MAGE-A genes as predictors of the outcome of laryngeal squamous cell carcinoma

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Abstract. Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignant tumors in the head and neck area. Melanoma-associated antigens A (MAGE-A) are strictly tumor-specific and are expressed in several types of tumors. To date, no studies have reported the potential of *MAGE-A* genes as markers for circulating tumor cells (CTCs) in patients with LSCC. The present study aimed to evaluate the expression and the possible prognostic significance of *MAGE-A* in the peripheral blood of patients with LSCC. In the present study, the expression of *MAGE-A* genes was determined by multiplex semi-nested PCR and restriction endonuclease treatment of the peripheral blood of patients with LSCC. The association between *MAGE-A* gene expression and clinicopathological parameters and prognosis was evaluated. The results demonstrated that the expression of *MAGE-A* was associated with the predictors that indicate poor prognosis. The expression levels of *MAGE-A* and each individual *MAGE-A* gene were also associated with a shorter overall survival time of patients with LSCC. In conclusion, the results of the present study suggested that the expression of *MAGE-A* genes may be a potential prognostic marker for patients with LSCC.

Introduction

Cancer is a major public health problem in China. Laryngeal squamous cell carcinoma (LSCC) is the most common malignant disease of the head and neck area, and is responsible for 99% of primary laryngeal carcinoma worldwide (1). Due to the increasing incidence rate (7 cases/100,000) (2), LSCC has gradually caused extensive concern among researchers and medical professionals for intensified research (3,4) and clinical trials (5-8). Although surgery combined with chemoradiotherapy has significantly improved the survival rate of patients (5-year survival rate is 63%) (2), recurrence and metastasis are still common and the prognosis remains poor, particularly in patients at the advanced stage. Therefore, the discovery of novel specific markers for early diagnosis and prognosis is urgently needed to improve patient survival.

Melanoma-associated antigen (MAGE), which was first discovered by Van der Bruggen (9) and termed MAGE-I, is a group of well-differentiated members of cancer/testicular antigens (CTA) (9). Thus far, ~60 members of MAGE have been discovered and investigated (10). The *MAGE* gene family encodes tumor antigens recognized by autologous cytotoxic T lymphocytes (11,12). Based on the difference in gene expression and genetic structure, the *MAGE* family is categorized into two subfamilies, *MAGE-I* (*MAGE-A*, *MAGE-B and MAGE-C*) and MAGE-II (*MAGE-D*) (13). The most widely studied gene is *MAGE-A*, which is strictly tumor-specific, and includes 12 family members, termed *MAGE-A1-12* (13). *MAGE-A* is expressed in several types of tumors, such as breast and gastric cancer as well as glioma (12,14).

The expression of *MAGE-A* genes in the peripheral blood of patients with LSCC remains unclear. Due to the high similarity in the sequences of *MAGE-A1, -A2, -A3, -A4* and-*A6*, it is difficult to design a unique primer to detect the different genes. In order to investigate the expression of the *MAGE-A* family genes in the peripheral blood of patients with LSCC and its association with prognosis, multiple *MAGE-A* genes in the peripheral blood of 104 patients with LSCC and 30 healthy volunteers were detected by multiple nested reverse transcription (RT)-PCR and restriction endonuclease treatment. The aim of the present study was to explore whether the expression of *MAGE-A*s in the peripheral blood circulating tumor cells (CTCs) may be used as a biomarker for guiding clinical treatment and monitoring prognosis in patients with LSCC.

Materials and methods

Patients and clinical parameters. A total of 104 patients with LSCC were recruited from the Department of Otolaryngology, The Fourth Hospital of Hebei Medical University between June 2011 and June 2012. In addition, 30 healthy volunteers with no history of carcinoma were enrolled in the present

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study during the same period. None of the patients underwent chemotherapy or radiotherapy prior to surgery. Written informed consent was provided by all participants before enrollment. The study protocol was approved by the Medical Ethics Committee of The Fourth Hospital of Hebei Medical University (approval no. 2011KY112).

The clinicopathological data of the patients were retrospectively collected, including age, smoking history, tumor size, clinical stage (8th edition of the American Joint Committee on Cancer) (15), clinical classification, pathological degree and lymph node metastasis.

Blood and tissue sample collection. Fresh blood samples (5 ml) were collected from the patients before surgery, as well as from the volunteers. All blood samples were immediately stored at 4˚C, and RNA extraction was performed on the day of sample collection. Blood samples were processed within 1 to 4 h after collection. Blood samples of healthy volunteers were used as the negative control for the RT-PCR assay.

Normal testicular tissue samples were collected from two patients undergoing castration at the Department of Urinary Surgery, The Fourth Hospital of Hebei Medical University. Written informed consent was provided by the two patients. The samples were stored at -80˚C until subsequent experiments.

RNA extraction and cDNA synthesis. Red blood cell lysis buffer was used to collect the peripheral blood cells. Total RNA was extracted from the peripheral blood cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Ultraviolet spectrophotometry and agarose gel electrophoresis were used to determine the quality of the RNA. The isolated RNA was stored at -80˚C. RNA (2 μg) was used to synthesize the first-strand cDNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) at 42˚C for 1 h.

