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From Cell Biology to Tissue Engineering

Correlation between the high expression levels of cancer-germline genes with clinical characteristics in esophageal squamous cell carcinoma

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Summary. Antigens encoded by cancer-germline genes are attractive targets for cancer immunotherapy. In this study, we aimed to evaluate the mRNA expression of cancer-germline genes, expression of the encoded proteins in patients with esophageal squamous cell carcinoma (ESCC) and their correlations with clinical characteristics. In addition, the effects of downregulation cancer-germline genes on ESCC cells were assessed *in vitro*. Our results showed that cancer-germline genes were frequently expressed in ESCC samples. The positive rates of in ESCC samples were: 87% of *MAGE-A3*, 60% of *MAGE-A4*, 65% of *MAGE-C2*, and 20% of *NY-ESO-1* at mRNA level. MAGE-A3 expression was associated with age, lymph node metastasis and tumor stage (all P<0.05), while MAGE-C2 expression was only associated with tumor stage (P<0.05). Furthermore, the MAGE-A3 expressing patients had a poorer overall survival (P<0.05). Multivariate analysis identified MAGE-A3 as an independent poor prognostic marker in ESCC. *In vitro* assay, ESCC cell lines treated with specific siRNAs to down-regulate *MAGE-A3* and *MAGE-C2* resulted in decreased colony-formation and migration ability (P<0.05). Epithelial marker *E-cadherin* was up-regulated in siRNA-*MAGE-A3/C2* cells compared to controls, whereas mesenchymal markers *Vimentin, N-cadherin* and *Slug* were downregulated (all P<0.05), suggesting a role for MAGE-A3/C2 in ESCC metastasis through inducing epithelial-mesenchymal transition. The present study revealed that cancer-germline genes and their encoded proteins were frequently expressed in ESCC tumor samples and were related to poor prognosis. Thus, cancer-germline genes may serve as useful biomarkers and potential targets for ESCC patients.

Key words: Esophageal squamous cell carcinoma (ESCC), Cancer-germline genes, Biomarker, Epithelial-mesenchymal transition

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Introduction

Esophageal carcinoma is the sixth leading cause of cancer death worldwide, and approximately 70% of the cases occur in China, with esophageal squamous cell carcinoma (ESCC) accounting for more than 90% of the cases (Song et al., 2014). In China, the incidence of ESCC shows regional distribution, with higher prevalence in some areas such as Henan Province (Yue et al., 2014). In spite of the progress in the therapy used for ESCC patients, the overall 5-year survival still remains poor (Hottenrott, 2013). To improve the survival rate and life quality, it is urgent to develop new strategies and targets for ESCC. Recently, immunotherapy was considered as potential strategy to improve the therapeutic effect of cancer (Coulie et al., 2014) and tumor associated antigen NY-ESO-1 as target of

genetically engineered T lymphocytes displayed surprising effect in myeloma (Rapoport et al., 2015).

Cancer-germline genes are a family of genes widely studied in the cancer immunotherapy field due to the restrictive expression pattern and immunogenicity of the encoded antigens in cancer patients (Scanlan et al., 2004; Simpson et al., 2005). Previous studies have shown that demethylating chemotherapy could facilitate their expression in tumor cells and a few HLA class I-restricted antigenic peptides have been identified (Van Der Bruggen et al., 2002; Cruz et al., 2011; Krishnadas et al., 2014). Thus, demethylating pre-treatment against tumor cells could enhance tumor-specific $CD8^+$ T-cell responses. The biological role of the proteins encoded by cancer-germline genes in tumors remain poorly understood. It has been reported that co-expression of cancer-germline genes in malignant tissues was correlated with tumor growth, patient survival and disease relapse (Simpson et al., 2005). Down-regulation of these genes could alter the morphology, adhesion and migration of tumor cells, which suggested that cancer-germline genes might contribute to oncogenesis (Caballero et al., 2013). Moreover, proteins encoded by cancer-germline genes expression was linked to advanced disease and a worse prognosis which indicated that these proteins may be used as poor prognostic markers in various cancers (Ayyoub et al., 2013; John et al., 2013). However, the characteristic of cancer-germline genes and their encoded protein expression and their prognostic value in ESCC patients have rarely been investigated (Liang et al., 2005; Forghanifard et al., 2011).

It has been reported that among the cancer-germline genes family, MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 are the major ones as promising targets for cancer immunotherapy (Caballero and Chen, 2009; Riener et al., 2009; Imai et al., 2012). In this study we firstly detected the four cancer-germline genes expression in ESCC tumor tissues at mRNA level. We found that the expressions of *MAGE-A3* and *MAGE-C2* (*MAGE-A3/C2*) were associated with advanced ESCC patients, thus we further evaluated the expression of MAGE-A3/C2 in ESCC tumor tissues by immunohistochemistry at protein level and analyzed the correlations between their expressions and the overall survivals of patients with ESCC. In addition, the biological effects of downregulation of *MAGE-A3/C2* in ESCC cells were detected *in vitro*.

Materials and methods

Cell line and tumor samples

ESCC cell lines EC1, KYSE70 and a normal esophagus epithollium cell Het-1a were used in the study. 193 fresh tumor samples were immediately harvested from patients with ESCC after surgery in the First Affiliated Hospital of Zhengzhou University, from November 2012 to January 2014. None of the patients received preoperative chemotherapy or radiotherapy. Clinical and pathological characteristics were taken from clinical database. 118 formalin-fixed paraffin-embedded ESCC tumor tissues and 5 paired neighboring non-cancerous tissues from the Department of Pathology, at the First Affiliated Hospital of Zhengzhou University, from 2008.10 to 2010.5, with available follow-up information, were used for immunohistochemical analysis.

RT-PCR and q RT-PCR

Total RNA was isolated from tissue samples and ESCC cells using Trizol reagent (Invitrogen, USA). The First-strand cDNA was synthesized from 1 μg of total RNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Canada). The expressions of cancer-germline genes were amplified using Premix (TaKaRa, Japan) by RT-PCR. GAPDH was used as a loading control. qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Japan) in Agilent Mx3005P to detect the mRNA expression of MAGE-A3, MAGE-C2 and EMT markers after transfected with negative control and si-MAGE-A3/C2. All the primer sequences are shown in Table 1.

MAGE-A3/C2 protein staining

Formalin-fixed paraffin embedded tissues were cut into 4μm sections. Samples were then incubated with anti-human MAGE-A3 (Abcam, England) and anti-human MAGE-C2 antibodies (Abcam, England) at the concentration of 1:150 and 1:400 respectively. For a negative control, samples were incubated with PBS instead of specific antibody. The detection of nuclear

Table 1. Primers used for RT-PCR.

Gene		Primer sequence	Product size(bp)
GAPDH	Forward	5'-GGAGCCAAAAGGGTCATCATCTC-3'	233
	Reverse	5'-GAGGGGCCATCCACAGTCTTCT-3'	
MAGE-A3	Forward	5'-AGTCCGAGTTCCAAGCAG-3'	239
	Reverse	5'-GCAGGTGGCAAAGATGTA-3'	
MAGE-A4	Forward	5'-TGCCTTACCCACTACCATC-3'	660
	Reverse	5'-TGCTCCAGGACTTTCACATA-3'	
MAGE-C2	Forward	5'-TGAGTTAGAAGACTGGGTAGATGC-3'	189
	Reverse	5'-ATGCTCTCGGTAAGATTTGGTATC-3'	
NY-ESO-1	Forward	5'-AGTTCTACCTCGCCATGCCT-3'	386
	Reverse	5'-TCCTCCTCCAGCGACAAACAA-3'	
E-cadherin	Forward	5'-TGCCCAGAAAATGAAAAAGG-3'	200
	Reverse	5'-GTGTATGTGGCAATGCGTTC-3'	
N-cadherin	Forward	5'-ACAGTGGCCACCTACAAAGG-3'	200
	Reverse	5'-CCGAGATGGGGTTGATAATG-3'	
Vimentin	Forward	5'-GAGAACTTTGCCGTTGAAGC-3'	163
	Reverse	5'-GCTTCCTGTAGGTGGCAATC-3'	
Slug	Forward	5'-GGGGAGAAGCCTTTTTCTTG-3'	158
	Reverse	5'-TCCTCATGTTTGTGCAGGAG-3'	

and/or cytoplasmic staining in any percentage of tumor cells was considered positive. Complete absence of staining was considered negative for each MAGE tested. Protein expression of MAGE-A3/C2 in Testis tissue was used as positive control.

