

Original Article

Correlation of ECM1 expression level with the pathogenesis and metastasis of laryngeal carcinoma

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Received March 17, 2013; Accepted April 7, 2013; Epub May 15, 2013; Published June 1, 2013

Abstract: Objective: To investigate the expression of extracellular matrix protein 1 (ECM1) in benign laryngeal lesions, precancerous lesions and malignant laryngeal lesions and analyze the clinical significance of ECM1 changes in the pathogenesis and metastasis of laryngeal carcinoma. Methods: A total of 46 patients with laryngeal lesions were recruited with a median age of 48.2 years (range: 33-67 years). Among these patients, 29 had laryngeal carcinoma (12 with metastasis and 17 without metastasis), 8 had benign laryngeal lesions and 9 had precancerous laryngeal lesions (laryngeal leukoplakia). Immunofluorescence staining was employed to detect the protein expression of ECM1 in benign laryngeal lesions, laryngeal leukoplakia and malignant laryngeal lesions; RT-PCR was used to measure the mRNA expression of ECM1 in laryngeal carcinoma and benign laryngeal lesions. Results: ECM1 expression was detected in 25% (2/8) of patients with benign laryngeal lesions, 78% (7/9) of patients with precancerous laryngeal lesions, and 100% (29/29) of patients with laryngeal carcinoma. Among the laryngeal carcinoma patients, high ECM1 expression (+++) was found in 64.7% (11/17) of patients without lymph node metastasis and 91.7% (11/12) of patients with lymph node metastasis. Increased ECM1 expression was found in laryngeal carcinoma when compared with other laryngeal lesions and the ECM1 expression in patients with metastasis was significantly higher than that patients without metastasis ($P < 0.01$). RT-PCR showed that the mRNA expression of ECM1 in laryngeal carcinoma was markedly higher than that in benign laryngeal lesions. Conclusion: ECM1 expression is in an increasing order in benign laryngeal lesions, precancerous laryngeal lesions and malignant laryngeal lesions. Meanwhile, the metastatic laryngeal carcinoma has higher ECM1 expression than laryngeal carcinoma without metastasis. Our findings suggest that ECM1 plays promotive roles in the occurrence, development and metastasis of laryngeal carcinoma.

Keywords: Extracellular matrix protein 1, laryngeal carcinoma, lymph node metastasis

Introduction

Laryngeal squamous cell carcinoma is one of common malignant tumors of head and neck and accounts for 7.9%-35.0% of all malignant tumors in otolaryngology. Invasion and metastasis of laryngeal carcinoma have important influence on the therapeutic efficacy and prognosis of laryngeal carcinoma patients [1, 2]. However, the invasion and metastasis are a complex process with multiple stages and steps. The degradation of extracellular matrix and basement membrane is one of key steps. Extracellular matrix protein-1 (ECM1) is a molecular basis of interaction between cancer

cells and surrounding microenvironment. (ECM1 is associated with the invasion and metastasis of a variety of malignant tumors. Recently, studies showed that ECM1 was highly expressed in the thyroid cancer, gastric cancer, bile duct cancer, colorectal cancer, and lung cancer [3, 4]. It is also found that ECM1 can promote the angiogenesis and proliferation of vascular endothelial cells, which are associated with the occurrence, progression and metastasis of many cancers [5].

In our previous study, our results showed ECM1 was highly expressed in laryngeal carcinoma, especially in the metastatic lymph nodes [6].

ECM1 expression level and laryngeal carcinoma

Table 1. Characteristics of laryngeal lesions

Lesions	Number	Clinical stage and pathologic grade (n)
benign lesions	8	/
precancerous lesions	9	/
malignant lesions (non-metastasis)	17	Stage: T ₁ (3), T ₂ (10), T ₃ (2), T ₄ (5); Grade: I (3), II (9), II-III (3), III (2);
malignant lesions (metastasis)	12	Stage: T ₂ (4), T ₃ (3), T ₄ (5); Grade II (5), II-III (4), III (3);