RT-PCR. cDNA was used for PCR amplification by using Go Taq Green Master Mix (Promega Corporation) with primers for the *MAGE-A9* and *MAGE-A11* genes. RNA integrity was confirmed by performing PCR amplification with the primer for the *GAPDH* gene. Agarose gel electrophoresis (2%) was used to identify the RT-PCR products, and the bands were observed under ultraviolet light. Each sample were measured three times. *GAPDH* was used as the internal reference gene. The primer sequences, product lengths, PCR cycle conditions, initial denaturation and final extension steps are presented in Table I.

Multiplex semi-nested PCR. GoTaq® Green Master mix (Promega Corporation) was used to amplify the cDNA. In the first cycle of PCR, the reaction mixture comprised 5 μl cDNA product of the reverse transcription, 0.5 μl MAGE-F1 (10 μM), 1 μl MAGE-R1 (10 μM), 0.2 μl MAGE-F2 (10 μM), 0.2 μl MAGE-R2 (10 μM), 25 μl 2X PCR Master mix and deionized water added up to 50 μl. In the second cycle of PCR, the reaction mixture comprised 5 μl external PCR product, 0.5 μl MAGE-F3 (10 μM), 1 μl MAGE-R3 (10 μM), 0.2 μl MAGE-F4 (10 μM), 0.2 μl MAGE-R4 (10 μM), 50 μl 2X PCR Master mix and deionized water added up to 100 μl. The primers are presented in Table II. *GAPDH* was used as the internal reference gene. The thermocycling conditions were as follows: (1)95˚C for 5 min; (2)32 cycles of 95˚C for 45 sec, 65˚C for 45 sec and 72˚C for 90 sec and 31 cycles of (2); (3)72˚C for 6 min. PCR product (6 μl) was used for electrophoresis on a 1.5% agarose gel. The amplification results were observed in the gel imaging system (Syngene Inc.).

Restriction endonuclease treatment. The products of multiplex semi-nested PCR were purified using the QIAquick PCR Product Purification kit (Qiagen China Co., Ltd.) according to the manufacturer's instructions. The purified products were digested by restriction endonucleases *Bcl*I, *Sph*I, *Eco*RI, *Eco47*III and *Afl*III, and gene fragments of *MAGE-A1*, *-A2*, *-A3*, *-A4* and *-A6* were obtained, respectively (Table III). The restriction fragment (6 μl) was analyzed by electrophoresis using a 1.5% agarose gel. The bands were observed in the gel imaging system.

Statistical analysis. SPSS v20.0 software (IBM Corp.) was used to analyze the data. The display strip is defined as high expression group and vice versa. χ^2 or Fisher's exact test were used to evaluate the potential association between the expression of all *MAGE-A* genes or single *MAGE-A* genes and patient clinicopathological characteristics. The Kaplan-Meier method was used to estimate the overall survival time of patients with LSCC. The Cox regression model was used for univariate and multivariate analysis of overall survival and prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of MAGE-A genes in the peripheral blood of patients with LSCC and healthy donors. Multiplex semi-nested RT-PCR and RT-PCR were used to detect the expression of *MAGE-A* genes, including *MAGE-A1*, *-A2*, *-A3*, *-A4*, *-A6*, *-A9* and *-A11*, in two normal testicular tissue samples (Fig. 1A). As the *MAGE-A* gene belongs to the *CTA* gene family, it is only expressed in normal testis and other germ cells, but not in other normal tissues (9). Therefore, testicular tissue was used in the present study as a positive control. Subsequently, the expression of *MAGE-A* in the blood samples of 104 patients with LSCC and 30 healthy donors was examined. The representative samples of the control and *MAGE-A* products in the peripheral blood of healthy donors (negative control) and patients with LSCC, as well as normal testicular tissue (positive control) are presented in Fig. 1A. The *MAGE-A* product was observed in the normal testicular tissues and in the blood samples of a number of patients with LSCC, but not in the blood samples from the healthy donors. The representative LSCC blood samples with positive *MAGE-A* gene expression after internal PCR (second PCR cycle) are presented in Fig. 1B.

As presented in Fig. 2, 31 of 104 patients with LSCC (29.8%) exhibited *MAGE-A* gene expression in the peripheral blood. The expression of *MAGE-A9* and *-A11* mRNA was detected by RT-PCR, whereas the expression pattern of other individual *MAGE-A* genes was identified by restriction

Table I. Primer sequences for reverse transcription-PCR.

Gene	Primer sequences (5'→3')	PCR cycle conditions	No. of cycles	PCR product length, bp
MAGE-A9	F: GTCTCTCGAGCAGAGGAGTCCGC R: CTCAGCCACCTTCAATTTCAGT	95˚C 30 sec; 58˚C 30sec, 72˚C 45 sec	35	340
MAGE-A11	F: ATGGAGACTCAGTTCCGAGA R: AAGAACTTTCATCTTGCTGG	95˚C 30 sec, 52˚C 30 sec, 72˚C 45 sec	35	878
GAPDH	F: ACCTGACCTGCCGTCTAGAA R: TCCACCACCCTGTTGCTGTA	95˚C 15 sec, 58˚C 15 sec, 72˚C 20 sec	28	247

MAGE, melanoma-associated antigen; F, forward; R, reverse.

