The role of Laminin111 and Matrix metalloproteinase-2 Immunohistochemistry Expression in Prediction of Laryngeal Squamous Cell Carcinoma Prognosis

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الخلاصة:
الهدف: البحث في دور LAM111 و MMP2 كموشرات تنبؤية في سرطان الحنجرة ذو الخلايا الحرشفية.
المواد وطرق: أجريت هذه الدراسة على 30 مريضا مصابون بسرطان الحنجرة ذو الخلايا الحرشفية في قسم جراحة الأنف والاذن والحنجرة، مستشفى الديوانية في محافظة القادسية - العراق. شملت البيانات السريرية والنسيجية; الجنس ،العمر ،الاعراض السريرية، مرحلة الورم،مبثوثات العقد الليمفاوية ، و تم إجراء الدراسة الكيميائية النسيجية المناعية باستخدام مضادات احادية النسل لتقيم تعبير LAM111 و MMP2. **النتائج:** كانت أعمار الغالبية العظمى من المرضى فوق 50 سنة (73.33٪)، الذكور أصيبوا اكثر من الإناث مع نسبة الذكور إلى الإناث 2.8: 1، وكانت 50٪ من ألاورام في منطقة لسان المزمار،ظهرت 86.67٪ منهم ككتل ورمية . صنفت معظم الأورام كمتمايزة الشكل (53.33٪)،وكانت نتائج العقد اللمفاوية لغالبية المرضى سلبية (76.67٪) ، وكان 14 (46.67٪) من المرضى في مرحلة المرض الثانية.اظهرت LAM111 ارتباط إيجابي كبير مع انبثاث العقد اللمفاوية (P = 0.027) ومرحلة الورم (P = 0.018) ، في حين كشفت MMP2 ارتباط إيجابي كبير فقط مع انبثاث العقد اللمفاوية (P = 0.029). كانت هناك علاقة طردية ذات دلالة إحصائية بين LAM111وMMP2 (P = 0.014) . **الخلاصة:** كل من LAM111 وMMP2 تعتبر مؤشرات تنبؤية سيئة لسرطان الحنجرة ذو الخلايا الحرشفية والتنبؤ بانبثاث العقد اللمفاوية.

ABSTRACT:
Objective: To investigate the role of laminin111 (LAM111) and matrix metalloproteinase-2 (MMP2) as a prognostic predictors in laryngeal squamous cell carcinoma (LSCC). **Materials and Methods:** The study was carried out on 30 patients with LSCC treated at the ENT Department, Al-Diwaniya Hospital in Al-Qadisiya province- Iraq. The clinical and histological data consisted of sex, age, site, clinical presentation, stage of the tumor, lymph node (LN) metastasis, and immunohistochemical studies (IHC). The IHC study was performed using anti LAM111and anti MMP2 monoclonal antibodies. **Results:** The majority of patients were above 50 years (73.33%), male affected more than the female with male to female ratio 2.8:1, 50% of tumors were located in glottic region, (86.67%) presented as mass. Most of the tumors were classified as well differentiated LSCC (53.33%), (76.67%) were recorded as negative LN metastasis, and 14 (46.67%) patients were in disease stage II. LAM111showed significant positive correlation with LN metastasis (P=0.027) and tumor stage (P=0.018), while MMP2 revealed significant positive correlation only with LN metastasis (P=0.029). There was a significant positive correlation between LAM111and MMP2 (P=0.014). **Conclusion:** Both LAM111 and MMP2 are poor prognostic markers for LSCC and predict LN metastasis.

Keywords: Laminin111; MMP2; Immunohistochemistry; Larynx; Carcinoma; Prognosis.

