RESEARCH COMMUNICATION

Expression of Type IV Collagen, Metalloproteinase-2, Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-1 in Laryngeal Squamous Cell Carcinomas

Xiao-Lin Cao[1*], Ru-Jun Xu[2], You-Yang Zheng[3], Jun Liu[4], Yao-Shu Teng[1], Yong Li[1], Jin Zhu[1]

Abstract

Objective: To investigate the significance of type IV collagen, metalloproteinase-2 (MMP-2), metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression in laryngeal squamous cell carcinomas (LSCCs). Methods: Expression was quantified in 44 LSCC and 22 adjacent non-cancer normal tissues using a streptavidin-peroxidase conjugated immunohistochemistry and associations between the levels of the four proteins and clinicopathological characteristics in LSCC were analyzed. Results: Significantly different expression of all four proteins was observed in LSCC and adjacent non-cancer normal tissues (P<0.05). Expression of type IV collagen correlated with primary cancer status (P = 0.04), clinical stage (P = 0.04) and histological grade (P = 0.01). Expression of MMP-9 correlated with the location of the tumor (P = 0.04), cervical node metastasis (P = 0.02) and prognosis (P = 0.02). The (MMP-2+MMP-9)/TIMP-1 score was associated with the prognosis of LSCC (P < 0.01). Conclusions: This study suggests that expression of type IV collagen and its regulators is strongly associated with the development of LSCC. Type IV collagen and MMP-9 may be more valuable than MMP-2 and TIMP-1 for the evaluation of clinical characteristics. Regulation of type IV collagen may contribute to the balance of MMPs and TIMPs in LSCC.

Keywords: Laryngeal SCC - collagen - metalloproteinase - tissue inhibitor of metalloproteinase

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common head and neck malignancies. In China, the LSCC morbidity rate is 1.7-1.8 per 100,000 males and 0.4 per 100,000 females (Yang et al., 2005) and the incidence of LSCC is increasing in many regions of the world. It is generally accepted that LSCC is strongly associated with tobacco smoking (Gandini et al., 2008), alcohol consumption (Islami et al., 2010) and viral infection (Marur et al., 2010). Early stage well-differentiated LSCC has a good prognosis, and the survival rate is significantly lower in patients with regional and distal metastasis; therefore, invasion and metastasis are important factors which greatly impact on prognosis in LSCC.

The procedures of invasion and metastasis in malignancy involve very complicated multi-step, multi-factorial and multi-gene processes. Degradation of the extracellular matrix (ECM) is an essential step which is required for the invasion of carcinoma cells. Basement membrane (BM) is the main component of EMC, and is composed of type IV collagen, laminin, entactin, proteoglycans and glycosaminoglycans (Nelson et al., 2000). Of these, type IV collagen is regarded as the predominate factor (Leblond et al., 1989) which prevents migration of carcinoma cells in the ECM (Krecicki et al., 2001).

The expression level of type IV collagen is regulated by matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Currently, there are at least 24 members of the MMP family, which can be classified into a number of subgroups, including collagenases, gelatinases, stromelysins and membrane-type MMPs (Nabeshima et al., 2002). The gelatinases comprise two subtypes, MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Gelatinases can induce partial or widespread loss of the BM by degradation of type IV collagen, and contribute to invasion and metastasis. Several studies have proven that MMP-2 and MMP-9 play an important role in the development of acute lymphoblastic leukemia (Klein et al., 2004), breast cancer (Figueira et al, 2009), prostate andenocarcinoma (Trudel et al., 2010), renal cancer (Kugler et al., 1988) and head and neck carcinoma (Kondratiev et al., 2008). The TIMP family contains four members which can restrain the

*[1]Department of Otolaryngology-Head and Neck Surgery, [2]Department of Pathology, Hangzhou First People's Hospital, [3]Department of Stomatology, the Second Affiliated Hospital of Zhejiang University, College of Medicine, Hangzhou, [4]Department of Otorhinolaryngology, Qilu Hospital of Shandong University, Jinan, China *For correspondence: hzdoctorcaoxiaolin@gmail.com*

invasion of neoplastic cells (Gomez et al., 1997). TIMP-1 can downregulate the expression of gelatinases, and is acknowledged to act as a protective factor against invasion and metastases in LSCC (Krecicki et al., 2003). However, some researchers have suggested that the expression level of type IV collagen is regulated by the balance of MMPs and TIMPs (Kugler et al., 1988; Suminoe et al., 2007; Figueira et al., 2009); therefore, a single MMPs or TIMP index cannot properly demonstrate the ability of tumors to invade and metastasize (Krecicki et al., 2001). As type IV collagen, MMP-2, MMP-9 and TIMP-1 interact to regulate the ECM, we designed this study to investigate the expression and significance of type IV collagen, MMP-2, MMP-9 and TIMP-1 in LSCC.