Table II. Primer sequences for multiplex semi-nested PCR.

Gene	Primer sequences (5'→3')	Fragment length, bp
MAGE-As first cycle	F1: ACTGGCCCTGGCTGCAAC R1: GCCCTGACCAGAGTCATCAT	993
	F2: ACTGGCCTTGGCTGCAAC R2: CGAGAGTCATCATG	965
MAGE-As second cycle	F3: ACTGGCCCTGGCTGCAAC R3: AGGCCCTGGGCCTGGTG	914
	F4: ACTGGCCTTGGCTGCAAC R4: AGGCCCTGGGCTTGGTG	893
GAPDH	F: ACCTGACCTGCCGTCTAGAA R: TCCACCACCCTGTTGCTGTA	247

MAGE, melanoma-associated antigen; F, forward; R, reverse.

Table III. Restriction endonuclease mixtures, multi-MAGE-A products and restriction fragments for each tested MAGE-A gene.

Restriction endonuclease	MAGE gene	PCR product length, bp	Fragment length, bp
BclI	A1	893	106,787
SphI	A2	914	21,22,151,720
EcoRI	A3	914	167,747
Eco47III	A4	917	375,542
AflIII	A6	914	22,172,282,438

MAGE, melanoma-associated antigen.

endonuclease treatment. *MAGE-A1* expression was positive in 19 of 104 (18.3%), *MAGE-A2* expression was positive in 21 of 104 (20.2%), *MAGE-A3* expression was positive in 21 of 104 (20.2%), *MAGE-A4* expression was positive in 16 of 104 (15.4%), *MAGE-A6* expression was positive in 12 of 104 (11.5%), MAGE-A9 expression was positive in 27 of 104 (26.0%) and *MAGE-A11* expression was positive in 29 of 104 (27.9%) patients with LSCC. The frequency of individual *MAGE-A* gene expression was in the following order: A11 > A9 > A2 = A3 > A1 > A4 > A6. A total of 18 patients were positive for only one *MAGE-A* gene, 12 patients were positive for two genes, seven patients were positive for three genes, eight patients were positive for four genes, eight patients were positive for five genes and two patients were positive for six genes. The genomic information of *MAGE-A1-12* for all 104 patients with LSCC is presented in Table SI.

Fig. 3 demonstrates the expression of the *MAGE-A* gene products from two patients following restriction endonuclease treatment. A multiple *MAGE-A*s product was observed in the peripheral blood of patients no. 17 and 45. Subsequently, the *MAGE-A* product (second PCR cycle) was digested with *Bcl*I, *Sph*I, *EcoR*I, *Eco47*III and *Afl*III, and the digested products were separated by agarose gel electrophoresis. The individual *MAGE-A* genes were identified by observing the fragment pattern. For patient 17 (Fig. 3A), the fragments of 787 bp was observed after *Bcl*I digestion, 720 bp was observed after