1. Introduction:

Despite the innovation of competent diagnostic and theraputic strategies related to laryngeal cancer (LC) is highly bushy, the survival rate remains virtually unchanged. LSCC is highly aggressive cancer and is the common histological type of LC, represented the 20th most malignant tumor in the world and more than 150,000 new cases are diagnosed every year [(1)]. In a review conducted by Bobdey *et al.*, 2015[(2)] concluded that tobacco smoking and alcohol are the major risk factors for LC. The majority

of LSCC are diagnosed between 50 to 75 years of age; the male-female ratio was from 6:1 to 10:1 in majority of cases [3]. Available approaches for early diagnosis of LSCC were proprely unserious. Due to the starving of effective early-diagnosis methods till now, only fewer than 50% of patients with LSCC were in early stage at diagnosis, [4]. LSCC extremly affects the life quality of patients, and the 5-year survival is about 60% regardless of stage, treatment planning, and tumor site [5].

Laminins are a group of glycoproteins that made up of one heavy α chain and two light β and γchains, is a major constituent of the basement membrane and have an important role in cell differentiation, adhesion, and immigration [6]. One of the first laminin isoforms to be discovered was laminin–1, but the nomenclature was recently changed to laminin–111(LAM111). The "111" identifies the isoform's chain composition of α1β1γ1 [7]. It has been found that laminin is strikingly expressed at the invasive front of the tumor and plays an important role in promote tumor growth and metastases [6, 8].

Matrix metalloproteinase-2 is a member of the matrix metalloproteinase (MMP) family, which is involved in the breakdown of extracellular matrix in normal physiological processes [9, 10], and in the tumor invasion and metastasis [11]. Christopoulos *et al.*,[12] believed that MMP2 could be clinically considered as an important pathological diagnostic parameter of LC because its protein expression emerges at the early stage of LC and gradually increases with disease progression. Significantly elevated expression levels of MMP2 protein with tumor progression and LN metastasis have been observed. As regards the tumor stage, Shehata *et al.*,[13] found that advanced tumor stages are associated with an increase in MMP2 expression; but this is not statistically significant, while the presence of LN node metastasis is significantly related to overexpression of MMP2 and concluded that increased production of MMP2 may be among the factors that increase the metastatic tendency in LC. Meanwhile, Liu *et al.*, [14] observed a non-significant relationship found between the expression of MMP-2 and clinico-pathological characters of LSCC, such as histological grade, primary site, T stage, N stage, and clinical stage, however, concluded that, the expression of MMP-2 could be used as a potential predictor for poor prognosis in patients with LSCC.

Regional LN metastasis and distant metastasis are critical in the prognosis of LSCC. This study investigates the roles of LAM111 and MMP2 as a prognostic predictors in LSCC.

2. Material and Methods:

This study was carried out on a conventional sample represented by thirty patients with LSCC involving various regions of the larynx (and at various stages) undergoing total Laryngectomy in ENT Department, Al-Diwaniya Hospital in Al-Qadisiya province- Iraq, from January 2014 through March 2016. The diagnosis of each case was proved by the histological examination of the hematoxylin and eosin (H&E) stained sections by mean of light microscope by two experienced pathologists, Figure (1). The clinical data consisted of age, sex, site, clinical presentation; tumor grade, stage and LN status were identified from surgical and pathological reports of the patients. Tumor Stages were carried out according to WHO classification schema, while tumor grades were established as follows: Grade I (well differentiated), Grade II (moderately differentiated), and Grade III (poorly differentiated) [15].

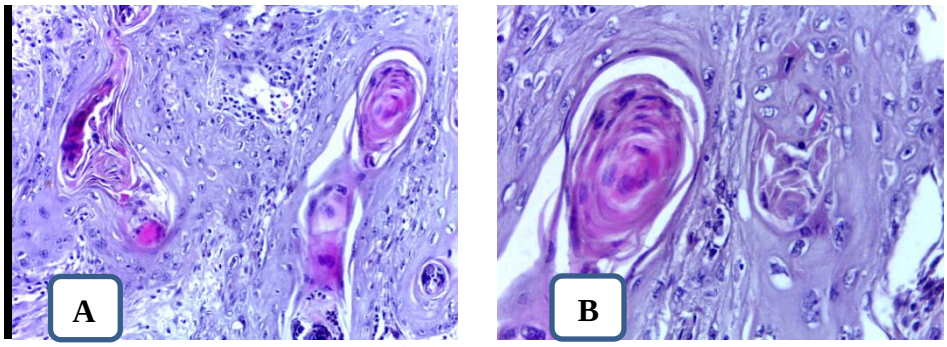

Figure1: Laryngeal SCC (A-20X) (B-40X) H&E.

