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Expression of Matrix Metalloproteinase-9 in Patients with Squamous Cell Carcinoma of the Larynx

Zaviša Čolović[1], Valdi Pešutić-Pisac[2], Nikola Kolja Poljak[1], Goran Račić[1], Draško Cikojević[1] and Mirko Kontić[1]

[1] University of Split, Split University Hospital Center, ENT Department, Split, Croatia
[2] University of Split, Split University Hospital Center, Department of Pathology, Split, Croatia

A B S T R A C T

Aim of this study was to investigate the correlation of matrix metalloproteinase-9 (MMP-9) expression with histopathologic and clinical characteristics of laryngeal squamous cell carcinoma, and to assess the role of MMP-9 expression in patient survival. Study included 196 patients with squamous cell carcinoma of the larynx treated at ENT Department, Split University Hospital Centre, from January 1, 2000 till December 31, 2009. The level of MMP-9 expression showed a statistically significant correlation ($p<0.001$) with the disease histopathologic grade, stage, metastatic potential, recurrence potential, and survival. Kaplan-Meier curve yielded a statistically significant survival difference ($p<0.001$) among the three patient groups with different levels of MMP-9 expression. The survival curve clearly showed the MMP-9 expression as a predictor of survival to be significantly ($p<0.001$) associated with survival. In this study, MMP-9 expression as a biological marker showed a potential predictive value in laryngeal squamous cell carcinoma.

***Key words**: matrix metalloproteinase-9, squamous cell carcinoma, larynx, survival*

Introduction

Matrix metalloproteinases (MMP) are a family of proteolytic zinc dependent enzymes that play an essential role in local invasion, and in regional and distal metastasizing of malignant tumours, breaking down the basal membrane and extracellular matrix components. MMP also influence tumour growth and tumour neoangiogenesis[1–7]. Gelatinases are a MMP subgroup that includes matrix metalloproteinases-9 (MMP-9) or gelatinase B. Gelatinases act by degrading denatured collagen, basal membrane collagen IV in particular[3,8–10]. MMP expression has been found to be typically low or absent in normal cells of adult body[8].

Tumour neoangiogenesis, which is important for further neoplasm growth, is facilitated by the action of MMP that stimulate the release of angiogenic factors and remodelling of tissue adhesiveness. MMP also play a major role in distal tumour metastasizing[11,12].

The effect of MMP-9 on tumour differentiation and progression in oral, oesophageal, lung, ovarian, colon carcinomas, thyroid papillary carcinoma and melanoma has been investigated by many authors[13–23], whereas only few studies addressed the effect of MMP-9 in squamous cell carcinoma of the larynx[13,24–26].

The aim of the present study was to assess the correlation of MMP-9 expression with histopathologic and clinical characteristics of laryngeal squamous cell carcinoma, and to determine the role of MMP-9 expression in disease prognosis and patient survival.

Materials and Methods

Patients

This study included 196 patients with squamous cell carcinoma of the larynx treated at University Department of ENT, Head and Neck Surgery, Split University Hospital Centre in Split, Croatia, from January 1, 2000 till December 31, 2009. The median patient age was 65, range 37–88 years. There were 189 (96.4%) male and seven (3.6%) female patients.

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Immunohistochemistry

Expression of MMP-9 in tumour cells was determined by immunohistochemistry (IH), where it is expressed as the percentage of stained tumour cells in the overall material, with the borderline positive value of 10%. Statistical analysis was performed by χ^2-test, whereas predictive value of the potential predictors was assessed by Cox regression analysis, preceded by Kaplan-Meier curve for analysis of survival. The level of significance was set at $p \leq 0.05$ in all tests.

Protocol of histology slides and immunohistochemistry staining

Tissue for histology analysis was obtained from operative material, fixed in 4% buffered formalin for 24 h, paraffin embedded, cut into 3- to 5-micron sections and stained by the standard hemalaun eosin (HE) method. Histologic preparations were selected, their paraffin sections were cut again and prepared for immunohistochemistry. Immunohistochemical study was performed on formalin fixed, paraffin embedded sections obtained from tumour specimens using streptavidin-avidin-biotin technique (DAKO, Glostrup, Denmark) with diaminobenzidine as chromogen and hematoxylin as counterstain. Pre-treatment of deparaffinised tissue sections with heat-induced epitope retrieval (HIER) is required using DAKO Target Retrieval Solution, pH 9 (codes S2368/S2367). HIER: 15 min at boiling point.

