ANTICANCER RESEARCH *39*: 6015-6023 (2019)
doi:10.21873/anticanres.13807

Expression, Function, and Prognostic Value of MAGE-D4 Protein in Esophageal Squamous Cell Carcinoma

YASUO UNO[1], MITSURO KANDA[1], YUSUKE SATO[2], DAI SHIMIZU[1], SHINICHI UMEDA[1], NORIFUMI HATTORI[1], MASAMICHI HAYASHI[1], CHIE TANAKA[1], DAISUKE KOBAYASHI[1], SUGURU YAMADA[1], GORO NAKAYAMA[1], SATORU MOTOYAMA[2], MASAHIKO KOIKE[1] and YASUHIRO KODERA[1]

[1]*Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan;*
[2]*Department of Thoracic Surgery (Surgery II), Akita University Graduate School of Medicine, Akita, Japan*

Abstract. *Background/Aim: We previously reported that expression of melanoma-associated antigen (MAGE)-D4 mRNA was a prognostic factor for esophageal squamous cell carcinoma (ESCC). The aim of this study was to validate the expression of MAGE-D4 in two additional patient cohorts, and to investigate its biological functions. Materials and Methods: The role of MAGE-D4 in cell proliferation, adhesion, and migration was determined by gene knockdown experiments in the KYSE590 cell line. MAGE-D4 protein expression was analyzed in ESCC tissues by immunohistochemistry. A second validation cohort consisted of an ESCC mRNA dataset from The Cancer Genome Atlas. Results: Knockdown of MAGE-D4 significantly decreased cell proliferation and migration. Expression of MAGE-D4 protein was significantly associated with disease-free survival. In the second validation cohort, high MAGE-D4 mRNA expression was associated with significantly shorter overall survival and disease-free survival. Conclusion: MAGE-D4 plays an important role in the malignant behavior of ESCC. MAGE-D4 was validated as a prognostic indicator in two independent ESCC patient cohorts.*

Esophageal cancer (EC) is the eighth most common cancer worldwide, with 456,000 new cases per year and about 400,000 deaths per year (1). Esophageal squamous cell carcinoma (ESCC) is more common in Asia and the developing world than in the West. Despite the improved multimodal treatments, there is no currently effective systemic therapy for advanced ESCC, and the prognosis remains poor (2). A better understanding of the mechanisms of ESCC development and progression is necessary to advance the development of novel diagnostic markers and therapeutic targets.

Members of the large melanoma-associated antigen (MAGE) family of proteins, so named because the first member was isolated from a melanoma cell line, have important physiological and pathological functions, and many members have been reported to play important roles in the progression of various malignant tumors (3-7). More than 50 *MAGE* genes have been identified to date (8), which can be broadly categorized into type I and type II genes based on differences in gene structure and tissue-specific expression (9). Type I MAGEs are expressed during germ cell development and are then silenced in normal mature somatic cells (10, 11). In contrast, relatively little is known about the localization and expression of type II MAGE proteins. Studies on type II MAGE-D4 have reported that its expression in normal tissues is restricted to the brain and ovary; however, it is overexpressed in several human malignancies, including lung, liver, oral, kidney, breast, and colorectal cancers, glioma (12-18), and ESCC, as previously reported by us (19). MAGE-D4 is a prominent tumor-associated antigen and has been shown to contribute to the proliferation, migration, and invasion of breast cancer and oral squamous cell carcinoma cells (14, 16). We have reported that high tumor expression of *MAGE-D4* was significantly associated with shorter overall survival (OS) of ESCC patients and that *MAGE-D4* overexpression was an independent prognostic factor (19). However, that study had several important limitations, including a relatively small sample size (n=65) from a single institute, no data on protein expression, and no functional analyses to understand the potential oncological roles of MAGE-D4 in ESCC.

The present study was designed to validate and extend our previous findings. To this end, we analyzed the biological functions of MAGE-D4 in ESCC cell lines *in vitro* and evaluated the relationship between survival and MAGE-D4

Correspondence to: Dr. Mitsuro Kanda, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: +81 527442249, Fax: +81 527442255, e-mail: m-kanda@med.nagoya-u.ac.jp

Key Words: Esophageal cancer, *MAGE-D4*, biomarker.

Table I. *Sequences of primers and siRNAs.*

	Experiment	Type	Sequence (5'-3')	Product size	Annealing temperature
MAGE-D4	qRT-PCR	Forward	ACTACGCAAGATGGGACTGC	109 bp	60˚C
		Reverse	TTGCTGTTGGGGATCTTCTT		
	siRNA	*siMAGE-D4-1*	UAUUUCACCAACUUAUUUGCC		
		siMAGE-D4-2	CAAAUAAGUUGGUGAAAUA		
		siMAGE-D4-3	AAUAUACAGGUGUUCUUCCUU		
GAPDH	qRT-PCR	Forward	GAAGGTGAAGGTCGGAGTC	226 bp	60˚C
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTC		

MAGE-D4: Melanoma-associated antigen-D4; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: quantitative reverse-transcription polymerase chain reaction; siRNA: small interfering RNA; bp: base pair.

expression at both the mRNA and protein levels using two additional patient cohorts.

Materials and Methods

Ethics. This study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects and was approved by the Institutional Review Board of Nagoya University, Japan (approval number 2014-0044) and the Ethics Committee of Akita University School of Medicine (number 1495). Written informed consent for the use of clinical samples and data was obtained from all patients, either directly or through the appropriate database.

Sample collection. Thirteen human ESCC cell lines (NUEC2, TE1, TE2, TE3, TT, TTn, WSSC, KYSE510, KYSE590, KYSE890, KYSE1170, KYSE1260, and KYSE1440) were obtained from the American Type Culture Collection (Manassas, VA, USA) or the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) (20). Cells were stored at –80˚C in a cell preservative (Cell Banker; LSI Medience Corporation, Tokyo, Japan) and cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an atmosphere containing 5% CO_2 at 37˚C.

