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Original Article

Synergistic regulation of methylation and SP1 on MAGE-D4 transcription in glioma

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Abstract: Background: The family of MAGE genes is well known due to the majority of MAGE genes expressing specifically in tumor tissues while restrictedly in normal tissues. MAGE-D4 is one of the MAGE family and considered as a promising target for glioma immunotherapy because of its overexpression in glioma and restricted expression in normal tissues. Whereas the mechanism of MAGE-D4 heterogeneous expression in glioma has not yet been elucidated. In this study, the transcriptional regulation mechanism of MAGE-D4 in glioma is focused from the perspectives of promoter methylation and SP1. Methods: Dual-luciferase reporter assay was performed to identify the core promoter of MAGE-D4 gene. Mass spectrometry was applied to quantify the methylation status of MAGE-D4 promoter in 50 glioma and 9 normal brain tissues. The influence of methylation and SP1 on MAGE-D4 transcriptional activity was evaluated by dual-luciferase reporter assay, qRT-PCR, western blot and ChIP-qPCR. Decitabine, an epigenetic drug, was used to treat the glioma cells. Then the treated cells were evaluated the influence of demethylation on SP1 binding to MAGE-D4 promoter. Results: The -358 to +172 bp region was identified as the core promoter of MAGE-D4 gene which demonstrated hypomethylated and negative correlation between methylation level and MAGE-D4 mRNA expression in glioma tissues. For single CpG unit analysis, 8 CpG units (CpG unit 1, 2, 3, 4, 5, 6, 9 and 12) in MAGE-D4 core promoter showed hypomethylated in glioma and the methylation level of CpG unit 6 was positively associated with the prognosis of glioma patients. Furthermore, the methylation level of CpG unit 1 and 6 was negative negatively correlated with MAGE-D4 mRNA expression. Then, the results demonstrated that the promoter activity of MAGE-D4 was decreased by methylation in glioma cell lines. In addition, SP1 can binds directly to the MAGE-D4 promoter leading to up-regulation of MAGE-D4 mRNA through activation of its promoter. Finally, demethylation of MAGE-D4 promoter could benefit the SP1 binding and resulting co-activation of MAGE-D4 promoter by demethylation and SP1 in glioma cell lines. Conclusion: These findings indicate that the synergies of promoter hypomethylation and SP1 up-regulated MAGE-D4 transcription in glioma, which implies a potential approach to resolve the heterogeneous expression of MAGE-D4 in order to establish foundation for the MAGE-D4 based glioma therapy.

Keywords: Glioma, MAGE-D4, methylation, SP1

Introduction

Glioma is the most common primary intracranial tumor, accounting for more than 60% of all adult primary intracranial tumors [1, 2]. The current treatment strategy of glioma is mainly surgery assisted with radiotherapy and chemotherapy. However, implementation of these therapies is not able to increase the survival rate of glioma patients [2, 3], and there are only 5% of patients who live for more than five years [4, 5]. As we known, early stage diagnosis and early treatment are two critical factors associating with the prognosis of glioma patients. Now, due to the lack of highly sensitive and specific targets for diagnosis and therapy, glioma treatment strategies like molecular therapy or immune therapy can not be utilized after effective complement of surgeries, which is also a reason for the unsatisfied prognosis of glioma patients. In addition, although some studies have revealed that some genetic factors

are involved in the pathogenesis of glioma, such as isocitrate dehydrogenase 1 (IDH1) and epidermal growth factor receptor (EGFR), the specific pathogenesis of glioma is still unclear [6-9], which not only interfered with the early diagnosis of glioma, but also hindered the development of new treatment strategy. Therefore, seeking potential target molecules has crucial significance on diagnosis and treatment for glioma. Meanwhile, developing molecular target therapy based on these target molecules is in accordance with the concept which is applying minimally surgical intervention to get best outcome for patients.

The family of MAGE genes is well known due to the majority of MAGE genes expressing specifically in tumor tissues while restrictedly in normal tissues [10, 11]. Therefore, MAGE genes regarded as the ideal target for tumor therapy. MAGE-D4 is a member of the MAGE gene family [12]. It has been reported that MAGE-D4 mRNA mostly expresses in glioma tissues, while low or absent in normal brain tissues. Moreover, the expression of MAGE-D4 is also found in other tumors such as breast cancer, oral squamous cell carcinoma and colorectal cancer [13-15]. At present, the functions of MAGE-D4 were believed to participate in proliferation, migration and invasion of tumor cells leading to unfavorable prognosis [13-15]. In addition, our previous study also has been testified up-regulation of MAGE-D4 in the tissues and cell lines of glioma, and low or absent expression in normal brain tissues; Moreover, we found that the expression of MAGE-D4 was more higher in high grade gliomas than that in low grade gliomas [16, 17]. These results suggested that MAGE-D4 could be a potential target of molecular or immune therapy for glioma. Nevertheless, heterogeneous intratumor expression of MAGE-D4 was observed in glioma [16], which might be an obstacle for MAGE-D4 based biotherapy or immunotherapy. Therefore, to explore the expression mechanism of MAGE-D4 in gliomas would be the essential prerequisite and foundation of utilizing MAGE-D4 as a therapeutic target for glioma.

It has been reported that the promoter region of most MAGE family genes exists CpG island, and demethylation of these CpG sites could promote the expressions of these genes [11, 18, 19]. Especially, the region of transcriptional start site of MAGE-D4 is considered to be present in CpG island as well [20]. In a previous study, we confirmed that the methylation level of MAGE-D4 promoter was negatively correlated with its expression, and decitabine (DAC), a DNA methyltransferase inhibitor, could promote MAGE-D4 expression in glioma cell lines [16]. These results indicated that the expression of MAGE-D4 might be affected by promoter methylation. Also, we speculated that promoter methylation might change the activity of MAGE-D4 promoter and then affect its expression. Therefore, in this study, we conducted a series of experiments to investigate the mechanism of promoter methylation which might adjust to MAGE-D4 expression.

As is known to all, transcriptional regulation of genes involves in many factors, one of which is transcription factor. So far, there is no any report for the transcription factor acting on the MAGE-D4. Therefore, we analyze the upstream sequence on 5' end of MAGE-D4 to search for potential binding sites of transcription factors, and finally lock in specificity protein 1 (SP1) among many other transcription factors. The reasons why selecting SP1 are as followed: Firstly, There are many putative SP1 binding sites on the MAGE-D4 promoter region based on the multiple online prediction websites (PROMO and HumanTFDB); Secondly, as SP1 binding sites and CpG sites have shared region on the MAGE-D4 promoter, when these CpG sites methylated it could interfere with SP1 binding, which may cause dis-regulation of MAGE-D4 transcription. Thus, we investigate the role of promoter methylation and SP1 on transcription of MAGE-D4 in glioma, respectively and together.

Materials and methods

Cell lines

Human glioma cell lines SHG44, U251, U87-MG and Human embryonic kidney cell line HEK293T were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere.

Glioma and normal brain tissues

A total of 50 glioma samples and 9 normal brain samples (from traumatic decompression

patients) were collected from the Department of Neurosurgery at the 1st Affiliated Hospital of Guangxi Medical University. The use of human materials obtained with informed consent was approved by the Clinical Research Ethics Committee of Guangxi Medical University.

Drug treatment

SHG44 cell line was treated with the DNA-demethylating agent 5-aza-2'-deoxycytidine (Decitabine, DAC; Sigma-Aldrich) at 1 μM every 24 h for 5 days. Then, the cells were washed with PBS and harvested for Chromatin Immunoprecipitation.

Transfection

SP1 cDNA (the sequence is obtained from the National Center of Biotechnology Information database) was subcloned into the plasmid pcDNA3.1 vector (pcDNA-SP1). siRNA targeting SP1 (SP1-siRNA, the sequences were listed in Supplemental Materials) was purchased from Transheep (Shanghai, China). The pcDNA-SP1 or the SP1-siRNA were transiently transfected into cell lines using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Quantitative analysis of DNA methylation

Genomic DNA was isolated from the glioma and normal brain samples using TIANamp Blood DNA Kit (Tiangen, China). Methylation status of MAGE-D4 promoter in glioma and normal brain tissues was quantified by Sequenom MassARRAY, which was performed by Beijing Honortech Co. Ltd., China. Methylation level was shown as the percentage of methylated cytosines over the total number of methylated and unmethylated cytosines. Glioma samples tested were divided into hypermethylated and hypomethylated groups according to the mean value of methylation level of MAGE-D4 promoter in normal brain tissues.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from glioma tissues and cell lines using a Universal RNA Extract Kit (TaKaRa). First-strand cDNA was synthesized from total RNA using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's protocols. qRT-PCR was performed on the StepOneTM Real-Time PCR System (Applied Biosystems) using SYBR Green I (Roche). MAGE-D4 and SP1 specific primers were designed and used for qRT-PCR. GAPDH mRNA expression was used as an internal control for normalization of target gene expression. The sequences of primers were listed in Supplemental Materials.

Western blot

Nuclear protein was extracted from glioma cells using a Nuclear Protein Extraction Kit (Solarbio) according to the manufacturer's protocol. Then the protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 5% non-fat dry milk, the membrane was sequentially incubated with the primary antibody SP1 and Lamin B1 (Abcam, ab231778 and ab16048, respectively) at a 1:800 dilution overnight at 4°C. Then, the membrane was washed by TBST for three times and applied to incubation of the second antibody (IRDye® 800CW-labelled goat anti-rabbit antibody, LI-COR Biosciences). The protein expression was represented by the band intensity analyzed by LI-COR Odyssey infrared imaging system (LI-COR Biosciences). Data were normalized by Lamin B1 protein level.

Dual-luciferase reporter assay

The upstream sequence of the MAGE-D4 gene gained in the NCBI and UCSC databases was input into different promoter prediction websites (Promoter Scan, FirstEF, PromoterInspector, etc). The approximate promoter region of MAGE-D4 was identified in the range of -1897~+172 bp relative to the transcriptional start site (TSS) of MAGE-D4 gene (Supplementary Table 1 and Supplementary Figure 1). According to this region, luciferase reporter constructs containing the MAGE-D4 truncated promoter sequences were inserted into the pGL3-basic vector (Promega), designated as pGL3-P1 (-1897~+172 bp), pGL3-P2 (-614~+172 bp), pGL3-P3 (-358~+172 bp), pGL3-P4 (-270~+172 bp) and pGL3-P5 (-90~+172 bp), respectively. In light of four potential SP1 binding sites in the sequence of pGL3-P3, pGL3-P3-MT1~4 was designated, respectively. The sequence of pGL3-P1~P5 and pGL3-P3-MT1~4 were listed in Supplemental Materials. pGL3-P1~P5 and

pGL3-P3-MT1~4 were transfected into HEK-293T, U251 and U87-MG cells. Firefly and Renilla luciferase activities were measured at 48 h post-transfection using a Dual-Luciferase reporter assay system (Promega). Relative promoter activation is represented as the ratio of firefly to Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed using a Pierce Agarose ChIP assay kit (Thermo). Briefly, the cells were fixed with 1% formaldehyde at 37°C for 10 min, washed twice with ice-cold PBS containing protease inhibitors, scraped, pelleted, and resuspended in ChIP lysis buffer. The cell lysate was subjected to enzyme digestion and then incubated overnight with anti-SP1 antibody (Abcam, ab231778). Normal rabbit IgG antibody (Thermo) was used as a negative control. After a series of washes, the bound DNA-protein complexes were eluted, and the cross-linking was reversed. MAGE-D4 promoter sequence in the resulting DNA fragments was amplified by qPCR. The values from the immunoprecipitated samples were normalized to that from the input DNA. Computational formula of % Input: ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] -Log2 (Input Dilution Factor))), % Input = 2^(-ΔCt [normalized ChIP]).

Statistical analysis

Each CpG unit methylation data of MAGE-D4 from 59 samples were used for stratified cluster analysis and describe by OmicShare Tools (https://www.omicshare.com/tools/) and GraphPad Prism 8. The methylation level of CpG units in the MAGE-D4 promoter was compared between Glioma and normal brain groups by student's t-test. The luciferase activity of pGL3-P1~P5 was compared by Kruskal-Wallis test. The association between CpG units methylation level and clinical parameters was determined by chi-square test or fisher's exact test. Spearman correlation test was used for analyzing the correlation between methylation level and MAGE-D4 mRNA expression.

Results

Identified MAGE-D4 core promoter contained multiple CpG sites

Luciferase assay demonstrated that pGL3-P3 had relatively higher luciferase activity than the others. Therefore, the region of -358~+172 nt was considered as MAGE-D4 core promoter (**Figure 1**). Interestingly, we found that there were 37 CpG sites in the region of MAGE-D4 core promoter. Based on our previous study, MAGE-D4 promoter methylation may reduce by methyltransferase inhibitor DAC, which resulted in MAGE-D4 up-regulation in glioma cell lines [16]. Thus, we infer that MAGE-D4 promoter methylation may also be present in glioma tissues.

MAGE-D4 promoter was hypomethylated in glioma tissues

A panel of glioma and normal brain tissues was applied to detect the methylation level of MAGE-D4 promoter. Of the 37 CpG sites in the MAGE-D4 core promoter region, a total of 29 CpG sites (from 2nd CpG site to 30th CpG site) were detected by methylation mass spectrometry, among which 22 CpG sites were successfully detected. The 22 CpG sites detected were grouped into 15 CpG units because some of CpG sites cannot be well distinguished due to their close distance (**Figure 2**; CpG units contained CpG sites were listed in Supplemental Materials). As **Figure 3A** shown, the total methylation level of 15 CpG units in the MAGE-D4 core promoter is significantly higher in normal brain tissues than that in glioma tissues. Of 15 CpG units, 8 CpG units demonstrated higher methylation levels in normal brain tissues compared to the glioma tissues (**Figure 3B**). Therefore, the methylation of these 8 CpG units may be crucial and possess clinical significance.

