Investigation into the expression levels of MAGEA6 in esophageal squamous cell carcinoma and esophageal adenocarcinoma tissues

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Abstract. Esophageal carcinoma (EC) is the sixth most deadly of all cancers. It is among the most malignant cancers due to its highly aggressive nature and low survival rate. The incidence of EC is high in Asia, particularly in Southern areas including China, Iran and Japan. There is a large body of evidence to suggest an association between the melanoma antigen gene (*MAGE*) family and the initiation of cancer; however, there is no clear evidence to suggest an association between EC and *MAGE*. Discovery of the chemical and physiological processes relevant to the occurrence of EC is vital for clinicians to diagnose and treat this highly aggressive cancer. The present study focused on the association of EC with the expression of MAGE family member A6 (MAGEA6) at the mRNA and protein levels using gene chip, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry. The expression of *MAGEA6* in human esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) tissue samples were compared with those in paracancerous tissue. The result of the gene chip assay revealed that as the generation grew, there was a significant increase in *MAGEA6* transcription in the esophageal epithelial cell line, SHEE Different ESC cell lines also exhibited a significantly higher transcription of *MAGEA6* compared with the HaCaT cell line, as determined via reverse transcription-quantitative PCR. An higher positive rate of *MAGEA6* expression in ESCC and EAC tissues was also revealed when compared with paracancerous tissues, as determined via immunohistochemistry. The results indicated that *MAGEA6* is highly transcribed and expressed in the development of EC and may therefore serve as a novel biomarker for the diagnosis or treatment of EC.

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Introduction

Esophageal carcinoma (EC) has the sixth highest lethality rate among cancers and is the eighth most common cancer worldwide (1). Developing countries in Asia, including China, Turkey and Iran, have the highest incidence rates of EC, where patients are mostly diagnosed with EC in the middle and late stages of the disease, with 15-25% survival after 5 years (2,3). However, early diagnosis, via highly developed endoscopic techniques, may increase survival rates by enabling the use of early treatment regimens and mitigation techniques (3,4).

The root cause of EC remains unclear; however, environment, sex, race and personal characteristics, including alcohol intake, smoking, viruses and genetic factors have all been linked to its initiation and progression (3). A number of susceptibility genes, such as *GSTT1* and *CYP1A1* (5,6), suppressor genes including *p53, p16, APC, Rb* and *EPB41L 3* (7,8), and oncogenes such as cyclin D1 and *EGFR* (9), have been shown to serve a role in the development of EC, and have provided significant data regarding the early diagnosis of EC.

Melanoma antigen gene (*MAGE*) is a family of tumor-associated antigens that was discovered in malignant tissues by van der Bruggen *et al* (10) through gene cloning experiments. Research has shown that the *MAGE* family A (*MAGEA*), which consists of a group of 12 genes named *MAGEA (1-12)*, is located in humans at the chromosomal location of ChrXq28 (11,12). Expression of MAGE1 has a close association with tumorigenesis, and certain members of the family have been studied as diagnostic markers for various types of carcinoma (12). Previous studies have also shown that the occurrence of EC is a consequence of environmental factors along with human papillomavirus (HPV) infection (13-15). The MAGEA family is highly expressed in different cancer tissues, particularly in ESCC tissues; various cancers in the lung, head and neck, esophagus, bladder, stomach, colorectal, breast, liver and ovary; as well as lymphocytic leukemia (12,16).

Research into the metastasis of esophageal epithelial cells has indicated that *MAGEA6* may be important in the occurrence of EC (12). However, the specific association between *MAGEA6* and the incidence of EC, as well as its mechanism remain unclear. Therefore, the present study focused on clarifying the biological association between the expression of MAGEA6 throughout different stages of the expression process (mRNA transcription and protein expression) and the occurrence of EC. Furthermore, a detailed analysis of clinically derived EC clinical samples and EC cell lines was conducted.

In the present study, *MAGEA6* was detected in clinical EC samples and EC cell lines. Gene chip and reverse transcription-quantitative (RT-q) PCR was designed to reveal the transcription levels of *MAGEA6* in different generations of human immortal esophageal epithelial cell lines and in different ESC cell lines, the results of which may indicate whether the transcription of *MAGEA6* is higher in EC cells than common human cell lines and if this changes during the development of EC. Immunohistochemistry detection was also designed to indicate the positive rate of MAGEA6 in EC tissue when compared with paracancerous tissue. The results of these experiments should reveal whether there is close association between EC and *MAGEA6*, and if *MAGEA6* could be treated as a novel biomarker of EC.

