Identification of potential glioma drug resistance target proteins based on ultra-performance liquid chromatography-mass spectrometry differential proteomics

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In this study, to screen for candidate markers of Temozolomide (TMZ) resistance in glioblastoma, we artificially established TMZ drug-resistant glioblastoma (GBM) cell lines, U251-TMZ and U87-TMZ. In the U251-TMZ and U87-TMZ cell lines, we screened and analyzed differentially expressed proteins using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) differential proteomics. Compared with the U251 and U87 control cell lines, 95 differential proteins were screened in the U251-TMZ and U87-TMZ cell lines, of which 28 proteins were upregulated and 67 proteins were down-regulated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the co-upregulated proteins showed that most of the differentially expressed proteins were located in the cytoplasm and were significantly upregulated in the biological processes related to vesicular transport in the intimal system and inflammatory response mediated by myeloid leukocytes. Seven candidates were identified as potential GBM markers of TMZ resistance. Combined with existing research findings, our study supports that UAP1L1 and BCKDK are promising potential markers of TMZ resistance in GBM. This is important for further understanding the molecular mechanisms that drive the development and enhancement of TMZ resistance.

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2 **target proteins based on ultra-performance liquid**
3 **chromatography-mass spectrometry differential**
4 **proteomics**
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Abstract

In this study, to screen for candidate markers of Temozolomide (TMZ) resistance in glioblastoma, we artificially established TMZ drug-resistant glioblastoma (GBM) cell lines, U251-TMZ and U87-TMZ. In the U251-TMZ and U87-TMZ cell lines, we screened and analyzed differentially expressed proteins using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) differential proteomics. Compared with the U251 and U87 control cell lines, 95 differential proteins were screened in the U251-TMZ and U87-TMZ cell lines, of which 28 proteins were upregulated and 67 proteins were down-regulated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the co-upregulated proteins showed that most of the differentially expressed proteins were located in the cytoplasm and were significantly upregulated in the biological processes related to vesicular transport in the intimal system and inflammatory response mediated by myeloid leukocytes. Seven candidates were identified as potential GBM markers of TMZ resistance. Combined with existing research findings, our study supports that UAP1L1 and BCKDK are promising potential markers of TMZ resistance in GBM. This is important for further understanding the molecular mechanisms that drive the development and enhancement of TMZ resistance.

Introduction

Glioblastoma (GBM) is one of the most common and recurrent primary malignant brain tumors. GBM accounts for approximately 46% of all diagnosed brain tumors. According to statistics, 2.7% of cancer-related deaths are caused by GBM[1] . The average survival time of GBM patients is only 12–15 months [2] . Extensive studies have been performed to understand and treat GBM, but the results remain unsatisfactory. The short survival time is related to the anatomical location of the tumor, heterogeneity of GBM cells, and rapid growth of GBM[3]. The incidence of GBM increases with age, and the incidence in men is slightly higher than that in women[4]. It is listed as the third most common cause of cancer-related death in patients aged 15–34 years. Clinically, standard GBM treatment includes the maximum safety of surgical resection of tumors, radiotherapy, and adjuvant chemotherapy[2].

Since Temozolomide (TMZ) was approved by the FDA in 2005, it has been widely used as a standard chemotherapy regimen for GBM diagnosis [6]. TMZ can cross the blood-brain barrier and can be administered orally. It is activated by conversion to the metabolite 5-(3-methyltriazen1-yl) imidazole-4-carboxamide (MTIC) at physiological pH values [7]. However, TMZ is also a key factor in drug resistance and recurrence. Due to the high heterogeneity and mutagenicity of GBM and extensive exposure to TMZ, > 50% of GBM patients treated with TMZ do not respond to treatment[8]. However, TMZ remains the key to the treatment of high-level gliomas. TMZ resistance is a significant issue that must be overcome for the successful treatment of GBM.

At present, proteomics technology can reflect the tumor phenotype more accurately, and its advantage is better than genome and transcriptome analyses[9]. The level of gene expression is not always related to the level of protein expression, and the process of post-translational repair

54 cannot be detected[10]. Because of the limited predictive markers of TMZ resistance[11], anti-TMZ

55 resistance has become more complex for GBM treatment[12]. Therefore, it is urgent to identify

56 reliable biomarkers that can be used to determine TMZ resistance.

57 In this study, two classic GBM cell lines, U251 and U87, were used to identify reliable

58 biomarkers for TMZ resistance. Firstly, TMZ-resistant U251 and U87 GBM cell lines were

59 constructed, and the protein expression profiles of U251-TMZ and U87-TMZ were compared

60 using the non-quantitative labeling proteomic technique ultra-performance liquid

61 chromatography-mass spectrometry (UPLC-MS). Twenty-seven upregulated differential proteins

62 were screened, among which seven were identified according to the expression difference

63 multiple and the credibility of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment

64 analysis. Bioinformatics analysis of differentially expressed proteins showed that the upregulated

65 differentially expressed proteins were mainly related to energy metabolism, ribosome synthesis,

66 and processing, and were enriched in endocytosis, carbon metabolism, and antibiotic synthesis.

