

# Analysis of the function of MAGE-A in esophageal carcinoma by bioinformatics

Xiaohua Chen, MD<sup>a,\*</sup>, Sina Cai, MM<sup>b</sup>, Liping Wang, MD<sup>c</sup>, Xiaona Zhang, MD<sup>d</sup>, Wenhui Li, BM<sup>a</sup>, Xiaolong Cao, MM<sup>a</sup>

## Abstract

**Background:** Melanoma-associated antigen-A (MAGE-A) was recognized as high-expressed in many solid tumors including esophageal carcinoma (EC), nevertheless, was reported to be low/not-expressed in normal tissues. Thus, it was considered as an extraordinary appropriate target for treatment especially in immunotherapy. Therefore, it demanded more detail knowledge on the precise function of MAGE-A.

**Methods:** In this study, we used the data from the Cancer Genome Atlas dataset (TCGA-ESCA) to analyze the expression and survival for MAGE A3/4/11 (the subtype of MAGE-A) using the online tool of UALCAN. Furthermore, the high-throughput sequencing data of the patients with esophageal squamous-cell carcinoma (ESCC) from TCGA dataset were performed to analyze the correlation test, gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of MAGE A3/4/9/11 using LinkeDomics (online tool) and ClueGO (inner software of Cytoscape). Finally, relative gene expressions of MAGE A3/4/9/11 were verified by quantitative real-time PCR (q-PCR) in the patients with EC.

**Results:** MAGE A3/4/11 was high-expressed in tissues of patients with ESCC, and there was no difference in survival time for patients between the high-expressed with the low/medium-expressed. The Go enrichment analysis showed that the 4 MAGE-A subtypes (MAGE-A3/4/9/11) were enriched in the regulation of the adaptive immune response, translational initiation, interleukin-4 production, response to type I interferon, and skin development, respectively. The KEGG results showed that they were enriched in T cell receptor signaling pathway (MAGE-A3), Th1 and Th2 differentiation, antigen processing and presentation (MAGE-A4), cytokine-cytokine receptor interaction (MAGE-A9), and chemokine signaling pathway (MAGE-A11).

**Conclusion:** MAGE A3/4/9/11 was high-expressed in EC, and were enrolled in the regulation of immune response. They may consider as candidate immune target for EC treatment and provided the messages for further research in the function of MAGE-A.

**Abbreviations:** EC = esophageal carcinoma, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, MAGE-A = Melanoma-associated antigen-A, q-PCR = quantitative real-time PCR, TCGA = the Cancer Genome Atlas.

**Keywords:** biological information, function, MAGE-A, regulation

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<sup>a</sup>Oncology of Panyu Central Hospital, Panyu Cancer Institute, <sup>b</sup>Oncology of The Hospital of Third Affiliated Southern Medical University, Guangzhou, Guangdong,

<sup>c</sup>The First People's Hospital of Chenzhou, Chenzhou, Hunan, <sup>d</sup>Graceland Medical Center, The Sixth Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong, China.

\* Correspondence: Xiaohua Chen, Department of Oncology, Panyu Central Hospital, Cancer Institute of Panyu, Guangzhou 511400, Guangdong, P.R. China (e-mail: cxh0663@126.com).

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## 1. Introduction

The incidence rate of esophageal cancer (EC) is increasing and is the fourth highest in China. About 90% of histology was squamous-cell carcinoma (SCC).<sup>[1]</sup> As most patients were diagnosed with advanced stage or even with distant metastasis, the 5-year survival rate has remained very low under traditional therapy (including surgery, chemotherapy, radiotherapy). Thus, it demands to search a new therapy for the patients.

Immunotherapy such as chimeric antigen receptor (CAR) T cell therapy (CAR-T) shows great special effect on tumor and has been proved to use in recurrent or refractory acute B cell type lymphoid leukemia (B-ALL) and refractory/recurrent invasive non-Hodgkin lymphoma (NHL) by American Food and Drug Administration (FDA). Nevertheless, the high immune efficiency depends on tumor-specific antigen.<sup>[2-4]</sup> The complete response rate (CR) was up to 82% of patients with B-ALL that depends on the specific antigen of CD19.<sup>[4]</sup> Thus, to find a new specific antigen was pivotal to develop the novel immunotherapy for EC.

MAGE antigen is a kind of protein with a peculiar expression profile and is regarded as cancer/testis antigens (CTAs), which is high-expressed in cancer cells and male germinal cells while low/not expressed in normal cells. As these cells lack the immunological target of HLA-molecules, thus, MAGE antigen

is considered academically as the proper target for immunotherapy. MAGE A is the subtype of MAGE family members. Recent studies reveal that MAGE A was high-expressed in many tumors and had great immunological effect in certain cancers. However, there is no detail regarding the regulatory role of these genes in EC.