Materials and methods

Malignant transformation of human esophageal epithelial cells detected via gene chip analysis. The present study was conducted with approval from the Ethics Committee of North China University of Science and Technology (Tangshan, China). The SHEE immortal esophageal epithelial cell line was obtained by HPV18E6E7 inducement at the Medical College of Shantou University (Shantou, China). A human embryonic esophageal epithelial cell line, obtained from the Medical College of Shantou University, was firstly infected with HPV18 E6E7-AVV and then treated with tumor-promoting factor (12-O-Tetradecanoylphorbol 13-Acetate, TPA) to induce transformation into malignant SHEE cells (17,18). This immortal esophageal epithelial cell line retains the characteristics of monolayer growth, contact inhibition and squamous epithelium origin, and when continually cultivated, readily undergoes malignant transformation (19). The normal esophageal epithelial cells were transformed into esophageal squamous epithelial cells after 55 generations. Changes of ~47,000 transcriptomes transcription within the cells were detected using gene chips. This involved detection of total RNA using the gene chips (GeneChip Human Genome U133 Plus 2.0; Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA) via their reverse transcribed cDNA (20). Finally, the transcription levels of MAGE family genes, including *MAGEA6*, were determined by comparing the Cq values detected by the gene chips in the 24, 48, 60 and 76th generations of the malignant-transformed esophageal epithelial cells.

Detection of MAGEA6 transcription using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) Preparation of cDNA. Four ESCC cell lines induced from esophageal epithelial cells, namely EC109, EC9706, CEC-3 and KYSE150, were used in the present study. The EC109 and EC9706 cell lines were obtained from the Chinese Academy of Medical Sciences, Peking Union Medical College (Beijing, China). The KYSE150 cell line was obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The Chinese Esophageal Cancer-3 (CEC-3) cell line, constructed by Professor Zeng Yi, was obtained from the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing, China). HaCaT cells, also obtained from Professor Yi Zeng, were used as a control. The EC109, EC9706, CEC-3, KYSE150 and HaCaT cells were lysed using TRIzol (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was extracted from the cells following lysis, dissolved in diethylpyrocarbonate water and then authenticated and measured through agarose gel electrophoresis to ensure the integrity of the RNA. The DNA that was mixed with the RNA during lysis was removed by the addition of RQ1 DNase. The cDNA of all the cell lines was then synthesized using the Reverse Transcription System MK (cat. no. A3500; Promega Corporation, Madison, WI, USA). The temperature protocol was as follows: 42˚C for 1 h, 95˚C for 5 min and 4˚C for 10 min.

RT-qPCR. SYBR-Green reagent (cat. no. 4472908; Applied Biosystems, Thermo Fisher Scientific, Inc.) was used for qPCR analysis [an 8 μl system containing 4 μl Fast MIX (a ready-to-use cocktail, containing antibody-mediated fast hot start Taq DNA Polymerase, SYBR-Green I fluorescent dye, $MgCl_2$, dNTP Mix and stabilizers), 0.25 μl of forward and reverse primers, 1.5 μl Nuclease-Free Water and 2 μl cDNA (synthesized as aforementioned)] (Table I). The thermocycling conditions were as follows: 40 cycles of denaturation at 95˚C for 10 min, annealing at 60˚C for 1 min and elongation at 72˚C for 1 min.

Human *GADPH* and some genes of the *MAGE* family were screened using RT-qPCR according to the results of the gene chip analysis. The primer sequences used in RT-qPCR (Table II) were designed using Beacon Designer 7.92 (Premier Biosoft International, Palto Alto, CA, USA). Optimally ranked primers were selected, and were synthesized by AuGCT Co., Ltd *GADPH* served as the internal control. Upon completion of the amplification, the obtained Cq values were used in the following formulae for analysis: $\Delta Cq=Cq$ (target gene)-Cq (internal control); $\Delta\Delta Cq=\Delta Cq$ (experimental group)-ΔCq (control group); relative expression of the target gene to the

Table I. Reverse transcription-quantitative polymerase chain reaction system.