The IHC study was carrying out using formalin-fixed and paraffin-embedded tissue sections. Rat monoclonal anti-LAM111 antibody and Rabbit monoclonal anti-MMP2 antibody were used for LAM111 and MMP2 respectively (Abcam). Positive controls were included in each IHC run. Tissue block of colon carcinoma was used for LAM111, and a tissue block of breast adenocarcinoma was used for MMP2 (according to antibodies manufacturer).

2.1 Evaluation of IHC Results

The immunoreactions evaluation analyzed according to the presence or absence of brown immunostaining in the cytoplasm and extracellular space. The expression of both markers was estimated semi-quantitatively. It was obtained by counting the number of tumor cells in 5 fields (using 40X objective in most represented areas of sections). Labeling index for each field was calculated using the following equation: (number of positive cells/ number of total cells); the mean value of labeling indices for the five fields was considered to be the label index for the case. For the LAM111 staining, four-point grading system was used: score 0= 0%; score 1= <10%; score 2=<50%; and score 3=>50% [6]. As regards the MMP2 protein expression, a semiquantitative analysis based on a 4-point scale was used depending on the percentage of positive cells staining: score 0=0%; score 1=<10% positive cells; score 2=10–50% positive cells; and score 3=>50% positive cells [13].

2.2 Statistical analysis:

Numeric variables were showed as mean±SD (standard deviation) whereas nominal variables which include: sex, sites, clinical presentation, grade, LN and stage were expressed as number and percentage. Spearman Rank coefficient of correlation (r) was used to find the relation between any two variables. Statistical analysis was done with SPSS (statistical package for social sciences) V20. The level of (≤0.05) was considered significant, while the level of (≤0.001) was considered highly significant for interpretation of P values.

3. Results:

3.1 Clinicopathologic characteristics:

This study it has been conducted on Thirty Iraqi patients, all of them are from Al-Qadisiya province. Clinical findings of LSCC cases were designed as follows: Most of the cases (22 =73.33%) were above 50 years with an age range from 40 to 80 years (mean ± SD = 58.23±9.38). Twenty two patients (68.4 %) were male; the rest 8 cases (26.67%) were female, with male to female ratio was 2.8:1. Primary sites were mainly identified in the glottic region 15 cases (50%), followed by the supraglottic region (12 cases= 40%), and only 3 cases was recorded in the subglottic region (10%). Twenty six of the studied cases (86.67%) presented as mass, the rest 4 cases (13.33%) were presented as ulcer, Table 1.

Table 1: Clinical characteristics

Characteristic		N (%)	Mean±SD (range)
Age (years)	≤50 years	8 (26.67%)	58.23±9.38 (40-80)
	>50 years	22 (73.33%)	
Sex	Male	22 (73.33%)	
	Female	8 (26.67%)	
Site	Glottic	15 (50%)	
	Supraglottic	12 (40%)	
	Subglottic	3 (10%)	
Clinical presentation	Mass	26 (86.67%)	
	Ulcer	4 (13.33%)	

N: Number of cases; SD: Standard deviation

Histological sections revealed that the majority of the cases had well differentiated SCC (16= 53.33%), followed by moderatily differentiated (11= 36.67%) then poorly differentiated SCC (3=10%).Twenty three cases (76.67%) it has been recorded as negative LN metastasis and 7 patients (23.33%) as positive LN metastasis. About half of the patients had stage II disease (14= 46.67%); stage III disease was seen in 8 patients (26.67%), while stage I and IV were seen in 4 patients (13.33%) respectively.Table 2.

