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Original Article

Significance of MMP11 and P14^{ARF} expressions in clinical outcomes of patients with laryngeal cancer

Zufei Li[1,2*], Shuo Ding[1], Qi Zhong[1], Guojun Li[3], Yang Zhang[1*], Xiaohong Chen[1] Zhigang Huang[1]

[1]Department of Otolaryngology Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Key Laboratory of Otolaryngology Head and Neck Surgery (Capital Medical University), Ministry of Education, Beijing 100730, China; [2]Department of Otolaryngology Head and Neck Surgery, Aerospace Center Hospital, Beijing 100039, China; [3]Department of Head and Neck Surgery and Epidemiology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA. [*]Equal contributors.

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Abstract: Background: To evaluate the association of MMP11 and P14^{ARF} expression in laryngeal squamous cell carcinoma (LSCC) with clinical pathological characteristics and survival. Methods: The mRNA and protein levels for both genes were determined in 65 LSCC patients. A log-rank test and Cox models were used to compare survival among different groups. Results: The mRNA expressions of MMP11 and P14^{ARF} were significantly different between LSCC and their corresponding adjacent tissues (All $P < 0.001$). The expressions of MMP11 and P14^{ARF} were correlated with several clinical characteristics (All $P < 0.05$). Patients with low MMP11 and high P14^{ARF} expression had significantly better survival compared with those with high MMP11 and low P14^{ARF} expression, respectively (All $P < 0.05$). The patients with surgery only had significantly better survival than those with chemoradiotherapy (log rank: $P = 0.016$), particularly in patients with low MMP11 and high P14^{ARF} expression (log rank: $P = 0.006$). Furthermore, multivariable analysis showed that patients with low MMP11 and high P14^{ARF} expression alone had a significantly reduced risk of death compared with those with high MMP11 and low P14^{ARF} expression. The reduced risk for overall death was pronounced for patients with low and high expression of both genes (HR, 0.2; 95% CI, 0.1-0.5) compared with any other co-expression status of both genes, particularly for patients with surgery only (HR, 0.1; 95% CI, 0.0-0.9). Conclusion: These results suggest that altered expression of MMP11 and P14^{ARF} in tumors may individually, or in combination, predict poor prognosis of LSCC, particularly for patients with surgery only.

Keywords: P14^{ARF}, MMP11, LSCC, survival, biomarker

Introduction

Head and neck squamous cell carcinomas (HNSCC) is the sixth most common type of cancer worldwide, with poor clinical outcomes [1] and laryngeal squamous cell carcinoma (LSCC) ranks second among the HNSCC [2]. It accounts for nearly half of all HNSCC cases in China [3]. The progression and metastasis of LSCC involve multiple factors and molecular processes, with a notably expression imbalance of numerous cellular molecules.

The matrix metalloproteinases (MMPs) is a family of extracellular or membrane-bound Zn^{2+}-dependent proteases that are capable of digesting various proteinaceous components of the extracellular matrix (ECM) which serves as a medium for cell-cell interactions and can directly signal cells through cell surface ECM receptors [4]. MMP11, also known as Stromelysin-3 (ST3), is one of members in MMPs family. It was first identified by its overexpression in primary breast cancer and isolated as a breast carcinoma-associated gene [5]. Extensive studies have shown that high expression of MMP11 were associated with tumor invasion, metastasis, tumor progression, and prognosis [6].

P14^{ARF}, located on human chromosome 9p21, is a tumor suppressor gene encoded by theunusual INK4a/p14^{ARF} locus, which is frequentlyinactivated in tumors [7]. Itis a multi-functional gene that is involved in many physic processes. P14^{ARF} can induce both G1 and G2 arrest due to

Table 1. Primers used in this study

Primers	Sequences
β-actin	F: 5-CACCCTTTCTTGACAAAACCT-3'
	R: 5'-AGTGGGGTGGCTTTTAGGA-3'
MMP11	F: 5-TGAGTGCCCGCAACCG 3'
	R: 5'-GGCGTCACATCGCTCCATA-3'
P14ARF	F: 5-TGGAGGCGGCGAGAACA-3'
	R: 5'-TCAGTAGCATCAGCACGAGGG-3'

its stabilizing effects on the p53 transcription factor, and is involved in cellular senescence and apoptosis [8]. Moreover, P14ARF has also significant association with autophagy and angiogenesis [9, 10].