Gene downregulation by siRNAs

SiRNAs specific to *MAGE-A3/C2* and scramble negative control siRNA were provided by Gene Pharma (China). SiRNA duplexes were used to transfect the ESCC cells using Lipofectamine 3000 (Invitrogen, USA) at a final concentration of 36 nM. Then we confirmed the effectiveness of gene downregulation with specific siRNAs by qRT-PCR.

Cell apoptosis and colony formation assay

After ESCC cells were transfected with siRNAs specific to MAGEs or scrambled siRNA for 48 hours, 10^5 transfected cells were collected to detect cell apoptosis using PI-Annexin V assay by Flow Cytomery. 1,000 cells were seeded in duplicate in 6-well low adhesion plates (Corning, China) and allowed to form colonies for 1 week. The number of colonies with 30 cells or larger than 1mm in diameter in each well was counted.

Migration assay

Cell migration was assessed in 24-well Boyden Chambers (Corning, China) according to the protocol of the manufacturer. Migrated cells were counted at 100 x magnification in 5 random areas.

Statistical analysis

Statistical analysis was performed by the SPSS program (SPSS 17.0). Pearson χ^2 test was used to compare the correlation between cancer-germline genes and their encoded proteins expressions and clinical characteristics. The Kaplan-Meier method was conducted to calculate the overall survival curve. In addition, to evaluate prognostic and predictive value of MAGE-A3/C2 expression both univariate and multivariate Cox proportional hazard ratio modeling analysis were performed in R. All the statistical significance was accepted at $P<0.05$.

Results

The expression of cancer-germline genes in ESCC tumor samples at mRNA level and the relationship with clinical characteristics

Among the four analyzed cancer-germline genes, none of them was expressed in normal esophageal mucosa cells. The most frequently expressed of these genes in ESCC samples was *MAGE-A3* with the positive rate 87% (168/193), followed by *MAGE-C2* 65% (125/193), *MAGE-A4* 60% (116/193) and *NY-ESO-1* 20% (39/193). Positive and negative expressions of each gene are shown in Fig. 1A. The relationship between these genes expression and clinicopathological characteristics of patients with ESCC are shown in Table 2. Higher frequency of MAGE-A3 was found in patients <65 years old at diagnosis (91%) compared to patients ≥65 years old at diagnosis (81%) ($P<0.05$). The incidence of MAGE-A3 expression was higher in patients with lymph node metastasis (95% vs.84%) ($P<0.05$). 83% of patients with clinical stage I-II and 96% of stage III-IV patients expressed *MAGE-A3* ($P<0.05$). In addition, *MAGE-C2* was significantly correlated with clinical stage ($P<0.05$), incidence of MAGE-C2 in stage I-II patients versus stage III-IV was 60% and 77%. There was no relationship between

Table 2. Relationship between cancer-germline genes expression and clinical pathological parameters.

Patients characteristics		N=193	Percentage of mRNA expression samples (%)							
			MAGE-A3	P value	MAGE-A4	P value	MAGE-C2	P value	NY-ESO-1	P value
Gender	male	131	86	0.412	60	0.528	64	0.458	21	0.501
	female	62	89		60		66		20	
Age (years old)	<65	113	91	0.037*	58	0.337	66	0.343	22	0.274
	≥65	80	81		63		63		18	
Tumor depth of invasion	T1-T2	86	90	0.187	64	0.203	61	0.166	21	0.481
	T3-T4	107	85		57		67		20	
Lymph node metastasis	positive	58	95	0.025*	67	0.121	72	0.097	24	0.241
	negative	135	84		57		62		19	
Histological grade	I	66	85	0.104	56	0.339	62	0.202	24	0.504
	II	70	84		63		60		17	
	III	57	93		61		83		19	
Stage number	I-II	137	83	0.016*	58	0.518	60	0.031*	17	0.076
	III-IV	56	96		64		77		28	

MAGE-A4 or *NY-ESO-1* and clinical characteristics (P>0.05).

Co-expressions of multiple cancer-germline genes in ESCC tissues at mRNA level

As shown in Fig. 1B, the positive rates of cancer-germline genes co-expression in ESCC tissues were: at least one was in 92% (178/193), whereas 8% (15/193) did not express any of the four cancer-germline genes. 15% (29/193) cases expressed one, 27% (52/193) samples expressed two, 38% (73/193) patients expressed three, and 13% (24/193) patients expressed four cancer-germline genes. The pattern of co-expression from all cases where could be evaluated was visualized in a Venn diagram (Fig. 1C).

The relationships among expressions of different cancer-germline genes

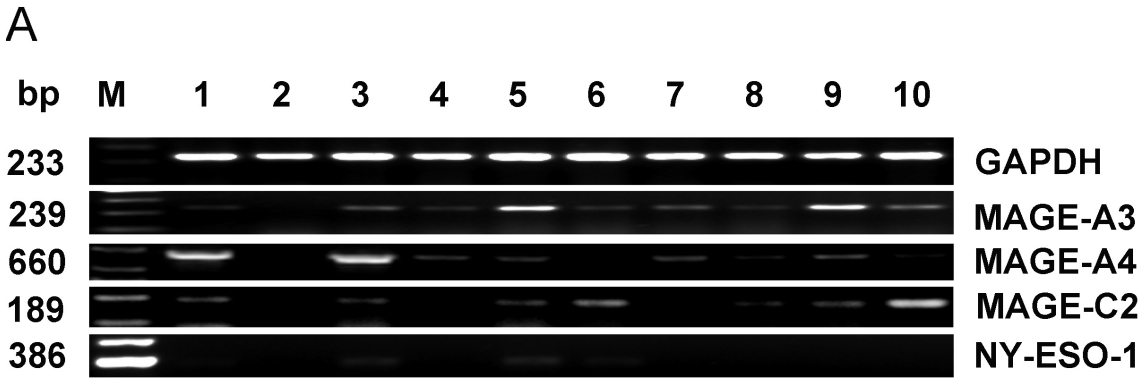
The analysis of the relationship among the expression of *MAGE-A3, MAGE-A4, MAGE-C2* and *NY-ESO-1* was performed by Pearson's correlation test (Table 3). Notably, the significant association was stronger for co-expression. Patients expressing *MAGE-A4* had significantly higher frequency of *MAGE-A3, MAGE-C2* and *NY-ESO-1* expression than *MAGE-A4* negative samples. We also found a statistically significant pattern of co-expression between *MAGE-A3* and *MAGE-C2* (P<0.05).

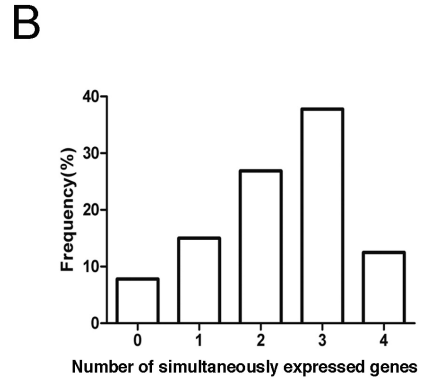
Detection of MAGE-A3/C2 at protein level by immunohistochemical staining

MAGE-A3/C2 was negatively expressed in non-

Table 3. Concomitant expression in 193 patients and associations between cancer-germline genes expression.

