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Oncology

Demethylation-mediated upregulation of melanoma-associated antigen-A11 correlates with malignant progression of esophageal squamous cell carcinoma

Lina Gu[a], Meixiang Sang[a,b,*], Juan Li[a], Fei Liu[a], Yunyan Wu[a], Shina Liu[a], Baoen Shan[a,b,*]

[a] Department of Research Center, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, People's Republic of China
[b] Tumor Research Institute, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, People's Republic of China

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ABSTRACT

Background: The expression and methylation status of oncogenes are closely related to the onset and progression of cancer.
Aims: To explore the role and methylation status of melanoma-associated antigen-A11 in the pathogenesis of esophageal squamous cell carcinoma.
Methods: 116 esophageal squamous cell carcinoma patients with tumor tissues and corresponding adjacent normal tissues were obtained. The expression level and methylation status of melanoma-associated antigen-A11 in esophageal cancer cell lines and esophageal squamous cell carcinoma tissues were determined respectively.
Results: Significant up-regulation of melanoma-associated antigen-A11 was detected in esophageal cancer cell lines and esophageal squamous cell carcinoma tissues. Up-regulation of melanoma-associated antigen-A11 contributed to proliferation and invasion in cancer cells. Hypomethylation of the CpG site was associated with pathological differentiation, clinical stage, tumor size, lymph node metastasis and distant metastasis. Esophageal squamous cell carcinoma patients in stage III and IV, with high expression of melanoma-associated antigen-A11 or hypomethylation of the CpG site within the promoter demonstrated poor survival.
Conclusion: Melanoma-associated antigen-A11 is up-regulated in esophageal squamous cell carcinoma at least partly by hypomethylation of the CpG site within the promoter and this hypomethylation may affect the prognosis of esophageal squamous cell carcinoma patients.

1. Introduction

Esophageal carcinoma is a malignant tumor that threatens human life and health, the incidence and mortality of which rank seventh in the world and forth in China [1,2]. The highest incidence was found in southeastern Shanxi and Hebei provinces in China. Adenocarcinoma (AC) and squamous cell carcinoma (SCC) are the main types of esophageal carcinoma. The incidence of AC has been on the rise in recent years. Due to the unique geographical and cultural environment, esophageal squamous cell carcinoma (ESCC) is still the major pathological type in China [3].

It is well known that the occurrence and development of malignant tumors are related to the inactivation and activation of oncogenes and tumor-suppressor genes. Recent studies showed that the methylation status of the promoter region is closely related to gene expression [4,5]. Melanoma-associated antigens (MAGE) are a group of well-differentiated cancer/testis antigens (CTA) [6]. MAGE-A, the most well-studied member in the family, is almost not expressed in adult tissues except for stem cells and various tumor tissues [7–10]. Some studies showed that the MAGE-A1 promoter is methylated in the human glioma cell line M059J, while the promoter is demethylated in another cell line, M059K. The MAGE-A1 expression level is opposite to its methylation status [11]. Our team reported that MAGE-A11 was an independent prognostic marker for laryngeal squamous cell carcinoma patients, and correlated with tumor progression and poor survival of patients with ESCC [8,12]. However, the relationship between MAGE-A11 expression and methylation status and their prognosis in ESCC is still unclear.

* Corresponding authors at: Research Center, The Fourth Hospital of Hebei Medical University, 050017, Shijiazhuang, Hebei, People's Republic of China.
E-mail addresses: mxsang@hotmail.com (M. Sang), baoenshan@hotmail.com (B. Shan).

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Therefore, it is necessary to detect the expression and methylation status of MAGE-A11 in esophageal cancer cell lines and ESCC tissues.

We assumed that the expression of MAGE-A11 may be associated with the oncology and progression of ESCC and abnormal CpG island methylation may be one of the mechanisms leading to the up-regulation of MAGE-A11 in ESCC. In the present study, we examined the function, expression level and methylation status of MAGE-A11 in esophageal cancer cell lines and tumor tissues by using methylation special PCR (MSP) and quantitative real-time Reverse Transcription-PCR to further elucidate the role of MAGE-A11 in the pathogenesis and prognosis of ESCC.

2. Materials and methods

2.1. Cell cultures and transfection

A total of three human esophageal cancer cell lines TE-1, Eca109 and Ec 9706 were examined in this study. All cell lines were maintained in RPMI1640 (GIBCO, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, USA), 50 units of penicillin and 50 μg/mL streptomycin. Cells were grown at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. Cells were seeded at a low density and incubated for 24 h prior to treatment with a DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-Aza-dC). All three esophageal cancer cells (2×10^5/mL) were treated with 5 μmol/L 5-Aza-dC (Sigma, St Louis, MO, USA) for 72 h and the medium containing 5-Aza-dC was changed every 24 h. Control cells received no drug treatment.