Although several studies have reported that these two genes may play important roles in development, progression and prognosis in human cancers [5-10], such potential roles and their relationship between MMP11 and P14ARF in LSCC is not unknown. This study aimed to compare the expression ofMMP11 and P14ARF in LSCC and their matched adjacent normal laryngeal tissues, explore their relationships with clinical pathological characteristics, and evaluate the potential association between MMP11 and P14ARF expression and survival of LSCC, which may thus lead to better understanding of the functional contribution of MMP11 and P14ARF to progress and prognosis of LSCC.

Materials and methods

Patients and biological samples

Sixty-five patients were included in this study and all biopsies were obtained with patients' consent. We included consecutive patients diagnosed with LSCC between 2009 and 2010. Immediately after surgical excision, a tumor sample was obtainedfrom the tumor area, while its corresponding peripheral normal laryngeal tissues were obtained from theassociated non-cancerous tissue within 5 cm of the tumor,without affecting the assessment of tumor margins. The biopsies were divided into two parts: one snap-frozen in liquid nitrogen immediately after surgery, and stored at -80°C; the other formalin fixed and paraffin embedded. Histopathological assessment was performed on the paraffin blocks. In addition, patients who were categorized as "ever drinkers" are related to those who had drunk at least one alcoholic beverage per week for at least 1 year during their lifetime, and patients who were categorized as "never drinkers" are related to those who had never had such a manner of drinking. Patients who were categorized as "ever smokers" are related to those who had smoked at least 100 cigarettes in their lifetime, and patients who had smoked fewer than 100 cigarettes in their lifetime were categorized as "never smokers". This study was approved by the ethics committee of Beijing Tongren Hospital of the Capital Medical University, and all of the patients had signed the informed consent forms.

Quantitative analysis

Total RNA was extracted for QPCR. The primers and internal reference primers were synthesized by Beijing Tiangen Biomedical Development Co., Ltd. (Beijing, China) and the Ct value comparison method was used to detect the gene expression level, with β-actin as an internal reference[9]. The relative expression levels of MMP11 and P14ARF were calculated with the Ct value referring to the number of cycles when the fluorescence signal reached the set threshold in each reaction tube. The primers used in this study are shown in **Table 1**.

Immunohistochemistry

MMP11 and P14ARF antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), while streptavidin-peroxidase, 4-dimethylaminoazobenzene, and the streptavidin-peroxidase immunohistochemical staining kit were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The LSCC tissues and its corresponding adjacent normal laryngeal tissues were preserved. A positive biopsy in the kit was used for the staining of the positive control, and the antibody was replaced by phosphate-buffered saline for the staining of the negative control. The immunohistochemical staining is shown in **Figure 1**. Yellow to brownish-yellow granules in cells were considered to indicate positive cells. Positive cells were counted in a total of 100 cells under high-power microscopy, and scored according to the positive expression rate (0 points for < 10%; 1 point for 11-20%; 3 points for 21-50%; 4 points for > 50%) and staining intensity (0 points for no staining; 2 points for weak; 3