67 Our study summarizes the protein expression profiles in U251 and U87TMZ drug-resistant cell

68 lines, which provides a basis for a better understanding of TMZ resistance in disease.

69

Materials & Methods

71 **Materials**

72 U251 and U87 cell lines were purchased from Tianjin Genink Biotechnology Co.,Ltd; DMEM and fetal

73 bovine serum were purchased from Gibco; Cell Counting Kit-8 was purchased from Dojindo

74 Laboratories.

75 **Screening of the U251 U87-TMZ cell line**

76 Glioma cells in the logarithmic phase were added with 200 μg/mL TMZ solution (diluted with DMEM

77 complete medium). After 24 h, the medium was replaced, and 200 μg/mL TMZ solution was added after

78 2 d of culture. The above procedure was repeated until there was no significant change in the proliferation

79 and morphology of glioma cells at this TMZ concentration. According to the above procedure, the

80 concentration of TMZ gradually increased (300, 400, and 500 μg/mL).

81 **Liquid-phase MS**

82 The type of liquid chromatography was EASY-nLC1200 (Thermo Fisher) and the tandem mass

83 spectrometer was Q Exactive HF (Thermo Fisher). The peptides were digested with phase A (0.1%

84 formic acid) loaded onto a self-made Trap column (100um × 2 cm C18 packing specification is 3umline

85 120A). Different gradients of phase B (80% acetonitrile, 0.1% formic acid, and 19.9% water) were used

86 to elute the trap column. The eluted peptides were separated using an analytical column (150um × 15 cm

87 C18 packing specification, 1.9um120A) to form a charged spray, which was detected using MS. The B

88 phase gradient was 0 min, 7%; 8 min, 14%; 60 min,28%; 72 min, 42%; 73 min, 95%; 78 min, 95%; and

89 79 min, 30%, and the flow rate was 600 nL/min. Mass spectrum parameters: The first-order detector type

90 was an orbital trap (Orbitrap) with a resolution of 120000; Orbitrap scan range, 300–1400m/z; max

91 injection time, 80ms; and AGC Target, 3E6. The second stage adopted a data-dependent scanning mode

92 and the top 20 precursor ions were selected for secondary acquisition in each scan. The secondary

93 detector was the same Orbitrap; resolution, 15000 at 120m/z; maximum injection time, 19 ms; AGC

94 Target, 2E4; scan range, 200–2000m/z; included charge state, 2–6; collision energy duration, 15 s; mass

95   tolerance, ±7 ppm; isolation window, 1.6 m/z; activation type, HCD; and normalized collision energy,
96   27%.
97   **Data processing and statistical analysis**
98   Microsoft Excel 2016 was used for preliminary data collation. We performed all statistical analyses using
99   GraphPad Prism 8. We used unpaired t test for comparison of cell viability between cell lines. The results
100   were expressed as means ± standard deviations, and $P < 0.05$ was considered significant. We considered p
101   < 0.05 to indicate statistical significance. *** $p < 0.001$.**** $p < 0.0001$.
102   MaxQuant software (version 1.6.1.0) was used to search the database of the original data collected using
103   MS. Restriction endonuclease digestion method: trypsin/P, and a maximum of two missing sites were
104   allowed. Variable modification: oxidation (M) and acetyl (protein N-term). Fixed modification:
105   Carbamidomethyl (C), a maximum of five variable modifications, was allowed for each peptide. The
106   first-level quality deviation was 20ppm. The second-level quality deviation was 20ppm. The database was
107   Uniprot's SWISS database (containing 20431 annotated proteins, released2019.07). The minimum peptide
108   length was seven amino acids, and the maximum peptide mass was 5000 Da. The false discovery rate
109   (FDR) of spectrum and protein matching was set to 1% and the protein quantitative value was obtained
110   using the iBAQ algorithm.
111   After the median normalization of the original quantitative value, log2 transformation was performed to
112   ensure that it met the normal distribution criteria, and the quantitative value of each sample met the
113   normal distribution criteria. Proteins that could be statistically analyzed were selected (quantification of at
114   least three samples in one group) and the minimum value for vacancies worthy of interpolation was
115   selected. The difference in protein was analyzed using the R software t-test and function, and the p value
116   was corrected using the Benjamini-Hochberg algorithm. Proteins with adjusted $P < 0.05$, and fold change
117   > 2 were considered differentially expressed proteins. The upregulated differential proteins were analyzed
118   using the Cluster Profiler Package for GO and KEGG pathway enrichment analyses. Protein-protein
119   interaction (PPI) analysis (STRING database, version 11.0) was also performed.
120
121   # Results
122   **Results 1. Screening of TMZ-resistant cell lines**
123   U251 and U87 glioma cell lines in the logarithmic growth phase were added to the TMZ solution and
124   cultured for 2 d, followed by the addition of TMZ solution. The above procedures were repeated until the
125   proliferation and morphology of glioma cells did not change significantly at this TMZ concentration.
126   Repeated operations increased the concentration of TMZ used to construct TMZ-resistant U251-TMZ and
127   U87-TMZ cell lines. In addition to observing the morphological changes in cells to confirm the state of
128   drug resistance, we also determined the OD value of cell-counting kit-8 (CCK8). As shown in Fig.1A and
129   B, there was no significant difference in morphology and quantity between the control and + TMZ groups
130   of the U251-TMZ and U87-TMZ cell lines. The OD values of CCK8 in the U251-TMZ and U87-TMZ
131   cell lines were significantly higher than those of their respective control groups. Therefore, we
132   successfully constructed and screened TMZ-resistant glioma U251 and U87 cell lines.
133
134   **Results 2. Data quality control**
135   To better analyze the differential protein expression between TMZ drug-resistant cell lines U251 and
136   U87, the quantitative data were normalized to the median. A comparison of the results before and after
137   normalization is shown in Fig.2A. Normalized data were analyzed using Pearson's correlation coefficient
138   (Fig.2B). The correlation coefficients between the samples were higher than those between the groups,