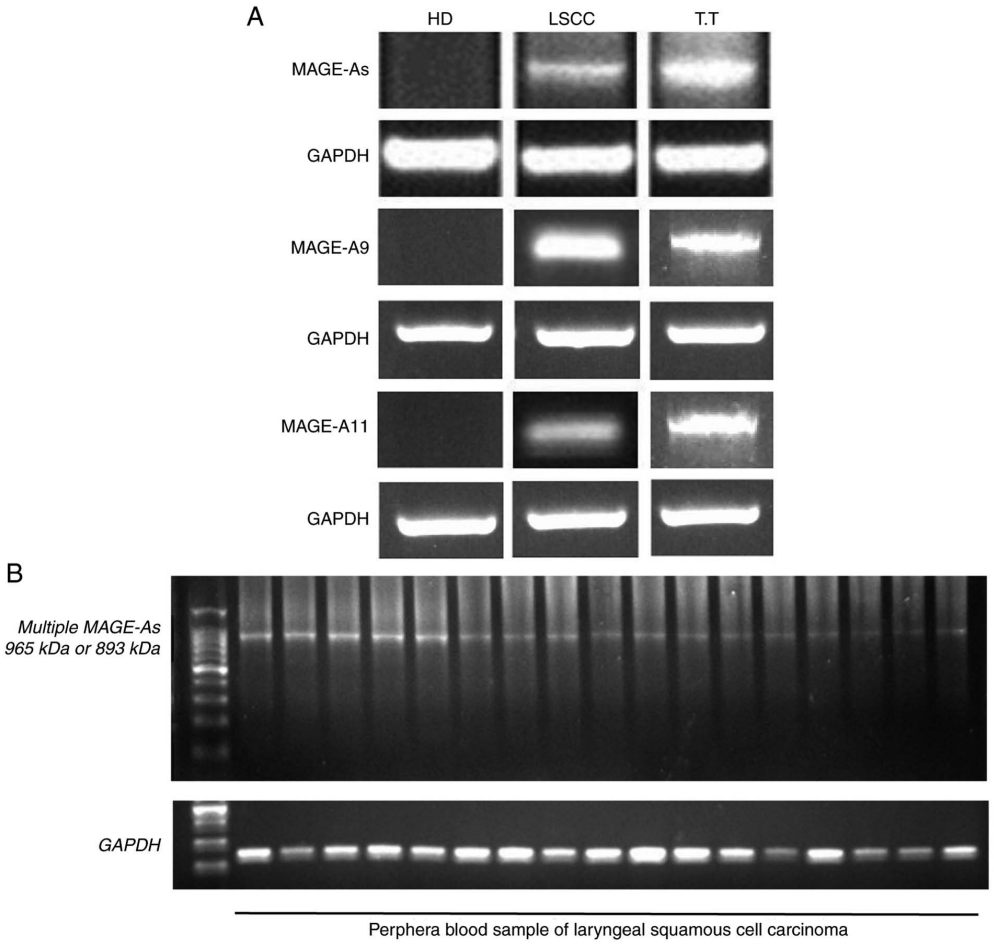

Figure 1. Expression of MAGE-A mRNA measured by reverse transcription-PCR in the peripheral blood of patients with LSCC and healthy volunteers. (A) Representative blots of multiple MAGE-As and GAPDH control products in the blood samples of patients with LSCC and healthy volunteers, as well as in normal testicular tissues. (B) Representative samples with positive MAGE-A gene products of the internal PCR (second PCR cycle) in patients with LSCC. MAGE, melanoma-associated antigen; LSCC, laryngeal squamous cell carcinoma; HD, healthy donor; T.T, normal testicular tissue.

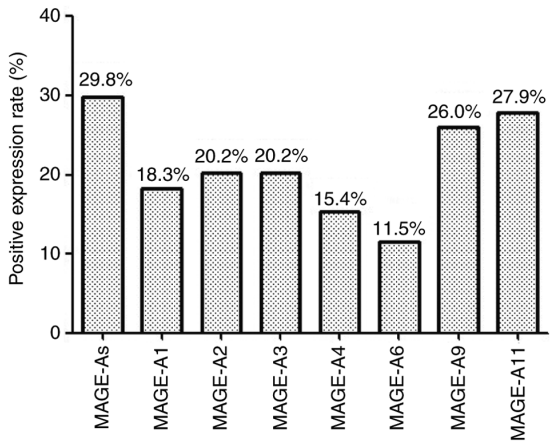

Figure 2. Expression patterns of MAGE-A1, -A2, -A3, -A4, -A6, -A9 and -A11 in the peripheral blood of patients with LSCC. MAGE, melanoma-associated antigen; LSCC, laryngeal squamous cell carcinoma.

*Sph*I digestion, 747 bp was observed after *Eco*RI digestion, and 438 bp was observed after *Afl*III digestion, indicating the presence of *MAGE-A1, -A2, -A3* and *-A6* in the PCR product. The expression pattern of *MAGE-A*s in patient 45 is presented in Fig. 3B; the expression of *MAGE-A1* and *-A6* was not observed, but the 720 bp band was observed after *Sph*I digestion, and the fragment of 747 bp was observed after *Eco*RI digestion, indicating the presence of *MAGE-A2* and *-A3* in the PCR product.

Association between MAGE-A gene expression in the peripheral blood and the clinicopathological characteristics of patients with LSCC. The association between *MAGE-A* gene expression in the peripheral blood and the clinicopathological characteristics of patients with LSCC was evaluated (Table IV). The expression of *MAGE-A* genes (*MAGE-A*s, *-A1*, *-A2*, *-A3*, *-A4*, *-A6*, *-A9* and *-A11*) were not associated with age, smoking history, tumor size and location, but was positively associated with lymph node metastasis (P=0.001, P=0.022, P<0.001, P=0.001, P=0.016, P<0.001, P=0.001 and P=0.016, respectively). High expression levels of *MAGE-A*s in LSCC were associated with the histological degree (P=0.007). Among individual *MAGE-A*s, positive *MAGE-A1*, *-A3*, *-A4* and *-A6* expression was more frequent in patients with histological grade G3 compared with those with histological grades G1/G2 (P=0.005, P=0.013, P=0.001 and P=0.001, respectively). In addition, more frequent positive expression of *MAGE-A3*, *-A6*, *-A9* and *-A11* was observed in patients

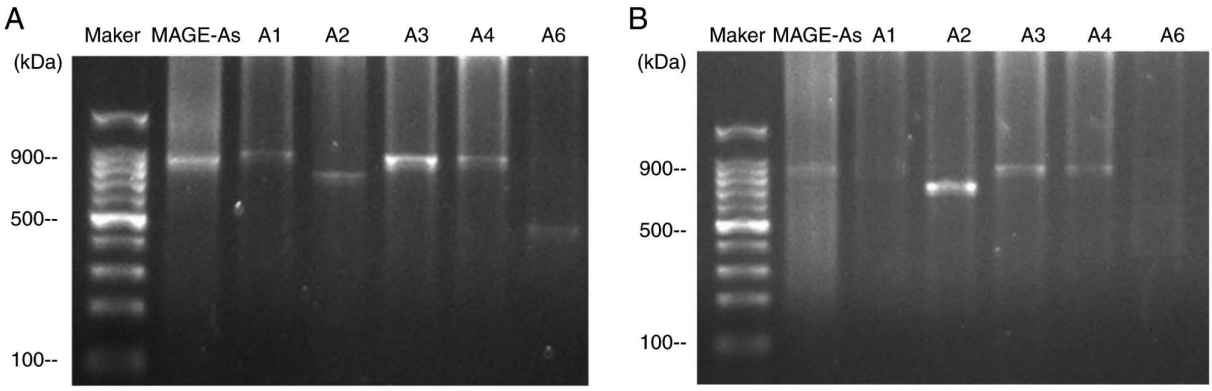

Figure 3. MAGE-A1, -A2, -A3, -A4 and-A6 expression in the peripheral blood of patients with LSCC no. (A) 17 and (B) 45. MAGE, melanoma-associated antigen; LSCC, laryngeal squamous cell carcinoma.

