstadt, Germany	MAB971	1:40	Breast cancer
p53	Dako®, Glostrup, Denmark	M7001	1:50	Breast cancer
Ki-67	Immunotech®, Westrook, NY, USA	M887	1:20	Tonsil tissue

Antibodies
Monoclonal antibodies used in this study are listed in Table 2.

Immunohistochemical staining
Immunohistochemistry was performed on sections of the 69 OSCC mounted on glass slides. Surgical specimens were fixed in 10% neutral-buffered formalin (pH 7.4) at 4°C for 72 h and then embedded in paraffin. About 4-μM-thick tissue sections of each specimen were prepared on poly-L-lysine-coated slides. Positive controls for each antibody were obtained from a tissue bank in the Department of Pathology, Central University Hospital of Asturias. Briefly, the sections were dewaxed with xylene, and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersion of slides in methanol with 0.03% hydrogen peroxide for 30 min. The sections were then heated in 10 mM citrate buffer (pH 6.0) three times for 10 min each in a microwave oven at 500 W to retrieve antigenicity. The sections were rinsed in distilled water, and then in phosphate-buffered saline (PBS). Non-specific conjugation was blocked with a solution of 20% rabbit serum (DAKO, Glostrup, Denmark) applied to the sections for 10 min. To establish the optimum concentration for the different antibodies, each antibody was tested using a range of dilutions.

The sections were incubated with primary antibodies at different dilutions (Table 2) for 30 min at room temperature and then they were rinsed twice in PBS for 4 min. Secondary antibody staining was performed using the EnVision polymer technology (K4001, DAKO, Carpinteria, CA, USA) for 30 min according to the manufacturer's instructions. After washing twice with PBS for 4 min, slides were incubated with 3,3′-diaminobenzidine-tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide for 5 min, and then lightly counterstained with Mayer's hematoxylin. One positive (Table 2) and one negative control were included in each batch of immunostained sections. For negative control, the primary antibody was omitted and replaced with non-immune mouse serum.

Assessment of immunohistochemical staining
All slides were scored by two investigators without knowledge of the clinical outcome. Occasional disagreements were discussed to reach a consensus. In cases of persistent differences between them, the sections were studied by a third independent observer and the majority decision was used. Staining for MMP-7, MT1-MMP, MMP-2, MMP-9, TIMP-1, and TIMP-2 was measured as the percentage of positively stained tumor cells, and assigned to four categories, whereby the intense immunoreaction observed in the corresponding control tissues (see Table 2) served as a positive control. The protein expression in tumor stromal cells was also registered. The degree of staining was scored as follows: 0, negative; 1, $<10\%$; 2, more than 10% and $<50\%$; 3, more than 50% positive staining. Group 0 was defined as negative expression, and groups 1, 2, and 3 as positive immunoexpression. Specifically, group 3 was designed as high expression. Areas with pronounced inflammation or necrosis were avoided. Staining for p53 was measured as the percentage of positively stained nuclei counting at least 700 tumor cells in consecutively chosen fields. MIB-1 were scored into four groups as follows: 0, negative; 1, low ($<25\%$); 2, intermediate (more than 25% and $<50\%$); and 3, high (more than 50% of positively stained nuclei).

Statistical analysis
SPSS for Windows (Version 11.5, SPSS Inc., Chicago, IL, USA) was used for statistical analyses. To study the association between clinical parameters (tumor size, regional lymph node status, tumor location, and histopathologic grade) and immunohistochemical results, pairwise comparisons were done, using the Mann–Whitney *U*-test, chi-square, Fisher's exact test (if $N < 5$), or multivariate logistic regression analysis. Differences between mean values of p53 immunostained cells in MMP-7 and MT1-MMP positive and negative cases were tested using the Student's *t*-test preceded by Levene's test. Survival analysis was performed with the Kaplan–Meier product limit method, and the log-rank test was used to compare survival among groups of patients. A Cox proportional hazards model was also used. Time was defined as the period from the treatment to the target event or last follow up. For overall survival, the target event was death or non-treatable recurrence. A significance level ≤ 0.05 was considered to be statistically significant.

