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Original Article

Methylation associated genes contribute to the favorable prognosis of gliomas with *isocitrate dehydrogenase 1* mutation

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Abstract: Gliomas, the most common primary brain tumors, are characterized by isocitrate dehydrogenase 1 mutation (IDH1-M). High mutation frequency of IDH1 indicates it's promoting role in tumorgenesis. However, the observation that patients with IDH1-M have better survival comparing with patients with IDH1 wild-type (IDH1-W) suggests that this alteration has other significant beneficial features for patients. Currently, temozolomide (TMZ) is a standard of care for patients which play a major role in DNA methylation that is similar with the role of IDH1-M in genome-wide methylation. In this study, we collected 323 gliomas samples with genome-wide methylation microarray, 502 samples with genome-wide mRNA expression microarray and 295 samples with RNA-seq. By significance analysis of microarray (SAM), we identified 18 genes which are hypermethylation and low expression in samples with IDH1-M comparing with IDH1-W (FDR<0.01). Furthermore, 18 candidate genes were downregulated in TMZ-treated samples. Finally, we obtained two candidate genes, F3 and RBP1. Survival analysis showed that hypermethylation or low expression of the two genes indicated a favorable prognosis, which was consistent with IDH1-M and administration of TMZ in glioma patients. F3 and RBP1 were further validated by qPCR on an independent validation cohort containing 145 samples. Our data suggest that these candidate genes were suppressed by TMZ or IDH1-M induced hypermethylation, resulting in the favorable prognosis of patients with gliomas.

Keywords: Gliomas, *IDH1* mutation, temozolomide, RNA-Seq, RNA microarray, methylation microarray

Introduction

Gliomas are the most common primary brain tumors and important cause of cancer-related mortality among adults. Aggressive surgery followed by adjuvant radiation and/or chemotherapy is considered the standard of care, but provides limited benefits [1]. Temozolomide (TMZ), a new oral alkylating agent, is currently the gold standard for adjuvant chemotherapy in patients with gliomas, and has been shown to significantly improve drug efficacy and safety. DNA methylation and failure of mismatch repair play a major role in TMZ's cytotoxicity. Addition of a methyl group to the O^6 position of guanine in genomic DNA results in the incorporation of thymine residue complementing O^6-methylguanine instead of the normal cytosine residue. The abnormal guanine-thymine pair leads to a pause in the DNA replication fork and triggers the DNA mismatch repair pathway, which eventually leads to cell cycle arrest and cell death [2]. However, many genes, like *MGMT*, can cause TMZ resistance in cancer cells by repairing the TMZ-induced DNA methylation [3]. Similarly, tumor cells present other genetic alterations (mutation, hypermethylation and overexpression) and do not undergo TMZ-induced G2 arrest; such cells are resistant to TMZ-induced cell death [4]. Moreover, patient survival is only prolonged by two months of TMZ administration (12.1 months with radiotherapy alone *vs* 14.6 months with radiotherapy plus TMZ) [1]. Therefore, TMZ-based chemotherapy

Table 1. Clinical characteristics of the *IDH1* status in CGGA samples

Variables	IDH1-M	IDH1-W	Total
Samples	241 (48.0%)	261 (52.0%)	502
Age (average)	46.4 (9-81)	38.8 (10-66)	42.8 (9-81)
Sex			
Male	137 (45.8%)	162 (54.2%)	299
Female	104 (51.2%)	99 (48.8%)	203
Histology			
A	67 (75.3%)	22 (24.4%)	89
O	27 (79.4%)	7 (20.6%)	34
OA	46 (66.7%)	23 (33.3%)	69
AA	13 (39.4%)	20 (60.6%)	33
AO	16 (84.2%)	3 (15.8%)	19
AOA	26 (51.0%)	25 (49.0%)	51
GBM	46 (22.2%)	161 (81.8%)	207
Location*			
Left hemisp	110 (46.6%)	126 (53.4%)	236
Right hemisp	110 (48.8%)	116 (51.2%)	226
Mixed	16 (57.1%)	12 (42.9%)	28

*12 samples lost the information.

must be improved in order to overcome the reduced sensitivity resulting from aberrant genes in a subgroup of glioma.

Recently, *IDH1* was shown to be mutated in up to 70% of low grade gliomas (grades II and III) and secondary glioblastomas (grade IV), making it an important marker to guide treatment decisions [5, 6]. Patients with *IDH1*-M have a favorable prognosis compared with those harboring *IDH1*-W [7]. However, most studies have proposed *IDH1*-M to be an oncogene in glioma development [8, 9], which is inconsistent with its prognostic value. Conversely, studies have found that expression of mutant *IDH1* causes increased hypermethylation of a large number of genes [10], which might reasonably explain the prognostic value of *IDH1*-M. IDH1 catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) [11]. *IDH1-M* is heterozygous and exclusively affects arginine at position 132. This mutation occurs at the arginine residue of the enzyme's active site and causes reduction of α-KG to D-2-hydroxyglutarate. Accumulation of this metabolite induces DNA hypermethylation, leading to genome-wide epigenetic changes [12]. Therefore, it is plausible that *IDH1-M* occurrence and TMZ administration play a similar role in gene methylation. It is well accepted that gene methylation and expression are closely related: methylation affects prognosis by regulating the expression of some genes. Consequently, identifying such methylation associated genes is important for decision-making when applying alkylating drugs to patients with gliomas.