139 and there were significant differences in protein expression between the two groups. Additionally, the
140 quantitative data were also analyzed by cluster analysis, and the results showed that each group of
141 biological repeats was clustered together, indicating high similarity and good data repeatability (Fig.2C).
142 Analysis of the coefficient of variation of the normalized data (Fig.2D) showed that the coefficient of
143 variation was low, and the quantitative stability of the protein was high.

144

145 **Results 3. Protein quantitative analysis**
146 In this study, some of the 4160 proteins detected were statistically available for analysis, including 3308
147 in the U251 group and 3621 in the U87 group. We screened the differentially expressed proteins in the
148 four groups, particularly the upregulated proteins, and focused on screening and analyzing the
149 differentially upregulated proteins in the U251-TMZ and U87-TMZ cell lines.

150

151 **Results 3.1 Cross-comparison of differential proteins between the U251-TMZ and U87-TMZ cell**
152 **lines**
153 A total of 1403 differential proteins were detected in the U251 group, of which 467 were upregulated and
154 936 were down-regulated in the U251-TMZ cell line. A total of 676 differentially expressed proteins were
155 detected in the U87 group, of which U87-TMZ upregulated 386 proteins and down-regulated 290 (see
156 Supplementary Data). Because of the heterogeneity of GBM, we only screened 28 proteins upregulated
157 from the upregulated proteins of the U251-TMZ and U87-TMZ cell lines (three repetitive samples), and
158 screened 67 down-regulated proteins from their respective down-regulated proteins. A total of 95
159 differential proteins were screened, as shown in the differential protein clustering thermogram (Fig. 3A).
160 Owing to the lack of statistical significance of the down-regulated proteins, we did not perform follow-up
161 bioinformatics analysis of the down-regulated proteins, but focused on the 28 proteins that were
162 upregulated. For each quantifiable protein, the fold change was logarithmically calculated from a base of
163 2, and the P value was logarithmically calculated from a base of 10 to create a volcano plot (Fig. 3B).

164

165 **Results 4. Functional annotation enrichment analysis of protein**
166 **4.1 GO and KEGG pathway analysis of the U251-TMZ and U87-TMZ cell lines**
167 A total of 28 proteins upregulated in the U251-TMZ and U87-TMZ cell lines were analyzed using GO
168 and KEGG pathways, respectively. Three categories, biological process (BP), cellular component (CC),
169 and molecular function (MF), were screened, and the possible pathways were screened. As shown in
170 Fig.4A, these differentially expressed proteins were significantly upregulated in biological processes
171 related to myeloid leukocyte activation, myeloid leukocyte-mediated immunity, leukocyte degranulation,
172 and inflammation. Based on GO cell composition analysis, most of them were located in the cytoplasm
173 and may be involved in vesicle transport in the intimal system. In addition, these proteins exhibited GO
174 molecular functions of "catalytic activity" and "binding," such as aminopeptidase activity and coenzyme
175 binding. These results suggest that the development of TMZ drug resistance may be related to
176 intracellular immune regulation and energy metabolism of leukocytes.
177 In addition, the enrichment of the 28 protein KEGG pathways upregulated in the U251-TMZ and U87-
178 TMZ cell lines mainly included carbon metabolism, antibiotic biosynthesis, secondary metabolite
179 biosynthesis, glyoxylic acid and dicarboxylic acid metabolism, and endocytosis (Fig.4B).
180 **Results 4.2 Differential protein interaction network**

