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Establishment of a Novel Temozolomide Resistant Subline of Glioblastoma Multiforme Cells and Comparative Transcriptome Analysis With Parental Cells

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Abstract. *Background/Aim: Glioblastoma multiforme (GBM) is a lethal disease with a high rate of chemoresistance to temozolomide (TMZ). The aim of the study was to establish a TMZ-resistant subline from the GBM-8401 cell line to determine the mechanisms of resistance and identify novel effective therapeutics for TMZ-resistant GBM. Materials and Methods: Comparative transcriptome analysis of GBM-8401/TMZR cells and the parental line was performed using Ion Torrent sequencing. Differentially expressed genes (DEGs) between the GBM-8401/TMZR and GBM-8401 cell lines were analyzed. Results: Transcriptomic profiling of GBM-8401/TMZR cells revealed DEGs involved in the retinoblastoma (RB) signaling, DNA damage response (DDR) pathway, and DNA repair mechanisms. Conclusion: In vitro and in vivo cell-based GBM models should be used in further biomedical studies to investigate the underlying mechanisms of TMZ-resistant GBM.*

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Glioblastoma multiforme (GBM) is the most common type of malignant glioma, characterized as an aggressive and lethal cancer (1, 2). According to recent reports, the 5-year survival rate of patients with GBM is about 5% (1, 3). The average survival time of patients with GBM following the first diagnosis is <15 months, which has not significantly improved in recent years (3-5). The standard treatment for GBM is surgical resection, followed by chemoradiation regimens. However, GBM often reoccurs after an average of 6-9 months, which is due to increased chemoresistance to the first-line treatment (5, 6). Therefore, further studies are required to improve the unsatisfactory therapeutic outcomes and GBM treatment efficacy.

Temolozomide (TMZ) is an important alkylating agent used for the standard treatment of GBM (7). TMZ-based chemotherapies are commonly used as they have been found to exert beneficial outcomes on the overall survival time of patients with GBM following the initial treatment course (8). However, according to a previous study, resistance to TMZ occurs in >50% of patients with GBM (6). Furthermore, recurrent GBM has been found to be more unresponsive to

TMZ treatment, with ~90% of patients failing to respond to repeated TMZ treatments (9). Thus, TMZ resistance is a significant obstacle for successful GBM treatment in the clinic, and novel treatment approaches or effective therapeutics are required to overcome the resistance, in addition to improving the overall GBM treatment outcome.

The GBM cell line, GBM-8401, was obtained from biopsies of adult Chinese patients with GBM in 1988 (10); this cell line is considered as a typical GBM cell line derived from Asian patients and has been commonly used in biomedical studies for developing anti-GBM therapies. Previous studies have predominantly investigated mechanisms associated with the induction of cell death, and the suppression of cell invasion and migration using GBM-8401 cells (11-13); however, to the best of our knowledge, studies investigating TMZ resistance in GBM-8401 cells have not been performed. Since the resistance to TMZ is an important factor reducing the effectiveness of current therapeutics and thereby, shortening the survival time of patients with GBM (6), an increasing number of studies have begun to investigate the mechanisms underlying TMZ resistance in GBM (5, 9, 14-16). Therefore, establishing a TMZ-resistant GBM cell subline may provide a novel *in vitro* model, not only for investigating mechanisms to improve current treatment outcomes, but also for identifying novel therapeutics for TMZ-resistant patients with GBM. The present study aimed to develop a TMZ-resistant subline from GBM-8401 cells and characterize the gene expression profile of the novel subline. The transcriptomic profiling analysis performed in the current study may provide a novel insight at the gene-transcriptional level into TMZ resistance in GBM cells, and enhance the current understanding of resistance mechanisms for future drug discovery studies investigating TMZ-resistant GBM.

Materials and Methods

Chemicals and reagents. DMSO and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA); Merck KGaA (Darmstadt, Germany). TMZ was obtained from TCI America, Inc. (Portland, OR, USA). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin G, and streptomycin were purchased from Gibco; Thermo Fisher Scientific, Inc. (Eugene, OR, USA).

Cell lines and culture. The GBM-8401 cell line was obtained from The Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were maintained in a 75-cm^2 culture flask at 37˚C with 5% CO_2 as previously described (13).

