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RESEARCH

Expressions of CD147, MMP-2 and MMP-9 in Laryngeal Carcinoma and its Correlation with Poor Prognosis

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Abstract The objectives of this study are to investigate the expressions of matrix metalloproteinase inducing factor (CD147), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9) in laryngeal tumor tissues and its significant correlation with tumor infiltration, metastasis and prognosis. Laryngeal tumor tissue from 48 laryngeal cancer patients with complete clinical information was collected. The laryngitis tissue from 15 patients were collected as control group. Immunohistochemical analysis for CD147, MMP-2 and MMP-9 was performed for all the tissue. The results showed the expression rates of CD147, MMP-2 and MMP-9 in laryngeal carcinoma were 87.5 %, 75.0 % and 79.2 % respectively, significantly higher than those (26.7 %, 6.7 %, and 33.3 % respectively) in the control group are ($P<0.01$). High expression of CD147, MMP-2 and MMP-9 related to the clinical stages and lymphatic metastasis of laryngeal carcinoma. Univariate survival analysis showed that the 5-year survival of laryngeal carcinoma patients with low expression of CD147, MMP-2 and MMP-9was 83.3 %, 83.3 % and 90 % respectively, while the patients with high expression had 5-year survival at 25 %, 7.7 % and 18.2 % respectively ($P<0.05$). Multiple regression analysis showed that high expression of MMP-9 was independently associated with poor prognosis ($P<0.05$). High expression of CD147, MMP-2 and MMP-9 were related with laryngeal carcinoma invasion and metastasis. High expressions of CD147, MMP-2 and MMP-9 were all predictive factors of poor prognosis of laryngeal carcinoma.

Keywords Laryngeal carcinoma · Extracellular matrix metalloproteinase induced factor · Matrix metalloproteinase-2 · Matrix metalloproteinase-9 · Immunohistochemistry

Introduction

Laryngeal carcinoma is a common malignant tumor in head and neck region with increasing morbidity. Infiltration and metastasis are important factors affecting treatment efficacy and prognosis of laryngeal carcinoma patients. The degradation of extracellular matrix components and basement membrane by cancer cells is the key step for tumor invasion and metastasis. The extracellular matrix metalloproteinase inducing factor (CD147) can trigger extracellular matrix metalloproteinase stimulate and induce the release of matrix metalloproteinase (MMPs), which then degrade the matrix and basement membrane [1–4]. It has been confirmed that CD147 plays an important role in invasion and metastasis of malignant melanoma, breast cancer and nasopharyngeal carcinoma. However, its role in laryngeal carcinoma has been rarely reported. In the present study, we investigated the expression of CD147 and MMPs in laryngeal carcinoma and demonstrated its relationship with the invasion and prognosis of laryngeal carcinoma.

Materials and Methods

Laryngeal Carcinoma and Laryngitis Tissue Samples

Tissue samples of laryngeal carcinoma from 48 patients preserved in our pathology department from November 1997 to

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January 2003 were selected, as well as tissue samples of laryngitis from 15 patients in corresponding period. All laryngeal carcinoma samples were pathologically confirmed as laryngeal squamous cell carcinoma. The median age of laryngeal carcinoma patients was 64 years (44 to 81 years). Their KPS score was above 70. The clinical stages of laryngeal carcinoma were determined according to sixth edition of AJCC criteria. Moreover, survival was defined as the period from confirmed diagnosis by biopsy or surgery to the last follow-up visit (March, 2008) or death caused by tumor recurrence or metastasis. The median time of follow-up was 58 months. None of the patients was given preoperative chemoradiotherapy. The involvement of all tissue samples in this study was approved by the institutional review board of the charging Hospital.

Therapeutic Regimens

Eleven cases only underwent operations. 3 cases only recived radiotherapy. One case had chemotherapy. 2 cases received chemotherapy combined with radiotherapy. 11 cases had surgery combined with radiotherapy and 20 cases had surgery combined with chemoradiotherapy. Surgical approach was as follows: 31 cases had partial laryngectomy, 11 cases had total laryngectomy. As for radiotherapy, 6MV of X-ray conventional radiotherapy was adopted with the dosage at 50~74Gy/5~8 W. The chemotherapy regimens included PF and TP regimens. Among the patients who had chemotherapy, 20 cases received PF(Cis-platinum 60~80 mg/m^2 iv, d1-2+fluorouracil 750 mg/m^2 d1, iv, sustained for 72~120 h) and 3 cases received TP (Cis-platinum 60~80 mg/m^2 iv, d1-2+paclitaxel 130 mg/m^2, iv, d1). A cycle was considered to include 21 to 28 days (median 3 weeks) and there were 2~6 cycles in total.