In this study, we collected 323, 502, and 295 samples with methylation microarray, mRNA microarray, and RNA-seq, respectively. By comparing samples with and without *IDH1*-M, and samples treated with TMZ and control untreated samples, we obtained 2 candidate genes associated with IDH1-M or TMZ induced hypermethylation. Survival analysis showed that hypermethylation or low expression of the two genes indicated a favorable prognosis, consistently with IDH1-M and TMZ treatment. Finally, the expression levels were further validated on an independent validation cohort. Overall, these findings provide a further basis for understanding the roles of IDH1-M and TMZ treatment in gliomas. The molecular mechanism of methylation may well be represented by these suppressed genes, which are novel potential interfering targets for treatment of the subgroup of patients with gliomas without IDH1-M.

Materials and method

Samples

Samples obtained from four datasets were listed in Table S1. The characteristics of patients from The Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn) are detailed in **Table 1**. These samples were used to perform whole genome expression profiling, genome-wide methylation profiling, RNA-sequencing, and survival analysis. All patients underwent surgical resection from January 2005 through December 2012. Patients were eligible for the study if their diagnosis was established histologically by 2 neuropathologists according to the 2007 WHO classification guidelines. Only samples with 80% tumor cells were selected for analysis. This study was approved by the institutional review boards, and wrote informed consent was obtained from each patient. The independent sample cohorts The Cancer Genome A*tlas* (TCGA) and GSE16011 are well *described in publ*ic databases (http://cancergenome.nih. gov and http: //www.ncbi.nlm.nih.gov/geo/query).

DNA pyro-sequencing for IDH1 mutation

Genomic DNA was isolated from frozen tumor tissues by using the QIAamp DNA Mini Kit (Qiagen). The genomic region spanning wild-type R132 of IDH1 was analyzed by pyrophosphate sequencing using the following primers: 5'-GCTTGTGAGTG-GATGGGTAAAAC-3' and 5'-Biotin-TTGCCAACATG ACT-TACTTGATC-3'. The PCR analysis was performed in duplicate in 40 μl reaction containing 1 μl of 10 μM each primer, 4 μl of 10× buffer, 3.2 μl of 2.5 mM dNTPs, 2.5 U hotstart Taq (Takara) and 2 μl of 10 μM DNA. The PCR was carried out on an ABI PCR system 9700 (Applied Biosystems) with the following program: 95°C for 3 min; 50 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 30 s; 72°C for 5 min. Single-stranded DNA was purified from the total PCR product and subjected to pyrosequencing on PyroMark Q96 ID System (QIAGEN) using the primer 5'-TGGATGGG TAAAACCT-3' and EpiTect Bisulfite Kit (QIAGEN).

Whole genome expression profiling (CGGA)

Microarray analysis was performed using an Agilent Whole Human Genome Array according to the manufacturer's instructions. The integrity of total RNA was checked with an Agilent 2100 Bioanalyzer (Agilent). cDNA and biotinylated cRNA were synthesized and hybridized to the array. Data were acquired using the Agilent G2565BA Microarray Scanner System and Agilent Feature Extraction Software (version 9.1). Probe intensities were normalized using GeneSpring GX 11.0. GSE16011 whole genome expression profiling data were downloaded from public database.

Genome-wide DNA methylation profiling (CGGA)

For methylation profiling, we used the Illumina Infinium Human Methylation 27 Bead-Chips (Illumina Inc.) as described previously [13]. The BeadChip contains 27,578 highly informative CpG sites covering more than 14,000 human RefSeq genes. This allows researchers to interrogate all these sites in samples at a single nucleotide resolution. Bisulfite modification of DNA, chip processing and data analysis were performed following the manufacturer's manual at the Wellcome Trust Centre for Human Genetics Genomics Lab, Oxford, UK. The results were analyzed with the BeadStudio software (Illumina). TCGA methylation profiling data were downloaded from the TCGA database.

RNA-seq (CGGA)

Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A pestle and a QIAshredder (Qiagen) tube were used to disrupt and homogenize the frozen tissues. RNA intensity was assessed using 2100 Bioanalyzer (Agilent Technologies); only high quality samples with RNA Integrity Number (RIN) values greater than or equal to 7.0 were used to construct the sequencing library. The subsequent steps included end repair, adapter ligation, size selection and polymerase chain reaction enrichment. The DNA fragment length was measured using a 2100 Bioanalyzer, and median insert sizes were 200 nucleotides. The libraries were sequenced on the Illumina HiSeq 2000 platform using the 101-bp pair-end sequencing strategy. Short sequence reads were aligned to the human reference genome (Hg19 Refseq) using the Burrows-Wheeler Aligner (BWA, Version 0.6.2-r126).