For the first validation cohort, primary ESCC tissues were collected from 177 patients who underwent esophageal resection for EC at the Department of Thoracic Surgery, Akita University Hospital between 2000 and 2011 (21). The patients had received no treatment before curative surgery. The tissue specimens were embedded in paraffin and a Tissue Microarray (TMA) was constructed at the Pathology Institute (Toyama, Japan) (22-24), and stained as described below. Specimens were classified histologically using the 8th edition of the Union for International Cancer Control (UICC) classification. Relevant clinicopathological parameters were acquired from medical records. For the second validation cohort, an RNA sequencing dataset from 184 ESCC patients was obtained from TCGA *via* cBioPortal (http://www.cbioportal.org/).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and PCR array analysis. MAGE-D4 mRNA expression was determined by qRT-PCR. Total RNA isolated using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) from cell lines. Reverse transcription was performed as follows: 10.5 μl 1 μg/μl RNA, 4 μl of 5X first strand buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 μl 100 mM dithiothreitol (Thermo Fisher Scientific, Inc.), 1 μl 10 mM dNTP mix (Promega Corporation, Madison, WI, USA), 1 μl random primers (Roche Diagnostics, Basel, Switzerland), 1 μl 200 U/μl Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Inc.) and 0.5 μl RNase inhibitor (Roche Diagnostics) were mixed and incubated for 60 min at 37˚C. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression levels (TaqMan; *GAPDH* control reagents; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were quantified, and the data were used to normalize the expression levels. qRT-PCR was performed using the SYBR Green PCR Core Reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: One cycle at 95˚C for 10 min; 40 cycles at 95˚C for 5 sec and 60˚C for 60 sec without a final extension step. The samples were tested in triplicate, and samples without a template were included in each PCR plate as a negative control. Real-time SYBR Green fluorescence was detected using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) (25) and the 2-ΔΔCq method was used for PCR quantification (26). The expression level of each sample is expressed as the value of the *MAGE-D4* amplicon divided by that of *GAPDH* (27). The PCR primers are listed in Table I. A Human Epithelial to Mesenchymal Transition (EMT) RT^2 Profiler PCR Array (Qiagen, Hilden, Germany) kit was used to assess expression of 84 key genes that encode transcription factors, ECM proteins as well as proteins involved in EMT, cell differentiation, morphogenesis, growth, proliferation, migration, cytoskeleton and major signalling pathways (27).

Small interfering RNA (siRNA)-mediated knockdown of MAGE-D4. KYSE590 cells were plated at 5×10^4 cells/ml in 24-well plates and incubated overnight. The cells were then transiently transfected with 30 nM of siRNA (siMAGE-D4 or siControl; sequences given in Table I) using LipoTrust EX Oligo (Hokkaido System Science, Sapporo, Japan). After transfection, cells were cultured in antibiotic-free RPMI-1640 with 10% FBS for 72 h before use in functional assays.

Cell proliferation assays. Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells (3×10^3 cells per well) were seeded into 96-

well plates with RPMI-1640 containing 2% FBS. The optical density of the solution in each well was measured 1, 3, and 5 days after seeding, 2 h after the addition of 10 ml of Cell CCK-8 solution (27).

Cell adhesion assay. A CytoSelect 48-Well Cell Adhesion Assay kit (Cell Biolabs, San Diego, CA, USA) was used to measure cell adhesion to the extracellular matrix (ECM) components fibronectin, collagen I, collagen IV, laminin I, and fibrinogen, with bovine serum albumin as a control. Cells (1.5×10^5 cells per well) were added to each well, and optical density at 560 nm of each well was measured after 1 h (28).

Wound-healing assay. Cell migration was evaluated using wound-healing assays. Cells (2×10^4 cells per well) were seeded into 12-well plates in RPMI-1640 containing 2% FBS using the ibidi Culture insert method (ibidi GmbH, Martinsried, Germany). After 24 h, the insert was removed, and the wound width was measured in 100-mm intervals (20 measurements per well, 40× magnification) (29).

Immunohistochemical (IHC) staining. TMA blocks were sectioned and incubated for 1 h at room temperature (25˚C) with a rabbit anti-MAGE-D4b polyclonal antibody (HPA003554, Sigma-Aldrich) diluted 1:1000 in ChemMate antibody diluent (Dako, Carpinteria, CA, USA). For semi-quantification of MAGE-D4 protein expression, two investigators who were blinded to the clinical data evaluated the tissue staining and assigned a score based on the HER2 IHC scoring system (American Society of Clinical Oncology/College of American Pathologists guidelines). Protein expression was scored as 3+ (intense cytoplasmic or nuclear staining in >30% of cells), 2+ (moderate cytoplasmic or nuclear staining in >10% of cells), 1+ (weak staining in <10% of cells), and 0 (no staining, negative) (21).

Statistical analysis. Data are presented as the means±standard deviation (SD) of the indicated number of replicates unless noted. The χ^2 test was used to analyze associations between MAGE-D4 expression and clinicopathological parameters. The significance of the difference between two variables was assessed using Spearman's rank correlation coefficient. OS and disease-free survival (DFS) were calculated using the Kaplan–Meier method, and differences were analyzed using the log-rank test. Prognostic factors were identified using Cox proportional hazards models, and variables with $p<0.05$ in the univariate analysis were entered into the final model. All analyses were performed using JMP 13 software (SAS Institute, Cary, NC, USA). $p<0.05$ was considered statistically significant.

Table II. *Tumor expression of MAGE-D4 and clinical characteristics of ESCC patients (n=177).*

Variables	*MAGED4* negative (n=83)	*MAGED4* positive (n=94)	*p*-Value
Age			
<70 year	51	58	0.972
≥70 year	32	36	
Gender			
Male	70	83	0.443
Female	13	11	
Brinkman index			
<1000	54	68	0.296
≥1000	29	26	
Alcohol consumption			
Absent	11	11	0.755
Present	72	83	
Tumor location			
Upper third	4	2	0.533
Middle third	52	64	
Lower third	27	28	
Tumor depth (UICC)			
pT2	16	15	0.842
pT3	63	74	
pT4a	4	5	
Differentiation			
Differentiated	60	60	0.229
Undifferentiated	23	34	
Lymphatic involvement			
Ly 0-1	30	28	0.368
Ly 2-3	53	66	
Vascular invasion			
V 0-1	24	23	0.468
V 2-3	57	70	
Lymph node metastasis			
Absent	26	23	0.309
Present	57	71	
UICC stage			
I	6	4	0.439
II	23	21	
III	54	69	

ESCC: Esophageal squamous cell carcinoma; *MAGE-D4*: melanoma-associated antigen-D4; UICC: Union for International Cancer Control.