Immunohistochemistry Staining

Dako EnVision Kit (Dako, Denmark) was applied for the immunohistochemical staining. Paraffine-embedded specimens were cut into pieces with thickness of 4-μm, mounted onto polylysine-coated slides, dewaxed in xylene, rehydrated in alcohol, and incubated with 3 % H_2O_2 mixed with methanol for 12 min to block endogenous peroxidase activity. For antigen retrieval, the pieces were treated with citric acid buffer (pH6.0). After treatment with PBS for 5 min per time and totally 3 times, the consecutive tissue pieces were incubated with Rabbit-anti-human polyclonal antibodies of CD147(1:100 dilution) or anti-MMP-2(1:50 dilution) or anti-MMP-9(1:50 dilution) for 12 h at 4 °C. The antibodies were all purchased from Beijing Zhong Shan -Golden Bridge Biological Technology Co., LTD. Then Envision reagent was added and incubated for 30 min in ambient temperature for chromogenic reaction. Finally, the slides were stained again but with haematoxylin. PBS was used to replace the primary antibody as the negative control.

Evaluation of Immunohistochemistry Staining

The pathological diagnosis of HE dyeing pieces were analyzed by experienced pathologist, who were blinded to the groups. The immunohistochemical staining pieces were observed under microscope. Meanwhile, percentage of positive cells was calculated. The positivity was confirmed according to two factors, the percentage of positive cells in the visual field and the dyeing intensity. Methods were as follows: (1) the percentages of positive cells were stratified into four levels: the percentage of positive cells ≤5 % scored as 0; 6–25 % as 1; 26–50 % as 2; >51 % as 3; (2) The non-staining was scored as 0, yellow as 1, claybank as 2, yellowish-brown as 3. Then, add up the results from procedure one and two and divided by 2. Ultimately, score 0 would be defined as negative (-), score 0.5 or 1 as weakly positive (+), score 1.5 or 2 as positive (++), score 2.5 or 3 as strongly positive (+++). All the results were confirmed by two pathologists in a double-blinded manner.

Statistical Analysis

Statistical software SPSS11.5 was applied to process the data, and χ^2 test was used. Spearman rank correlation was adopted to analyze the relevance between the three proteins and disease prognosis. Moreover, Kaplan-meier Curve was used for univariate survival analysis, and log-rank test was adopted to test the significance of difference. In addition, Cox proportional hazard model was applied in multivariate survival analysis. $P<0.05$ was considered to have statistical significance.

Results

CD147, MMP-2 and MMP-9 Highly Expressed in Laryngeal Carcinoma Tissues

The positive staining of CD147 were charactered by intense and diffuse brown staining on tumor cell membrane (Fig. 1d). Some cases also showed weak or inconsecutive staining in cytoplasm (Fig. 1b, c). CD147 expression was very weak or negative in the laryngitis tissue (Fig. 1a). The positive expression rates of CD147 in laryngeal carcinoma and laryngitis groups were 87.5 % and 26.7 %, respectively ($P<0.01$). Additionally, negative (-), weakly positive (+), positive (++), strongly positive (+++) expression rates of CD147 in laryngeal carcinoma were 12.5 %, 22.9 %, 22.9 % and 41.7 % respectively.