	MAGE-A3	MAGE-C2	NY-ESO-1
MAGE-A4	109(57%)	90(47%)	34(18%)
P value	0.001*	0.000*	0.001*
Pearson correlation	0.253	0.329	0.278
NY-ESO-1	37(19%)	29(15%)	
P value	0.079	0.111	
Pearson correlation	0.117	0.101	
MAGE-C2	116(60%)		
P value	0.002*		
Pearson correlation	0.232		





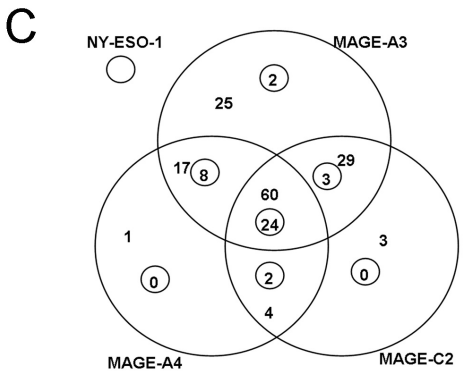


Fig. 1. RT-PCR analysis of cancer-germline genes expression in ESCC tissues. **A.** The expression of cancer-germline genes in ESCC tissues. M-marker, 1-positive control (testis), 2-negative control (Het-1a), 3-10-ESCC tissues. GAPDH was used as an internal quality control. **B.** Simultaneous expression of multiple genes in ESCC tissues. The proportion of ESCC tissues expressing at least one and up to four genes was shown as a percentage of 193 ESCC tissues. **C.** Venn diagram demonstrating the overlap between MAGE-A3, MAGE-A4, MAGE-C2 and NY-ESO-1 expression in ESCC specimens. Numbers represent positive tumor among the total of 193 ESCC specimens included in the analysis. Numbers in the small circles indicate the NY-ESO-1-positive tissue samples.

malignant esophageal mucosa tissues. The positive rate of MAGE-A3 protein was 71 % (84/118) in cancer tissues, and MAGE-C2 was 56% (66/118) (Fig. 2). Although heterogeneous, MAGE-A3/C2 positive staining samples showed that more than 75% of tumor cells were positive. At the cellular level, MAGE-A3/C2 staining was both cytoplasmic and nuclear, but a nuclear localization was slightly predominant. We analyzed the relationship between the protein expression of MAGE-A3/C2 and the clinical characteristics (Table 4). MAGE-A3 was associated with lymph node metastasis and tumor stage, whereas MAGE-C2 was only correlated

Table 4. Correlation of protein expression of MAGE-A3/C2 with clinical characteristics of ESCC.

Patients characteristics		N=118	Percentage of protein expression samples (%)					
			MAGE-A3	P value	MAGE-C2	P value	co-expression	P value
Age at diagnosis	<65years	70	77	0.084	63	0.089	44	0.705
	≥65years	48	63		46		40	
Gender	Male	76	74	0.161	57	0.849	40	0.439
	Female	42	67		55		48	
Tumor depth of invasion	T1-T2	58	66	0.224	52	0.458	35	0.097
	T3-T4	60	77		60		50	
Lymph node metastasis	Positive	30	83	0.036*	70	0.090	67	0.003*
	Negative	88	67		51		34	
Histological grade	G1	46	65	0.211	57	0.839	40	0.793
	G2	40	73		53		43	
	G3	32	78		59		47	
Stage number	I-II	84	64	0.050	48	0.004*	32	0.001*
	III-IV	34	88		76		68	

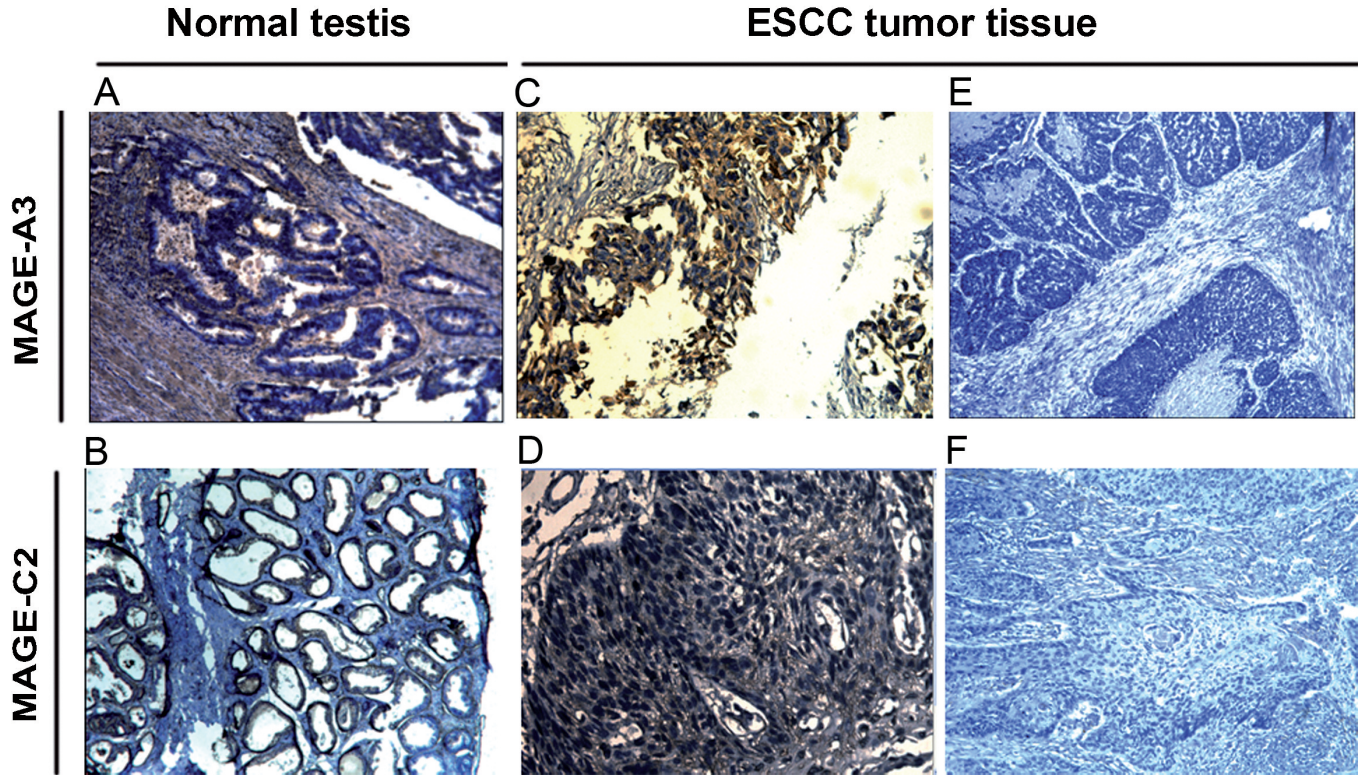


Fig. 2. Protein expression of MAGE-A3/C2 in testis tissue and ESCC tumors. Immunohistochemical analysis of MAGE-A3/C2 expression in clinical ESCC specimens. Representative tumor staining with monoclonal antibodies recognizing MAGE-A3/C2. Normal testis was included as control **(A, B)**. MAGE-A3/C2 positive expression **(C, D)** and MAGE-A3/C2 negative expression **(E, F)**. × 200.

with tumor stage (P<0.05). Co-expression of MAGE-A3/C2 was more frequently observed in tumors with lymph node metastasis and advanced stage (P<0.05).

