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Original Article

Expression and prognostic value of MAGE-A9 in laryngeal squamous cell carcinoma

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Abstract: Background: *Melanoma-associated antigen* (*MAGE*) family genes are reported to play important roles in the development of human cancers. However, the relationship between the expression of MAGE-A9 and clinicopathological characteristics in human laryngeal carcinoma remains unclear. This study aimed to examine the expression of MAGE-A9, and to evaluate the clinical significance of its expression in human laryngeal squamous cell carcinoma (LSCC). Methods: Quantitative real-time reverse transcription-PCR (qPCR) and immunohistochemistry (IHC) were performed to characterize the expression of MAGE-A9 in LSCC tissues and tumor-adjacent normal tissues. Kaplan-Meier survival and Cox regression analyses were performed to evaluate the prognosis of patients with LSCC. Results: The expression of MAGE-A9 was significantly higher in LSCC than in tumor-adjacent normal tissues. Cytoplasmic expression of MAGE-A9 was detected in 70 of 123 (56.9%) LSCC specimens. Levels of MAGE-A9 in LSCC were related to histopathological grade ($P = 0.024$). Kaplan-Meier survival and Cox regression analysis revealed that MAGE-A9 expression level and lymph node metastasis were independent prognostic factors of LSCC ($P = 0.005$; $P = 0.001$, respectively). Conclusions: Our study suggests that MAGE-A9 expression is a prognostic biomarker for LSCC patients. High expression of MAGE-A9 indicates unfavorable survival outcome in LSCC patients.

Keywords: Laryngeal squamous cell carcinoma, MAGE-A9, prognosis

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common head and neck tumor. There are more than 500 000 new cases of LSCC each year, constituting approximately 1.2% of all cancers, 25% of head and neck cancers and 99% of laryngeal malignant tumors [1-3]. The incidence of LSCC has increased in recent years. Although therapeutic strategies targeting LSCC have improved, including surgery, radiotherapy and chemotherapy, the mortality rate of LSCC has not changed [4-6]. The recurrence rate is still as high as 50%, with a local recurrence rate of 5-25% in patients with tumor stage I and 15-50% in patients with tumor stage II [7, 8]. Therefore, the identification of novel biomarkers for LSCC tumor staging and new treatment strategies is necessary.

Members of the *MAGE* gene family are tumor-associated antigens, which are commonly expressed in various tumors of epithelial origin, including breast cancer, lung cancer and colorectal cancer [9-12]. *MAGE-A*, a subset of highly homologous *MAGE* genes, belongs to the chromosome X-clustered cancer/testis antigens [13, 14]. The *MAGE-A* subfamily, which contains 12 genes, is also detected in the human germ line and in various cancers [15-17]. However, their biological functions remain largely unknown. *MAGE-A9*, which is expressed in high-risk bladder cancer [18], is a cancer-testis gene, which exhibits restricted expression in normal tissue, but is frequently expressed in cancer and testicular germ cells. Previous studies indicated the oncogenic characteristics of MAGE-A9 during the development and progression of malignant tumors. However, the relationship between MAGE-A9 expression and clinicopathological outcome in LSCC remains unclear.

In this study, we examined the expression of *MAGE-A9* mRNA in LSCC and tumor-adjacent

Table 1. Association of MAGE-A9 expression with clinicopathological factors of LSCC