For transfection, cells were transfected with the mammalian expression vector pcDNA3.1 and pCMV-AC-MAGEA11-GFP (Origen Technologies, Austin, TX) using LipofectAMINE 2000 transfection reagent (Invitrogen).Cells (4×10^5 cells/well in 6 well plates) were transfected with 1 μg of pcDNA3.1, or pCMV-AC-MAGEA11-GFP. Mixed the 100 μL of OPTI culture media (Invitogen) with 2 μL of LipofectAMINE 2000 transfection reagent for 5 min. DNA was added to the above mixtures. After 20 min incubation, LipofectAMINE 2000 DNA mixture was added to each well containing 1 mL medium. After 6 h incubation, cells were washed with phosphate buffered saline (PBS) and 1 mL/well medium with 10% fetal bovine serum was added and incubated for 42 h at 37 °C. 48 h after transfection, cells were washed with PBS for the follow-up experiments.

2.2. Patients and specimens

Primary ESCC tissues and corresponding adjacent normal tissues were obtained from 116 ESCC patients, who received surgical treatment in the Fourth Affiliated Hospital, Hebei Medical University from January 2012 to October 2012. All of the tissues were frozen and stored at −80 ° prior to genomic RNA extraction. All study subjects were ethnically homogeneous Han nationality and residents of Hebei Province. Informed consent was obtained from all recruited patients. One (or more) first- or second-degree relatives with esophageal/cardia/gastric cancer were defined as having family history of upper gastrointestinal cancers (UGIC). Information on clinicopathologic characteristics was available from hospital recordings and pathological diagnosis. The study was approved by the Ethics Committee of the Fourth Affiliated Hospital, Hebei Medical University.

2.3. RNA preparation, reverse transcription, regular reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR assays

Standard methods using Trizol reagent (Invitrogen, Carlsbad, CA, USA) were adopted to isolate total RNA from 5-Aza-dC treated and untreated cell lines, frozen tumor and corresponding normal tissues according to the manufacturer's recommended protocol. Two microgram RNA was used to synthesize single-stranded cDNA using the advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) with oligo (dT) priming as recommended in the protocol. The cDNA from each sample was used as regular RT-PCR and quantitative real-time RT-PCR template. The primers and reaction conditions were listed in Supplementary Table S1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and β-actin were used as internal controls. For quantitative real-time RT-PCR, power SYBR Green PCR Master Mix (Life Technology, Foster City, CA, USA) was used as amplification reaction mixture according to the manufacturer's instructions. The fold changes of the target gene expression were calculated using the $2^{-\triangle\triangle CT}$ method [13]. All samples were run in triplicate.

2.4. Cell proliferation assay

The proliferation of 5-Aza-dC-treated and transfected tumor cells were measured by the cell-counting kit-8 (CCK-8) assay. Cells were seeded into 96-well plates (5×10^3 cells per well). Before proliferation was detected, 10 μg/L of CCK8 (Dojindo, Japan) was added to the 100 μL cultured cells. After incubating for 72 h in a humidified incubator containing 5% CO_2 at 37 °, the absorption of each well was detected at 450 nm. The experiments were performed in triplicate.

2.5. In vitro cell invasion assay

The invasiveness of 5-Aza-dC treated and transfected tumor cells were evaluated in 24-well transwell chambers (Corning, Kennebunk, ME, USA) according to the manufacturer's instructions. Briefly, the transwell chambers were coated with 25 μL Matrigeland and incubated at 37 ° overnight. 1.5×10^5 cells in serum-free RPMI 1640 were placed into the upper compartment of wells, and the RPMI1640 medium with 10% FBS was injected into the bottom well. After harvest, the bottom of the insert was stained with giemsa. The number of cells invaded through the membrane to the lower surface was counted in five microscopic fields (at 200× magnification) per filter. The experiments were repeated three times.

2.6. DNA preparation, sodium bisulfite treatment, methylation specific PCR (MSP), and bisulfite sequencing PCR (BSP)

Genomic DNA was isolated using a simplified Proteinase K (Merck, Darmstadt, Germany) digestion method with a TIANamp Genomic DNA Kit (column based, Tiangen Biotech Co., Ltd., Beijing, China). Bisulfite modification of DNA (1 μg) was performed using an EZ DNA methylation-direct kit (Zymo Research, Irvine, CA, USA). The methylation status of CpG island of MAGE-A11 promoter was detected by MSP. The primers for MSP were shown in Supplementary Table S1 and Fig. S2A. MSP was performed by PCR using GoTaq G2 Green Master Mix (Promega, USA). The methylation status of each CpG site was confirmed by BSP. Bisulfite-modified DNA was subjected to PCR amplification. The PCR products were cloned into pGEM-T vectors (Promega, CA, USA) and 12 clones of each sample were selected for sequencing. The BSP primers were designed to recognize sodium bisulfite-converted DNA, encompassing CpG island within the human MAGE-A11 (from −140 to +6 bp) (Supplementary Table S1).

