

## Original Article

# Overexpression of MAGE-D4 in colorectal cancer is a potentially prognostic biomarker and immunotherapy target

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**Abstract:** Melanoma-associated antigen D4 (MAGE-D4) is a novel member of MAGE family. This study aimed to examine the expression and immunogenicity of MAGE-D4 in colorectal cancer (CRC) to determine its potential as a prognosis and immunotherapeutic target. The expression of MAGE-D4 mRNA and protein was determined by RT-PCR and immunohistochemistry (IHC) in CRCs with paired adjacent non-tumor tissues, colorectal adenomas and normal colorectal tissues, respectively. Sera from 64 CRC patients were tested for MAGE-D4 antibody by ELISA. MAGE-D4 mRNA was more frequently expressed in CRCs (76.7%, 46/60) than in adjacent non-tumor tissues (15.0%, 9/60). MAGE-D4 protein was detected in all the CRC tissues tested, 70.0% of which showed high expression. There was no MAGE-D4 protein detected in any paired adjacent non-tumor tissue. No MAGE-D4 expression was found in colorectal adenomas and normal colorectal tissues by either RT-PCR or immunohistochemistry. Patients with high MAGE-D4 protein expression had significantly shorter overall survival than those with low MAGE-D4 protein expression (median, 68.6 vs 122.2 months;  $P=0.030$ ). Furthermore, multivariate analysis exhibited high MAGE-D4 protein expression had a trend toward an independent prognostic factor (hazard ratio: 6.124;  $P=0.050$ ). Humoral immunity to MAGE-D4 was detected in 12 of 64 (18.8%) CRC patients' sera but not in 77 healthy donors. There was no correlation between MAGE-D4 expression, serum antibody and clinicopathological parameters. These findings suggest MAGE-D4 may serve as a potentially prognostic biomarker and an attractive target of immunotherapy in CRC.

**Keywords:** Melanoma-associated antigen, MAGE-D4, colorectal cancer, serum immunoreactivity

## Introduction

Colorectal cancer (CRC) is the third most common malignancy and fourth leading cause of cancer mortality worldwide, with more than a million individuals diagnosed and about half million deaths annually [1, 2]. Although there are many established therapeutic strategies including surgery, chemotherapy and radiotherapy, its prognosis remains unsatisfactory due to late diagnosis [3]. Thus, novel therapeutic strategies are urgently needed for this malignancy. Immunotherapy is an attractive approach among novel therapeutic strategies [4, 5]. This approach requires the identification of tumor specific antigens. Currently, a number of such antigens are encoded by the genes of

melanoma-associated antigen (MAGE) family [6, 7].

MAGE is a large gene family including more than 60 members, in which some genes encode tumor-specific antigens with characteristics of broad expression in various tumors but restricted in normal tissues, and recognized by cytotoxic T lymphocytes (CTLs) [8, 9]. Some of MAGE antigens and their epitope peptides constitute important targets for antitumor immunotherapy with a number of clinical studies already completed or underway [10-12]. Unfortunately, it has been found that CRC expressed some of MAGE antigen with low frequency, especially those (Mage-A1 and -A3) which had been applied on clinical trial [13-18]. Therefore, iden-

tification of other MAGE antigens with high expression in CRC is essential.

MAGE-D4, originally named MAGE-E1, is a novel member of MAGE family. It has been reported restricted expression in normal tissues except brain and ovary and overexpression in some human malignancies, including lung, liver, oral, kidney, breast, esophagus cancer and glioma [19-25]. A MHC class I ligand from MAGE-D4 presented by HLA-A on tumor tissue was also reported by Kramer et al [20]. Several previous studies have shown that MAGE-D4 contributes to proliferation, migration, and invasion of tumor cells in breast cancer and oral squamous cell carcinoma [22, 23]. Recent studies have shown that MAGE-D4 is a marker of poor prognosis in hepatocellular, esophagus and breast carcinoma [23-25]. To some extent, these preliminary findings have been suggested that MAGE-D4 may play an important role in the progression of tumors and may be a potentially promising target for tumor prognosis and treatment.