In this study, the data of EC from the Cancer Genome Atlas (TCGA) dataset (TCGA-ESCA) were downloaded and the online tool of UALCAN (<http://ualcan.path.uab.edu/index.html>) and LinkeDomics (<http://www.linkedomics.org>) was used to analyze the correlation, survival, Gene ontology, and pathway enrichment, and verified the relative gene expression of MAGE-A3/4/9/11 in the patients with EC. The aim of the study was to obtain further information regarding the function of MAGE-A3/4/9/11 and provide candidate target genes for ESCC immunotherapy.

## 2. Methods

### 2.1. The expression of MAGE A3/4/11 in EC

Data of TCGA-ESCA were used for MAGE A3/4/11 expression and survival analysis. There are 185 patients enrolled in this data profile, including 11 of normal, 89 of adenocarcinoma, and 95 of SCC in patients with EC. The analysis was performed on the online tool of UALCAN<sup>[5]</sup> based on tumor histology.

### 2.2. Correlation, gene ontology, and pathway enrichment analysis

High-throughput sequencing data (HiSeq RNA 01/28/2016) of TCGA-ESCA were used for MAGE A3/4/9/11 correlation analyses and were performed using the online tool LinkeDomics.<sup>[6]</sup> The patients enrolled in these data included 158 males and 27 females, and there were 96 patients with SCC and 89 patients with adenocarcinoma. Tissues from patients were sent for HiSeq RNA detection. The data of the patients with SCC were chosen for analysis. Top 50 correlated genes (positively and negatively regulated) were screened on the basis of the Pearson correlation coefficient (PCC) to make the heat map. The gene enrichment analysis, including biological processes (BPs), cell component (CC), and molecular function (MF), was processed by Gene Set Enrichment Analysis (GSEA) of the online tool LinkeDomics. Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was predicted by ClueGO in Cytoscape3.6.1.<sup>[7,8]</sup>

### 2.3. PCR samples collecting

All samples used for quantitative real-time PCR (q-PCR) were collected from the Panyu Central Hospital and the Third Affiliated Hospital of Southern Medical University from February 8, 2014, to October 16, 2017. The patients enrolled in this study should be diagnosed with clinical pathology of EC. Twenty-four patients were male, and 1 patient was female. This study was approved by the Ethics Committee of Panyu Central Hospital. All patients agreed to participate in this study. The tissues were stored at -80°C after they were collected. These samples, including 25 tumors and 25 normal adjacent tissues as controls, were used for detecting the gene expression of MAGE-A3/4/9/11 by q-PCR.

### 2.4. q-PCR assay

Total RNA was extracted from all samples according to the instruction of the process.

**Table 1**

**The primer sequence for MAGE-A3/4/9/11 and  $\beta$ -actin.**

Gene	Primer (5'- 3')
MAGEA3-F: CCCATCGGGCCACTTGATACAT	R: CGCCCTCTCTTGCATTATG
MAGEA4-F: TGCCTTACCCACTACCATCA	R: AGTGCTTCTCGGAACAAGGA
MAGEA9-F: GATTGGGTGCAGGAAAATA	R: GGTAGCAGATGGGCTCTCTT
MAGEA11-F: TGTGGGAAGTCTGAGCATT	R: ACTCATAGCATGCAGGATCAGT
$\beta$ -actin-F: ACTCTCCAGCCTTCTTCC	R: GTACTTGCCTCAGGAGGAG

In brief, first-strand cDNA was synthesized with 1  $\mu$ g total RNA per sample (Genecopoeia, Inc, Rockville, MD) and then amplified (Genecopoeia, Inc, Rockville, MD) in a final volume of 20  $\mu$ L under the ABI Vii7 dx detector (ABI, Vernon, CA). The amplifications were performed as follows: predestination for 2 minutes at 50°C, denaturation for 30 seconds at 95°C, followed by 40 cycles of 95°C for 5 seconds, and 65°C for 34 seconds. The experiments were carried out in triplicate and  $\beta$ -actin was used as endogenous reference control. The relative gene expression level was calculated according to the  $2^{-\Delta\Delta C_t}$  method.<sup>[9]</sup> The primer pairs for MAGE A3/4/9/11 and  $\beta$ -actin are summarized in Table 1.

### 2.5. Statistical analysis

$P < .05$  was considered as a statistically significant difference. The false discovery rate (FDR) method was used to adjust the  $P$  value for multiple hypothesis testing. FDR  $< 0.05$  was established as the threshold.<sup>[10,11]</sup> The Pearson correlation test was performed to analyze the correlation between MAGE A3/4/9/11 and the other genes. Independent  $t$  tests were used to analyze the PCR results using SPSS software (version 16.0; SPSS, Inc., Chicago, IL).