2.7. Statistical analysis

Statistical analysis was performed with SPSS22.0 software (SPSS Inc, Chicago, IL, USA). The RT-PCR and real-time RT-PCR results were expressed as the mean ± S.D. Student's t-test was used to compare

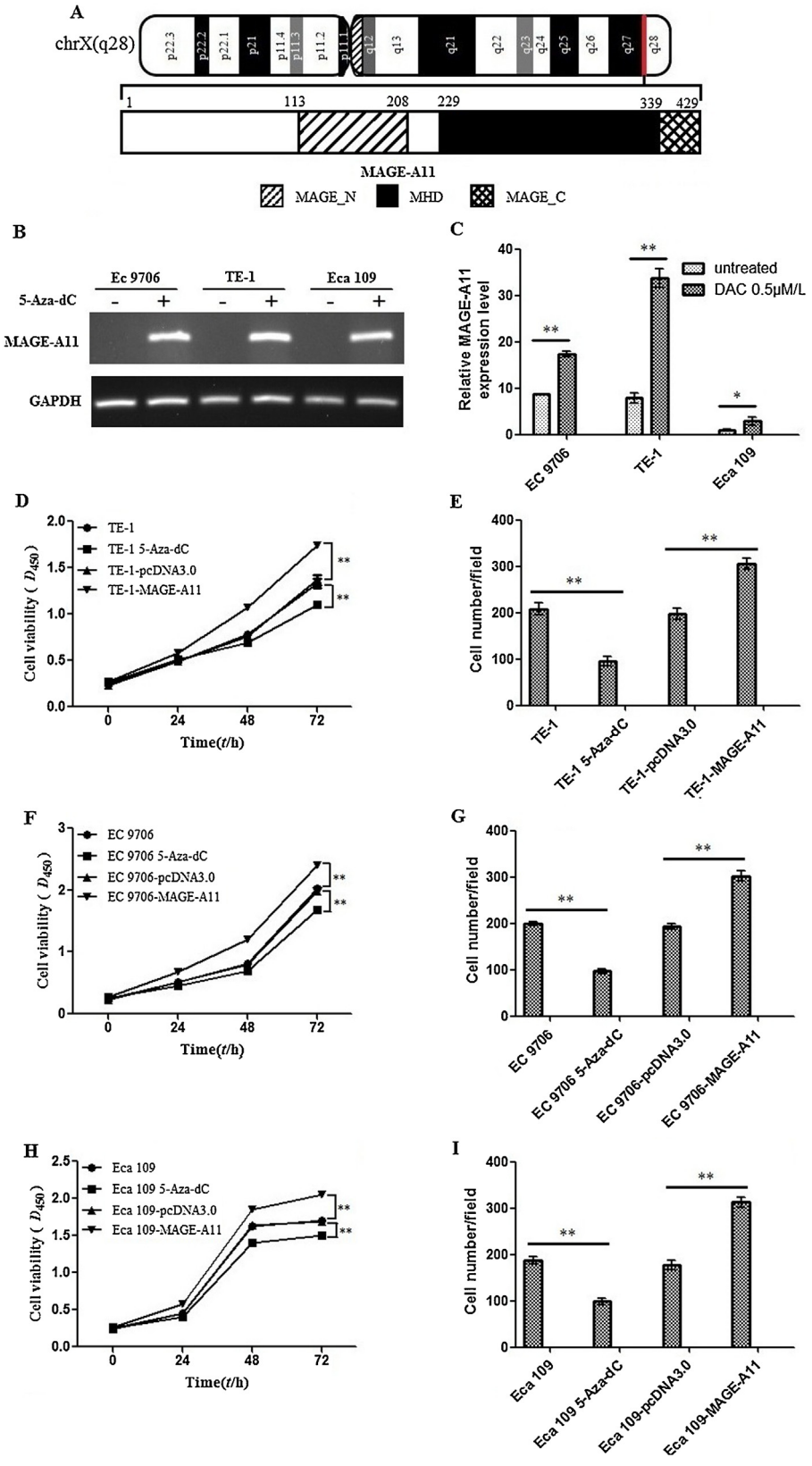

Fig. 1. The expression and effect of MAGE-A11 on proliferation and invasion in human esophageal cancer cell lines. (A) Schematic representation of genomic structures of MAGE-A11. (B) Expression of MAGE-A11 in three esophageal cancer cell lines treated or untreated with 5-Aza-dC. (C) Relative expression of MAGE-A11 in three esophageal cancer cell lines treated or untreated with 5-Aza-dC, and the levels were normalized to GAPDH. (D) CCK-8 assay results in TE-1 cell line treated or untreated with 5-Aza-dC or transfected with MAGE-A11. (E) The in vitro invasiveness of TE-1 cell line treated or untreated with 5-Aza-dC or transfected with MAGE-A11. The results were measured by determined cell counts that penetrated through Matrigel-coated transwell chambers (8-μm pore size). (F) CCK-8 assay results in EC 9706 cell line treated or untreated with 5-Aza-dC or transfected with MAGE-A11. (G) The in vitro invasiveness of EC 9706 cell line treated or untreated with 5-Aza-dC or transfected with MAGE-A11. The results were measured by determined cell counts that penetrated through Matrigel-coated transwell chambers (8-μm pore size). (H) CCK-8 assay results in Eca 109 cell line

the expression means between different groups. The statuses of gene methylation between different groups were analyzed using Chi-square test. Relationships between variables were tested by Spearman correlation analysis. Survival curves were made plotted by using the Kaplan–Meier method and the Log-rank or the Breslow tests were used as needed for the univariate comparison of MAGE-A11 expression and methylation categories. The Cox proportional hazards model was used for multivariate analysis to examine the potential prognostic value of different variables on overall survival. All statistical tests were two sided and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Up-regulation of MAGE-A11 by 5-Aza-dC treatment in esophageal cancer cell lines