Component	Volume (μl)
Fast mix	4
F Primer	0.25
R Primer	0.25
Nuclease-free water	1.5
cDNA	2
Total	8

control=$2^{-\Delta\Delta Cq}$ (21). *GAPDH* was regarded as having an expression level of 1 and target gene expression was calculated relative to that of *GAPDH*.

Detection of MAGEA expression using immunohistochemistry (IHC). Fresh surgically resected tissue slices from 107 patients (average age, 56 years; age range, 41-77 years; 58 male and 49 female, only tissues from 98 patients were successfully produced into slice samples for IHC detection) were used. The samples included 51 slices of ESCC tissue, 47 slices of EAC tissue and 98 paracancerous tissue slices as the control group, every paracancerous tissues samples was took around the EC tissues we used. The samples were obtained from January 2015 to December 2016 after each specimen donor provided signed consent. All specimen donors were pathologically diagnosed with EC and treated at the Pathology Department of Tangshan People's hospital in Tangshan, Hebei Province. The ESCC and EAC tissue slices (2 μm thick), together with the paracancerous tissue, slices were fixed with 4% paraformaldehyde for 24 h at 25˚C, then sealed with paraffin wax for further use. Sealed slices were subsequently incubated at 56˚C overnight prior to paraffin wax washing with 100% xylene twice for 15 min. Subsequent rinsing with 100, 95 and 70% ethanol for 5 min respectively was conducted to clean the slices. The cleaned slices were boiled in a pressure cooker with 0.01 M citrate buffer solution 5 times (2 min each) to activate and retrieve the antigens on the surface, and then cooled to 25˚C. Afterwards, the slices were immersed in 3% hydrogen peroxide at 25˚C for 20 min to block endogenous peroxidase activity. Rabbit anti-human MAGEA6 polyclonal antibodies (primary antibodies; 1:50; cat. no. 14602-1-AP) from ProteinTech Group, Inc., Chicago, IL, USA were then added and the slices were maintained at 4˚C overnight. Then, the slices were washed with phosphate-buffered saline (PBS) 4 times (4 min each) and primary antibody enhancer [Boster Biological Technology; Ready-to-use SABC-POD (mouse/rabbit IgG) kit; cat. no. SA1020] was added. The slices were incubated with secondary antibodies and stained for 0.5-1 min with the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (DAB; cat. no. K500711). Then samples were treated with hematoxylin for 30 sec at 25˚C. Following thorough dehydration and soaking in xylene, the stained slices were sealed and observed under a microscope.

The data from the IHC assay were then analyzed by two double-blinded people (Dr Minglian Wang and Miss Zhu Jiang).

Cells with positive MAGEA6 protein staining primarily appeared brown-yellow, and the strength of color indicated the extent of protein expression. Cells with no clear staining were considered negative. The total positive rates for MAGEA6 in the ESCC, EAC and paracancerous tissues were first calculated. The total EC positive rate was also calculated. The sensitivity and specificity of MAGEA6 IHC positive staining for ESCC and EAC tissues compared with paracancerous tissues were then analyzed, and the positive and negative predictive values were calculated in order to determine whether the expression of MAGEA6 has the potential to serve as a marker of ESCC and EAC.

Statistical analysis. The differences in *MAGEA6* expression level in the EC cell lines compared with the HaCaT cell line were analyzed with Duncan's multiple range test (α=0.05) using SPSS19 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Malignant transformation in different generations of human esophageal epithelial cells detected by gene chip analysis. Through gene chip analysis, ~47,000 transcriptomes of total RNA were detected in the esophageal epithelial cells and specific transcripts were observed to undergo gradual changes as the number of cell generations increased. Among them, a change in *MAGEA6* transcription was clearly detected. The results demonstrated that the transcription level of *MAGEA6* was markedly increased after the 48th generation from <8.9 to >1,097 in the 60th generation (Fig. 1). Furthermore, *MAGEA1*, *MAGEA3* and *MAGEB2*, which were highly transcribed in different types of carcinoma, exhibited almost no transcription in EC109 and EC9706 cell lines.