After thermal treatment, the buffer and slides were cooled for 20 min at room temperature and the sections were rinsed with deionised water. For MMP-9 identification, the MMP-9 A0150 antibody (polyclonal rabbit, anti-human; DAKO, Glostrup, Denmark), 1:75 dilution, 1-h incubation, was employed according to the staining procedure recommended.

The level of MMP-9 expression referred to the intensity of tumour cell staining: 0 = no staining; 1 = weak staining in more than 10%; and 2 = intensive staining in more than 10%[27] (Figures 1 and 2). According to the manufacturer's instructions, normal lung tissue served as positive control.

Statistical analysis

Excel 2010 (MS Office 2010, Microsoft, USA) and Statistica 10.0 (StatSoft Inc., Tulsa, USA) were used on data processing and analysis. The χ^2-test was employed to assess the association of the potential predictors/variables (clinical characteristics and histopathologic features with MMP-9 expression). Predictive value of the potential predictors for disease outcome and patient survival was assessed by Cox regression analysis and survival analysis (Kaplan-Meier curve). Statistical significance of particular tests was determined at the level of 95% ($p \leq 0.05$).

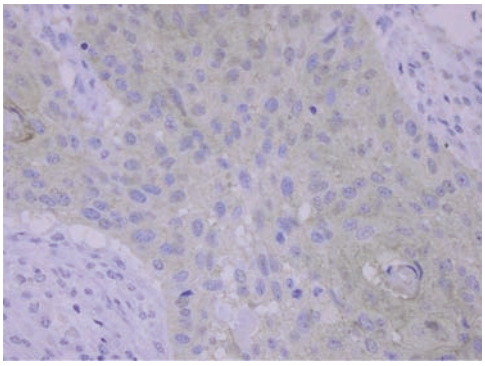

Fig. 1. Weak MMP-9 expression in a well-differentiated squamous cell carcinoma (IH, x200).

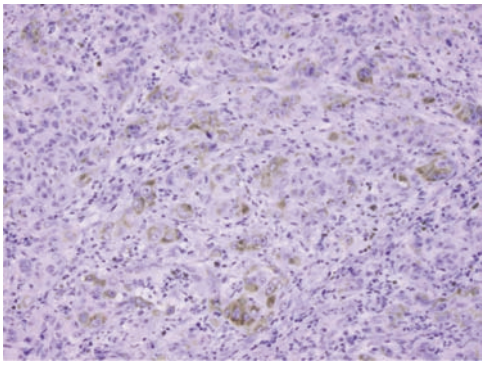

Fig. 2. Intensive MMP-9 expression in poorly differentiated squamous cell carcinoma (IH, x200).

Results

Out of 196 study patients, MMP-9 expression was demonstrated in 95 (48.4%) and high MMP-9 expression in 62 (31.6%) patients. Negative (level 0) MMP-9 expression was recorded in 101 (51.5%) and positive MMP-9 expression in 95 (48.4%) patients: level 1 MMP-9 expression in 33 (16.8%) and level 2 MMP-9 expression in 62 (31.6%) patients (Figure 3).

Table 1 shows correlation of tumour grade, stage, metastasis, relapse and mortality according to the level of MMP-9 expression. Results showed a statistically significant correlation among the study variables ($p<0.001$). The higher the tumour grade, the higher was the MMP-9

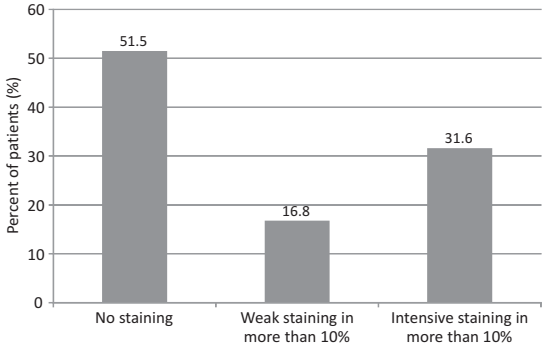

Fig. 3. MMP-9 expression in 196 patients with squamous cell carcinoma (%).

TABLE 1
CORRELATION OF MATRIX METALLOPROTEINASE-9 (MMP-9) EXPRESSION WITH CLINICAL AND HISTOPATHOLOGIC CHARACTERISTICS OF 196 PATIENTS WITH LARYNGEAL SQUAMOUS CELL CARCINOMA