Groups	No.	MAGE-A9 expression in cancer cells				No.	MAGE-A9 in stromal cells			
		Low expression (%)	High Expression (%)	Pearson 2	*p* value		Low expression (%)	High Expression (%)	Pearson 2	*p* value
Total	123	53 (41.3)	70 (56.9)			89	70 (78.7)	19 (21.3)		
Gender										
Female	2	1 (50.0)	1 (50.0)	0.04	0.842	1	1 (100.0)	0 (0.0)	0.275	0.6
Male	121	52 (43.0)	69 (57.0)			88	69 (78.4)	19 (21.6)		
Age										
≤ 60 years	45	20 (44.4)	25 (55.6)	0.053	0.818	28	23 (82.1)	5 (17.9)	0.297	0.586
> 60 years	78	33 (42.3)	45 (57.7)			61	47 (77.0)	14 (23.0)		
Smoking										
No	32	14 (43.7)	18 (56.3)	0.66	0.416	24	22 (91.7)	2 (8.3)	1.816	0.178
Yes	68	24 (35.3)	44 (64.7)			53	42 (79.2)	11 (20.8)		
Unknown	23	15 (65.2)	8 (34.8)			12	6 (50.0)	6 (50.0)		
Alcohol										
No	50	20 (40.0)	30 (60.0)	0.17	0.68	40	31 (77.5)	9 (22.5)	1.872	0.171
Yes	50	18 (36.0)	32 (64.0)			37	33 (89.2)	4 (10.8)		
Unknown	23	15 (65.2)	8 (34.8)			12	6 (50.0)	6 (50.0)		
pTNM										
T1	13	6 (46.1)	7 (53.9)	1.621	0.655	11	10 (90.9)	1 (9.1)	0.863	0.834
T2	54	20 (37.0)	34 (63.0)			40	33 (82.5)	7 (17.5)		
T3	31	12 (38.7)	19 (61.3)			25	20 (80.0)	5 (20.0)		
T4	2	0 (0.0)	2 (100.0)			1	1 (100.0)	0 (0.0)		
Unknown	23	15 (65.2)	8 (34.8)			12	6 (50.0)	6 (50.0)		
Lymph node metastasis										
No	103	44 (42.7)	59 (57.3)	0.036	0.85	77	61 (79.2)	16 (20.8)	0.11	0.74
Yes	20	9 (45.0)	11 (55.0)			12	9 (75.0)	3 (25.0)		
Histopathological grade										
High	55	31 (56.4)	24 (43.6)	7.487	0.024*	38	27 (71.0)	11 (29.0)	3.088	0.214
Middle	55	18 (31.5)	37 (68.5)			45	39 (86.7)	6 (13.3)		
Low	11	3 (27.3)	8 (72.7)			5	4 (80.0)	1 (20.0)		
Unknown	2	1 (50.0)	1 (50.0)			1	0 (0.0)	1 (100.0)		

*$P < 0.05$.

normal tissues via one-step quantitative reverse transcription-polymerase chain reaction (qPCR). Furthermore, we evaluated the expression of MAGE-A9 protein in LSCC by tissue microarray (TMA). Finally, we evaluated the clinical significance of MAGE-A9 expression in LSCC.

Materials and methods

Specimen collection

A total of 123 paraffin-embedded LSCC tissues and 22 tumor-adjacent normal tissue samples were collected from the archives of the Department of Pathology at the Affiliated Hospital of Nantong University, between January 2000 and May 2010. Histological diagnosis of LSCC was performed according to the latest World Health Organization (WHO) criteria [19]. All patients were typed in accordance with the TNM stage classification system (UICC 2009) [20]. Clinical data including gender, age, alcohol consumption, tobacco use, pTNM stage, lymph node metastasis and histopathological grade were retrospectively collected from hospital medical records. Clinical characteristics of 123 patients with LSCC are shown in **Table 1**. All patients received radical surgery. None of the patients received radiotherapy chemotherapy, and/or immunotherapy. Ethical approval to perform this study was obtained from the Human Research Ethics Committee of the local hospital, and written, informed consent was obtained from all patients participating in this study.