Real time quantitative PCR (qPCR)

Gene expression levels of *F3* and *RBP1* in 145 samples were analyzed by real-time quantitative PCR using the SYBR Supermix Kit (Bio-Rad, Hercules, CA). PCR reactions included the following components: 100 nM each primer, diluted cDNA templates, and iQ SYBR Green supermix. The PCR efficiency was examined by assessing serial dilutions of the template cDNA, and melting curve data were collected to evaluate specificity. Each cDNA sample was analyzed in triplicate. ACTIN was used for normalization, and relative mRNA level was determined as 2-[Ct (ACTIN)-Ct (gene of interest)]. The primer sequences: *F3*-F: 5'CCGA CGAGATTGTGAAGGATGTGA-3', *F3*-R: 5'-TCCGAGG TTTGTCTCC AGGTAAGG-3'; *RBP1*-F: 5'-TCCAGT ACTCCCCGAAATG-3', *RBP1*-R: 5'-AGGTACTCCTCGAAATTCTCGTT-3'.

Statistical analysis

Differentially expression genes in microarray and RNA-seq were detected by significance analysis of microarray (SAM) (FDR<0.01). Differentially expression genes in TMZ-treated

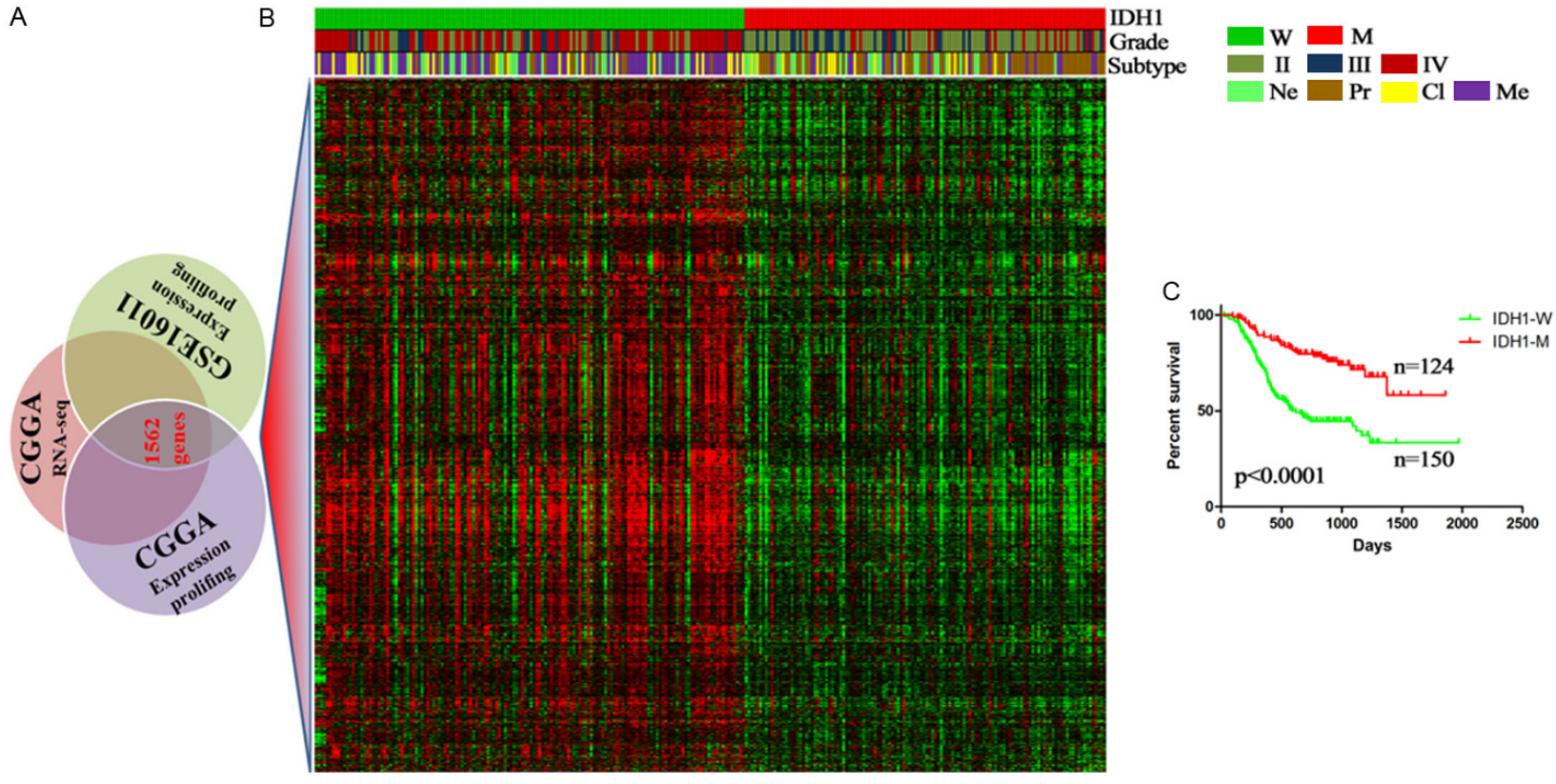

Figure 1. *IDH1*-M specific gene expression profiling based on 1562 genes. A. By overlapping the data from the CGGA mRNA microarry and RNA-seq, and GSE16011 mRNA microarry dataset, a total of 1562 genes were identified by comparing the gene expression levels between samples with and without *IDH1*-M (FDR<0.01). B. Heat map was created using the 1562 genes using CGGA dataset; *IDH1* status, glioma grade of samples and TCGA subtypes were indicated with different colors. C. Kaplan-Meier plots of the overall survival based on *IDH1* status are shown. The overall survival data were analyzed using log-rank tests. W-*IDH1* wild-type, M-*IDH1* mutation; Ne-Neural, Pr-Proneural, Cl-Classical, Me-Mesenchymal.

