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Experimental Research

Pathology Section

Immunohistochemical Study of Metalloproteinases (MMP-2 and MMP-9) Expression in Laryngeal Squamous Cell Carcinoma

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ABSTRACT

Introduction: Laryngeal squamous cell carcinoma (LSCC) is the second most common head and neck Squamous cell carcinoma (SCC). Among more than 24 members of the Matrix metalloproteinases (MMPs) family, MMP-2 and MMP-9 are proved to have the capacity to degrade the most important elements of Extracellular matrix (ECM) and basement membrane, thus contributing to the invasion and metastases of the malignant tumours.

Aim: To evaluate and compare Immunohistochemistry (IHC) stained sections of MMP-2 and MMP-9 in both in situ and invasive LSCC and to study its relation with other clinicial and pathological features.

Materials and Methods: This was a retrospective cross-sectional study conducted in Pathology department, Facutly of Medicine, Cairo University. A total of 60 specimens LSCC were studied obtained from laryngsopyic biopsies, partial and total laryngectomy specimens with selective neck dissection over one year duration. Both Haematoxylin and Eosin (H&E) and IHC (MMP-2 and MMP-9) stained sections were evaluated. IHC results of MMP-2 and MMP-9 were correlated with clinicopathological parameters. Data was analysed using SPSS software version 17.0. The p-value <0.05 was considered as statistically significant.

Results: Thirty five (35) cases (58.3%) were considered MMP-2 expressors and 36 cases (60%) were considered MMP-9 expressors. A significant relations were detected between MMP-2 and MMP-9 expressions on one hand and tumour grading, tumour site, infiltration of thyroid cartilage, infiltration of resected margins, lymph node metastasis and tumour staging on the other hand (p-value <0.05).

Conclusion: MMP-2 and MMP-9 overexpression could be a useful prognostic marker for predicting LSCC with a potential for metastatic behaviour. This allows easier identification of patients who are at higher risk of metastatic carcinoma and should be controlled more frequently. Larger studies on a wider scale of patients are needed based on long term follow-up.

Keywords: Head and neck, Metalloproteinase 2, Metalloproteinase 9, Squamous cell carcinoma

INTRODUCTION

Head and neck SCC is considered the sixth malignant tumour worldwide. Laryngeal SCCis thought to be the second most common of the head and neck cancers [1]. Tumour invasion has multiple steps, involves interactions of cells with the Extracellular matrix (ECM). During invasion, malignant cells invade the basement membranes and ECM [2], at the early phase of malignant tumour, malignant cells infiltrate the normal tissues and degrade the stromal ECM and basement membrane, including laminin, type IV collagen and fibronectin [3]. Matrix metalloproteinases (MMPs), have a very important roles in physiological and pathological tissue remodelling. Gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are proven to have great ability in degradation of important elements of ECM and basement membrane, which helps in the invasion and metastases of cancers [4]. They are the most important MMPs found in head and neck cancers [5].

Many studies work to prove the importance of MMP-2 and MMP-9 expressions as a hallmark in laryngeal SCC at which expressions of MMP-2 and MMP-9 are up-regulated in poorly differentiated laryngeal SCC with lymph node metastasis, suggesting that MMPs could be used in the detection of occult lymph node metastasis but more research is needed to prove this [6-8]. Additionally some of the therapeutic agents are tried to induce MMP-2 and MMP-9 degradation. These studies try to prove that the induction of MMP-2 and MMP-9 degradation can offer a useful avenue for therapeutic intervention [9-12].

The aim of this study was to evaluate immunohistochemical expression of MMP-2 and MMP-9 in cases of laryngeal SCC and their relation with other clinical and pathological features, also to compare between the expression of the MMP-2 and MMP-9 in both insitu and invasive laryngeal SCC. The novality of this study that it detect the difference between the markers' expression in insitu and invasive tumours which is not studied in many other studies, which proves that they play an important role in invasion mechanism.