CpG unit 6 was associated with patients' survival

To understand the influence of MAGE-D4 core promoter methylation on patients' prognosis, we performed a Kaplan-Meier analysis. First, total methylation level of 15 units was analyzed. As shown in **Figure 3C**, there was no significant difference in prognosis between the hypermethylated and hypomethylated MAGE-D4 core promoter. Then, each of 8 CpG units, which were identified from above showing differential methylation level in gliomas and normal brain tissues, was analyzed. The result demonstrated that the methylation status of CpG unit 6 is associated with prognosis instead of others. The hypermethylation of CpG unit 6 had a better outcome than that hypomethylation one (**Figure 3D**, Supplementary Figures 2, 3, 4, 5, 6, 7, 8).

On the other hand, we performed a Kaplan-Meier analysis to understand the influence of

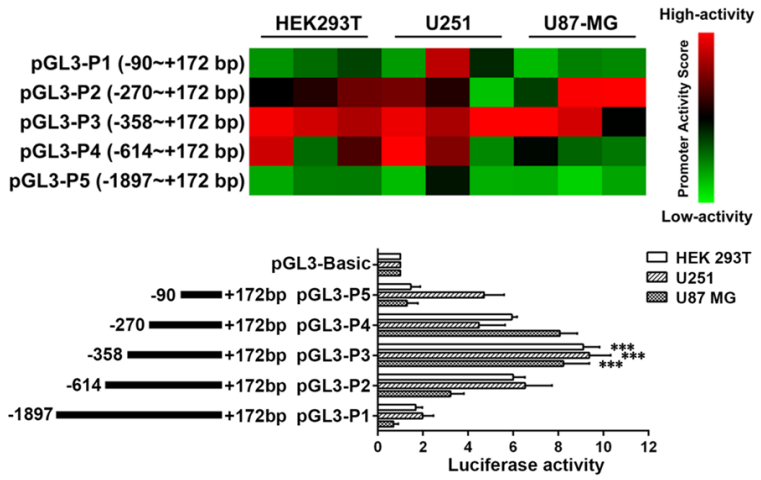

Figure 1. Identification of MAGE-D4 core promoter. Upper part: The heatmap showed the differential luciferase activity of pGL3-P1~P5 in HKE293T, U251 and U87-MG cell lines. The red and green scales represented higher or lower luciferase activity, respectively. Each line described the luciferase activity of pGL3-P1~P5 in three cell lines and each column represented an independent experiment. Lower part: Luciferase activities of pGL3-P1~P5 were measured and normalized to pGL3-Basic. The error bars represented the standard deviations of three independent experiments. ***P<0.001.

clinical parameters (Supplementary Table 2) on the prognosis of patients and the results indicated that WHO grade, pathological type, KPS score, tumor size and MAGE-D4 mRNA expression may be the influence factors of prognosis in glioma patients (Supplementary Figures 9, 10, 11, 12, 13, 14, 15, 16, 17). Further, a cox regression model was established which was shown in Supplementary Table 3 and the results demonstrated that WHO grade, KPS score and MAGE-D4 mRNA expression were independent risk factors of prognosis in glioma patients.

MAGE-D4 promoter methylation was correlated with its expression and clinical parameters

We next figure out whether methylation of MAGE-D4 promoter will affect its expression. The results indicated that the total methylation level of MAGE-D4 core promoter was negatively correlated with its mRNA expression (**Table 1** and **Figure 3E**). Further, the methylation level of 8 CpG units was validated, respectively. The results of chi-square test showed that the methylation level of 6 CpG units (CpG unit 1, CpG unit 3, CpG unit 4, CpG unit 5, CpG unit 6 and CpG unit 9) was associated with MAGE-D4 mRNA expression (Supplementary Tables 4, 6, 7, 8, 9, 10). Of these 6 CpG units, the methylation level of CpG unit 1 and CpG unit 6 was significantly negatively correlated with MAGE-D4 mRNA expression by spearman correlation test (**Figure 3F** and **3G**), others were no statistically significance (Supplementary Figures 18, 19, 20, 21).

Then, we analyzed the possible correlation between the methylation of MAGE-D4 core promoter and clinicopathological parameters. The results were summarized in **Table 1**, which indicated there was a significant difference between the gender of patients. Particularly, the methylation level in all of the 8 CpG units was different between male and female patients (Supplementary Tables 4, 5, 6, 7, 8, 9, 10, 11). And CpG unit 12 was correlated with age (Supplementary Table 11).

Methylation reduced MAGE-D4 promoter activity

Based on our previous result that demethylation by DAC led to up-regulation of MAGE-D4 in glioma cells [16] and above results, it is reasonable to infer that methylation may affect MAGE-D4 promoter activity and cause a change of its expression in glioma. To figure out this, a luciferase assay was performed through pGL3-P3 contained the core promoter region of MAGE-D4. Firstly, pGL3-P3 was methylated by methylase and then digested by the methylation-sensitive restriction enzyme (HhaI) to confirm its methylated status which was shown in Supplementary Figure 22. Subsequently, both of methylated and unmethylated pGL3-P3 (M-pGL3-P3 and UM-pGL3-P3) were transfected into cells, respectively. As shown in **Figure 3H**, the luciferase activity of M-pGL3-P3 was significantly lower than that of UM-pGL3-P3, suggesting that methylation may reduce MAGE-D4 promoter activity.

SP1 may influence MAGE-D4 expression in glioma cells

Besides the role of methylation on MAGE-D4 expression in glioma, some transcription factors may involve in the regulation of MAGE-D4 expression. Therefore, the potential transcription factors binding sites on the MAGE-D4 core

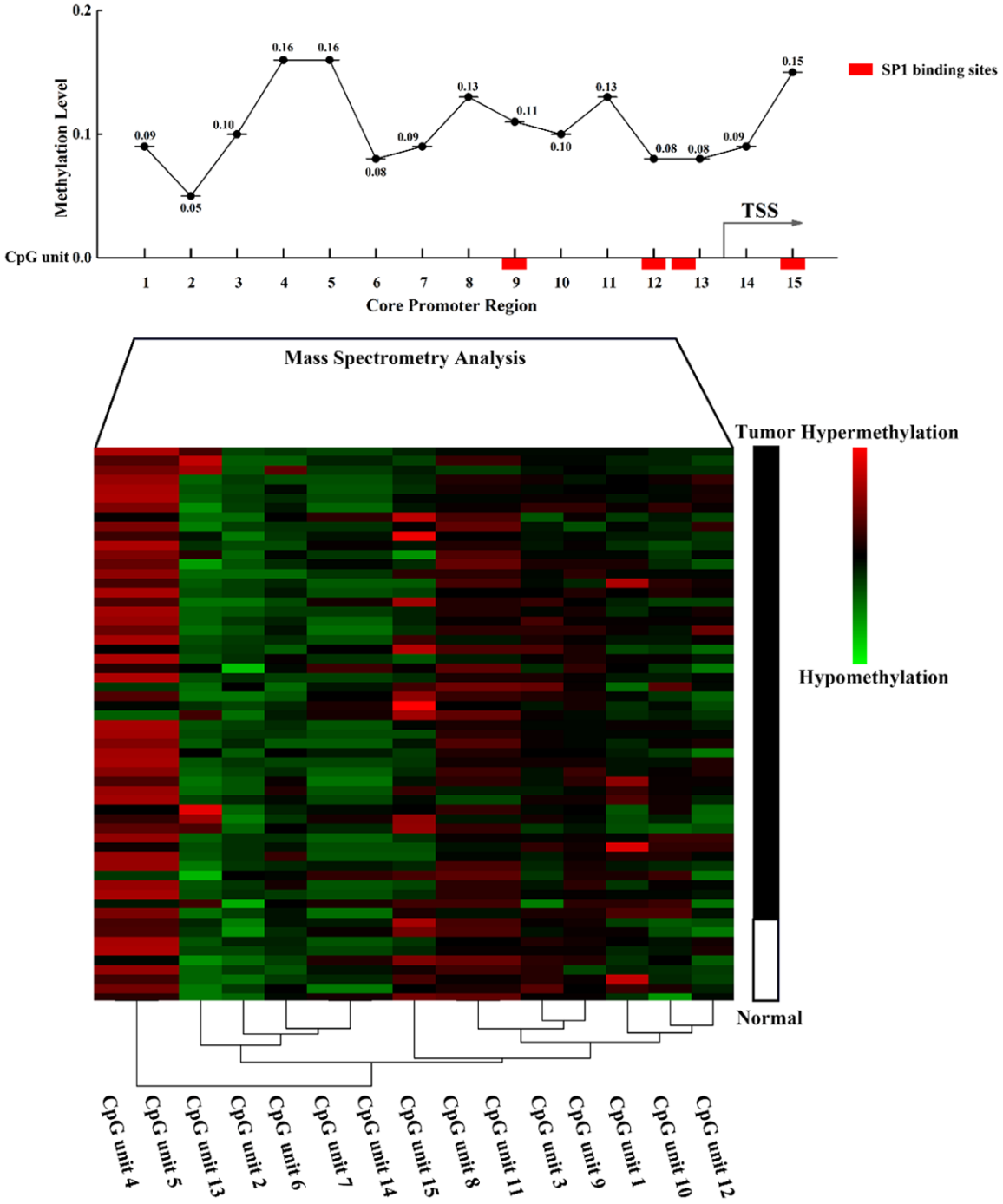

Figure 2. Genomic structure and CpG methylation profiles of MAGE-D4 promoter in glioma and normal brain tissues. Upper part: Mass Spectrometry analysis showed the differential methylation level of each CpG unit tested in MAGE-D4 promoter. The ordinate served as a scale of methylation level. The numbers under the abscissa represented the 15 CpG units in MAGE-D4 promoter and their methylation levels were shown with black solid points. TSS, transcriptional start site. Lower part: The heatmap showed the CpG methylation profiles of MAGE-D4 promoter in 50 glioma and 9 normal brain tissues. The location of Mass Spectrometry analysis was from CpG unit 1 to CpG unit 15. The red and green scales represented higher or lower methylation levels, respectively. Each line represented the methylation level of CpG units in a sample and columns represented different CpG units. Methylation levels of the CpG units were performed in hierarchical cluster analysis.

promoter were searched with online prediction tools (Supplementary Figure 23 and Supplementary Table 12). As shown in **Figure 2**, there were 4 potential binding sites of SP1. It is

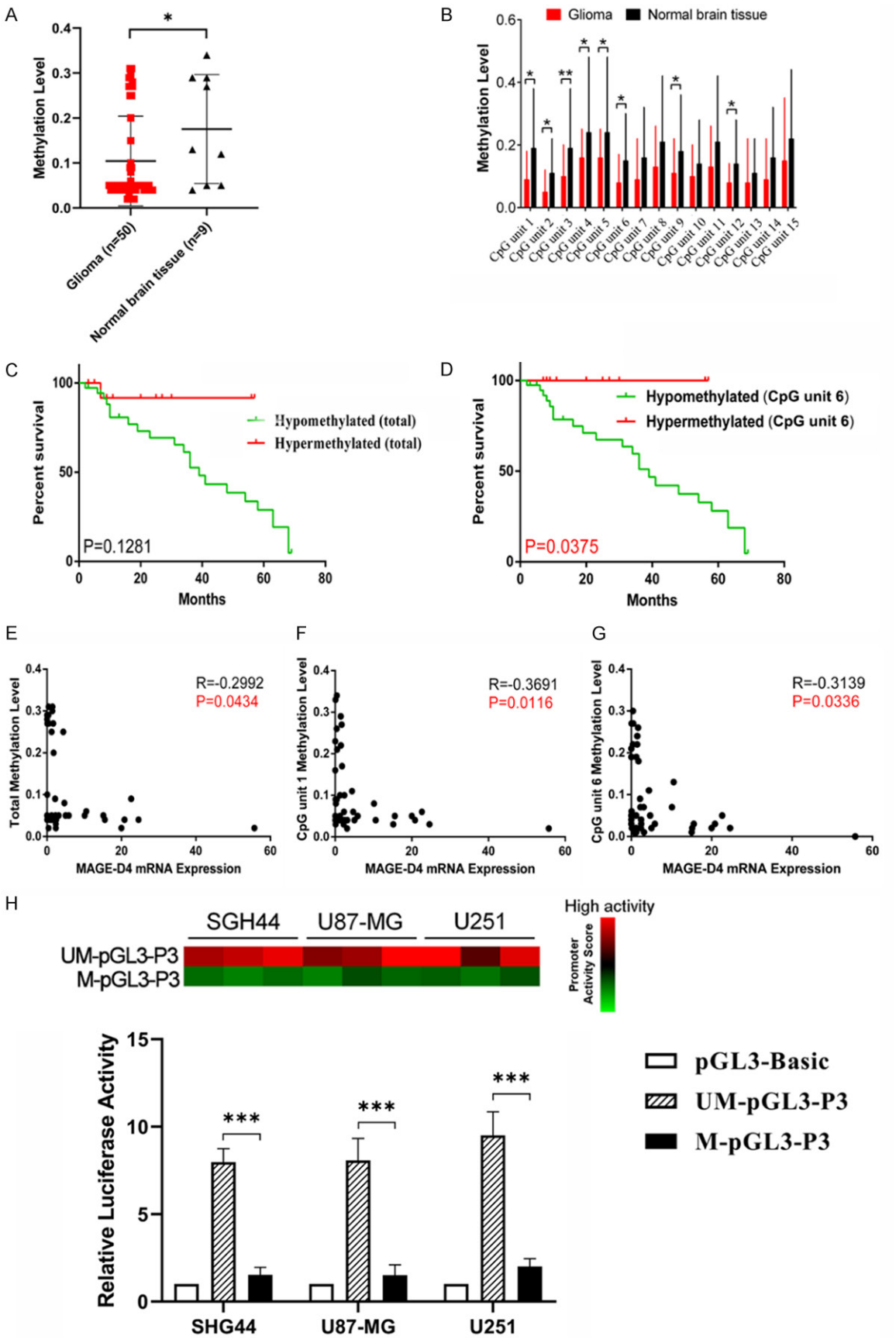
A
Methylation Level
Glioma (n=50)
Normal brain tissue (n=9)
B
Glioma
Normal brain tissue
Methylation Level
CpG unit 1
CpG unit 2
CpG unit 3
CpG unit 4
CpG unit 5
CpG unit 6
CpG unit 7
CpG unit 8
CpG unit 9
CpG unit 10
CpG unit 11
CpG unit 12
CpG unit 13
CpG unit 14
CpG unit 15
C
Percent survival
Hypomethylated (total)
Hypermethylated (total)
P=0.1281
Months
D
Percent survival
Hypomethylated (CpG unit 6)
Hypermethylated (CpG unit 6)
P=0.0375
Months
E
Total Methylation Level
R=-0.2992
P=0.0434
MAGE-D4 mRNA Expression
F
CpG unit 1 Methylation Level
R=-0.3691
P=0.0116
MAGE-D4 mRNA Expression
G
CpG unit 6 Methylation Level
R=-0.3139
P=0.0336
MAGE-D4 mRNA Expression
H
SGH44
U87-MG
U251
UM-pGL3-P3
M-pGL3-P3
High activity
Promoter Activity Score
Relative Luciferase Activity
pGL3-Basic
UM-pGL3-P3
M-pGL3-P3
SHG44
U87-MG
U251