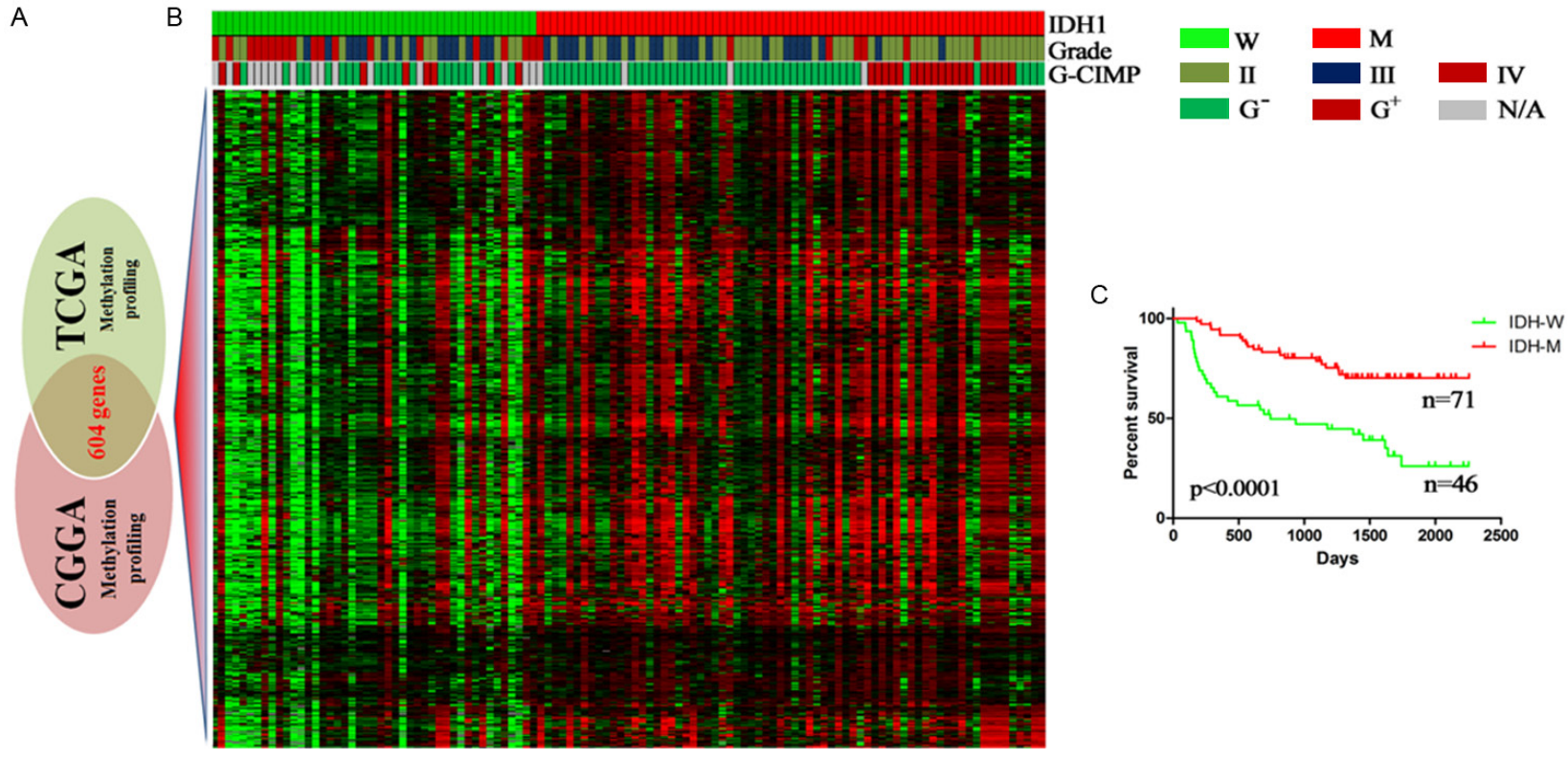

Figure 2. *IDH1*-M specific hypermethylation profiling based on 604 genes. A. By overlapping the data from CGGA methylation profiling and TCGA methylation profiling, a list of 604 hypermethylatied genes were identified by comparing the methylation levels between samples with and without *IDH1*-M (FDR<0.01). B. Heat map was created using the 604 genes using CGGA dataset which had high methylation in samples with *IDH*-M; *IDH1* status, grade of samples and G-CIMP subtypes were indicated with different colors. C. Kaplan-Meier plots of the overall survival based on *IDH1* status are shown.

samples and untreated samples were detected by unpaired Student's t-test. Kaplan-Meier survival analysis was used to estimate the survival distributions. The log-rank test was applied to assess the statistical significance between stratified survival groups using the GraphPad Prism version 4.0 statistical software. KEGG pathway analysis was performed using DAVID (http://david.abcc.ncifcrf.gov/) [14]. Heat maps of different grade ODs were constructed by Gene Cluster 3.0 and Gene Tree View software using the differentially expression genes. Multivariate Cox models were used after univariate analysis using SPSS, version 13.0 (SPSS). A two-sided *p* value <0.05 was regarded as significant.