MATERIALS AND METHODS

The study was a retrospective cross-sectional study. The material of this study consisted of the paraffin blocks and clinical data of 60 laryngeal SCC cases. The specimens collected were: laryngoscopic biopsies (20 cases), partial and total laryngectomy specimens with selective neck dissection (40 cases) which were obtained from the Department of Pathology, Kasr El-Ainy Faculty of Medicine Hospital, within the period from March 2016 to January 2017. The specimens were anonymous for confidentiality and replaced by numbers. The procedures followed were in accordance with the ethical standards of the institutional committee and are approved by their ethics committe.

Histopathological evaluation: Each paraffin block was re-cut by rotatory microtome at 5 microns thickness then mounted on glass slides to be stained by Haematoxylin and Eosin (H&E) for evaluation of the histopathological grades, tumour infiltration of underlying cartilage, tumour infiltration of resected margins, presence of lymph node metastasis and tumour stage.

Immunohistochemical procedure: Each paraffin block was re-cut by rotatory microtome at 5 microns thickness. The sections were mounted on positively charged slides, deparaffinized in xylene, then were hydrated through a series of graded alcohols (95%-70%), distilled water and phosphate buffered saline (at pH 7.6). After a 20 minute cooling period, the endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide (H_2O_2) for 5 minutes. The slides were then immersed in 10 mm of citrate buffer (pH 6) and were twice pretreated by microwaving (oven 800W) for 4 then 8 minutes. Between each period of heating, evaporated fluid was replenished. Protein blocking was performed. After washing with Tris-buffered saline, the sections were incubated with the primary antibody for 1 hour at room temperature. The primary antibody was a rabbit polyclonal antihuman MMP-2 (8B4) and MMP-9 (SB15c) antibodies, manufactured by Thermo scientific, UK at dilution of 1:50. The sections were washed in Tris-buffer and incubated with avidin-biotin peroxidase system (Thermo Scientific) for 30 minutes. Peroxidase reaction was detected by addition of di amino benzidine tetra hydrochloride. All slides were rinsed well in tap water for 5 minutes then slightly counterstained with hematoxylin for 1-2 minutes and dehydrated in ascending alcohol. The slides were cleared in xylene for 3 changes then cover slips were applied. Transitional cell carcinoma in urinary bladder was used as positive control for MMP-2 and placenta was used as positive control for MMP-9.

Assessment of MMP-2 and MMP-9 immunostaining: Sections were examined under high power field (400x) to observe immunoreactivity. Expression of MMP-2 and MMP-9 was identified as cytoplasmic and nuclear staining in tumour cells. The immunohistochemical reactions were analysed by two observers and scored applying the following criteria [6,13]:

As regards the MMP-2 and MMP-9 protein expression, a semiquantitative analysis based on a 4-point scale was used depending on the percentage of positively stained cells:

0=No staining.

1=Weak expression (>10% positive cells).

2=Moderate expression (10-50% positive cells).

3=Strong expression (<50% positive cells).

STATISTICAL ANALYSIS

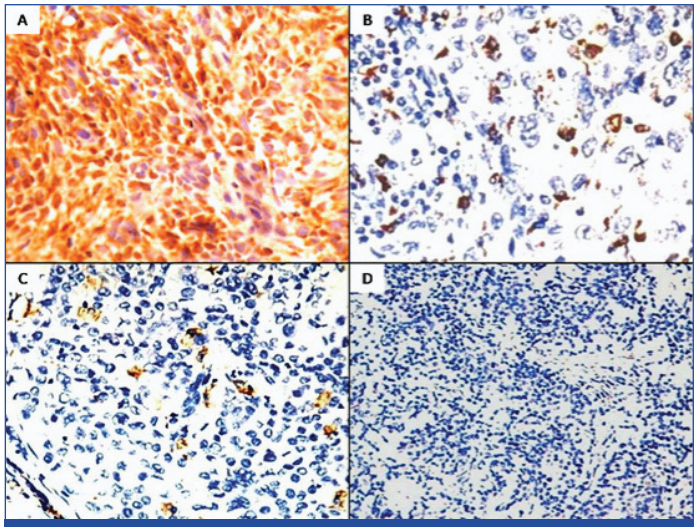
Data was analysed using SPSS (statistical package for social science). Version 17.0 (SPSS, Inc., Chicago, Ill., USA) for windows. The significance of the results was assessed by determining the probability factor p-value using the chi-square test. p-value <0.05 is considered statistically significant. Data were statistically described in terms of mean, +/- standard deviation (SD), or frequencies (number of cases) and percentages when appropriate. Confidence interval for the mean and the mean differences were also calculated to assess sample size and significance.