## 3. Results

### 3.1. The expression of MAGE A3/4/11 in EC

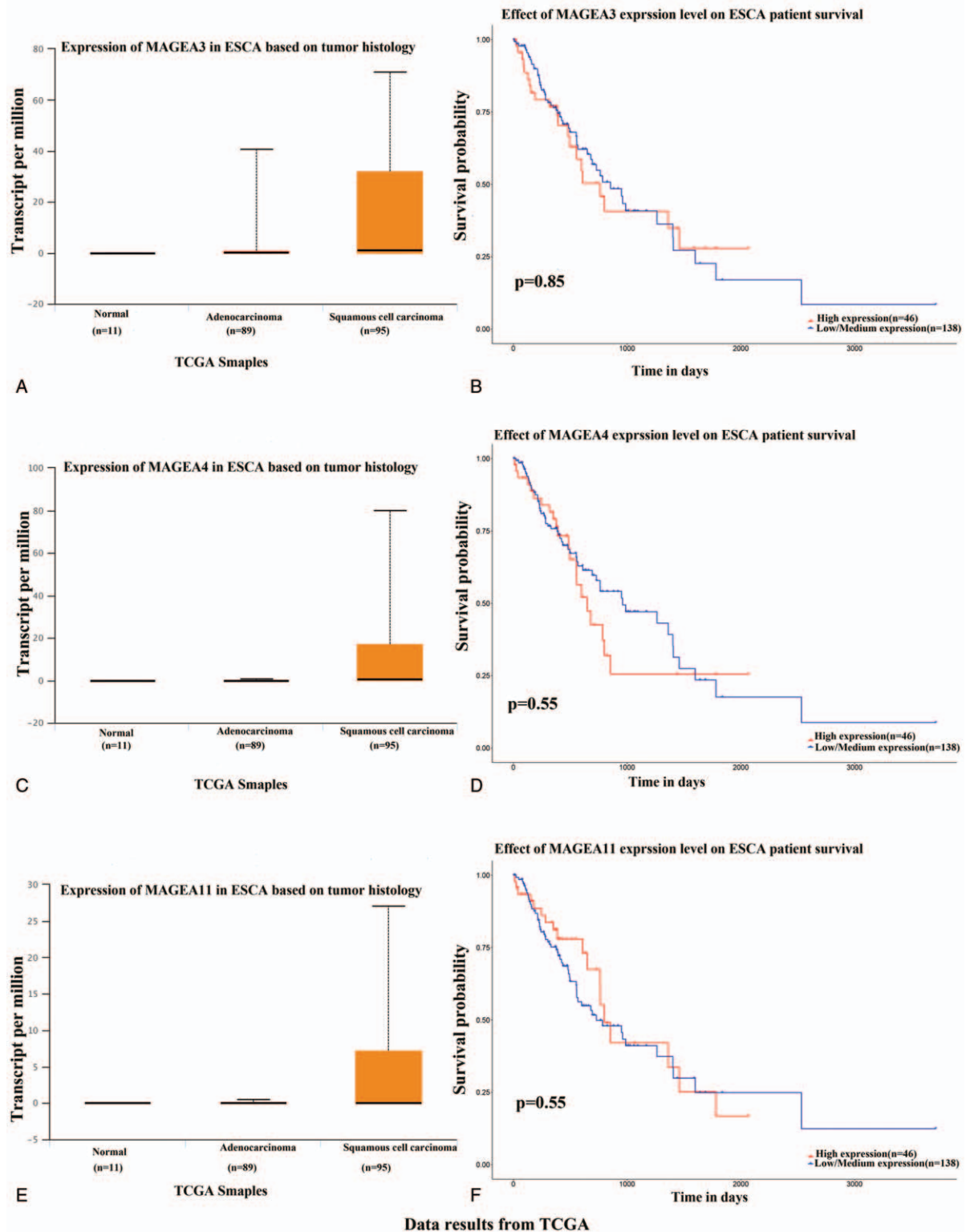
The results showed that MAGE A3/4/11 was high-expressed in tissues of patients with SCC (n=95) compared with normal (n=11), and was also significantly increased in the patients with adenocarcinoma (n=89) compared with the normal, although there was no significant difference between the patients with squamous and adenocarcinoma (Fig. 1). The survival time of patients between the high-expressed and the low/medium-expressed was not different (Fig. 1).

### 3.2. Correlation analysis

In the Pearson correlation test, there were 20,104 genes that showed correlation of MAGE-A3/4/9/11, and there were 32, 40, 24, and 79 genes that showed significant correlation with MAGEA 3/4/9/11, respectively. The 50 top correlated genes (including 50 positively regulated and 50 negatively regulated) were screened and made the heat map (Figs. 2–5).