Results

Coordinated expression of MAGE-D4 and cancer-related genes in ESCC cell lines. In order to investigate the expression and function of MAGE-D4 in ESCC, we first examined the mRNA levels of MAGE-D4 and a panel of 84 EMT-related genes in 13 ESCC cell lines of differentiated (n=6), undifferentiated (n=3), and unknown differentiation (n=4) status. qRT-PCR analysis revealed no significant difference in *MAGE-D4* mRNA levels among the ESCC cell lines (Figure 1A). PCR array analysis of 84 EMT-related genes identified strong correlations (Spearman coefficient ≥0.6) between the expression of MAGE-D4 and five EMT-related genes; namely, matrix metalloproteinase 9 (*MMP9*), transforming growth factor β1 (*TGFB1*), transmembrane protein 132A (*TMEM132A*), snail family transcriptional repressor 3 (*SNAI3*), and versican (*VCAN*) (Figure 1B, C).

Effect of MAGE-D4 knockdown on the biological activities of ESCC cells. Given the importance of the EMT in the development of malignant behavior, we next evaluated the importance of MAGE-D4 in cell proliferation, migration, and adhesion. The KYSE590 cell line, which expressed the highest levels of *MAGE-D4* mRNA, was transfected with a

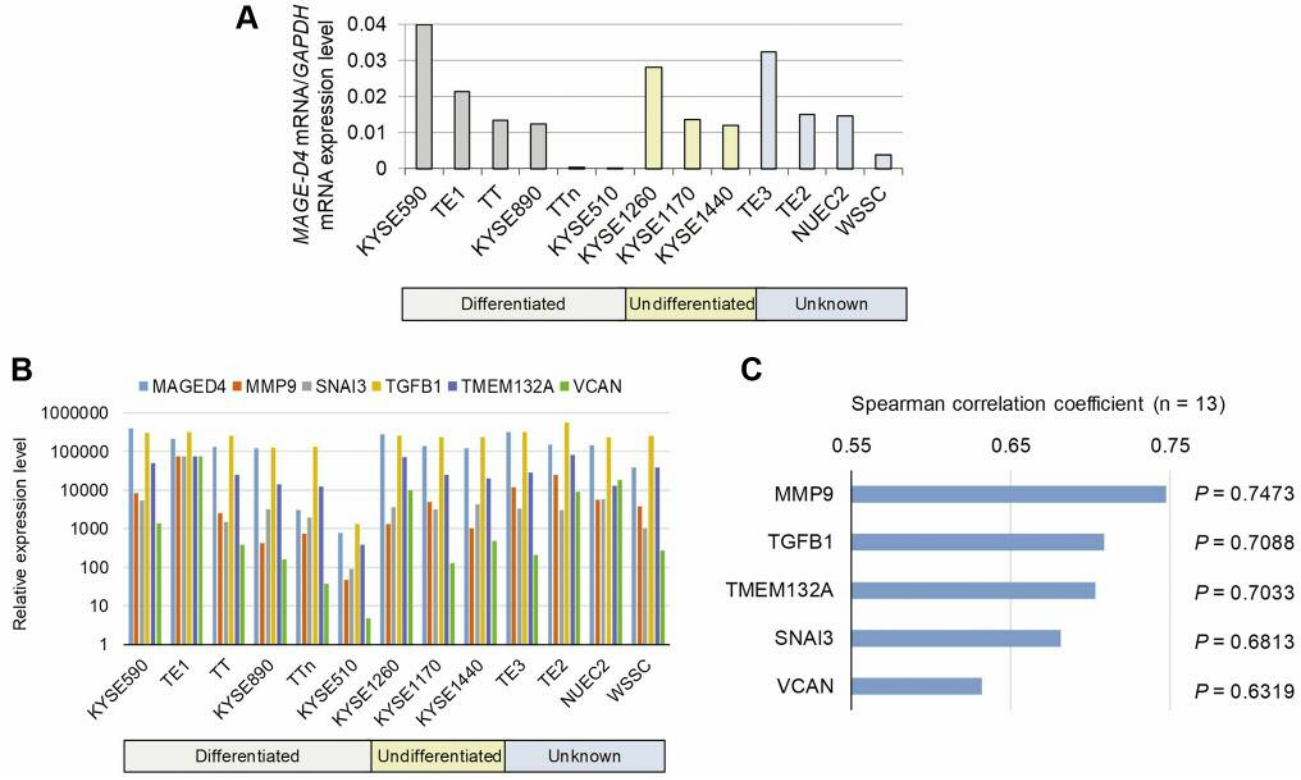

Figure 1. *Analysis of MAGE-D4 expression in 13 ESCC cell lines. (A) qRT-PCR analysis of MAGE-D4 mRNA normalized to GAPDH mRNA levels. (B) PCR array analysis of MAGE-D4 and EMT-related genes. Data were normalized to TE1 mRNA levels. (C) Correlation between MAGE-D4 and MMP9, TGFB1, TMEM132A, SNAI3, and VCAN mRNA levels, using Spearman's rank correlation coefficient.*

control sequence (siControl) or *MAGE-D4*-targeting siRNA (siMAGE-D4) and analyzed by qRT-PCR to confirm effective gene silencing (Figure 2A). Next, we evaluated cell proliferation using the CCK-8 assay, interactions with five ECM proteins (fibronectin, collagen I, collagen IV, laminin I, and fibrinogen) using an adhesion assay, and cell migration using a wound-healing assay. Notably, compared with the control cells, MAGE-D4 knockdown significantly decreased the proliferation of KYSE590 cells at 72 h and 120 h after transfection (Figure 2B). Similarly, cell migration at 24 h and 36 h after transfection was significantly suppressed by *MAGE-D4* knockdown (Figure 2C). However, MAGE-D4 knockdown had little influence on the ability of KYSE590 cells to adhere to the five ECM proteins. Thus, MAGE-D4 plays a crucial role in ESCC cell proliferation and migration; two fundamental behaviors associated with tumor growth and metastasis.