Current knowledge about MAGE-D4 gene expression in CRC was only based on mRNA level from one previous report [26]. However, MAGE-D4 protein expression has not been elucidated including its immunogenicity. In the present study, we examined the expression of MAGE-D4 at mRNA and protein levels in CRC tissues, as well as the serum antibody against MAGE-D4 in a subset of CRC patients. Furthermore, whether MAGE-D4 protein can be a prognostic factor was analyzed, and the correlation among MAGE-D4 expression, serum antibody and clinicopathological parameters in CRC patients were also investigated.

### Materials and methods

#### *Tissue and serum samples*

All tissue and serum samples were collected from the First Affiliated Hospital of Guangxi Medical University with the informed consent of patients and approved by Hospital Ethic Review Committee. A total of 154 tissue samples, including 60 primary CRCs with adjacent non-tumor tissues (42 men and 18 women; mean age, 56.37±14.74 years; age ranging, 30-86 years), 24 colorectal adenomas and 10 normal colorectal tissues, were analyzed by RT-PCR. 82 of paraffin-embedded tissue sections for immunohistochemical analysis was construct-

ed from 30 primary CRCs with paired adjacent non-tumor tissues (17 men and 13 women; mean age, 56.45±11.43 years; age ranging, 35-76 years), 12 colorectal adenomas and 10 normal colorectal tissues. CRC patients were followed up for 1-130 months (mean, 79.6±52.6 months). Overall survival was defined as the time from diagnosis to the date of death or last follow-up. All CRC and paired non-tumor tissues were surgically removed without undergoing preoperative treatment including chemotherapy and radiation. The colorectal adenomas were available from the patients by endoscopic polypectomy and normal colorectal tissues were obtained at autopsy. Sera were collected from 64 patients with CRC (47 men and 17 women; mean age, 56.03±14.96 years; age ranging, 26-93 years) at diagnosis prior of therapy and sera of 77 healthy donors from routine physical examination of students of Guangxi Medical University were used as controls. All tumor samples were classified according to the TNM classification of the Union for International Cancer Control [27]. Tumor samples were coded and assessed in a blinded manner.

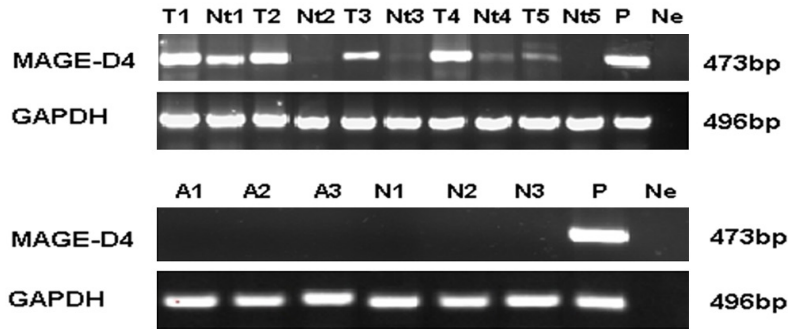
#### *RT-PCR analysis*

Total RNA was prepared by Trizol reagent (Invitrogen, CA) and 2.5 µg RNA was reverse transcribed into cDNA with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. cDNA was then tested for integrity by amplification of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. RT-PCR was carried out with MAGE-D4 specific primers as previously reported [19]. The cycling parameters were as following: initial denaturation at 94°C for 5 min followed by 30 sec at 94°C, 30 sec at (64°C for MAGE-D4, 55°C for GAPDH), and 30 sec at 72°C for 35 cycles, and a final extension for 10 min at 72°C. Target bands were visualized on a 1.5% agarose gel with ethidium bromide staining. The expression of MAGE-D4 was counted as positive, only if the RT-PCR reaction repeated at least twice with same result.