### 3.3. Gene ontology and pathway enrichment analysis

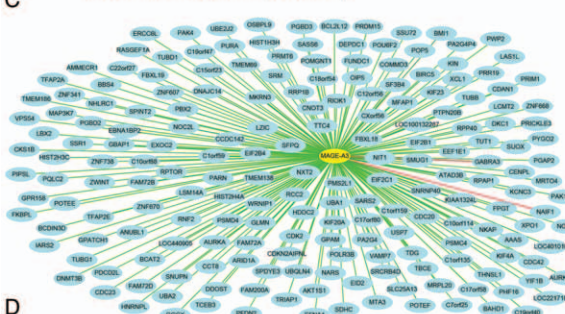
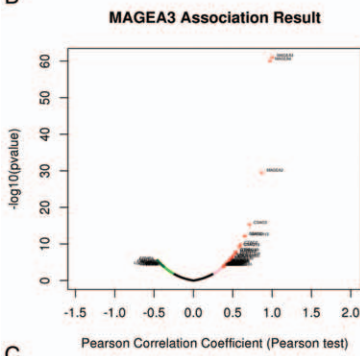
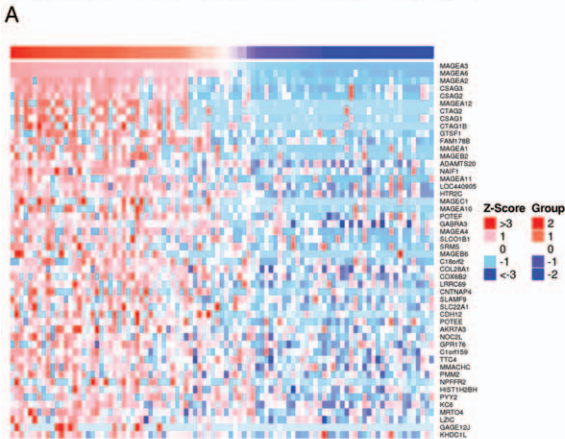
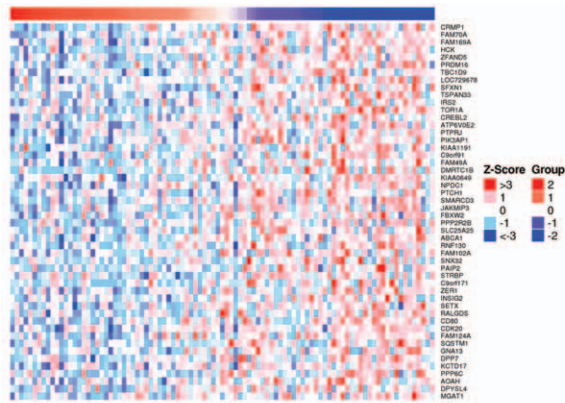
The Go enrichment analysis showed that MAGE-A3 was enriched mostly in the adaptive immune response regulation (Table 2). MAGE-A4 was enriched in regulation of translational initiation, translational elongation, rRNA metabolic process, ribonucleoprotein complex biogenesis, mitochondrial translation, ncRNA processing, DNA damage response, detection of DNA damage,



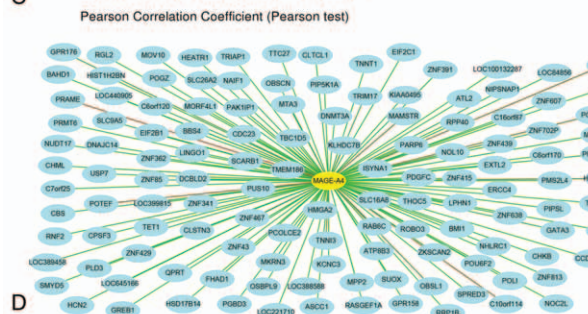
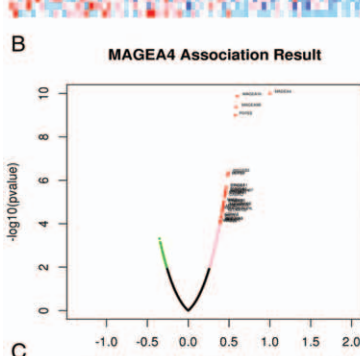
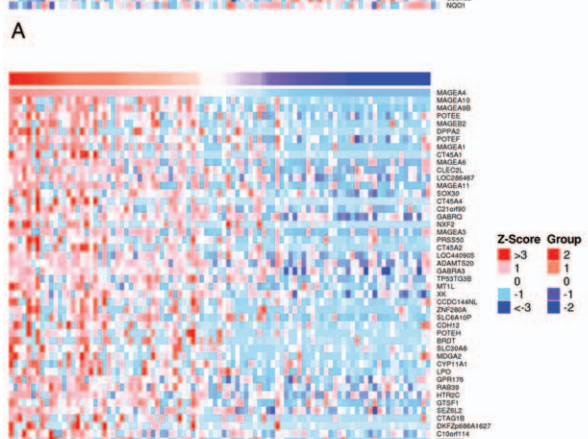
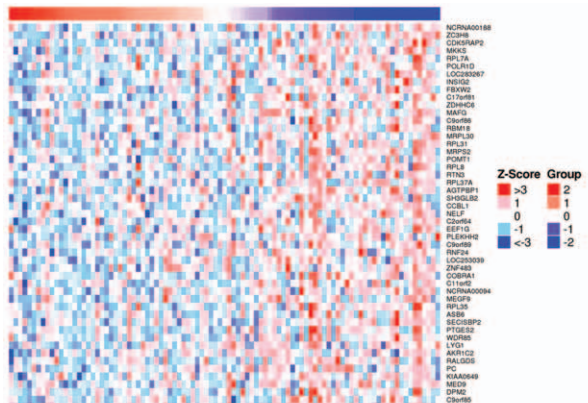
**Figure 1.** The tissue expression and survival of MAGE-A3/4/11 in patients with esophageal carcinoma using the online tool of UALCAN. MAGE A3/4/11 was high-expressed in tissues of patients with squamous cell carcinoma (n=95) compared with normal (n=11), and significantly increased in the patients with adenocarcinoma (n=89) compared with the normal, although there was no significant difference between the patients with squamous and adenocarcinoma (A, C, E). The survival time of patients between the high-expressed and the low/medium-expressed was also not different (B, D, F).