	MMP-9 expression (n, %)				
	0	1	2	Total	p
Grade					
I	64 (83.1)	4 (5.2)	9 (11.7)	77 (100)	
II	36 (38.7)	26 (28)	31 (33.3)	93 (100)	<0.001
III	1 (3.8)	3 (11.5)	22 (84.6)	26 (100)	
Stage					
I	51 (87.9)	6 (10.3)	1 (1.7)	58 (100)	
II	14 (73.7)	4 (21.1)	1 (5.3)	19 (100)	<0.001
III	34 (41)	20 (24.1)	29 (34.9)	83 (100)	
IV	2 (5.6)	3 (8.3)	31 (86.1)	36 (100)	
Meta					
No	100 (73)	28 (20.4)	9 (6.6)	137 (100)	<0.001
Yes	1 (1.7)	5 (8.5)	53 (89.9)	59 (100)	
Relapse					
No	100 (78.7)	22 (17.3)	5 (3.9)	127 (100)	<0.001
Yes	1 (1.4)	11 (15.9)	57 (82.6)	69 (100)	
Censor					
Death from primary disease	0 (0)	10 (14.9)	57 (85.1)	67 (100)	
Death from other diseases	31 (77.5)	9 (22.5)	0 (0)	40 (100)	<0.001
Alive	70 (78.7)	14 (15.7)	5 (5.6)	89 (100)	
Total	101 (51.5)	33 (16.8)	62 (31.6)	196 (100)	

TABLE 2
REGRESSION ANALYSIS (COX REGRESSION) OF CLINICAL AND HISTOPATHOLOGIC PREDICTORS OF SURVIVAL DURING THE STUDY PERIOD

	B	SE	Wald	p	Exp (B)	95.0% CI for Exp(B)	
						Lower	Upper
Stage I			0.6	0.892			
Stage II (1)	8.5	39.5	0.04	0.830	4852.963	0.000	1.89E37
Stage III (2)	8.8	39.5	0.05	0.824	6622.129	0.000	2.52E37
Stage IV (3)	8.6	39.5	0.05	0.827	5437.795	0.000	2.07E37
Grade I			0.7	0.719			
Grade II (1)	–0.3	0.4	0.6	0.447	0.756	0.367	1.55
Grade III (2)	–0.1	0.4	0.1	0.724	0.867	0.393	1.91
MMP-9 0			14.2	0.001			
MMP-9 1 (1)	10.7	47.8	0.1	0.822	46394.594	0.000	2.30E45
MMP-9 2 (2)	12.2	47.8	0.1	0.798	207880.355	0.000	1.03E46

expression, present in about 85% of patients (N=22). The same applied to tumour staging, where level 2 of MMP-9 expression was recorded in 35% (N=29) of stage III patients and as many as 86.6% (N=31) of stage IV patients. Level 2 of MMP-9 expression was present in 90% of patients with metastases and only 7% of those free from metastases. In addition, level 2 of MMP-9 expression was significantly more common in patients with relapses (N=57; 83%) *versus* only 4% of those free from relapse. Significant correlation was also found between mortality rate and MMP-9 expression, with level 2 of MMP-9 expression recorded in more than 80% of patients having died from primary disease.

Kaplan-Meier curve (Figure 4) showed a statistically significant difference in survival among the three patient groups divided according to the level of MMP-9 expression (χ^2=152.6; p<0.001). Out of 62 patients with level 2 of MMP-9 expression, 57 (92%) patients had died and

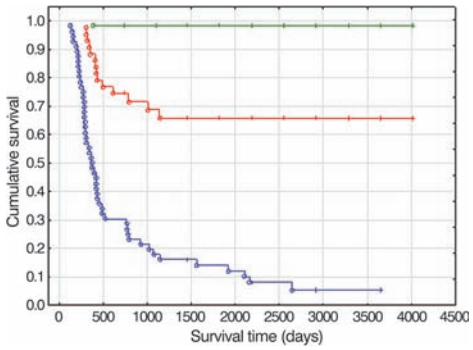

Fig. 4. Survival curve according to the level of MMP-9 expression (0, blue color = no staining; 1, red color = weak staining in more than 10%; 2, green color = intensive staining in more than 10%).

only five (8%) were alive at the end of the study period. On the other hand, all patients without MMP-9 expression were alive at the same time point.

Table 2 shows regression coefficients obtained by regression analysis of clinical and histopathologic survival predictors during the study period. MMP-9 expression as a survival predictor showed higher correlation ($p=0.001$) with patient survival than either tumour stage or tumour grade.

Discussion and Conclusion

The survival of patients with squamous cell carcinoma of the larynx has remained unchanged over the last three decades.[28] In the present study, the value of the biological marker MMP-9 expression as a prognostic factor of survival was evaluated in a large group of patients with squamous cell carcinoma of the larynx. MMP-9 expression was demonstrated in 95 (48.5%) of 196 study patients, which is consistent with the results reported by Bogusiewicz et al. and Uloza et al.[26,29]. In our study, MMP-9 expression was presented in three levels (0, 1 and 2). Level 0 (negative) MMP-9 expression was recorded in 101 (51.5%) and positive MMP-9 expression in 95 (48.5%) patients: level 1 in 33 (16.8%) and level 2 in 62 (31.6%) patients.