#### *Immunohistochemistry (IHC)*

Immunohistochemistry (IHC) was performed with minor modification as previous report by Luo et al [28]. In brief, formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated under the regular condition. Subsequently, the sections were heated in eth-

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**Figure 1.** RT-PCR analysis of MAGE-D4 mRNA expression in CRC tissues (T) with paired adjacent non-tumor tissues (Nt), colorectal adenomas (A) and normal colorectal tissues (N). GAPDH was used as internal control for the parallel PCR analysis of the same sample. P, positive control (glioma); Ne, negative control (no cDNA template).

ylene diamine tetraacetic acid (EDTA, pH8.0) for antigen retrieval. After the inactivation of endogenous peroxidase, the sections were treated with normal goat serum for blocking and then immunostained with anti-MAGE-D4 polyclonal antibody (1:500 dilution, Santa Cruz Biotechnology, USA) overnight at 4°C. Negative controls using rabbit serum collected before immunization were also incubated in parallel. Then horseradish peroxidase-conjugated goat anti-rabbit IgG (ZSGB-BIO, China) was added as the secondary antibody. Immunoreactivity was visualized with 3, 3'-diaminobenzidine (DAB) (Maixin Biotechnology, China) followed by hematoxylin counterstain.

Positive immunoreactivity was assessed by two independent pathologists who did not know patients' clinical information and recorded semi-quantitatively according to the staining intensity, in combination with the percentage of positive cells. The staining intensity was quantified using the following scores: 0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining. The percentage of positive tumor cells was defined as follows: 0=0-5%, 1=6-25%, 2=26-50%, 3=51-75%, 4=76-100%. According to the sum of both points, each section was assigned as no MAGE-D4 expression when the sum score was 0, low MAGE-D4 expression when the sum score was between 1 to 4, and high MAGE-D4 expression when the sum score was more than 4 [21, 29].

### ELISA

MAGE-D4 recombinant protein was generated according to a previous report by He et al [30].

ELISA was performed as previous report by Zhou et al [31]. In brief, MAGE-D4 protein (1 µg/ml) was coated on the 96-well plates (Corning, USA) at 4°C overnight. Maltose binding protein (MBP) protein was used as a blank control. The plates were blocked with 5% nonfat milk 37°C for 1 h, then 1:800 diluted serum were added and incubated at 37°C for 1 h. Streptavidin-biotinylated horseradish peroxidase complex (KPL, USA) was

used as secondary antibody. Detection was accomplished using tetramethylbenzidine substrate, followed by adding sulfuric acid to stop the reaction. The absorbance at 450 nm with 630nm as reference filter was determined with a microplate reader (Bio-Rad, USA). All serum samples were performed from triplicates and indicated as mean. An optical density (OD) value that exceeds three standard deviations (SDs) above the mean OD value of sera from healthy donors was defined as positive. Specificity of each positive serum sample was examined by testing reactivity after pre-incubating with recombinant MAGE-D4 protein.

