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Original Article

Upregulated TIMP-1 correlates with poor prognosis of laryngeal squamous cell carcinoma

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Abstract: The tissue inhibitor of metalloproteinase-1 (TIMP-1) is an endogenous inhibitor of matrix metalloproteinases and potential biomarker of various types of human cancers. However, the association between TIMP-1 expression and the clinical features of laryngeal squamous cell carcinoma (LSCC) is barely investigated. In this study, one-step quantitative reverse transcription-polymerase chain reaction and immunohistochemical staining with tissue microarrays were employed to evaluate the relationship between TIMP-1 expression and the clinicopathological characteristics of LSCC. Results showed that the TIMP-1 mRNA and protein expression levels were significantly higher in LSCC than in the corresponding non-cancerous tissues ($p<0.05$). TIMP-1 protein expression in LSCC was associated with tumor differentiation ($p=0.012$) and overall survival ($p=0.043$). Kaplan-Meier method and Cox multi-factor analysis suggested that high TIMP-1 expression ($p=0.008$) and positive lymph node metastasis ($p=0.029$) were significantly associated with the poor survival of patients with LSCC. These data indicated that TIMP-1 may be identified as a prognostic marker of LSCC.

Keywords: TIMP-1, LSCC, qPCR, immunohistochemistry, prognosis

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common type of head and neck malignancy. LSCC accounts for approximately 25% of all types of head and neck cancers and is considered the second most common malignancy in the respiratory tract [1]. In recent years, the incidence rate of LSCC has consistently increased; the infiltration and metastasis of LSCC are the primary factors that severely affect patients' quality of life and overall survival [2]. LSCC usually originates from laryngeal epithelial cells, and the clinical symptoms often depend on the site of origin and size of tumor [3]. Carcinogenic factors from habits such as cigarette smoking and alcohol consumption also contribute to LSCC. Other exogenic and endogenic factors are considered as promoters or carcinogens of carcinogenesis [4]. Thus far, surgery and radiotherapy are the two major treatment strategies for LSCC. Function preservation has also gained increasing importance, such that chemotherapy is a significant component of several therapeutic regimens [5]. Although LSCC treatments have been developed, a satisfactory therapeutic outcome has not been achieved yet, and the five-year survival rate of LSCC is only 60% [6]. Therefore, novel and valuable markers should be determined to identify patients with LSCC exhibiting poor survival and provide information regarding surgery, chemotherapy, and radiotherapy.

The tissue inhibitor of metalloproteinase-1 (TIMP-1), a 28.5 kDa glycoprotein that belongs

to the TMIPs family, is one of the endogenous inhibitors of matrix metalloproteinases (MMPs) [7]. TIMP-1 regulates the activity of MMPs, which are enzymes involved in the degradation of the extracellular matrix (ECM); hence, TIMP-1 may be involved in cancer invasion and metastasis [8]. Recently, TIMP-1 has been brought forward as a potential prognostic biomarker of different types of human cancers, such as breast cancer, colon cancer, gastric cancer, hepatocellular cancer, rectal cancer, glioblastoma, multiple myeloma and lymphoma [9-16]. Several studies have also reported that TIMP-1 is highly expressed in head and neck squamous cell carcinomas, including LSCC, as well as tumor growth and invasion [17-19]. However, studies have rarely been conducted to determine the association between TIMP-1 expression and clinical characteristics of LSCC; its clinicopathological significance, particularly prognostic attributes, has barely been investigated. Further examination is required to verify whether or not TIMP-1 can be used as a biomarker of poor survival and candidate for molecular targeted therapy of LSCC.

In this current study, we detected the expression of TIMP-1 mRNA in fresh LSCC tissue via one-step quantitative reverse transcription-polymerase chain reaction (qPCR). Moreover, we evaluated the expression of TIMP-1 protein in LSCC with tissue microarray (TMA) by immunohistochemistry (IHC). Finally, we investigated the correlation of TIMP-1 expression with the clinicopathologic attributes and survival in LSCC.