Figure 3. Feature and significance of MAGE-D4 promoter methylation in glioma. A. Total methylation level of MAGE-D4 promoter in glioma and normal brain tissues. The mean values of total methylation level of MAGE-D4 promoter in glioma (n=50) and normal brain tissues (n=9) were compared by Student's t test. The results showed total methylation level of the MAGE-D4 promoter in normal brain tissues is significantly higher than that in glioma tissues. B. CpG units methylation level of MAGE-D4 promoter in glioma and normal brain tissues. The mean values of each 15 CpG units in glioma and normal brain tissues were compared by Student's t test. Eight CpG units demonstrated higher methylation levels in normal brain tissues compared to the glioma tissues. C and D. The Correlation between total or CpG unit 6 methylation level and overall survival of glioma patients. Glioma samples were divided into hypermethylated and hypomethylated groups according to the mean value of methylation level of MAGE-D4 promoter in normal brain tissues. Kaplan-Meier curve was performed to evaluate the correlation between methylation and overall survival of glioma patients. *P* values were noted in the figures. E-G. The correlations between methylation and MAGE-D4 mRNA expression. Spearman correlation test was performed to evaluate the correlation between methylation and MAGE-D4 mRNA expression. The correlation coefficient and *P* values are noted in the figures. H. Influence of methylation on MAGE-D4 promoter activity. The Heatmap and histogram showed differential luciferase activities of UM-pGL3-P3 and M-pGL3-P3 in SHG44, U87-MG, U251 cell lines. The result indicated that methylation may reduced MAGE-D4 promoter activity. The red and green scales represented higher or lower luciferase activity, respectively. Each line described the luciferase activity of UM-pGL3-P3 and M-pGL3-P3 in three cell lines and each column represented an independent experiment. *P<0.05, **P<0.01, ***P<0.001.

interesting that these SP1 binding sites which were co-localized with 3 of CpG units (CpG unit 9, CpG unit 12 and CpG unit 15). It is worth noting that both CpG unit 9 and CpG unit 12 are differential methylation in normal brain tissues and glioma tissues as shown above, which implied some links between CpG methylation and SP1. On the other hand, highly expressed SP1 in glioma was positively correlated with MAGE-D4 mRNA expression (**Figure 4A-C**), which indicated that SP1 may participate in the regulation of MAGE-D4 mRNA expression.

To verify the effect of SP1 on MAGE-D4 mRNA expression, up-regulated and down-regulated SP1 were modulated in cell lines, respectively. Firstly, the profile of SP1 mRNA and protein expression in different cells was determined by qRT-PCR and Western blot. As shown in **Figure 4D** and **4E**, the lowest SP1 mRNA and protein expression were in U87-MG cells, followed by U251 cells, and the highest expression in SHG44 cells. Therefore, U87-MG and SHG44 were transfected by pcDNA-SP1 and SP1-siRNA, respectively, and U251 was used to transfect pcDNA-SP1 as well as SP1-siRNA, respectively (**Figure 4F-I**). The results showed that an increase of SP1 resulted in the up-regulation of MAGE-D4 mRNA (**Figure 4J**). Inversely, knocking down the SP1 caused reduce of MAGE-D4 mRNA in U251, instead of SHG44 (**Figure 4K**). These results suggested that SP1 may promote MAGE-D4 transcription in some of glioma cell lines.

MAGE-D4 promoter was activated by binding of SP1

We further confirmed that the up-regulation of MAGE-D4 resulted from the enhancement of its promoter activity through SP1 binding. As shown in **Figure 5A**, the luciferase assay demonstrated increasing MAGE-D4 promoter activity in U87-MG after co-transfecting pGL3-P3 and pcDNA-SP1. On the contrary, MAGE-D4 promoter activity was reduced in SHG44 after co-transfecting pGL3-P3 and SP1-siRNA. Additionally, both increased and reduced MAGE-D4 promoter activity was demonstrated in U251 after co-transfecting pGL3-P3 with pcDNA-SP1 and SP1-siRNA, respectively.

Next, to determine whether the four potential SP1 binding sites have a role in the transcriptional activation of MAGE-D4 core promoter, these individual binding sites were mutated respectively. The result showed that abrogation of the first and fourth binding sites of SP1 (pGL3-P3-MT1 and pGL3-P3-MT4) led to significantly reduce luciferase activity compared to the wild type binding site (pGL3-P3-WT) (**Figure 5B**). Thus it can be concluded that these two sites may involve in the activation of MAGE-D4 core promoter instead of the others.

Then, SP1 bound to MAGE-D4 promoter was confirmed by ChIP-qPCR (**Figure 5C**). Taken together, the above results provide a proof that SP1 binds directly to the MAGE-D4 promoter leading up-regulation of MAGE-D4 mRNA through activation of its promoter.

MAGE-D4 promoter was activated by the synergistic role of demethylation and SP1

As shown from the above results that up-regulation of MAGE-D4 resulted from its promoter demethylation and SP1 binding, respectively,

Table 1. Correlation between total methylation level of MAGE-D4 promoter and clinical parameters in glioma patients

Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	1 (8.3)	35 (92.1)	
Female	11 (91.7)	3 (7.9)	
Age (years)			0.185
≤37	8 (66.7)	17 (44.7)	
>37	4 (33.3)	21 (55.3)	
WHO Grade			0.750
I/II (Low grades)	6 (50.0)	21 (55.3)	
III/IV (High grades)	6 (50.0)	17 (44.7)	
Pathological type			0.505
astrocytoma	7 (58.3)	25 (65.8)	
anaplastic astrocytoma	0 (0.0)	3 (7.9)	
oligodendroglioma	1 (8.3)	1 (2.6)	
glioblastoma	4 (33.3)	9 (23.7)	
KPS Score			0.768
≤70	10 (83.3)	28 (73.7)	
>70	2 (16.7)	10 (26.3)	
P53 Protein			1.000
-	6 (50.0)	19 (50.0)	
+	6 (50.0)	19 (50.0)	
Ki-67 (%)			1.000
<10	9 (75.0)	27 (71.1)	
≥10	3 (25.0)	11 (28.9)	
Tumor Size (cm)			0.508
<5	5 (41.7)	20 (52.6)	
≥5	7 (58.3)	18 (47.4)	
MAGE-D4 mRNA			0.001
High	1 (8.3)	24 (63.2)	
Low	11 (91.7)	14 (36.8)	

we are curious about the co-effect of SP1 and methylation on MAGE-D4 promoter. U87-MG with relatively lower SP1 expression was used to co-transfect pcDNA-SP1 and pGL3-P3 (methylated or unmethylated status). The results showed that co-transfecting unmethylated pGL3-P3 (UM-pGL3-P3) and pcDNA-SP1 cause the highest luciferase activity compared to others (**Figure 6A**), suggesting that MAGE-D4 promoter activation depended on the cooperation of promoter demethylation and SP1.

Interestingly, we have noticed that the first and fourth SP1 binding sites were coincident with the CpG units on the core promoter region of MAGE-D4 (**Figure 2**). It is reasonable to ask whether the methylation of MAGE-D4 promoter will affect the biding of SP1 to MAGE-D4 promoter. To answer this question, we treated SHG44 cells with DAC to reduce the methylation level of the MAGE-D4 promoter. It should be mentioned that SHG44 cell was used for DAC treatment because of its relatively high SP1 expression, and thus the effect of DAC on SP1 expression could be ignored. Additionally, our previous study has revealed a relatively high methylation level of the MAGE-D4 promoter in SHG44 [16], and thus DAC may significantly reduce its methylation level. Subsequently, DAC-treated SHG44 was used to ChIP-qPCR. The MAGE-D4 promoter was able to be amplified by qPCR from SP1 immunoprecipitation. As demonstrated in **Figure 6B**, PCR amplificated product of MAGE-D4 promoter was much higher in DAC-treated cells than those in DAC-untreated cells, which implied demethylation of MAGE-D4 promoter will benefit the SP1 binding. Overall, MAGE-D4 promoter demethylation and SP1 may play synergistic effects on the activation of MAGE-D4 promoter.

Discussion

Based on our previous study, MAGE-D4 specifically expressing in glioma, we consider that it may be an ideal target for molecular therapy or immunotherapy in glioma [16, 17]. But the heterogeneity in the expression of MAGE-D4 may be a barrier to serve it as the target for targeted-therapy in glioma. Thus, the purpose of investigating the expression mechanism of MAGE-D4 is to find out a way to eliminate the heterogeneity of MAGE-D4 expression in glioma and to make it as an ideal therapeutic target for practical application.

The promoter methylation is a universal epigenetic modification to control gene expression under the circumstances of DNA sequence unchanged. Previous studies have demonstrated that abnormal epigenetic modification and promoter methylation participate in the generation and development of multiple tumor types [21-23]. At present study, Mass spectrum analysis was applied to testing the methylation level of MAGE-D4 promoter in glioma and normal brain tissues. The result reveals that the total methylation level of MAGE-D4 promoter is lower in glioma tissues than that in normal brain tissues. Therefore, we further analyzed the correlation between the methylation level of MAGE-D4 promoter and clinical parameters

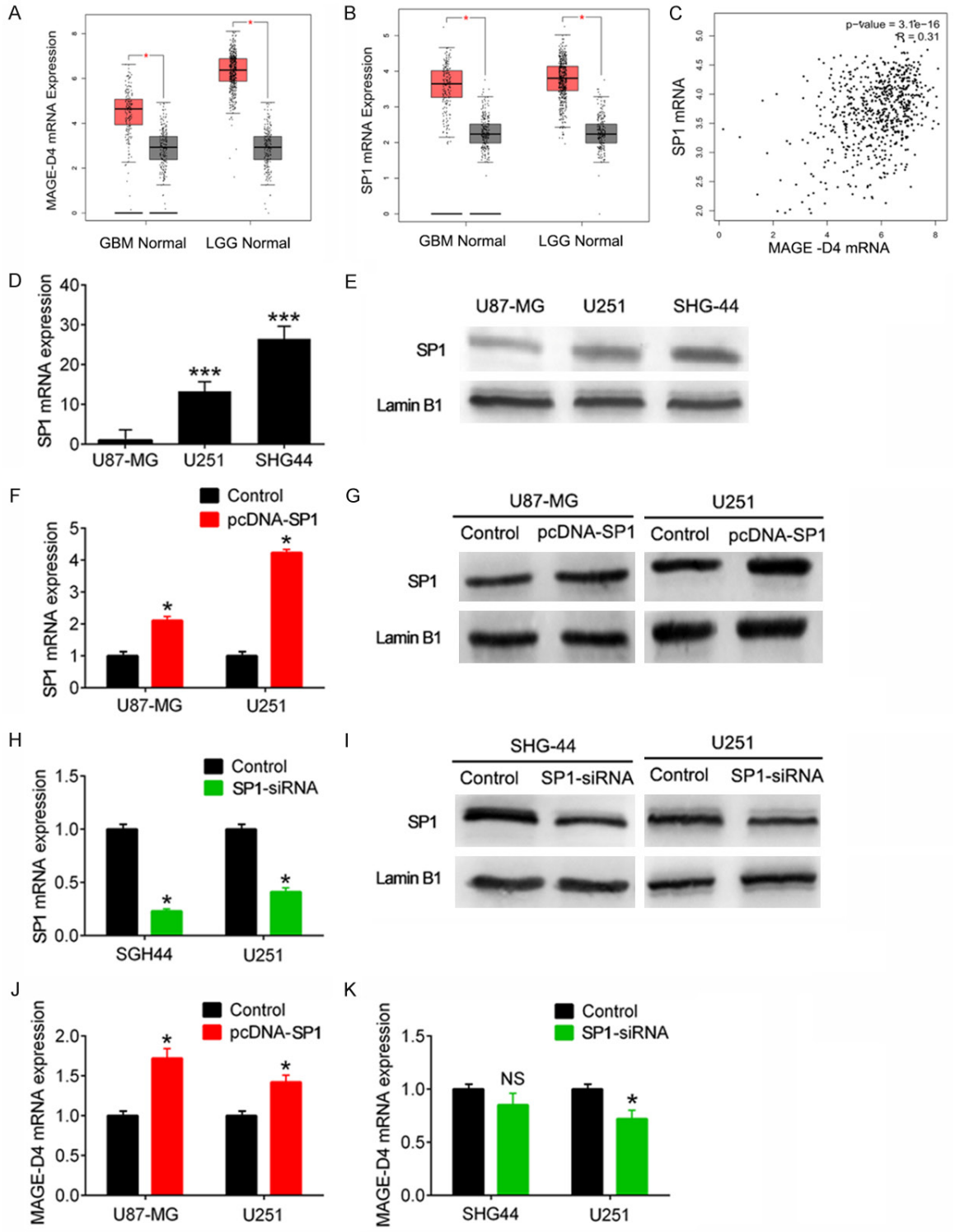

Figure 4. SP1 regulates MAGE-D4 mRNA expression in glioma cell lines. A and B. The mRNA expression of MAGE-D4 and SP1 in glioblastoma multiforme (GBM), low-grade glioma (LGG) and normal brain tissues. The mRNA expression of MAGE-D4 and SP1 in GBM (n=163) and LGG (n=518) was significantly higher than that in normal brain tissues (n=207). The data was gained from the GEPIA database (http://gepia.cancer-pku.cn/index.html). C. The correlations between MAGE-D4 and SP1 mRNA expression in glioma. Spearman correlation test was performed to evaluate the correlation between MAGE-D4 and SP1 mRNA expression in glioma (GBM and LGG, n=681). The result showed SP1 in glioma was positively correlated with MAGE-D4 mRNA expression. The data was gained from the GEPIA database. The correlation coefficient and *P* values are noted in the figure. D-K. MAGE-D4 mRNA expression

after up- and down-regulating SP1 in glioma cell lines. The profile of SP1 mRNA and protein expression in different cells was determined by qRT-PCR and Western blot. The result showed the lowest SP1 mRNA and protein expression was in U87-MG cells, followed by U251 cells, and the highest expression in SHG44 cells. U87-MG and SHG44 were transfected by pcDNA-SP1 and SP1-siRNA, respectively, and U251 was used to transfect pcDNA-SP1 as well as SP1-siRNA, respectively. The qRT-PCR showed increase of SP1 resulted in the up-regulation of MAGE-D4 mRNA. And knocking down the SP1 caused reduce of MAGE-D4 mRNA in U251, instead of SHG44. The cell lines of control group were transfected by non-specific siRNA or empty vector of pcDNA. GAPDH and Lamin B1 were used as internal controls for qRT-PCR and Western blot, respectively. The error bars represented the standard deviations of three independent experiments. *P<0.05, ***P<0.001. ns: no significance.