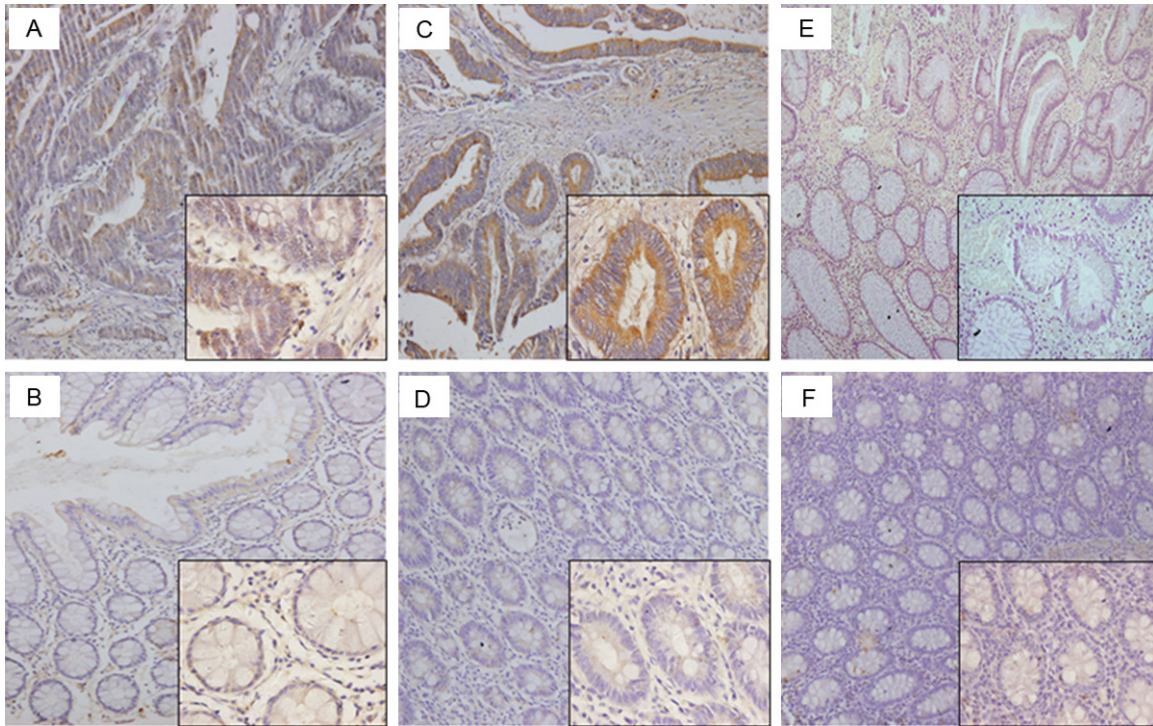
### Statistical analysis

Statistical analyses were conducted by SPSS software (version 16). Statistical significance was defined as  $P < 0.05$ . Relationships between MAGE-D4 expression and antibody response with clinicopathological parameters were tested by  $\chi^2$  test or Fisher's exact test. Overall survival rates were calculated using the Kaplan-Meier method, and differences in survival curves were compared using the log-rank test. Multivariable regression analysis was performed to detect prognostic factors using Cox proportional hazards models, and variables with a two-sided  $P$  value of  $< 0.05$  were entered into the final model.

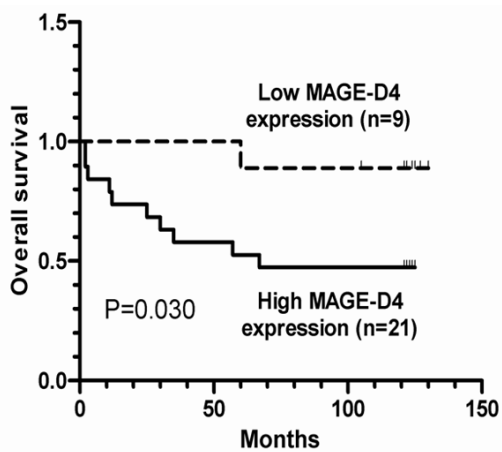
## Results

### Expression of MAGE-D4 mRNA

We investigated the expression of MAGE-D4 mRNA in 60 CRCs with paired adjacent non-tumor tissues, 24 colorectal adenomas and 10



**Figure 2.** Representative immunohistochemical staining of MAGE-D4 protein in CRC tissues (A, C), paired adjacent non-tumor tissues (B, D), colorectal adenomas (E) and normal colorectal tissues (F). Low and high immunoreactivity of MAGE-D4 protein immunostaining were shown in (A and C) respectively, using polyclonal MAGE-D4 antibody. No positive reactivity was observed in paired adjacent non-tumor tissues (B, D), colorectal adenomas (E) and normal colorectal tissues (F). Original magnification,  $\times 100$  ( $\times 400$  in the right down corner).



**Figure 3.** Kaplan-Meier analysis of MAGE-D4 protein in 30 patients with CRC, categorized as having low and high expression of MAGE-D4. Patients with high MAGE-D4 protein expression in CRC tissues had significantly shorter overall survival than patients with low MAGE-D4 protein expression.

normal colorectal tissues by RT-PCR. MAGE-D4 mRNA was detected in 46 of 60 (76.7%) CRCs and 9 of 60 (15.0%) of adjacent non-tumor tissues (**Figure 1**). Statistical analysis revealed

that there was significant difference between CRCs and adjacent non-tumor tissues ( $P=0.000$ ). No positive expression was detected in any of both colorectal adenomas and normal colorectal tissues (**Figure 1**).