Materials and Methods

Study subjects

Between June 2003 and May 2004, 44 patients with histologically confirmed, previously untreated LSCC were recruited at Hangzhou First People's Hospital. In total, 41 patients were male and three were female. The age of the patients ranged from 46 to 84 years old, and the median patient age was 67 years. Twenty two cases of LSCC were located in the supraglottic area, 21 cases in the glottic area and one case in the subglottic area. According to the UICC classification (2002), 7, 9, 9 and 19 tumors were staged T1, T2, T3 and T4, respectively; 26, 10, 6 and 1 tumors were staged N0, N1, N2 and N3, respectively and 7, 6, 10 and 21 tumors had a histological grade of I, II, III and IV, respectively. The number of well, moderately and poorly differentiated tumors was 10, 33 and 1 respectively. The follow-up time was more than 5 years, during which time 12 cases recurred and 32 cases survived without cancer. In addition, 22 adjacent non-cancer normal tissues (20 male and 2 female; age 46 to 73 years, median 58 years) were obtained from contralateral semi-laryngeal normal tissue or mucosal tissue more than 1.5 cm distal to LSCC tumors, and were confirmed by H&E staining. This study was approved by the Institutional Review Board of Hangzhou First People's Hospital.

Immunohistochemical staining

All specimens were fixed in 10% formalin, paraffin embedded and 5 μm continuous sections were prepared. All 44 LSCCs tissues and 22 adjacent non-caner normal tissues were reconfirmed by H&E staining before immunohistochemical analysis.

A streptavidin peroxidase-conjugated immunohistochemical technique was performed to determine the expression of type IV collagen, MMP-2, MMP-9 and TIMP-1 using the SP kit (Zymed Laboratories, San Francisco, CA, USA) following the manufacturer's instructions with the following mouse monoclonal antibodies: anti-human type IV collagen (1:100 dilution; 1 h incubation); anti-human MMP-2 (1:50 dilution; 1 h incubation); anti-human MMP-9 (1:50 dilution; 1 h incubation) and anti-human TIMP-1 (1:50 dilution; 1 h incubation). All antibodies were purchased from Zymed Laboratories.

For type IV collagen, positive staining of the BM of endothelial vessels or perineurium in the same tissue section was used as the positive control. Human breast cancer sections were used as a positive control for MMP-2, MMP-9 and TIMP-1. Negative controls were performed by substituting the primary antibody with PBS.

The immunohistochemical staining was scored independently by two pathologists. Each pathologist randomly selected five high power fields of view (×100) from each section and the extent of staining was semi-quantitatively scored using a four-point scale: "-", negative (<5% positive linear structure or cells); "+", weak expression (5%-25% positive linear structure or cells); "2+", moderate expression (26%-50% positive linear structure or cells); "3+", strong expression (>50% positive linear structure or cells). The average score for each section was calculated. In order to determine an overall index, the scores obtained for MMP-2, NMP-9 and TIMP-1 were combined using the following formula: (MMP-2+MMP-9)/TIMP-1.

Statistical analysis

All data was analyzed using SPSS 10.0. The main statistical methods included the Chi-square test, t-test and Fisher exact test.

Results

Expression of type IV collagen

Positive type IV collagen staining was observed in 21/44 (42.73%) of the primary LSCCs and 16/22 (72.73%)