Survival analysis according to MAGE-A3/C2 expression

With regard to MAGE-A3 expression, 34 of 118 patients with negative expression were evaluated and 12 died, whereas 84 of 118 patients with positive expression were evaluated and 57 died. As shown in Fig. 3A, MAGE-A3 expression was correlated with overall survival (P=0.0002). According to MAGE-C2 expression, 52 of 118 patients with negative expression were evaluated and 28 died. 66 of 118 patients with positive expression were analyzed and 41 died. The overall survival was not associated with MAGE-C2 expression (P=0.0589; Fig. 3B). Co-expression of MAGE-A3 and MAGE-C2 was associated with overall survival (P=0.0124; Fig. 3C). The univariate analysis showed that lymph node metastasis, tumor stage and MAGE-A3 expression were significantly correlated with poor outcome (Table 5). In multivariate analysis, MAGE-A3 expression was significantly related to poorer overall survival (HR, 2.812, 95%CI 1.046~7.559; P=0.040) (Table 5).

si-MAGEs strongly and specifically suppressed corresponding gene expression in ESCC cell lines

Based on the correlation between *MAGE-A3/C2* expression and tumor progression, we tried to understand how *MAGE-A3/C2* expression influences ESCC cell biology. We selected the ESCC cell lines EC1 and KYSE70 which express high levels of *MAGE-A3* and *MAGE-C2*. To down-regulate *MAGE-A3/C2* expressions by using siRNAs specific to *MAGE-A3/C2*, both siRNAs were individually introduced into the ESCC cell lines and the effect on mRNA level examined by real-time quantitative RT-PCR analysis 48 hours post transfection. The si-*MAGE-A3/C2* were examined with 80-90% reduction at mRNA levels compared with the negative control sample transfected with scrambled siRNA (P<0.05; Fig. 4A).

Effects of MAGEs knockdown on ESCC cell apoptosis and clonogenic formation

To investigate the biological characteristics after *MAGEs* downregulation by siRNAs, we examined growth phenotypes of the EC1 and KYSE70. The knockdown of *MAGEs* genes did not exert effects on cell

Table 5. Univariate and multivariate analysis of factors associated with survival in patients with ESCC.

Patients	Univariate analysis			Multivariate analysis		
Characteristics	HR	CI (95%)	P value	HR	CI (95%)	P value
Age	0.573	0.289~1.135	0.110			
Gender	0.969	0.450~2.085	0.935			
Tumor invasion	1.652	0.808~3.378	0.169			
Lymph Node metastasis	2.225	1.081~4.579	0.030*	1.137	0.473~2.737	0.774
TNM stage	2.811	1.499~5.272	0.001*	1.361	0.518~3.578	0.532
Tumor grade	1.380	0.874~2.178	0.167			
MAGE-A3	2.552	1.557~4.183	0.000*	2.812	1.046~7.559	0.040*
MAGE-C2	1.583	0.983~2.551	0.059			

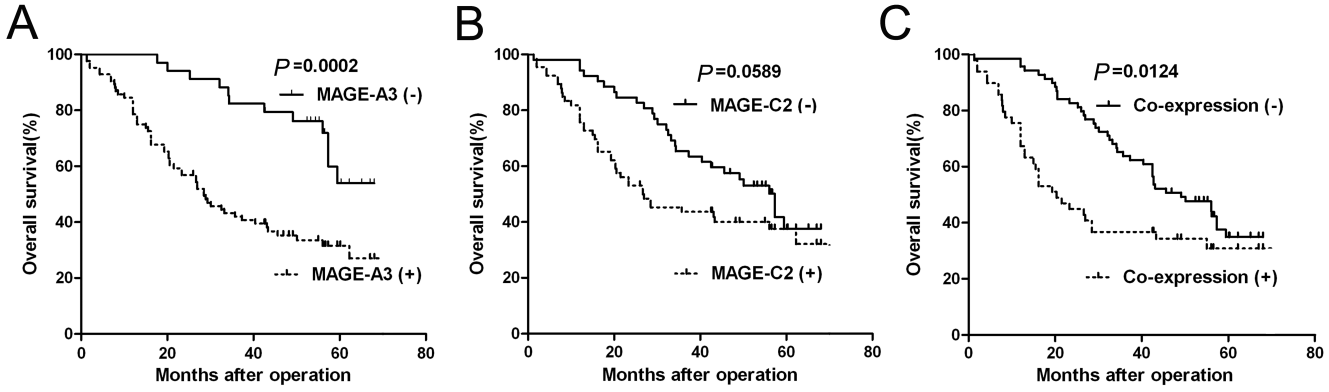


Fig. 3. Kaplan–Meier survival curves for overall survival of patients with ESCC according to MAGE-A3/C2 expression. MAGE-A3 **(A)**, MAGE-C2 **(B)** expression and co-expression of both proteins **(C)**.

apoptosis (P>0.05; Fig. 4B). We next analyzed the ability of si-*MAGE-A3/C2* cells to form colonies 7 days after transfection. In addition, down-regulations of *MAGE-A3/C2* significantly reduced the colony-forming ability of EC1 and KYSE70 cells to 50% less than control levels (P<0.05; Fig. 4C).

Effects of MAGEs knockdown on ESCC cell migration and epithelial mesenchymal transition

To determine the possible role of *MAGEs* in the migration of ESCC cell lines, we used a transwell assay. Btoh siRNAs specific to *MAGE-A3/C2* significantly inhibited migration of ESCC cells (P<0.05; Fig. 4D). Then several well-known EMT-associated factors, such as *E-cadherin* (*E-cad*), *N-cadherin* (*N-cad*), *Vimentin* (*Vim*) and *Slug* were detected. As expected, in si-*MAGE-A3/C2*-treated ESCC cells, the expression of *E-cad* was significantly increased compared to scrambled control (P<0.05), while the mesenchymal markers, *N-cad, Vim* and *Slug* were decreased (P<0.05; Fig. 4E).

Discussion

Cancer-germline genes were first discovered in 1991, and initially described limited expression in normal human germ cell lines and tumor cells (van der Bruggen et al., 1991). Recently, many studies have shown that these genes and their encoded proteins can also be frequently expressed in various malignant tumors and affect patients prognosis (Simpson et al., 2005; Caballero and Chen, 2009). It has been documented that cancer-germline genes expression is epigenetically repressed by promoter region methylation in most cells (Yang et al., 2007). Cancer-germline genes expression in cancer probably results from global hypomethylation (De Smet and Loriot, 2013), and induction of their expression has been achieved using hypomethylating