#### Expression of MAGE-D4 protein

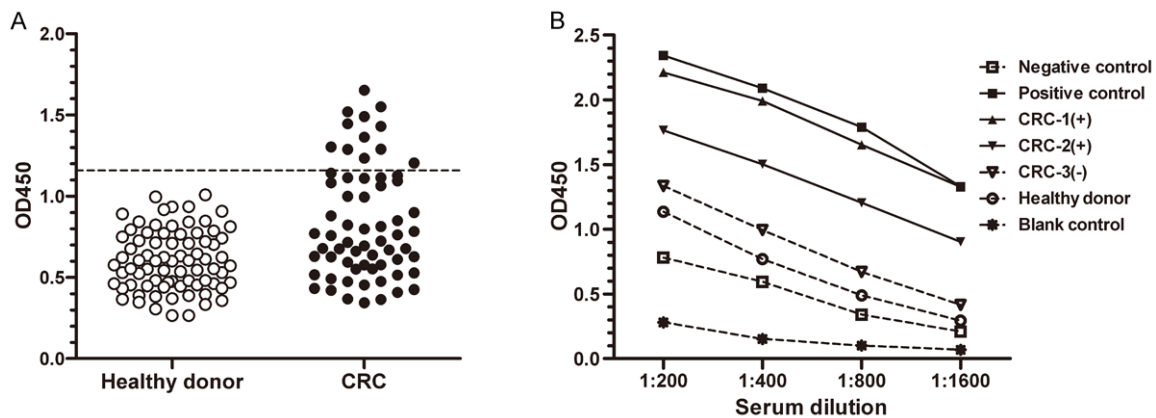
MAGE-D4 protein expression was examined in 30 CRCs with paired adjacent non-tumor tissues, 12 colorectal adenomas and 10 normal colorectal tissues by IHC. Positive staining was observed with different staining intensity in all the CRC samples tested and primarily located in the cytoplasm. High MAGE-D4 protein expression was demonstrated in 21 of 30 patients (70.0%, **Figure 2C**) and low in the other 9 patients (30.0%, **Figure 2A**). Notably, a rather heterogeneous expression was present in a significant number of CRC samples, which varied from individual positive cells, foci of stained cells to uniform staining of tumor cells. None of paired adjacent non-tumor tissues (**Figure 2B** and **2D**), colorectal adenomas (**Figure 2E**) and normal colorectal tissues (**Figure 2F**) was positive for MAGE-D4 protein immunostaining.

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**Table 1.** Prognostic factors in 30 patients with CRC

Variable	n	Univariate		Multivariate	
		HR (95% CI)	P value	HR (95% CI)	P value
Gender (Male)	17	1.416 (0.426-4.708)	0.567	-	-
Age $\geq$ 56 (years)	18	0.669 (0.212-2.116)	0.490	-	-
Tumor location (Colon)	24	1.407 (0.307-6.444)	0.658	-	-
Tumor size $\geq$ 5 cm)	17	1.037 (0.334-3.244)	0.950	-	-
CEA (>5 ng/mL)	17	5.296 (1.153-24.323)	0.016*	4.891 (0.930-25.728)	0.174
Depth of tumor Invasion (T4)	5	2.237 (0.601-8.325)	0.216	-	-
Lymph node metastasis	23	4.514 (0.582-35.030)	0.113	-	-
Distant metastasis	12	4.951 (1.470-16.672)	0.004*	2.663 (0.582-12.173)	0.010*
TNM stage (III+IV)	23	4.514 (0.582-35.030)	0.113	-	-
Histological type (Non-mucin-producing)	27	0.040 (0.00-62.396)	0.179	-	-
Histological grade (Moderate and poor)	22	1.587 (0.347-7.254)	0.546	-	-
MAGE-D4 protein expression (High)	21	7.001 (0.900-54.451)	0.030*	6.124 (0.780-48.095)	0.050

Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. \*Statistically significant ( $P < 0.05$ ). HR, Hazard ratio; 95 percent CI, 95 percent confidence interval for relative risk; CEA, carcinoembryonic antigen.