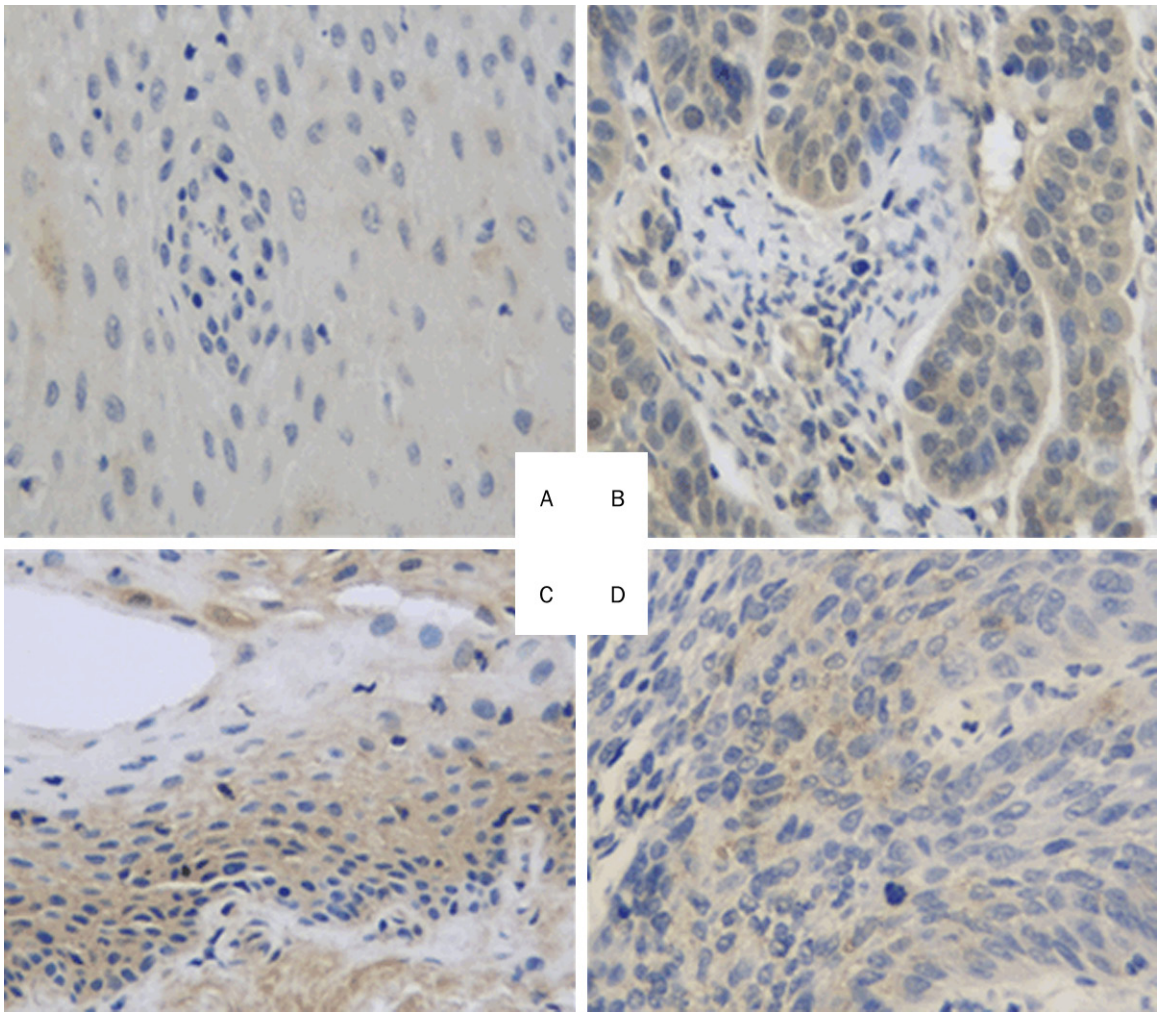

Figure 1. Immunostaining of MMP11 and P14ARF in laryngeal carcinoma tissues. A. MMP11 weakly expressed in the adjacent tissue; B. Laryngeal carcinoma tissues with high MMP11 expression in the cytoplasm; C. P14ARF positively expressed in the adjacent tissue; D. P14ARF weekly expressed in the laryngeal carcinoma tissue.

points for strong). The sum of the points for staining intensity and positive expression rate was used as the expression score. A score ≥ 3 was considered to indicate a positive case while a score of 0-2 was classified as a negative case.

Statistical analysis

The primary endpoint in this study is overall death. Overall survival (OS) was defined as the time from first appointment to death from any cause or date of last follow-up. Participants who were alive at the end of the study period or lost to follow-up were considered censored. Medical record review for follow-up status of all patients was performed under direct supervision of staff head and neck surgeon. Primary tumor subsite, clinical stage, treatment, and vital status were reviewed from medical records as assessed between the initial and final patient contact recorded. Data were analyzed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL). Measurement data were compared using a paired t-test, while the correlation of MMP11 and P14ARF expression was analyzed using Spearman's rank correlation analysis. Survival analysis was performed by using Kaplan-Meier analysis, and the significance was analyzed with the log-rank test. Univariate cox proportional hazards regressions were applied to estimate the individual hazard ratio (HR) for the overall survival. The difference was considered significant when the *P* value was less than 0.05.