with high clinical stage compared with that in patients with low clinical stage (P=0.044, P=0.048, P=0.01 and P=0.045, respectively). No associations were observed between each individual *MAGE-A* genes expression and other clinicopathological factors.

Association between MAGE-A gene expression in the peripheral blood and the overall survival of patients with LSCC. All 104 patients with LSCC were followed up for 18-65 months. Kaplan-Meier analysis was performed to determine the association between *MAGE-A* gene (*MAGE-A1*, *-A2*, *-A3*, *-A4*, *-A6*, *-A9* and *-A11*) expression levels and the overall survival of patients with LSCC. Overall survival of patients with high *MAGE-A* gene expression in the peripheral blood was significantly lower compared with those with low expression (P=0.020, P=0.061, P=0.010, P=0.036, P=0.039, P<0.001, P<0.001 and P<0.001, respectively; Fig. 4). To further evaluate the prognostic significance of *MAGE-A* expression, univariate analysis of clinicopathological factors and overall survival was performed. High expression of *MAGE-A*s (P=0.024), *-A2* (P=0.014), *-A3* (P=0.041), *-A4* (P=0.046), *-A6* (P<0.001), *-A9* (P<0.001) and *-A11* (P<0.001), as well as lymph node metastasis (P<0.01), low clinical stage (P=0.013) and high histological grade (P=0.028) were demonstrated to be predictors of poor overall survival (Table V). clinical stage, which includes tumor size and lymph node metastasis, and *MAGE-A*s, which includes *MAGE-A1*, *-A2*, *-A3*, *-A4* and *-A6*, were not considered as independent prognostic factors. Lymph node metastasis, histological grade and the expression of *MAGE-A2*, *-A3*, *-A4*, *-A6*, *-A9* and *-A11* were further analyzed by multivariate Cox regression analysis. As demonstrated in Table V, high expression of *MAGE-A11* (P=0.032) and lymph node metastasis (P<0.01) were determined to be independent prognostic factors for poor prognosis of patients with LSCC.

Discussion

Clinical imaging is often the first to identify the tumor and is important for the diagnosis of recurrence and metastasis of the auxiliary technology (2). Failure to identify the primary or metastatic tumors by imaging means that the best time for diagnosis and treatment is missed, and late metastasis of the tumor has no effective treatment and the prognosis is poor (2). The process by which tumor cells invade the body remains unclear and may involve the active invasion of cells via epithelial-to-mesenchymal transition (16) and passively shed individual cells or clusters of tumor cells from impaired tumor blood vessels (17,18). The presence of CTCs in the peripheral blood is considered to be the key factor of tumor metastasis (19). A number of studies have evaluated the clinical application value of CTCs for metastatic breast (20,21), prostate (22), colon (23) and lung (24,25) cancer. Using the FDA-approved Cell Search system (Veridex), the peripheral blood CTC count test and analysis of the prognosis of patients suggest that CTCs can be used as an independent prognostic indicator (26,27). The technology detects the biomarkers on the cell surface to identify the CTCs (26,27). The downregulation or absence of epithelial markers should have an effect on detecting CTCs (19). Thus, the discovery of new tumor markers may help identify CTCs in the peripheral blood of patients with malignant tumors.

RT-PCR, which has a wide range of applications, is considered the most sensitive method for detecting CTCs in the peripheral blood (28). However, the following issues may exist: i) Large amounts of water in the peripheral blood may dilute the normal mRNA and cause false negative results (29); ii) contamination of the target gene in the peripheral blood, leading to DNA amplification, may result in false positive results (30), and iii) low levels of abnormal transcription and amplification of target genes in the peripheral blood tumor cells may also cause false-positive results (30). Improving the detection technology, designing more suitable primers and selecting accurate tumor markers may help avoid the aforementioned issues. Previous studies have used RT-nested PCR to detect the mRNA expression of CTCs in the peripheral blood of non-cancerous and colorectal cancer cells, and demonstrated high sensitivity and specificity (31,32). It has been demonstrated that the expression of *MAGE-A* genes can be used as a biomarker of CTCs in colorectal, breast and gastric cancer (33). The results of the previous study demonstrated that the MAGE-A antigen is a tumor-associated antigen of LSCC (34). Therefore, *MAGE-A* mRNA may be used to detect CTCs in the peripheral blood of patients with LSCC as a specific tumor marker, which may guide the diagnosis and prognosis of LSCC. In the present study, fresh blood was used for further analysis of *MAGE-A* gene expression;

Table IV. Clinicopathological characteristics and MAGE-A gene expression in the peripheral blood from 104 patients with laryngeal squamous cell carcinoma.