Establishment of a subline of TMZ-resistant cells. TMZ-sensitive GBM-8401 cells were treated with increasing concentrations of TMZ (10, 25, 50, 75, 100 and 200 μM) under the cell culturing conditions described above within 6 passages. As shown in Figure 1A, each passage comprised five cycles of repetitive exposure to TMZ at the designated concentration within a 72-h timeframe each week, and then surviving cells were selected for subsequent passage. Several samples were cloned independently to ensure the rigidity of the method. The generated TMZ-resistant subline consisted of cells collected after the selective process at the end of passage 6, which were named as GBM-8401/TMZR cells.

Cell viability assay. GBM-8401 and GBM-8401/TMZR cells were seeded into 96-well plates. Then, they were subsequently treated with 0, 10, 25, 50, 75, 100, 200 or 500 μM TMZ diluted in 0.1% DMSO and incubated for 24 h, at 37˚C. Following incubation, 100 μl MTT solution (0.5 mg/ml) were added/well and incubated at 37˚C for 3 h. Subsequently, 100 μl 0.04 N HCl dissolved in isopropanol were added/well and the absorbance was measured at a wavelength of 570 nm as previously described (13).

Sample preparation and RNA extraction. Samples from the parental cell line (samples named as GBM-8401-1 and GBM-8401-2) and the newly established TMZ-resistant cell line (samples named as GBM-8401-R-1 and GBM-8401-R-2) were prepared and cultured independently in identical conditions as previously described in the aforementioned section. Total RNA was extracted from cells using a MagNA Pure Compact RNA Isolation kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. The process was performed on a fully automated MagNA Pure Compact instrument (Roche Applied Science). DNA contamination was eliminated by treatment with DNase I for 30 min at 37˚C and consequently by treatment with EDTA (25 mM) for 5 min for enzyme inactivation.

Ion torrent sequencing. Sequencing was conducted as previously described (17). Briefly, Ion Torrent sequencing libraries were prepared using the AmpliSeq Library prep kit 2.0 (Life Technologies, Saint-Aubin, France) according to the manufacturer's protocol. Total RNA (10 ng) was reverse transcribed into cDNA using the SuperScript IV VILO Master Mix. The obtained cDNA was subsequently amplified *via* 12 cycles under standard thermal cycling conditions using a TaqMan PCR Master mix (QuantStudio, Applied Biosystems, Foster City, CA, USA) with an AmpliSeq human transcriptome gene expression primer pool (provided in the AmpliSeq Library prep kit). The proprietary FuPa enzyme was used for the digestion of the amplicons. Barcoded adapters (Ion Xpress) were subsequently ligated to the target amplicons. The barcodes used for each sample are listed in Table I. Library amplicons were then purified and quantified *via* quantitative PCR. Individual libraries were diluted to a concentration of 50 pM, and then combined before further processing. Using an Ion Chef instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA), Emulsion PCR mixture, templates, and the PI chip were loaded for the sequencing step. The Ion 540™ Chip Kit (Thermo Fisher Scientific, Inc.), which is supported by the Ion GeneStudio™ S5 Prime system (Thermo Fisher Scientific, Inc.) was used for sequencing, according to the manufacturer's protocol. The built-in Torrent Suite v5.10.0 software (Thermo Fisher Scientific, Inc.) was used for base calling. Finally, transcriptome analysis console software 4.0 (Thermo Fisher Scientific, Inc.) was used for the analysis of RNA expression levels (18).

Signaling pathway enrichment analysis. Within the graphic user interface of the Transcriptome Analysis Console software 4.0