Results

Expression of MMP-7 and MT1-MMP in OSCC
For immunohistochemical analysis, 69 oral cancer specimens were stained. Protein expression of MMP-7

was located in the cytoplasm of tumor cells, and it was detected in 54 (78.3%) of 69 cancer tissues. Immunolocalization of matrilysin in carcinoma cells was located in the inner part of tumor islands or cords in 33 cases (47.8%), in the periphery of tumoral tissue in 10 (14.5%) cases and diffusely through the tumoral tissue in the 11 (15.9%) remaining specimens. Staining in all specimens was restricted to the tumor cells, with no staining of the stroma.

Immunostaining for MT1-MMP was seen in 45 of 69 (65.2%) specimens. Of these 45 cases, 11 (15.9%) showed a weak staining for MT1-MMP; 19 (27.5%) depicted a moderate staining, and the remaining 15 positive cases (21.7%) strongly expressed this protease. Conversely to matrilysin, MT1-MMP was immunoexpressed in both tumoral tissue and stroma. MT1-MMP staining was diffuse and as a consequence, a classification of the immunoexpression was not possible.

Carcinoma cells showed intracytoplasmic staining for MMP-7 and MT1-MMP, but with MT1-MMP, cell membranes were also positively stained (Fig. 1).

Relationship between MMP-7 and/or MT1-MMP expression and other immunohistochemical and clinicopathologic parameters

The expression of MMP-7 and MT1-MMP proteins were correlated with the clinicopathologic features summarized in Table 3. The data indicated that expression of MMP-7, but not MT1-MMP, was significantly higher in carcinoma tissues of patients with neck node metastasis than in those without them [$P = 0.03$; relative risk (RR) of 3.2; Table 3]. The multivariate logistic regression analysis related to lymph node metastasis shows that positivity of MMP-7, but not any other variable, was found to be an independent risk factor affecting lymph node metastasis in the present series. Hence, the mathematical model that predicts the metastasis in neck nodes from oral cavity cancers is $P(N = N+) = 1/1 + e^{-[-1.872+1.573(MMP-7=1)]} = 0.4258$. When MMP-7 is negative, we observed lymph node metastasis, as the following model shows, in 13% of cases: $P(N = N0) = 1/1 + e^{-[-1.872+1.573(MMP-7=0)]} = 0.1332$. Thus, when MMP-7 immunostaining is positive, 42.5% of cases are