181   In the interaction network of proteins upregulated in the U251-TMZ and U87-TMZ cell lines (Fig.4C),
182   the changes in these proteins are related to immune regulation and vesicular transport, as well as to energy
183   metabolism, such as the mitochondrial stress response.
184   **Results 5 Potential GBM markers of TMZ resistance**
185   In the MS analysis of differentially expressed proteins in the U251/U251-TMZ and U87/U87-TMZ cell
186   lines, 28 proteins were upregulated. Through manual screening, seven proteins with the highest
187   differential expression in the two groups were identified: UGT1A7, BCKDK, UAP1L1, RAB3GAP1,
188   VWA8, HLA-B, and VPS26B(Table.1). At present, there is no research on the relationship between these
189   seven proteins and TMZ resistance. Among these proteins, only UAP1L1 was related to GBM studies,
190   and the expression of UAP1L1 in glioma tissues was significantly upregulated. Increased expression of
191   UAP1L1 is associated with higher tumor grade and poor prognosis. UAP1L1 may promote the
192   proliferation of glioma cells by regulating glycosylation of key proteins[13].
193   Another noteworthy protein, BCKDK, has recently been found to regulate the catabolism of branched
194   chain amino acids (BCAA) and enhance MEK/ERK signal transduction, which is a key pathway driving
195   the growth and proliferation of cancer cells[14]. Among the remaining five candidate proteins, it has been
196   reported that UGT1A7 may affect the progression of pancreatic cancer[15]. Rab3GAP1 was first isolated
197   from the synaptic soluble section of the rat brain as an Rab3A-binding protein, and it exhibits GAP
198   activity against Rab3[16]. Rab3GAP deficiency leads to neurological disorders[17]. VWA8 is expressed in
199   organs with high energy requirements, including those with a high density of mitochondria[18].
200   Upregulation of VWA8 expression was negatively correlated with brain metastasis associated with breast
201   cancer[19]. HLA is necessary for T-cell activation and is associated with multiple sclerosis [20]. VPS26 is
202   one of the components of the cargo recognition core of the reverse transcriptase complex, which is
203   significantly decreased in the hippocampus of patients with Alzheimer's disease. Changes in reverse
204   transcriptase composition are related to an increased risk of Alzheimer's disease[21,22].
205   **Results 6 Identification of potential markers of TMZ resistance using western blotting**
206   To verify the results of MS analysis of the U251/U251-TMZ and U87/U87-TMZ cell lines, we performed
207   western blotting on seven selected markers: UGT1A7, BCKDK, UAP1L1, RAB3GAP1, VWA8, HLA-B,
208   and VPS26B. The results showed that in the four cell lines, the above seven proteins were successfully
209   upregulated in the U251-TMZ and U87-TMZ cell lines compared to the control groups (Fig. 5).
210

Discussion

212   TMZ is the preferred alkylating agent for the treatment of GBM, and it induces cell death through DNA
213   damage. Most patients develop TMZ resistance during treatment. Activation of the DNA repair pathway
214   is the main mechanism underlying this phenomenon. With the deepening of research, newly proposed
215   mechanisms of drug resistance, such as the regulation of microRNAs, membrane transporters, gap
216   junction activity, and autophagy, still cannot solve this issue. There is a lack of effective treatments to
217   maintain the effects of TMZ. In the present study, we screened and analyzed the differentially expressed
218   proteins using UPLC-MS differential proteomics in two TMZ-resistant GBM cell lines, namely U251-
219   TMZ and U87-TMZ. Compared with the U251 and U87 cell lines, 95 differential proteins were screened
220   in the U251-TMZ and U87-TMZ cell lines, of which 28 proteins were upregulated and 67 proteins were
221   down-regulated. GO and KEGG pathway analyses of the co-upregulated proteins showed that most of the
222   co-upregulated differentially expressed proteins were located in the cytoplasm and were significantly
223   upregulated in inflammation-related biological processes such as endocytosis and myeloid leukocyte-
224   mediated immunity, and may be involved in vesicle transport in the intimal system. These findings

225 suggest that TMZ resistance may be related to immune regulation of leukocytes and intimal system
226 transport. Through manual screening, we further identified seven proteins as potential GZM markers of
227 TMZ resistance. Following this investigation and analysis of existing studies, we propose for the first
228 time that UAP1L1 and BCKDK are potential markers of TMZ resistance in GBM.
229 UAP1L1 is a collateral homologue of UAP1 that participates in protein glycosylation and promotes the
230 proliferation of some tumors. UAP1L1 is significantly upregulated in hepatocellular carcinoma tissues,
231 and a high level of UAP1L1 expression is associated with poor prognosis [23]. Recent studies have found
232 that the expression of UAP1L1 is significantly upregulated in glioma tissues. Increased expression of
233 UAP1L1 is associated with higher tumor grade and poor prognosis. UAP1L1 may promote the
234 proliferation of glioma cells by regulating glycosylation of key proteins[13]. However, the specific
235 mechanism by which UAP1L1 regulates glioma cell proliferation, apoptosis, and TMZ drug resistance is
236 still poorly understood. BCKDK is a key regulator of branched-chain ketoacid dehydrogenase complex
237 activity, and inactivates branched-chain ketoacid dehydrogenase through phosphorylation. Recent studies
238 have shown that BCKDK regulates the catabolism of branched chain amino acids (BCAA) and enhances
239 MEK/ERK signal transduction, which is the key pathway that drives the growth and proliferation of
240 cancer cells[14]. In patients with colorectal cancer, high BCKDK expression is associated with poor
241 prognosis[24]. In addition, BCKDK can alter the metabolism of non-small cell lung cancer[24]. Owing to
242 time constraints, we found only these two possible markers. In a follow-up study, it will be necessary to
243 conduct an in-depth study of BCKDK and UAP1L1 based on the mechanism of TMZ resistance in GBM.
244 This will be of great significance for the diagnosis, development, and application of TMZ resistance in
245 GBM in the future.
246