Materials and methods

Patient specimens

A total of 109 paraffin-embedded LSCC tissue samples and 28 control samples were collected from the archives of the Department of Pathology, the Affiliated Hospital of Nantong University, between January 2002 and May 2009. Diagnosis of LSCC was confirmed according to the latest WHO criteria [20] and TNM stage classification (UICC 2002). All patients were underwent laryngectomy and/or neck dissection (unilateral or bilateral, radical or functional, based on clinical and surgical findings). Nodal metastasis was confirmed by postoperative histological examination. All the cases were reevaluated for tumor differentiation and TNM stage by two independent pathologists (L. Xiong and H. Zhu). The original clinical data were obtained from hospital medical records, including patient gender and age, tobacco use, alcohol consumption, tumor differentiation, TNM stage, lymph node metastasis, and overall survival. None of the patients had received preoperative radiotherapy or chemotherapy before surgery. Written informed consent was obtained from the patient for publication of this study and any accompanying images. Ethical approval to perform this research was approved from the Human Research Ethics Committee of local hospital.

One-step quantitative real-time reverse transcription-polymerase chain reaction (qPCR)

12 samples of fresh LSCC tissues and matched tumor-adjacent tissues were collected from the Department of Otolaryngology-Head and Neck Surgery, Yiji Shan Hospital of Wannan Medical College and the Department of Otolaryngology-Head and Neck Surgery, the Second Affiliated Hospital of Nanjing Medical University. Total RNA from LSCC tissues and tumor-adjacent tissues were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. One-step qPCR analysis was performed with a SensiMixTM One-Step Kit (Quantace, London, UK) using a Real Time PCR system (Bio-Rad IQ5, Hercules, CA, USA) according to the standard protocol. The primers for TIMP-1 which designed by Primer Express Software were as follows: forward primer 5'-CTG TTG TTG CTG TGG CTG ATA G-3'; reverse primer 5'-CGC TGG TAT AAG GTG GTC TGG-3'. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers for GAPDH were as follows: forward primer 5'-TGC ACC ACC AAC TGC TTA GC-3' and reverse primer 3'-GGC ATG GAC TGT GGT CAT GAG-5'. Total RNA extraction, amplification conditions and one-step qPCR procedure were described in our previous publication [21].

Tissue microarrays (TMA) construction and Immunohistochemistry (IHC)

109 LSCC tissues were prepared and TMA was produced by Xinchao Biotech Co., Ltd (Shanghai, China). Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded sections and arranged in the new recipient paraffin blocks. The TMA was cut into 4 μm sec-

Table 1. Association of TIMP-1 expression with clinical parameters of 109 LSCC patients

Groups	No.	TIMP-1		X^2	*p* value
		+	%		
Gender					
Male	107	55	51.40	0.0015	0.969
Female	2	1	50.00		
Age (years)					
≤60 y	45	22	48.89	0.1898	0.663
>60 y	64	34	53.13		
Tobacco consumption					
Yes	77	39	50.65	0.0555	0.814
No	32	17	53.13		
Alcohol consumption					
Yes	53	26	49.06	0.2222	0.637
No	56	30	53.57		
Tumor differentiation					
Well	51	19	37.25	8.8907	0.012*
Moderate	53	35	66.04		
Poor	5	2	40.00		
TNM stage					
Stage I, II	65	31	47.69	0.8747	0.350
Stage III, IV	44	25	56.82		
Lymph node metastasis					
Yes	19	13	68.42	2.6762	0.102
No	90	43	47.78		
Overall survival					
Yes	72	32	44.44	4.0797	0.043*
No	37	24	64.86		

*$p<0.05$.

tions and placed on super frost charged glass microscope slides. IHC analysis was performed as described previously [22, 23]. TMA sections were incubated with a primary monoclonal mouse anti-TIMP-1 antibody (1:200, Abcam, England) in TBS and then incubated with horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) after washing. The results of IHC were evaluated with a double-blind method and the staining results were determined under an optical microscope by two pathologists independently (L. Xiong and H. Zhu). In the case of disagreement, the slides were reviewed by a third pathologist (N. Xu) until a consensus score was established. Expression levels of TIMP-1 protein were assessed using immunohistochemistry score (IHS). Briefly, the IHS was determined by the evaluation of both staining intensity and density. The staining intensity of TIMP-1 was scored as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The percentage of TIMP-1-positive cells was also scored according to the following four levels, where 1 was given for 0% to 10%, 2 for 11% to 50%, 3 for 51% to 80%, and 4 for 81% to 100%. The sum of the intensity and the percentage scores led to the final IHS: samples with a score below 3 (IHS≤3) were considered as low TIMP-1 expression while those with a score above 4 (IHS≥4) as high TIMP-1 expression.