As shown in Fig. 1B and C, the expression of MAGE-A11 mRNA in three ESCC cells was different. Whether GAPDH or beta-actin were used as internal reference genes, these results were similar. After treating with DNA methylase inhibitor 5-Aza-dC, the increases in expression of MAGE-A11 were different in the three esophageal cancer cell lines, indicating the important role of promoter hypomethylation in the over expression of MAGE-A11 in esophageal cancer cell lines.

3.2. The effect of MAGE-A11 on promoting esophageal cancer cell proliferation and invasion

To explore the influence of MAGE-A11 on esophageal cancer cell proliferation and invasiveness, CCK-8 assay and in vitro cell invasion assay were done on 5-Aza-dC-treated or transfected esophageal cancer cells. As shown in Fig. 1D, F and H, MAGE-A11 increased esophageal cancer cell proliferation. Although 5-Aza-dC treatment increased MAGE-A11 expression in esophageal cancer cells, it decreased the proliferation of cells in all three esophageal cancer cell lines. As shown in Fig. 1E, G and I, transfection of MAGE-A11 increased invasiveness of cells in all three esophageal cancer cell lines while 5-Aza-dC treatment decreased the invasiveness of cells in all three esophageal cancer cell lines. 5-Aza-dC treatment may cause demethylation of some tumor suppressors which are always aberrantly hypermethylated in tumor cells [14,15]. To prove this point, tumor-suppressor genes (RASSF2 [16], RASSF5A [17], DACT1 and DACT2 [18]) were detected by using real time PCR after 5-Aza-dC treatment and MAGE-A11 treatment. As shown in Supplementary Fig. S2, tumor-suppressor genes (RASSF2, RASSF5A, DACT1 and DACT2) were increased after 5-Aza-dC treatment while transfection of MAGE-A11 did not influenced the expression of those genes.

3.3. The aberrant methylation of MAGE-A11 induces decrease of its expression

As shown in Fig. 2A, a CpG island of the MAGE-A11 promoter was predicted by Meth Primer. To further analyze whether CpG islands have a vital function in the regulation of MAGE-A11 expression, the BSP method was performed in three esophageal cancer cell lines to detect the methylation status of the CpG island from −140 to +1 on MAGE-A11 promoters. As shown in Fig. 2B, this CpG island was moderately to highly methylated in TE-1 and Eca 109 cells which had relatively low levels of MAGE-A11 expression, while this region was hypomethylated in Ec9706 cells expressing high level of MAGE-A11. After the 5-Aza-dC treatment, the aberrant methylation status of the esophageal cancer cells was reversed. We found similar results from the MSP assay. As shown in Fig. 2C, the three esophageal cancer cell lines were fully or partially methylated at the regions studied.

3.4. Increased expression of MAGE-A11 in clinical specimens of ESCC tumor tissues

Previously, we have detected MAGE-A11 expression in ESCC tissues by using TMA-based immunohistochemistry and found MAGE-A11 is an independent poor prognostic marker for ESCC. In order to confirm the expression pattern and clinical relevance of MAGE-A11 in ESCC patients, we detected the mRNA level of MAGE-A11 using quantitative real-time RT-PCR assays in 116 pairs of ESCC tissues and the corresponding adjacent normal esophageal epithelial tissues. As shown in Fig. 3A, the expression of MAGE-A11 in ESCC tumor tissues was significantly increased compared to corresponding normal tissues ($P < 0.01$). Furthermore, we found that MAGE-A11 expression was not associated with age, gender and family history, However, MAGE-A11 expression was positively associated with clinical stage, pathological differentiation, tumor size, lymph node metastasis and distant metastasis ($P < 0.05$) (Fig. 3B).

3.5. Aberrant methylation of MAGE-A11 in clinical specimens of ESCC tumor tissues

The methylation analysis was performed by the MSP method in clinical specimens of ESCC tumor tissues (Fig. 3C). In primary tumor tissues and corresponding normal tissues, hypermethylation at MAGE-A11 CpG island was observed in 23.28% (27/116) and 78.45% (91/116), respectively. Furthermore, we found that the methylation status of MAGE-A11 CpG island was not associated with age, gender and family history, but was positively associated with pathological differentiation, clinical stage, tumor size, lymph node metastasis and distant metastasis ($P < 0.05$) (Table 1). On this basis, we detected the relationship between the expression and methylation status of MAGE-A11 in ESCC tissues. As shown in Fig. 3D, the expression of MAGE-A11 in ESCC tissues with unmethylated CpG island was significantly higher than that with hypermethylation of CpG island ($P < 0.05$).