RESULTS

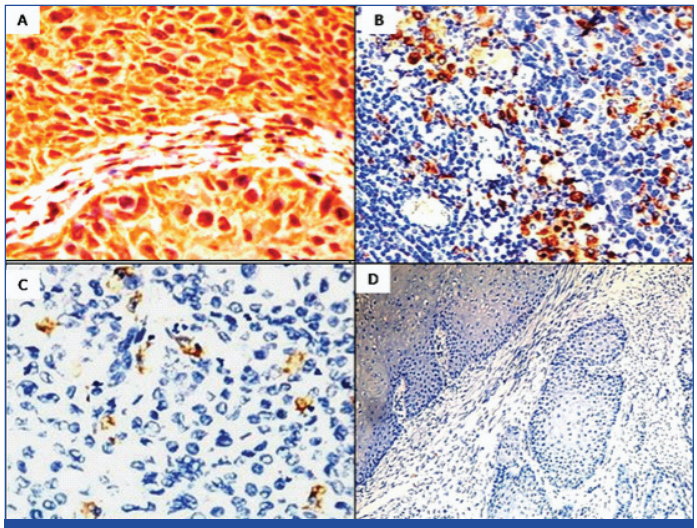
The age of patients ranged between 36 and 86 years with a mean age of 60.28±9.94 SD.

Thirty five (58.3%) cases were expressors for MMP-2 [Table/Fig-1a-c]. and twenty five (41.7%) were non-expressors [Table/Fig-1d]. Thirty six (60%) cases were expressors for MMP-9 [Table/Fig-2a-c]. and twenty four (40%) were non-expressors [Table/Fig-2d]. Correlation between MMP-2 expression and clinicopathological variables is summarised in [Table/Fig-3,4]. Correlation between MMP-9 expression and clinicopathological variables is summurised in [Table/Fig-5,6].

A significant relations were detected between MMP-2 and MMP-9 expression on one hand and invasion, tumour grading (degree of differentiation), anatomical site, positive lymph node metastasis, infiltration of underlying thyroid cartilage, infiltration of resected margins and tumour staging on the other hand in the studied cases of laryngeal SCC [Table/Fig-3-6].



[Table/Fig-1]: IHC MMP-2 immunstaining: a) Poorly differentiated laryngeal SCC, shows staining in ≥50% of tumour cells, considered as strong MMP-2 expression (IHC, X200); b) Poorly differentiated laryngeal SCC, shows staining in 10-50% of tumour cells, considered as moderate MMP-2 expression (IHC, X400); c) Well-differentiated laryngeal SCC, shows staining in <10% of tumour cells, considered as weak MMP-2 expression (IHC, X 200); d) Well-differentiated laryngeal SCC, shows absent MMP-2 immunstaining staining, considered as negative MMP-2 expression (IHC, X100).



[Table/Fig-2]: IHC MMP-9 immunstaining; a) Moderately differentiated laryngeal SCC, shows staining in ≥50% of tumour cells, considered as strong MMP-9 expression (IHC, X400); b) Moderately differentiated laryngeal SCC, shows staining in 10-50% of tumour cells, considered as moderate MMP-9 expression (IHC,X100). c) Well-differentiated laryngeal SCC, shows staining in <10% of tumour cells, considered as weak MMP-9 expression (IHC,X400); d) Carcinoma insitu with microinvasion showed absent MMP-9 immunstaining, considered as negative MMP-9 expression (IHC, X100).

DISCUSSION

The current study showed MMP-2 positive expression in 58.3% of the studied cases. These results were close to that reported by Zhou B et al., who stated that 57.5% their cases expressed MMP-2 [14]. However, other studies found MMP-2 expression in 77% and 75% of their cases respectively [7,13]. Statistical analysis revealed no significant correlation between the patients 'sex and age on one hand and MMP-2 expression on the other hand. Accordingly, several studies showed no significant correlation as well [6,7]. The present study showed a statistical significant relationship between MMP-2 expression and tumour site (p-value=0.000). Similarly, results reported by Zhou B et al., showed a significant relation with p-value=0.002 [14]. On the other hand, our findings were different from those reported Lotfi A et al., and Cao XL et al., which showed no significant relation between MMP-2 expression and tumor site [12,13].