The level of MMP-9 expression yielded a statistically significant correlation ($p<0.001$) with histopathologic grade, stage, metastasizing potential, relapsing potential and patient survival.

Higher MMP-9 expression was determined in cases with a higher histopathologic grade, higher, i.e. advanced stage of disease, and those with high relapsing potential. In their study, Gou et al. pointed to positive correlation of the increased MMP-9 expression with the stage of disease and metastasizing potential. These authors also demonstrated the increased MMP-9 expression to be associated with a reduced patient survival[30]. O-Charoenrat et al. report on a significant correlation between the increased MMP-9 expression and advanced stages of disease, however, they observed correlation with all squamous cell carcinomas of the head and neck, not only laryngeal carcinoma[25]. Xie et al. found significant correlation between the increased MMP-9 expression and metastasizing to lymph nodes[2]. Wittekindt et al. report on a significant correlation of MMP-9 expression with histopathologic grade (degree of differentiation), but not with the stage of disease, metastasizing potential and tumour relapsing potential[31]. Liu et al. did not found significant correlation between MMP-9 expression and clinicopathologic characteristics of squamous cell carcinoma (histologic grade, TNM clinical stage and primary seat of disease)[32].

In our study, Kaplan-Meier curve yielded a statistically significant survival difference ($\chi^2=152.6$; $p<0.001$) among three patient groups divided according to the level of MMP-9 expression. In the group of patients with level 2 MMP-9 expression, 92% of patients had died and only 8% survived. In contrast, in the group of patients with level 0 (negative) MMP-9 expression, the rate of survival was 100%. Regression analysis of clinical and histopathologic survival predictors during the study period indicated the predictor of MMP-9 expression to be more significantly ($p=0.001$) associated with survival than tumour grade and tumour stage.

Odds ratio could be extremely high when the probability of event for one of the groups is very low, as in our regression model. Similar results have been reported by Gou et al.; in their study, the higher MMP-9 expression was associated with shorter patient survival[30].

Significant correlation between MMP-9 expression and overall survival was also recorded by Smilek et al. in their study population of 217 patients with head and neck carcinoma[33]. Thus, the higher the MMP-9 expression, the poorer is patient survival.

In our study, expression of the biological marker MMP-9 showed a potential predictive value for squamous cell carcinoma of the larynx. With a relatively large study population, the results of the present study contribute to further clarification of MMP-9 as a prognostic biological marker to predict tumour aggressiveness and consequentially to tailor therapeutic approach accordingly. As currently there are no reliable markers for laryngeal carcinoma, we believe that research should be focused on this potential marker, in order to establish MMP-9 as a biological marker predicting tumour aggressiveness. The modality and extent of therapeutic approach could then be based on the level of MMP-9 expression.

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Z. Čolović

University of Split, Split University Hospital Center, ENT Department, Spinčićeva 1, 21000 Split, Croatia
e-mail: zcolovic@kbsplit.hr

IZRAŽAJNOST MATRIKS METALOPROTEINAZE-9 U BOLESNIKA S PLOČASTIM KARCINOMOM GRKLJANA

S A Ž E T A K

Cilj ove studije bio je istražiti povezanost izražajnosti matriks metaloproteinaze-9 (MMP-9) s histopatološkim i kliničkim osobinama pločastog karcinoma grkljana, te značajnost izražajnosti MMP-9 na preživljavanje pacijenata. U studiju je uključeno 196 bolesnika s pločastim karcinomom grkljana liječenih u ENT odjelu Kliničkog bolničkog centra Split u periodu od 1.siječnja 2000. do 31. prosinca 2009. godine. Dokazali smo statistički značajnu povezanost ($p<0,001$) između stupnja izražajnosti MMP 9 i atohistološkog gradusa, stadija bolesti, sklonosti metastaziranju, sklonosti recidiviranju i preživljenja. Kaplan-Meier krivulja pokazuje statistički značajnu razliku ($p<0,001$) u preživljenju između 3 skupine bolesnika koji imaju različiti stupanj izražajnosti MMP 9. U krivulji preživljenja vidljivo je da je prediktor izražajnost MMP 9 značajno ($p=0,001$) povezan s preživljenjem. Izražajnost MMP 9 biološkog markera u našoj studiji pokazala je potencijalnu prediktivnu vrijednost za planocelularne karcinome grkljana.