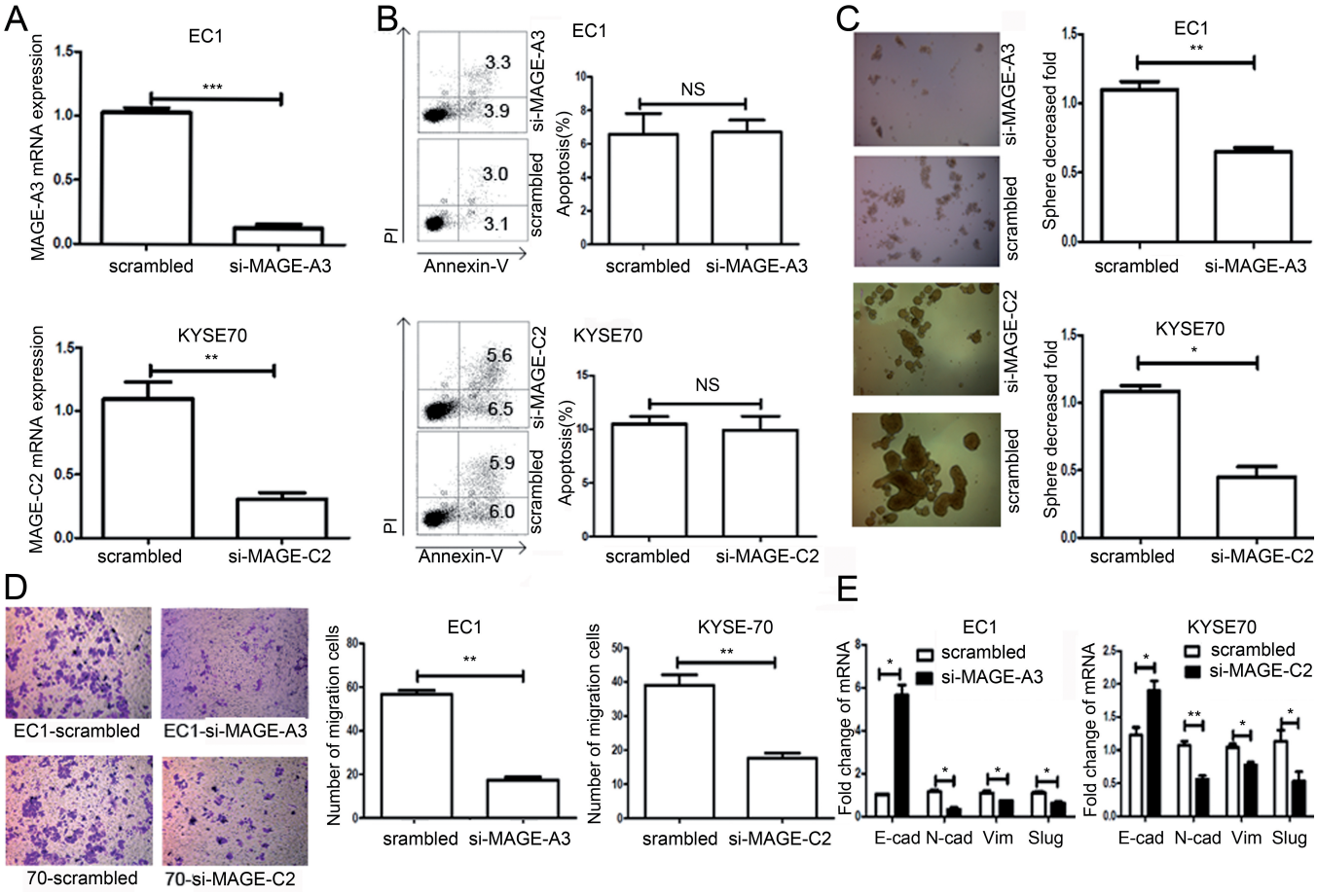


Fig. 4. Effects of down-regulation MAGE-A3 and MAGE-C2 on ESCC cell lines. **A.** Efficiency of siRNAs-mediated MAGE-A3/C2 knockdown: real-time PCR analysis was to examine the effect of the specific siRNAs on MAGEs expression at mRNA level. **B.** Effect of MAGEs knockdown on cell apoptosis was detected by Flow Cytometry. **C.** Depletion of MAGEs inhibited colony formation of ESCC cells. **D.** Down-regulation of MAGEs in ESCC cells resulted in reduced migration. **E.** Effect of MAGEs knockdown on cell EMT markers was detected by qRT-PCR. All the experiments were repeated at least three times. Bars, SD.*P<0.05, **P<0.01 and ***P<0.001 relative to scrambled negative control siRNA.

agents such as DAC (Guo et al., 2006). In addition, cancer-germline genes showed preferential expression in cancer stem-like cells and cancer-initiating cells (Yamada et al., 2013; Yin et al., 2014). Thus, immunotherapy of targeting their encoded antigens might effectively eliminate cancer stem cells to improve antitumor activity.

To assess the relevance of cancer-germline genes in patients with ESCC, we determined the expression of *MAGE-A3, MAGE-A4, MAGE-C2* and *NY-ESO-1* in ESCC *tissues*. *MAGE-A3, MAGE-A4* and *MAGE-C2* were frequently expressed in ESCC tumor tissues. According to the results, 92% of ESCC patients would be eligible for specific immunotherapeutic approaches with at least one cancer-germline gene. It has been reported that 97.5% of ESCC samples showed an over expression of at least one cancer-germline gene (Forghanifard et al., 2011). Co-expression of multiple cancer-germline genes provided a possibility of polyvalent vaccinations for ESCC. In our study, we were particularly intrigued by the expression frequency of MAGE-A3/C2. MAGE-A3 has been a leading target for immunotherapeutic approaches for cancer, due to its frequent expression in multiple tumor types. We also found the highest frequency of *MAGE-A3* expression in ESCC patients among the four cancer-germline genes. Our data indicated that *MAGE-A3* expression correlated with age, lymph node metastasis and tumor stage. Therefore, *MAGE-A3* might be involved in the development and progress of the ESCC. We further explored the protein expression of MAGE-A3 using immunohistochemistry. MAGE-A3 protein expression was significantly correlated with poor survival in ESCC. Unvaried and Multivariate analysis of factors associated with survival in patients with ESCC indicated that MAGE-A3 was an independent poor prognostic marker for ESCC. These are similar to previous reports. A high level of MAGE-A3 expression is associated with later tumor stage and a poor prognosis in lung cancer (Gure et al., 2005). MAGE-A3 was found to be expressed in all lymph node metastases in ESCC and associated with lymph node metastases in oral squamous cell carcinoma (Bujas et al., 2011; Brisam et al., 2016). Higher MAGE-A expression was found in metastatic versus primary tumors (Park et al., 2016). The antigens encoded by cancer-germline genes were correlated with advanced clinical stage but not associated with survival in squamous cell carcinoma of the larynx (Figueiredo et al., 2011). Although heterogeneous, MAGE positive staining samples showed that more than 75% of tumor cells were positive. At the cellular level, MAGE staining was both cytoplasmic and nuclear, but a nuclear localization was slightly predominant. Cancer testis antigens were expressed in nuclear and/or cytoplasm (Takahashi et al., 1995; Lin et al., 2004). In addition, a similar study reported that MAGE was positive in more than 80% cells also mainly with nuclear localization (Cuffel et al., 2011). In general, the antigen peptides were degraded in the cytoplasm and presented by HLA in APCs. MAGE peptides were tumor associated antigens presented by tumor cells expressing the immunoproteasome (Schultz et al., 2002). As in previous reports, both cytoplasm protein and nuclear protein could be degraded and presented by HLA molecules (Pavlovic et al., 2014; Berkers et al., 2015; Liu et al., 2015). MAGE-A3 positive samples showed that more than 75% of tumor cells were positive, which indicated that most tumor cells would respond to cancer immunotherapy. Thus, detection of MAGE-A3 expression in the postoperative patients with ESCC could guide the antigen specific T cell therapy to improve the survival rate, above all in advanced stage of disease. However, the positive rate of MAGE-A3 expression was lower in protein level than that in mRNA level and the same phenomenon has been reported in melanoma and colorectal cancer (Vaughan et al., 2004; Roeder et al., 2005; Akcakanat et al., 2006; Shantha Kumara et al., 2012). The reasons for this may be as follows: First, the PCR-based technique is more sensitive than immunohistochemistry method. Second, the samples used in RT-PCR and IHC methods were from different groups. In addition, the difference between RNA expression level and protein level may be due to post transcriptional regulation (Pagotto et al., 2013; Minges et al., 2015). However, in other studies, similar results of different methods (RT-PCR and IHC) used to detect the expression of cancer-germline genes in the same samples were reported in head and neck squamous cell carcinoma and lung cancer (Jungbluth et al., 2000; Cuffel et al., 2011; Wang et al., 2015). Increased lymph node metastasis and poor prognosis in patients with *MAGE-A3* expression may be explained by increased colony formation and migration ability in the *MAGE-A3* expressing ESCC cell lines shown in this study. Downregulation of *MAGE-A3* decreased migration and colony formation ability and influenced the progress of *EMT* of ESCC cells. In addition, there have been several reports on possible involvement of MAGE-A3 in cancer development and cell proliferation (Liu et al., 2008; Xie et al., 2016). These findings support MAGE-A3 not only as a useful prognostic factor but also a potential target for cancer immunotherapy.