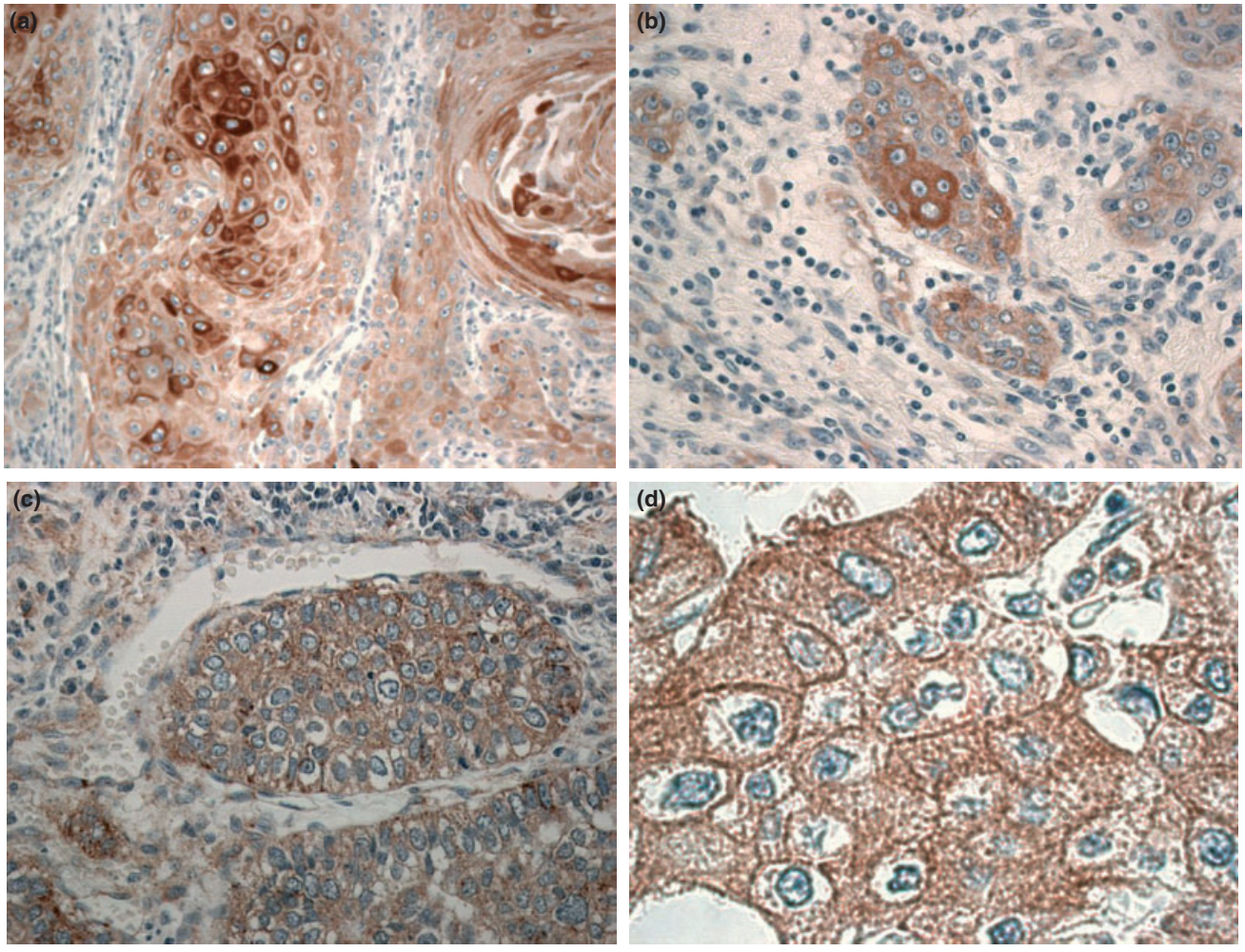

Figure 1 Immunostaining of MMP-7 and MT1-MMP in oral squamous cell carcinoma. (a) MMP-7 is localized in cancer nests, mainly in cells located in the inner part of them. (b) Matrilysin-positive immunostaining in neck lymph node metastasis. (c and d) MT1-MMP immunostaining is located in the cytoplasm and also in cell membranes of cells in carcinoma nests (a–c, 200×; d, 600×).

Table 3 Correlation between immunostaining of MMP-7 and MT1-MMP and clinicopathologic features

		MMP-7			*MT1-MMP*		
Variable	*Number*	*Positive*	*Negative*	*P-value*	*Positive*	*Negative*	*P-value*
Age				0.69			0.35
Sex							
Male	54	40	14	0.24	34	20	0.82
Female	15	14	1		11	4	
Tumor location							
Lip	4	2	2	0.62	3	1	0.40
Tongue	29	23	6		20	9	
Floor	18	15	3		11	7	
Gum	8	7	1		5	3	
Palate	4	2	2		2	2	
Buccal	6	5	1		4	2	
Tumor status							
T1	19	16	3	0.11	12	7	0.97
T2	28	20	8		20	8	
T3	6	6	0		3	3	
T4	16	12	4		10	6	
Nodal status							
N0	44	31	13	0.03	28	16	0.14
N+	25	23	2		17	8	
TNM stage							
I	14	11	3	0.19	9	5	0.64
II	19	13	6		13	6	
III	12	11	1		6	6	
IV	24	19	5		17	7	
Tumor recurrence							
No	40	31	9	0.85[a]	27	13	0.05
Yes	29	23	6		18	11	
Histologic grade							
Poor	6	4	2	0.95	5	1	0.91
Moderate	18	11	7		9	9	
Well	45	32	13		28	17	
Tobacco							
No	22	18	4	0.86	16	6	0.72
Yes	47	36	11		29	18	
Alcohol							
No	24	19	5	0.95	18	6	0.42
Yes	45	35	10		27	18	

[a]MMP-7 protein localization in the peripheral cells of tumor tissue is correlated with the probability of disease recurrence ($P = 0.02$).

associated with regional metastasis, and when MMP-7 expression is negative, 13.3% of tumors are associated with node metastasis.