3.6. Survival analysis of MAGE-A11 in ESCC

As shown in Fig. 4A and B, MAGE-A11 expression was negatively correlated with ESCC patients' survival, while MAGE-A11 CpG island methylation was positively correlated with ESCC patients' survival. ESCC cases with both high expression and CpG island unmethylation of MAGE-A11 showed worse survival rates compared to the ESCC cases with both low expression and CpG island hypermethylation of MAGE-A11 (Fig. 4C). Combining clinical stage and MAGE-A11 expression or CpG island methylation status in analysis, we observed that ESCC patients of stage III and IV, with high expression or CpG island unmethylation of MAGE-A11, were the most likely to develop metastatic and also indicated the worst survival (Fig. 4D and E). Cox multivariate analysis revealed that MAGE-A11 expression, MAGE-A11 methylation, tumor size, lymph

treated or untreated with 5-Aza-dC or transfected with MAGE-A11. (I) The in vitro invasiveness of Eca 109 cell line treated or untreated with 5-Aza-dC or transfected with MAGE-A11. The results were measured by determined cell counts that penetrated through Matrigel-coated transwell chambers (8-μm pore size). Compared with untreated cells, $^{*}P < 0.05$. $^{**}P < 0.01$. The experiments are representative of three independent experiments with similar results.

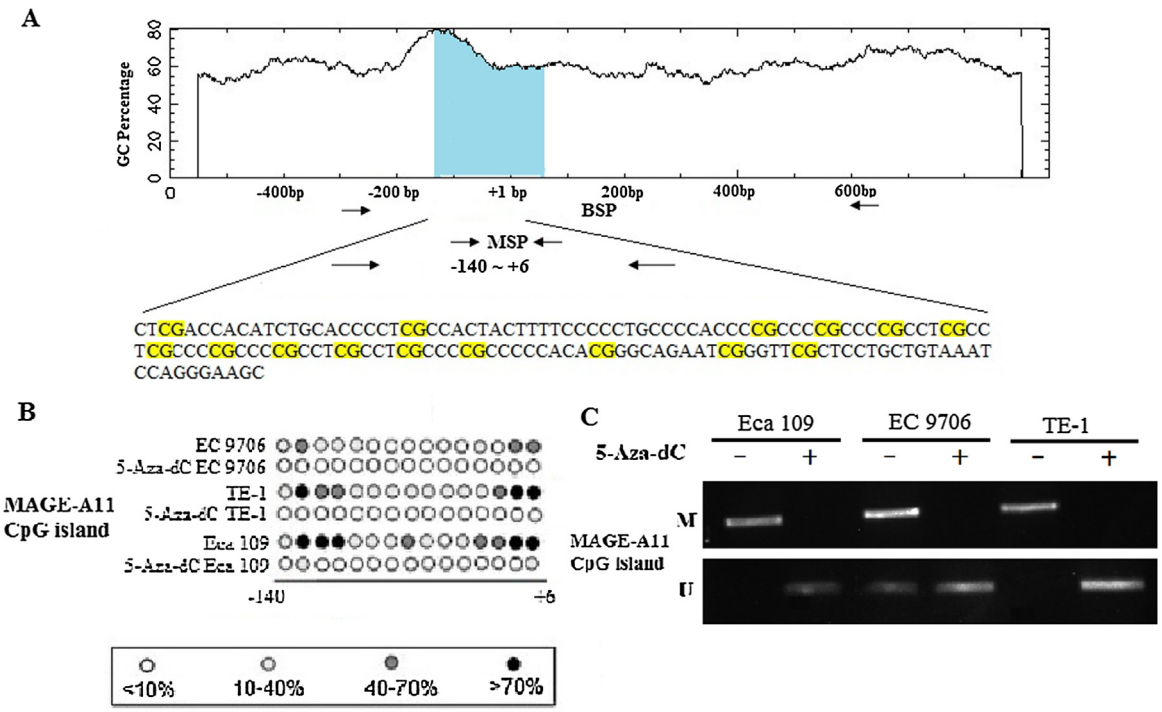

Fig. 2. Methylation status of MAGE-A11 in human esophageal cancer cell lines. (A) Schematic structure and sequence of MAGE-A11 CpG islands. (B) High-resolution mapping of the methylationstatus of every CpG site in the MAGE-A11 CpG islands in three esophageal cancer cell lines. Each CpG site is shown at the top row as an individual number. Percentage methylation was determined as percentage of methylated cytosines from 10 sequenced colonies. The color of circles for each CpG site represents the percentage of methylation. (C) The methylation status of three regions of MAGE-A11 detected by MSP analysis in esophageal cancer cell lines with or without 5-Aza-dC treatment. M, methylated; U, unmethylated.