Detection of MAGEA6 expression at the transcription level in EC cell lines using RT-qPCR. The expression of *MAGEA6* mRNA was detected using RT-qPCR in four EC cell lines (EC109, EC9706, CEC-3 and KYSE150) and in the human immortalized epidermal HaCaT cell line, using *GAPDH* as an internal control. The amplification of target genes was divided into fluorescent background signal, fluorescent signal exponential amplification and platform stages, and the RT-qPCR melting curve signified the high specificity of the primers for *MAGEA6* and *GADPH*. These results indicated that the RT-qPCR method used in the present study was reliable (data not shown).

Comparison of the transcription of *MAGEA6* and *GAPDH* in the four esophageal cancer cell lines and HaCaT cells using Duncan's multiple range test revealed that the P value between groups was $P<0.001$, indicating that the transcription level of *MAGEA6* (relative to *GADPH*) in the four esophageal cancer cell lines was significantly higher compared with that in the HaCaT cell line (Fig. 2).

Detection of MAGEA6 expression in carcinoma and paracancerous tissues using IHC. In the present study, 196 tissue specimens were analyzed using IHC and the results compared. These specimens included 51 specimens of ESCC tissue, 47 specimens of EAC tissue and 98 specimens of paracancerous tissue.

Table II. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Primer	Direction	Sequence
GAPDH	F	5'-ACCACAGTCCATGCCATCAC-3'
	R	5'-TCCACCACCCTGTTGCTGTA-3'
MAGEA6	F	5'-CGGTCACAAAGGCAGAAAT-3'
	R	5'-AGGCAGGTGGCAAAGATG-3'
MAGEA1	F	5'-CGGCCGAAGGAACCTGACCCAG-3'
	R	5'-GCTGGAACCCTCACTGGGTTGCC-3'
MAGEA3	F	5'-TGGAGGACCAGAGGCCCCC-3'
	R	5'-GGACGATTATCAGGAGGCCTGC-3'
MAGEB2	F	5'-CTGACTTCCGCTTTGGAGGC-3'
	R	5'-GCACCCCCAGAAACAGAAGAGGAACA-3'

MAGE, melanoma antigen gene.
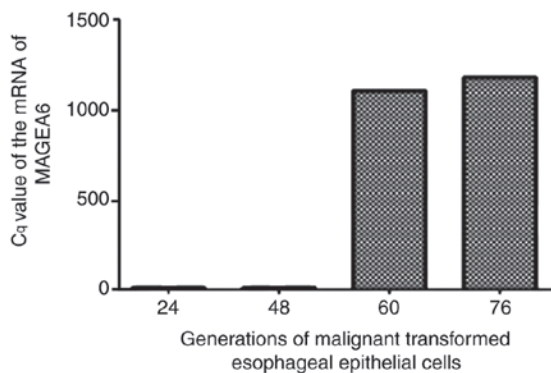
Figure 1. Changes in the transcription level of MAGEA6 gene. Gene expression was analyzed as the generations of a malignantly transformed esophageal epithelial cell line increased, using gene chip analysis. The transcription levels of *MAGEA6* underwent the greatest changes from the 24th to the 76th generation; the Cq value rose markedly from <8.9 in the 40th generation to >1,097 in the 60th generation. MAGE, melanoma antigen gene.

Figure 2. Transcription levels of *MAGEA6* in various cell lines detected by reverse transcription-quantitative polymerase chain reaction. In the EC109, EC9706, KYSE150 and CEC-3 esophageal carcinoma cell lines, the expression of *MAGEA6* mRNA was significantly higher compared with that in the HaCaT cell line. The expression ratios were as follows: EC109, 118.9/1; EC9706, 179.6/1; KYSE150, 688.3/1; CEC-3, 402.1/1. $^{*}P<0.05$; $^{**}P<0.01$. MAGE, melanoma antigen gene.

Analysis of the ESCC tissues and corresponding paracancerous tissues revealed that 46 ESCC tissues were MAGEA6 positive and 5 were MAGEA6 negative; all 51 samples of paracancerous tissues were MAGEA6 negative. Therefore, when analyzing these two types of tissue, the sensitivity was 90.2% (46/51), the specificity was 100% (51/51), the positive predictive value was 100% (46/46) and the negative predictive value was 91.1% (51/56).