multiorganism metabolic process, and protein localization to endoplasmic reticulum (Table 3). MAGE-A9 was shown to be enriched in translational initiation, NADH dehydrogenase

complex assembly, mitochondrial translation, interleukin-4 production, multiorganism metabolic process, protein localization to endoplasmic reticulum, and mitochondrial respiratory chain



**Figure 2.** Correlation analysis of MAGE-A3 in patients with esophageal squamous cell carcinoma (ESCC). (A) Fifty top negatively related genes; (B) 50 top positively related genes; (C) Pearson correlation test of MAGE-A3 and its related genes. (D) The positive genes correlated with MAGE-A3 as  $0.3 < PCC$ . The line color of blue to red represents the PCC value from low to high.



**Figure 3.** Correlation analysis of MAGE-A4 in patients with esophageal squamous cell carcinoma (ESCC). (A) Fifty top negatively related genes; (B) 50 top positively related genes; (C) Pearson correlation test of MAGE-A4 and its related genes. (D) The positively related genes correlated with MAGE-A4 as  $0.35 < PCC$ . The line color of blue to red represents the PCC value from low to high.



**Table 2****The enrichment analysis of MAGE-A3 in esophageal squamous carcinoma.**

GO term	Function	P	FDR
GO:0002250	Adaptive immune response	0	0
GO:0035587	Purinergic receptor signaling pathway	0	0.0016
GO:0034341	Response to interferon-gamma	0	0.0019
GO:0070661	Leukocyte proliferation	0	0.0024
GO:0002263	Cell activation involved in immune response	0	0.0042
GO:0007159	Leukocyte cell-cell adhesion	0	0.0046
GO:0002253	Activation of immune response	0	0.0050
GO:0050865	Regulation of cell activation	0	0.0050
GO:0032633	Interleukin-4 production	0	0.0052
GO:0002764	Immune response regulating signaling pathway	0	0.0055
GO:0090077	Foam cell differentiation	0	0.0055
GO:0002697	Regulation of immune effector process	0	0.0056
GO:0060326	Cell chemotaxis	0	0.0058
GO:0002831	Regulation of response to biotic stimulus	0	0.0059
GO:0002521	Leukocyte differentiation	0	0.0060
GO:0032613	Interleukin-10 production	.0037	0.0075
GO:0007224	Smoothed signaling pathway	0	0.0075
GO:0042113	B cell activation	0	0.0075
GO:0032103	Positive regulation of response to external stimulus	0	0.0076
GO:0032609	Interferon-gamma production	0	0.0076
GO:0050920	Regulation of chemotaxis	0	0.0077
GO:0034340	Response to type I interferon	0	0.0088
GO:0002274	Myeloid leukocyte activation	0	0.0088
GO:0070098	Chemokine-mediated signaling pathway	0	0.0093
GO:0002683	Negative regulation of immune system process	0	0.0097

**Table 3****The enrichment analysis of MAGE-A4 in esophageal squamous carcinoma.**

Gene	Primer (5'- 3')	P	FDR
GO term	Function	P	FDR
GO:0006413	Translational initiation	0	0
GO:0006414	Translational elongation	0	0
GO:0016072	rRNA metabolic process	0	0
GO:0022613	Ribonucleoprotein complex biogenesis	0	0
GO:0032543	Mitochondrial translation	0	0
GO:0034470	ncRNA processing	0	0
GO:0042769	DNA damage response	0	0
GO:0044033	Multicellular organism metabolic process	0	0
GO:0070972	Protein localization to endoplasmic reticulum	0	0
GO:0002181	Cytoplasmic translation	0	0.0002
GO:0015949	Nucleobase-containing small molecule interconversion	0	0.0008
GO:0006353	DNA-templated transcription	0	0.0012
GO:0071826	Ribonucleoprotein complex subunit organization	0	0.0017
GO:0016073	snRNA metabolic process	0	0.0019
GO:0090305	Nucleic acid phosphodiester bond hydrolysis	0	0.0026
GO:0010257	NADH dehydrogenase complex assembly	.0037	0.0030
GO:0033108	Mitochondrial respiratory chain complex assembly	0	0.0031
GO:0032069	Regulation of nuclease activity	0	0.0031
GO:0032103	Positive regulation of response to external stimulus	0	0.0045
GO:0032609	Interferon-gamma production	0	0.0031
GO:0008334	Histone mRNA metabolic process	0	0.0045
GO:0098534	Centriole assembly	0	0.0063
GO:0097031	Mitochondrial respiratory chain complex I biogenesis	0	0.0086
GO:0006383	Transcription from RNA polymerase III promoter	.0227	0.0116
GO:0098781	ncRNA transcription	0	0.0129
	misfolded or incompletely synthesized		0.0116
GO:0006515	Protein catabolic process	0	0.0129
GO:0031123	RNA 3'-end processing	0	0.0177