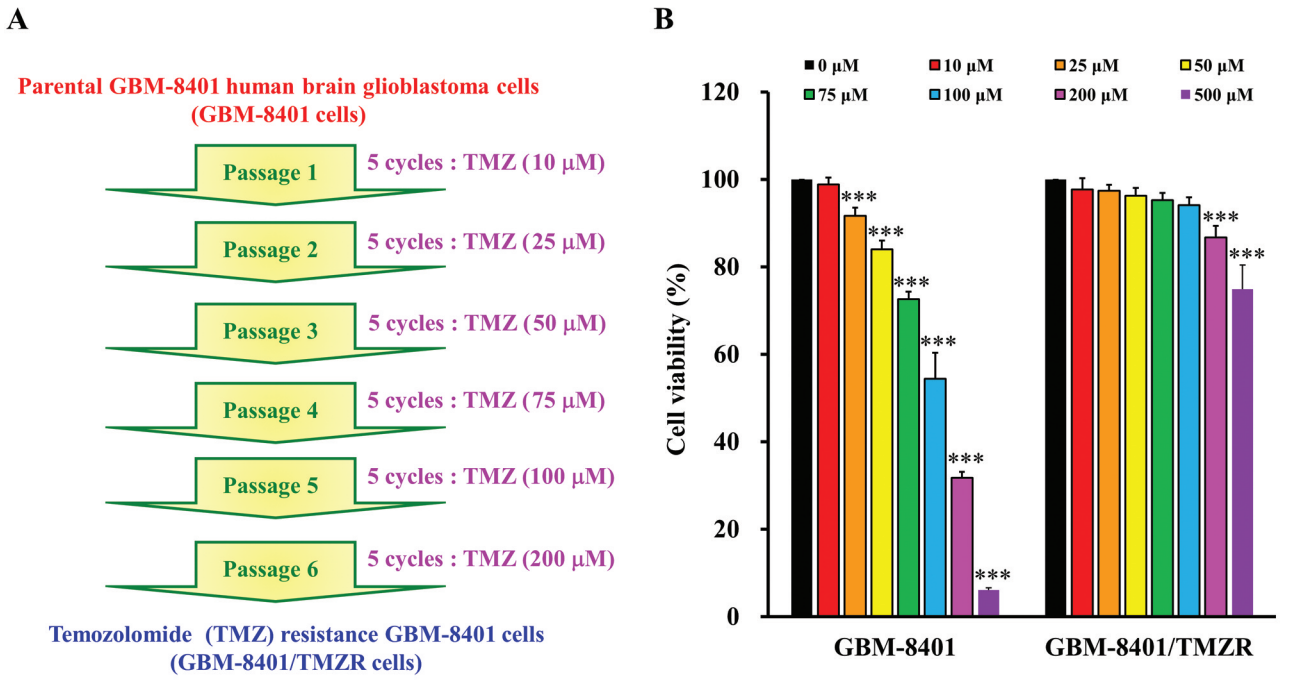

Figure 1. *Establishment of GBM-8401/TMZR cells from the parental cell line, GBM-8401. (A) Passage and TMZ treatment concentration of each cycle. (B) Different concentrations of TMZ induced cytotoxicity in GBM-8401 and GBM-8401/TMZR cells. GBM-8401/TMZR cells: GBM-8401 cells that are resistant to TMZ. TMZ: Temozolomide.*

(Thermo Fisher Scientific, Inc.), the integrated wikipathways database (19) was selected for the analysis of signaling pathway enrichment on the dataset obtained from Ion Torrent sequencing results. The relevant pathways were associated with the significant DEGs using designated cutoff parameters (fold-change of <2 and $p>0.05$). The output images for the target pathways were downloaded for analysis.

Statistical analysis. For the MTT assay results, statistical analysis was performed using SPSS 16.0 software (SPSS, Inc.). To compare the sensitivity of the cells to TMZ treatment, the percentage values for cell viability were collected for each experiment and presented as the mean±SD of three replicates/group. Statistical differences between treated groups and the control group were determined using a one-way ANOVA followed by a Dunnett's *post hoc* test, or an unpaired Student's *t*-test; $^{***}p<0.001$. For the comparison of transcriptomic data, the software Transcriptome Analysis Console software 4.0 (Thermo Fisher Scientific, Inc.) was used for the statistical analysis with the integrated algorithm, and $p<0.05$ was considered to indicate a statistically significant difference.

Results

TMZ induces significant cytotoxicity on the GBM-8401 parental cell line, but has negligible effects on the newly established GBM8401/TMZR cells. The sensitivity of GBM-8401 cells to TMZ treatment was found to be concentration-dependent (Figure 1B). The results revealed that the newly established GBM-8401/TMZR cell subline had a deceased sensitivity to TMZ compared with the parental cells. The viability of GBM-8401/TMZR cells was not significantly altered following treatment with 10-100 μM TMZ; however, following treatment with high concentrations of TMZ (200-500 μM), the viability was markedly decreased to ~70-90% compared with the nontreated group. These results suggested that the sensitivity of GBM-8401/TMZR cells to TMZ is markedly reduced following long-term TMZ exposure with gradually increasing concentrations.