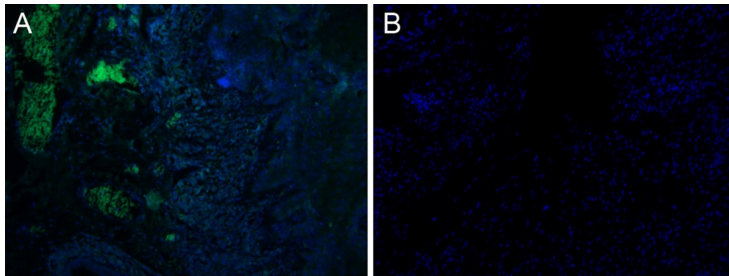


Figure 1. Immunofluorescence staining of ECM1 in laryngeal carcinoma ($\times 200$). A. Laryngeal carcinoma was positive for ECM1; B. negative control. Green fluorescence represented ECM1 expression. Blue staining was cell nuclei.

However, the ECM1 expression in different stages of laryngeal carcinoma is still poorly understood. In this study, immunofluorescence staining and RT-PCR were performed to detect the ECM1 expression in benign laryngeal lesions, precancerous laryngeal lesions and malignant laryngeal lesions. Our results revealed that the ECM1 expression was positively correlated with the severity of laryngeal carcinoma. It is important to further study the role of ECM1 in the pathogenesis of laryngeal carcinoma and understand the clinical significance of ECM1 in laryngeal carcinoma.

Materials and methods

Materials

Rabbit anti-human ECM1 polyclonal antibody was from Sigma. Chicken anti-rabbit IgG antibody conjugated with Alexa Fluor 488 was from Molecular Probes Company.

Sample collection

A total of 46 patients with laryngeal lesions undergoing laryngeal surgery were recruited from our hospital. The average age was 48.2 years (range: 33-67 years). All the patients had clear and complete clinical data. They were not

treated with radiotherapy, chemotherapy and other anti-tumor treatment. These patients had no other medical history or family history of cancers. Lesions were collected for histopathological examination. Among them, 29 were diagnosed with laryngeal carcinoma, 8 with benign laryngeal lesions and 9 with precancerous laryngeal lesions (laryngeal leukoplakia). Of the laryngeal carcinoma patients, 17 had no metastasis and 12 developed metastasis. Lesions were collected during the surgery and fixed in 10% formaldehyde.

Immunofluorescence staining

Paraffin embedded sections were deparaffinized with xylene. Antigen retrieval was done at 95-98°C in 0.01 M citric acid buffer for 15 min. Then, sections were incubated in 3% H₂O₂ and washed thrice with PBS for 5 min to inactivate endogenous peroxidase. Sections were incubated with 5% BSA for 15 min to block non-specific binding. Then rabbit primary anti-ECM1 (1:100) antibody was added followed by incubation at 4°C over night. After that, the sections were washed thrice with PBS for 5 min and incubated with chicken anti-rabbit IgG antibody conjugated with Alexa Fluor 488 at room temperature for 30 min. DAPI was added to stain the nuclei. Images were captured under microscope. Cells with green fluorescence were recognized as to be positive for ECM-1. The fluorescence intensity was classified as negative (-), weak positive (+), positive (++) and strong positive (+++). The primary antibody was replaced with PBS in the negative control group.

RT-PCR

Total RNA was extracted with routine method and then underwent reversed transcription into cDNA. The primers were as follows: ECM-1: 5'-GACCTGCCATTTCCAGAAC AG-3' (forward), 5'-GGGACCACACAGATCATTGATG-3' (reverse); GAPDH: 5'-CCATCAATGACCCCTTCATTG-3' (forward), 5'-CATG GGTG GAATCATATTGGAAC-3'

ECM1 expression level and laryngeal carcinoma

Table 2. ECM1 expression in different laryngeal lesions

Lesions	n	ECM1 positive (n)	ECM1 negative (n)	Positive rate (%)	X ²	P
benign lesions	8	2	6	25		
precancerous lesions	9	7	2	78		4.26E-6
malignant lesions	29	29	0	100	24.73	0.0001