Likewise, MMP-7 protein expression did not correlate with tumor recurrence, but the localization of MMP-7 expression in the periphery of tumor islands and cords was significantly associated with disease recurrence ($P = 0.02$; Table 3). Thus, the RR of recurrence was 1.8 when comparing cases in which MMP-7 was located in invasive front of tumoral tissue to all other cases considered as a group. On the other hand, MT1-MMP immunostaining showed an association with the number of recurrences ($P = 0.05$).

However, no relationship was observed between age, gender, site of primary tumor, tumor size, clinical stage, histologic grade of differentiation, alcohol or tobacco consumption and MMP-7 or MT1-MMP expression.

MMP-2 protein expression was detected in 19 of 69 cases (28%) and mainly in the advancing front of neoplastic tissue. In the majority of these cases, some fibroblast-like cells of tumor stroma expressed MMP-2 protein. MMP-9 immunostaining was detected in 12 of 69 cases (17%) and both in cancer cells and in the surrounding stroma. In fact, MMP-9 staining was mainly localized in the stroma rather than within the tumor areas. TIMP-1 immunostaining was detected in 45 of 69 cases (65%) and all of them showed staining in tumor tissue, and 19 (27%) expressed TIMP-1 also in the surrounding stroma. Expression of TIMP-2 was detected in 38 of 69 cases (55%). All these cases expressed the protein in cancer cells, and nine (13%) stroma also in adjacent.

MMP-7 protein expression showed a significant and inverse relationship with that of MT1-MMP ($P = 0.04$). MMP-7 immunostaining also showed a direct significant association with that of TIMP-1 (Fisher's exact test, $P < 0.0005$) and TIMP-2 ($P = 0.006$). Furthermore, in tumors with neck node metastasis only, there was an inverse correlation between MMP-7 immunostaining and gelatinases A ($P = 0.03$) and B ($P = 0.03$) (Table 4).

When we divided the sample into two groups regarding the expression of p53, considering the cut point of 20% of positively stained cells, MT1-MMP but not MMP-7, showed a relationship with p53. When MT1-MMP is negative 43% of cells are positively stained for p53, but when MT1-MMP is positive only 23% of

Table 4 Correlation between immunostaining of MMP-7 and MT1-MMP and gelatinases and TIMPs

		MMP-7			*MT1-MMP*		
Variable	*Number*	*Positive*	*Negative*	*P-value*	*Positive*	*Negative*	*P-value*
MMP-2							
Negative	49	41	8	0.08*	34	15	0.25
Positive	20	13	7		11	9	
MMP-9							
Negative	55	44	11	0.48**	37	18	0.47
Positive	14	10	4		8	6	
TIMP-1							
Negative	23	12	11	< 0.0005	15	8	0.61
Positive	46	42	4		30	16	
TIMP-2							
Negative	29	18	11	0.008	20	9	0.58***
Positive	40	36	4		25	15	

*MMP-7/MMP-2 ($P = 0.03$), **MMP-7/MMP-9 ($P = 0.03$), and ***MT1-MMP/TIMP-2 ($P = 0.04$) in tumors with neck node-positive cases.

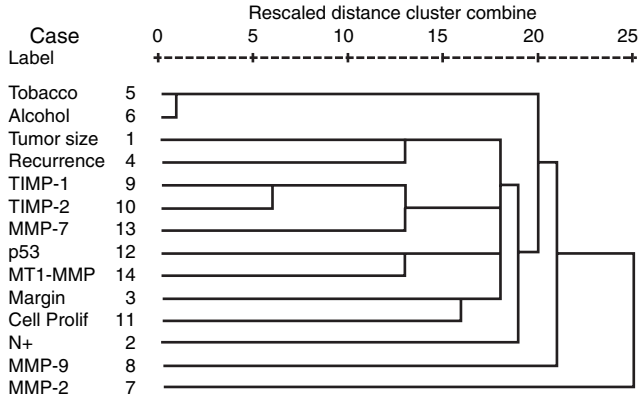

Figure 2 Cluster analysis of variables included in this study.

tumor cells are p53 positive ($P = 0.01$). MT1-MMP also showed a relationship with TIMP-2 staining, but only in neck node-positive cases (Fisher's exact test, $P = 0.04$).