Variable	N	MAGE-As, n (%)	P	MAGE-A1, n (%)	P	MAGE-A2, n (%)	P	MAGE-A3, n (%)	P	MAGE-A4, n (%)	P	MAGE-A6, n (%)	P	MAGE-A9, n (%)	P	MAGE-A11, n (%)	P
Age, years																	
<60	45	12 (26.7)	0.541	6 (13.3)	0.255	6 (13.3)	0.128	9 (20.0)	0.966	6 (13.3)	0.613	4 (8.9)	0.460	9 (20.0)	0.226	9 (20.0)	0.117
≥60	59	19 (32.2)		13 (22.0)		15 (25.4)		12 (20.3)		10 (16.9)		8 (13.6)		18 (30.5)		20 (33.9)	
Tumor location																	
Glottic	65	17 (26.2)	0.293	12 (18.5)	0.948	10 (15.4)	0.115	10 (15.4)	0.115	11 (16.9)	0.575	8 (12.3)	0.751	13 (20.0)	0.073	18 (27.7)	0.955
Supraglottic	39	14 (35.9)		7 (17.9)		11 (28.2)		11 (28.2)		5 (12.8)		4 (10.3)		14 (35.9)		11 (28.2)	
Smoking index																	
<400	41	9 (22.0)	0.158	5 (12.2)	0.196	6 (14.6)	0.255	5 (12.2)	0.101	3 (7.3)	0.066	5 (12.2)	0.866	7 (17.1)	0.095	8 (19.5)	0.125
≥400	63	22 (34.9)		14 (22.2)		15 (23.8)		16 (25.4)		13 (20.6)		7 (11.1)		20 (31.7)		21 (33.3)	
Tumor size, cm																	
<2	43	13 (30.2)	0.937	7 (16.3)	0.659	7 (16.3)	0.404	10 (23.3)	0.513	9 (20.9)	0.188	4 (9.3)	0.549	8 (18.6)	0.151	10 (23.3)	0.377
≥2	61	18 (29.5)		12 (19.7)		14 (23.0)		11 (18.0)		7 (11.5)		8 (13.1)		19 (31.1)		19 (31.1)	
Lymph node metastasis																	
N	32	17 (53.1)	0.001	10 (31.3)	0.022	14 (43.8)	<0.001	13 (40.6)	0.001	9 (28.1)	0.016	9 (28.1)	<0.001	15 (46.9)	0.001	14 (43.8)	0.016
N0	72	14 (43.8)[a]		9 (12.5)[a]		7 (9.7)[a]		8 (11.1)[a]		7 (9.7)[a]		3 (4.2)[a]		12 (16.7)[a]		15 (20.8)[a]	
Histological grade																	
G1/G2	91	23 (25.3)	0.007	13 (14.3)	0.005	17 (18.7)	0.292	15 (16.5)	0.013	10 (11.0)	0.001	7 (7.7)	0.001	22 (24.2)	0.315	25 (27.5)	0.752
G3	13	8 (61.5)[a]		6 (46.2)[a]		4 (30.8)		6 (46.2)[a]		6 (46.2)[a]		5 (38.5)[a]		5 (38.5)		4 (30.8)	
Clinical stage																	
I/II	45	10 (22.2)	0.140	7 (15.6)	0.532	7 (15.6)	0.304	5 (11.1)	0.044	5 (11.1)	0.291	2 (4.4)	0.048	6 (13.3)	0.010	8 (17.8)	0.045
III/IV	59	21 (35.6)		12 (20.3)		14 (23.7)		16 (27.1)[a]		11 (18.6)		10 (16.9)[a]		21 (35.6)[a]		21 (35.6)[a]	

[a]P<0.05. MAGE, melanoma-associated antigen; N, patients with lymph node metastasis.

Table V. Univariate and multivariate analyses of prognostic factors for overall survival of patients with laryngeal squamous cell carcinoma.

	Univariate analysis			Multivariate analysis		
Variables	HR	P-value	95% CI	HR	P-value	95% CI
Expression of MAGE-As, high vs. low	2.048	0.024[a]	1.099-3.816			
Expression of MAGE-A1, high vs. low	1.948	0.068	0.951-3.990			
Expression of MAGE-A2, high vs. low	2.297	0.014[a]	1.187-4.443	2.137	0.050	1.001-4.561
Expression of MAGE-A3, high vs. low	2.016	0.041[a]	1.028-3.954	1.207	0.731	0.412-3.540
Expression of MAGE-A4, high vs. low	2.131	0.046[a]	1.013-4.482	2.339	0.174	0.686-7.970
Expression of MAGE-A6, high vs. low	4.050	<0.001[a]	1.958-8.376	1.410	0.480	0.543-3.665
Expression of MAGE-A9, high vs. low	3.204	<0.001[a]	1.722-5.961	2.082	0.065	0.954-4.543
Expression of MAGE-A11, high vs. low	3.019	<0.001[a]	1.636-5.573	2.438	0.032[a]	1.080-5.504
Age, years, <60 vs. ≥60	1.369	0.327	0.730-2.566			
Tumor location, supraglottic vs. glottic	1.839	0.053	0.992-3.409			
Smoking index, <400 vs. ≥400	1.335	0.381	0.700-2.547			
Tumor size, cm, <2 vs. ≥2	1.924	0.057	0.981-3.777			
Histological grade, G1/G2 vs. G3	2.013	0.028[a]	1.078-3.760	1.290	0.544	0.568-2.931
Clinical stage (AJCC), I/II vs. III/IV	2.345	0.013[a]	1.194-4.604			
Metastatic state of lymph node, N vs. N0	10.788	<0.001[a]	5.036-23.110	21.112	<0.001[a]	7.927-56.226