Table 2: Stage, grade and LN involvement

Characteristic		N (%)
Grade	I	16 (53.33%)
	II	11 (36.67%)
	III	3 (10%)
LN	Positive	7 (23.33%)
	Negative	23 (76.67%)
Stage	I	4 (13.33%)
	II	14 (46.67%)
	III	8 (26.67%)
	IV	4 (13.33%)

3.2 Immunohistochemistry evaluation:

Immunohistochemical staining was detected as a brown staining in the cytoplasm and (or) extracellular space of target antigen cells. Positive IHC expression was found in all 30 cases (100%) in both markers. Regarding LAM111 about Two-thirds of the cases were reported as score 2 (21 cases=70%), followed by score 1(8cases=26.67%), while score 3 was recorded in only one case (3.33%), with a mean score range of 1.77±0.50. The IHC expression of MMP2 was distributed as follows: The majority of the cases expressed MMP2 in score 2 (17 cases =56.67%), followed by score 3 (11 cases =36.67%) whereas 2 cases (6.67%) had score 3, with a mean score range of 2.3±0.60. Table: 3 and Figure: 2, 3 and 4.

Table 3: Immunohisochemical expression scores of LAM111and MMP2

	LAM111		MMP2	
Score	N	%	N	%
1	8	26.67	2	6.67
2	21	70.00	17	56.67
3	1	3.33	11	36.67
Mean score (range)	1.77±0.50		2.3±0.60	

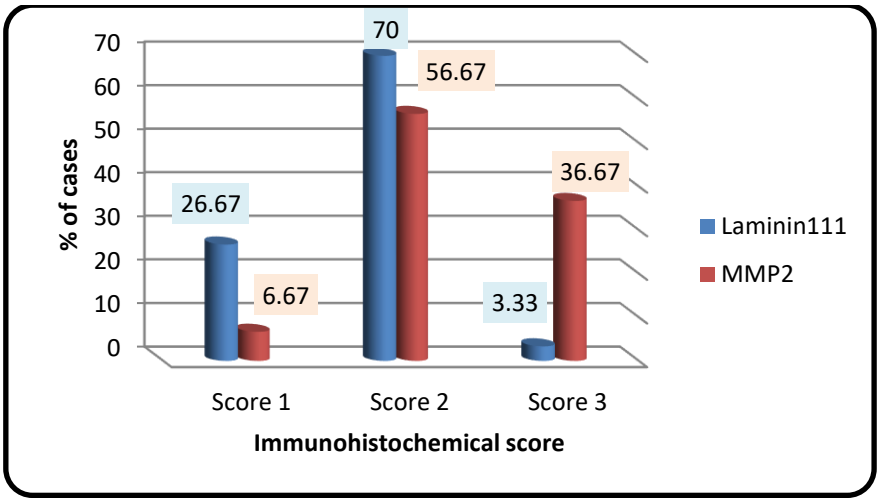

Figure 2: Immunohistochemical expression scores of LAM111and MMP2

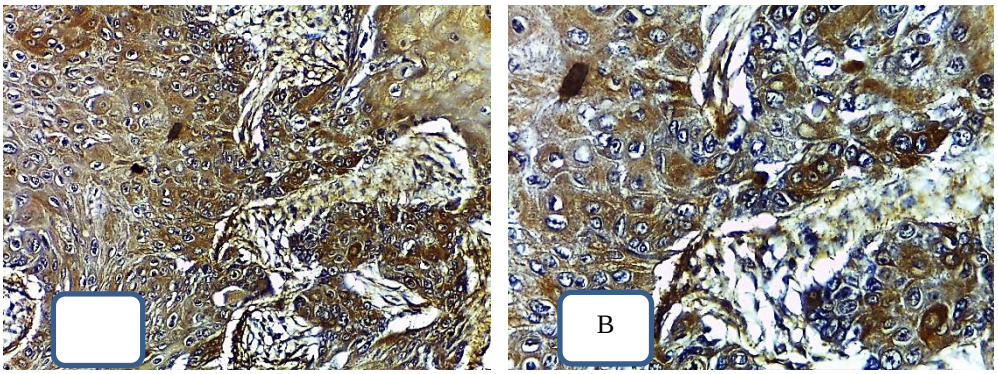

Figure 3: LAM111immunohistochemical expression (A-20X) (B-40X)