Results

Gene expression levels in samples with IDH1-M

IDH1 mutation increases gene methylation levels and results in downregulation of these genes [10, 12]. To identify the downregulated genes induced by *IDH1-M*, we compared gene expression levels in 276 samples with and without *IDH1*-M from the CGGA dataset (FDR<0.01). By SAM analysis, a total of 4543 genes (5952 probes) were identified with lower expression in 126 samples with *IDH*-M compared with 150 *IDH1*-W samples. We projected these genes into 295 samples with RNA-seq from the CGGA dataset and 226 samples with mRNA microarray from the GSE16011 dataset (**Figure 1A**). Finally, 1568 out of 4543 genes were validated on 232 samples with *IDH1*-M and 289 samples with *IDH*-W (**Figure 1B**). As expected, the majority of samples with *IDH1*-M (53.2% and 84.1%) were classified into proneural subtype and lower grade gliomas (II and III). Among these genes, *MGMT* has been described in previous studies by us or others: its high methylation and low expression indicate a favorable outcome in patients with gliomas [15].

Hypermethylation status in samples with IDH1-M

To identify the hypermethylated genes induced by *IDH1*-M, we compared the genome-wide methylation levels in 118 samples with and without *IDH1*-M from the CGGA dataset (FDR <0.01). A total of 1055 genes (1211 probes) showed higher methylation levels in 72 samples with *IDH*-M in comparison with 46 samples with *IDH1*-W. The list was projected into 205 samples with methylation microarray from the TCGA dataset (FDR<0.01) (**Figure 2A**). Finally, 606 out of 1055 genes showed higher methylation levels in 22 samples with *IDH1*-M compared with 183 *IDH*-W samples (**Figure 2B**). As expected, the majority of samples with G-CIMP$^+$ were concentrated in the *IDH1*-M group. Among the genes, *ALDH1A3* has been reported in our previous study with higher methylation levels in patients with prolonged survival time compared with those with short survival. A significant negative correlation was observed between *ALDH1A3* methylation status and protein expression [13].

Candidate genes were downregulated in samples from patients treated with TMZ

To identify the genes downregulated as a result of hypermethylation, we overlapped the hypermethylated genes and downregulated genes based on the *IDH1-M* status. Finally, 156 genes were obtained with higher methylation and lower expression in samples with *IDH1*-M in comparison with the IDH1-W group. TMZ play a major role in DNA methylation that leads to cytotoxicity. To further confirm that these genes were affected by methylation, we collected 23 samples (recurrent gliomas) from patients who received at least one course of TMZ and 114 samples without any treatment on the CGGA dataset. We compared the gene expression levels and found that 18 out of 156 candidate genes were downregulated after treatment with TMZ (**Figure 3A** and **3B**). Downregulation of these 18 candidate genes might result from the hypermethylation status and contribute to the favorable prognosis observed in the *IDH1*-M group and TMZ treatment (**Figures 1C**, **2C** and **3C**).

High methylation and low expression confer a better clinical outcome

Increasing evidence has showed that *IDH1*-M and administration of TMZ are beneficial for glioma patients [1, 16]. However, whether the candidate genes obtained by analysis of *IDH1-M* and methylation status are associated with patient survival is unknown. Multivariate Cox analyses showed that two candidate genes, *F3* (*P*=0.037; 95% CI: 0.986-1.026) and *RBP1* (*P*=0.003; 95% CI: 1.063-1.339), were signifi-