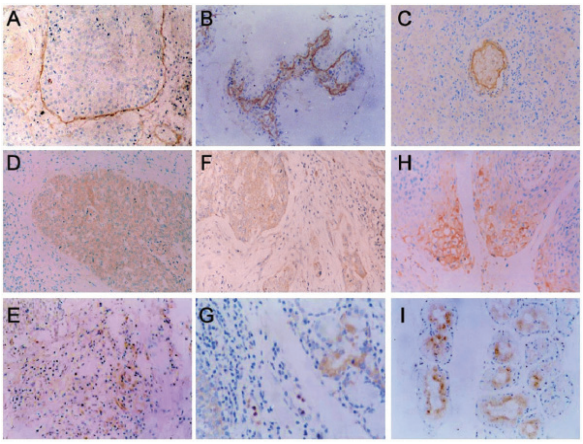

Figure 1. AIHC Staining of Type IV Collagen, MMP-2, MMP-9 and TIMP-1 in LSCC. Figure A-C. Representative images of strong (3+) type IV collagen stainging in (A) well-differentiated LSCC, SP×200 and (B) in the adjacent non-cancer normal tissue, SP×100. (C) Representative images showing negative type IV collagen staining in poorly-differentiated LSCC, and strong staining (3+) in the perineurium (at the center of the picture), which was used as the positive control, SP×200, Figure D-E. Representative images of (D) strong MMP-2 staining (3+) in well-differentiated LSCC, SP×200 and (E) weak MMP-2 staining (+) in the adjacent non-cancer normal tissue, SP×400, Figure F-G. Representative images of (F) moderate MMP-9 staining "2+" in moderately-differentiated LSCC, SP ×200 and (G) weak MMP-9 staining "2+" in the adjacent non-cancer normal tissue, SP×400, Figure H-I. Representative images of (H) moderate TIMP-1 staining "2+" in moderately-differentiated LSCC, SP ×200 and (I) the adjacent non-cancer normal tissues, SP ×200

Table 1. Relation Between Expression of Type IV Collagen, its Regulators and Clinicopathological Characteristics of Laryngeal SCCs

	Type IV collagen				X^2	MMP-2				X^2	MMP-9				X^2	TIMP-1		X^2
	0	1	2	3	P value	0	1	2	3	P value	0	1	2	3	P value	0	1	P value
Tissue																		
LSCC	23	12	8	1	12.27	10	11	18	5	10.27	12	9	15	8	11.40	21	23	4.10
Normal1	6	2	12	2	0.02	13	2	7	0	0.04	14	1	7	0	0.02	17	5	0.04
Location																		
Supraglottic	15	5	2	0	5.20	3	6	11	2	3.07	3	4	8	7	10.16	10	12	0.21
Glottic	8	6	6	1	0.27	7	4	7	3	0.55	9	5	7	0	0.04	11	10	0.65
T category																		
T1-2	4	6	6	0	10.27	7	3	6	0	8.21	5	5	4	2	2.63	7	9	0.01
T3-4	19	6	2	1	0.04	3	8	12	5	0.08	7	4	11	6	0.62	14	14	0.93
N category																		
N0	11	9	7	0	6.61	6	7	10	4	1.02	10	7	9	1	11.53	12	15	0.30
N1-3	12	3	1	1	0.16	4	4	8	1	0.91	2	2	6	7	0.02	9	8	0.58
Clinical Stage																		
I-II	4	3	6	0	10.11	5	3	5	0	4.16	4	3	4	2	0.28	5	8	0.64
III-IV	19	9	2	1	0.04	5	8	13	5	0.39	8	6	11	6	0.99	16	15	0.43
Histological grade																		
G1	2	2	5	1	13.44	5	2	3	0	6.21	3	3	4	0	3.10	5	5	0.03
G2-3	21	10	3	0	0.01	5	9	15	5	0.18	9	6	11	8	0.54	16	18	0.87
Recurrence																		
Yes	10	2	0	0	7.10	1	3	5	3	4.21	1	0	6	5	11.78	7	5	0.74
No	13	10	8	1	0.13	9	8	13	2	0.38	11	9	9	3	0.02	14	18	0.39

[1]Normal adjacent non-cancer tissues were confirmed by H&E staining, X^2 and P indicated the value of Chi-squared test and represented the comparison of expression levels within different clinicopathological characteristics.