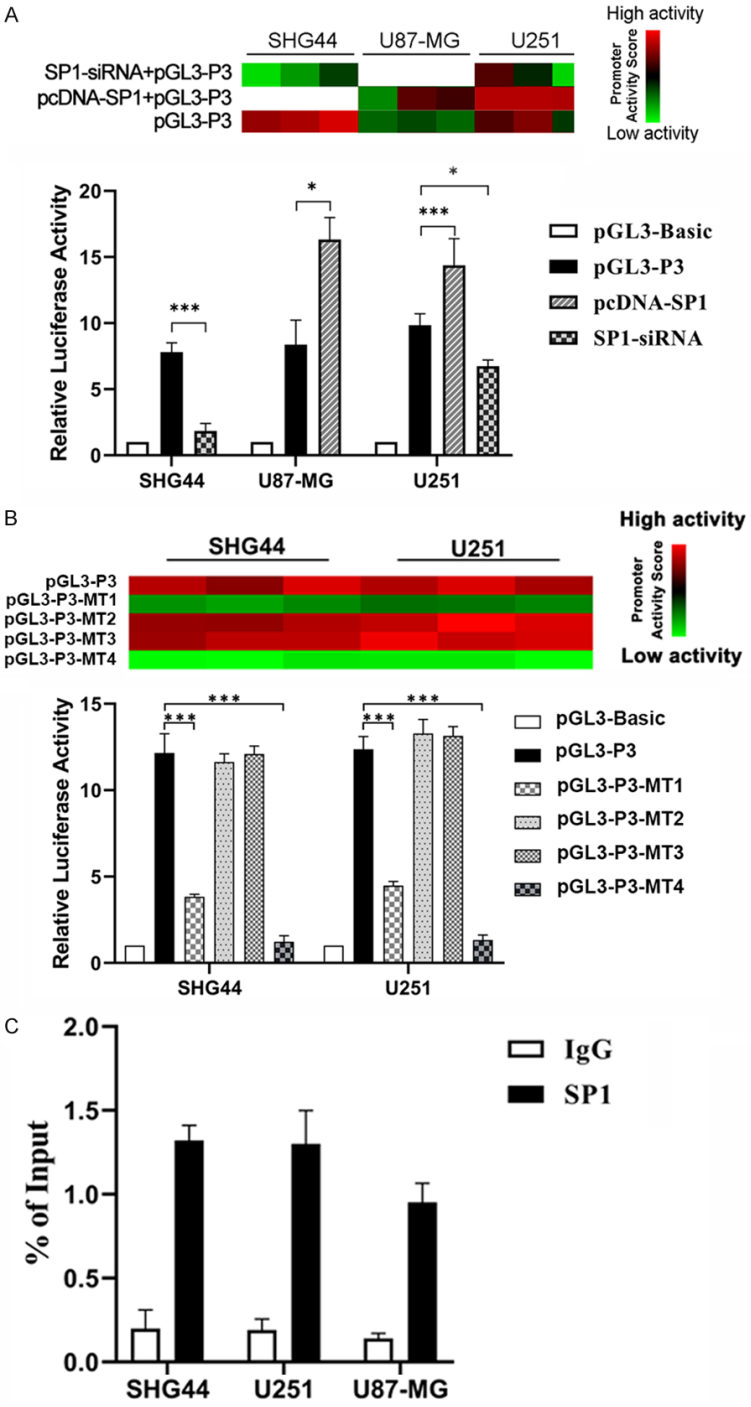

Figure 5. Influence of SP1 on MAGE-D4 promoter activity. A. SP1 activates MAGE-D4 promoter. The Heatmap and histogram showed differential luciferase activities of pGL3-P3 under the different SP1 status in SHG44, U87-MG, U251 cell lines. The result showed increasing MAGE-D4 promoter activity in U87-MG after co-transfecting pGL3-P3 and pcDNA-SP1. MAGE-D4 promoter activity was reduced in SHG44 after co-transfecting pGL3-P3 and SP1-siRNA. And both increased and reduced MAGE-D4 promoter activity was demonstrated in U251 after co-transfecting pGL3-P3 with pcDNA-SP1 and SP1-siRNA, respectively. The red and green scales represented higher or lower luciferase activity, respectively. Each line described the luciferase activity of pGL3-P3 in three cell lines and each column represented an independent experiment. B. The effect of mutation of SP1 sites on the activity of MAGE-D4 promoter. Mutations of the individual SP1 binding sites of pGL3-P3 were made. Luciferase activity of pGL3-P3-MT1 and pGL3-P3-MT4 were significantly repressed and the other two (pGL3-P3-MT2 and pGL3-P3-MT3) with no significant changes. Each line described the luciferase activity of pGL3-P3 and pGL3-P3-MT1~4 in two cell lines and each column represented an independent experiment. C. SP1 binding to MAGE-D4 promoter. ChIP-qPCR was performed to confirm the binding of SP1 to MAGE-D4 promoter in SHG44, U87-MG, U251 cell lines. IgG group was used as negative controls. *P<0.05, ***P<0.001.

of the patients to figure out the clinical significance of MAGE-D4 promoter methylation. Of 15 CpG units tested, 8 CpG

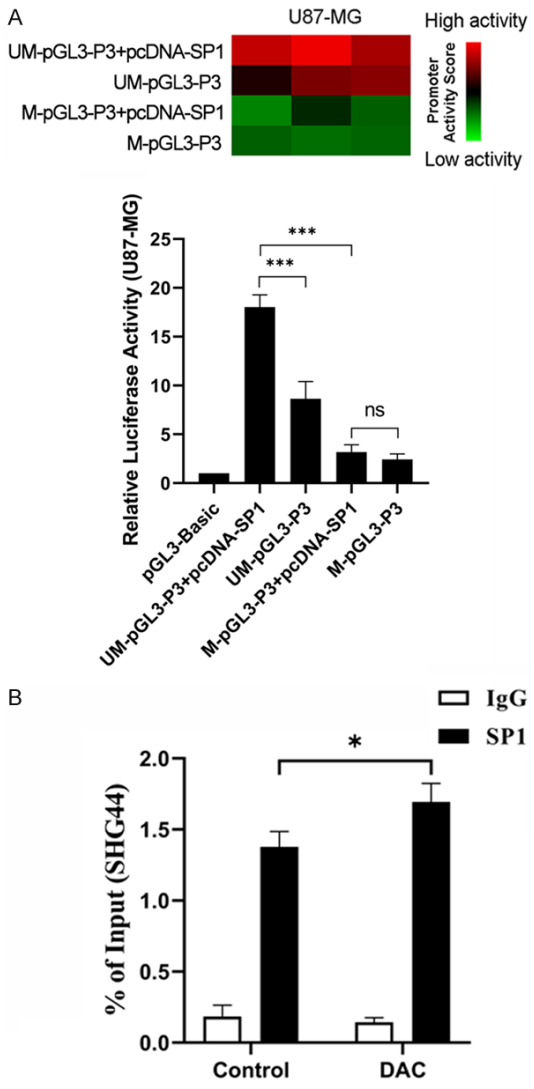

Figure 6. Co-effect of SP1 and methylation on MAGE-D4 promoter activity. A. MAGE-D4 promoter was activated by synergistic role of demethylation and SP1. The Heatmap and histogram showed differential luciferase activities of methylated or unmethylated pGL3-P3 (UM- or M-) under the different SP1 status in U87-MG cell line. The result showed that co-transfecting UM-pGL3-P3 and pcDNA-SP1 cause highest luciferase activity compared to others. Each line described the luciferase activity of UM-pGL3-P3 or M-pGL3-P3 in U87-MG and each column represented an independent experiment. B. Increase of SP1 binding to MAGE-D4 promoter through demethylation by DAC. DAC-treated SHG44 cell line was performed ChIP-qPCR to evaluate the binding capacity of SP1 to MAGE-D4 promoter. Cells treated with phosphate buffer saline served as a control group. IgG group was used as negative controls in ChIP. *P<0.05, ***P<0.001.

units were demonstrated significantly lower methylation levels in glioma tissues when compared with normal brain tissues, and this implied that these 8 CpG units may be crucial. Then, the further analysis indicated different methylation levels of these 8 CpG units in gender. As for CpG unit 12 and CpG unit 6, their methylation level was associated with age. It should be noted that the methylation level of CpG unit 6 may affect the patients' prognosis with Kaplan-Meier estimation. Interestingly, our result demonstrated that the correlation between the MAGE-D4 mRNA expression and methylation level of 6 CpG units (CpG unit 1, CpG unit 3, CpG unit 4, CpG unit 5, CpG unit 6 and CpG unit 9), which may play a significant role in the process of controlling of MAGE-D4 expression. Combined with MAGE-D4 mRNA expression associated with its promoter methylation, we considered that high expression of MAGE-D4 in glioma may result from hypomethylation of its promoter. And further luciferase assay validated that methylated modification of CpG sites on MAGE-D4 promoter could decrease the activity of MAGE-D4 promoter. These results suggested that demethylation of MAGE-D4 promoter may promote MAGE-D4 expression through promoting the activity of its promoter in glioma.

It's well known that the transcription factor may play an important role in the regulation of gene expression. In the present study, we discover multiple SP1 binding sites in MAGE-D4 promoter region and a significant correlation between expressions of SP1 and MAGE-D4. We further confirm SP1 binds to the MAGE-D4 promoter region and promotes its activity. It has been reported that gliomas express high level of SP1 that activated multiple target genes, which involved in malignant behavior of glioma, such as proliferation, migration, invasion, inflammatory reaction and drug-resistance as well as poor prognosis of patients [24-27]. Previous studies have indicated that overexpression of MAGE-D4 increases cell migration and growth in oral squamous cell carcinoma and lung cancer [14, 28], and is associated with poor disease outcome in breast cancer and esophageal cancer [13, 29]. Here, MAGE-D4 is added in the list of SP1 activated genes.

SP1 is a universal transcription factor, which is usually binds to the enriched CpG sequence on the promoter region. As above mentioned, on the one hand, SP1 can activate transcription of

its target genes through binding to the promoter of target genes. On the other hand, the methylation of CpG site in the binding area of SP1 also can inhibit the transcriptional activation of its target genes [27, 30-34]. Thus, functioning of SP1 is closed to the status of the promoter methylation of its target genes. In MAGE-D4 core promoter identified, the three SP1 binding sites overlap with CpG units. We hypothesized that the low methylation of MAGE-D4 promoter is more likely to bind with SP1. To test our hypothesis, we selected glioma cell line SHG44 treated with DAC to detect the binding ability of SP1. It should be pointed out that the reason why we choose SHG44 cell line instead of the other two cell lines is that the expression of SP1 in SHG44 is relatively high compared to the others. If the cell line with low SP1 expression was chosen, SP1 will increase after DAC treatment, which will cause false positive results of ChIP-qPCR. The other reason is that SHG44 has higher methylation level than others in MAGE-D4 promoter [16]. Ultimately, experimental results showed that demethylated promoter of MAGE-D4 is more likely to bind with SP1.

As a DNA methylation transferase inhibitor, DAC, was initially used to reverse the abnormal hypermethylation of tumor suppressor genes, and restore their expression to achieve tumor inhibition ultimately [35]. At present, some studies have confirmed that DAC can enhance the capacity of immunological cells to identify and kill glioma cells [36, 37]. So, we consider that DAC may serve as an adjunct of MAGE-D4-targeted immunotherapy. The reasons, first, DAC can enhance MAGE-D4 expression in glioma cells via demethylation of its promoter. Second, DAC may further homogenize the expression of MAGE-D4 by promoting the expression of SP1 in the samples with low SP1 expression [38], and facilitate SP1 binding to MAGE-D4 promoter to adjusting its expression.

In this study, exploring the expression mechanism of MAGE-D4 in glioma from the perspectives of promoter methylation and SP1 in order to establish foundation for the MAGE-D4 based glioma therapy. But the other manner for regulating MAGE-D4 expression is still unclear and glioma immunotherapy by targeting MAGE-D4 combined with DAC needs further research. At last, we hope our study could provide new ideas for the strategy of glioma treatment.

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Disclosure of conflict of interest

None.

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References

[1] Jemal A, Siegel R, Xu J and Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60: 277-300.

[2] Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG and Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. The Lancet Oncology 2009; 10: 459-466.

[3] Minniti G, Muni R, Lanzetta G, Marchetti P and Enrici RM. Chemotherapy for glioblastoma: current treatment and future perspectives for cytotoxic and targeted agents. Anticancer Res 2009; 29: 5171-5184.