[a]P<0.05. MAGE, melanoma-associated antigen; HR, hazard ratio; CI, confidence interval; N, patients with lymph node metastasis; AJCC, American Joint Committee on Cancer.

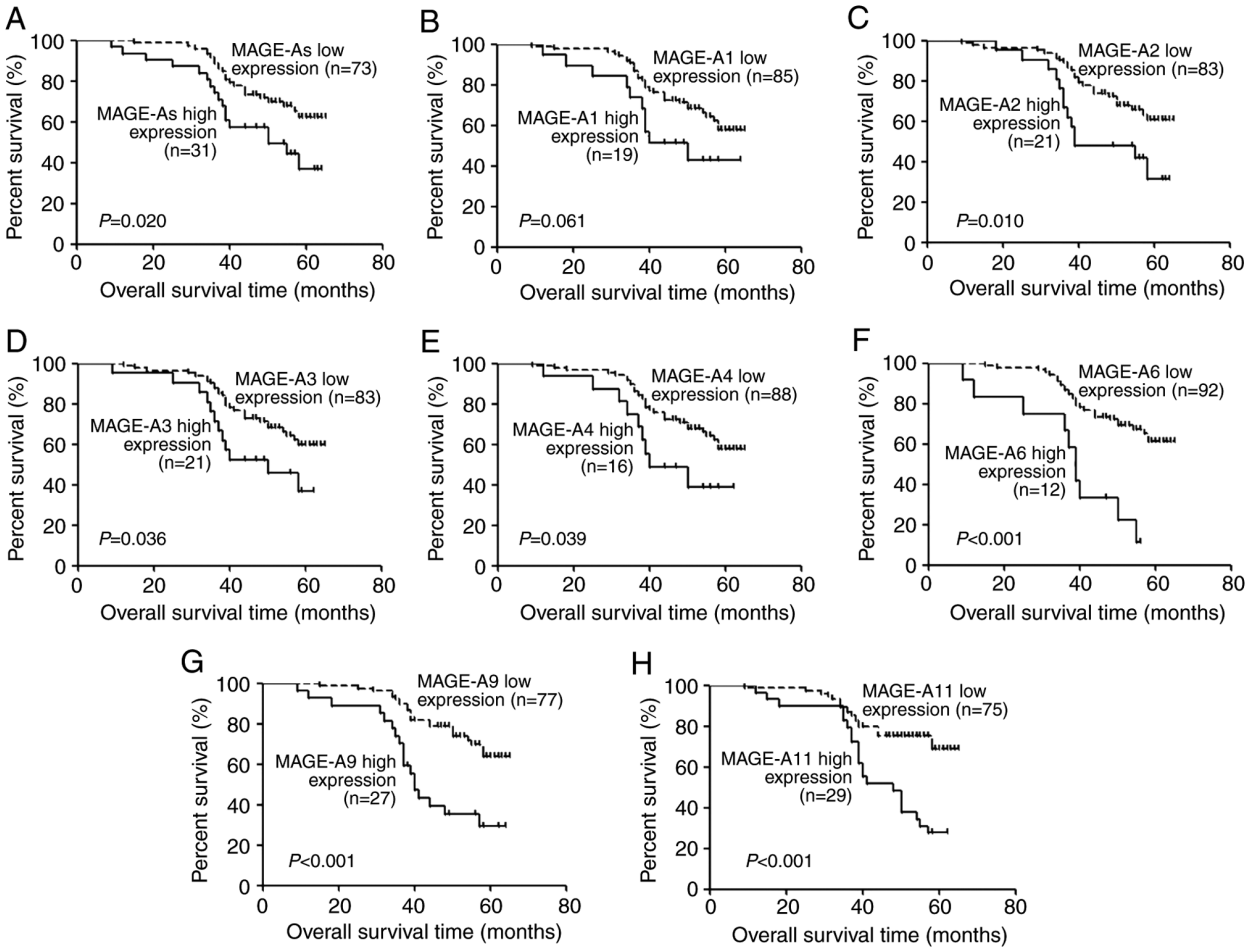

Figure 4. Kaplan-Meier curve analysis of the associations between (A) MAGE-As, as well as (B-H) each MAGE-A member and the 5-year overall survival rate of patients with laryngeal squamous cell carcinoma. MAGE, melanoma-associated antigen.

fresh blood is easy to collect and observe, allowing for timely guidance on clinical diagnosis and treatment.