Statistical analysis

The TIMP-1 mRNA expression in fresh LSCC tissues compared with matched tumor-adjacent tissues was analyzed with the Wilcoxon signed rank nonparametric test. The influence of TIMP-1 expression on clinicopathological parameters of LSCC was analyzed by chi-square test. The Kaplan-Meier method was used to evaluate the associations between TIMP-1 expression and LSCC outcomes. Univariate and multivariate analyses were performed using Cox proportional hazards regression models to determine factors that were independently associated with overall survival. For all tests, the significance level for statistical analysis was set at $p<0.05$. All the statistical analyses were conducted by using STATA Version 12.0 (Stata Corporation, College Station, TX) and SPSS 18.0 statistic software (SPSS Inc, Chicago, IL).

Result

Clinical characteristics of 109 patients with LSCC

The main clinicopathological characteristics of patients with LSCC are shown in **Table 1**. A total of 109 tumor samples were collected from 107 men and 2 women. The mean age of the patients at the time of diagnosis was 60.8 years (ranging from 29 years to 87 years). A history of cigarette smoking was noted in 77 patients, whereas the other 32 patients did not smoke. Alcohol consumption was noted in 53 patients, whereas the remaining 56 patients did not drink. A total of 51 patients suffered

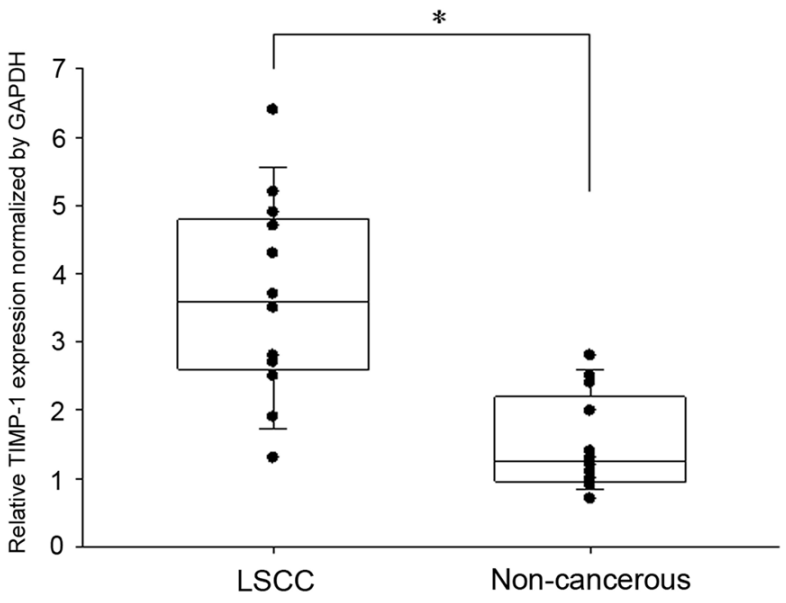

Figure 1. One-step quantitative real-time polymerase chain reaction (qPCR) was employed to evaluate TIMP-1 mRNA expression levels in LSCC (cancer) compared with matched tumor adjacent tissues (non-cancerous). Normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels, the TIMP-1 mRNA level in LSCC tissue is significantly higher than that in corresponding non-cancerous tissue (*p=0.001).

from a well-differentiated tumor, 53 had moderate tumor differentiation, and 5 had poor tumor differentiation. Basing from the TNM staging system, we found that 65 patients were in stages I and II, whereas the remaining 44 patients were in stages III and IV. Positive lymph node metastasis was observed in 90 patients; negative lymph node metastasis was detected in the remaining 19 patients. Among these 109 cases, 72 patients survived and 37 patients died.

Analysis of TIMP-1 mRNA expression in LSCC by qPCR

Total RNA was extracted from the LSCC tissues and subjected to one-step qPCR to evaluate the expression of TIMP-1 mRNA. We also detected samples from the normal tumor-adjacent tissues for comparing the expression of TIMP-1 mRNA with that of non-cancerous tissue. When normalized to GAPDH, the means of TIMP-1 mRNA in LSCC and corresponding tumor-adjacent tissues were 3.66±1.496 and 1.52±0.716 respectively (t=4.475, p=0.001). The TIMP-1 expression averaged 2.41-fold higher in the LSCC samples than in non-cancerous tissues (**Figure 1**).