[4] Wen PY and Kesari S. Malignant gliomas in adults. N Engl J Med 2008; 359: 492-507.
[5] Mrugala MM. Advances and challenges in the treatment of glioblastoma: a clinician's perspective. Discov Med 2013; 15: 221-230.
[6] Nagane M, Huang HJ and Cavenee WK. Advances in the molecular genetics of gliomas. Curr Opin Oncol 1997; 9: 215-222.
[7] Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, Friedman H, Friedman A, Reardon D, Herndon J, Kinzler KW, Velculescu VE, Vogelstein B and Bigner DD. IDH1 and IDH2 mutations in gliomas. N Engl J Med 2009; 360: 765-773.
[8] Melin B. Genetic causes of glioma: new leads in the labyrinth. Curr Opin Oncol 2011; 23: 643-647.
[9] Nakada M, Kita D, Watanabe T, Hayashi Y, Teng L, Pyko IV and Hamada J. Aberrant signaling pathways in glioma. Cancers (Basel) 2011; 3: 3242-3278.
[10] Inoue H, Mori M, Honda M, Li J, Shibuta K, Mimori K, Ueo H and Akiyoshi T. The expression of tumor-rejection antigen "MAGE" genes in human gastric carcinoma. Gastroenterology 1995; 109: 1522-1525.
[11] Wischnewski F, Pantel K and Schwarzenbach H. Promoter demethylation and histone acetylation mediate gene expression of MAGE-A1, -A2, -A3, and -A12 in human cancer cells. Mol Cancer Res 2006; 4: 339-349.
[12] Sasaki M, Nakahira K, Kawano Y, Katakura H, Yoshimine T, Shimizu K, Kim SU and Ikenaka K. MAGE-E1, a new member of the melanoma-associated antigen gene family and its expression in human glioma. Cancer Res 2001; 61: 4809-4814.
[13] 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 2012; 130: 1991-2002.
[14] Chong CE, Lim KP, Gan CP, Marsh CA, Zain RB, Abraham MT, Prime SS, Teo SH, Silvio Gutkind J, Patel V and Cheong SC. Over-expression of MAGED4B increases cell migration and growth in oral squamous cell carcinoma and is associated with poor disease outcome. Cancer Lett 2012; 321: 18-26.
[15] 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 2014; 7: 3918-3927.
[16] Zhang QM, Shen N, Xie S, Bi SQ, Luo B, Lin YD, Fu J, Zhou SF, Luo GR, Xie XX and Xiao SW. MAGED4 expression in glioma and upregulation in glioma cell lines with 5-aza-2'-deoxycytidine treatment. Asian Pac J Cancer Prev 2014; 15: 3495-3501.
[17] He SJ, Gu YY, Yu L, Luo B, Fan R, Lin WZ, Lan XW, Lin YD, Zhang QM, Xiao SW and Xie XX. High expression and frequently humoral immune response of melanoma-associated antigen D4 in glioma. Int J Clin Exp Pathol 2014; 7: 2350-2360.
[18] Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J and Rosenberg SA. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. Cancer Res 1994; 54: 1766-1771.
[19] Serrano A, García A, Abril E, Garrido F and Ruiz-Cabello F. Methylated CpG points identified within MAGE-1 promoter are involved in gene repression. Int J Cancer 1996; 68: 464-470.
[20] Kawano Y, Sasaki M, Nakahira K, Yoshimine T, Shimizu K, Wada H and Ikenaka K. Structural characterization and chromosomal localization of the MAGE-E1 gene. Gene 2001; 277: 129-137.
[21] Valastyan S and Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell 2011; 147: 275-292.
[22] Wu CY, Tsai YP, Wu MZ, Teng SC and Wu KJ. Epigenetic reprogramming and post-transcriptional regulation during the epithelial-mesenchymal transition. Trends Genet 2012; 28: 454-463.
[23] Huang Y and Rao A. Connections between TET proteins and aberrant DNA modification in cancer. Trends Genet 2014; 30: 464-474.
[24] Safe S, Abbruzzese J, Abdelrahim M and Hedrick E. Specificity Protein transcription factors and cancer: opportunities for drug development. Cancer Prev Res (Phila) 2018; 11: 371-382.
[25] Luo J, Wang X, Xia Z, Yang L, Ding Z, Chen S, Lai B and Zhang N. Transcriptional factor specificity protein 1 (SP1) promotes the proliferation of glioma cells by up-regulating midkine (MDK). Mol Biol Cell 2015; 26: 430-439.
[26] Dong Q, Cai N, Tao T, Zhang R, Yan W, Li R, Zhang J, Luo H, Shi Y, Luan W, Zhang Y, You Y, Wang Y and Liu N. An axis involving SNAI1, microRNA-128 and SP1 modulates glioma progression. PLoS One 2014; 9: e98651.
[27] Gopisetty G, Xu J, Sampath D, Colman H and Puduvalli VK. Epigenetic regulation of CD133/PROM1 expression in glioma stem cells by Sp1/myc and promoter methylation. Oncogene 2013; 32: 3119-3129.
[28] 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 2006; 51: 79-88.
[29] Uno Y, Kanda M, Sato Y, Shimizu D, Umeda S, Hattori N, Hayashi M, Tanaka C, Kobayashi D, Yamada S, Nakayama G, Motoyama S, Koike M and Kodera Y. Expression, function, and prognostic value of MAGE-D4 protein in esophageal squamous cell carcinoma. Anticancer Res 2019; 39: 6015-6023.
[30] Tian HP, Lun SM, Huang HJ, He R, Kong PZ, Wang QS, Li XQ and Feng YM. DNA methylation affects the SP1-regulated transcription of FOXF2 in breast cancer cells. J Biol Chem 2015; 290: 19173-19183.
[31] Wang Y, Zhang ZX, Chen S, Qiu GB, Xu ZM and Fu WN. Methylation status of SP1 Sites within miR-23a-27a-24-2 promoter region influences laryngeal cancer cell proliferation and apoptosis. Biomed Res Int 2016; 2016: 2061248.
[32] Reamon-Buettner SM and Borlak J. Epigenetic silencing of cell adhesion molecule 1 in different cancer progenitor cells of transgenic c-Myc and c-Raf mouse lung tumors. Cancer Res 2008; 68: 7587-7596.
[33] Guo D, Wu B, Yan J, Li X, Sun H and Zhou D. A possible gene silencing mechanism: hypermethylation of the Keap1 promoter abrogates binding of the transcription factor Sp1 in lung cancer cells. Biochem Biophys Res Commun 2012; 428: 80-85.
[34] Shen L, Qu X, Ma Y, Zheng J, Chu D, Liu B, Li X, Wang M, Xu C, Liu N, Yao L and Zhang J. Tumor suppressor NDRG2 tips the balance of oncogenic TGF-β via EMT inhibition in colorectal cancer. Oncogenesis 2014; 3: e86.
[35] Pinto A and Zagonel V. 5-Aza-2'-deoxycytidine (Decitabine) and 5-azacytidine in the treatment of acute myeloid leukemias and myelodysplastic syndromes: past, present and future trends. Leukemia 1993; 7 Suppl 1: 51-60.
[36] Riccadonna C, Yacoub Maroun C, Vuillefroy de Silly R, Boehler M, Calvo Tardón M, Jueliger S, Taverna P, Barba L, Marinari E, Pellegatta S, Bassoy EY, Martinvalet D, Dietrich PY and Walker PR. Decitabine treatment of glioma-initiating cells enhances immune recognition and killing. PLoS One 2016; 11: e0162105.
[37] Konkankit VV, Kim W, Koya RC, Eskin A, Dam MA, Nelson S, Ribas A, Liau LM and Prins RM. Decitabine immunosensitizes human gliomas to NY-ESO-1 specific T lymphocyte targeting through the Fas/Fas ligand pathway. J Transl Med 2011; 9: 192.
[38] Venkatasubbarao K, Ammanamanchi S, Brattain MG, Mimari D and Freeman JW. Reversion of transcriptional repression of Sp1 by 5 aza-2' deoxycytidine restores TGF-beta type II receptor expression in the pancreatic cancer cell line MIA PaCa-2. Cancer Res 2001; 61: 6239-6247.

Supplemental Materials

1. Sequences of SP1-siRNA

Sense strand: 5'-GCAAGUUCUGACAGGACUATT-3'

Antisense strand: 5'-UAGUCCUGUCAGAACUUGCTT-3'

2. Primers of MAGE-D4, SP1 and GAPDH

	Forward	Reverse
MAGE-D4	CAGGATGGGAGGCAAGAGGACC	CCAAGGAGGCGAGCTGAGGAGT
SP1	ATCCCACAGTTCCAGACCGT	ATGTTGCCTCCACTTCCTCG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

3. Primer of MAGE-D4 promoter and diagram

AAAGGGCAGGGCAGGACAGAGAAGCATTGTGCAAAAGTGGGGGGACTGAGAAGAGATGATTGGGTGGGTCGAGCTGCACTG-CAGGGTATGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGCGGGAAGAGCCCCCC-GCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAGGAAAGGGCTTTT-GTTGGGAAAGCGGGCGGGCTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACGGAGAAAAGGGGC-GGGGTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAGGACGGAC-GCCATTGGAGTTCCCGAGAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACACGCTGC-GCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAG

Sequence of bases: pGL3-P3

Red: primer of MAGE-D4 promoter

Yellow: potential SP1 binding sites

4. Sequence of pGL3-P1~P5 and pGL3-P3-MT1~4

pGL3-P1 (-1897~+172 bp):

5'-GCTCTGGAGACCCTTCAGAGCTTGCCCTTCTGGATGCAGCGTATCAGAAACGGGGAGTGGTGATCGATGACTCCCAGTTTCTG-GAGTGAGTGGTTGTGTGGATAATGGCGCCATTTACTGAGACTGTGAAGATGGGTTGGGGGGGGGGGGAACAGGAGC-GATGGGGATGTGTGAGTGTATGGGAGGAGTGAGTATACCAAGTGACCAGTTTTGGACAGGCAAACCATGAAGAGCCCTT-GAGACTTCCAAGTGGAGATGCCACGTAGGTGGGTGAATGTATGTACTTGGACCTCAAAAGTGAAGTTCAGACTGGGGTTG-TAGATTTTTTGGAGTGATTAGCACGTAGCACTTTCTGAAGGCTTTCGTTTGTAGGGTGTGTTCCTCTCCCGCAGAGGATATGT-TACAGGGGAAGGGCTAAAGAGGCACAAGCAGCTGACAGGAGAGATTGATATATAACAGCTGGAGAAAACCACAGAGCCAG-GCAGAATGGATACTGGTCAGCGGAGAGTAAAAGCTGCTGTCCTAGCAATACCTTCCATTCATACACAGGGGTCCTGGTGAGGCT-GACATAAAAACAAATATAAAAATCTACTACGAATTTTACAAACAGTCCAGGTTCGACTGCAGATGAGACCACAGCATTTTCTGCCT-CAAAAGACATCCAAGTGGAGATGTCACGTAGGCAGGTGAATGTACTATGCACTTGGACCTCAAAAGTGAAGTTTGAACTGGGCTT-GTAGATTGTGGAGTAATTAGCTCAGACACACAGGTGTCTAATTGTCCTGGCGGACACCAGGAGGGTCTCAGCGGCAGCGTGATT-GTATCGCTGATAAGGAAGAGGCCTGACAATCTGAGTAGAGAGTGGACCCGGTGGAGCAATTCTCATACCTGGATGGACCCA-CATAGAGCCGCAGAGGCTGGGGGCGAGGCGGGGGGAGGCTCTGTCTCCCTGTGTCCTGAATGCGGAGCATGAACTTAG-GCCTTCTACAGCACAAACCTTGCTTCTTTGGGACAGATGTTGGGGTGGGGGAGGCCTGTCACTCAGACGGAGGAGGCG-GTGCCTCTGTCCCCAGGCCAGCAGGAGCAGGAGGTGGACTTTGGAGTCCGGAAGACCCAGGTTCAAATTTTGCATTTTCT-GTTTCCTGGCTCTGTGGTCTAGTTCATGCAATGTGTCTGTGCCTCAGCTTCCTGACACGCCAAATGGGGATGCTGACATC-CACTTCCCAGCGCTGCTGTGAGAGGAAGAAAAGCCCCAGCACAGATCCCTCTGTGACATACAAGCTGCATAAAGGGTAGCTGAG-GAAGCAGATGTTCCCAGTATGTCTGGGGGCCAGAGAGTTGGCTAGTGGAGAAGCACACAAAGCGAAGTGCCATCCTCTGGCCAT-GTCCATTTCGTAGCCCCGCAGGTTGAGGATTTCCACTTGTTGCAGTTAGAGACCCAGCTTATTAATTGTGAGACCTCACTAATTGT-GACCTAAGGGGTCTTGCCGGGGAAACGGGGCATAGTGTATCCCAGACCAGGCTGGAGGAGTTTGGGTGAAAGGGCAGGGCAG-GACAGAGAAGCATTGTGCAAAAGTGGGGGGACTGAGAAGAGATGATTGGGTGGGTCGAGCTGCACTGCAGGGTATGAGTGT-GCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGCGGGAAGAGCCCCCCGCACCCTACCTTC-GACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAGGAAAGGGCTTTTGTTGGGAAAGCGGGC-GGGCTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACGGAGAAAAGGGGCGGGGTGGTCCGGGCT-GCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAGGACGGACGCCATTGGAGTTCCCGA-GAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACACGCTGCGCCCTCCCTCCGCCTCCG-GATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAG-3'

pGL3-P2 (-614~+172 bp):

5’-GCATAAAGGGTAGCTGAGGAAGCAGATGTTCCCAGTATGTCTGGGGGCCAGAGAGTTGGCTAGTGGAGAAGCACACAAAGC-
GAAGTGCCATCCTCTGGCCATGTCCATTTCGTAGCCCCGCAGGTTGAGGATTTCCACTTGTTGCAGTTAGAGACCCAGCTTAT-
TAATTGTGAGACCTCACTAATTGTGACCTAAGGGGTCTTGCCGGGGAAACGGGGCATAGTGTATCCCAGACCAGGCTGGAG-
GAGTTTGGGTGAAAGGGCAGGGCAGGACAGAGAAGCATTGTGCAAAAGTGGGGGGACTGAGAAGAGATGATTGGGTGGGTC-
GAGCTGCACTGCAGGGTATGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGC-
GGGAAGAGCCCCCCGCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAG-
GAAAGGGCTTTTGTTGGGAAAGCGGGCGGGCTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACG-
GAGAAAAGGGGCGGGGTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAG-
GACGGACGCCATTGGAGTTCCCGAGAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACAC-
GCTGCGCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAG-3’

pGL3-P3 (-358~+172 bp):