Asignificant relation was found between MMP-2 expression and degree of differentiation (p-value=0.000). These results

Variables		Total number	MMP-2 expression				p-value
			Absent	Weak	Moderate	Strong	
Age	<60	30 (50%)	14 (56%)	9 (56%)	3 (25%)	4 (57%)	0.15
	≥60	30 (50%)	11 (44%)	7 (44%)	9 (75%)	3 (43%)	
Sex	Male	54 (90%)	22 (88%)	14 (87.5%)	11 (91.7%)	7 (100%)	0.7
	Female	6 (10%)	3 (12%)	2 (12.5%)	1 (8.3%)	0	
Grading	I	7 (17.5%)	2 (40%)	5 (31%)	0	0	0.000*
	II	25 (62.5%)	3 (60%)	11 (69%)	7 (58.3%)	4 (57%)	
	III	8 (20%)	0	0	5 (41.7%)	3 (43%)	
Site	Glottic	35 (58.3%)	25 (100%)	10 (62.5%)	0	0	0.000[1]
	Supraglottic	9 (15%)	0	1 (6.3%)	8 (66.7%)	0	
	Subglottic	5 (8.3%)	0	5 (31.2%)	0	0	
	Transglottic	11 (18.4%)	0	0	4 (33.3%)	7 (100%)	
Lymph node metastasis	Positive	10 (25%)	0	1 (6.3%)	4 (33.3%)	5 (71.5%)	0.000*
	Negative	30 (75%)	5 (100%)	15 (93.7%)	8 (66.7%)	2 (28.5%)	
Infiltration of thyroid cartilage	Positive	23 (57.5%)	1 (20%)	6 (37.5%)	9 (75%)	7 (100%)	0.000*
	Negative	17 (42.5%)	4 (80%)	10 (62.5%)	3 (25%)	0	
Infiltration of the resected margins	Positive	11 (27.5%)	1 (20%)	2 (12.5%)	4 (33.3%)	4 (57%)	0.005*
	Negative	29 (72.5%)	4 (80%)	14 (87.5%)	8 (66.7%)	3 (43%)	
Tumour staging	Stage 0	20 (33.3%)	20 (80%)	0	0	0	0.000*
	Stage I	4 (6.7%)	3 (12%)	1 (6.25%)	0	0	
	Stage II	10 (16.7%)	1 (4%)	9 (56.25%)	0	0	
	Stage III	6 (10%)	0	4 (25%)	2 (16.7%)	0	
	Stage IVa	20 (33.3%)	1 (4%)	2 (12.5%)	10 (83.3%)	7 (100%)	

[Table/Fig-3]: Relation between MMP-2 expression and clinicopathological variables.
*Statistically significant

Clinical pathological variables		Total	MMP-2 expression				p-value
			Absent	Weak	Moderate	Strong	
Variant	Invasive carcinoma	40	5 (20%)	16 (100%)	12 (100%)	7 (100%)	<0.01
	Micro-invasive	10	10 (40%)	0	0	0	
	Non-invasive	10	10 (40%)	0	0	0	
Total		60	25	16	12	7	

[Table/Fig-4]: Relation between invasiveness and MMP-2 expression.