MAGE-C2 was considered as a diagnostic, prognostic and immunotherapeutic target in patients with multiple myeloma and was an independent predictor of recurrence in prostate cancer (Pabst et al., 2010; Karbach et al., 2011). MAGE-C2 may be a good candidate for immunotherapy in patients with hepatocellular carcinoma (Wang et al., 2002). Previous studies have shown that MAGE-C2 is able to induce specific T cell response, and cytotoxic T lymphocytes directed against MAGE-C2 epitopes have been identified in melanoma patients (Ma et al., 2004). In this study, MAGE-C2 was associated with tumor stage, but not correlated with a poor prognosis. However, co-expression of MAGE-A3 and MAGE-C2 protein was correlated with poor prognostic factors and shorter survival. The frequency of multiple cancer-germline genes expression was significantly increased with disease progression in ESCC

(Liang et al., 2005). In addition, we found *MAGE-C2* also influenced the colony formation and migration ability of ESCC cells through inducing the EMT of tumor cells. MAGE-C2 was reported as an EMT inducer and associated with breast cancer metastasis (Yang et al., 2014).

MAGE-A4 was significantly associated with an advanced stage, lymph node metastasis and high grade tumors (Kocher et al., 2002). MAGE-A4 may be an optional therapeutic target for T cell receptor gene therapy to treat patients with MAGE-A4 expressing tumors (Shirakura et al., 2012). Correlation between the expressions of cancer-germline genes has been reported in other malignancies (Lethe et al., 1998). In our study, *MAGE-A4* was expressed in 58.0% patients with ESCC, but not correlated with disease progression. The mRNA expression of *MAGE-A4* was significantly associated with that of *MAGE-A3, MAGE-C2* and *NY-ESO-1*. Patients with concomitant expression of *MAGE-A4* had significantly higher frequency of *MAGE-A3, MAGE-C2* and *NY-ESO-1*. However, in another report, MAGE-A4 was associated with lymph node metastasis and advanced stage, moreover, a significant direct correlation between MAGE-A4 expression and LAGE1/NY-ESO-1 levels expression was detected in patients with ESCC (Forghanifard et al., 2011). It has been reported that coordinated promoter demethylation is associated with the aberrant co-expression of cancer-germline genes (Akers et al., 2010).

NY-ESO-1 was identified in 1998 and extensively used as a target in clinical trials including vaccines and adoptive transfer of gene-modified T cells (Chen et al., 1998; Le Gal et al., 2005; Zhao et al., 2005; Rapoport et al., 2015). NY-ESO-1 expression was reported in 15% to 50% of highly prevalent tumors and effective therapies that target NY-ESO-1 could potentially be applied to the large population of patients with these malignancies (Robbins et al., 2011). NY-ESO-1 mRNA expression was reported in 21%-40.1% ESCC specimens (Mashino et al., 2001; Fujita et al., 2004; Forghanifard et al., 2011). Among the four cancer-germline genes, *NY-ESO-1* has the lowest positive rate in ESCC. However, we did not detect any significant correlations among *NY-ESO-1* expression and clinicopathological features. However, Bujas T et al. found that NY-ESO-1 expression was directly correlated with lymph node metastasis in ESCC (Bujas et al., 2011). The difference may result from the different patient groups, number and method.

Taken together, our results showed that MAGE-A3, MAGE-C2 and MAGE-A4 were frequently expressed in patients with ESCC. Therefore, combining current treatment with immunotherapy targeting antigens encoded by cancer-germline genes, especially multi-antigen vaccine and tumor antigen–specific T cells, and may significantly improve the efficacy and life quality of ESCC patients. Furthermore, cancer-germline genes were shown as EMT inducers, and associated with ESCC metastasis. MAGE-A3 was correlated with the progress of ESCC and a poor prognosis. Thus, MAGE-A3 may be used as a potential prognostic marker for patients with ESCC.

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References

Akcakanat A., Kanda T., Tanabe T., Komukai S., Yajima K., Nakagawa S., Ohashi M. and Hatakeyama K. (2006). Heterogeneous expression of GAGE, NY-ESO-1, MAGE-A and SSX proteins in esophageal cancer: Implications for immunotherapy. Int. J. Cancer 118, 123-128.

Akers S.N., Odunsi K. and Karpf A.R. (2010). Regulation of cancer germline antigen gene expression: implications for cancer immunotherapy. Future Oncol. 6, 717-732.

Ayyoub M., Pignon P., Classe J.M., Odunsi K. and Valmori D. (2013). CD4+ T effectors specific for the tumor antigen NY-ESO-1 are highly enriched at ovarian cancer sites and coexist with, but are distinct from, tumor-associated Treg. Cancer Immunol. Res. 1, 303-308.

Berkers C.R., de Jong A., Schuurman K.G., Linnemann C., Geenevasen J.A., Schumacher T.N., Rodenko B. and Ovaa H. (2015). Peptide splicing in the proteasome creates a novel type of antigen with an isopeptide linkage. J. Immunol. 195, 4075-4084.

Brisam M., Rauthe S., Hartmann S., Linz C., Brands R.C., Kubler A.C., Rosenwald A. and Muller-Richter U.D. (2016). Expression of MAGE-A1-A12 subgroups in the invasive tumor front and tumor center in oral squamous cell carcinoma. Oncol. Rep. 35, 1979-1986.

Bujas T., Marusic Z., Peric Balja M., Mijic A., Kruslin B. and Tomas D. (2011). MAGE-A3/4 and NY-ESO-1 antigens expression in metastatic esophageal squamous cell carcinoma. Eur. J. Histochem. 55, e7.

Caballero O.L. and Chen Y.T. (2009). Cancer/testis (CT) antigens: potential targets for immunotherapy. Cancer Sci. 100, 2014-2021.

Caballero O.L., Cohen T., Gurung S., Chua R., Lee P., Chen Y.T., Jat P. and Simpson A.J. (2013). Effects of CT-Xp gene knock down in melanoma cell lines. Oncotarget 4, 531-541.

Chen Y.T., Gure A.O., Tsang S., Stockert E., Jager E., Knuth A. and Old L.J. (1998). Identification of multiple cancer/testis antigens by allogeneic antibody screening of a melanoma cell line library. Proc. Natl. Acad. Sci. USA 95, 6919-6923.

Coulie P.G., Van den Eynde B.J., van der Bruggen P. and Boon T. (2014). Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. Nat. Rev. Cancer 14, 135-146.

Cruz C.R., Gerdemann U., Leen A.M., Shafer J.A., Ku S., Tzou B., Horton T.M., Sheehan A., Copeland A., Younes A., Rooney C.M., Heslop H.E. and Bollard C.M. (2011). Improving T-cell therapy for relapsed EBV-negative Hodgkin lymphoma by targeting upregulated MAGE-A4. Clin. Cancer Res. 17, 7058-7066.

Cuffel C., Rivals J.P., Zaugg Y., Salvi S., Seelentag W., Speiser D.E., Lienard D., Monnier P., Romero P., Bron L. and Rimoldi D. (2011). Pattern and clinical significance of cancer-testis gene expression in head and neck squamous cell carcinoma. Int. J. Cancer 128, 2625-2634.

De Smet C. and Loriot A. (2013). DNA hypomethylation and activation of germline-specific genes in cancer. Adv. Exp. Med. Biol. 754, 149-166.

Figueiredo D.L., Mamede R.C., Spagnoli G.C., Silva W.A. Jr, Zago M., Neder L., Jungbluth A.A. and Saggioro F.P. (2011). High expression of cancer testis antigens MAGE-A, MAGE-C1/CT7, MAGE-C2/CT10, NY-ESO-1, and gage in advanced squamous cell carcinoma of the larynx. Head Neck 33, 702-707.