5’-AAAGGGCAGGGCAGGACAGAGAAGCATTGTGCAAAAGTGGGGGGACTGAGAAGAGATGATTGGGTGGGTCGAGCTGCACT-
GCAGGGTATGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGCGGGAAGAGCCCCCC-
GCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAGGAAAGGGCTTTT-
GTTGGGAAAGCGGGCGGGCTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACGGAGAAAAGGGGC-
GGGGTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAGGACGGAC-
GCCATTGGAGTTCCCGAGAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACACGCTGC-
GCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAG-3’

pGL3-P4 (-270~+172 bp):

5’-TGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGCGGGAAGAGCCCCCC-
GCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAGGAAAGGGCTTTT-
GTTGGGAAAGCGGGCGGGCTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACGGAGAAAAGGGGC-
GGGGTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAGGACGGAC-
GCCATTGGAGTTCCCGAGAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACACGCTGC-
GCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAG-3’

pGL3-P5 (-90~+172 bp):

5’-CGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACGGAGAAAAGGGGCGGGGTGGTCCGGGCTGCTGTGCTG-
GCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAGGACGGACGCCATTGGAGTTCCCGAGAAGGTAAG-
GATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACACGCTGCGCCCTCCCTCCGCCTCCGGATCTGAA-
CAAAGCCCAAGCACTCAGAACCGGAACCCCATTAG-3’

pGL3-P3-MT1:

GgtaccAAAGGGCAGGGCAGGACAGAGAAGCATTGTGCAAAAGTGGGGGGACTGAGAAGAGATGATTGGGTGGGTC-
GAGCTGCACTGCAGGGTATGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGC-
GGGAAGAGCCCCCCGCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAG-
GAAAGGGCTTTTGTTGGGAAAGTTTTTTTTTTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACG-
GAGAAAAGGGGCGGGGTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAG-
GACGGACGCCATTGGAGTTCCCGAGAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACAC-
GCTGCGCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAGaagctt

pGL3-P3-MT2:

ggtaccAAAGGGCAGGGCAGGACAGAGAAGCATTGTGCAAAAGTGGGGGGACTGAGAAGAGATGATTGGGTGGGTC-
GAGCTGCACTGCAGGGTATGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGC-
GGGAAGAGCCCCCCGCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAG-
GAAAGGGCTTTTGTTGGGAAAGCGGGCGGGCTGGAGGGGTCCGCGCATGCGCAGGCTATTTTTTTTTGGGGGGGTGCACG-
GAGAAAAGGGGCGGGGTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAG-
GACGGACGCCATTGGAGTTCCCGAGAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACAC-
GCTGCGCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAGaagctt

pGL3-P3-MT3:

GgtaccAAAGGGCAGGGCAGGACAGAGAAGCATTGTGCAAAAGTGGGGGGACTGAGAAGAGATGATTGGGTGGGTC-
GAGCTGCACTGCAGGGTATGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGC-
GGGAAGAGCCCCCCGCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAG-
GAAAGGGCTTTTGTTGGGAAAGCGGGCGGGCTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACG-
GAGAAAATTTTTTTTTTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAGGAC-
GGACGCCATTGGAGTTCCCGAGAAGGTAAGGATCCAGCCCCAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACACGCTGC-
GCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAGaagctt

pGL3-P3-MT4:

GgtaccAAAGGGCAGGGCAGGACAGAGAAGCATTGTGCAAAAGTGGGGGGGACTGAGAAGAGATGATTGGGTGGGTC-GAGCTGCACTGCAGGGTATGAGTGTGCGCGCCCGCGGTAGGAGGAGGAGGAGGAGGAGAGGTGAAGTGGATCTGGC-GGGAAGAGCCCCCCGCACCCTACCTTCGACCCCACCGCCCACCCACCCGCGGAATCGCATGCGCACTGGAGACCTGGAG-GAAAGGGCTTTTGTTGGGAAAGCGGGCGGGCTGGAGGGGTCCGCGCATGCGCAGGCTACCCAGCCGCGGGGGGTGCACG-GAGAAAAGGGGCGGGGTGGTCCGGGCTGCTGTGCTGGCAGCAGTAGGCGAGGGCGCGGCTGCGGGGTTCCTGGTGCTGAG-GACGGACGCCATTGGAGTTCCCGAGAAGGTAAGGATTTTTTTTTAGACAGGACCGGGAGAGGGCGAGTGGAACCCGACACGCT-GCGCCCTCCCTCCGCCTCCGGATCTGAACAAAGCCCAAGCACTCAGAACCGGAACCCCATTAGaagctt

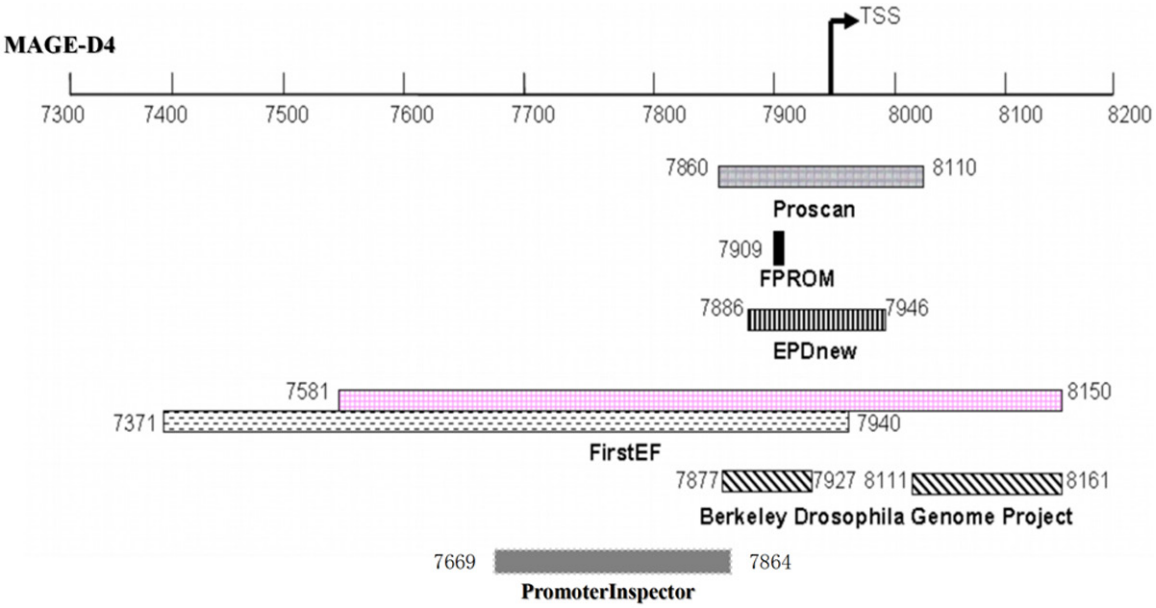
Red: mutation sites

5. CpG units contained CpG sit

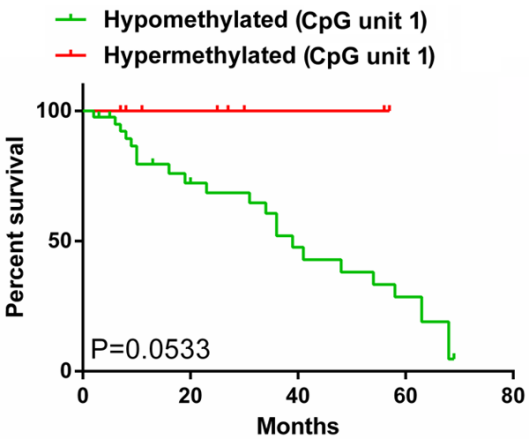
CpG unit (number)	CpG site
CpG unit 1	CpG 2.3.4.5
CpG unit 2	CpG 6
CpG unit 3	CpG 7
CpG unit 4	CpG 8
CpG unit 5	CpG 9
CpG unit 6	CpG 10.11
CpG unit 7	CpG 12
CpG unit 8	CpG 13
CpG unit 9	CpG 14.15
CpG unit 10	CpG 16.17
CpG unit 11	CpG 18
CpG unit 12	CpG 19.20
CpG unit 13	CpG 24
CpG unit 14	CpG 29
CpG unit 15	CpG 30

Supplementary Table 1. Prediction of MAGE-D4 promoter by bioinformatics

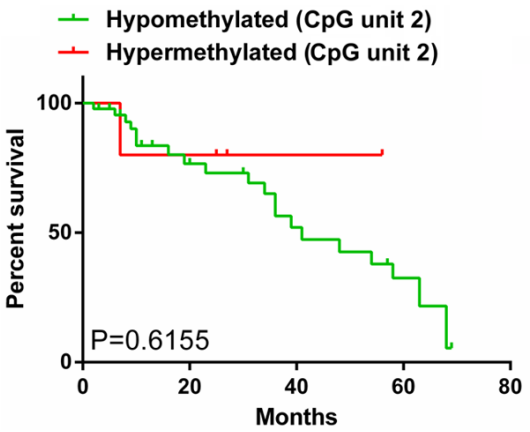
Database	Position of MAGE-D4 Promoter (bp)	Website
Promoter Scan	7860-8110	http://www-bimas.cit.nih.gov/molbio/proscan/
FirstEF	7581-8150	http://rulai.cshl.org/tools/FirstEF/
	7371-7940	
PromoterInspector	7669-7864	http://www.genomatix.de/products/PromoterInspector/PromoterInspector2.html
FPROM	7909	http://www.softberry.com/berry.phtml?topic=fprom&group=programs&subgroup=promoter
EPDnew	7886-7946	http://epd.vital-it.ch
Berkeley Drosophila Genome Project	7877-7927	https://insitu.fruitfly.org/cgi-bin/ex/insitu.pl
	8111-8161	



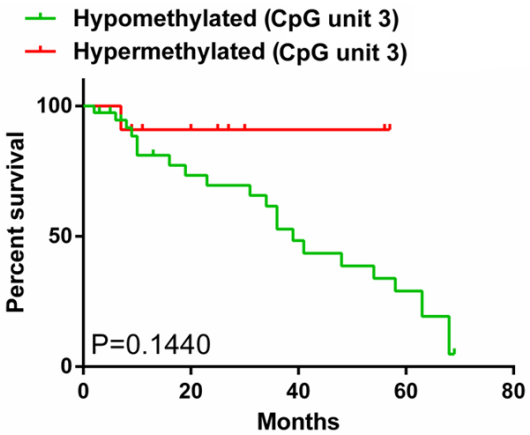
Supplementary Figure 1. Schematic diagram of MAGE-D4 promoter prediction.



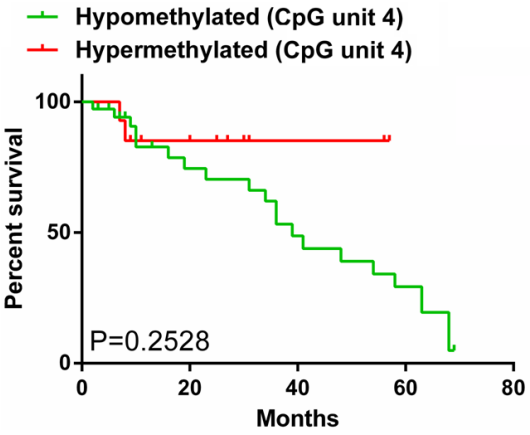
Supplementary Figure 2. Survival analysis with CpG unit 1 methylation level of MAGE-D4 promoter.



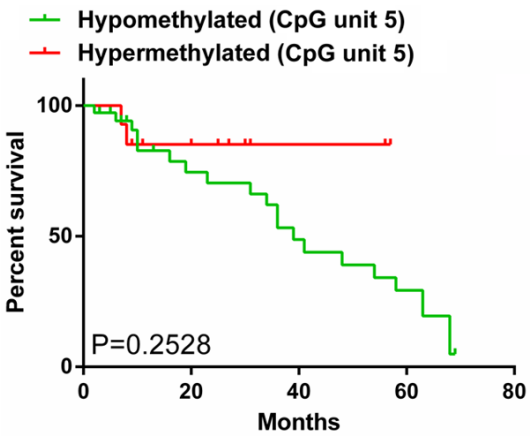
Supplementary Figure 3. Survival analysis with CpG unit 2 methylation level of MAGE-D4 promoter.



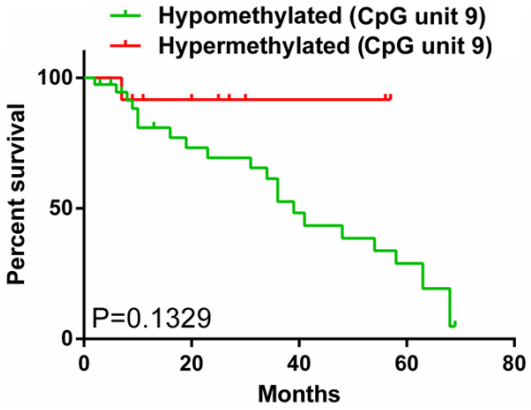
Supplementary Figure 4. Survival analysis with CpG unit 3 methylation level of MAGE-D4 promoter.



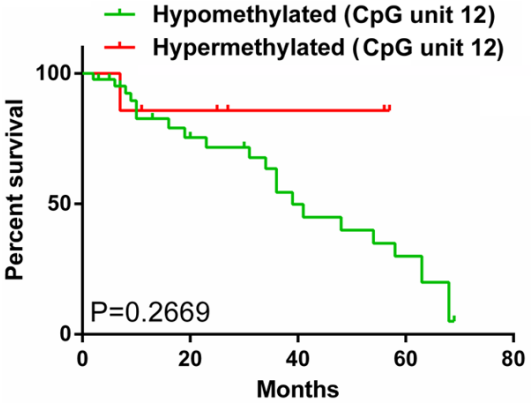
Supplementary Figure 5. Survival analysis with CpG unit 4 methylation level of MAGE-D4 promoter.