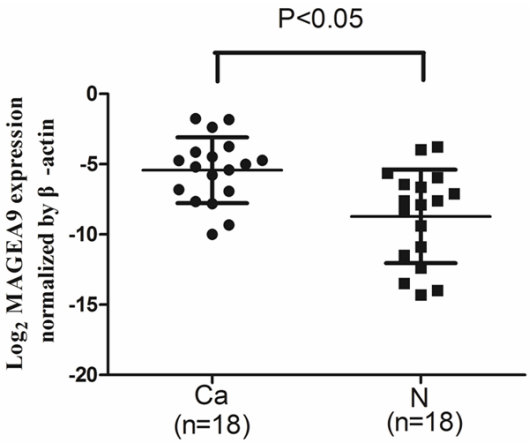

Figure 1. One-step quantitative reverse transcription-polymerase chain reaction (qPCR) was employed to evaluate *MAGE-A9* mRNA expression levels in LSCC (Ca) compared with tumor adjacent tissue (N). Levels of *MAGE-A9* mRNA in LSCC and tumor-adjacent normal tissues were 0.064 ± 0.0086 and 0.0123 ± 0.0045, respectively (t = 2.032, P = 0.028) after normalizing to *β-actin*.

One-step qPCR analysis

Eighteen samples of fresh LSCC tissues and matched tumor-adjacent normal tissues were collected. Total RNA was extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (2 mg) was reverse transcribed into cDNA using Moloney murine leukemia virus retrotranscriptase (Promega, USA). RT-PCR primers were designed with the assistance of Beacon Designer 7.7 software and are as follows: *MAGEA9* forward: 5'-CACTGTATGTCATCTCTG-3'; *MAGEA9* reverse: 5'-ACTACTGTCATTCATTAACT-3'; *β-actin* forward: 5'-TTAATCTTCGCCTTAATACTT-3'; *β-actin* reverse: 5'-AGCCTTCATACATCTCAA-3'. qPCR was performed using SYBR green dye and a Bio-Rad iQ50 Real-time PCR system in accordance with the manufacturer's instructions. Real-time PCR cycling parameters were as follows: denaturation at 95°C for 20 s, annealing at 56°C for 30 s and extension at 72°C for 30 s. Expression data were normalized to the geometric mean of the *β-actin* housekeeping gene and analyzed using the 2-Delta Delta Ct method as previously described.

Tissue microarray (TMA) construction and IHC analysis

Formalin-fixed, paraffin-embedded tumor samples (n = 123) and normal tumor-adjacent tissue specimens (n = 22) were prepared and TMAs were produced by Xinchao Biotech Co., Ltd (Shanghai, China). The TMA was cut into 4-μm sections and placed on super frost charged glass microscope slides.

IHC streptavidin peroxidase (SP) staining was performed as previously described [21]. Tissue microarray sections were incubated with rabbit polyclonal anti-MAGE-A9 antibody (AP6170a, 2.5 μg/ml dilution; Abgent, San Diego, CA, USA) overnight at 4°C, followed by incubation with biotinylated anti-rabbit secondary antibody at 37°C for 30 min. The same isotype of rabbit IgG was used as a negative control. Sections were then incubated with a streptavidin-horseradish peroxidase complex, colorized with 3,3-diaminobenzidine (DAB) chromogen solution and counterstained with hematoxylin. Results were analyzed as previously described [22]. Briefly, the percentage of MAGE-A9 positive cells was scored as follow: 0 for 0%, 1 for 1-33%, 2 for 34-66% and 3 for 67-100%. The intensity of MAGE-A9 staining was also scored as follows: 0 for negative staining, 1 for yellow color staining, 2 for light brown color staining and 3 for brown color staining. Samples with a sum score of 0-2 were considered to exhibit low MAGE-A9 expression, and those with a sum score of 3-6 were considered to exhibit high MAGE-A9 expression.

Statistical analysis

Statistical analysis was performed using STATA 12.0 software (Stata Corporation, College Station, TX, USA). Comparison of *MAGE-A9* mRNA expression in fresh-frozen LSCC tissues with tumor-adjacent normal tissues was analyzed with the Wilcoxon signed rank nonparametric test. The association between MAGE-A9 expression and clinicopathologic variables was examined by chi-square test. Survival rate was estimated by Kaplan-Meier method and log-rank test. Multivariate analysis was performed using Cox's proportional hazard regression model. For all tests, a two-tailed P value of less than 0.05 was considered statistically significant.