Analysis of the EAC tissues and paracancerous tissues revealed that 46 EAC tissue samples were MAGEA6 positive and 1 was MAGEA6 negative; all 47 paracancerous tissue samples were MAGEA6 negative. Therefore, for distinguishing between these two types of tissue, the sensitivity was 97.9% (46/47), the specificity was 100% (47/47), the positive predictive value was 100% (46/46) and the negative predictive value was 97.9% (47/48). Representative images of IHC staining are presented in Figs. 3-7, the positive and negative rates of MAGEA6 in the tissues as determined by IHC are shown in Table III and the sensitivity, specificity, positive and negative predictive values of MAGEA6 expression analysis using IHC are shown in Table IV.
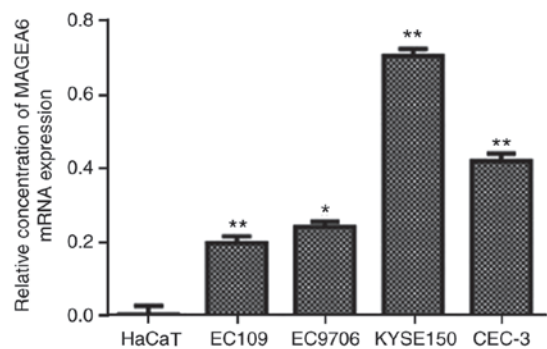
Discussion

The causes of EC are highly complex, and there are limited options for its early diagnosis and treatment (3,4,22). EC is typically diagnosed in the middle and late stages of the disease (22,23), with conventional therapy using radiotherapy, chemotherapy and surgery (24,25). Progress has been made via the detection of a number of susceptibility genes and specific oncogenes (7,9), which provide an important starting point for research into target drug discovery.

The *MAGEA* family has garnered much interest in the research community for its potential as a target for new therapeutics. Genes belonging to the *MAGEA* family have been demonstrated to be specifically expressed in numerous types of tumor cells and tissues (13,26); for example, the *MAGEA3* is highly expressed in pancreatic cancer (27). Therefore, *MAGEA* is a significant target in tumor-targeting therapy (13). Although preliminary research has documented an association between the *MAGEA* family and the occurrence of EC (28,29), the specific relationship remains unclear, with no firm data available to clarify the connection.
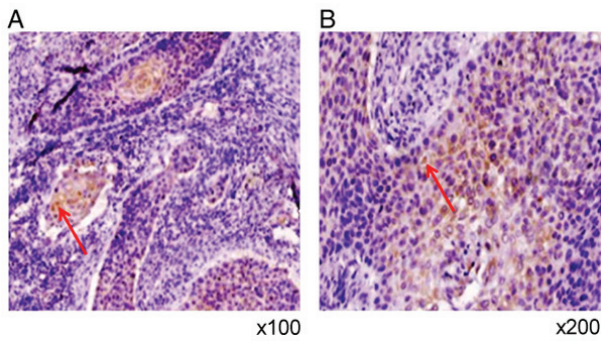
Figure 3. Light microscopy images of the positive immunohistochemical staining of *MAGEA6* in esophageal squamous cell carcinoma. Original magnification (A) x100 and (B) x200. MAGEA6, melanoma antigen gene A6. Red arrows indicate positive staining of MAGEA6 protein (Red-yellow in color).
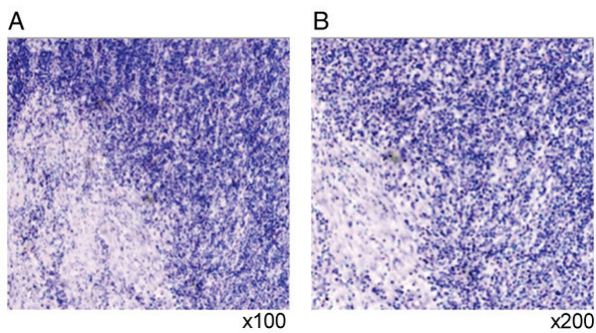