Supplementary Figure 6. Survival analysis with CpG unit 5 methylation level of MAGE-D4 promoter.



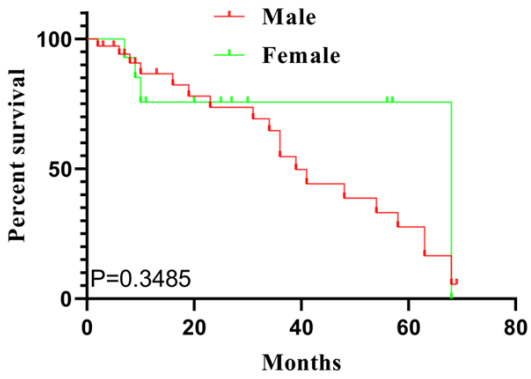
Supplementary Figure 7. Survival analysis with CpG unit 9 methylation level of MAGE-D4 promoter.



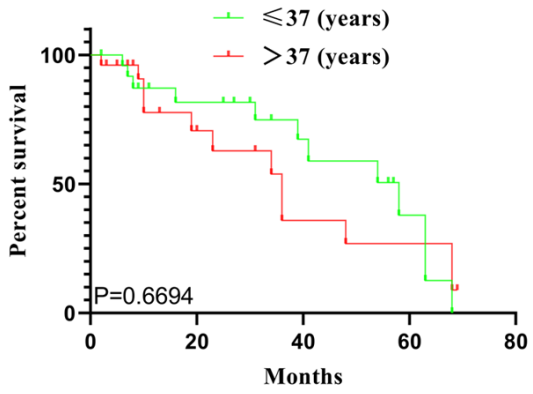
Supplementary Figure 8. Survival analysis with CpG unit 12 methylation level of MAGE-D4 promoter.

Supplementary Table 2. General information of glioma patients

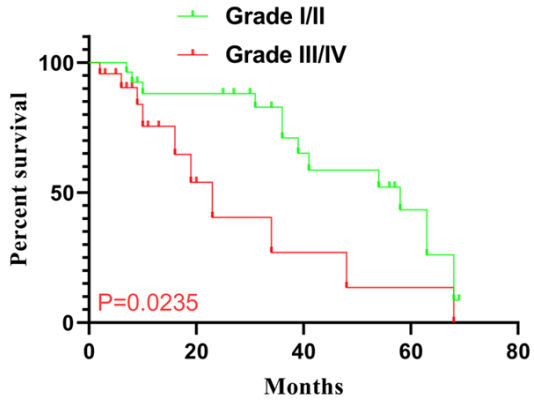
Parameters	Numbers
Gender	
Male	36
Female	14
Age (years)	
≤37	25
>37	25
WHO Grade	
I/II (Low grades)	27
III/IV (High grades)	23
Pathological type	
astrocytoma	32
anaplastic astrocytoma	3
oligodendroglioma	2
glioblastoma	13
KPS Score	
≤70	38
>70	12
P53 Protein	
-	25
+	25
Ki-67 (%)	
<10	36
≥10	14
Tumor Size (cm)	
<5	25
≥5	25
MAGE-D4 mRNA	
High	25
Low	25



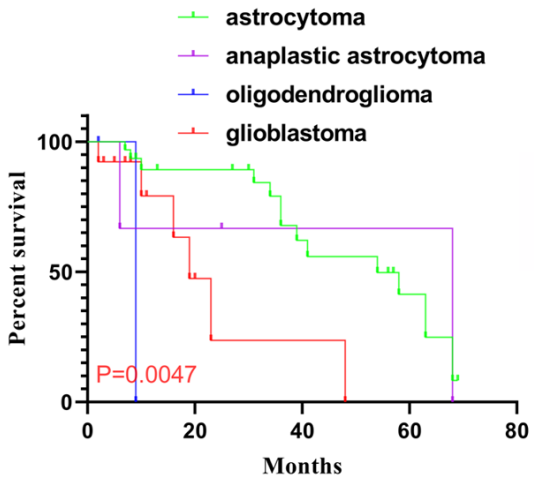
Supplementary Figure 9. Survival analysis with gender in glioma patients.



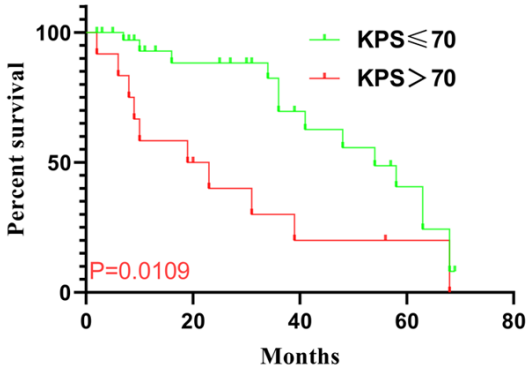
Supplementary Figure 10. Survival analysis with age in glioma patients.



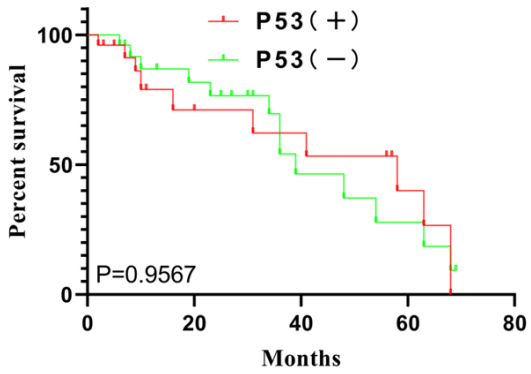
Supplementary Figure 11. Survival analysis with WHO grade in glioma patients.



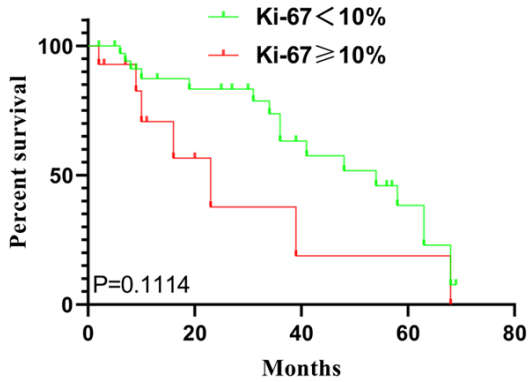
Supplementary Figure 12. Survival analysis with pathological type in glioma patients.



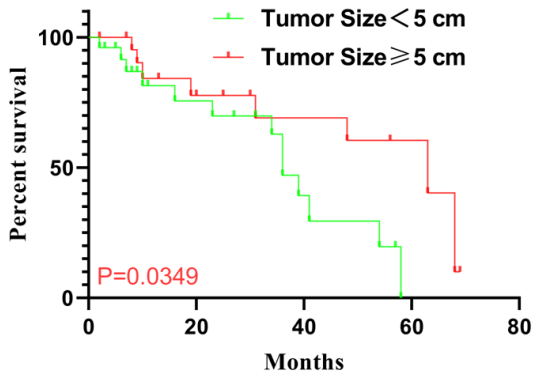
Supplementary Figure 13. Survival analysis with KPS score in glioma patients.



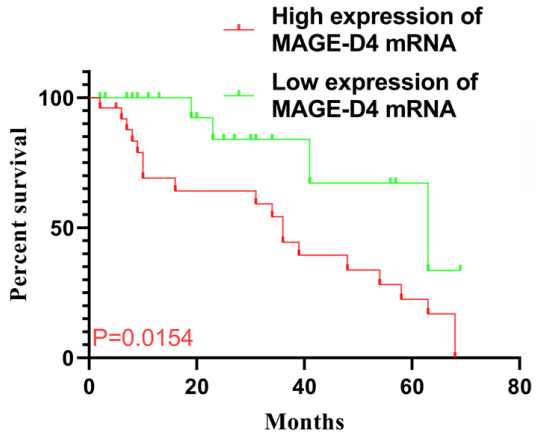
Supplementary Figure 14. Survival analysis with P53 protein expression in glioma patients.



Supplementary Figure 15. Survival analysis with Ki-67 expression in glioma patients.



Supplementary Figure 16. Survival analysis with tumor size in glioma patients.



Supplementary Figure 17. Survival analysis with MAGE-D4 mRNA expression in glioma patients.

Supplementary Table 3. Cox regression model for the prognosis of glioma patients

Parameters	Univariate					Multivariable					
	Median Survival Time			χ^2	P	B	SE	Wald	HR	95% CI	P
	Estimate	SE	95% CI								
Gender	54	10.995	32.450-75.550	0.565	0.452						
Male	48	12.221	24.047-71.953								
Female	68	0									
Age (years)	54	10.995	32.450-75.550	2.175	0.14						
≤37	63	2.864	57.386-68.614								
>37	34	10.691	13.045-54.955								
WHO Grades	54	10.995	32.450-75.550	9.006	0.003	1.602	0.6	7.14	4.965	1.533-16.081	0.008
I/II (Low Grades)	63	3.347	56.441-69.559								
III/IV (High Grades)	20	2.873	14.368-25.632								
P53 Protein	54	10.995	32.450-75.550	0.001	0.977						
-	48	8.738	30.873-65.127								
+/++/+++	58	22.665	13.577-102.423								
Ki-67 (%)	54	10.995	32.450-75.550	5.334	0.021	-0.227	0.56	0.164	0.797	0.366-2.389	0.686
<10	58	4.105	49.955-66.045								
≥10	20	5.042	10.118-29.882								
KPS Score	54	10.995	32.450-75.550	7.911	0.005	1.44	0.486	8.78	4.22	1.628-10.938	0.003
>70	58	4.289	49.594-66.406								
≤70	19	8.66	2.026-35.974								
Tumor Size (cm)	54	10.995	32.450-75.550	2.732	0.098						
<5	39	8.276	22.779-55.221								
≥5	63	10.617	42.191-83.809								
MAGE-D4 mRNA	54	10.995	32.450-75.550	5.059	0.025	1.253	0.596	4.417	3.501	1.088-11.266	0.036
Low	63	28.93	6.298-119.702								
High	39	8.737	21.876-56.124								

Supplementary Table 4. Correlation between CpG unit 1 methylation level and clinical parameters in glioma patients

Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	1 (12.5)	35 (83.3)	
Female	7 (87.5)	7 (16.7)	
Age (years)			0.054
≤37	7 (87.5)	18 (42.9)	
>37	1 (12.5)	24 (57.1)	
WHO Grade			0.889
I/II (Low grades)	5 (62.5)	22 (52.4)	
III/IV (High grades)	3 (37.5)	20 (47.6)	
Pathological type			0.318
astrocytoma	4 (50.0)	28 (66.7)	
anaplastic astrocytoma	0 (0.0)	3 (7.1)	
oligodendroglioma	1 (12.5)	1 (2.4)	
glioblastoma	3 (37.5)	10 (23.8)	
KPS Score			0.704
≤70	7 (87.5)	31 (73.8)	
>70	1 (12.5)	11 (26.2)	
P53 Protein			0.700
-	5 (62.5)	20 (47.6)	
+	3 (37.5)	22 (52.4)	
Ki-67 (%)			0.525
<10	7 (87.5)	29 (69.0)	
≥10	1 (12.5)	13 (31.0)	
Tumor Size (cm)			1.000
<5	4 (50.0)	21 (50.0)	
≥5	4 (50.0)	21 (50.0)	
MAGE-D4 mRNA			0.007
High	0 (0.0)	25 (59.5)	
Low	8 (100.0)	17 (40.5)	

Supplementary Table 5. Correlation between CpG unit 2 methylation level and clinical parameters in glioma patients

Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.001
Male	0 (0.0)	36 (80.0)	
Female	5 (100.0)	9 (20.0)	
Age (years)			0.059
≤37	5 (100.0)	20 (44.4)	
>37	0 (0.0)	25 (55.6)	
WHO Grade			0.449
I/II (Low grades)	4 (80.0)	23 (51.1)	
III/IV (High grades)	1 (20.0)	22 (48.9)	
Pathological type			0.343
astrocytoma	3 (60.0)	29 (64.4)	
anaplastic astrocytoma	0 (0.0)	3 (6.7)	
oligodendroglioma	1 (20.0)	1 (2.2)	
glioblastoma	1 (20.0)	12 (26.7)	
KPS Score			1.000
≤70	4 (80.0)	34 (75.6)	
>70	1 (20.0)	11 (24.4)	
P53 Protein			1.000
-	3 (60.0)	22 (48.9)	
+	2 (40.0)	23 (51.1)	
Ki-67 (%)			0.345
<10	5 (100.0)	31 (68.9)	
≥10	0 (0.0)	14 (31.1)	
Tumor Size (cm)			1.000
<5	2 (40.0)	23 (51.1)	
≥5	3 (60.0)	22 (48.9)	
MAGE-D4 mRNA			0.346
High	1 (20.0)	24 (53.3)	
Low	4 (80.0)	21 (46.7)	

Supplementary Table 6. Correlation between CpG unit 3 methylation level and clinical parameters in glioma patients

Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	0 (0.0)	36 (92.3)	
Female	11 (100.0)	3 (7.7)	
Age (years)			0.088
≤37	8 (72.7)	17 (43.6)	
>37	3 (27.3)	22 (56.4)	
WHO Grade			0.967
I/II (Low grades)	6 (54.5)	21 (53.8)	
III/IV (High grades)	5 (45.5)	18 (46.2)	
Pathological type			0.601
astrocytoma	7 (63.6)	25 (64.1)	
anaplastic astrocytoma	0 (0.0)	3 (7.7)	
oligodendroglioma	1 (9.1)	1 (2.6)	
glioblastoma	3 (27.3)	10 (25.6)	
KPS Score			0.911
≤70	9 (81.8)	29 (74.4)	
>70	2 (18.2)	10 (25.6)	
P53 Protein			0.733
-	5 (45.5)	20 (51.3)	
+	6 (54.5)	19 (48.7)	
Ki-67 (%)			1.000
<10	8 (72.7)	28 (71.8)	
≥10	3 (27.3)	11 (28.2)	
Tumor Size (cm)			0.306
<5	4 (36.4)	21 (53.8)	
≥5	7 (63.6)	18 (46.2)	
MAGE-D4 mRNA			0.002
High	1 (9.1)	24 (61.5)	
Low	10 (90.9)	15 (38.5)	