Table 2. Relationship Between Prognosis and (MMP-2+MMP-9)/TIMP-1 Score in 44 Cases of LSCC

Recurrence	Number of cases	(MMP-2+MMP-9) /TIMP-1	T Value	P
Yes	12	2.29±0.86	2.74	<0.01
No	32	1.66±0.18		

of the adjacent non-cancer tissue normal tissues. Intact and continuous expression of type IV collagen was observed in normal tissues and well-differentiated LSCC, whereas partial and entire loss of type IV collagen structure was observed in poorly differentiated LSCC tissues (Fig. A-C). The type IV collagen expression level in normal tissues was significantly higher than LSCC (P = 0.02). In LSCC, the expression of type IV collagen was related to the T category, clinical stage and pathological grade. The expression of type IV collagen was higher in T1-2 LSCC (12/16, 75.00%) than T3-4 LSCC (9/28, 32.14%; P = 0.04). The expression of type IV collagen was higher in stage I-II LSCC (9/13, 69.23%) than stage III-IV LSCC (12/31, 38.71%; P = 0.04). The expression of type IV collagen was higher in G1 LSCC (8/10, 80.00%) than G2-3 LSCC (13/34, 38.24%; P = 0.01). No significant correlations between type IV collagen expression and localization, and the N category or prognosis of LSCC were observed (P>0.05; Table 1).

Expression of MMP-2

Positive MMP-2 staining was significantly more frequent in LSCC (34/44, 77.27%) than the adjacent non-cancer normal tissues (9/22, 40.91%; P = 0.04; Fig. D-E). No significant correlation between the expression of MMP-2 and tumor location, T category, N category, clinical stage, pathological grade or the prognosis of LSCC were observed (P>0.05; Table 1).

Expression of MMP-9

MMP-9 staining was significantly higher in LSCC (32/44, 72.73%) than the adjacent non-cancer normal tissues (8/22, 36.36%; P = 0.02; Figure1 F-G). The expression of MMP-9 was related to the location of LSCC, N category and prognosis. MMP-9 staining was significantly higher in supraglottic LSCC (19/22; 72.72%) than glottic LSCC (12/21; 57.14%; P=0.04). MMP-9 staining was significantly lower in N1-2 disease (17/27; 62.96%) than N3-4 disease (15/17; 88.24%; P=0.02). MMP-9 staining was significantly higher in the LSCC tissues of patients who recurred during follow-up (11/12, 91.67%) than the tissues of patients who did not recur (21/32, 65.63%; P = 0.02). No significant correlations between MMP-9 expression and T category, clinical stage or pathological grade were observed in LSCC (P>0.05; Table 1).

Expression of TIMP-1

TIMP-1 expression was significantly higher in LSCC (23/44; 52.27%) than the adjacent non-cancer normal tissues (5/22, 22.73%; P = 0.04; Figure1 H-I). No significant correlations were observed between the expression and localization of TIMP-1 and T category, N category, clinical stage, pathological grade or prognosis on LSCC.

(MMP-2+MMP-9)/TIMP-1 score and prognosis

Significant differences in the (MMP-2+MMP-9)/TIMP-1 score of LSCC patients who recurred during follow up (2.29±0.86) and those who did not recur (1.66±0.18) were observed (t = 2.74, P<0.01).

Discussion

Liotta et al. (1983) described a three-step hypothesis for carcinoma invasion, which includes attachment, degradation and locomotion. BM serves as a barrier to separate epithelial cells from the underlying stroma, and tumor cells can penetrate the BM by secreting proteinases to invade the surrounding tissue and metastasize to distant organs. Degradation of the BM is regarded as an essential step in the metastasis of carcinoma cells, and type IV collagen is the main framework of the BM. In benign laryngeal lesions, type IV collagen is expressed as an intact and continuous structure (Courey et al., 1996; Martins et al., 2010); however, type IV collagen expression is discontinuous and partially or wholly lost in LSCC (Wang et al., 2011). Similar to these previous reports, we also observed a loss of type IV collagen in LSCC, and found that type IV collagen was strongly associated with T category, clinical stage and the degree of differentiation in LSCC. These results indicate that there may be an increased proteinase activity in LSCC, especially in poorly differentiated tissues. The extent of type IV collagen loss may reflect the potential of tumors to metastasize, as loss of type IV collagen may provide the foundation for invasion and metastasis.