**Figure 4.** Anti-MAGE-D4 antibody in sera by ELISA. A. Detection of anti-MAGE-D4 antibody in the sera from 64 CRC patients and 77 normal donors. Three standard deviations above the mean absorbance in the sera from normal donors were used as cutoff (1.1593) for a positive result (dotted line). B. Sera titration curves of dilution series of recombinant MAGE-D4 protein against four different concentrations. Sera from three CRC patients ( $\blacktriangle$ ,  $\blacktriangledown$ ,  $\blacktriangledown$ ) and one healthy donor ( $\circ$ ) were shown, together with a positive control ( $\blacksquare$ ) and a negative control ( $\square$ ). MBP protein ( $*$ ) was used as a blank control. Patient 1 and 2 demonstrated the highest ( $\blacktriangle$ ) and the lowest ( $\blacktriangledown$ ) MAGE-D4 antibody titer among 12 seropositive CRC patients, respectively.

### Prognostic impact of high MAGE-D4 protein expression

Prognostic impact of MAGE-D4 protein expression in patients with CRC was analyzed as a continuous variable in a regression analysis. The result showed that mean overall survival (68.6 vs 122.2 months) was significantly shorter in patients with high MAGE-D4 protein expression than in those with low expression ( $P = 0.030$ , **Figure 3**). Furthermore, Univariate analysis identified carcinoembryonic antigen (CEA)  $>5$  ng/mL, distant metastasis and high

MAGE-D4 protein expression as significant prognostic factors (**Table 1**). In multivariate analysis, although distant metastasis exhibited the only independent prognostic factor, high MAGE-D4 protein expression tended to be as an independent prognostic factor (hazard ratio: 6.124;  $P = 0.050$ ; **Table 1**).

### Serum antibody against MAGE-D4

Serum antibody against MAGE-D4 was investigated using recombinant MAGE-D4 protein by ELISA. MAGE-D4 was immunogenic in 12 of 64

## MAGE-D4 in colorectal cancer

**Table 2.** Correlation among MAGE-D4 expression, serum antibody and clinicopathological parameters in CRC patients

Clinicopathological parameters	mRNA		Protein		Antibody	
	Positive/Total (%)	P value	High-expression/Total (%)	P value	Positive/Total (%)	P value
<b>Gender</b>						
Male	34/42 (81.0)	0.319	13/17 (76.5)	0.443	10/47 (21.3)	0.490
Female	12/18 (66.7)		8/13 (61.5)		2/17 (11.8)	
<b>Age (year)</b>						
<56	22/28 (78.6)	0.744	8/12 (66.7)	1.000	3/32 (9.4)	0.055
≥56	24/32 (75.0)		13/18 (72.2)		9/32 (28.1)	
<b>Tumor location</b>						
Colon	28/36 (77.8)	0.803	17/24 (70.8)	1.000	6/32 (18.8)	1.000
Rectum	18/24 (75.0)		4/6 (66.7)		6/32 (18.8)	
<b>Tumor size (cm)</b>						
<5	23/27 (85.2)	0.158	10/13 (76.9)	0.691	7/41 (17.1)	0.742
≥5	23/33 (69.7)		11/17 (64.7)		5/23 (21.7)	
<b>CEA (ng/mL)<sup>a</sup></b>						
≤5	25/31 (80.6)	0.451	10/13 (76.9)	0.691	4/25 (16.0)	0.751
>5	21/29 (72.4)		11/17 (64.7)		8/39 (20.5)	
<b>Depth of tumor invasion</b>						
T1-T2	8/10 (80.0)	1.000	0/0 (0)	-	2/18 (11.1)	0.483
T3-T4	38/50 (76.0)		21/30 (70.0)		10/46 (21.7)	
<b>Lymph node metastasis</b>						
N0	27/35 (77.1)	0.918	4/7 (57.1)	0.640	8/43 (18.6)	1.000
N1-3	19/25 (76.0)		17/23 (73.9)		4/21 (19.0)	
<b>Distant metastasis</b>						
M0	36/48 (75.0)	0.713	11/18 (61.1)	0.249	10/57 (17.5)	0.607
M1	10/12 (83.3)		10/12 (83.3)		2/7 (19.0)	
<b>TNM stage</b>						
I + II	27/35 (77.1)	0.918	4/7 (57.1)	0.640	8/43 (18.6)	1.000
III + IV	19/25 (76.0)		17/23 (73.9)		4/21 (19.0)	
<b>Histological type<sup>a</sup></b>						
Non-mucin-producing	41/54 (75.9)	1.000	20/27 (74.1)	0.207	10/54 (18.5)	1.000
Mucin-producing	5/6 (83.3)		1/3 (33.3)		2/10 (20.0)	
<b>Histological grade<sup>b</sup></b>						
G1	13/17 (76.5)	0.862	5/8 (62.5)	0.484	1/16 (6.2)	0.259
G2	20/27 (74.1)		14/18 (77.8)		6/28 (21.4)	
G3	13/16 (81.2)		2/4 (50.0)		5/20 (25.0)	