Supplementary Table 7. Correlation between CpG unit 4 methylation level and clinical parameters in glioma patients

Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	4 (28.6)	32 (88.9)	
Female	10 (71.4)	4 (11.1)	
Age (years)			0.059
≤37	10 (71.4)	15 (41.7)	
>37	4 (28.6)	21 (58.3)	
WHO Grade			0.363
I/II (Low grades)	9 (64.3)	18 (50.0)	
III/IV (High grades)	5 (35.7)	18 (50.0)	
Pathological type			0.685
astrocytoma	9 (64.3)	23 (63.9)	
anaplastic astrocytoma	0 (0.0)	3 (8.3)	
oligodendroglioma	1 (7.1)	1 (2.8)	
glioblastoma	4 (28.6)	9 (25.0)	
KPS Score			1.000
≤70	11 (78.6)	27 (75.0)	
>70	3 (21.4)	9 (25.0)	
P53 Protein			1.000
-	7 (50.0)	18 (50.0)	
+	7 (50.0)	18 (50.0)	
Ki-67 (%)			0.319
<10	12 (85.7)	24 (66.7)	
≥10	2 (14.3)	12 (33.3)	
Tumor Size (cm)			1.000
<5	7 (50.0)	18 (50.0)	
≥5	7 (50.0)	18 (50.0)	
MAGE-D4 mRNA			0.012
High	3 (21.4)	22 (61.1)	
Low	11 (78.6)	14 (38.9)	

Supplementary Table 8. Correlation between CpG unit 5 methylation level and clinical parameters in glioma patients

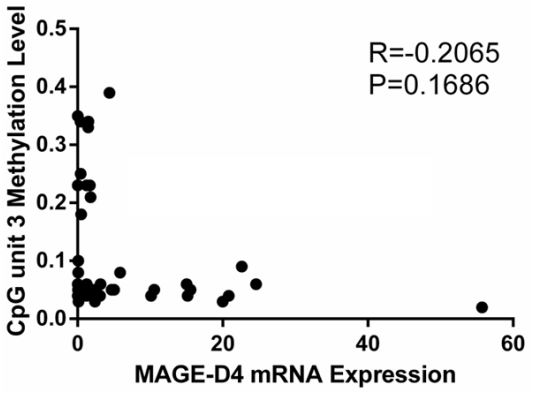
Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	4 (28.6)	32 (88.9)	
Female	10 (71.4)	4 (11.1)	
Age (years)			0.059
≤37	10 (71.4)	15 (41.7)	
>37	4 (28.6)	21 (58.3)	
WHO Grade			0.363
I/II (Low grades)	9 (64.3)	18 (50.0)	
III/IV (High grades)	5 (35.7)	18 (50.0)	
Pathological type			0.685
astrocytoma	9 (64.3)	23 (63.9)	
anaplastic astrocytoma	0 (0.0)	3 (8.3)	
oligodendroglioma	1 (7.1)	1 (2.8)	
glioblastoma	4 (28.6)	9 (25.0)	
KPS Score			1.000
≤70	11 (78.6)	27 (75.0)	
>70	3 (21.4)	9 (25.0)	
P53 Protein			1.000
-	7 (50.0)	18 (50.0)	
+	7 (50.0)	18 (50.0)	
Ki-67 (%)			0.319
<10	12 (85.7)	24 (66.7)	
≥10	2 (14.3)	12 (33.3)	
Tumor Size (cm)			1.000
<5	7 (50.0)	18 (50.0)	
≥5	7 (50.0)	18 (50.0)	
MAGE-D4 mRNA			0.012
High	3 (21.4)	22 (61.1)	
Low	11 (78.6)	14 (38.9)	

Supplementary Table 9. Correlation between CpG unit 6 methylation level and clinical parameters in glioma patients

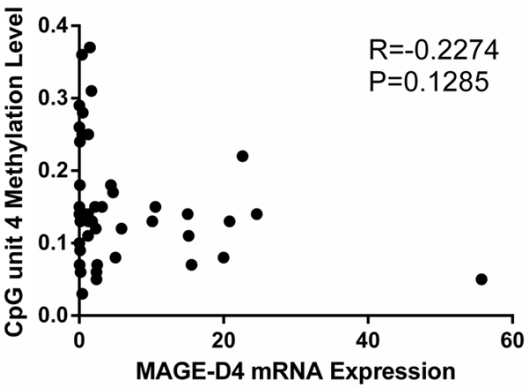
Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	1 (9.1)	35 (89.7)	
Female	10 (90.9)	4 (10.3)	
Age (years)			0.306
≤37	7 (63.6)	18 (46.2)	
>37	4 (36.4)	21 (53.8)	
WHO Grade			0.52
I/II (Low grades)	5 (45.5)	22 (56.4)	
III/IV (High grades)	6 (54.5)	17 (43.6)	
Pathological type			0.422
astrocytoma	6 (54.5)	26 (66.7)	
anaplastic astrocytoma	0 (0.0)	3 (7.7)	
oligodendroglioma	1 (9.1)	1 (2.6)	
glioblastoma	4 (36.4)	9 (23.1)	
KPS Score			0.911
≤70	9 (81.8)	29 (74.4)	
>70	2 (18.2)	10 (25.6)	
P53 Protein			0.733
-	6 (54.5)	19 (48.7)	
+	5 (45.5)	20 (51.3)	
Ki-67 (%)			1.000
<10	8 (72.7)	28 (71.8)	
≥10	3 (27.3)	11 (28.2)	
Tumor Size (cm)			0.306
<5	4 (36.4)	21 (53.8)	
≥5	7 (63.6)	18 (46.2)	
MAGE-D4 mRNA			0.000
High	0 (0.0)	25 (64.1)	
Low	11 (100.0)	14 (35.9)	

Supplementary Table 10. Correlation between CpG unit 9 methylation level and clinical parameters in glioma patients

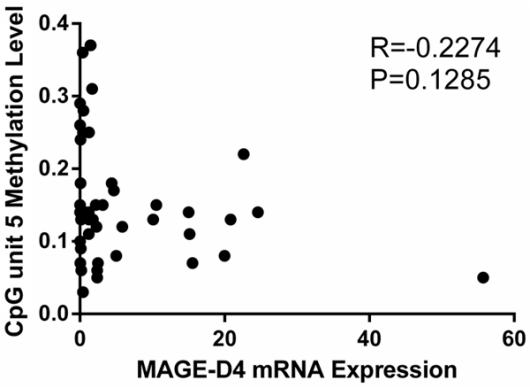
Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	1 (8.3)	35 (92.1)	
Female	11 (91.7)	3 (7.9)	
Age (years)			0.185
≤37	8 (66.7)	17 (44.7)	
>37	4 (33.3)	21 (55.3)	
WHO Grade			0.750
I/II (Low grades)	6 (50.0)	21 (55.3)	
III/IV (High grades)	6 (50.0)	17 (44.7)	
Pathological type			0.505
astrocytoma	7 (58.3)	25 (65.8)	
anaplastic astrocytoma	0 (0.0)	3 (7.9)	
oligodendroglioma	1 (8.3)	1 (2.6)	
glioblastoma	4 (33.3)	9 (23.7)	
KPS Score			0.768
≤70	10 (83.3)	28 (73.7)	
>70	2 (16.7)	10 (26.3)	
P53 Protein			1.000
-	6 (50.0)	19 (50.0)	
+	6 (50.0)	19 (50.0)	
Ki-67 (%)			1.000
<10	9 (75.0)	27 (71.1)	
≥10	3 (25.0)	11 (28.9)	
Tumor Size (cm)			0.508
<5	5 (41.7)	20 (52.6)	
≥5	7 (58.3)	18 (47.4)	
MAGE-D4 mRNA			0.001
High	1 (8.3)	24 (63.2)	
Low	11 (91.7)	14 (36.8)	



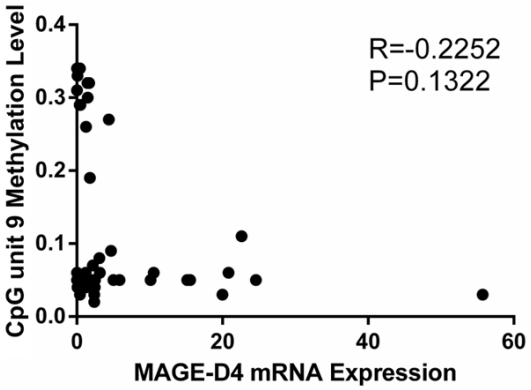
Supplementary Figure 18. Correlation between CpG unit 3 methylation level and MAGE-D4 mRNA expression.



Supplementary Figure 19. Correlation between CpG unit 4 methylation level and MAGE-D4 mRNA expression.



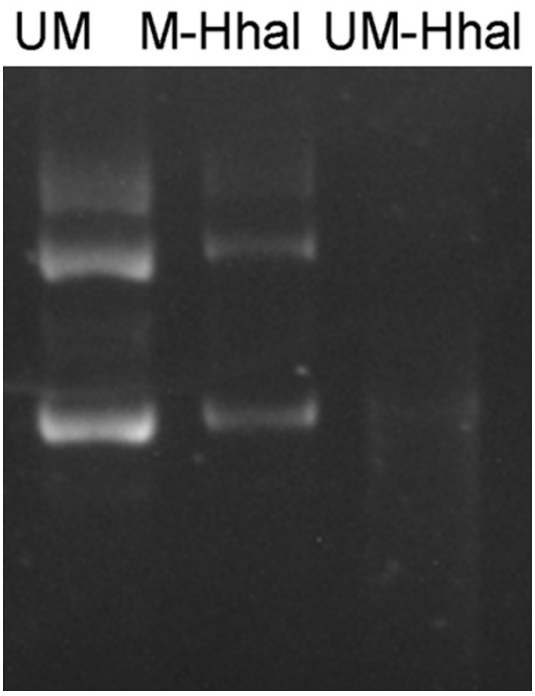
Supplementary Figure 20. Correlation between CpG unit 5 methylation level and MAGE-D4 mRNA expression.



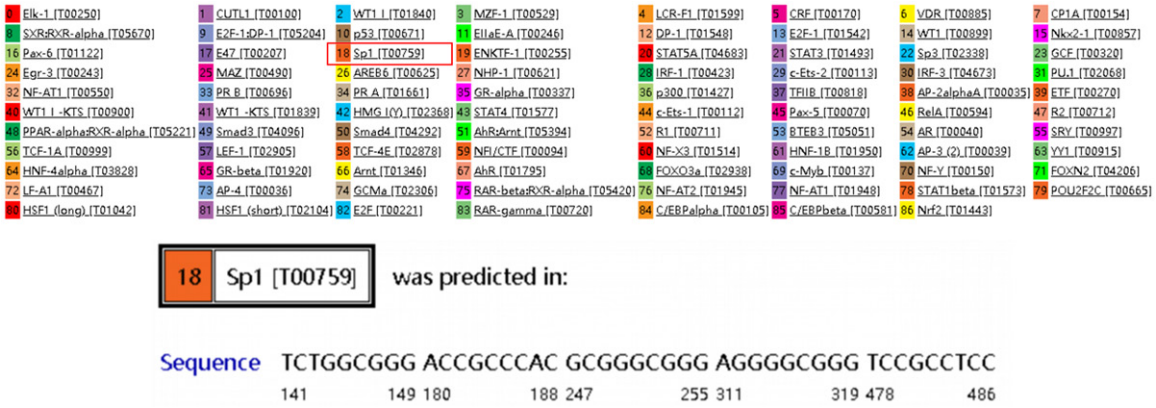
Supplementary Figure 21. Correlation between CpG unit 9 methylation level and MAGE-D4 mRNA expression.

Supplementary Table 11. Correlation between CpG unit 12 methylation level and clinical parameters in glioma patients

Parameters	Methylation n (%)		*P* value
	High	Low	
Gender			0.000
Male	0 (0.0)	36 (83.7)	
Female	7 (100.0)	7 (16.3)	
Age (years)			0.014
≤37	7 (100.0)	18 (41.9)	
>37	0 (0.0)	25 (58.1)	
WHO Grade			0.556
I/II (Low grades)	5 (71.4)	22 (51.2)	
III/IV (High grades)	2 (28.6)	21 (48.8)	
Pathological type			0.459
astrocytoma	4 (57.1)	28 (65.1)	
anaplastic astrocytoma	0 (0.0)	3 (7.0)	
oligodendroglioma	1 (14.3)	1 (2.3)	
glioblastoma	2 (28.6)	11 (25.6)	
KPS Score			0.864
≤70	6 (85.7)	32 (74.4)	
>70	1 (14.3)	11 (25.6)	
P53 Protein			1.000
-	3 (42.9)	22 (51.2)	
+	4 (57.1)	21 (48.8)	
Ki-67 (%)			0.676
<10	6 (85.7)	30 (69.8)	
≥10	1 (14.3)	13 (30.2)	
Tumor Size (cm)			1.000
<5	4 (57.1)	21 (48.8)	
≥5	3 (42.9)	22 (51.2)	
MAGE-D4 mRNA			0.103
High	1 (14.3)	24 (55.8)	
Low	6 (85.7)	19 (44.2)	



Supplementary Figure 22. Methylated pGL3-P3 was digested by HhaI.



Supplementary Figure 23. Predicted transcription factors of MAGE-D4 promoter by PROMO. (PROMO: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

Supplementary Table 12. Predicted SP1 binding sites on MAGE-D4 promoter by HumanTFDB (HumanTFDB: http://bioinfo.life.hust.edu.cn/HumanTFDB#!/)

TF	Source	Sequence	Start	Stop	Strand	Score	*P*-value	Q-value	Matched Sequence
SP1	database	MAGED4	178	188	-	10.9438	5.96E-05	0.00635	GTGGGCGGTGG
SP1	hTFtarget	MAGED4	246	257	-	12.6053	2.17E-05	0.00841	AGCCCGCCCGCT
SP1	hTFtarget	MAGED4	311	322	-	15.3421	4.11E-06	0.00331	CACCCCGCCCCT
SP1	database	MAGED4	476	486	-	13.0899	1.52E-05	0.00491	GGAGGCGGAGG