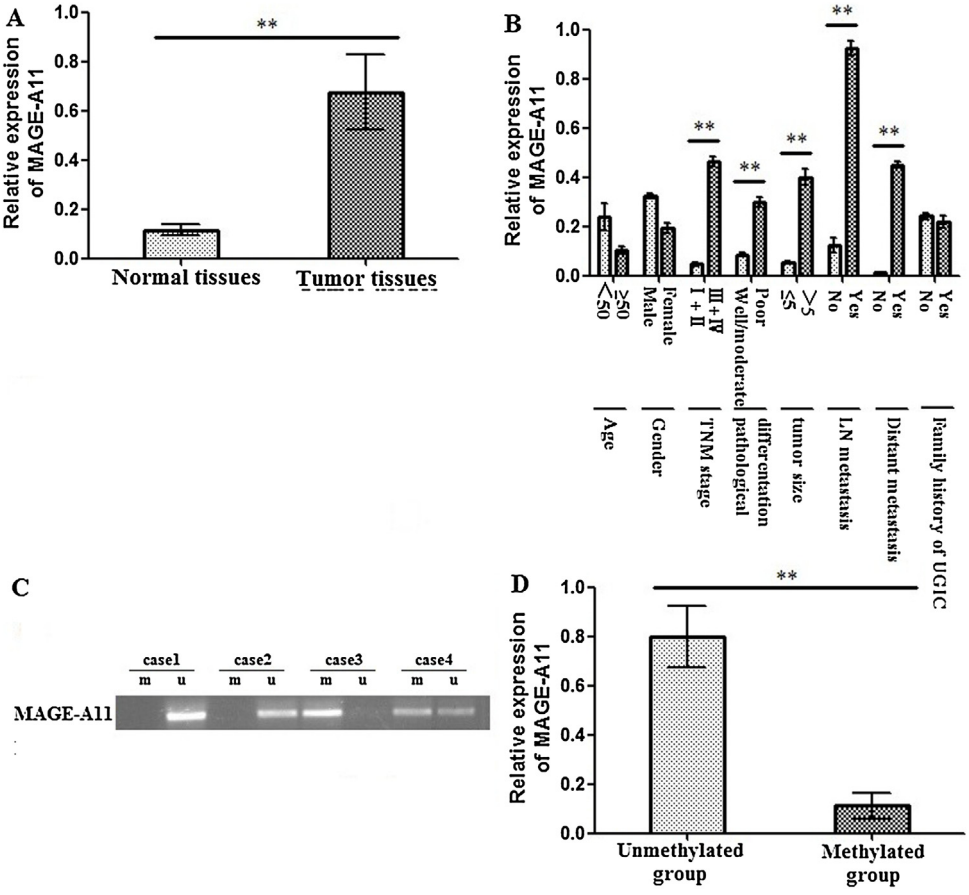

Fig. 3. Expression and methylation status of MAGE-A11 in ESCC tissues. (A) Relative expression of MAGE-A11 in ESCC tumor tissues and corresponding normal tissues. $^{*}P < 0.05$. (B) Relative expression of MAGE-A11 in different subgroups. $^{*}P < 0.05$. (C) The methylation status of three regions of MAGE-A11 determined by BS-MSP analysis in ESCC tumor tissues. M: methylated; U: unmethylated. (D) Relative expression of MAGE-A11 in the tumor tissues with and without methylation of the promoter. $^{*}P < 0.05$.

Table 1
Methylation status and expression of MAGE-A11 in ESCC tissues.

Clinicopathological factors	N	MAGE-A11 expression		χ^2	*P*	MAGE-A11 methylation		χ^2	*P*
		Low	High			Low	High		
Gender				0.154	0.694			0.307	0.579
Men	90	42	48			68	22		
Woman	26	11	15			21	5		
Age/year				0.293	0.589			0.005	0.942
<50	38	16	22			29	9		
≥50	78	37	41			60	18		
Family history of UGIC				0.150	0.698			0.101	0.751
No	70	33	37			53	17		
Yes	46	20	26			36	10		
Pathological differentiation				16.461	<0.01			6.069	0.014
Well/moderate	81	47	34			57	24		
Poor	35	6	29			32	3		
Clinical stage				24.629	<0.01			27.624	<0.01
I–II	52	37	15			28	24		
III-IV	64	16	48			61	3		
Tunmor size(cm)				18.677	<0. 01			19.466	<0.01
≤5	60	39	21			36	24		
>5	56	14	42			53	3		
Lymph node metastasis				39.834	<0.01			110.463	<0.01
N_0	26	26	0			0	26		
N_+	90	27	63			89	1		
Distant metastasis				38.275	<0.01			32.845	<0.01
M_0	60	44	16			33	27		
M_+	56	9	47			56	0		

Table 2
Univariate and multivariable analyses of prognostic factors in ESCC for overall survival.

Variable	Univariate analysis			Multivariate analysis		
	HR	*P* value	95% CI	HR	*P* value	95% CI
MAGE-A11 expression High vs low	4.159	<0.01	2.573–6.724	1.944	0.023	1.098–3.440
MAGE-A11 methylationa High vs low	0.168	<0.01	0.090–0.312	0.070	0.016	0.008-–0.614
Gender Male vs female	1.355	0.207	0.8459–2.171			
Age (years) <50 vs ≥50	1.376	0.135	0.905–2.092			
Family history of UGIC Yes vs no	1.350	0.149	0.898–2.028			
Pathological differentiation Well/moderate vs poor	1.312	0.235	0.838–2.054			
Tumor size (cm) ≤5 vs >5	1.533	0.044	1.012–2.322	1.248	0.366	0.772–2.017
Clinical stage I and II vs III and IV	2.620	<0.01	1.703–4.029			
Metastatic state of lymph node Yes vs no	6.496	<0.01	3.433–12.290	46.867	<0.01	5.065–43.644
Distant metastasis Yes vs no	25.207	<0.01	11.625–54.658	17.908	<0.01	7.755–41.356

node metastasis and distant metastasis were independently associated with ESCC patients' survival (Table 2).