Table I. *The barcodes used for the samples used in this study.*

Sample	Barcode	Barcode sequence
GBM-8401-1	IonXpress_001	CTAAGGTAAC
GBM-8401-2	IonXpress_002	TAAGGAGAAC
GBM-8401-R-1	IonXpress_003	AAGAGGATTC
GBM-8401-R-2	IonXpress_004	TACCAAGATC

Expression levels of genes are significantly altered in GBM-8401/TMZR cells compared with GBM-8401 parental cells.

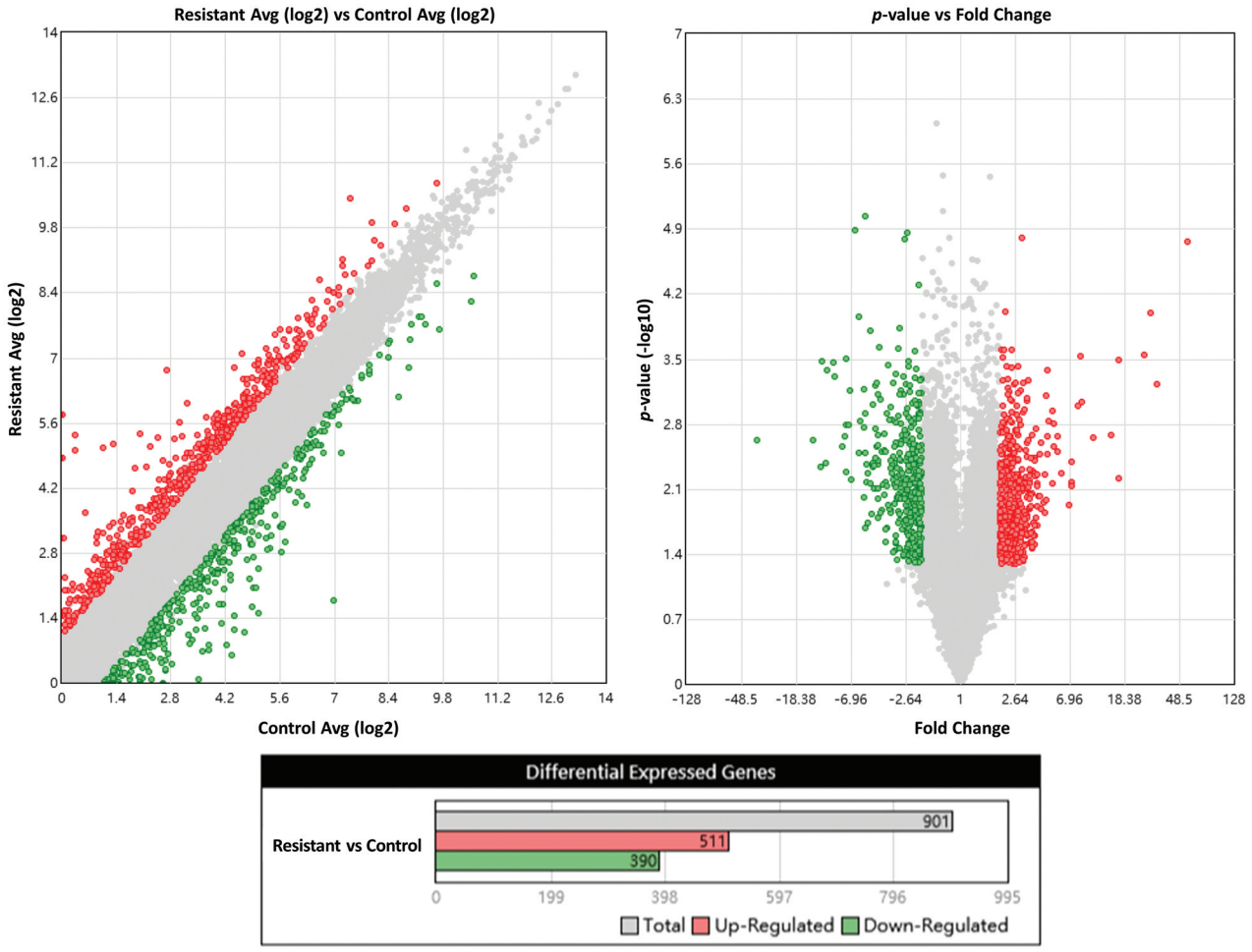

Figure 2. *Identification of significantly DEGs in GBM-8401/TMZR cells compared with the parental cell line. Upper-left panel: Scatter plots of DEGs in the resistant cell line compared with the parental cell line. Data are expressed as log2 values. Upper-right panel: Volcano plot of fold-change values of DEGs in the resistant cell line compared with the parental cell line. Data are expressed as p-value (log10). DEGs: Differentially expressed genes; GBM-8401/TMZR cells: GBM-8401 cells that are resistant to TMZ; TMZ: temozolomide.*