Results

Analysis of MAGE-A9 mRNA expression in LSCC by qPCR

To investigate the expression of *MAGE-A9* mRNA in LSCC, we performed qPCR on RNA

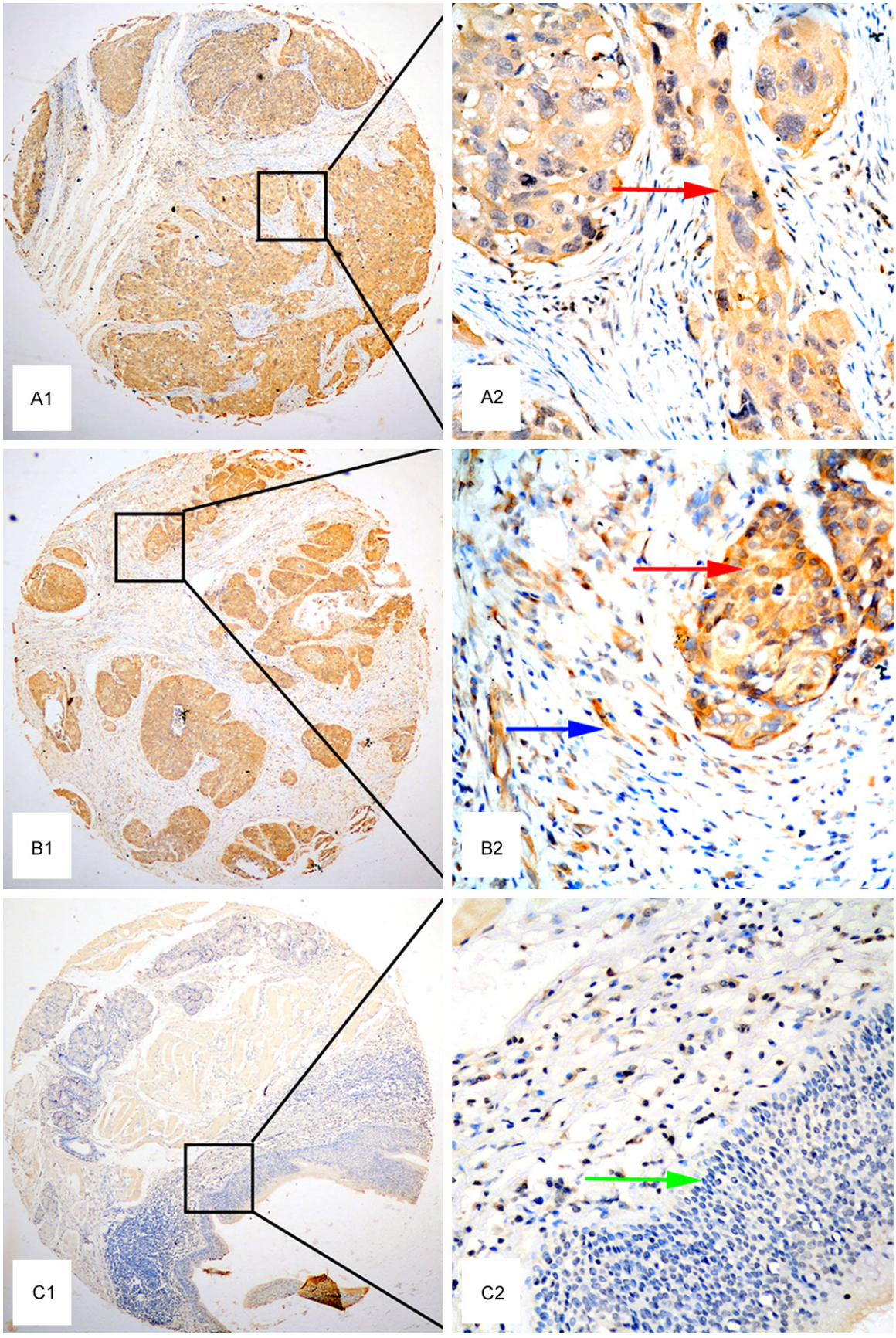
A1
A2
B1
B2
C1
C2

Figure 2. Representative images of MAGE-A9 expression in LSCC and tumor-adjacent normal tissues. A1 and A2. Immunohistochemical staining for MAGE-A9 showing positive cytoplasmic staining in LSCC. Red arrow indicates positive cytoplasmic staining of MAGE-A9 in LSCC cells. B1 and B2. Positive cytoplasmic staining of MAGE-A9 in cancer cells and stromal cells. Red arrow indicates positive cytoplasmic staining of MAGE-A9 in LSCC cells; blue arrow indicates positive MAGE-A9 staining in stromal cells. C1 and C2. Negative staining of MAGE-A9 in tumor-adjacent normal tissue. Green arrow indicates negative MAGE-A9 staining in epithelial cells. Original magnification ×40 in A1, B1 and C1; ×400 in A2, B2 and C2.