The expression of MMP-2 and MMP-9 appeared to be in the cytoplasm of tumor cells and interstitial cells (Fig. 1h, l), which were rarely expressed in laryngitis tissue (Fig. 1e, i). The total expression rate of MMP-2 in laryngeal carcinoma was 87.5 %, which was significantly higher than that in the laryngitis group (26.7 %, $P<0.01$). Specifically, expression rates of MMP-2 in levels of negative (-), weakly positive (+),

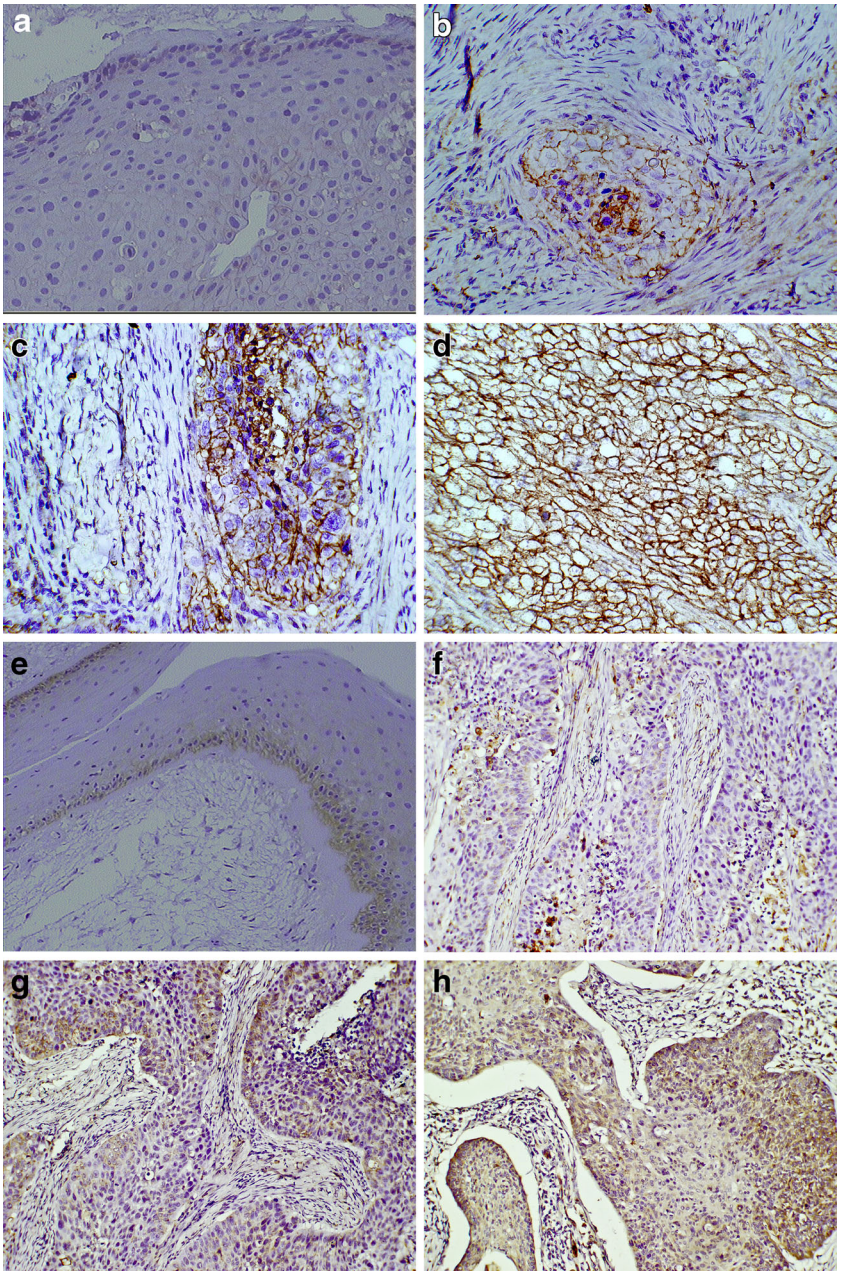

Fig. 1 Immunohistochemical staining for CD147,MMP-2 and MMP-9 in laryngitis (EnVision×200). CD147 positive expression was found in cell membrane at various levels (+), (++), (+++) in laryngeal carcinoma (**b**, **c**, **d**), respectively, staining in non-tumor larynx tissues (**a**), MMP-2,MMP-9 positive expression was found in cytoplasm at various levels in laryngeal carcinoma (**f**, **g**, **h**, **j**, **k**, **l**), respectively,MMP-2,MMP-9 staining in non-tumor larynx tissues (**e**, **i**)

positive (++) and strongly positive (+++) were 25.0 %, 27.1 %, 20.8 % and 27.1 %, respectively. The total expression rate of MMP-9 was 75.0 %, which was also significantly higher than that in the laryngitis group (6.7 %, $P<0.01$). The expression rates of MMP-9 in levels of negative (-), weakly positive (+), positive (++) and strongly positive (+++) were 20.8 %, 25.0 %, 31.3 % and 22.9 % respectively.