Due to the increased tolerance of GBM-8401/TMZR cells to TMZ, the present study sought to determine whether the expression of genes was altered at the transcriptional level between the newly generated subline and parental cells. Transcriptomic analysis was performed to identify DEGs between GBM-8401 and GBM-8401/TMZR cells. As shown in Figure 2, scatter and volcano graphs were used to demonstrate the significantly up-regulated (red color) and down-regulated (green color) genes in GBM-8401/TMZR cells compared with GBM-8401 cells. Genes in which the expression levels were not significantly altered, with an absolute fold-change of <2 and $p>0.05$, were filtered out (grey color). In total, 901 DEGs were identified, including 511 down-regulated and 390 up-regulated DEGs (Supplementary data). These results indicated that the expression levels of a number of genes were significantly altered following transformation of GBM-8401 cells into GBM-8401/TMZR cells. The most down-regulated genes (fold change value) were CHI3L1 (–36.67), FRAS1 (–13.58), and CYB5R2 (–12.10). whereas, the top up-regulated genes (fold change value) were NRK (55.38), SDPR (32.11), and MGMT (28.73).

The gene expression heatmap with hierarchical clustering data for the four examined samples (GBM-8401-1, GBM-8401-2, GBM-8401-R-1 and GBM-8401-R-2 cells) is presented in Figure 3. The significantly up-regulated or down-regulated genes in the resistant cells compared with the sensitive cells can be observed by the different heatmap colors. The top up-regulated and down-regulated genes are listed correspondingly in Tables II and III. In addition, the similarities between TMZ-resistant samples (GBM-8401-R-1 and GBM-8401-R-2 cells) demonstrated the steady