Table 3. Grades of ECM1 expression in different laryngeal lesions

Lesions	n	Grades of ECM1 expression				Pe
		-	+	++	+++	
benign lesions	8	6	1	1	0	
precancerous lesions	9	2	1	1	5	
malignant lesions	29	0	1	8	20	

(reverse). These primers were synthesized in Sangon Shanghai Co., Ltd. Amplification of ECM-1 was done in 15 µl of mixture (rTaq polymerase: 0.1 µl; cDNA: 2 µl; DNTP: 1 µl; 10×buffer: 1.5 µl; 10 pmol primers: 0.2 µl; double distilled water). Reaction was done for 35 cycles. The mixture for amplification of GAPDH was similar to that of ECM-1 except for 0.5 µl of cDNA. In addition, the reaction was done for 28 cycles in the amplification of GAPDH.

Statistical analysis

Qualitative data were expressed as percentage to the control group. Chi square test or Fisher test was used to compare these quantitative data among groups. Statistical analysis was performed with SPSS version 13.0.0 for Windows. A value of $P < 0.05$ was considered statistically significant.

Results

Characteristics of laryngeal lesions

The characteristics of laryngeal lesions are summarized in **Table 1**.

ECM1 expression in different laryngeal lesions

Immunofluorescence staining was done to measure the protein expression of ECM1 in benign laryngeal lesions, precancerous laryngeal lesion and malignant laryngeal lesions. Our results showed that, in laryngeal carcinoma, ECM1 was mainly expressed in the cytoplasm, and partially in cell membrane and

extracellular matrix. The ECM1 expression in benign laryngeal lesions, precancerous laryngeal lesions and malignant laryngeal lesions is summarized in **Table 2**. ECM1 expression was in 100% (29/29) of patients with malignant laryngeal lesions, 78% (7/9) of patients with in precancerous laryngeal lesions and 25% (2/8) of patients with benign laryngeal lesions. In addition, the ECM1 expression in malignant laryngeal lesions was significantly higher than that in benign laryngeal lesions ($P < 0.05$). However, the ECM1 expression was comparable between precancerous laryngeal lesions and benign laryngeal lesions ($P = 0.051$) (**Figure 1**).

Grades of ECM1 expression in different laryngeal lesions

As shown in immunofluorescence staining, ECM1 was expressed in benign laryngeal lesions, precancerous laryngeal lesions and malignant lesions (both non-metastasis and metastasis) (**Figure 2**). The correlation between ECM1 expression and pathological grade was evaluated in different laryngeal lesions. As described in methods, the expression level of ECM1 in each sample was labeled with negative (-), weak positive (+), positive (++), or strong positive (+++) according to the intensity of the green fluorescence. Results showed the proportion of malignant laryngeal lesions was markedly higher than that of precancerous laryngeal lesions and benign laryngeal lesions among laryngeal lesions strong positive for ECM1 ($P < 0.05$) (**Table 3**).

Relationship between ECM-1 expression and metastasis of laryngeal carcinoma

ECM1 was expressed in laryngeal carcinoma with and without metastasis. In addition, among laryngeal carcinomas strong positive for ECM-1, the proportion of laryngeal carcinoma with lymph node metastasis was markedly