Table 2. mRNA expression of MMP11 and P14ARF in LSCC and adjacent tissues

Tissue types	n	MMP11	*P*	P14ARF	*P*
Adjacent tissues	65	0.961 ± 0.294		1.050 ± 0.202	
Tumor tissues	65	2.346 ± 1.995	< 0.001	0.794 ± 0.248	< 0.001

Table 3. MMP11 and P14ARF protein expression level in LSCC and adjacent tissues

Tissue type	n	MMP11 (+) N %	*P*	P14ARF (+) N %	*P*
Adjacent tissues	65	14 (21.5)		53 (81.5)	
Tumor tissues	65	50 (76.9)	< 0.001	28 (43.1)	< 0.001

Results

mRNA levels of MMP11 and P14ARF

QPCR was performed to detect the potential changes in the mRNA levels of MMP11 and P14ARF expression in LSCC and its corresponding adjacent normal laryngeal tissues. The QPCR results showed significant gene expression level differences between the cancerous and adjacent tissues ($P < 0.05$). The expression of MMP11 in the cancerous tissue was significantly increased ($P < 0.05$) while the P14ARF mRNA expression in the cancerous tissue was notably decreased compared with that in the adjacent tissue ($P < 0.05$), as shown in **Table 2**. These results indicate that the mRNA levels of MMP11 in LSCC are increased while the mRNA levels of P14ARF are decreased compared with those in the adjacent tissues.

Protein expression of MMP11 and P14ARF

Immunohistochemical analyses of MMP11 and P14ARF expression in LSCC and their corresponding adjacent normal laryngeal tissues were performed to detect the expression of MMP11 and P14ARF proteins (**Table 3**). Both MMP11 and P14ARF proteins were expressed in the cytoplasm. The positive expression rates and scores of MMP11 were significantly increased in the cancerous tissue compared with those in the adjacent tissue ($P < 0.05$). For P14ARF, however, its protein expression levels were significantly decreased in LSCC compared with those in the adjacent tissues ($P < 0.05$). These results indicate that the expression levels of MMP11 in LSCC are increased while the levels of P14ARF are decreased compared with those in adjacent tissues.

Correlation between the mRNA and protein expression of MMP11 and P14ARF and the clinical pathological characteristics

The mRNA expression levels were analyzed to explore the potential relationship between the protein expression and mRNA levels of MMP11 and P14ARF and their relationships with clinical pathological characteristics (**Table 4**). It was revealed that MMP11 and P14ARF were negatively correlated (correlation coefficient, -0.376, $P = 0.002$). In the 65 cases of LSCC tissues, MMP11 was correlated with the tumor stage, lymph node metastasis; and the patients with later tumor stage and lymph node metastasis had shown a much stronger MMP11 expression compared with those with early tumor stage and negative lymph node metastasis ($P < 0.05$). In contrast, P14ARF expression was also associated with the tumor stage; and the patients in the later stage had shown a much lower expression level ($P < 0.05$). Both MMP11 and P14ARF had a significant correlation with tobacco smoking. These results suggest that the elevated expression of MMP11 may be correlated with the malignancy of tumors and that P14ARF expression may be negatively correlated with the degree of malignancy.

Association of MMP11 and P14ARF expression with survival in LSCC

Figure 2 shows the univariate Kaplan-Meier analyses of survival with respect to the death from all causes. Among the 65 patients, 45 had surgery only and 20 received chemotherapy, radiation or their combination (**Table 4**). At a median follow-up time of 67 months (range, 4-67 months), 15 deaths occurred from any causes. There were 10 deaths from 45 patients with surgery only and 5 deaths from 20 patients with chemoradiotherapy or in combination. The patients with low MPP11 and high *P14ARF* expression had a significantly better overall survival than patients with high MPP11 and low *P14ARF* expression (Log-rank: $P = 0.035$ for

Table 4. Correlations between the protein/mRNA expressions of MMP11 and P14ARF with clinical pathological characteristics in 65 LSCC cases