Expression and prognostic value of MAGE-D4 protein in the first validation cohort. To investigate the expression of MAGE-D4 protein and its relationship to clinicopathological characteristics we performed IHC staining on TMA of tumor and normal tissue samples collected from 177 patients undergoing curative surgery for ESCC. The population was composed of 153 males and 24 females (66±8.2 years, mean±SD; range=38-82 years) with pathologically diagnosed differentiated (n=120) and undifferentiated (n=57) ESCC. Based on the UICC classification (eighth edition), 10, 44, and 123 patients had stage I, II, and III ESCC, respectively.

IHC staining of MAGE-D4 protein was evaluated using a semiquantitative scoring system that evaluates the localization, intensity, and extent of protein expression. Representative photomicrographs of specimens with negative (0) and positive (3+, 2+, 1+) expression are shown in Figure 3A. Of the 177 patient samples, 83 (46.9%) scored 0, 61 (34.5%) scored 1+, 31 (17.5%) scored 2+, and 2 (1.1%) scored 3+. For evaluation of the prognostic value of MAGE-D4 protein expression, patients were dichotomized into a MAGE-D4-positive group (scores 1+, 2+, or 3+; n=83) and a MAGE-D4-negative group (score 0; n=94), and OS and DFS were compared using the Kaplan–Meier method. We found that the 5-year OS rate of the MAGE-D4-positive group was significantly smaller than that of the MAGE-D4-negative group (37% *vs*. 65%,

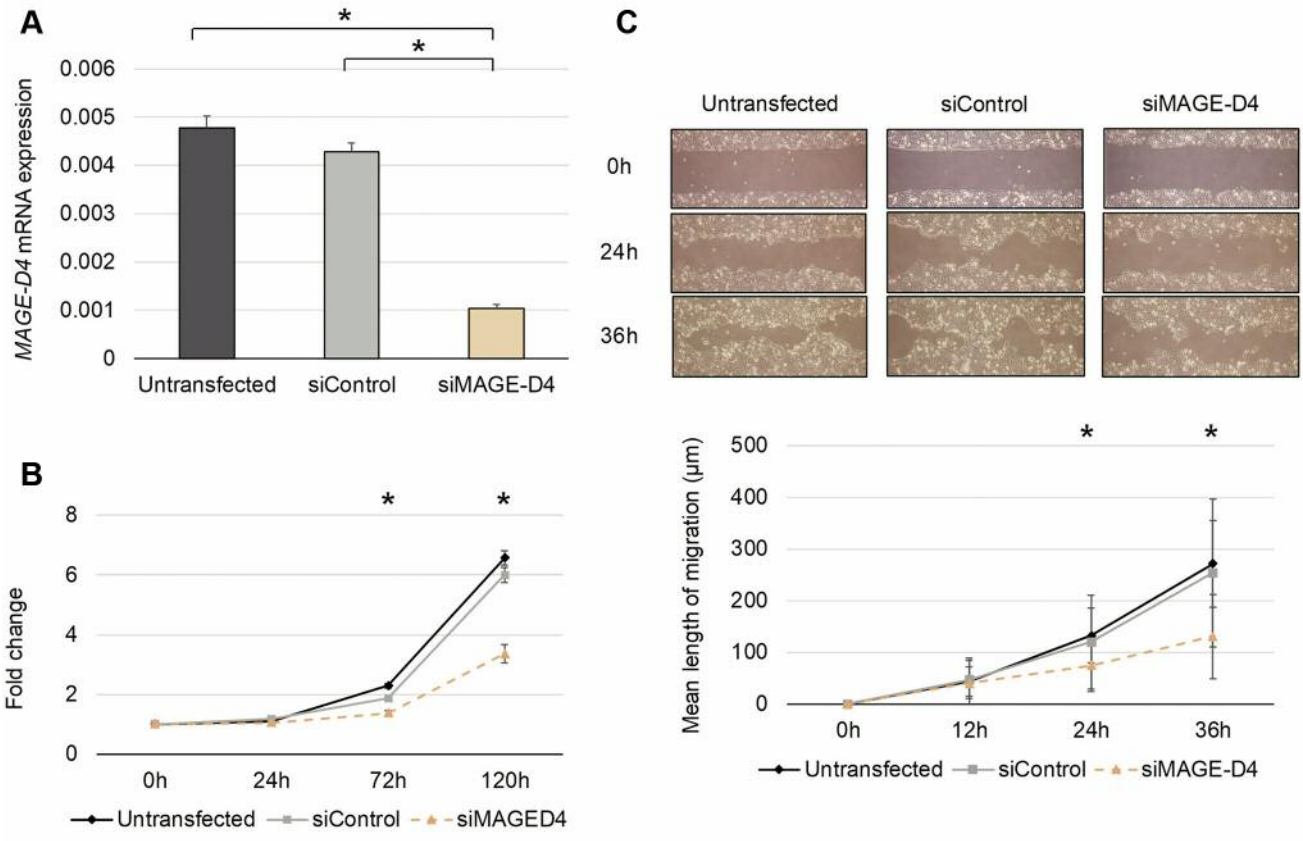

Figure 2. *Effects of siRNA-mediated knockdown of MAGE-D4 on ESCC cell function. (A) qRT-PCR analysis of MAGE-D4 expression in untransfected or siRNA-transfected KYSE590 cells. *p<0.05. (B) CCK-8 cell proliferation assay of untransfected or siRNA-transfected KYSE590 cells. Mean±SD. *p<0.05. (c) Representative images of migration and the mean migration distance from wound-healing assays of untransfected or siRNA-transfected KYSE590 cells. Mean±SD. *p<0.05.*

p=0.0001), and the same outcome was obtained when the 5-year DFS rates were compared (42% *vs*. 66%, p=0.0015; Figure 3B).