Variables		Total number	MMP-9 expression				p-value
			Absent	Weak	Moderate	Strong	
Age	<60	30 (50%)	13 (54%)	10 (59%)	4 (33.3%)	3 (43%)	0.12
	≥60	30 (50%)	11 (46%)	7 (41%)	8 (66.7%)	4 (57%)	
Sex	Male	54 (90%)	21(87.5%)	15 (88%)	11 (91.7%)	7 (100%)	0.6
	Female	6 (10%)	3 (12.5%)	2 (12%)	1 (8.3%)	0	
Grading	I	7 (17.5%)	3 (75%)	4 (23.5%)	0	0	0.000*
	II	25 (62.5%)	1 (25%)	13 (76.5%)	8 (66.7%)	3 (43%)	
	III	8 (20%)	0	0	4 (33.3%)	4 (57%)	
Site	Glottic	35 (58.3%)	24 (100%)	11 (64.7%)	0	0	0.000*
	Supraglottic	9 (15%)	0	1 (58.8%)	8 (66.7%)	0	
	Subglottic	5 (8.3%)	0	5 (29.4%)	0	0	
	Transglottic	11 (18.4%)	0	0	4 (33.3%)	7 (100%)	
Lymph node metastasis	Positive	10 (25%)	0	1 (6%)	4 (33.3%)	5 (71.5%)	0.000*
	Negative	30 (75%)	4 (100%)	16 (94%)	8 (66.7%)	2 (28.5%)	
Infiltration of thyroid cartilage	Positive	23 (57.5%)	1 (25%)	6 (35%)	10 (83.3%)	6 (86%)	0.000*
	Negative	17 (42.5%)	3 (75%)	11 (65%)	2 (16.7%)	1 (14%)	
Infiltration of the resected margins	Positive	11 (27.5%)	1 (25%)	2 (12%)	4 (33.3%)	4 (57%)	0.006*
	Negative	29 (72.5%)	3 (75%)	15 (88%)	8 (66.7%)	3 (43%)	

Tumour staging	Stage 0	20 (33.3%)	20 (80%)	0	0	0	0.000*
	Stage I	4 (6.7%)	3 (12%)	1 (6.25%)	0	0	
	Stage II	10 (16.7%)	1 (4%)	9 (56.25%)	0	0	
	Stage III	6 (10%)	0	4 (25%)	2 (16.7%)	0	
	Stage IVa	20 (33.3%)	1 (4%)	2 (12.5%)	10 (83.3%)	7 (100%)	

[Table/Fig-5]: Relation between MMP-9 expression and clinicopathological variables.

Clinical pathological variables		Total	MMP-9 expression				p-value
			Absent	Weak	Moderate	Strong	
Variant	Invasive carcinoma	40	4 (16.6%)	17 (100%)	12 (100%)	7(100%)	<0.01
	Micro-invasive	10	10 (41.7%)	0	0	0	
	Non-invasive	10	10 (41.7%)	0	0	0	
Total		60	24	17	12	7	

[Table/Fig-6]: Relation between invasiveness and MMP-9 expression.

were consistent with those reported by Uloza V et al., and Pietruszewska W et al., in which a significant relation was found with p-value=0.001 and 0.006 respectively [8,15]. Althoughother studies found an insignificant relation between MMP-2 expression and degree of differentiation with p-value = 0.1 and 0.8 respectively [13,16]. May be increase in tumour grades leads to increased cell aggressiveness causing increased ability of invasion which increase MMPs expression. This suggest the great role of MMP-2 in tumour invasion.

As regards the correlation between MMP-2 expression and Lymph node (LN) status, significant relation was found between LN metastasis and MMP-2 expression (p-value=0.000). Our data were consistent with many studies that found a significant correlation between MMP-2 expression and lymph node metastasis with p-values= 0.03, 0.01, 0.03, 0.04, 0.034 and 0.01 respectively [6,8,12,14-16]. However, other investigators stated no significant relation between MMP-2 expression and LN metastasis with p-value=0.9 and 0.3 respectively [7,13].

The present study illustrated a significant correlations between MMP-2 expression on one hand and and infiltration of the thyroid cartilage (p-value = 0.000) and infiltration of the resected margins (p-value=0.005) on the other hand . Our findings were in agreement with those reported by Gou X et al., who stated the presence of a significant relation between MMP-2 expression on one hand and thyroid cartilage infiltration (p-value=0.012) and infiltration of the resected margins (p-value=0.007) on the other hand [7].

A statistically significant relation between MMP-2 expression and stagingwas found in the current study with p-value=0.000. Those results were near to those reported by Gou X et al., and Lotfi A et al., with p-values=0.012 and 0.04 respectively [7,12]. But other studies revealed no statistically significant relation between MMP-2 expression and staging in spite of increased MMP-2 expression in cases of stage III and IV compared to cases of stage I and II (p-value=0.07 and 0.28 respectively) [6,15].