It was shown in correlation analysis that concordance rates of expression level between CD147 and MMP-2, CD147 and MMP-9, MMP-2 and MMP-9 in laryngeal carcinoma tissue were 72.9 %, 56.3 % and 50.0 %, respectively. Expressions of the three proteins in laryngeal carcinoma were positively correlated ($r=0.436\sim0.741$, $P<0.01$).

Expressions of CD147, MMP-2 and MMP-9 and Clinicopathological Features in Laryngeal Carcinoma

The data indicated that the expressions of CD147, MMP-2 and MMP-9 in laryngeal carcinoma had nothing to do with the age of patients. However, expression of CD147, MMP-2 and

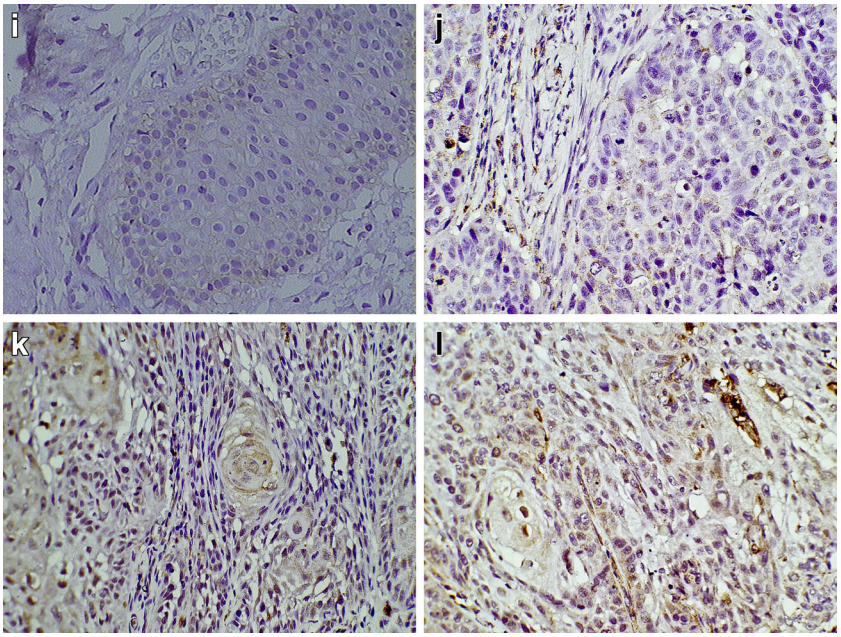

Fig. 1 (continued)

MMP-9 varied between patients with different T stages. Their positive expression rates of primary lesion in advanced stages (T3+T4) was significantly higher than those of primary lesion in early stages (T1+T2), (Table 1, $p<0.05$). The total positive rate of CD147, MMP-2 and MMP-9 expression in laryngeal carcinoma seemed not related with lymphatic metastasis, compared with that in non-metastasis group ($P>0.05$), whereas the strongly positive (+++) expression rates of CD147, MMP-2 and MMP-9 were significantly higher than that in non-metastasis group (Table 2, $P<0.05$). Expressions of CD147, MMP-2 and MMP-9 varied in the laryngeal carcinoma of different clinical stages, and positive expression rates in stage III to IV diseases were significantly higher than those in stage I to II diseases ($P<0.05$).

Relationship Between the Expressions of CD147, MMP-2 and MMP-9 and the Prognosis of Laryngeal Carcinoma

Eleven cases underwent operation only. Eleven cases received postoperative radiotherapy and 20 cases had postoperative chemo-radiotherapy. All the cases were followed up for more than 5 years. And 5-year overall survival was 62.5 % (30/48) in total. 5-year overall survival in operation-only, post-operation radiotherapy, and post-operation chemo-radiotherapy group were 45.4 %, 72.7 % and 65.0 % respectively. There were no statistically significant difference among overall survival of patients with different treatment ($P>0.05$).