We examined the potential correlation between MAGE-D4 protein expression and patient clinicopathological characteristics (Table II) and found no significant differences between the MAGE-D4-positive and -negative patient groups with respect to age, sex, Brinkman index, alcohol consumption, tumor location, tumor depth, differentiation, lymphatic involvement, vascular invasion, lymph node metastasis, or UICC Stage. However, univariate analysis identified age ≥70 years, undifferentiated tumor, lymphatic involvement, lymph node metastasis, stage III disease, and positive tumor expression of MAGE-D4 protein as significant indicators of poor OS (Table III). In multivariate analysis, age ≥70 [hazard ratio (HR)=1.65, 95% confidence interval (95%CI)=1.06-2.53; p<0.025] and MAGE-D4 protein expression (HR=2.36, 95%CI=1.52-3.75; p<0.001) remained the only independent prognostic factors.

Expression and prognostic value of MAGE-D4 mRNA in the second validation cohort. To confirm these results, we analyzed a second validation cohort consisting of a TCGA RNA sequencing dataset from 184 patients with ESCC. The patients were dichotomized into low (n=152) and high (n=32) *MAGE-D4* mRNA expression groups using a cut-off value of 0.7 (log). Patients with high *MAGE-D4* mRNA expression had significantly shorter OS and DFS than the patients with low tumor mRNA levels (p=0.0082 and p=0.0327, respectively; Figure 3C), consistent with the findings from the first validation cohort.

Discussion

In this study, we sought to increase our understanding of the functions of MAGE-D4 in ESCC and to determine the prognostic value of MAGE-D4 mRNA and protein expression in two independent patient cohorts, thereby validating the results of our previous study. In the *in vitro* analyses, we found that expression of MAGE-D4 correlated

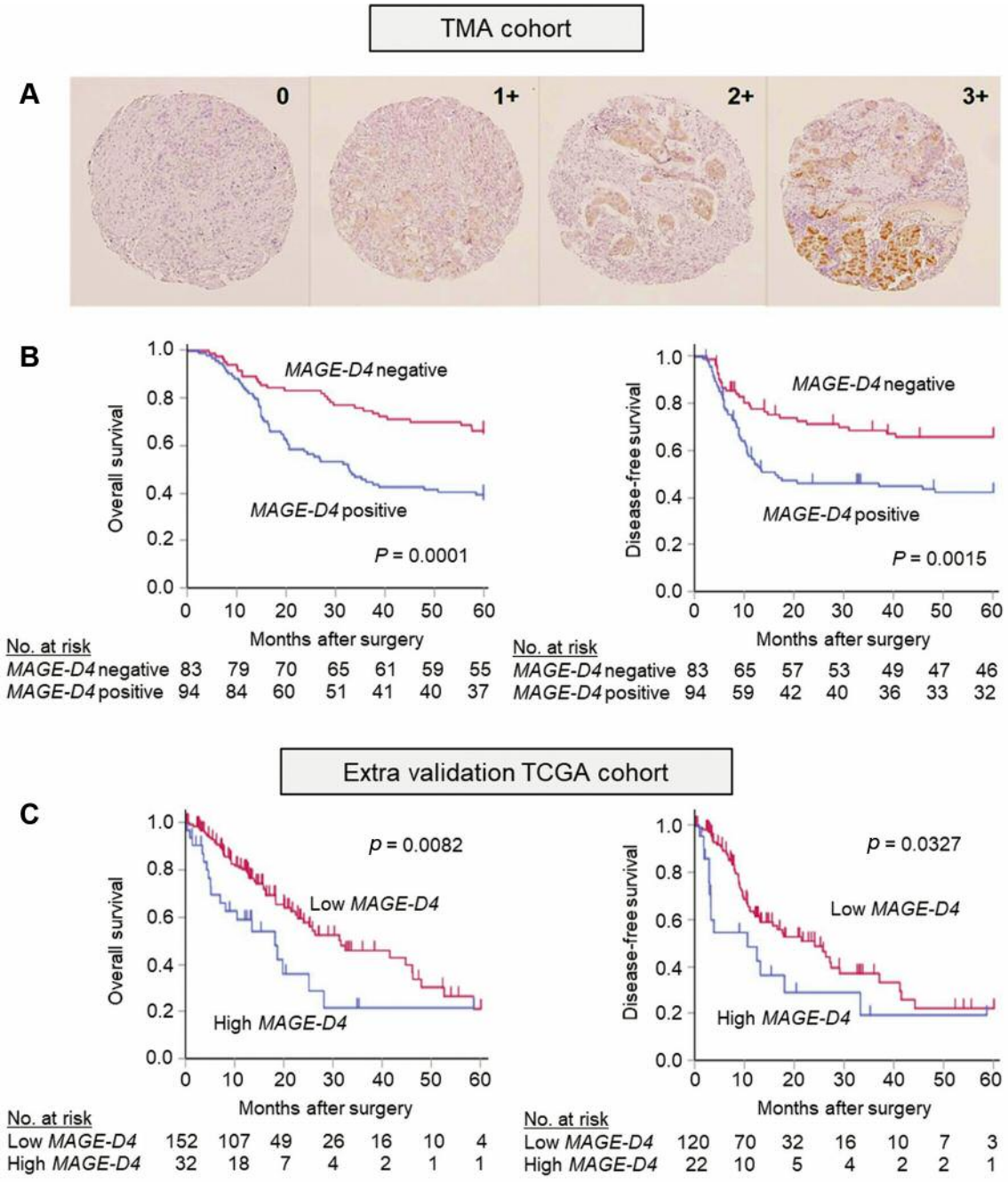

Figure 3. *Analysis of MAGE-D4 protein expression in ESCC and correlation with patient survival. (A) Representative images of MAGE-D4 immunohistochemical staining in a TMA from the first validation cohort. Examples of negative (0) and positive (1+, 2+, and 3+) expression are shown (100× magnification). (B) Overall survival and disease-free survival of the first validation cohort (n=177) stratified by positive (n=83) or negative (n=94) MAGE-D4 protein expression. (C) Overall survival and disease-free survival periods of the second validation cohort (TCGA, n=184) stratified by high (n=32) or low (n=152) MAGE-D4 mRNA expression.*

positively with that of five EMT-related genes (*MMP9, TGFB1, TMEM132A, SNAI3,* and *VCAN*) and that silencing of MAGE-D4 expression significantly decreased the proliferation and migration abilities of ESCC cells. In the clinical study, MAGE-D4 protein expression was an independent prognostic factor for OS in ESCC.