Due to the high homology, it is difficult to design specific primers for a single *MAGE-A* gene. Therefore, a pair of primer sequences containing two forward primers and two reverse primers were designed in the present study for the amplification of a mixture of *MAGE-A1*, *-A2*, *-A3*, *-A4* and *-A6* genes by multi-RT-nested PCR. The expression of *MAGE-A9* and *-A11* mRNA was detected by RT-PCR. MAGE-A mRNA was expressed in different degrees in peripheral blood of patients with LSCC, while *MAGE-A* expression was not detected in the healthy donors. These are consistent with previous studies (35,36). A total of 18 patients were positive for only one *MAGE-A* gene, 12 patients were positive for two genes, seven patients were positive for three genes, eight patients were positive for four genes, eight patients were positive for five genes, and two patients were positive for six genes. Since the MAGE-A gene only exists in tumor cells (9), if MAGE-A gene expression is detected in peripheral blood, they must be expressed in tumor cells. In view of the definition of CTC, the results of the present study indicated that MAGE-A is expressed in CTCs. The results of the present study suggested that *MAGE-A* mRNA was only expressed in tumor cells and may be detected only in the presence of tumor cells in the peripheral blood of patients with LSCC. Therefore, the expression of *MAGE-A* genes may be used as a specific marker for detecting CTCs in the peripheral blood of patients with LSCC.

The association between *MAGE-A* gene expression in the peripheral blood and the clinicopathological features of patients with LSCC was statistically analyzed in the present study. The expression of *MAGE-A1*, *-A3*, *-A4* and *-A6* were more frequently detected in patients with histological grade G3 tumors compared with patients with tumor grades G1/G2. A previous study has demonstrated that histological grade and tumor prognosis are significantly associated in breast cancer (37). Therefore, the expression of *MAGE-A1*, *-A3*, *-A4* and *-A6* genes may be an important indicator of the prognosis of LSCC. In the present study, the expression of *MAGE-A3*, *-A6*, *-A9* and *-A11* was observed in patients with a high clinical stage more frequently compared with that in patients with a low clinical stage. Patients with late clinical staging usually have a poor prognosis (37). In addition, the expression of *MAGE-A*s in the peripheral blood of patients with LSCC was positively associated with the lymph node metastasis status in the present study. For each individual *MAGE-A* gene, including *MAGE-A1*, *-A2*, *-A3*, *-A4*, *-A6*, *-A9* and *-A11*, the expression frequency in patients with lymph node metastasis was significantly higher compared with that in patients without lymph node metastasis. Previous studies have demonstrated that CTCs are commonly present in advanced metastatic malignancies, with more CTCs in the peripheral blood of distantly metastatic breast cancer compared with early-stage breast cancer (38), and similar observations in ovarian cancer (39). In the present univariate analysis, individual *MAGE-A* expression, metastatic state of the lymph nodes, clinical stage and histological grade qualified to enter the regression model; however, as clinical stage includes the metastatic state of the lymph nodes and distant metastasis, only the metastatic state of the lymph nodes was entered into the regression model. The results demonstrated that multiple *MAGE-A*s expression, the metastatic state of the lymph nodes and distant metastasis were risk factors for the 5-year survival of patients with LSCC.

To date, serological hallmark and high-resolution imaging technology still cannot identify micrometastasis and reflect the efficacy of treatments. CTCs detected in the peripheral blood suggest the possibility of early occult micrometastasis (26,27). CTCs cannot only be used to study the biological characteristics of malignant tumors, but they overcome the disadvantage of traditional tissue biopsy and allow real-time dynamic monitoring of the changes in the tumor (40). Previous studies have demonstrated that the changes in the number of CTCs can reflect the efficacy of treatments and provide the basis for individual treatment. Qiao *et al* (41), reported the quantitative variation of CTCs in a patient with ESCC before and after surgery and during a 5-year follow-up period; the results demonstrated that the number of CTCs before and the initial period after surgery remained high. By contrast, following combined treatment, the number of CTCs deceased, and after 117 weeks, the number gradually stabilized at a low level (41). Thus, a change in the number of peripheral blood CTCs may be used to monitor disease status and treatment efficacy. Another previous study reported that clinically acquired drug resistance did not become resistant at the cellular level during the course of treatment, but clinically acquired drug resistance was the selective reaction of heterogeneous cancer cells to target cells (42). Through the dynamic supervision of certain molecular indicators of CTCs, the endpoint for therapy can be determined (43). CTCs in the peripheral blood of patients with LSCC can be examined to dynamically monitor the changes and development of the tumor in order to achieve an improved understanding of personalized therapy. CTCs in the peripheral blood of patients with LSCC may be monitored by detecting the *MAGE-A* genes to guide clinical treatment and the judgement of prognosis.

In conclusion, the results of the present study identified the expression patterns of multiple *MAGE-A* genes in the peripheral blood of patients with LSCC. *MAGE-A* gene expression in the peripheral blood may therefore be used as a molecular marker for guiding the treatment and monitoring the prognosis of patients with LSCC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BS and MS contributed to the experimental design and fund-raising. YZ and YX performed some of the expriments. RZ and LG assisted in the data analysis. SL analyzed the data and drafted the initial manuscript.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of The Fourth Hospital of Hebei Medical University (Shijiazhuang, China; approval no. 2011KY112). Written informed consent was provided by all participants before enrollment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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