To study the relationship among the variables included in this study we have performed a hierarchical cluster analysis. The distances among the variables and groups resulting of the analysis are the observed in the dendrogram of the Fig. 2. On the one hand, MMP-7 forms a cluster with tissue inhibitors of MMPs, TIMP-1 and TIMP-2, and on the other hand, MT1-MMP is clustered with p53 protein expression. Both clusters are associated between them in a posterior step and with the clusters formed by the remaining variables (tumor size and recurrence, cell proliferation measured by Ki-67 immunostaining and status of resection margin). Regional lymph node metastases are added to the model before the addition of tobacco and alcohol, and finally MMP-9 and MMP-2, were included.

Overall survival rate was 60% at 5 years and 47% at 10 years. In univariate Kaplan–Meier analysis, tumor size, node status, TNM stage, and positive status of the surgical margin were significantly associated with shortened survival (Table 5). When considering all 69 cases as a group, there was no difference in survival between MMP-7 and MT1-MMP positive and negative cases

Table 5 Relationship of clinical and immunohistochemical variables to survival rates in 69 patients with squamous cell carcinoma of the oral cavity (Kaplan–Meier analysis)

Variable	*Number*	*Survival number and rate (%)*	*Mean survival time in months (95% CI)*	*Log-rank P-value*
Tumor size				
T1	19	15 (78.95)	108 (91–126)	0.0003
T2	28	19 (67.86)	90 (72–109)	
T3	6	1 (16.67)	45 (25–65)	
T4	16	6 (37.50)	27 (5–49)	
Lymph node status				
N0	44	30 (68.18)	94 (79–109)	0.02
N+	25	11 (44.00)	64 (43–86)	
Stage				
I	14	12 (85.71)	117 (103–131)	< 0.00001
II	19	14 (73.68)	92 (71–113)	
III	12	8 (66.67)	94 (67–121)	
IV	24	7 (29.17)	38 (10–38)	
Resection margin status				
Negative	66	41 (62)	86 (73–99)	0.05
Positive	3	0 (0)	30 (22–38)	
MMP-7				
Negative	15	9 (60.0)	79 (53–105)	0.97
Positive	54	32 (59.3)	83 (69–98)	
MMP-7[a]				
Negative, low	19	10 (52.63)	75 (50–100)	0.04
High	6	1 (16.67)	23 (2–43)	
MT1-MMP				
Negative	24	13 (54.17)	82 (61–103)	0.90
Positive	45	28 (62.22)	80 (65–95)	

[a]N+ cases.

(Figs 3 and 4). However, in patients with neck node metastases (N+) MMP-7, but not MT1-MMP, showed a significant relationship with survival. Thus, patients with weak or negative MMP-7 immunoexpression had better prognosis than those with tumors of high MMP-7 immunostaining ($P = 0.04$; Fig. 5). In a multivariate Cox regression analysis, tumor size ($P = 0.001$), neck node status ($P = 0.004$), status of the resection margin ($P = 0.016$), and MMP-7 ($P = 0.008$) showed a significant relationship with survival (Table 6). However, MMP-7 showed a negative association with survival, seeming to play a role as a poor prognostic factor. But