4. Discussion

Aberrations in DNA methylation play a causal role in tumor. Tumor suppressors tend to be highly methylated while oncogenes tend to be hypomethylated. Aberrations in DNA methylation disrupt many cellular activities, causing transcriptional repression, disorder of cell cycle, aberrant activation or inactivation of signaling pathway, increased cell invasion, abnormal apoptosis, activation of oncogene, and promoting tumorigenesis [19–21]. Methylation of the gene promoter region is closely related to malignancies, which has been reported in a large number of literature, such as *Caspase8*, *Apaf1*, *APC*, *RASSF1A*, *AOX1*, *RARB2*, *RRG*, *ADAMTS9* and *FXE1* [22–25]. So far researchers have focused mainly on *MAGE-A11* in prostatic carcinoma. Little is known about the role of *MAGE-A11* in esophageal cancer. In the present study, we found the expression of MAGE-A11 was increased in esophageal cancer cell lines. Treatment with 5-Aza-dC and transfection of MAGE-A11 in esophageal cancer cell lines led to the up-regulation of MAGE-A11 and contributed to the invasiveness of these cells. The findings of the association between MAGE-A11 expression, methylation and tumor size, lymph node metastasis, distant metastasis and ESCC patients' survival further identified the role of MAGE-A11 as an oncogene and may be used as a marker for ESCC tumor progression and prognosis.

It is generally known that the expression of CTA is negative in normal somatic cells. Research has suggested that DNA methylation is the major epigenetic mechanism, and CTA genes, DNA demethylating agents or knockdown of DNA methyltransferases (DNMTs) can induce the expression of CTA genes in cancer cells [26]. As showed by James et al., MAGE-A11 protein is rarely expressed in benign prostate intraepithelial neoplasia, but the CpG island of