**Table 4**  
The enrichment analysis of MAGE-A9 in esophageal squamous carcinoma.

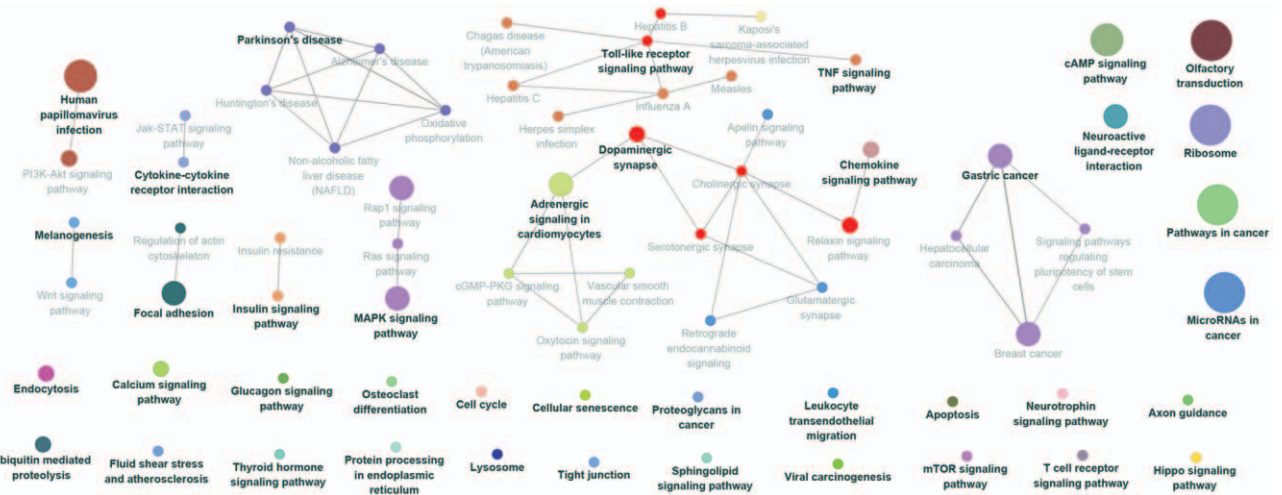
GO term	Function	P	FDR
GO:0006413	Translational initiation	0	0
GO:0010257	NADH dehydrogenase complex assembly	0	0
GO:0032543	Mitochondrial translation	0	0
GO:0032633	Interleukin-4 production	0	0
GO:0044033	Multiorganism metabolic process	0	0
GO:0070972	Protein localization to endoplasmic reticulum	0	0
GO:0097031	Mitochondrial respiratory chain complex I biogenesis	0	0
GO:0033108	Mitochondrial respiratory chain complex assembly	0	0.0098
GO:0032609	Interferon-gamma production	0	0.0148
GO:0006414	Translational elongation	0	0.0157
GO:0016072	rRNA metabolic process	0	0.0353
GO:0019439	Aromatic compound catabolic process	0	0.0353

**Table 5**  
The enrichment analysis of MAGE-A11 in esophageal squamous carcinoma.

GO term	Function	P	FDR
GO:0008544	Epidermis development	0	0
GO:0009593	Epidermis development	0	0
GO:0018149	Peptide cross-linking	0	0
GO:0034340	Response to type I interferon	0	0
GO:0043588	Skin development	0	0
GO:0007606	Sensory perception of chemical stimulus	0	0.0021
GO:0034341	Response to interferon-gamma	0	0.0025
GO:0050906	Detection of stimulus involved in sensory perception	0	0.0047
GO:0030104	Water homeostasis	0	0.0078
GO:0097031	Mitochondrial respiratory chain complex I biogenesis	0	0.0185
GO:0010257	NADH dehydrogenase complex assembly	0	0.0193
GO:0032543	Mitochondrial translation	0	0.0304
GO:0072376	Protein activation cascade	0	0.0438
GO:0033108	Mitochondrial respiratory chain complex assembly	0	0.047
GO:0006414	Translational elongation	0	0.049

complex I biogenesis (Table 4). MAGE-A11 regulated the epidermis development, detection of chemical stimulus, peptide cross-linking, response to type I interferon, and skin development (Table 5). In KEGG analysis, the genes were enriched in T cell