Detection of TIMP-1 expression in LSCC by IHC

IHC analysis was executed to investigate the expression of TIMP-1 in LSCC. Elevated LSCC expression was detected in 63 of 109 (57.8%) LSCC tissues, while only 7 cases of 28 normal tumor-adjacent tissues (25.0%) exhibited TIMP-1 expression. There was significant difference in high expression rate of TIMP-1 between LSCC tissues and normal tumor-adjacent tissues (p<0.001). Positive staining was mainly localized in the cytoplasm of cancer cells while strong staining was not observed in the non-cancerous tumor-adjacent areas. Typically observed IHC staining patterns of TIMP-1 in LSCC were shown in **Figure 2**.

Relationship between TIMP-1 expression and clinical parameters

The relationship between high expression of TIMP-1 protein and the clinical parameters of 109 LSCC patients was displayed in **Table 1**. High TIMP-1 expression was remarkably associated with tumor differentiation (p=0.012) and overall survival (p=0.043). In contrast, no significant correlation was discovered between TIMP-1 expression and other clinical items, such as gender, age, tobacco and alcohol consumption, TNM stage and lymph node metastasis.

Survival analysis

Univariate analysis showed that the life span of LSCC patients was correlated with high TIMP-1 expression (p=0.001), TNM stage (p=0.004), tumor differentiation (p=0.023) and lymph node metastasis (p=0.001). Multivariate analysis with the Cox regression model suggested that high TIMP-1 protein level and lymph node metastasis may serve as two independent prognostic factors for overall survival (p=0.008 and p=0.029, respectively) (**Table 2**). Kaplan-Meier survival curves exhibited that LSCC patients with high TIMP-1 expression and posi-