Forghanifard M.M., Gholamin M., Farshchian M., Moaven O. Memar B., Forghani M.N., Dadkhah E., Naseh H., Moghbeli M., Raeisossadati R. and Abbaszadegan M.R. (2011). Cancer-testis gene expression profiling in esophageal squamous cell carcinoma: identification of specific tumor marker and potential targets for immunotherapy. Cancer Biol. Ther. 12, 191-197.

Fujita S., Wada H., Jungbluth A.A., Sato S., Nakata T., Noguchi Y., Doki Y., Yasui M., Sugita Y., Yasuda T., Yano M., Ono T., Chen Y.T., Higashiyama M., Gnjatic S., Old L.J., Nakayama E. and Monden M. (2004). NY-ESO-1 expression and immunogenicity in esophageal cancer. Clin. Cancer Res. 10, 6551-6558.

Guo Z.S., Hong J.A., Irvine K.R., Chen G.A., Spiess P.J., Liu Y., Zeng G., Wunderlich J.R., Nguyen D.M., Restifo N.P. and Schrump D.S. (2006). De novo induction of a cancer/testis antigen by 5-aza-2'-deoxycytidine augments adoptive immunotherapy in a murine tumor model. Cancer Res. 66, 1105-1113.

Gure A.O., Chua R., Williamson B., Gonen M., Ferrera C.A., Gnjatic S., Ritter G., Simpson A.J., Chen, Y.T., Old L.J. and Altorki N.K. (2005). Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. Clin. Cancer Res. 11, 8055-8062.

Hottenrott C. (2013). Predictive medicine and esophageal cancer response to preoperative chemotherapy. Surg. Endosc. 27, 347-348.

Imai N., Ikeda H. and Shiku H. (2012). Targeting cancer antigen (MAGE-A4, NY-ESO -1) for immunotherapy. Nihon Rinsho 70, 2125-2129. (in Japanese).

John T., Starmans M.H., Chen Y.T., Russell P.A., Barnett S.A., White S.C., Mitchell P.L., Walkiewicz M., Azad A., Lambin P., Tsao M.S., Deb S., Altorki N., Wright G., Knight S., Boutros P.C. and Cebon J.S. (2013). The role of Cancer-Testis antigens as predictive and prognostic markers in non-small cell lung cancer. PloS one 8, e67876.

Jungbluth A.A., Stockert E., Chen Y.T., Kolb D., Iversen K., Coplan K., Williamson B., Altorki N., Busam K.J. and Old L.J. (2000). Monoclonal antibody MA454 reveals a heterogeneous expression pattern of MAGE-1 antigen in formalin-fixed paraffin embedded lung tumours. Br. J. Cancer 83, 493-497.

Karbach J., Neumann A., Atmaca A., Wahle C., Brand K., von Boehmer L., Knuth A., Bender A., Ritter G., Old L.J. and Jager E. (2011). Efficient *in vivo* priming by vaccination with recombinant NY-ESO-1 protein and CpG in antigen naive prostate cancer patients. Clin. Cancer Res. 17, 861-870.

Kocher T., Zheng M., Bolli M., Simon R., Forster T., Schultz-Thater E., Remmel E., Noppen C., Schmid U., Ackermann D., Mihatsch M.J., Gasser T., Heberer M., Sauter G. and Spagnoli G.C. (2002). Prognostic relevance of MAGE-A4 tumor antigen expression in transitional cell carcinoma of the urinary bladder: a tissue microarray study. Int. J. Cancer 100, 702-705.

Krishnadas D.K., Bao L., Bai F., Chencheri S.C. and Lucas K. (2014). Decitabine facilitates immune recognition of sarcoma cells by upregulating CT antigens, MHC molecules, and ICAM-1. Tumour Biol. 35, 5753-5762.

Le Gal F.A., Ayyoub M., Dutoit V., Widmer V., Jager E., Cerottini J.C., Dietrich P.Y. and Valmori D. (2005). Distinct structural TCR repertoires in naturally occurring versus vaccine-induced CD8+ T-cell responses to the tumor-specific antigen NY-ESO-1. J. Immunother. 28, 252-257.

Lethe B., Lucas S., Michaux L., De Smet C., Godelaine D., Serrano A., De Plaen E. and Boon T. (1998). LAGE-1, a new gene with tumor specificity. Int. J. Cancer 76, 903-908.

Liang Z., Sun Z.Y., Yuan Y.H., Han Y., Wang Y., Gu J. and Zhang L.J. (2005). The expression of 11 cancer/testis (CT) antigen genes in esophageal carcinoma. Zhonghua Zhong Liu Za Zhi 27, 534-537. (in chinese).

Lin J., Lin L., Thomas D.G., Greenson J.K., Giordano T.J., Robinson G.S., Barve R.A., Weishaar F.A., Taylor J.M., Orringer M.B. and Beer D.G. (2004). Melanoma-associated antigens in esophageal adenocarcinoma: identification of novel MAGE-A10 splice variants. Clin. Cancer Res. 10, 5708-5716.

Liu W., Cheng S., Asa S.L. and Ezzat S. (2008). The melanoma-associated antigen A3 mediates fibronectin-controlled cancer progression and metastasis. Cancer Res. 68, 8104-8112.

Liu X., Sun N., Dong Y., Li J., Liu Y., Ren Y., Yang C., Zhang L., Zhou Y., Tong Z., Ding J., Zhao X., Liu Y., He R., Xu K., Wu Y., Huang Y., Wang H., Tao X. and Liu Y. (2015). Anticancer effects of adenovirus-mediated calreticulin and melanoma-associated antigen 3 expression on non-small cell lung cancer cells. Int. Immunopharmacol. 25, 416-424.

Ma W., Germeau C., Vigneron N., Maernoudt A.S., Morel S., Boon T., Coulie P.G. and Van den Eynde B.J. (2004). Two new tumor-specific antigenic peptides encoded by gene MAGE-C2 and presented to cytolytic T lymphocytes by HLA-A2. Int. J. Cancer 109, 698-702.

Mashino K., Sadanaga N., Tanaka F., Yamaguchi H., Nagashima H., Inoue H., Sugimachi K. and Mori M. (2001). Expression of multiple cancer-testis antigen genes in gastrointestinal and breast carcinomas. Br. J. Cancer 85, 713-720.

Minges J.T., Grossman G., Zhang P., Kafri T. and Wilson E.M. (2015). Post-translational Down-regulation of Melanoma Antigen-A11 (MAGE-A11) by Human p14-ARF Tumor Suppressor. J. Biol. Chem. 290, 25174-25187.

Pabst C., Zustin J., Jacobsen F., Luetkens T., Kroger N., Schilling G., Bokemeyer C., Sauter G., Atanackovic D. and Marx A. (2010). Expression and prognostic relevance of MAGE-C1/CT7 and MAGE-C2/CT10 in osteolytic lesions of patients with multiple myeloma. Exp. Mol. Pathol. 89, 175-181.

Pagotto A., Caballero O.L., Volkmar N., Devalle S., Simpson A.J., Lu X. and Christianson J.C. (2013). Centrosomal localisation of the cancer/testis (CT) antigens NY-ESO-1 and MAGE-C1 is regulated by proteasome activity in tumour cells. PloS One 8, e83212.

Park T.S., Groh E.M., Patel K., Kerkar S.P., Lee C.C. and Rosenberg S.A. (2016). Expression of MAGE-A and NY-ESO-1 in Primary and Metastatic Cancers. J. Immunother. 39, 1-7.

Pavlovic M.D., Jandrlic D.R. and Mitic N.S. (2014). Epitope distribution

in ordered and disordered protein regions. Part B - Ordered regions and disordered binding sites are targets of T- and B-cell immunity. J. Immunolog. Met. 407, 90-107.