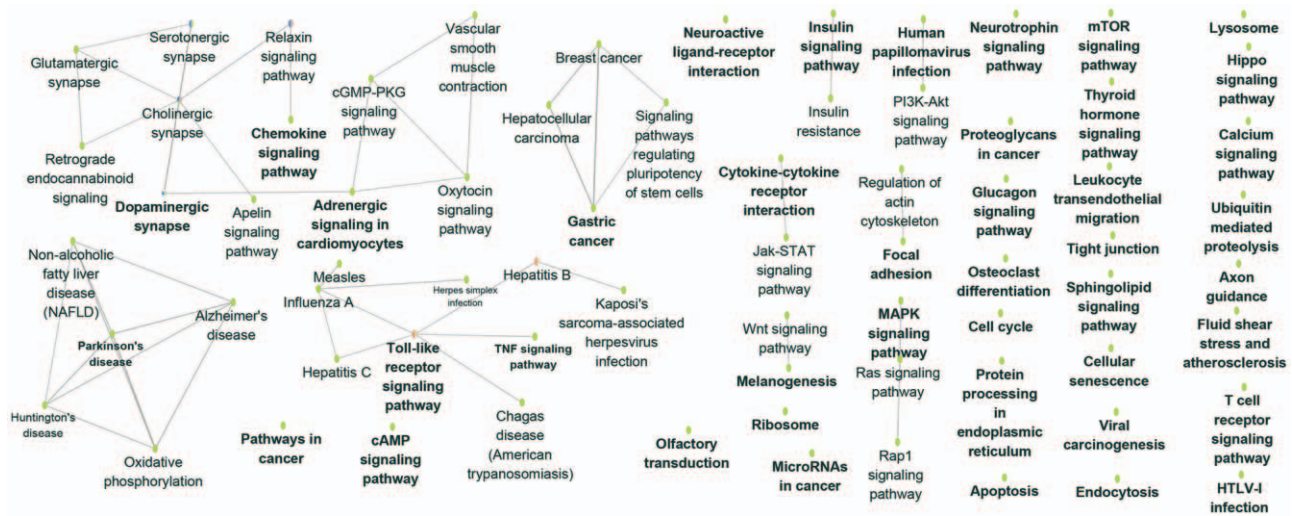
receptor signaling pathway (MAGE-A3), Th1 and Th2 differentiation, antigen processing and presentation (MAGE-A4), cytokine-cytokine receptor interaction (MAGE-A9), and chemokine signaling pathway (MAGE-A11) ( $P < .05$ ) (Figs. 6–9).



**Figure 6.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of MAGE A3 in patients with esophageal squamous cell carcinoma (ESCC). The result showed that A3 was enriched in T cell receptor signaling pathway. The more large volume of the circle represents the lower P value.



**Figure 7.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of MAGE A4 in patients with esophageal squamous cell carcinoma (ESCC). The result showed that A4 was enriched in Th1 and Th2 differentiation, antigen processing, and presentation. The more large volume of the cycle represents the lower *P* value.



**Figure 8.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of MAGE A9 in patients with esophageal squamous cell carcinoma (ESCC). The result showed that A9 was enriched in cytokine-cytokine receptor interaction. The more large volume of the cycle represents the lower *P* value.

**3.4. PCR results**

The patients enrolled in this study were diagnosed with clinical pathology (24 patients with squamous esophageal carcinoma and 1 patient with adenocarcinoma) and with the mean age of 62.08 ± 6.70 years. The results showed that the expression of MAGE-A3/4/9/11 was significantly increased in the tumor samples according to the normal adjacent tissues of esophageal carcinoma patients (*P* < .05, Fig. 10).

**4. Discussion**

A recent study reported that immunotherapy had a profound effect on cancer and may become the new method in curing certain cancers.<sup>[12–14]</sup> Clinical results of immune checkpoint inhibitors (ICBs) show high effective rate and had been proved to be used in certain cancers (such as lung cancer, malignant melanoma, intestinal cancer, lymphadenoma, and so on) by American FDA.<sup>[15–19]</sup> Car-T is another significant clinical progress in cancer immunotherapy and has been used in certain hematological oncologies.<sup>[3,20,21]</sup> Nevertheless, the effect of

Car-T on cancers depends on their specific antigen<sup>[2,22–24]</sup>; thus, it is extremely important to find the proper antigen to develop the particular immune therapy.

As reported, CTAs are the most attractive targets for cancer immunotherapy among all the tumor antigens. MAGE-A was regarded as one of best CTA, as they were highly expressed in certain tumor tissues while low/not expressed in normal tissues.<sup>[25–28]</sup> MAGE-A subfamily includes 12 closely related genes located at Xq28.<sup>[2,5]</sup> MAGE proteins contain the epitopes of cytotoxic T-lymphocyte (CTL), which attracts tumor-specific CTLs.<sup>[18,29]</sup>

In present study, the results of the data analysis of TCGA-ESCA showed that MAGE-A3/4/11 were all highly expressed in esophageal squamous carcinoma compared with the normal tissues (almost not expressed in the normal) and their expression level was not correlated with the survival time. These indicated that MAGE-A3/4/11 may be a potential immune target for EC.