Conclusions

248 In this study, we established TMZ drug-resistant cell lines, U251-TMZ and U87-TMZ, in GBM. Through
249 the screening and analysis of differential proteins using UPLC-MS differential proteomics technology, 95
250 differential proteins were screened using the U251-TMZ and U87-TMZ cell lines, of which 28 proteins
251 were upregulated and 67 proteins were down-regulated. The co-upregulated proteins were studied using
252 GO and KEGG analyses. Most of the co-upregulated differentially expressed proteins were located in the
253 cytoplasm and were significantly upregulated in biological processes related to endocytosis and myeloid
254 leukocyte-mediated immune response, and may be involved in vesicle transport in the intimal system.
255 These findings suggest that TMZ resistance may be related to immune regulation of leukocytes and
256 intimal system transport. Subsequently, we screened and identified seven potential candidates for TMZ
257 resistance. Combined with existing research, we propose for the first time that UAP1L1 and BCKDK are
258 promising potential markers of TMZ resistance in GBM.
259

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307   [24] Wang Y, Xiao J, Jiang W, et al. BCKDK alters the metabolism of non-small cell lung cancer[J].
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Figure 1

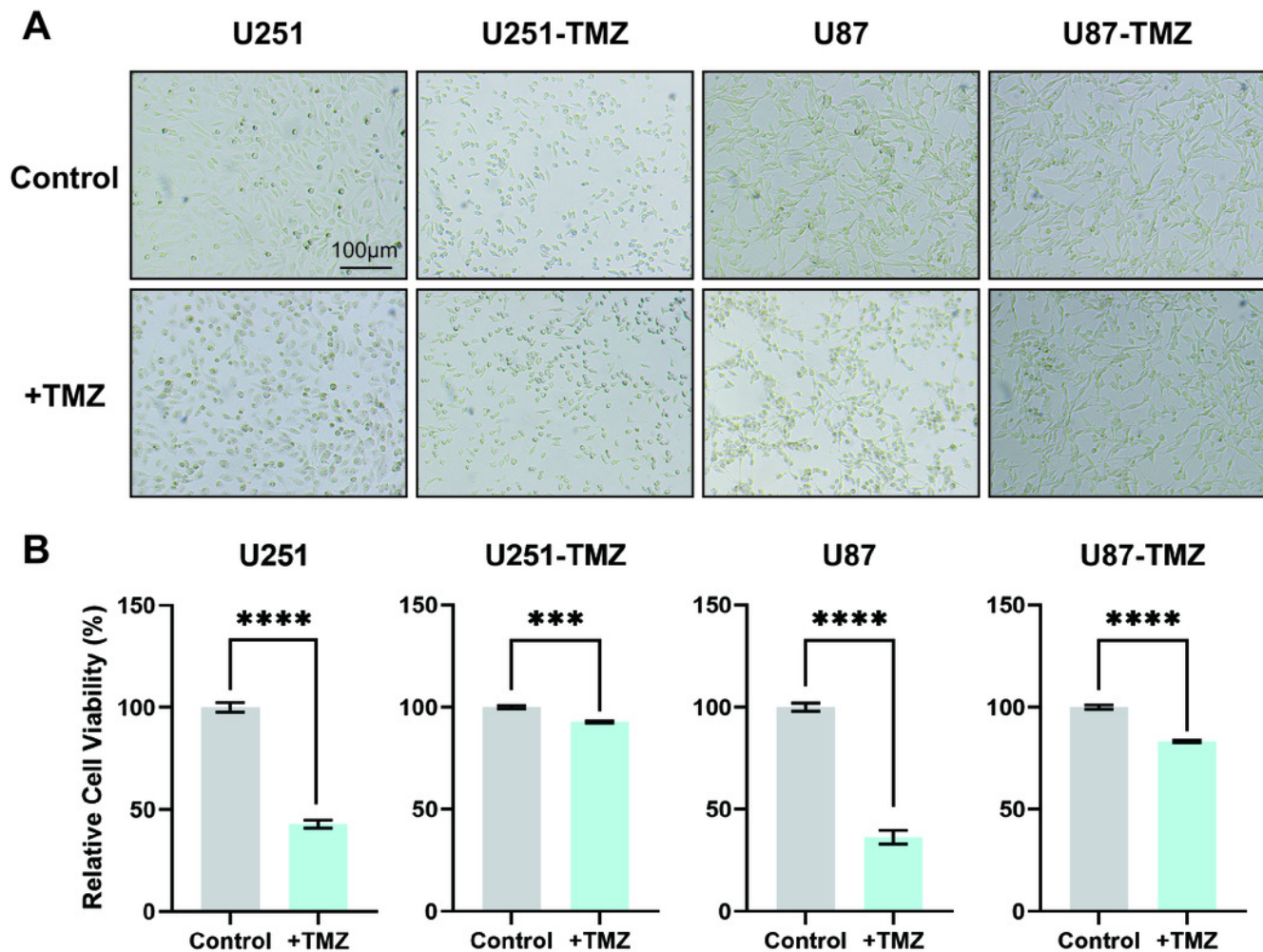Screening of TMZ-resistant glioma cell lines U251 and U87.