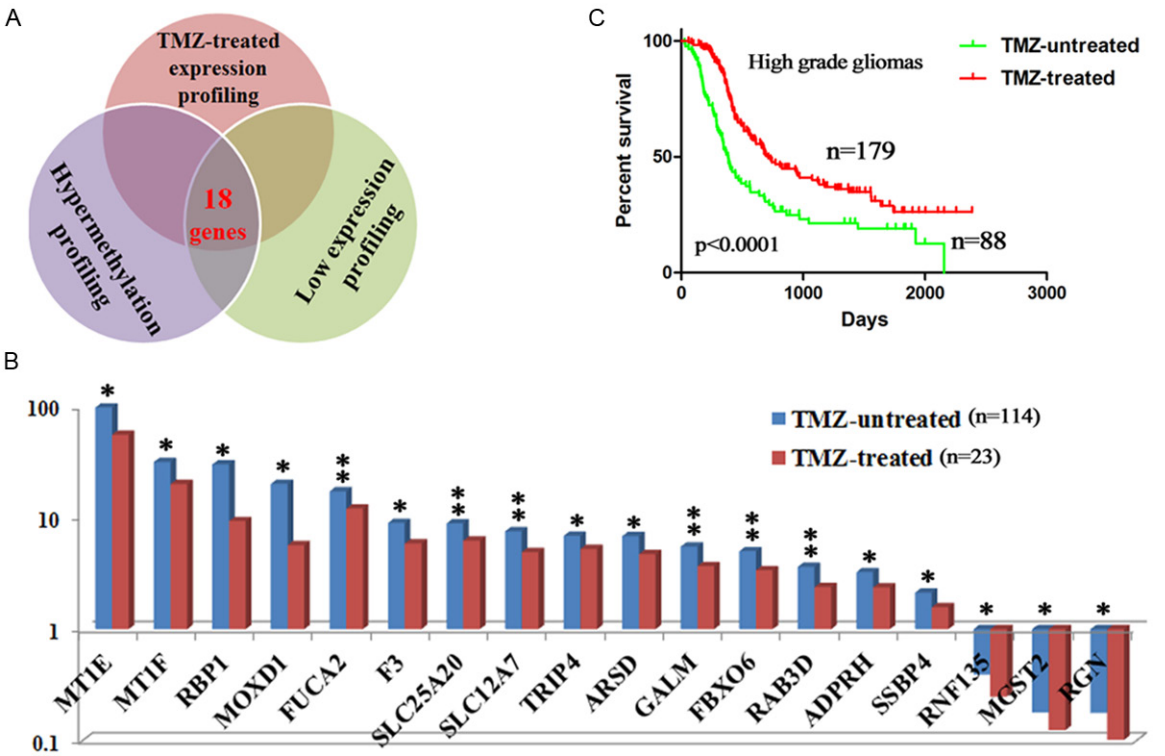

Figure 3. 18 candidate genes were downregulated in TMZ-treated samples. A and B. After overlapping the above two *IDH1*-M specific gene profiling, 18 genes were found to be downregulated in TMZ-treated samples ($P<0.05$). These patients were treated with TMZ chemotherapy or radiation after the first surgery and underwent second operation, and samples were collected. C. TMZ-treated patients with gliomas have significantly longer survival than untreated patients in the CGGA dataset.

cantly associated with OS independent of tumor grades and patients age (Tables S2, S3 and S4). Kaplan-Meier survival analysis showed that high methylation or low expression of *F3* and *RBP1* conferred a better clinical outcome that was similar with *IDH1-M* and TMZ administration of gliomas (**Figure 4A**). The result was further validated in TCGA methylation data and GSE16011 gene expression data (**Figure 4B**).

Candidate genes were validated on an independent validation cohort

Semi-quantitative RT-PCR was performed to assess *F3* and *RBP1*. The expression levels of the two genes were significantly lower in 75 samples with *IDH*-M than 70 samples with *IDH1*-W (**Figure 5**). These results provided further evidence supporting the downregulated genes resulted from *IDH1*-M. These data demonstrated that the candidate genes were affected by *IDH1*-M or TMZ, their alterations contributing to the prognostic value of *IDH1*-M and TMZ. These findings provide new targets for subgroups of patients without *IDH1*-W or those insensitive to TMZ.

Discussion

Gliomas are the most common primary brain tumors with various genetic alterations, such as *TP53* mutation, *PTEN* mutation, *EGFR* amplification and 1p/19q loss. Currently, *IDH1*-M is a hot topic in glioma research. Direct sequencing in a series of 685 brain tumors revealed the highest frequencies of *IDH1*-M in gliomas (68-88%) [5]. The high frequency of *IDH1*-M suggests a role in early tumor development. Therefore, *IDH1*-M was thought to have protumorigenic potential [8, 9]. However, glioma patients with *IDH1*-M have a better survival outcome compared with those harboring wild-type *IDH1*. *IDH1*-M acts as a predictive biomarker in gliomas and exhibits a better response to TMZ [17]. These data suggest that *IDH1*-M is a double-edged sword in gliomas. Recent studies have found that *IDH1*-M resulted in increased levels of D-2-hydroxyglutarate that induces