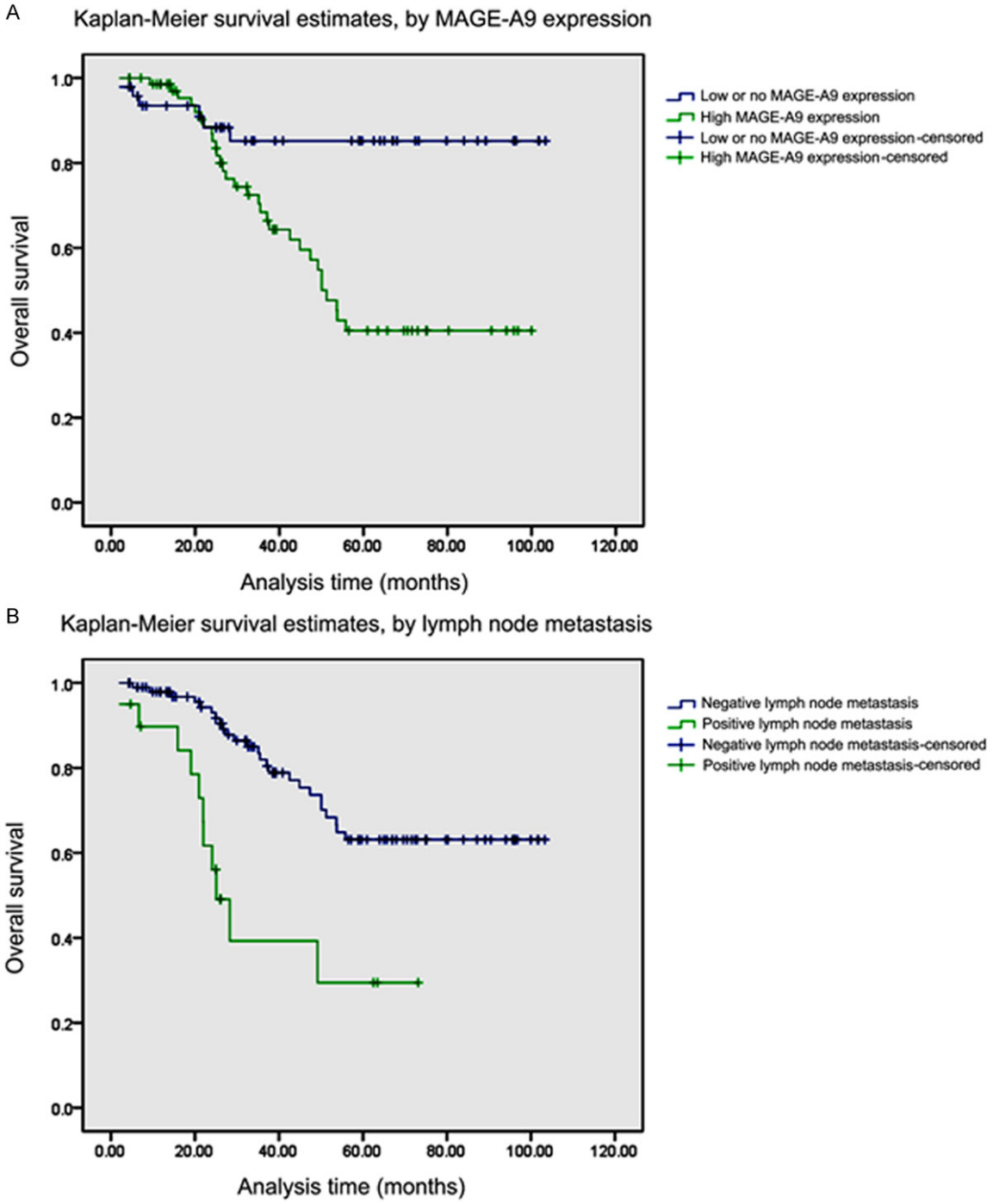

Figure 3. Survival analysis of LSCC patients (n = 123) by Kaplan-Meier method. A. The overall survival rate in patients with high MAGE-A9 expression (green line) was significantly lower than that in patients with low MAGE-A9 expression (blue line). B. The overall survival rate in patients with positive lymph node metastasis (green line) was significantly lower than that of patients with negative lymph node metastasis (blue line).

Table 2. Univariate and multivariate analysis of prognostic factors in LSCC for 5-year survival

Variable	Univariate analysis			Multivariate analysis		
	HR	*P* value	95% CI	HR	*P* value	95% CI
Expression of MAGE-A9 in cancer cells						
High vs. low	3.74	0.003*	1.554-9.016	3.57	0.005*	1.457-8.762
Expression of MAGE-A9 in stromal cells						
High vs. low	1.97	0.118	0.842-4.619			
Age (years)						
≤ 60 vs. > 60	1.71	0.151	0.823-3.544			
Smoking						
Yes vs. no	0.66	0.255	0.318-1.355			
Alcohol						
Yes vs.no	0.89	0.728	0.452-1.740			
pTNM						
T1 vs. T2 vs. T3 vs. T4	1.56	0.069	0.966-2.528			
Lymph node metastasis						
Yes vs. no	3.89	0.001*	1.889-8.002	4.40	0.001*	2.120-9.129
Histopathological grade						
High vs. middle vs. low	1.91	0.007*	1.189-3.063	1.61	0.063	0.975-2.672

*$P < 0.05$.

extracted from fresh LSCC tissues (n = 18) and matched tumor-adjacent normal tissues. Following normalization to *β-actin*, we observed a significant increase in *MAGE-A9* mRNA in LSCC compared with tumor-adjacent normal tissues (0.064 ± 0.0086 vs 0.0123 ± 0.0045, respectively, $t = 2.032$, $P = 0.028$). The average level of *MAGE-A9* mRNA was 5.18-fold higher in LSCC compared with tumor-adjacent normal tissues (**Figure 1**).