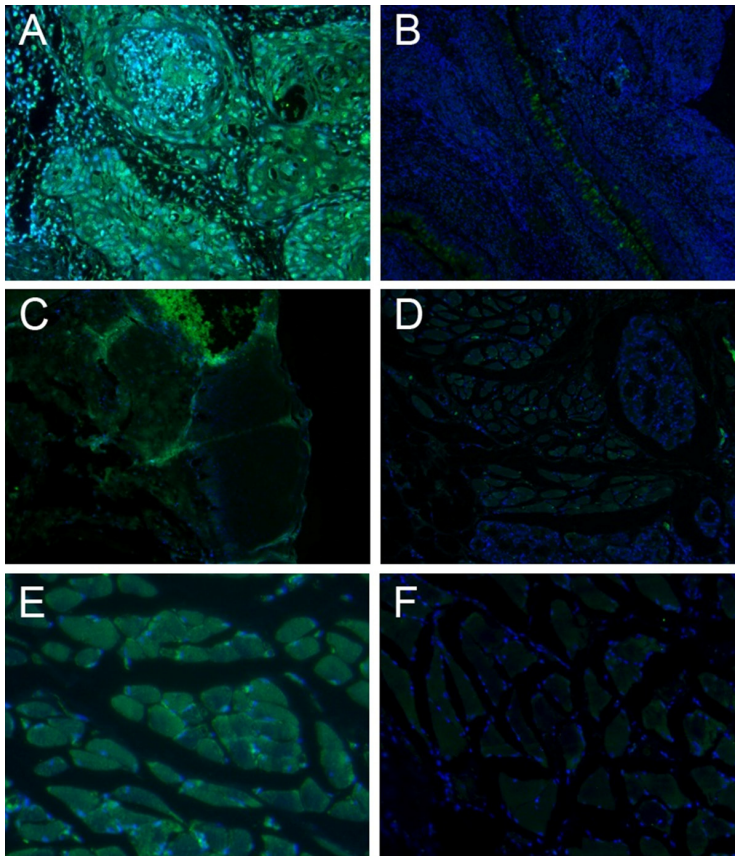


Figure 2. Immunofluorescence staining of different laryngeal lesions positive for ECM1 ($\times 200$). A. Malignant laryngeal lesions B. Precancerous laryngeal lesions C. Benign laryngeal lesions D. Recurrent laryngeal carcinoma E. Metastatic lymph nodes in laryngeal carcinoma F. Lymph nodes without metastasis in laryngeal carcinoma. (Green: ECM1; blue: Dapi stained nuclei).

Table 4. Relationship between ECM1 expression and metastasis of laryngeal carcinoma

Laryngeal carcinoma	n	Grades of ECM1 expression			
		-	+	++	+++
Metastatic	12	0	0	1	11
Non-metastatic	17	0	1	7	9

higher than that of laryngeal carcinoma without metastasis (Table 4).

mRNA expression of ECM1 in laryngeal carcinoma and benign laryngeal lesions

In addition, the mRNA expression of ECM-1 was also detected in 4 patients with benign laryngeal lesions and 4 with laryngeal carcinoma by RT-PCR (GAPDH as an internal control). The length of ECM1 and GAPDH was 249 bp and

251 bp respectively. Results showed the mRNA expression of ECM1 in laryngeal carcinoma was significantly higher than that in benign laryngeal lesions. Only one benign laryngeal lesion showed high ECM1 expression, which might be attributed to the individual difference or presence of precancerous lesion.

Discussion

Extracellular matrix is to support and maintain the structure and functions of tissues and plays important roles in morphogenesis, remodeling, repair, fibrosis, cell proliferation and migration. Proliferation, invasion, and metastasis of cancer cells have been found to be related to the changes in extracellular matrix. Recently, studies show that cancer cells can produce many factors acting on extracellular matrix, and thereby causing the change in surrounding components, to benefit their own activities. The extracellular matrix limits the activity of cancer cells. Proteolytic enzymes secreted by cancer cells can degrade some components of extracellular matrix including collagen, which promotes the invasion and migration of cancer cells through basement membrane and into circulation

resulting in distant metastasis. Cancer cells also secrete some molecules, which function through extracellular matrix, such as induction of angiogenesis and reduction of cancer cell adhesion.

ECM1 is a secretory glycoprotein first isolated from rat osteoblastic stromal cell line (MN7 cells) [1]. ECM1 has a hydrophobic signal peptide and several glycosylation sites. It has been found to be involved in a variety of biological

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processes, including cell proliferation, angiogenesis, embryonic cartilage formation, skin differentiation and tumor formation. ECM1 is considered to have close relationship with cancers [3, 5]. It plays an important role in the initiation and progression of cancers. ECM1 affects not only the cancer cell proliferation, but the cancer cell migration and severity of malignant tumors [7, 8]. Recent work suggests that frame-shift mutations in ECM1 lead to disorders in lymphangiogenesis in human mucosa of skin, indicating the potential role of ECM1 in lymphangiogenesis [9, 10]. Over-expression of ECM1 in a variety of malignant epithelial tumors has been recognized as a clinical indicator of poor prognosis [11-13]. However, the relationship between ECM1 expression and malignant lymph node metastasis remains poorly understood.