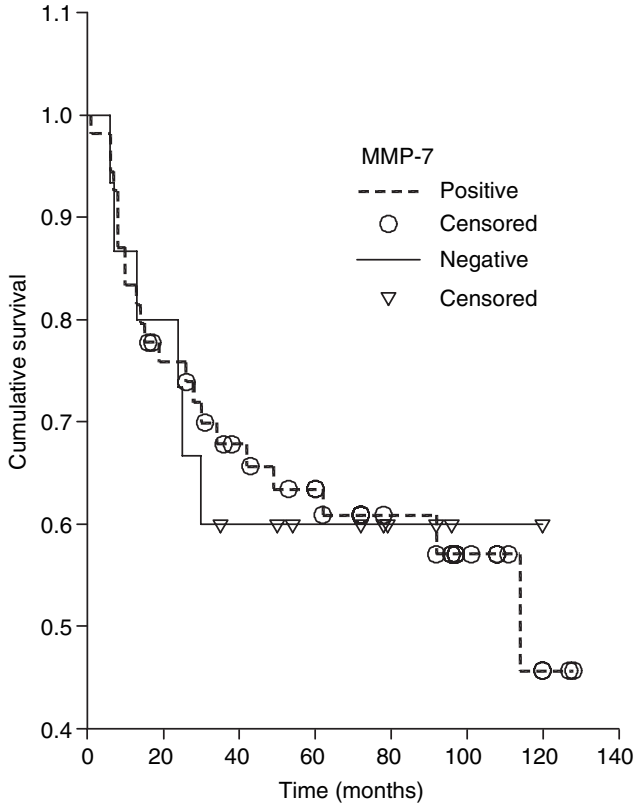

Figure 3 Survival curve for 69 patients with oral squamous cell carcinoma according to the immunostaining of MMP-7 ($P = 0.97$).

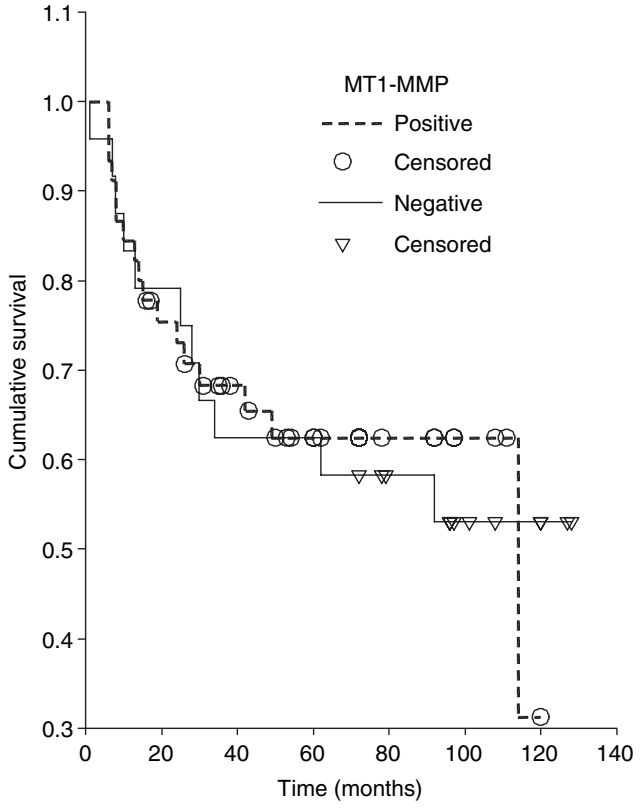

Figure 4 Survival curve for 69 patients with oral squamous cell carcinoma according to the immunostaining of MT1-MMP ($P = 0.90$).

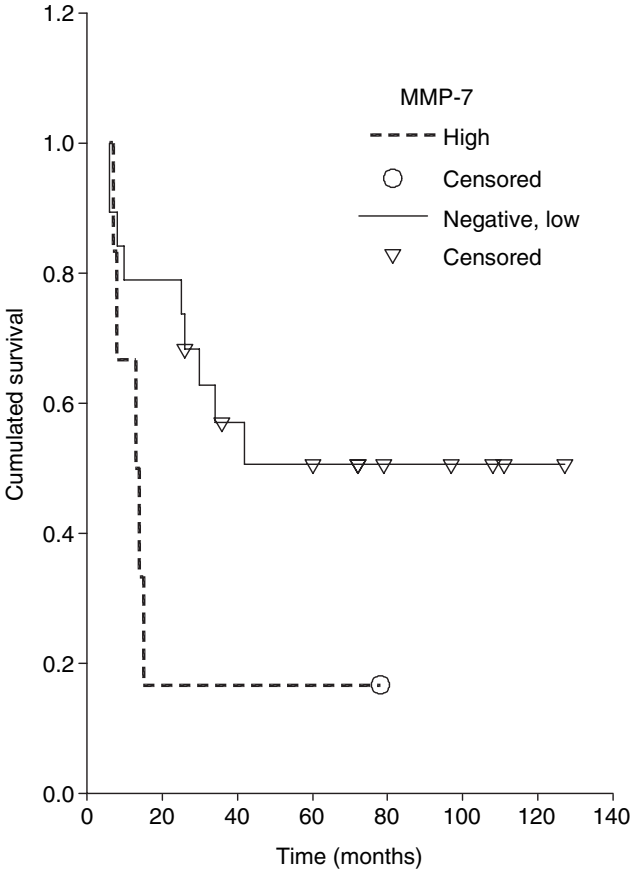

Figure 5 Survival curve for 25 patients with oral squamous cell carcinoma and neck node metastasis according to the immunostaining of MMP-7 ($P = 0.04$).