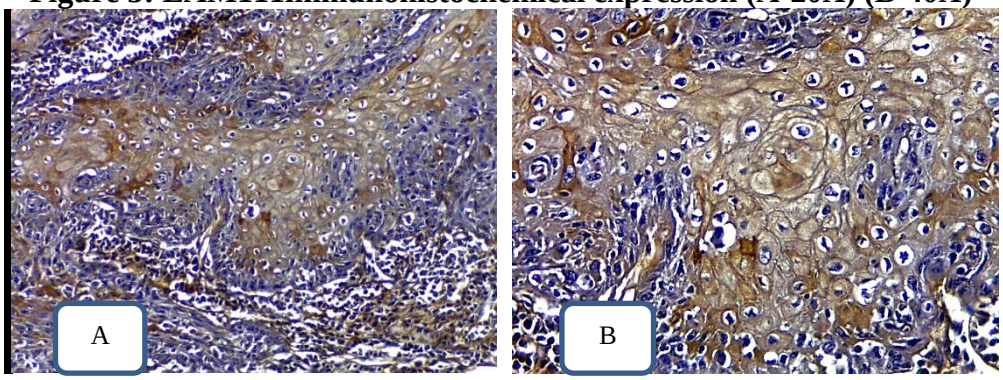

Figure 4: MMP2 immunohistochemical expression (A-20X) (B-40X)

According to Chi-Square test, the IHC expression of LAM111 showed a significant positive correlation with both LN metastasis (r=0.404, P=0.027) and

tumor stage (r=0.428, P=0.018). Whereas, MMP2 revealed significant positive correlation only with LN metastasis (r=0.4, P=0.029). Moreover, There was a significant positive correlation between LAM111and MMP2 (r=0.444, P=0.014). Table: 4.

Table 4: Correlation between clinicopathologic characteristics and immunohistochemical expression of LAM111and MMP2

Parameter	Correlation	Age	Sex	Site	Clinically	Grade	LN	Stage	MMP2
LAM111	r	0.003	0.147	0.146	-0.007	-0.03	0.404	0.428	0.444
	P	0.985	0.438	0.443	0.97	0.875	0.027	0.018	0.014
MMP2	r	-0.146	0.06	0.042	0.129	-0.013	0.4	0.282	
	P	0.443	0.754	0.825	0.496	0.945	0.029	0.131	

*r: Correlation coefficient; P: level of significance

4. Discussion:

For the best of our knowledge this is the first study that deals with IHC expression of LAM111 and MMP2 in LSCC in Al-Qadisiya province -Iraq.

Concerning the clinical finding, the present study showed that most of the patients were more than 50 years of age and that the tumor showed male predilection. These findings are similar to the results of several studies [1, 3, 16, 17]. The occurrence of disease in such relatively advanced age reflects the nature of pathogenesis for such tumor development which requires the accumulation of multiple genetic mutations that favor the emergence of neoplastic epithelial laryngeal cells, and this requires the accumulation of effect of environmental factors namely habitual smoking. The high rate of the tumor in male in comparison with females may be attributed to the relatively high exposure to carcinogenic agents by the male compared to female patients (smoking and alcoholism). The most prevalent site recorded in the current work was glottis region and this agrees with studies conducted by Calkovsky *et al*., 2016 [1] and Markou *et al*., 2013[18], while disagrees with the results of Butler *et al*., 2016 [19] and Siddiqui *et al*., 2012[20] who found in their study which took place in Bihar, a state of Eastern India, that the supraglottic area was commonly affected site, and attributed it to a widespread unawareness, low healthcare serving with virtually non-existent cancer programs.