Among patients who survived more than 5 years, expression of CD147, MMP-2 and MMP-9 of their tissue samples

Table 1 Expressions of CD147, MMP-2 and MMP-9clinicopathological features in laryngeal carcinoma

Factor	Cases	CD147 positive		MMP-2 positive		MMP-9 positive	
		n (%)	*p* value	n (%)	*p* value	n (%)	*p* value
Age							
<65岁	24	21(87.5)		18(75)		18(75)	
≥65岁	24	21(87.5)	1.000	18(75)	1.000	20(83.3)	0.477
T stages							
T_1+T_2	21	16(76.2)		12(57.1)		13(61.9)	
T_3+T_4	27	26(96.3)	0.037	24(88.9)	0.012	25(92.6)	0.025
N stages							
N_0	33	27(81.8)		23(69.7)		25(75.8)	
N_1+N_2	15	26(96.3)	0.195	13(86.7)	0.369	13(86.7)	0.632
Clinical stages							
I+II	20	15(75.0)		11(55.0)		12(60.0)	
III+IV	28	27(96.4)	0.3	25(89.3)	0.007	26(92.9)	0.016

Table 2 Strongly positive expression of CD147, MMP-2 and MMP-9 and lymphatic metastasis in laryngeal carcinoma

	CD147 strongly positive			MMP-2 strongly positive			MMP-9 strongly positive		
		n (%)	*p* value		n (%)	*p* value		n (%)	*p* value
N_0	10/33	30.3		5/33	15.2		4/33	12.1	
N_1+N_2	10/15	66.7	0.018	8/15	53.3	0.016	7/15	46.7	0.023

were 25 (83.3 %), 20 (66.7 %) and 21 (70.0 %) respectively. However, among the patients who survived less than 5 years, expressions of the three proteins were 17 (94.4 %), 16 (88.9 %) and 17 (94.4 %), respectively. The notifying difference is that MMP-9 expression had significant difference between patients who survived more than 5 years and those who survived less than 5 years ($P<0.05$).

In Kaplan-Meier survival analysis, 5-year survival of laryngeal carcinoma patients with negative CD147, MMP-2 and MMP-9 expression groups was 83.3 %, 83.3 % and 90 % respectively, higher than that in patients with strongly positive expression (25 %, 7.7 % and 18.2 %, respectively; log-rank= 5.24, 4.09 and 6.74; $P<0.05$, Figs. 2, 3, and 4). The higher expression of CD147, MMP-2 and MMP-9, the less survival of patients and the worse prognosis they had as well. Moreover, strongly positive expression rates of CD147, MMP-2 and MMP-9 were 80 %, 60 % and 60 % respectively in patients with recurrence and metastasis of laryngeal carcinoma, significantly higher than those of the three proteins in tumor-free patients (31.6 %, 18.4 % and 13.2 % respectively; $P<0.05$, Table 3). In addition, multivariate analysis by Cox model demonstrated that the expression of MMP-9 rather than CD147 and MMP2, was the independent factor which affected the prognosis and overall survival of laryngeal carcinoma ($P<0.05$) (Table 3).

Discussion

Degradation of extracellular matrix components and basement membrane by tumor cells is an important step in the invasion and metastasis of tumor. MMPs are a group of zinc ion dependent endopeptidases. Among them, type IV collagenase (MMP-2, MMP-9) plays a pivotal role [5], and its high expression has also been found closely related with metastasis and poor prognosis of carcinoma, such as human colorectal cancer, gastric carcinoma, breast cancer, lung cancer, prostatic carcinoma and head and neck cancer, etc. [6].

CD147 is a new cell surface adhesion molecule, which mediates the adhesion effect between tumor cells, or between tumor cells and stromal cells. It can also induce the expression and activation of matrix metalloproteinase on the surface of tumor cells and stimulates the tumor cells and interstitial cells to produce MMP-2 and MMP-9. Thus, it is a up-stream molecule causing the degradation of extracellular matrix and