Type IV collagen proteinases include the proteolytic zinc-containing enzymes MMP-2 and MMP-9, which are associated with invasion and metastasis. Dong et al. reported that high levels of MMP-2 protein expression positively correlate with tumor size, lymph node metastasis, distant metastasis and clinical stage in colorectal cancer (Dong et al., 2011); however, other studies have reached different conclusions. For example, Krecicki et al. (2001) observed no significant relationship between MMP-2 expression and clinicopathological parameters, including localization, T category, N category and histological grade in laryngeal cancer. In this study, we observed that MMP-2 was expressed at significantly higher levels in LSCC than normal tissues, but did not observe a significant relationship between MMP-2 and any clinicopathological features of LSCC. MMP-9 can degrade type IV collagen and lead to an increased density of blood vessels (Wittekindt et al., 2011), which can increase cancer cell infiltration as tumor cells expressing high levels of MMP-9 are more likely to invade (Peschos et al., 2006). In our study, we found that the expression level of MMP-9 could serve as an index to assess lymph node metastasis, clinical stage and prognosis in LSCC. Additionally, increased expression of MMP-9 was observed in tumors from the supraglottic area compared to the glottic area, indicating that supraglottic LSCC cells may secrete increased levels of MMP-9, possibly due to the different cell types in each laryngeal area, which may explain the increased lymph node metastasis rate of supraglottic LSCC. Taken together, the expression level of MMP-9 appears to be a much more useful marker for evaluation of the characteristics of LSCC than MMP-2.

The expression of MMP2 and MMP-9 is intricately positively and negatively regulated by epidermal growth factors and hepatocyte growth factor (Rosenthal et al., 1998; Overall et al., 2002). Overexpression of MMP-9 is strongly associated with p53 mutations (Franchi et al., 2002), which are a frequent genetic alteration in head and neck squamous cell carcinoma (Maestro et al., 1992). TIMP-1 is also an important regulator of MMP-2 and MMP-9 expression, and TIMP-1 can inhibit the invasion of cancer cells in vitro and vivo (Gomez et al., 1997). Increased TIMP-1 expression is related to reduced nodal metastases and a lower differentiation grade in LSCC (Krecicki et al., 2003). Woessner et al. (1994) reported a significant relationship between TIMP-1 expression and histological grade, but not T category, N category, clinical stage or distal metastasis. Other studies have also found no significant correlation between TIMP-1 expression and clininopathological characteristics in prostate and laryngeal cancer (Peschos et al., 2006; Trudel et al., 2010), however, Shi et al. (1999) observed that higher levels of TIMP-1 mRNA were observed in advanced thyroid carcinoma. Similarly, in this study, we observed that TIMP-1 protein expression was increased in LSCC compared to normal tissues. The increased expression levels of TIMP-1 in LSCC suggest that stromal or cancer cells may increase expression of TIMP-1, in order to maintain a balance between degradation and remodeling of the BM.

Discovery of an index to properly evaluate the prognosis of cancer patients would be very useful. Local invasion and distant metastasis are strongly associated with a poorer prognosis in LSCC; therefore, it is possible that the expression levels of the BM and it regulators may serve as prognostic factors in LSCC. Currently, no consensus has been reached on the prognostic ability of BM regulators. Aref et al. (2007) and Molica et al. (2003) suggested that high MMP-2 and MMP-9 expression levels were associated with a poorer prognosis, while Travaglino et al. (2008) found no correlation between MMP-9 and prognosis in acute myeloid leukaemias. This study indicates that only MMP-9 can be regarded as a useful prognostic factor in LSCC. The network which regulates the ECM is rather complicated, and the differing results reported in these studies indicate the involvement of several associated factors. The final action of MMPs and TIMPs may depend on the balance of MMPs and TIMPs. Suminoe et al. (2007) suggested that the balance of different BM regulators may be more strongly associated with the infiltration of leukemia cells than any single factor, and similar results were reported in breast (Figueira et al., 2009) and renal cancer (Kugler et al., 1988). In this study, we observed a positive correlation between the (MMP-2+MMP-9)/TIMP-1 score and prognosis in LSCC, indicating that this index may be useful for the evaluation of prognosis in LSCC.

Although significant results were obtained in our study, the results need to be interpreted with caution. Firstly, the number of subjects in this study was relatively small, and further studies with larger sample sizes will be performed to confirm the results. Secondly, as the regulation of the BM is rather complex, further studies are required to focus on the role of the network of MMPs and TIMPs which regulate BM deposition and degradation.

In conclusion, altered expression of the BM and its regulators may be strongly associated with the

development of laryngeal carcinoma. Type IV collagen and MMP-9 may be more valuable than MMP-2 and TIMP-1 for the evaluation of clinical characteristics of LSCC. The balance of MMPs and TIMPs may contribute to the regulation of type IV collagen, and the (MMP-2 + MMP-9)/TIMP-1 score may be helpful useful index to assess prognosis in LSCC.

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