Factors	Cases (%)	MMP11		P14ARF	
		Protein (+)	High mRNA	Protein (+)	High mRNA
		N, %	N, %	N, %	N, %
Age (years)					
≥ 60	28 (43.1)	22 (78.6)	28 (100)[a]	10 (35.7)	8 (28.6)
< 60	37 (56.9)	28 (75.7)	30 (81.1)	18 (48.6)	5 (13.5)
Gender					
Male	58 (89.2)	45 (77.6)	52 (89.7)	25 (43.1)	12 (20.7)
Female	7 (10.8)	5 (71.4)	6 (85.7)	3 (42.9)	1 (14.3)
Tumor stage					
I-II	20 (30.8)	11 (55.0)	14 (70.0)	15 (75.0)	10 (50.0)
III-IV	45 (69.2)	39 (86.7)[a]	44 (97.8)[a]	13 (28.9)[a]	3 (6.7)[a]
LNM					
Absent	26 (38.2)	16 (61.5)	20 (76.9)	8 (30.8)	6 (23.1)
Present	39 (61.8)	34 (87.8)[a]	38 (97.4)[a]	20 (51.3)	7 (17.9)
Differentiation					
High	23 (35.4)	16 (69.6)	20 (86.9)	13 (56.5)	6 (26.1)
M./low	42 (64.6)	34 (81.0)	38 (90.5)	15 (36.7)	7 (16.7)
Treatment					
Surgery only	45 (63.1)	32 (71.1)[a]	43 (95.5)[a]	24 (53.3)[a]	12 (26.7)[a]
C/X/CX/CXS	20 (36.9)	18 (90.0)	15 (75.0)	4 (20.0)	1 (5.0)
Smoking					
Ever	51 (78.5)	44 (88.0)	49 (96.1)	16 (31.4)	5 (9.8)
Never	14 (21.5)	6 (42.9)[a]	9 (64.3)[a]	12 (85.7)[a]	8 (57.1)[a]
Alcohol					
Ever	55 (84.6)	42 (76.4)	50 (90.9)	23 (41.8)	10 (18.2)
Never	10 (15.4)	8 (80.0)	8 (80.0)	5 (50.0)	3 (30.0)

[a]P < 0.05: significant for either MMP and P14ARF protein or mRNA expressions between then comparison groups for the factors. LNM: lymph node metastasis; M: moderate; C: chemotherapy; X: radiation; S: surgery.

MMP11 and P = 0.005 for *P14ARF*, **Figure 2A**, **2B**); and patients with low and high expression of both *MMP11 and P14ARF* had a significantly better survival than patients with high and low expression of either of genes or any other co-expression status of both genes (*P* = 0.009, **Figure 2C**). Furthermore, the patients with surgery only had significantly better survival than those with chemoradiotherapy or their combination with surgery (log rank, P = 0.016) (**Figure 3A**). Among the patients with surgery only, the patients with low MMP11 and high *P14ARF* expressions had significantly better survival than those with high and low expression of either of genes or any other co-expression status of both genes (*P* = 0.006, **Figure 3B**). The multivariable Cox proportional hazards regression analysis regarding the association between expression of both genes and risk of overall death are shown in **Table 5**. Estimates of association were adjusted for potential confounders including age, gender, tumor differentiation, TNM stage, postoperative treatment, smoking and alcohol use, and Lymph node metastasis. Compared with patients having high and low expression of either of genes, the patients with low and high expression had significantly reduced risk of overall death (HR, 0.5; 95% CI, 0.2-0.8 for MMP11 and HR, 0.4; 95% CI, 0.2-0.7 for *P14ARF*). The reduced risk for overall death was pronounced for patients with low and high expression of both genes (HR, 0.2; 95% CI, 0.1-0.5) compared with any other co-expression status of both genes (**Table 5**), particularly for patients with surgery only (HR, 0.1; 95% CI, 0.0-0.9) (data not shown).