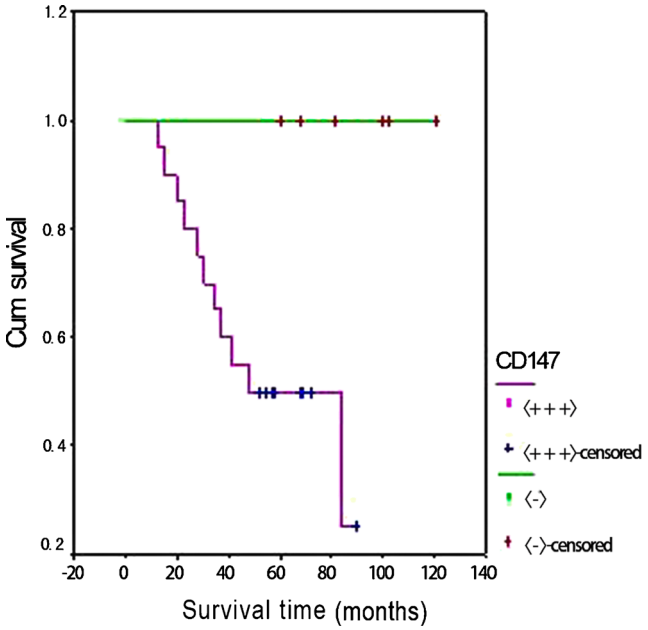

Fig. 2 Survival curves of laryngeal carcinoma patients with negative or intensive positive expression of CD147 (log-rank=5.24, $P=0.0220$)

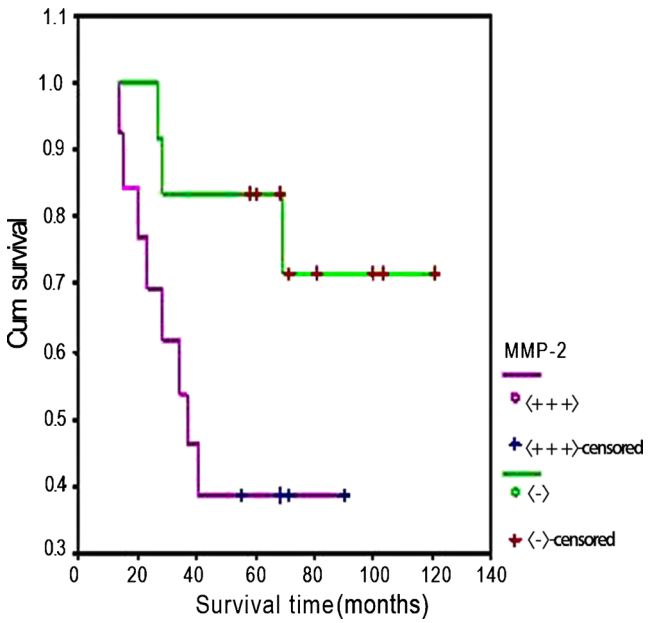

Fig. 3 Survival curves of laryngeal carcinoma patients with negative or intensive positive expression of MMP-2 (log-rank=4.09, $P=0.0432$)

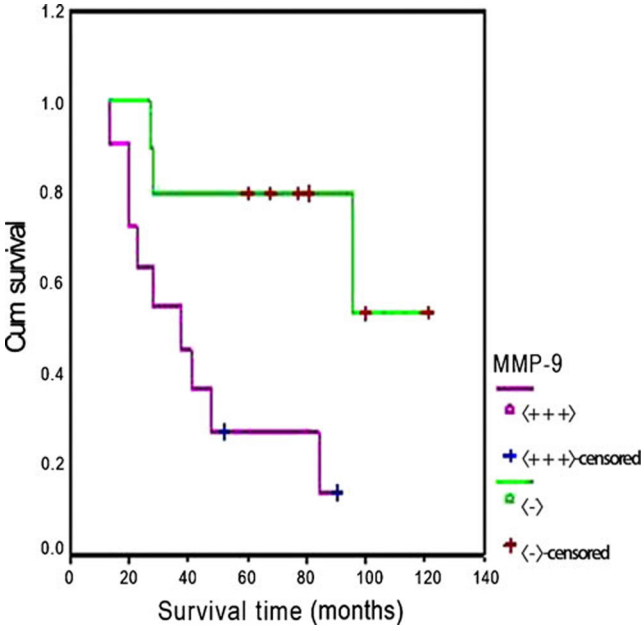

Fig. 4 Survival curves of laryngeal carcinoma patients with negative or intensive positive expression of MMP-9 (log-rank=6.74, P=0.0094)