Fig. 1. Screening of TMZ-resistant glioma cell lines U251 and U87. The culture status of U251-TMZ, U87-TMZ cells and control cell lines (A). The relative cell viability of U251-TMZ, U87-TMZ cells and control cell lines, as measured by CCK-8 assay (B).
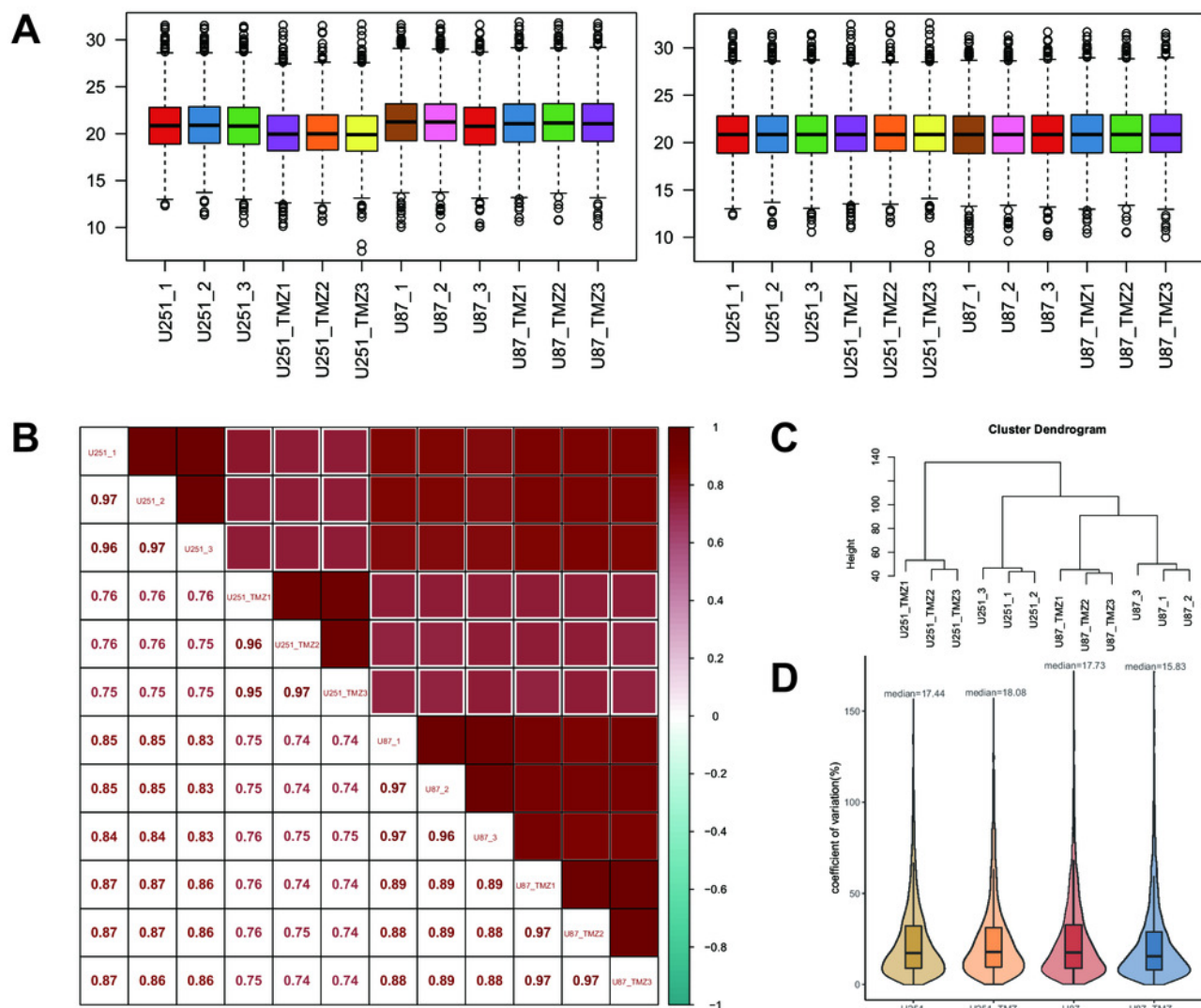
Figure 2

Quantitative protein data quality control.

Fig. 2. Quantitative protein data quality control. Before and after the median normalization (A left and right). The correlation between samples (B). The correlation between U251-1 and U251-2 is 0.97, the correlation between U251-1 and U251-3 is 0.96, the correlation between U251-2 and U251-3 is 0.97, the higher the correlation is, the closer the sample is. Cluster analysis of U251, U87, U251-TMZ and U87-TMZ samples (C). The coefficients of variation of U251, U87, U251-TMZ and U87-TMZ proteins were analyzed (D).