Discussion

Few studies have comprehensively evaluated the role of MMP11 and P14ARF in LSCC. Our current study evidently shows that both MMP11 and P14ARF individually, or in combination, affect survival and might be served as prognostic biomarkers of LSCC patients, particularly for those with surgery only. There is increasing evidence that genetic differences are involved in the occurrence of cancer and are independent predictors of cancer outcome in many cancer types. Characterizing patients according to their difference could help in clinical management and enhance personalized therapy. In the current study, we explored the contribution of MMP11 and P14ARF expressions in LSCC, which we believe is a step toward personalized prevention and treatment of thyroid cancer. We did find that the combined expressions of both genes were significantly associated with survival and disease stage of patients with LSCC.

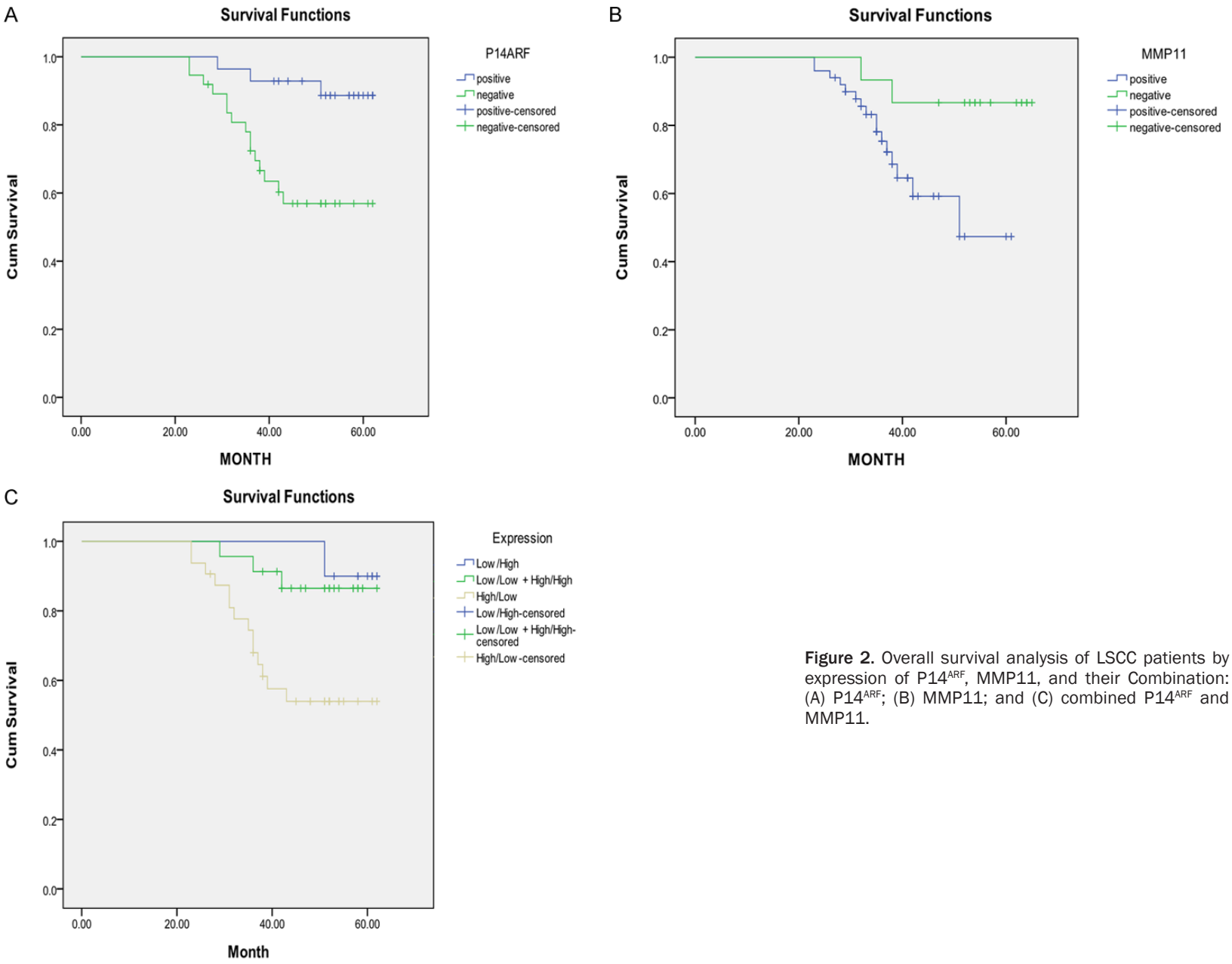

Figure 2. Overall survival analysis of LSCC patients by expression of P14ARF, MMP11, and their Combination: (A) P14ARF; (B) MMP11; and (C) combined P14ARF and MMP11.