Zhili reported that microRNA-539 could inhabit EMT in TE3 cells through *TWIST1*-mediated regulation of MAGE-

Table III. *Prognostic factors for overall survival in ESCC patients (n=177).*

Variables	n	Univariate			Multivariate		
		Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
Age (≥70)	68	1.57	1.03-2.39	0.036	1.65	1.06-2.53	0.025
Gender (male)	153	1.82	0.93-4.10	0.078			
Brinkman index (≥1000)	55	1.30	0.82-2.00	0.251			
Alcohol consumption	155	1.24	0.67-2.54	0.517			
Tumor location (lower third)	55	1.06	0.67-1.64	0.801			
Tumor depth (pT4, UICC)	9	1.49	0.52-3.31	0.414			
Tumor differentiation (undifferentiated)	57	1.90	1.23-2.90	0.004	1.19	0.76-1.84	0.454
Lymphatic involvement (Ly 2,3)	119	2.08	1.29-3.51	0.002	1.45	0.88-2.50	0.145
Vascular invasion (V 2,3)	127	1.16	0.72-1.95	0.538			
Lymph node metastasis	49	5.76	2.96-13.0	<0.001	2.78	0.89-8.76	0.078
Stage (≥Stage III, UICC)	123	5.16	2.80-10.6	<0.001	2.00	0.77-6.19	0.169
MAGED4 positive	94	2.33	1.50-3.68	<0.001	2.36	1.52-3.75	<0.001

ESCC: Esophageal squamous cell carcinoma; *MAGE-D4*: melanoma-associated antigen-D4; CI: confidence interval; UICC: Union for International Cancer Control.

A4 (30). To our knowledge, this is the first report of an association between MAGE-D4 and the EMT. Because the EMT is the process through which cells switch their differentiation and behavior from an adhesive to an invasive/migratory phenotype, it is a crucial event in the progression of cancer and is highly regulated at the transcriptional level (31, 32). In this study, we found that expression of the EMT-related genes *MMP9, TGFB1, TMEM132A, SNAI3*, and *VCAN* correlated strongly with that of *MAGE-D4* in ESCC cells. *MMPs* have established roles in the development of human malignancies through remodeling of the ECM. Degradation of collagen IV in the basement membrane and ECM facilitates tumor invasion, metastasis, growth, and angiogenesis, thereby promoting tumor progression (33). *TGF-β* is a multifunctional cytokine that controls the proliferation and differentiation of many cell types, among other functions. *TGF-β* secreted within the tumor microenvironment is one of several factors known to promote the EMT (34). *TMEM132A* is a poorly characterized protein, but it is thought to promote neuronal cell survival by regulating stress-related genes (35). The *Snail* family protein *SNAI3* is a transcriptional repressor implicated in embryogenesis and carcinogenesis. During development, *SNAI3* represses transcription of the adhesion molecule E-cadherin and regulates the EMT (36). The role of *VCAN* in cell adhesion, migration, and proliferation has been extensively studied. An increase in *VCAN* expression is detected in many tumors, including cancers of the breast, brain, ovary, gastrointestinal tract, and prostate, as well as melanoma, sarcoma, and peritoneal mesothelioma (37). Our data suggest that, in ESCC, MAGE-D4 participates in the EMT through its coordinated expression with other EMT-regulating molecules. However, further work will be necessary to fully understand the involvement of MAGE-D4 in the EMT in ESCC and other cancers.

In the present study, we showed that knockdown of MAGE-D4 expression reduced the proliferation and migration abilities of KYSE590 ESCC cells, which is indicative of an active role of MAGE-D4 in the malignant behavior of cells and is consistent with our finding that high MAGE-D4 expression is associated with poor prognosis. Our results have some important translational implications for ESCC. For example, patients with positive MAGE-D4 expression detected in endoscopic biopsies or surgical specimens might benefit from intensive postoperative surveillance and perioperative systemic treatment to anticipate early recurrence and subsequent adverse prognosis.

Several limitations of this study should be acknowledged. First, experimental analysis of MAGE-D4 expression was performed retrospectively on tissues collected from a relatively small number of patients. Second, pathway analyses of the MAGE-D4 network would strengthen the results and further our understanding of the functions of this gene in ESCC biology. Finally, *in vivo* experiments using mouse xenograft models would be helpful in clarifying the role of MAGE-D4 in ESCC pathogenesis.

In conclusion, our findings support an important role for MAGE-D4 in the malignant behavior of ESCC. Our study of two independent cohorts totaling 361 patients also robustly confirms the validity of our previous finding that *MAGE-D4* expression is an independent prognostic factor in ESCC.

Conflicts of Interest

The Authors declare no competing financial interests regarding this study.

Authors' Contributions

YU, MKa, and SU performed the experiments and the data analysis. YU, MKa, YS, DS, SU, NH, MH, CT, DK, SY GN, SM, MKo and YK collected the cases and the clinical data. YU and MKa conceived and designed the study and prepared the initial manuscript. YK supervised the project. All Authors contributed to the final manuscript. All Authors read and approved the final manuscript.