when the analysis is performed only in tumors accompanied by neck node metastases, both MMP-7 ($P = 0.02$) and MT1-MMP ($P = 0.03$) showed a negative relationship with survival. Thus, in this study MMP-7 showed a dual relationship with survival regarding the neck node status and MT1-MMP depicted a survival relevance only in N+ cases.

Discussion

Several studies have shown the expression of MMPs in head and neck carcinomas, and more specifically in OSCC, as well as their involvement in tumor progression, but until now, their clinical significance and prognostic value have been controversial. Because the ECM shows a complex structure, the combined action of several MMPs is crucial for the degradation of this physical barrier during the metastatic process. We therefore examined the expression of two MMPs (-7, -14) and their relationship with other MMPs (-2, -9) and with their tissue inhibitors (TIMP-1, TIMP-2). We found that matrilysin is associated with neck node metastasis, and both MMP-7 and MMP-14 showed a correlation with tumor recurrence, and in neck node-positive cases, with poor survival. Our study also showed that OSCC is heterogeneous for the potential

Table 6 Multivariate survival Cox analysis

	69 cases of OSCC			*25 cases of OSCC with neck node metastases (N+)*		
Variable	*B coefficient*	*B exponent*	*P-value*	*B coefficient*	*B exponent*	*P-value*
Tumor size (T)	1.097	2.995	0.015	0.463	1.589	0.430
N status	1.736	5.673	0.004			
Margin status	2.260	0.104	0.016	2.787	16.230	0.039
MMP-7	−1.947	0.143	0.008	1.613	5.020	0.023
MT1-MMP	−0.431	0.650	0.425	2.328	10.258	0.035

to produce these proteases and that when matrilysin-1 is located in the invasive front of the tumor, disease recurrence is more frequent.

The process of metastasis is now believed to begin early in the growth of the primary tumor (21), but the genetic changes responsible of tumorigenesis do not, by themselves, produce the metastatic phenotype (22). Basement membrane and ECM degradation mediated by proteases, mainly MMPs, contribute to the formation of a microenvironment that promotes tumor growth, invasion, and metastasis (23). But beyond the classical roles, MMPs have additional functions that include activation of growth factors, suppression of tumor cell apoptosis, destruction of chemokine gradients, and release of angiogenic factors (24). Furthermore, protease activity seems not to be always essential for tumor invasion due to the existence of protease-independent cell migration (25).

Several studies have explored the relationships between the expression of MMP-7 or MT1-MMP and clinical behavior of cancers, but the results are still inconclusive. In cancer tissues, the activation of pro-MMP-2 is mediated by a combination of TIMP-2 and MT1-MMP (proMMP-2/TIMP-2/MT1-MMP system) that correlates well with the progression of many cancers such as breast carcinomas, thyroid papillary carcinomas, gastric adenocarcinomas, OSCCs, and gliomas, whereas MMP-7 plays a role in the metastasis of endometrial, gastrointestinal, and other carcinomas (26). A member of the transmembrane 4 superfamily (TM4SF) captures proMMP-7 on the carcinoma cell membranes through interaction with its propeptide, leading to its pericellular activation, and it is strongly suggested that proteolysis due to the proMMP-2/TIMP-2/MT1-MMP and proMMP-7/TM4SF systems plays crucial roles in the progression of human cancers (26).