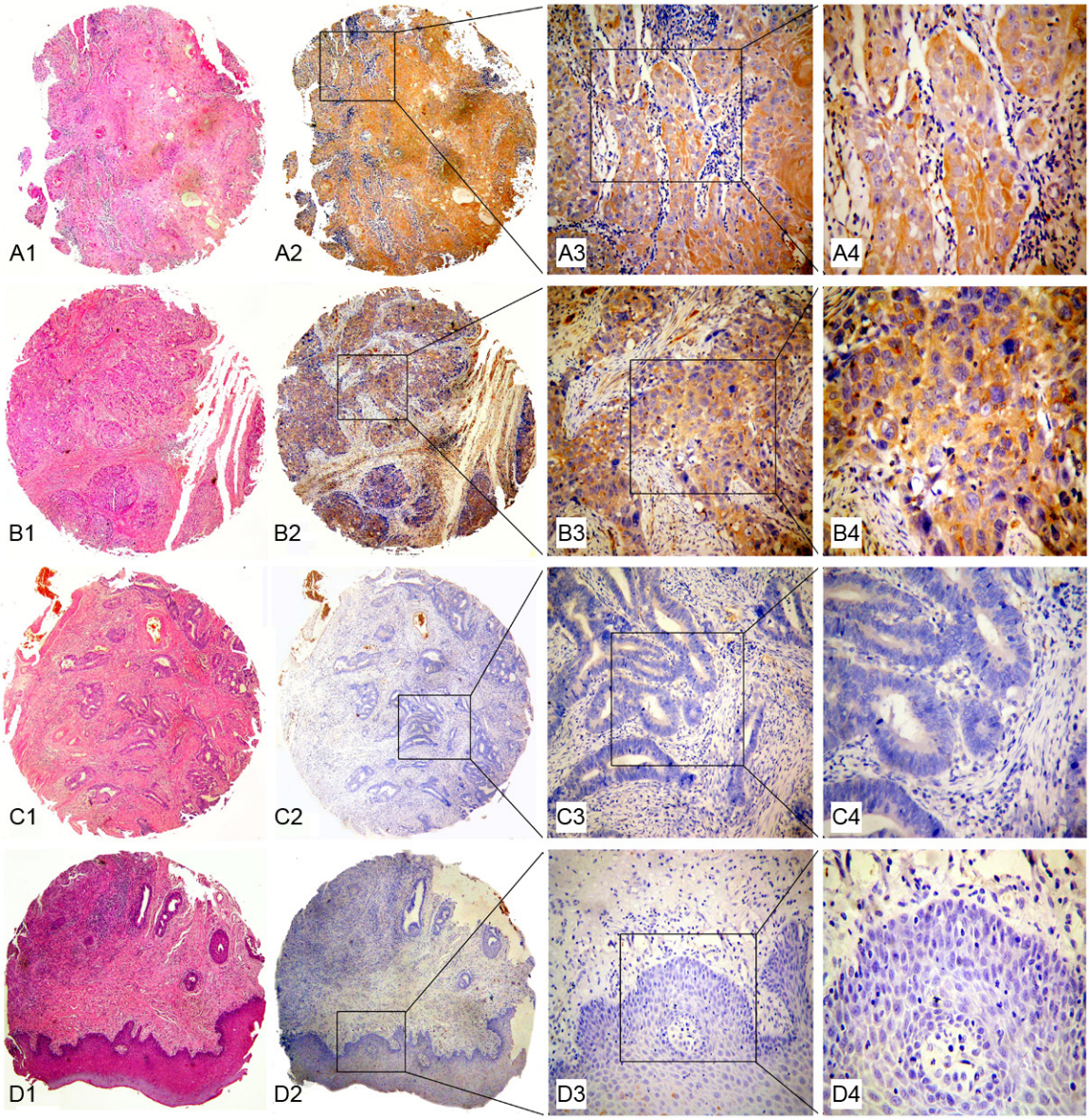

Figure 2. Representative hematoxylin-eosin (H&E) staining and pattern of TIMP-1 protein expression in laryngeal squamous cell carcinoma (LSCC) and adjacent non-cancerous tissue with tissue microarray (TMA) sections. A1: H&E staining in well-moderately differentiated LSCC tissue sample. A2-A4: Strong immunohistochemical (IHC) staining of TIMP-1 in well-moderately differentiated LSCC tissue sample. B1: H&E staining in poorly differentiated LSCC tissue sample. B2-B4: Strong IHC staining of TIMP-1 in poorly differentiated LSCC tissue sample. C1: H&E staining in LSCC tissue sample. C2-C4: Negative IHC staining of TIMP-1 in LSCC tissue sample. D1: H&E staining in tumor adjacent non-cancerous tissue sample. D2-D4: Negative IHC staining of TIMP-1 in tumor adjacent non-cancerous tissue sample. Original magnification ×40 in A1, B1, C1, D1, A2, B2, C2, and D2; ×200 in A3, B3, C3 and D3; ×400 in A4, B4, C4 and D4.

tive lymph node metastasis showed the significantly poor survival time (**Figure 3**).

Discussion

TIMPs are important inhibitors of MMPs and comprise a family of four structurally related proteins (TIMP-1 to TIMP-4). TIMPs inhibit metalloproteinase activity associated with tumor angiogenesis and progression. TIMPs play significant roles in several cellular processes, such as cell growth, apoptosis, migration, and differentiation [24]. TIMP-1 is also a multifunctional protein and proteolytic enzyme that degrades the ECM. TIMP-1 characteristics are normally identified as either MMP dependent or

Table 2. Univariate and multivariate analysis of prognostic factors in LSCC for overall survival

	Univariate analysis			Multivariate analysis		
	HR	$p>\|z\|$	95% CI	HR	$p>\|z\|$	95% CI
TIMP-1 expression						
High versus Low	3.412	0.001*	1.713-6.796	2.660	0.008*	1.295-5.463
Age (years)						
≤60 y versus >60 y	0.624	0.181	0.313-1.244			
Tobacco consumption						
Yes versus No	0.667	0.263	0.328-1.356			
Alcohol consumption						
Yes versus No	0.938	0.847	0.491-1.791			
TNM stage						
Stage I, II versus Stage III, IV	0.378	0.004*	0.195-0.733	0.548	0.127	0.253-1.186
Tumor differentiation						
Well versus Moderate to Poor	0.467	0.023*	0.243-0.898	0.657	0.221	0.335-1.288
Radiotherapy						
Yes versus No	0.682	0.362	0.299-1.554			
Lymph node metastasis						
Yes versus No	4.670	0.001*	2.228-9.793	2.594	0.029*	1.104-6.095

*$p<0.05$.