<sup>a</sup>Non-mucin-producing cancer includes tubular and (or) papillary adenocarcinoma, Mucin-producing cancer includes mucinous cancer and signet-ring cell cancer. <sup>b</sup>Histological grade (G). G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

(18.8%) CRC patients, but not in every healthy controls (**Figure 4A**). The titration curves of selected MAGE-D4 antibody-positive and -negative sera were illustrated in **Figure 4B**. Antibody against MBP protein failed to be detected in sera from MAGE-D4 seropositive patients (data not shown). Of 64 sera screened, 25 sera were collected from CRC patients whose tissues were also assessed for MAGE-D4

mRNA, in which 21 was MAGE-D4 mRNA positive and 4 was negative. 3 of 21 (14.3%) CRC patients with MAGE-D4 mRNA-positive tumors had serum antibody against MAGE-D4, while the remaining 4 patients with MAGE-D4 mRNA-negative tumors were failed to detect serum antibody against MAGE-D4. Furthermore, the three sero-positive patients showed high-MAGE-D4 protein expression.

### *Associations among MAGE-D4 expression, serum antibody and clinicopathological parameters in CRC*

Associations among MAGE-D4 expression, serum antibody and clinicopathological parameters including gender, age, tumor location and size, CEA level, depth of tumor invasion, Lymph node metastasis and distant metastasis, TMN and histological type and grade were statistically evaluated. As shown in **Table 2**, No significant correlations were observed among MAGE-D4 expression, serum antibody and those clinical parameters.

### **Discussion**

One of the major barriers to antigen-specific immunotherapy in CRC is the lack of well-defined immunogenic tumor antigens. It is urgent and challenging to identify and characterize tumor-specific antigens in CRC. In the present study, we characterized both expression pattern and humoral immune response of MAGE-D4, a new member of the MAGE family, to assess its potential as a target for prognosis and immunotherapy of CRC.

Our results demonstrated that the expression frequency of MAGE-D4 mRNA in CRC tissues was significantly higher than that in adjacent non-tumor tissues ( $P=0.000$ ). It is in disagreement with the report by Chung et al, they showed no differential expression of MAGE-D4 mRNA between CRC tissues and adjacent non-tumor tissues [26]. This discrepancy may result from different clinical samples, the heterogeneity of gene expression in tumors and different research method. In general, RT-PCR analysis applied in present study turned out to be more sensitive than the cDNA microarray hybridization used by Chung et al [32]. Furthermore, MAGE-D4 protein was declared positive in all of CRC tissues, 70% of which exhibited high-expression. Previous studies have reported that CRC is a poor MAGE antigen expresser [13-18]. Our data explored that either mRNA or protein of MAGE-D4 has a higher expression frequency as compared to other MAGE antigens in CRC, suggesting that MAGE-D4 may be a more promising target for CRC immunotherapy than other MAGE antigens, at least in Chinese.

In analyzing MAGE-D4 expression in non-tumor tissue setting, we found that MAGE-D4 mRNA

was positive in a small portion of adjacent non-tumor tissues (15.0%, 9/60) and absent in normal colorectal tissues. MAGE-D4 positivity in adjacent non-tumor tissues might be associated with infiltrating tumor cells. Jeon et al [33] examined MAGE mRNA expression in 46 CRC tissues and matched normal mucosal tissues within 20 mm, 20 to 50 mm and more than 50 mm from tumors. They found that the MAGE expression rates were greatly decreased as the tissues collected far from tumor site. Therefore, when adjacent non-tumor tissues were collected, their locations from tumor are an important issue. In addition, we also found that colorectal adenoma tissues showed no MAGE-D4 expression by either RT-PCR or immunohistochemistry. Many previous studies demonstrated colorectal adenoma as a precursor lesion for CRC [28, 34], thus MAGE-D4 seems not to be involved in transformation from benign to malignancy.