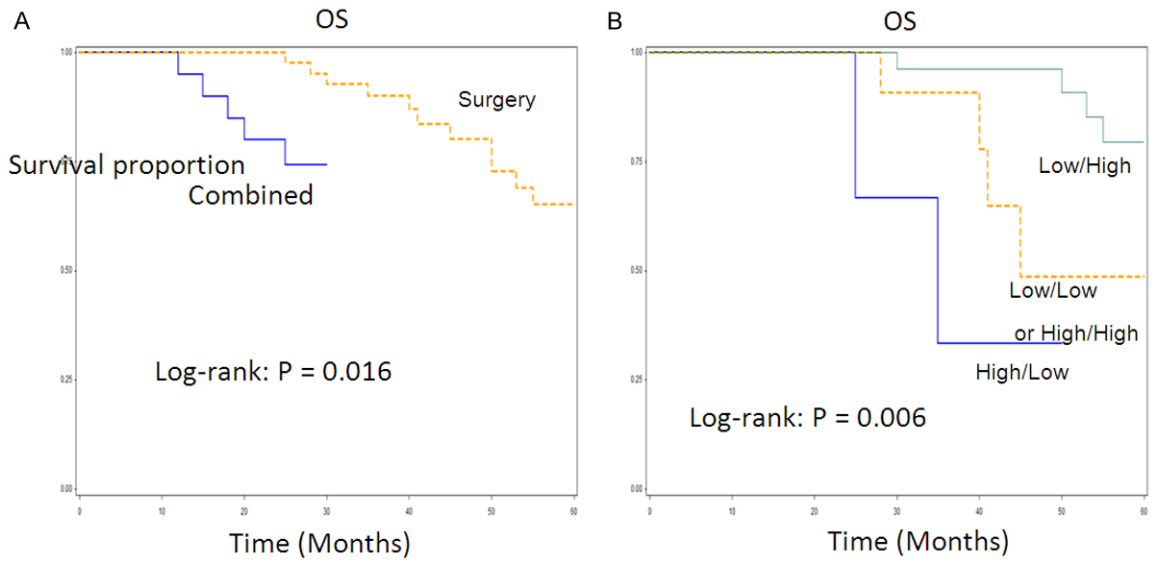

Figure 3. A. Overall survival analysis of LSCC patients by treatments among all 65 patients. B. Overall survival analysis of LSCC patients by expression of P14ARF in combination. With MMP11 among 45 patients with surgery only.

Table 5. Multivariable survival analysis by expression of MMP11 and P14ARF alone or in combination in 65 LSCC patients

Gene expression	Events/Total (15/65)	Survival (OS) Crude HR, 95% CI	Survival (OS) aHR, 95% CI
MMP11			
High	13/50	1.0	1.0
Low	2/15	0.6 (0.3-0.9)	0.5 (0.2-0.8)
P14ARF			
Low	12/37	1.0	1.0
High	3/28	0.4 (0.3-0.8)	0.4 (0.2-0.7)
Combined MMP11 and P14ARF			
High/Low	5/12	1.0	1.0
Low/Low + High/High	6/25	0.5 (0.2-0.9)	0.6 (0.3-1.0)
Low/High	4/28	0.3 (0.2-0.7)	0.2 (0.1-0.5)

Adjusted for age, sex, smoking, alcohol, overall stage, differentiation, node metastasis, and treatment in Cox's models.

Such findings could have implications for early detection, diagnosis, and treatment strategies and may help provide clinicians with additional information for personalized treatment, such as surgery only or surgery plus adjuvant treatment (chemotherapy/radiation).