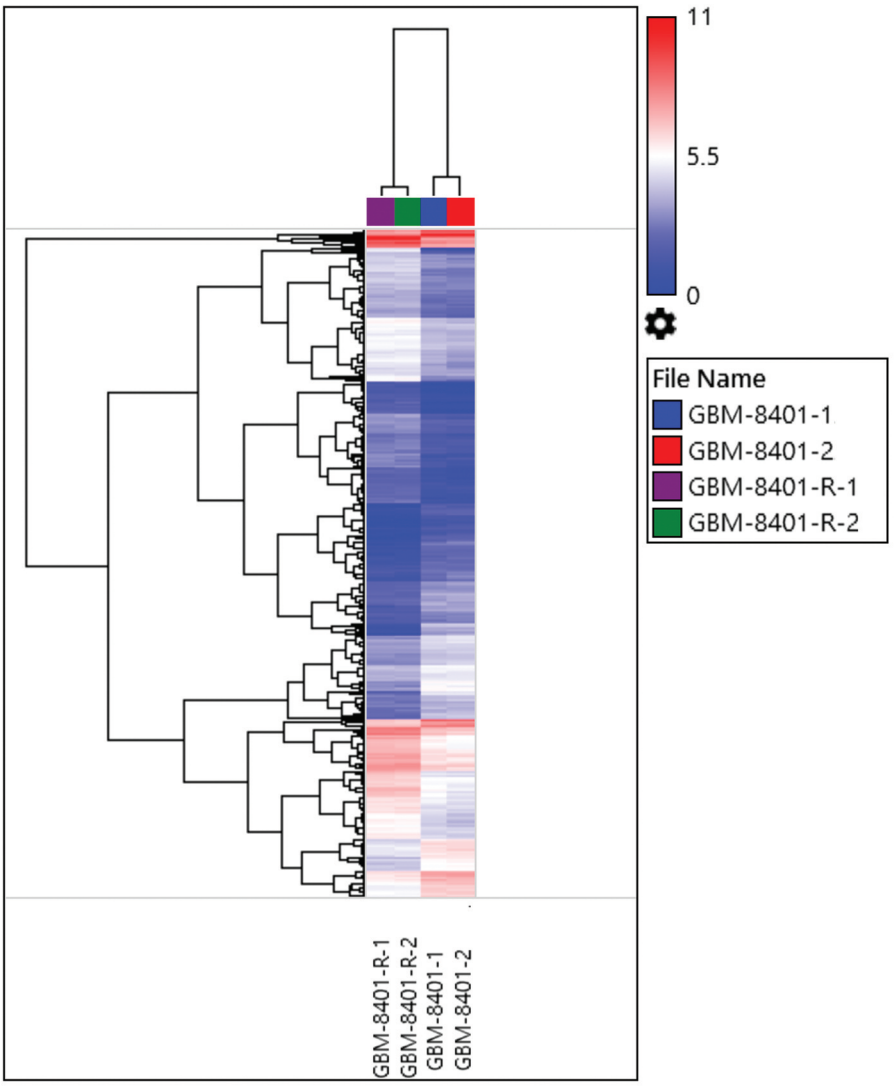

Figure 3. *Phylogenetic tree and gene heatmap of the four samples: GBM-8401-1, GBM-8401-2, GBM-8401-R-1 and GBM-8401-R-2. R: Resistant.*

evolution of the cells during the establishment of the new subline and the clonality between the samples. The alterations in gene expression levels between TMZ-sensitive and -resistant cells were statistically significant, and did not occur at random.

The retinoblastoma (RB) cancer pathway is potentially involved in the development of TMZ resistance in GBM-8401/TMZR cells. Understanding how pathways are affected during the development of chemotherapy resistance in cells may enhance the current understanding of drug resistance mechanisms and provide novel targets for molecular-targeted therapies to overcome resistance. The relevant biological pathways of GBM-8401/TMZR cells were analyzed to determine the transcriptional profile of the newly established subline of cells (Tables IV and V). The RB gene encodes the RB1 protein, which has been widely reported to act as a tumor suppressor protein (20). Therefore, the RB cancer pathway has been suggested as a potential target for antitumor treatment, including for GBM (4). In GBM-8401/TMZR cells, the expression levels of RB were not significantly altered compared with the parental cells; however, the expression levels of 25 genes associated with the RB signaling pathway and have previously reported to be involved in cancer were found to be up-regulated in GBM-8401/TMZR cells compared with the parental cells (Figure 4). Notably, the expression levels of proteins with central roles in the RB signaling pathway, such as E2F transcription factor 2 (E2F2), CDK2,

Table II. *Top 20 up-regulated genes in GBM-8401/TMZR cells compared with GBM-8401 cells.*

ID	Resistant avg (log2)	Control avg (log2)	Fold change	*p*-Value
NRK	5.79	0.00	55.38	<0.0001
SDPR	5.33	0.33	32.11	0.0006
MGMT	4.84	0.00	28.73	0.0001
SPRR2D	5.03	0.33	25.96	0.0003
SERPINB2	5.08	1.04	16.50	0.0003
LCN2	6.73	2.68	16.48	0.006
ADAM23	5.16	1.30	14.50	0.0021
CSF3	5.38	2.00	10.43	0.0022
GAP43	3.13	0.04	8.52	0.0009
VCAN	3.67	0.60	8.44	0.0003
IL1B	10.43	7.42	8.05	0.001
IL11	5.29	2.45	7.18	0.0072
HLA-DRB1	5.09	2.26	7.07	0.004
ALOX5AP	6.03	3.21	7.05	0.0067
C12orf39	4.63	1.87	6.81	0.0119
TNFRSF9	5.61	3.03	5.98	0.0054
LOC100507410	4.65	2.17	5.60	0.0021
TXNIP	7.09	4.61	5.58	0.0031
CNTD1	5.26	2.88	5.20	0.0027
MOCOS	6.80	4.44	5.16	0.0015

Table III. *Top 20 down-regulated genes in GBM-8401/TMZR cells compared with GBM-8401 cells.*