Taken together, MAGE-D4 expression analysis suggested that MAGE-D4 may be a CRC-specific antigen. However, heterogeneous intratumor expression of MAGE-D4 was observed, which may hamper the effectiveness of MAGE-D4-based immunotherapy for CRC. This phenomenon is also frequent in other MAGE antigens [35, 36]. The underlying reason is still largely unknown. Generally, most MAGE family genes belong to epigenetic-mediated regulation genes, by which the heterogeneity may be improved to a certain extent through epigenetic modulation [9, 37, 38]. Our previous study has shown that DNA methylation in MAGE-D4 promoter region is an important mechanism in regulation of MAGE-D4 expression, and treatment of 5-AZA-CdR, a DNA methyltransferase (DNMT) inhibitor, can enhance MAGE-D4 expression in MAGE-D4-negative glioma cells [39]. Whether the heterogeneous expression of MAGE-D4 in CRC is also related to DNA methylation, it needs further investigation.

Here, we fail to observe significant correlations between MAGE-D4 expression and clinicopathological parameters of CRC patients. However, we found that MAGE-D4 protein expression correlated to CRC clinical outcome. Interestingly, patients with high-MAGE-D4 expression were associated with a poorer survival outcome, whereas low-MAGE-D4 expression was associated with a better survival outcome. Therefore, MAGE-D4 expression tends to be an indepen-

dent prognostic factor, suggesting that MAGE-D4 may have the potential value as a prognostic marker of CRC. However, the sample size is still small for longer period of follow-up. Further studies are needed to confirm its prognosis value by enlarging the sample size.

It has been noted that overexpression of many tumor antigens may cause humoral immune response in patients with tumor burden [40-43]. So did patients with CRC [15, 28, 44]. Monitoring these antibodies in patients' sera could have potential diagnostic and prognostic significance [45]. Considering MAGE-D4 overexpression in CRC mentioned above, it is necessary to uncover a possibly immune response against MAGE-D4 in patients with CRC. Accordingly, we further tested MAGE-D4 seroreactivity in CRC patients and healthy individuals. The results showed that sera antibody against MAGE-D4 was found in 18.8% (12/64) of patients with CRC and not in healthy individuals. In our survey of 25 CRC patients where tumor and serum samples from the same patients were available, 3 of 21 CRC patients with MAGE-D4 mRNA-positive tumors had antibodies against MAGE-D4. Furthermore, the three sero-positive patients showed high-MAGE-D4 protein expression, suggesting that preferential, high expression of this antigen may give rise to the specific humoral immune response in patients with CRC. Although we have not tested antigen-specific CD8<sup>+</sup> T cell responses to MAGE-D4 in the present study, humoral immune response to MAGE-D4 in CRC patients may be a predictive of cellular immune response, resembling the case of MAGE-A1 and MAGE-A3 [46]. A HLA-A\*25/MHC I-binding peptide of MAGE-D4 protein has been identified [20]. In a recent study in other cancer, we also found that MAGE-D4 protein can be recognized by CD8<sup>+</sup> T cells in vitro and induce cytotoxic reaction against glioma cell line which expresses MAGE-D4 (unpublished data). Clearly, further investigation will be needed to explore MAGE-D4-specific cytotoxic T cells in CRC.

In conclusion, our findings demonstrate that MAGE-D4 is frequently expressed in CRC and show inherent immunogenicity. Hence, MAGE-D4 may be a potential target for prognostic marker and specific immunotherapy in CRC. The role of MAGE-D4 in CRC progression remains to be elucidated. Further studies of functional analysis should be done to clarify the

molecular mechanisms underlying the biological activities of MAGE-D4 in CRC.

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### Disclosure of conflict of interest

All disclosures will be published if the manuscript is accepted.

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