Rapoport A.P., Stadtmauer E.A., Binder-Scholl G.K., Goloubeva O., Vogl D.T., Lacey S.F., Badros A.Z., Garfall A., Weiss B., Finklestein J., Kulikovskaya I., Sinha S.K., Kronsberg S., Gupta M., Bond S., Melchiori L., Brewer J.E., Bennett A.D., Gerry A.B., Pumphrey N.J., Williams D., Tayton-Martin H.K., Ribeiro L., Holdich T., Yanovich S., Hardy N., Yared J., Kerr N., Philip S., Westphal S., Siegel D.L., Levine B.L., Jakobsen B.K., Kalos M. and June C.H. (2015). NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat. Med. 21, 914-921.

Riener M.O., Wild P.J., Soll C., Knuth A., Jin B., Jungbluth A., Hellerbrand C., Clavien P.A., Moch H. and Jochum W. (2009). Frequent expression of the novel cancer testis antigen MAGE-C2/CT-10 in hepatocellular carcinoma. Int. J. Cancer 124, 352-357.

Robbins P.F., Morgan R.A., Feldman S.A., Yang J.C., Sherry R.M., Dudley M.E., Wunderlich J.R., Nahvi A.V., Helman L.J., Mackall C.L., Kammula U.S., Hughes M.S., Restifo N.P., Raffeld M., Lee C.C., Levy C.L., Li Y.F., El-Gamil M., Schwarz S.L., Laurencot C. and Rosenberg S.A. (2011). Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J. Clin. Oncol. 29, 917-924.

Roeder C., Schuler-Thurner B., Berchtold S., Vieth G., Driesch P., Schuler G. and Luftl M. (2005). MAGE-A3 is a frequent tumor antigen of metastasized melanoma. Arch. Dermatol. Res. 296, 314-319.

Scanlan M.J., Simpson A.J. and Old L.J. (2004). The cancer/testis genes: review, standardization, and commentary. Cancer Immun. 4, 1.

Schultz E.S., Chapiro J., Lurquin C., Claverol S., Burlet-Schiltz O., Warnier G., Russo V., Morel S., Levy F., Boon T., Van den Eynde B.J. and van der Bruggen P. (2002). The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immunoproteasome. J. Exp. Med. 195, 391-399.

Shantha Kumara H.M., Grieco M.J., Caballero O.L., Su T., Ahmed A., Ritter E., Gnjatic S., Cekic V., Old L.J., Simpson A.J., Cordon-Cardo C. and Whelan R.L. (2012). MAGE-A3 is highly expressed in a subset of colorectal cancer patients. Cancer Immun. 12, 16.

Shirakura Y., Mizuno Y., Wang L., Imai N., Amaike C., Sato E., Ito M., Nukaya I., Mineno J., Takesako K., Ikeda H. and Shiku H. (2012). T-cell receptor gene therapy targeting melanoma-associated antigen-A4 inhibits human tumor growth in non-obese diabetic/SCID/ γcnull mice. Cancer Sci. 103, 17-25.

Simpson A.J., Caballero O.L., Jungbluth A., Chen Y.T. and Old L.J. (2005). Cancer/testis antigens, gametogenesis and cancer. Nat. Rev. Cancer 5, 615-625.

Song Y., Li L., Ou Y., Gao Z., Li E., Li X., Zhang W., Wang J., Xu L., Zhou Y., Ma X., Liu L., Zhao Z., Huang X., Fan J., Dong L., Chen G., Ma L., Yang J., Chen L., He M., Li M., Zhuang X., Huang K., Qiu K., Yin G., Guo G., Feng Q., Chen P., Wu Z., Wu J., Zhao J., Luo L., Fu M., Xu B., Chen B., Li Y., Tong T., Wang M., Liu Z., Lin D., Zhang X., Yang H. and Zhan Q. (2014). Identification of genomic alterations in oesophageal squamous cell cancer. Nature 509, 91-95.

Takahashi K., Shichijo S., Noguchi M., Hirohata M. and Itoh K. (1995). Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. Cancer Res. 55, 3478-3482.

van der Bruggen P., Traversari C., Chomez P., Lurquin C., De Plaen E., Van den Eynde B., Knuth A. and Boon T. (1991). A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254, 1643-1647.

Van Der Bruggen P., Zhang Y., Chaux P., Stroobant V., Panichelli C., Schultz E.S., Chapiro J., Van Den Eynde B.J., Brasseur F. and Boon T. (2002). Tumor-specific shared antigenic peptides recognized by human T cells. Immunol. Rev. 188, 51-64.

Vaughan H.A., Svobodova S., Macgregor D., Sturrock S., Jungbluth A.A., Browning J., Davis I.D., Parente P., Chen Y.T., Stockert E., St Clair F., Old L.J. and Cebon, J. (2004). Immunohistochemical and molecular analysis of human melanomas for expression of the human cancer-testis antigens NY-ESO-1 and LAGE-1. Clin. Cancer Res. 10, 8396-8404.

Wang J.Y., Cao M., Guo M.R., Li S., Yang X.F., Wang M., Fang J. and Zhao J. (2015). Expression and antibody generation of the cancer-testis antigen, BIOT2-S. Genet. Mol. Res. 14, 8685-8693.

Wang Y., Han K.J., Pang X.W., Vaughan H.A., Qu W., Dong X.Y., Peng J.R., Zhao H.T., Rui J.A., Leng X.S., Cebon J., Burgess A.W. and Chen W.F. (2002). Large scale identification of human hepatocellular carcinoma-associated antigens by autoantibodies. J. Immunol. 169, 1102-1109.

Xie C., Subhash V.V., Datta A., Liem N., Tan S.H., Yeo M.S., Tan W.L., Koh V., Yan F.L., Wong F.Y., Wong W.K., So J., Tan I.B., Padmanabhan N., Yap C.T., Tan P., Goh L.K. and Yong, W.P. (2016). Melanoma associated antigen (MAGE)-A3 promotes cell proliferation and chemotherapeutic drug resistance in gastric cancer. Cell. Oncol. 39, 175-186.

Yamada R., Takahashi A., Torigoe T., Morita R., Tamura Y., Tsukahara T., Kanaseki T., Kubo T., Watarai K., Kondo T., Hirohashi Y. and Sato N. (2013). Preferential expression of cancer/testis genes in cancer stem-like cells: proposal of a novel sub-category, cancer/testis/stem gene. Tissue Antigens 81, 428-434.

Yang B., Wu J., Maddodi N., Ma Y., Setaluri V. and Longley B.J. (2007). Epigenetic control of MAGE gene expression by the KIT tyrosine kinase. J. Invest. Dermatol. 127, 2123-2128.

Yang F., Zhou X., Miao X., Zhang T., Hang X., Tie R., Liu N., Tian F., Wang F. and Yuan J. (2014). MAGEC2, an epithelial-mesenchymal transition inducer, is associated with breast cancer metastasis. Breast Cancer Res. Treat. 145, 23-32.

Yin B., Zeng Y., Liu G., Wang X., Wang P. and Song Y. (2014). MAGE-A3 is highly expressed in a cancer stem cell-like side population of bladder cancer cells. Int. J. Clin. Exp. Pathol. 7, 2934-2941.

Yue D., Fan Q., Chen X., Li F., Wang L., Huang L., Dong W., Zhang Z., Liu J., Wang F., Wang M., Zhang B. and Zhang Y. (2014). Epigenetic inactivation of SPINT2 is associated with tumor suppressive function in esophageal squamous cell carcinoma. Exp. Cell. Res. 322, 149-158.

Zhao Y., Zheng Z., Robbins P.F., Khong H.T., Rosenberg S.A. and Morgan R.A. (2005). Primary human lymphocytes transduced with NY-ESO-1 antigen-specific TCR genes recognize and kill diverse human tumor cell lines. J. Immunol. 174, 4415-4423.

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