Figure 3

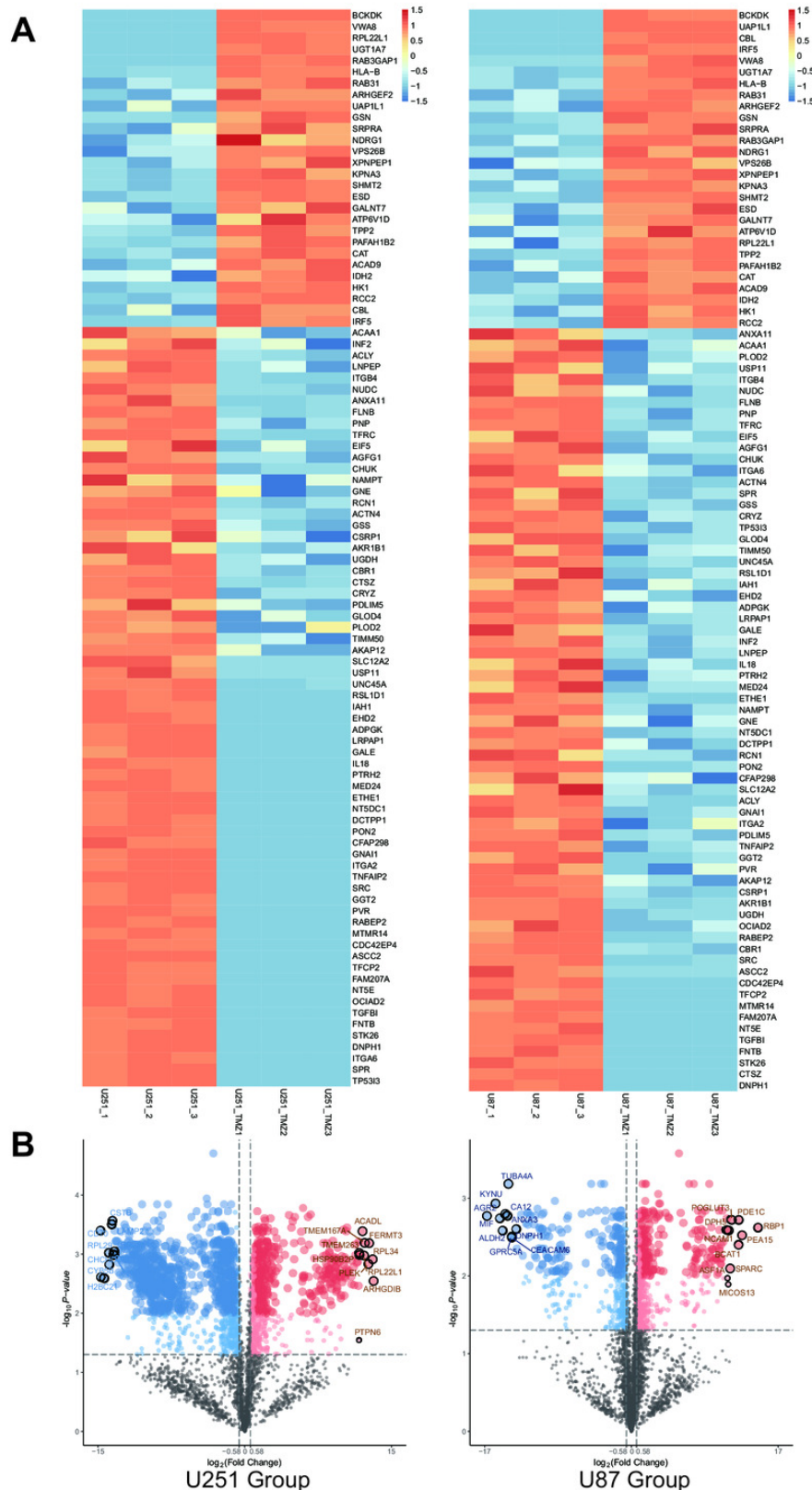Differential protein cross-comparison.

Fig. 3. Differential protein cross-comparison. The differential proteins of U251-TMZ and U251 (left), U87-TMZ and U87 (right) were clustered respectively. U251-TMZ and U87-TMZ up-regulated 28 proteins and down-regulated 67 proteins (A). U251-TMZ and U251, U87-TMZ and U87 can be statistically analyzed protein volcano diagram (B).