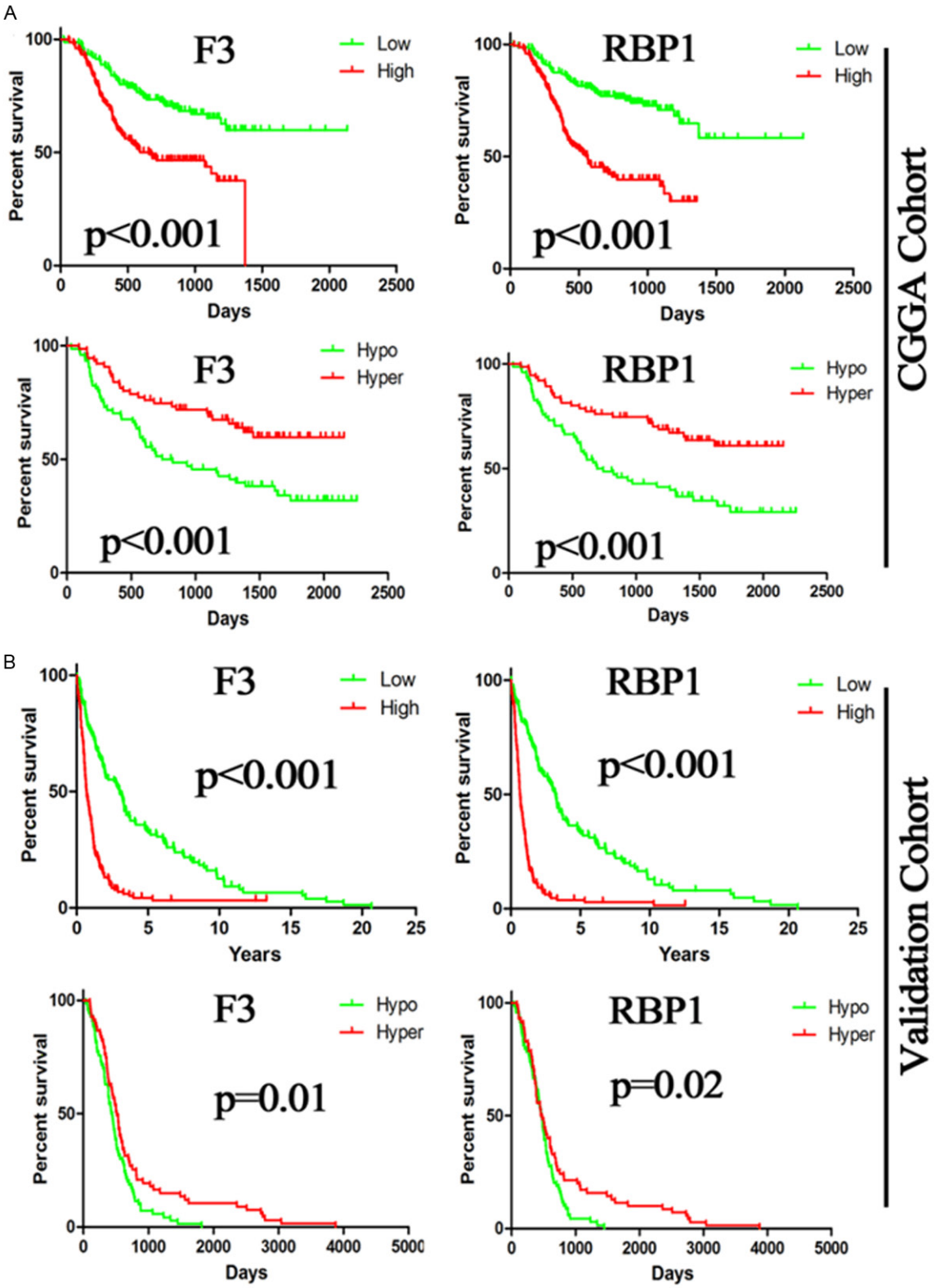

Figure 4. Candidate genes were tightly associated with prognosis in both CGGA cohort and validation cohort. A. Low expression or hypermethylation of candidate genes indicates longer survival than high expression or hypomethylation based on CGGA dataset. B. The prognostic value of candidate genes was validated on GSE16011 mRNA microarray dataset and TCGA methylation microarray dataset.

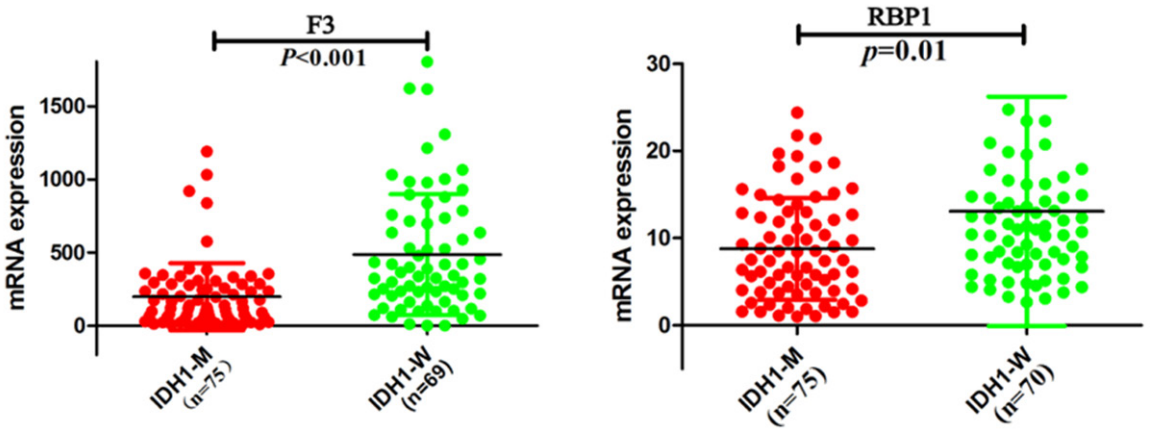

Figure 5. Candidate genes were validated by qPCR in 145 additional samples. The expression levels of *F3* and *RBP1* were lower in samples with *IDH1*-M than in those with *IDH1*-W on an independent validation cohort containing 145 samples ($P<0.05$).