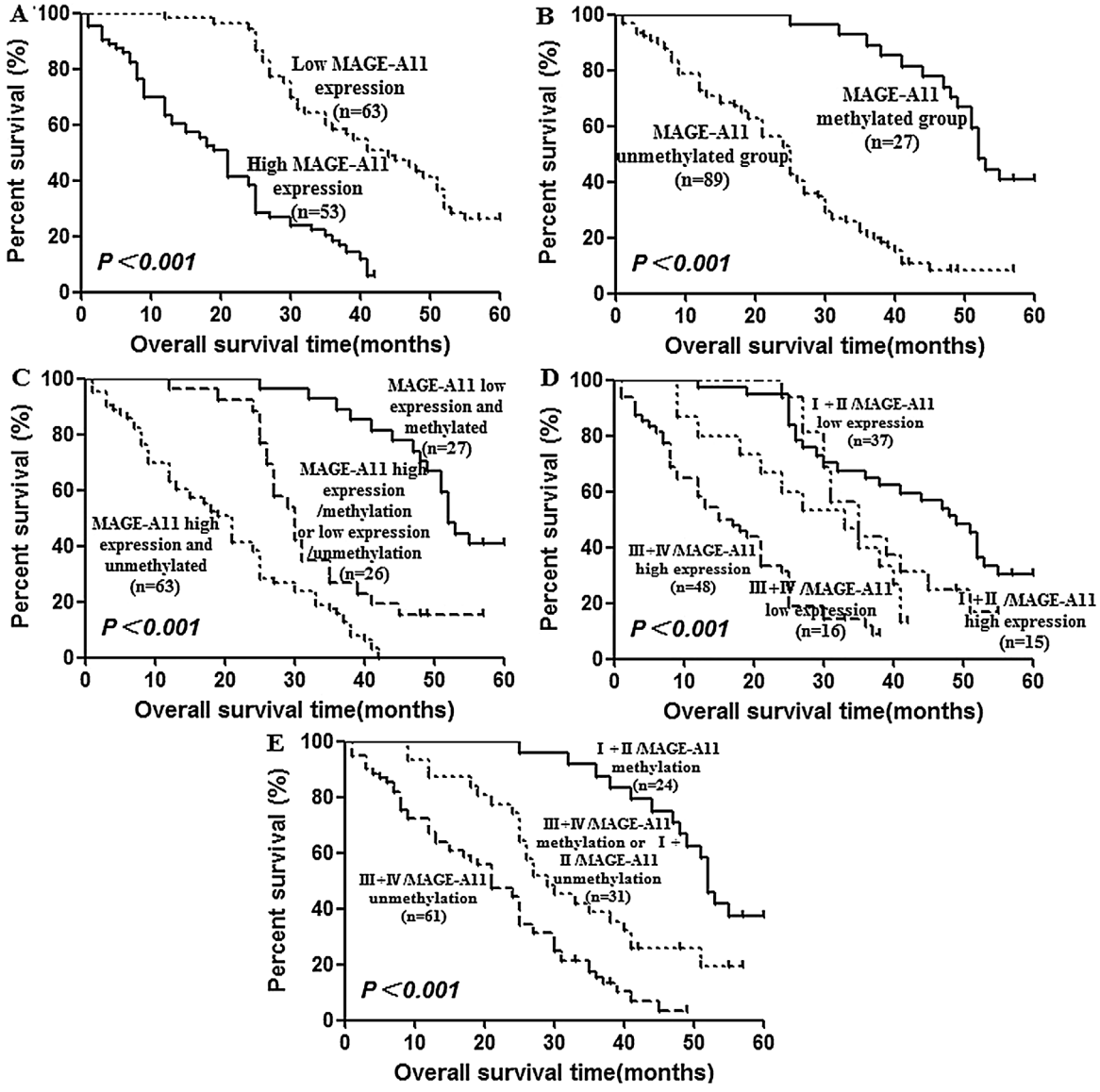

Fig. 4. Kaplan–Meier survival analysis of MAGE-A11 expression and methylation in ESCC cases. (A) Showing a direct correlation between low MAGE-A11 expression and poor patient survival. (B) Showing consistently a direct correlation between MAGE-A11 promoter methylation and poor survival. (C) ESCC cases with simultaneous low expression and promoter methylation of MAGE-A11 showing poor survival. (D) ESCC cases in stage III and IV and with low expression of MAGE-A11 showing poor survival. (E) ESCC cases in stage III and IV and with promoter methylation of MAGE-A11 showing poor survival.

MAGE-A11 promoter is hypermethylated. In contrast, MAGE-A11 protein is highly expressed in prostate cancer, but the CpG island of MAGE-A11 promoter is hypomethylated, especially at the transcription start site (TSS)-resident CpG sites [27,28]. The present study found hypomethylated profiles of MAGE-A11 gene promoter in esophageal cancer cell lines with upregulated MAGE-A11 expression such as in TE1, EC9706, and Eca109 cells. Kallikrein-related peptidase 4 (KLK4) as an oncogene, like MAGE-A11, was regulated by DNA methylation of promoter regions [29,30]. It is further confirmed that DNA methylation plays an important role in regulating gene expression. The determination of the CpG sites is essential for regulating gene transcription and expression in tumor-related genes.

We found that the expression and methylation status in the promoter of MAGE-A11 were closely related to pathological differentiation, clinical stage, tumor size, lymph node metastasis and distant metastasis, and MAGE-A11 increased esophageal cancer cell proliferation and invasion. The above results indicated MAGE-A11 plays an important role in promoting ESCC progression. In further survival analysis, we found that MAGE-A11 expression and promoter hypomethylation were associated with ESCC patients' survival. ESCC patients with simultaneous high expression and hypomethylation of MAGE-A11 had a worse prognosis. Others investigation have shown that methylation status of oncogene may be used to assess clinical outcomes of carcinomas or responses to chemotherapeutic agents [21–24,30]. Due to the limitations of single gene, co-expression and methylation status of MAGE-A11 can better predict tumor progression and prognosis of ESCC. Thus, MAGE-A11 expression and hypomethylation may be potential markers for progression and prognosis of ESCC tumor.

We have reported that MAGE-A11 peptides induce cytotoxic T-lymphocyte (CTL), responses against cancer cells that expressed MAGE-A11 [31]. MAGE-A11 expression in tumor cells is heterogenous, and the MAGE-A11 gene is not expressed in some tumor patients, leading to tumor escape. If it is possible to induce high levels of MAGE-A11 expression in all tumor cells, these limitations can be overcome. In recent years, researchers have reported that the recognition and cytotoxicity of antigen-specific T cells

can be improved by increasing the expression of tumor antigen [32]. In this study, although 5-Aza-dC treatment could increase the expression of MAGE-A11, it reduced the proliferation of esophageal cancer cells. We suspect that 5-Aza-dC treatment may cause demethylation of some other tumor suppressors which are always aberrantly hypermethylated in tumor cells [14,15]. To prove this point, RASSF2, RASSF5A, DACT1 and DACT2 were detected by using real time PCR after 5-Aza-dC treatment and MAGE-A11 treatment. We found that tumor-suppressor genes were increased after 5-Aza-dC treatment while transfection of MAGE-A11 did not influenced the expression of those genes. If there are epigenetic-modifying drugs which could specifically hypomethylate the promoter region of MAGE-A11, the cytotoxicity of MAGE-A11 peptides on tumor cells can be enhanced and inhibit angiogenesis can be inhibited. We will look for specific or other more efficient drugs to reduce the methylation of MAGE-A11 promoter region, which will lay the foundation for the clinical application of MAGE-A11 peptides.

In summary, MAGE-A11 may act as an oncogene and hypomethylation at the CpG sites of its promoter is essential for the overexpression of MAGE-A11. In addition, MAGE-A11 may be used as a potential biomarker in predicting ESCC progression and prognosis.

Conflict of interest

None declared. The authors alone are responsible for the content and writing of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dld.2019.04.018.

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