Detection of MAGE-A9 expression in LSCC by IHC

We next investigated the expression of MAGEA9 protein in LSCC by IHC. MAGE-A9-positive staining was predominantly localized in the cytoplasm of cancer cells and stromal cells. Expression of MAGE-A9 was significantly higher in LSCC tissues compared with tumor-adjacent normal tissues ($P < 0.001$). High expression of MAGEA9 was detected in 70 of 123 (56.9%) LSCC tissues, while only 6 of 22 (27.3%) tumor-adjacent normal tissues exhibited high expression. High expression of MAGE-A9 in stromal cells was detected in 19 of 89 (21.3%) LSCC tissues, compared with 2 of 22 (9.1%) tumor-adjacent normal tissues. Representative IHC staining patterns of MAGE-A9 in LSCC are shown in **Figure 2**.

Relationship between MAGE-A9 expression and clinical parameters

The relationship between high expression of MAGE-A9 protein and LSCC patient clinical parameters is displayed in **Table 1**. High MAGE-A9 expression in cancer cells was significantly associated with histopathological grade ($P = 0.024$), while no significant correlation with other clinical parameters, including gender, age, tobacco and alcohol consumption, TNM stage and lymph node metastasis, was observed. In contrast, MAGE-A9 expression in stromal cells was not correlated with any clinicopathological factors.