Figure 4. Light microscopy images of the negative immunohistochemical staining of *MAGEA6* in esophageal squamous cell carcinoma. Original magnification (A) x100 and (B) x200. Cells were stained for MAGEA6, but appeared negative. MAGEA6, melanoma antigen gene A6.
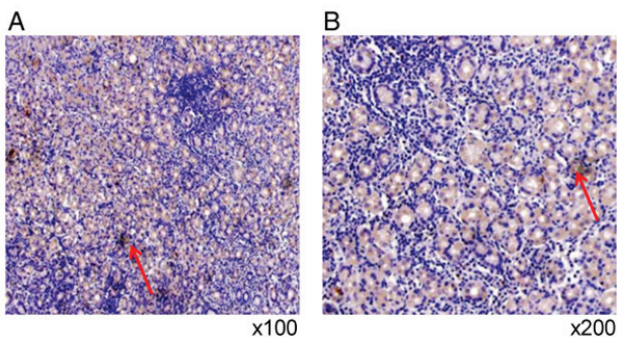

Figure 5. Light microscopy images of the positive immunohistochemical staining of *MAGEA6* in esophageal adenocarcinoma. Original magnification (A) x100 and (B) x200. Red arrows indicate positive staining of MAGEA6 protein (Red-yellow in color). MAGEA6, melanoma antigen gene A6.
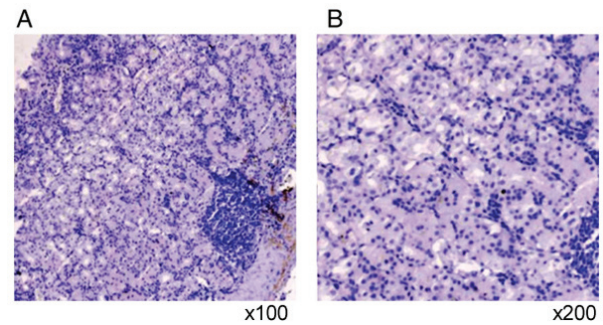

Figure 6. Light microscopy images of the negative immunohistochemical staining of *MAGEA6* in esophageal adenocarcinoma. Original magnification (A) x100 and (B) x200. Cells were stained for MAGEA6, but appeared negative. MAGEA6, melanoma antigen gene A6.
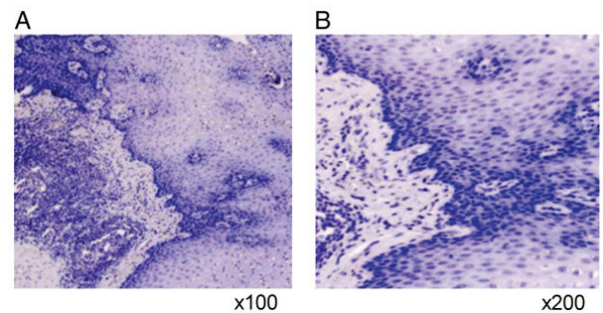

Figure 7. Light microscopy of the negative immunohistochemical staining of *MAGEA6* in paracancerous tissue. Original magnification (A) x100 and (B) x200. Cells were stained for MAGEA6, but appeared negative. MAGEA6, melanoma antigen gene A6.

The current research team has a continued interest in EC, and has previously reported a malignant transformation model for esophageal epithelial cells (17,18). Using this model, changes in gene transcript levels over increasing cell generations were analyzed with a gene chip assay in the present study. The results demonstrated that the transcription level of *MAGEA6* rapidly increased as malignant transformation occurred in the esophageal epithelial cells from the 48th generation. From this, it may be speculated that the transcription of *MAGEA6*

is closely associated with the occurrence of EC. However, since only one set of gene chip detection data was obtained, additional data concerning the transcription of MAGEA6 in different generations of malignantly transformed esophageal epithelial cells are required.

To obtain further evidence of MAGEA6 expression at the mRNA level, RT-qPCR analysis was conducted. The ratio of the transcription level of *MAGEA6* in KYSE150 and HaCaT cells was calculated to be 688.3. This cell line exhibited the highest *MAGEA6* transcription level among all the carcinoma cell lines that were tested. In the EC109, EC9706 and CEC-3 cell lines, the ratios were 118.9, 179.6 and 402.1, respectively, also indicating much higher transcription levels of *MAGEA6* than were observed in the HaCaT cells. This result demonstrated that MAGEA6 was generally expressed among these most prevalent esophageal cell lines, and provided good evidence for the close association between the expression of MAGEA6 and the occurrence of EC.