To date, little is known on the relationship between ECM-1 expression and head and neck cancers, especially the laryngeal carcinoma as a leading head and neck cancer [5, 14]. In the present study, laryngeal carcinoma patients were recruited, and immunofluorescence staining was performed to detect the protein expression of ECM-1 in laryngeal carcinoma with and without lymph node metastasis, precancerous laryngeal lesions and benign laryngeal lesions. Our results showed the ECM-1 expression in laryngeal carcinoma was markedly higher than that in benign laryngeal lesions ($P < 0.05$). In addition, the ECM-1 expression was dramatically increased in laryngeal carcinoma with metastasis when compared with laryngeal carcinoma without metastasis ($P < 0.05$). Our findings provide evidence for the role of ECM1 in the metastasis of laryngeal carcinoma. Currently, the mechanisms underlying the metastasis of laryngeal carcinoma are poorly understood. There are following explanations: 1) ECM-1 promotes the cancer metastasis via mediating the interaction between cells and extracellular matrix; 2) ECM-1 may stimulate the migration of endothelial cells and initiate angiogenesis which is crucial for the formation of new cancer foci [8]. 3) ECM-1 together with vascular endothelial growth factor C (VEGF-C) may affect lymphangiogenesis which then promotes the lymphatic metastasis of cancers [9].

In the present study, the ECM-1 expression was detected in different laryngeal lesions. Our results showed ECM-1 expression in laryngeal

carcinoma was significantly higher than that in benign laryngeal lesions. In addition, RT-PCR was employed to detect the mRNA expression of ECM-1. Results revealed that the mRNA expression of ECM-1 in laryngeal carcinoma was also markedly higher than that in benign laryngeal lesions. This demonstrates that the increased protein expression of ECM-1 is attributed to the elevated transcription of ECM-1 gene. Moreover, there was no marked difference in the ECM-1 expression between laryngeal carcinoma and laryngeal leukoplakia ($P = 0.051$). The above findings suggest that the ECM-1 expression varies among different laryngeal lesions. In our study, results also indicate that the ECM-1 expression in laryngeal carcinoma with lymph node metastasis was dramatically increased when compared with laryngeal carcinoma without metastasis. These findings imply that ECM-1 expression is related to the pathogenesis of tumors, especially the malignant tumors, and their lymph node metastasis, but the specific role of ECM-1 in malignancies is largely unclear, and more studies are required. ECM-1 may bind to its receptors to exert effects. Thus, to block the binding of ECM-1 to its receptor might be promising to prevent growth and metastasis of cancers, which may be a new anti-tumor therapy. Detection of serum ECM-1 (a secretory protein) may aid the diagnosis and determination of prognosis of diseases. However, this study had a small sample size, and whether ECM-1 may serve as a marker of cancers is still unclear. Further studies with large sample size are needed to confirm it.

Taken together, the ECM1 expression is in an increasing order in benign laryngeal lesions, precancerous laryngeal lesions and malignant laryngeal lesions, and the ECM-1 expression in laryngeal carcinoma with lymph node metastasis is significantly higher than that in laryngeal carcinoma without metastasis. These findings suggest that ECM-1 play promotive roles in the initiation, progression and metastasis of laryngeal carcinoma. Our findings together with previous results demonstrate that the ECM-1 expression in laryngeal carcinoma is related to the growth and migration of cancer cells. Further studies are required to clarify whether ECM-1 may serve as a marker of cancers.

Acknowledgment

The study was supported by Youth Scientific Project of Shanghai Health Administration (No.

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2009Y120). The author Meizhen Gu greatly appreciates her previous staff from Department of Otolaryngology, Shibei Hospital of Zhabei District, Shanghai, China.

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