Survival analysis

Kaplan-Meier survival analyses revealed that LSCC patients with high cytoplasmic expression of MAGE-A9 and positive lymph node metastasis exhibited significantly poorer survival (**Figure 3**). Multivariate analysis using the Cox regression model indicated that high MAGE-A9 expression ($P = 0.005$) and lymph node metastasis ($P = 0.001$) were independent prognostic factors for overall survival (**Table 2**).

Discussion

Recently, a growing number of studies have reported that expression of MAGE family pro-

teins is associated with tumor progression and overall survival in various cancers [23-27]. *MAGE-A9* is a member of the *MAGE-A* gene family, which is located on chromosome X, and encodes a protein of approximately 35 kDa [28]. Although several *MAGE-A* family members have been reported to be potential candidates for tumor therapy [29, 30], the relationship between MAGE-A9 and LSCC remains unclear, and whether MAGE-A9 may be useful for diagnosis and as a therapeutic target in LSCC, requires further investigation. In the present study, the clinicopathological significance of MAGE-A9 expression in patients with LSCC was evaluated, particularly the prognostic attributes of MAGE-A9.

The results of qPCR indicated that *MAGE-A9* mRNA expression was higher in LSCC tissues than in normal cells of tumor-adjacent tissues. This result is consistent with previous studies, in which *MAGE-A9* mRNA expression was significantly increased in bladder cancer tissue compared with adjacent normal tissue [17]. In this study, we also conducted IHC analysis to evaluate MAGE-A9 protein expression in LSCC TMA specimens. This analysis revealed higher MAGE-A9 expression in the cytoplasm and mesenchyme of LSCC compared with normal tumor-adjacent tissues. Previous IHC analyses demonstrated that MAGE-A is expressed in mesenchymal stem cells (hMSC-TERT20) [31], suggesting that MAGEA-9 may be a mesenchymal stem cell marker. In addition to LSCC, high MAGE-A9 protein expression has also been identified in malignant tumors [17, 18]. In our study, we also demonstrate that high cytoplasmic expression of MAGE-A9 in LSCC is correlated with histopathological grade.

To date, studies investigating the prognostic value of MAGE-A9 are rare; therefore, we investigated the correlation between MAGE-A9 expression and overall survival in LSCC patients. Univariate analysis indicated that in addition to cytoplasmic expression of MAGE-A9, lymph node metastasis and histopathological grade were also correlated with LSCC patient survival. Multivariate analysis further demonstrated that cytoplasmic MAGE-A9 expression and lymph node metastasis were also independent factors of poor prognosis in patients with LSCC. These data are in keeping with recent studies showing that high MAGE-A9 expression is independently associated with poor survival in patients with renal cell carcinoma (RCC) [32]. Interestingly, previous studies have reported nuclear MAGE-A9 staining by IHC analysis [17, 18, 28, 29]. In contrast, we did not observe MAGE-A9 expression in the nucleus of LSCC cells, although positive expression in the mesenchyme was observed. In all 123 case of LSCC, 89 cases were witness mesenchyme tissue and 19 of 89 cases showed positive mesenchyme expression of MAGE-A9. These conflicting results may be owing to the differences in the pathological samples or the antibodies used. Although mesenchymal expression of MAGE-A9 was detected in our study, this expression was not significantly associated with pathological attributes in LSCC patients, including patient survival.

In conclusion, this study is the first to evaluate *MAGE-A9* mRNA expression by qPCR and protein expression with TMAs in LSCC. The present findings demonstrate high expression of MAGE-A9 in LSCC tissues, which is associated with a poor prognosis in LSCC patients. MAGE-A9 may represent a valuable prognostic biomarker of LSCC. Further research is necessary to elucidate the mechanisms of action of MAGE-A9 in LSCC.

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Disclosure of conflict of interest

None.

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