MMP-7, a 'minimal domain MMP' has been shown to play a role in the degradation of the basement membrane that separates epithelium from stroma allowing tumor cells to intravasate into the vessels and metastasize. Recently, Tan et al. (27) have demonstrated that MMP-7 is involved in cell dissociation and invasion of pancreatic cancer cell by disruption of tight junction structures, and activation of the epidermal growth factor receptor (EGFR). During the metastasis formation in squamous cell carcinomas, the tumor cells have to penetrate into the lymph ducts. MMP-2 and MMP-9 can digest the basement membrane in the wall of lymph ducts, whereas MMP-7 can degrade elastin, the main component of this wall (28). MMP-7 was found to correlate with metastasis in digestive system cancers (29, 30), whereas other studies (31) showed no difference in MMP-7 expression between carcinomas with and without node metastasis. In oral cancer, MMP-7 has been found in squamous, but not in verrucous carcinomas (15). Our results show a clear relationship between MMP-7, nodal metastasis (RR = 3), and poor prognosis in N+ cases. Thus, MMP-7 could be a valuable marker for predicting prognosis in cases of OSCC with neck node metastases. The evaluation of this protease in squamous cell carcinomas of the oral cavity at the time of diagnosis, might allow identification of a subset of patients who are prone to regional metastasis and support a more aggressive management of the neck in the surgical treatment of them.

It remains unclear if the expression of a particular MMP reflects its functional role in the malignant process or if such expression is a sign of the host response to the tumor itself. Mylona et al. (32) observed in breast cancers the presence of MMP-7 in cancer and stromal cells. MMPs are usually located in both type of cells, but specifically, MMP-7 is considered the lone MMP exclusively expressed in tumoral cells (7). Our results support this notion because in none of our tissue samples was MMP-7 found in non-cancerous cells. The role played by MMP-7 in tumor progression is not fully understood. The death receptor Fas is one of the major apoptosis inducers. Engagement of Fas by its ligand FasL initiates the recruitment of the adaptator protein Fas-associated death domain (FADD; 33) that provides a surface for activation of FADD-like interleukin-1β converting enzyme or caspase-8 (34), which triggers a caspase cascade that in turn cleaves vital components of the cell and lead to its demise. MMP-7 sheds the ectodomain of membrane-bound FasL (mFasL) from the tumor cell surface generating soluble FasL (sFasL) which displays reduced proapoptotic potency compared with its cell surface precursor. Thus, MMP-7-mediated FasL cleavage could provide tumor cells with a mechanism to evade apoptosis, thereby facilitating tumor survival and progression. This supports the notion that tumor progression associated with MMP-7 activity may not be attributable to proteolytic degradation of ECM but rather could be related to the regulation of tumor cell death itself (35).

Several studies have examined relationships between the expression of MT1-MMP and invasiveness in human cancers, but the results are still inconclusive. In this study, no significant correlation was found between MT1-MMP and lymph node involvement, results that

are in agreement with those of O-charoenrat et al. (30), but not with other that observed a relation between this protease and metastasis in OSCC (36), prostate (37), and breast cancer (38). Discrepancies observed among several studies could be attributed to the use of different methods in the study of MMPs expression, as well as in sample sizes or a possible tissue specificity on which MMPs regulate ECM function under specific microenvironments. We have investigated here the expression of MMPs using immunohistochemistry because it allow to establish direct relationship between MMPs and morphology, which has a functional relevance, and it can be performed on paraffin-embedded specimen of patients follow up for a long period of time. We have also found that MMP-7 and MT1-MMP were related with tumor recurrence and both of them with poor survival rate in multivariate analysis, highlighting a multifunctional role played by these proteases. Furthermore, MMP-7 and MT1-MMP expression was inversely associated with that of MMP-2 and MMP-9, suggesting a sequential action of these proteases in different moments of invasive process.

It has been reported that members of the MMP family are p53 target genes subject to repression, whereas mutant p53 activates MMP gene transcription (39). The signal transduction pathways that mediate the activity of MMP transcriptional activators are diverse, and among the regulatory elements in the promoter regions of human MMP genes, p53 has been described only regarding MMP-2. As far as we know, we reported here the first relation between p53 and MT1-MMP expression, and we speculate here that among the promoters of MT1-MMP (NF-κB, C/EBP, PEA3, EGR1, SP1; 40) could also be p53 protein.

In conclusion, the results of this study suggest that MMP-7 and MT1-MMP are useful markers for progression in OSCC, and that they may be useful in identifying patients who would benefit from treatments based on MMP inhibitors. We believe that the findings of this investigation support that MMP-7 and MMP-14 combined with other markers may be used to predict the metastatic potential of OSCC.

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