ID	Resistant avg (log2)	Control avg (log2)	Fold change	*p*-Value
CHI3L1	1.79	6.99	–36.67	0.0023
FRAS1	0.61	4.37	–13.58	0.0023
CYB5R2	1.10	4.70	–12.10	0.0045
OVCH2	1.52	5.06	–11.64	0.0003
PADI1	0.07	3.51	–10.82	0.0042
CXorf48	0.82	4.23	–10.66	0.0004
ANXA3	1.14	4.41	–9.64	0.0003
ARNT2	1.66	4.87	–9.29	0.0005
IFI30	0.72	3.76	–8.22	0.0027
ICAM2	1.46	4.42	–7.77	0.0021
COL8A1	0.82	3.76	–7.70	0.0016
TMEM246	0.68	3.60	–7.58	0.0003
OR5P3	2.05	4.97	–7.57	0.0052
KRT86	2.04	4.92	–7.32	0.0016
CCDC71L	2.82	5.65	–7.14	0.0007
GALC	1.29	4.10	–7.01	0.0063
TNFRSF11B	3.04	5.74	–6.49	<0.0001
OXTR	2.23	4.84	–6.07	0.0032
CACNA2D1	0.00	2.59	–6.04	0.0001
ARL4C	2.47	5.05	–5.99	0.0015

cell division cycle 25A (CDC25A), CDK1, cyclin B1 (CCNB1), and cyclin B2 (CCNB2) were up-regulated in GBM-8401/TMZR cells compared with GBM-8401 cells. Furthermore, no genes associated with the RB signaling pathway were significantly down-regulated in GBM-8401/TMZR cells compared with GBM-8401 cells.

Alterations in the expression levels of genes associated with the DNA damage response (DDR) pathway in GBM-8401/TMZR cells. The RB signaling pathway is not only associated with cell cycle transition, but it also regulates other pathways, such as those involved in DDR and DNA repair (20). Notably, previous studies have reported that the mechanism of TMZ-induced cytotoxicity was associated with the DDR pathway (8, 21); therefore, the present study focused on the DDR pathway in further detail. As shown in Figure 5, several important genes associated with the DDR pathway were up-regulated in GBM-8401/TMZR cells, including CDC25A, CCNB1, damage specific DNA binding protein 2 (DDB2), H2A.X variant histone (H2AFX), FA complementation group D2 (FANCD2), RAD51 recombinase, CDK2, BRCA1, CCNB2, CDC25A, and cyclin E2. Conversely, the expression levels of Growth arrest and DNA damage inducible β (GADD45B) were found to be down-regulated in GBM-8401/TMZR cells.

Increased expression of genes associated with DNA repair pathways in GBM-8401/TMZR cells. The aforementioned results indicate that the observed changes in the expression levels of these DDR-associated genes may induce cellular responses involving DNA repair pathways (Figure 6). Transcriptomic analysis revealed that the following genes associated with the DNA repair pathways were up-regulated in GBM-8401/TMZR cells: Replication factor C subunit (*RFC*)1, *RFC2*, *RFC3, RFC4*, exonuclease 1 (*EXO1*), *DDB2*, high mobility group box 1 (*HMGB1*), *BRCA2*, *FANCD2*, FA complementation group I (*FANCI*), FA complementation group G (*FANCG*), *BRCA1, RAD51* paralog C and O6-methylguanine (O6-MeG)-DNA methyltransferase (*MGMT*). In contrast, the expression levels of FA complementation group F were down-regulated in GBM-8401/TMZR cells. The expression levels of *MGMT* and *EXO1* were up-regulated by 28.73- and 3.63-fold, respectively (Table II), in GBM-8401/TMZR cells compared with GBM-8401 cells, which indicated that *MGMT* and *EXO1* may play an important role in responding to TMZ exposure over a long period of time. Therefore, DNA repair mechanisms associated with MGMT activity and mismatch repair (MMR), which is regulated by EXO1, may be responsible for the chemoresistance of GBM-8401/TMZR cells.

Changes in the levels of genes involved in DNA MMR in GBM-8401/TMZR cells. TMZ-resistance in GBM cells has been associated with two main mechanisms of DNA repair, MGMT-mediated and MMR pathways (21, 22). Therefore,

Table IV. *Top 10 enriched pathways by gene count in GBM-8401/TMZR cells compared with GBM-8401 cells*.