Figure 4

Functional analysis of proteins up-regulated by U251-TMZ and U87-TMZ.
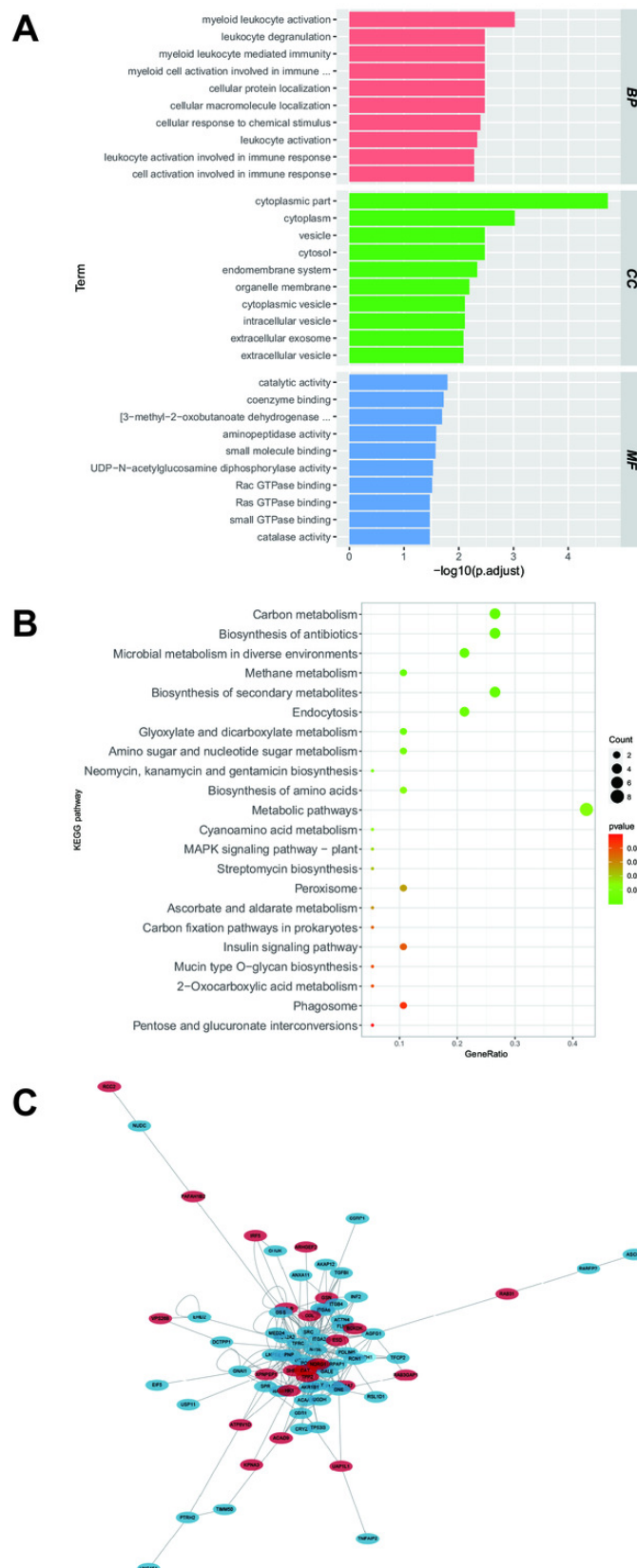
Fig. 4. Functional analysis of proteins up-regulated by U251-TMZ and U87-TMZ. GO analysis of proteins up-regulated by U251-TMZ and U87-TMZ, including biological processes (Biological Process BP), cellular components (CC), and molecular function MF (A). KEGG signal pathway enrichment analysis of proteins up-regulated by U251-TMZ and U87-TMZ (B). Differential protein interaction network (C).

Figure 5

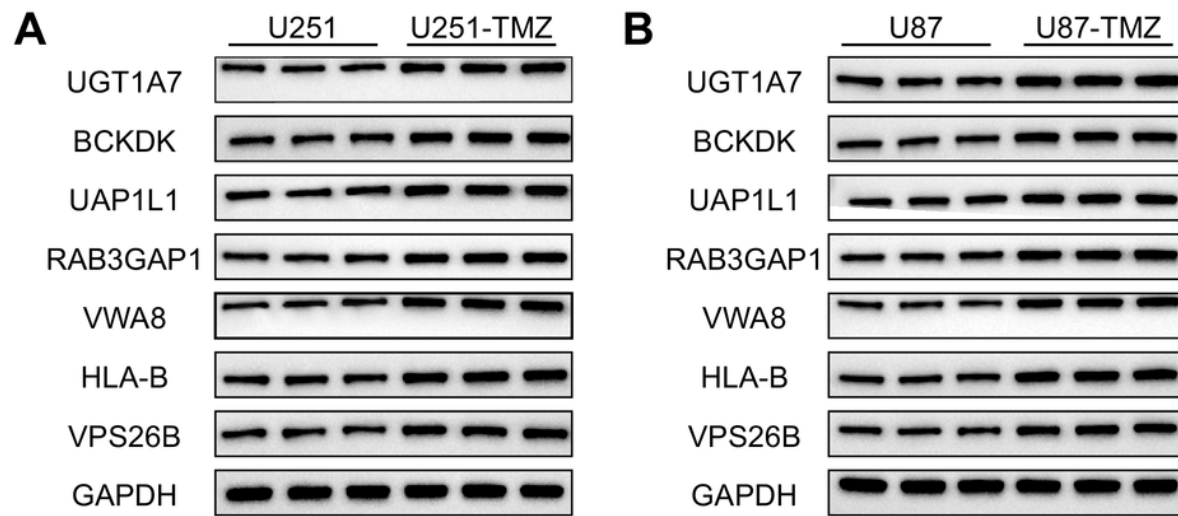Western Blotting showing the levels of upregulated proteins in U251/U251-TMZ and U87/U87-TMZ.

Fig. 5. Western Blotting showing the levels of upregulated proteins in U251/U251-TMZ and

Table 1(on next page)

Potential markers for TMZ resistance in GZM

1

Gene Symbol	Gene ID	Description
UGT1A7	54577	UDP glucuronosyltransferase family 1 member A7
BCKDK	10295	branched chain keto acld dehydrogenase kinase
UAP1L1	91373	UDP-N-acelylglucosamine pyrophosphorylase 1 like 1
RAB3GAP1	22930	RAB3 GTPase aclivating protein catalyic subunit 1
WWA8	23078	von Willebrand factor A domain containing 8
HL A-B	3106	major histocompatblity complex, class I, B
VPS26B	112936	VPS26, retromer complex component B

Table 1. Potential markers for TMZ resistance in GZM

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