All the twenty cases of carcinoma insitu and carcinoma insitu with microinvasion were non-expressors (100%). While 35 out of the 40 cases with invasive carcinoma showed MMP 2 expression (87.5%), so a significant relation was found between MMP-2 expression and invasion (p-value <0.01) which suggest the great role of MMP-2 in tumour invasion.

Regarding the immunohistochemical staining for MMP-9 in the current study; 60% of the cases showed MMP-9 positive expression. Similarly, Xie M et al., reported that 63% of their cases expressed MMP-9 [17]. Nevertheless, other investigators found MMP-9 expression in 72% and 79% of their cases respectively [7,13].

No significant correlation was detected between the patients' age and sex on one hand and MMP-9 expressions on the other hand. This is similar to the observations made by Uloza V et al., [8]. A significant relation between MMP-9 expression and tumour site was proved (p-value = 0.000). Similarly, Cao XL et al., stated a significant relation between MMP-9 expression and tumor site (p-value=0.04) [13]. Nevertheless Lotfi A et al., reported no significant relation (p-value = 0.72) [12]. Also, a significant relation was detected between MMP-9 expression and degree of differentiation (p-value=0.000). Those results were close to several studies with p-value=0.01 & 0.001 respectively [8, 15]. Regarding MMP-9 expression and LN metastasis, a significant relation existed (p-value=0.000). Also many studies found a significant correlation between MMP-9 expression and LN metastasis with p-values=0.02 and 0.01 respectively [12,13]. Neverthess, other studies did not find a significant relation between MMP-9 expression and LN metastasis with p-values=0.6, 0.1 and 0.8 respectively [7,8,15]. A significant correlation was found between MMP-9 expression on one hand and infiltration of the thyroid cartilage (p-value=0.000) and infiltration of the resected margins (p-value=0.006) on the other hand. Similar findings were reported by Gou X et al., who stated a significant relation between MMP-9 expression on one hand and infiltration of the thyroid cartilage (p-value=0.025) and infiltration of the resected margins (p-value=0.016) on the other hand [7]. The current study showed significant relation between MMP-9 expression and staging with p-value=0.000. These findings were consistent with other studies results with p-values=0.025 and 0.01 respectively [7,12]. Although, Pietruszewska W et al., showed no statistically significant relation between MMP-9 expression and staging (p-value=0.32) [15].

All the twenty cases of carcinoma insitu and carcinoma insitu with microinvasion were non-expressors (100%). While 36 out of the 40 cases with invasive carcinoma showed MMP-9 expression (90%), so a significant relation was found between MMP-9 expression and invasion (p-value <0.01) which suggest the great role of MMP-9 in tumour invasion.

Statistical analysis proved a significant relation between MMP-2 and MMP-9 expression on one hand and tumour grading (degree of differentiation), anatomical site, positive lymph node metastasis, infiltration of underlying thyroid cartilage, infiltration of resected margins and tumour staging, invasion on the other hand in the studied cases of laryngeal SCC. These differences between studies could be due to different study sample, variable stage of the disease and absence or presence of metastasis as well as the method of evaluating these markers. However, it is obvious that MMPs have considerable role in evaluating the advanced or early stage of the tumours and may be used as early predictor of their prognosis.

LIMITATION

The current study has the following limitations: Small sample size and lack of reliable registry of the patients and follow-up for detection of distant metastasis and survival-rate, so we could not comment on prognosis.

CONCLUSION

To sum up, in this study, the relation between MMP-2 and MMP-9 expression on one hand and degree of differentiation, tumour site, extent of ivasion, lymph node metastasis and stage on the other hand was proved to be statistically significant. MMP-2 and MMP-9 may be a novel and worthwhile therapeutic target for cancer. Future studies should be carried out on larger scale in correlation with other factors as survival rate and therapeutic effect in attempt to develop novel therapeutic strategy targeting MMP-2 and MMP-9 antigen and also for more investigations of their possible role in differentiation between neoplastic and non-neoplastic laryngeal lesions.

Future recommendations: Further studies are required in a larger number of patients with respect to follow-up and distant metastasis, so the prognostic role of MMP-2 and MMP-9 can be highlighted.

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