Acknowledgements

The Authors would like to thank Anne M. O'Rourke, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

References

1 Ferlay J, Shin H, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer *127*: 2893-2917, 2010. PMID: 21351269. DOI: 10.1002/ijc.25516

2 Kamangar F, Dores GM and Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol *24*: 2137-2150, 2006. PMID: 16682732. DOI: 10.1200/JCO.2005.05.2308

3 Atanackovic D, Hildebrandt Y, Jadczak A, Cao Y, Luetkens T, Meyer S, Kobold S, Bartels K, Pabst C, Lajmi N, Gordic M, Stahl T, Zander AR, Bokemeyer C and Kröger N: Cancer-testis antigens MAGE-C1/CT7 and MAGE-A3 promote the survival of multiple myeloma cells. Haematologica *95*: 785-793, 2010. PMID: 20015885. DOI: 10.3324/haematol.2009.014464

4 Bergeron A, Picard V, LaRue H, Harel F, Hovington H, Lacombe L and Fradet Y: High frequency of MAGE-A4 and MAGE-A9 expression in high-risk bladder cancer. Int J Cancer *125*: 1365-1371, 2009. PMID: 19533752. DOI: 10.1002/ijc.24503

5 Karpf AR, Bai S, James SR, Mohler JL and Wilson EM: Increased expression of androgen receptor coregulator MAGE-11 in prostate cancer by DNA hypomethylation and cyclic AMP. Mol Cancer Res *7*: 523-535, 2009. PMID: 19372581. DOI: 10.1158/1541-7786.MCR-08-0400

6 Pastorcic-Grgic M, Sarcevic B, Dosen D, Juretic A, Spagnoli GC and Grgic M: Prognostic value of MAGE-A and NYESO-1 expression in pharyngeal cancer. Head Neck *32*: 1178-1184, 2010. PMID: 20029985. DOI: 10.1002/hed.21314

7 Ries J, Mollaoglu N, Toyoshima T, Vairaktaris E, Neukam FW, Ponader S and Nkenke E: A novel multiple-marker method for the early diagnosis of oral squamous cell carcinoma. Dis Markers *27*: 75-84, 2009. PMID: 19893202. DOI: 10.3233/DMA-2009-0652

8 van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A and Boon T: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science *254*: 1643-1647, 1991. PMID: 1840703. DOI: 10.1126/science.1840703

9 Barker P A and Salehi A: The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. J Neurosci Res *67*: 705-712, 2002. PMID: 11891783. DOI: 10.1002/jnr.10160

10 Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T and Lucas S: An overview of the MAGE gene family with the identification of all human members of the family. Cancer Res *61*: 5544-5551, 2001. PMID: 11454705.

11 Xiao J and Chen HS: Biological functions of melanoma-associated antigens. World J Gastroenterol *10*: 1849-1853, 2004. PMID: 15222021. DOI: 10.3748/wjg.v10.i13.1849

12 Ito S, Kawano Y, Katakura H, Takenaka K, Adachi M, Sasaki M, Shimizu K, Ikenaka K, Wada H and Tanaka F: Expression of MAGE-D4, a novel MAGE family antigen, is correlated with tumor-cell proliferation of non-small cell lung cancer. Lung Cancer *51*: 79-88, 2006. PMID: 16225959. DOI: 10.1016/j.lungcan.2005.08.012

13 Takami H, Kanda M, Oya H, Hibino S, Sugimoto H, Suenaga M, Yamada S, Nishikawa Y, Asai M, Fujii T, Nomoto S and Kodera Y: Evaluation of MAGE-D4 expression in hepatocellular carcinoma in Japanese patients. J Surg Oncol *108*: 557-562, 2013. PMID: 24068544. DOI: 10.1002/jso.23440

14 Cheong SC, Chandramouli GV, Saleh A, Zain RB, Lau SH, Sivakumaren S, Pathmanathan R, Prime SS, Teo SH, Patel V and Gutkind JS: Gene expression in human oral squamous cell carcinoma is influenced by risk factor exposure. Oral Oncol *45*: 712-719, 2009. PMID: 19147396. DOI: 10.1016/j.oraloncology.2008.11.002

15 Kramer BF, Schoor O, Kruger T, Reichle C, Müller M, Weinschenk T, Hennenlotter J, Stenzl A, Rammensee HG and Stevanovic S: MAGED4-expression in renal cell carcinoma and identification of an HLA-A*25-restricted MHC class I ligand from solid tumor tissue. Cancer Biol Ther *4*: 943-948, 2005. PMID: 16082191. DOI: 10.4161/cbt.4.9.1907

16 Germano S, Kennedy S, Rani S, Gleeson G, Clynes M, Doolan P, McDonnell S, Hughes L, Crown J and O'Driscoll L: MAGE-D4B is a novel marker of poor prognosis and potential therapeutic target involved in breast cancer tumorigenesis. Int J Cancer *130*: 1991-2002, 2012. PMID: 21618523. DOI: 10.1002/ijc.26200

17 Zhang QM, He SJ, Shen N, Luo B, Fan R, Fu J, Luo GR, Zhou SF, Xiao SW and Xie XX: Overexpression of MAGE-D4 in colorectal cancer is a potentially prognostic biomarker and immunotherapy target. Int J Clin Exp Pathol *7*: 3918-3927, 2014. PMID: 25120768.

18 Yan J, Wen J, Wei ZD, Li XS, Li P and Xiao SW: Prognostic and clinicopathological value of melanoma-associated antigen D4 in patients with glioma. Oncology Letters *15*: 4151-4160, 2018. PMID: 29541180. DOI: 10.3892/ol.2018.7884

19 Oya H, Kanda M, Takami H, Hibino S, Shimizu D, Niwa Y, Koike M, Nomoto S, Yamada S, Nishikawa Y, Asai M, Fujii T, Nakayama G, Sugimoto H, Fujiwara M and Kodera Y: Overexpression of melanoma-associated antigen D4 is an independent prognostic factor in squamous cell carcinoma of the esophagus. Dis Esophagus *28*: 188-195, 2015. PMID: 24147998. DOI: 10.1111/dote.12156

20 Tanaka H, Shimada Y, Imamura M, Shibagaki I and Ishizaki K: Multiple types of aberrations in the p16 (INK4a) and the p15(INK4b) genes in 30 esophageal squamous-cell-carcinoma cell lines. Int J Cancer *70*: 437-442, 1997. PMID: 9033652. DOI: 10.1002/(sici)1097-0215(19970207)70:4<437::aid-ijc11>3.0.co;2-c