With regard to histological findings, this study revealed that majority of the cases had well differentiated squamous cell carcinoma. This is in accordance with results of Menach *et al*., 2014 [21] in a demographic study ln Kenya. However, Markou *et al*., 2013[18] recorded relatively a similar rate of grade I and grade II in their epidemologic study on 1088 cases of LSCC in Northern Greece. The most frequent stage in the present study was stage II, this in harmony with the result of Dechaphunkul, 2011 [22]. Whereas, Calkovsky *et al*., 2016 [1], in their study that carryed out in Northern Slovakia, found that the majority of the cases with LC were admitted to the hospital in advanced stages, and they found that (68 %) of patients were in disease stage III and IV, Likewise, the results of Menach *et al*., 2014 [21]. Most of our cases had no LN metastasis and the rate of positive LN involvement was 23.33%; this probably due to that the most of our cases are diagnosed in stage

stage II. Relatively a lower percentage of LN involvement (13.3%) was recorded by Markou *et al*., 2013[18]. However, (28.1%) was observed by Ma et al 2014[23], furthermore a higher rate of LN metastasis (50%) was recorded by Mielcarek-Kuchta *et al*., 2008 [6].

Regarding Imunohistochemical evaluation, both LAM111 and MMP2 overexpression came up with a predominant score 2 (70%) and (56.67%), respectively. This is in agrement with the results of previous studies [6, 24, 25, 26, 27,28]. Concerning LAM111 biomarker, the results of the present study revealed non significant correlation of LAM111 with age, sex, site, clinical presentation and grade, while it correlated significantly with LN metastasis and tumor stage, and this is consistent with results of Zhou *et al*., 2006 [24] and Hagedorn *et al*., 1998 [29]. This means that LAM111 is a poor prognostic factor since its higher expression predicts higer stage and LN involvement. The proposed explanation for this finding in our opinion is that the higher the expression of LAM111 the more is the ability of neoplastic cell to invade and breach basement membrane and surrounding tissue. Contrarliy, Mielcarek-Kuchta *et al*., 2008 [6] concluded that there was statistically non significant correlation (P = 0.09) between laminin and occurrence of LN metastases.

Regarding MMP2 biomarker, Statistical significance could not be proved between MMP2 expression and the clinico-pathological features of the disease except with LN involvement. This result goes with the results of previous studies [11,13,25,27,28]. This indicates a bad prognostic value for MMP2 in LSCC. The higher the expression of MMP2 by neoplastic cell make them more capable of degrading surrounding matrix tissue and breaching lymphatic channels and for an unknown reason the breaching of vascular channels will be less efficient. In the same context, Zhou *et al*., 2015 [26] reviewed Seventy-three surgical specimens from patients with LSCC, found a significant correlation with site of the tumor, clinical stage, and LN metastasis, while age, sex, pathological differentiation revealed non significant correlation, concluded that, MMP2 was related with worse overall disease survival and could be considered as a possible marker of poor prognosis. However, Liu *et al*., 2015[28] in their meta-analysis study on LSCC, found that MMP2 protein expression revealed a highly significant correlation with tumor differentiation (P<0.001).

Metastatic spread depends on the ability of tumor cells to penetrate basement membranes by elaborating and secreting specific proteolytic enzymes such as MMP2. Laminin is a major constituent of the extracellular matrix that can elicit production of MMP2 in metastatic cells,in addition laminin-induced stimulation of phospholipase D and consequent generation of phosphatidic acid are involved in a signal producing pathway leading to induction of MMP2 and enhanced invasiveness of metastatic tumor cells[30]. It has been shown that LAM111 can be processed by different MMPs and a variety of other enzymes and that such modification are mainly related to cell immigration because cleavage of laminin results in a loosened basement membrane [31]. Moreover, MMP2 is known to have an tendency for laminin and type IV collagen, which are the main compositions of the basal lamina, this indicates that MMP2 may be related to the destruction of the basal lamina, inducing the process of malignant cell invasion [32]. Our data is in consistent with these studies; as the LAM111 and MMP2 showed a significant positive correlation (r=0.444, P=0.014). The results from this study showed that the

high expression of LAM111 and MMP2 protein are poor prognostic markers for LSCC and predict LN metastasis.

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