In order to learn the function of MAGE-A in EC, the HiSeq RNA data of EC were downloaded from TCGA. The correlation test of MAGE-A and its related genes, Go, and KEGG enrichment



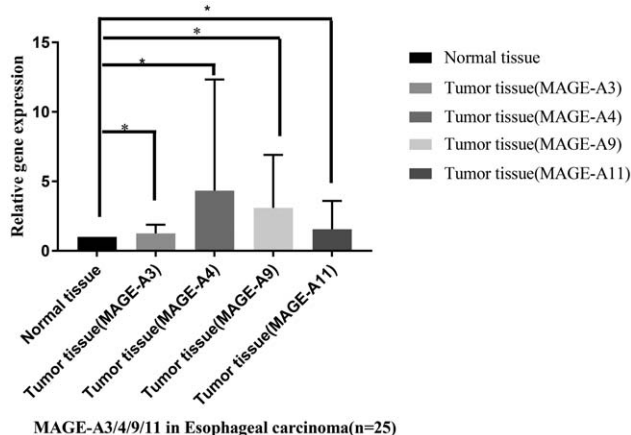


**Figure 9.** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of MAGE A11 in patients with esophageal squamous cell carcinoma (ESCC). The result showed that A11 was enriched in chemokine signaling pathway. The more large volume of the circle represents the lower *P* value.

analysis was performed on the basis of the HiSeq data. The results showed that there were 20,104 genes that showed correlation of MAGE-A3/4/9/11, and there were 32, 40, 24, and 79 genes that showed significant correlation with MAGE-A 3/4/9/11 respectively. Go enrichment results showed that MAGE-A subtypes were enriched in regulation of adaptive immune response (MAGE-A3), immune process (such as interleukin-4 production) (MAGE-A9), and response to type I interferon and skin development (MAGE-A11). Furthermore, KEGG pathway

analysis revealed that they were enriched in T cell receptor signaling pathway (MAGE-A3), Th1 and Th2 differentiation, antigen processing and presentation (MAGE-A4), cytokine-cytokine receptor interaction (MAGE-A9), and chemokine signaling pathway (A11). Recent studies showed that administering autologous CD4 (+) T cells could express an MHC class II restricted antitumor TCR that targets MAGE-A3.<sup>[30]</sup> Wu et al<sup>[31]</sup> found that the peptide p286–1Y2L9L of CD8+ T cell epitope was derived from cancer-testis antigen MAGE-4.<sup>[31]</sup> The above results indicated that MAGE-A3/4/9/11 played an important role in regulating the immune response and process. The 4 antigens showed theoretically proper immune target for EC immunotherapy.

Finally, the relative gene expression of MAGE-A3/4/9/11 was detected in the tumor tissues according to normal adjacent tissues of patients with EC by q-PCR. The results showed that the expressions of MAGE-A3/4/9/11 all significantly increased in the tumor tissues. This was consistent with the results of recent studies, which indicated that MAGE-A3/4/9/11 was high-expressed in EC tumor tissues while low/not expressed in normal tissues. Thus, it can be regarded as one of the best CTA for EC immunotherapy.<sup>[31–35]</sup>



**Figure 10.** The relative gene expressions of MAGE-A3/4/9/11 in patients with esophageal carcinoma. Twenty-five patients with esophageal carcinoma were enrolled in the study. The adjacent normal tissue was calculated as the control samples. The gene expression of MAGE-A3/4/9/11 was validated by the gene expressions by q-PCR in the tumor tissues compared with normal adjacent tissues. The results showed that the 4 genes were significantly increased in the tumor tissues (*P* < .05).

### 5. Conclusion

MAGE-A (A3/4/9/11) played an important role in regulation of immune response (MAGE-A3), immune process (MAGE-A4, MAGE-A11) and T cell receptor signaling pathway (MAGE-A3), Th1 and Th2 differentiation, antigen processing and presentation (MAGE-A4), cytokine-cytokine receptor interaction (MAGE-A9), and chemokine signaling pathway (A11). The relative gene expressions of MAGE-A3/4/9/11 were significantly increased in the tumor tissues according to normal adjacent tissues of patients with EC. The present results revealed that MAGE-A can be considered as a candidate immune target for EC treatment and

provided the messages for further research in the function of MAGE-A.

### Author contributions

**Data curation:** Sina Cai.

**Formal analysis:** Sina Cai.

**Investigation:** Liping Wang.

**Methodology:** Xiaona Zhang.

**Project administration:** Xiaohua Chen, Sina Cai, Xiaona Zhang.

**Software:** Liping Wang, Wenhui Li.

**Validation:** Wenhui Li.

**Writing – original draft:** Xiaohua Chen.

**Writing – review & editing:** Xiaolong Cao.

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