To further investigate the association between EC and *MAGEA6*, specimens of ESCC and EAC tissues were obtained from patients in one of the aforementioned geographical areas with a high prevalence of EC. The MAGEA6 positive rates of the tissues were detected via IHC and, notably, the positive rate differed significantly between the EC tissues and paracancerous tissues. Paracancerous tissues exhibited no expression of MAGEA6 while the total EC tissues presented a very high positive rate of 93.9%. Positive rates were 90.2 and 97.9%

Table III. Summary of *MAGEA6* immunohistochemical staining results.

Tissues	Positive cases (n)	Negative cases (n)	Positive rate (%)	Total positive rate (%)
Esophageal squamous cell carcinoma	46	5	90.2	93.9[a]
Esophageal adenocarcinoma	46	1	97.9	
Paracancerous tissues	0	98	0	0

[a]Total positive rate for esophageal carcinoma (esophageal squamous cell carcinoma and esophageal adenocarcinoma). MAGEA6, melanoma antigen gene A6.

Table IV. Sensitivity, specificity, positive and negative values of *MAGEA6* expression.

Parameter	Esophageal squamous cell carcinoma vs. paracancerous tissues (%)	Esophageal adenocarcinoma vs. paracancerous tissues (%)
Sensitivity	90.2	97.8
Specificity	100	100
Positive predictive value	100	100
Negative predictive value	91.1	97.9

MAGEA6, melanoma antigen gene A6.

in the ESCC and EAC tissues, respectively. The sensitivity of the MAGEA6 IHC detection for ESCC and EAC tissues compared with paracancerous tissues was 90.2 and 97.9%, respectively. Furthermore, the specificity for ESCC and EAC tissues compared with paracancerous tissues and the positive predictive values were all 100%. The negative predictive values for ESCC and EAC tissues were also high at 91.1 and 97.9%, respectively. From this analysis, it may be concluded that the sensitivity and specificity were sufficiently high to indicate that MAGEA6 expression at the protein level may be treated as an indicator of the occurrence of ESCC and EAC. This is supported by the high positive and negative predictive values. These results indicate that MAGEA6 is highly expressed in malignantly transformed cells and scarcely expressed in para-cancerous tissues.

From the aforementioned results, it may be concluded that the expression of MAGEA6 in EC cells is much higher than that in normal esophageal epidermal cells, and may be easily detected using various methods. These results serve as an important reference to support the use of *MAGEA6* as a new drug target for EC treatment, and the expression level may also be an important prognostic index of EC.

In conclusion, the present study demonstrated via gene chip analysis, that the transcription level *MAGEA6* in embryonic esophageal epithelial cells increases during carcinogenesis. The greatest increase in the transcription level of *MAGEA6* occurred from the 48th to the 60th cell generation, during which the Cq level of transcription increased from <8.9 to >1,097. The markedly higher transcription levels identified in all the studied esophageal cell lines strongly indicate the high general level of MAGEA6 expression from the transcription level. The quantity of the MAGEA6 translation product is also much higher in EC tissue than in paracancerous tissues. Therefore, it is concluded that the expression of MAGEA6 has

a close association with the occurrence of EC, and *MAGEA6* is strongly suggested to be a new oncogene in EC.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JH wrote the manuscript, designed RT-qPCR, performed the RT-qPCR with the help of ZJ, and took the photos of immunohistochemical analysis. YW performed the gene chip and the RT-qPCR, contributed to the writing of the paper. ZJ and RJ performed immunohistochemical analysis, performed the literature search and designed the RT-qPCR procedure. MW performed immunohistochemical analysis, helped operated RT-qPCR and wrote the manuscript. SL obtained

patient tissue, performed immunhistochemical analysis and delivered samples to Beijing Key Laboratory of Environmental and Viral Oncology. JL designed the gene chip experiment and completed gene sequencing with help from MG and YW, who collectively analysed the results of gene chip analysis and provided assistance with immunohistochemistry. MG and JL also helped with the writing of manuscript. RJ performed the literature search and wrote the manuscript. SC performed all the statistical analysis. YZ determined how to detect the transcription and expression of MAGEA6 and performed the literature search. MB helped revised some parts of the manuscript.

Ethics approval and consent to participate

The present study was conducted with approval from the Ethics Committee of North China University of Science and Technology (approval no. 2015119 and 2018216). All participants provided signed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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