promoting the invasion and metastasis of tumor cells [7–9]. Further investigation by Tang et.al [2]found that the increase of MMP could promote the expression of dissolvable CD147 in tumor matrix as well. The critical role of CD147 in the invasion and metastasis of liver cancer and malignant melanoma was confirmed [6, 10, 11]. Moreover, Su et al. [12] demonstrated that the interaction of highly expressed CD147 and MCT1, MCT4 would promote the glycolysis of tumor cells, and accelerate the progression of malignant melanoma. In addition, siRNA was applied to silence A375 cells in CD147/basigin, then the proliferation, invasion and generation of VEGF was inhibited by down-regulating glycolysis.

Our study demonstrated that CD147 was only expressed by the stratum basale cells in laryngitis tissue, and its expression, mainly on the surface of cell membrane, was increased in the laryngeal carcinoma tissuemembrane. MMP-2 and MMP-9 were mainly expressed in the cytoplasm of tumor cells and interstitial cells. The results indicated that CD147 was mainly expressed in the tumor cells, while MMP-2 and MMP-9 were either secreted by fibroblast stimulated by tumor cells with CD147 on the surface of their membrane; or alternatively secreted by tumor cells themselves. Positive expression rates of CD147, MMP-2 and MMP-9 in laryngeal carcinoma were significantly higher than those of the three proteins in laryngitis group (P<0.01), and even more significantly higher in T and N stage cancers. Meanwhile, there was significant difference between stage I/II and stage III/IV diseases in terms of expression of the three proteins. These results confirmed that high expression of CD147 was related with the invasion and metastasis of tumor. The expressions of CD147, MMP-2 and MMP-9 had positive correlation with each other in laryngeal carcinoma tissue, which indicated that the expression level of CD147 in laryngeal carcinoma cells was related with the quantity and activity of MMP-2 and MMP-9 produced by fibroblast around. Meanwhile, there was synergetic effect between MMP-2 and MMP-9 in promotion of tumor invasion, while its mechanism remained unclear.

Table 3 Cox model analysis for 48 cases of laryngeal carcinoma

	Regression coefficient B	Standard error of mean	Wald chi-square value	Exp(B)	P value
CD147	0.181	0.371	0.238	1.199	0.626
MMP-2	0.216	0.275	0.615	1.241	0.433
MMP-9	0.629	0.288	4.754	1.875	0.029

Five-year overall survival of laryngeal carcinoma patients in the 48 cases of experimental group was 62.5 %. The high expression of CD147, MMP-2 and MMP-9 indicated the poor prognosis of laryngeal carcinoma patients. Among them, MMP-9 would probably be the independent predictive factor. It was shown that high expression of MMP-9 was related to lower survival of patients with lung cancer, rectal carcinoma, and esophageal carcinoma. Moreover, it was associated with the severity of tumor invasion, lymphatic metastasis, venous tumor thrombus and pathological stages. Although CD147 was not a risk factor of prognosis, it was demonstrated that there was interaction among the CD147 molecules in multidrug-resistant cells of human oral squamous carcinoma. And siRNA against CD147 could induce the apoptosis of KB/V cells, multidrug resistence (MDR) derivative cell lines of human oral squamous cancer KB cells, which might be associated with the consumption of XIAP, X-linked inhibitor of apoptosis protein [13]. siRNA to silence CD147 in rodents lymphoma of P388D1 cell could lead to inhibition to tumor progression and enhance the sensitivity to chemotherapy as well [14]. Hence, CD147 would probably become one of effective therapeutic targets in inhibiting metastasis and treatment-resistance of tumor cells. Our study did not take drug resistance into consideration, and it remained unclear whether CD147 in laryngeal carcinoma had correlation with drug resistance or not.

In conclusion, our research showed that CD147, MMP-2 and MMP-9 were significantly overexpressed in laryngeal carcinoma, and were related with poor prognosis. More importantly, MMP was identified as independent predictive factor of laryngeal carcinoma. It has the potential as a new approach to improve therapeutic effect and prognosis of laryngeal carcinoma.

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Conflict of interest There is no conflict of interests between the authors.

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