DNA hypermethylation, leading to genome-wide epigenetic changes [11]. Researchers have found that glioma-CpG island methylator phenotype (G-CIMP$^+$) is tightly associated with *IDH1*-M. This alteration along with G-CIMP$^+$, defines a relatively favorable subtype (Proneural) [10]. Therefore, *IDH1*-M increases gene methylation levels and results in downregulation of some genes that might contribute to the favorable value of *IDH1*-M. However, the role of *IDH1*-M on the gene methylation might be similar to TMZ effect in gliomas. TMZ is a standard of care for patients which play a major role in DNA methylation.

In our study, we analyzed the genome-wide methylation profiling, whole genome expression profiling and RNA-seq to identify genes suppressed by the *IDH1*-M induced hypermethylation. A total of 18 genes were identified with higher methylation and lower expression in samples with *IDH1*-M compared with those without *IDH1*-M. Moreover, these candidate genes were also downregulated in TMZ-treated samples: ADPRH, ARSD, F3, FBXO6, FUCA2, GALM, MGST2, MOXD1, MT1E, MT1F, RAB3D, RBP1, RGN, RNF135, SLC12A7, SLC25A20, SSBP4 and TRIP4. Among the 18 genes, *F3* and *RBP1*were independent prognostic markers for patients with gliomas and hypermethylation or low expression of the two genes indicated a favorable prognosis.

RBP1 had been reported before that hypermethylation was described in nearly all *IDH1* and *IDH2* mutated gliomas (79/82) and shown to be associated with improved patient survival [17, 18]. Indeed, decreased RBP1 expression was noted in glioma patients with long term survival. More importantly, reduction of methylation was closely related to increasing mRNA expression in the demethylated cell lines. These data suggest that *RBP1* is regulated by methylation results from *IDH1*-M or administration of TMZ. But the exact functional effect of *RBP1* hypermethylation in gliomas has yet to be elucidated. *F3* encodes the coagulation factor III, a cell surface glycoprotein. It functions as a high-affinity receptor for coagulation factor VII and enables cells to initiate the blood coagulation cascades [19]. *F3* mRNA and protein expression levels had proven to be increased in tumors from patients with clear cell carcinoma. Full-length *F3* is overexpressed in breast cancer, and alternatively, spliced *F3* promotes breast cancer growth in a β1 integrin-dependent manner. These findings show that *F3* is an oncogene that leads to tumor development. However, these two genes have been rarely reported in human gliomas and their exact regulatory mechanisms remain to be elucidated. In the present study, these genes for the first time were reported by their association with *IDH1*-M and methylation in gliomas. Our results indicate that gene methylation induced by *IDH1*-M or TMZ administration might lead to low expression of these candidate genes, which contributes to favorable survival outcome.

Overall, these findings provide new insights for understanding the fundamental basis of *IDH1*-M and TMZ roles in gliomas. The molecu-

lar mechanism of methylation may well be represented by these genes. Finally, these genes constitute novel interfering targets for glioma patients.

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Disclosure of conflict of interest

None.

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Table S1. Numbers of enrolled samples from five datasets

Datasets	IDH1-M	IDH1-W
CGGA microarray	126	150
CGGA RNA-Seq	149	146
GSE16011 microarray	83	143
CGGA methylation microarray	46	72
TCGA methylation microarray	22	183

Table S2. Univariable regression analyses of candidate genes and other characteristics in relation to overall survival in gliomas

Variable	Univariable Regression	
	HR (95% CI)	*P* value
Gender (female vs. male)	0.839 (0.565-1.245)	0.382
Age	1.043 (1.025-1.061)	<0.001
Grade	3.55 (2.677-4.709)	<0.001
1p loss	12.208 (3.005-49.601)	<0.001
19q loss	6.427 (2.358-17.520)	<0.001
F3 (High vs. Low)	1.456 (1.265-1.676)	<0.001
RBP1 (High vs. Low)	1.443 (1.314-1.584)	<0.001

Table S3. Multivariable regression analyses of *F3* and other characteristics in relation to overall survival in gliomas

	Multivariable Regression	
	HR (95% CI)	*P* value
Age	1.006 (0.986-1.026)	0.549
Grade	2.771 (1.989-3.862)	<0.001
1p loss	8.413 (1.153-61.416)	0.036
19q loss	0.626 (0.152-2.581)	0.517
F3 (High vs. Low)	1.179 (1.01-1.376)	0.037

Table S4. Multivariable regression analyses of *RBP1* and other characteristics in relation to overall survival in gliomas

	Multivariable Regression	
	HR (95% CI)	*P* value
Age	1.002 (0.982-1.022)	0.862
Grade	2.557 (1.816-3.601)	<0.001
1p loss	8.523 (1.168-62.602)	0.035
19q loss	0.609 (0.148-2.503)	0.492
RBP1 (High vs. Low)	1.193 (1.063-1.339)	0.003