21 Sato Y, Motoyama S, Wakita A, Kawakita Y, Liu J, Nagaki Y, Nanjo H, Terata K, Imai K, Saito H and Minamiya Y: TLR3 expression status predicts prognosis in patients with advanced

thoracic esophageal squamous cell carcinoma after esophagectomy. Am J Surg *216*: 319-325, 2018. PMID: 29395019. DOI: 10.1016/j.amjsurg.2018.01.038

22 Sato Y, Motoyama S, Nanjo H, Ito S, Yoshino K, Sasaki T, Kuribayashi K, Nagaki Y, Imai K, Saito H, Minamiya Y and Ogawa J: REG1A expression status suggests chemosensitivity among advanced thoracic esophageal squamous cell carcinoma patients treated with esophagectomy followed by adjuvant chemotherapy. Ann Surg Oncol *20*: 3044-3051, 2013. PMID: 23645481. DOI: 10.1245/s10434-013-2983-9

23 Sato Y, Marzese DM, Ohta K, Huang SK, Sim MS, Chong K and Hoon DS: Epigenetic regulation of REG1A and chemosensitivity of cutaneous melanoma. Epigenetics *8*: 1043-1052, 2013. PMID: 23903855. DOI: 10.4161/epi.25810

24 Sato Y, Motoyama S, Nanjo H, Wakita A, Yoshino K, Sasaki T, Nagaki Y, Liu J, Imai K, Saito H and Minamiya Y: CXCL10 expression status is prognostic in patients with advanced thoracic esophageal squamous cell carcinoma. Ann Surg Oncol *23*: 936-942, 2016. PMID: 26464192. DOI: 10.1245/s10434-015-4909-1

25 Shimizu D, Kanda M, Tanaka H, Kobayashi D, Tanaka C, Hayashi M, Iwata N, Niwa Y, Takami H, Yamada S, Fujii T, Nakayama G, Fujiwara M and Kodera Y: GPR155 serves as a predictive biomarker for hematogenous metastasis in patients with gastric cancer. Sci Rep *7*: 42089, 2017. PMID: 28165032. DOI: 10.1038/srep42089

26 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods *25*: 402-408, 2001. PMID: 11846609. DOI: 10.1006/meth.2001.1262

27 Kanda M, Shimizu D, Fujii T, Sueoka S, Tanaka Y, Ezaka K, Takami H, Tanaka H, Hashimoto R, Iwata N, Kobayashi D, Tanaka C, Yamada S, Nakayama G, Sugimoto H, Koike M, Fujiwara M and Kodera Y: Function and diagnostic value of Anosmin-1 in gastric cancer progression. Int J Cancer *138*: 721-730, 2016. PMID: 26270236. DOI: 10.1002/ijc.29803

28 Miwa T, Kanda M, Tanaka H, Tanaka C, Kobayashi D, Umeda S, Iwata N, Hayashi M, Yamada S, Fujii T, Fujiwara M and Kodera Y: FBXO50 enhances the malignant behavior of gastric cancer cells. Ann Surg Oncol *24*: 3771-3779, 2017. PMID: 28560594. DOI: 10.1245/s10434-017-5882-7

29 Shimizu D, Kanda M, Tanaka H, Shibata M, Tanaka H, Takami H, Iwata N, Hayashi M, Tanaka C, Kobayashi D, Yamada S, Nakayama G, Koike M, Fujiwara M, Fujii T and Kodera Y: The protein arginine methyltransferase 5 promotes malignant phenotype of hepatocellular carcinoma cells and is associated with adverse patient outcomes after curative hepatectomy. Int J Oncol *50*: 381-386, 2017. PMID: 28101581. DOI: 10.3892/ijo.2017.3833

30 Cao Z, Zheng X, Cao L and Liang N: MicroRNA-539 Inhibits the epithelial-mesenchymal transition of esophageal cancer cells by twist-related protein 1-mediated modulation of melanoma-associated antigen A4. Oncol Res *26*: 529-536, 2018. PMID: 28653599. DOI: 10.3727/096504017X14972679378357

31 Wu Q, Hou X, Xia J, Qian X, Miele L, Sarkar FH and Wang Z: Emerging roles of PDGF-D in EMT progression during tumorigenesis. Cancer Treat Rev *39*: 640-646, 2013. PMID: 23261166. DOI: 10.1016/j.ctrv.2012.11.006

32 Lamouille S, Xu J and Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol *15*: 178-196, 2014. PMID: 24556840. DOI: 10.1038/nrm3758

33 Groblewska M, Siewko M, Mroczko B and Szmitkowski M: The role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. Folia Histochem Cytobiol *50*: 12-19, 2012. PMID: 22532131. DOI: 10.2478/18691

34 Gao J, Zhu Y, Nilsson M and Sundfeldt K: TGF-beta isoforms induce EMT independent migration of ovarian cancer cells. Cancer Cell Int *14*: 72, 2014. PMID: 25278811. DOI: 10.1186/s12935-014-0072-1

35 Oh-Hashi,K. Sone A, Hikiji T, Hirata Y, Vitiello M, Fedele M, Ishigaki S, Sobue G and Kiuchi K: Transcriptional and post-transcriptional regulation of transmembrane protein 132A. Mol Cell Biochem *405*: 291-299, 2015. PMID: 25926156. DOI: 10.1007/s11010-015-2419-x

36 Kaufhold S and Bonavida B: Central role of Snail1 in the regulation of EMT and resistance in cancer: a target for therapeutic intervention. J Exp Clin Cancer Res *33*: 62, 2014. PMID: 25084828. DOI: 10.1186/s13046-014-0062-0

37 Kischel P, Waltregny D, Dumont B, Turtoi A, Greffe Y, Kirsch S, De Pauw E and Castronovo V: Versican overexpression in human breast cancer lesions: known and new isoforms for stromal tumor targeting. Int J Cancer *126*: 640-650, 2010. PMID: 19662655. DOI: 10.1002/ijc.24812

Received September 25, 2019
Revised October 8, 2019
Accepted October 9, 2019