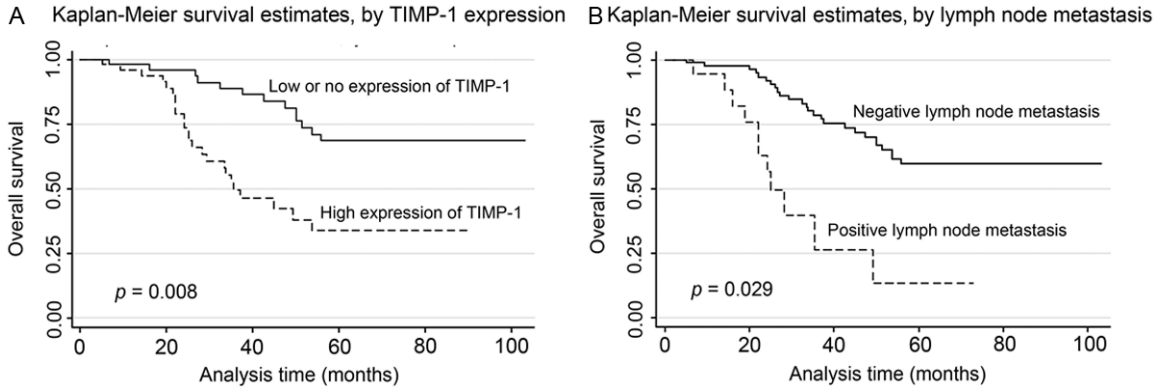

Figure 3. Survival analysis of laryngeal squamous cell carcinoma (LSCC) patients by Kaplan-Meier method. A: Overall survival rate in patients with high TIMP-1 expression (dashed line) was significantly lower than that in patients with low and no TIMP-1 expression (solid line). B: Overall survival rate in patients with positive lymph node metastasis (dashed line) was significantly lower than that in patients with negative lymph node metastasis (solid line).

MMP independent [25, 26]. Hence, not only known for the MMP-inhibitory function, TIMP-1 is also reported to promote cell growth, inhibit apoptosis and regulate angiogenesis [27]. The regulation of TIMP-1 in cellular processes is complicated and depends on various conditions and environments. Certain regulatory process also involves contradictory mechanisms. For instance, an increased MMP activity promotes tumor progression; high TIMP-1 levels suppress tumor invasion by inhibiting MMP and preventing the facilitated breakdown of the ECM and basement membranes [11]. However, high TIMP-1 levels have been detected in aggressive tumors and elevated TIMP-1 expressions are associated with shorter overall survival [9-16]. Several studies have also revealed controversial results on TIMP-1 in head and neck squamous cell carcinomas [17-19, 28]. In the present study, the clinicopathological significance of TIMP-1 in patients with LSCC was evaluated, particularly the prognostic function of TIMP-1.

The results of qPCR indicated that the mRNA expression levels of TIMP-1 in LSCC tissues

were higher than those in normal cells of the tumor-adjacent tissues. This result is consistent with that in a previous study, in which the mRNA expression of TIMP-1 is significantly increased in colorectal cancer tissue compared with that in adjacent normal mucosa [29]. We further investigated this association by preparing TMA from LSCC specimens. IHC results showed a higher TIMP-1 protein expression in LSCC tissues than in normal tumor-adjacent tissues. This result is consistent with that in previous studies, in which a TIMP-1 protein expression is found in malignant tumors [19, 30].

TIMP-1 overexpression (such as in serum, plasma, and tissue) is also associated with the poor prognosis of various cancers, including esophageal cancer [24], breast cancer [27], prostate cancer [31], lung cancer [25], glioblastoma [14], and myeloma [15]. In our study, high TIMP-1 expression in LSCC was correlated with two clinical pathological characteristics, namely, tumor differentiation and overall survival. Univariate analysis showed that the life span of LSCC patients was correlated with TNM stage, tumor differentiation, lymph node metastasis, and high TIMP-1 expression. Moreover, multivariate analysis revealed a prognostic value for TIMP-1 overexpression, indicating that the strong expression of TIMP-1 and positive lymph node metastasis correspond to the poor prognosis of patients with LSCC. The Kaplan-Meier curve also indicated that the patients with LSCC exhibiting low or no TIMP-1 expression and who were negative for lymph node metastasis demonstrated a favorable overall survival. These findings suggested that TIMP-1 expression may be associated with the development and progression of LSCC; hence high TIMP-1 expression may serve as an independent prognostic marker of LSCC.

Interestingly, other studies have suggested that TIMP-1 is not a reliable prognostic marker of cancer [28, 32]. Our present data differ from those in previous studies. These conflicting results may be attributed to the differences in tumor type and pathological samples, antibodies used, or experimental methods. Further studies that enroll larger scale of clinical samples of LSCC will be necessary and are of great interest.

In conclusion, this study is the first to evaluate the TIMP-1 mRNA expression with qPCR and protein expression with TMA in LSCC. Our results showed that high TIMP-1 expression was correlated with an aggressive malignant phenotype of LSCC. Therefore, TIMP-1 may be used as a novel and valuable prognostic marker of LSCC and targeting TIMP-1 may provide a rational strategy for LSCC treatment.

Disclosure of conflict of interest

None.

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