The MMPs and play important roles in angiogenesis, tumor cell invasion and malignant cell proliferation [11-16]. Normal MMP11 expression has been shown to be involved in tissue remodeling during embryogenesis, tissue involution, wound healing and metamorphosis [17, 18]. Recent studies have shown that the expression level of MMP-11 was elevated in several cancers, including lung, colorectal, and ovariancarcinomas [19-21]. MMP11 expression has also been observed in HNSCC [22, 23]. The tumor-suppressor P14ARF is a multifunctional gene which is involved in a lot of physic processes, such as cell cycle and apoptosis [8, 24-26]. To date, whether the expression level of P14ARF during tumor progression is increased or decreased is still controversial. Zhang Y *et al.* demonstrated that P14ARF promoter genetic polymorphisms, which may affect expression of P14ARF, were associated with the susceptibility to second primary malignancy in patients with index squamous cell carcinoma of the head and neck [27]. Some studies suggest that p14ARF is widely down-regulated in several solid tumors, including breast, urinary bladder, pancreatic and esophageal carcinomas, as well as gliomas [28, 29]. On the contrary, others indicate that that P14ARF expression level was up-regulated in some tumors [30].

Our study illustrated that MMP11 was over-expressed while the expression level of P14ARF was decreased in LSCC in both mRNA and protein level. MMP11 was correlated with the tumor stage, lymph node metastasis of LSCC patients while P14ARF was associated with tumor stage. For MMP11, its expression was much higher in patients with deeper tumor stage and lymph node metastasis, suggesting that MMP11 was strongly associated with tumor malignance. For P14ARF, the expression was decreased and negatively correlated with tumor stage, which is in agreement with the investigation in HNSCC [31].The possible mechanisms that cause altered expression could be due to its promoter hypermethylation, genomic loss, and epigenetic repression [32, 33]. In addition, both MMP11 and P14ARF had a significant relationship with tobacco smoking, patients with ever tobacco smoking resulted in higher MMP11 expression as well as lower P14ARF expression than those who were never smoking, suggesting that tobacco smoking is another influence factor which affect the abnormal expression of MMP11 and P14ARF in LSCC.

Moreover, our data showed that patients with high-level MMP11 expression had significantly lower survival compared with those with low levels of MMP11. Several studies have demonstrated that over-expression of MMP-11 is related to a lower survival among patients with human breast cancer and non-small cell lung cancer [34, 35]. The function of MMP-11 in cellular is closely associated with decreasing cancer cells death through necrosis and apoptosis during malignancy [36]. In contrast, patients with low-level of P14ARF had significantly shorter survival compared with those with low expression levels. One study found similar result in hepatocellular carcinoma [37]. These results thus might indicate that both MMP11 and P14ARF might individually or jointly affect the prognosis of LSCC.

Interestingly, in this study we found that there exists a negative correlation between the expression levels of MMP11 and P14ARF. The potential mechanism may due to the fact that P14ARF may inhibit angiogenesis by down-regulating the expression of VEGF [38, 39], while the knockdown of MMP11 inhibited the proliferative activities and invasive potential of SGC-7901 GAC cells with decreased expression of VEGF [40]. Therefore, more studies are needed to further investigations to understand the relationship between these two genes.

In conclusion, over-expressed MMP11 and down-regulated P14ARF in LSCC were correlated with malignancy and prognosis of LSCC. Moreover, patients with low MMP11 and high P14ARF expressions had better survival and reduced risk of overall deaths, particularly for LSCC patients with surgery only. In addition, we found a negative correlation between the expression levels of MMP11 and P14ARF. Therefore, our results may provide evidence that both MMP11 and P14ARF may contribute to development, progression and prognosis of LSCC. However, further larger studies are required for validation of our findings and an exploration of the molecular mechanisms underlying the observed associations.

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Disclosure of conflict of interest

None.

Abbreviations

LSCC, laryngeal squamous cell carcinoma; HNSCC, head and neck squamous cell carcinomas; MMPs, matrix metalloproteinases; RT-PCR, reverse transcription polymerase chain reaction; HR, hazard ratio.

Address correspondence to: Xiaohong Chen and Zhigang Huang, Department of Otolaryngology Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Key Laboratory of Otolaryngology Head and Neck Surgery (Capital Medical University), Ministry of Education, Beijing 100730, China. E-mail: trchxh@163.com (XHC); enthzg@trhos.com (ZGH)

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