Pathway	#Total	#Up	Up list	#Down	Down list	Significance	*p*-Value
Nuclear receptors meta-pathway	25	11	SDPR, IL11, IL1B, SERPINA1, ALOX5AP, FGF13, SERPINB2, DNER, CDK1, SLC6A9, SLC6A17	14	CCL2, TGFB2, PDK4, ABCC3, PPARA, CCL20, PDGFB, CDC42EP3, GADD45B, AGER, TNS4, ANKRD1, SLC5A3, SCD	2.29	0.005151
Retinoblastoma gene in cancer	25	25	CDK2, CCNA2, SUV39H1, CCNE2, CDC25A, CDK1, CCNB1, CCNB2, RFC4, TOP2A, CDC45, RFC3, MCM4, PRIM1, ORC1, CDT1, TTK, RRM1, RRM2, DHFR, HMGB2, TYMS, HMGB1, FANCG, E2F2	0		13.33	0
Cell cycle	24	22	PLK1, CDK2, CCNA1, CCNA2, CDC45, PTTG1, CDK1, PKMYT1, CDC20, CDC25C, CCNE2, CDC6, MCM4, ORC1, ORC6, BUB1, CCNB2, CCNB1, E2F2, CDC25A, TTK, SMC1B	2	TGFB2, GADD45B	9.46	0
PI3K-Akt signaling pathway	22	11	CCNE2, CSF3, TNC, COL6A2, KITLG, FGF13, GNGT2, PPP2R3B, IL6, CDK2, BRCA1	11	ITGA10, IL7R, LAMA5, TNXB, THBS1, IL2RG, FGF1, PDGFB, IGF1R, PDGFRB, F2R	1.23	0.058719
VEGFA-VEGFR2 signaling pathway	21	11	SH2D2A, ITPR1, TXNIP, GRB10, RND1, HDAC9, BIRC5, HLX, ADAMTS9, HMGB1, IDH2	10	CDH5, CCL2, AMOT, MMP2, F3, TNXB, CTGF, FGG, CACNA2D1, TFCP2	0.2	0.634865
Focal adhesion-PI3K-Akt-mTOR-signaling pathway	20	7	CSF3, COL5A1, TNC, COL6A2, FGF13, KITLG, GNGT2	13	COL11A2, ITGB2, ITGA10, IL7R, LAMA5, TNXB, THBS1, IL2RG, FGF1, PDGFB, IGF1R, PDGFRB, F2R	1.21	0.061836
Endothelin pathway	16	8	VCAN, COL5A1, ACTA2, IL1B, IL11, CCNB1, IL6, CDK2	8	PLOD2, MMP2, THBS1, IGFBP4, CCL2, EDN1, EDNRB, LEPR	0.66	0.217956
miR-targeted genes in lymphocytes - TarBase	16	10	TYMS, UBE2S, POLA2, PLK1, MLLT11, PRIM1, AURKB, GFPT1, TRIP13, DHFR	6	CACNA2D1, TPM2, PLAG1, MAT2A, TNFAIP2, CDCP1	0.65	0.22334
Mitotic G2-G2/M phases	16	16	CDC25C, MYBL2, TPX2, PKMYT1, CCNB1, CDC25A, PLK1, BORA, FBXL7, FOXM1, CCNB2, CDK1, AURKA, CENPF, GTSE1, HMMR	0		0.78	0.164084
Mitotic G1-G1/S phases	16	16	MCM10, TOP2A, CDC25A, CCNA1, CDC45, CCNA2, RRM2, CDK1, MYBL2, DHFR, CDC6, ORC1, TK1, TYMS, CDK2, CDT1	0		1.13	0.074309

Table V. *Top 10 most enriched pathways by significance in GBM-8401/TMZR cells compared with GBM-8401 cells*.

Pathway	#Total	#Up	Up list	#Down	Down list	Significance	*p*-Value
Retinoblastoma gene in cancer	25	25	CDK2, CCNA2, SUV39H1, CCNE2, CDC25A, CDK1, CCNB1, CCNB2, RFC4, TOP2A, CDC45, RFC3, MCM4, PRIM1, ORC1, CDT1, TTK, RRM1, RRM2, DHFR, HMGB2, TYMS, HMGB1, FANCG, E2F2	0		13.33	0
Cell cycle	24	22	PLK1, CDK2, CCNA1, CCNA2, CDC45, PTTG1, CDK1, PKMYT1, CDC20, CDC25C, CCNE2, CDC6, MCM4, ORC1, ORC6, BUB1, CCNB2, CCNB1, E2F2, CDC25A, TTK, SMC1B	2	TGFB2, GADD45B	9.46	0
O-linked glycosylation	1	0		1	ST6GAL1	7.68	0
Glycosaminoglycan metabolism	1	0		1	GLCE	7.19	0
Gastric Cancer Network 1	10	10	KIF15, AURKA, MYBL2, CENPF, MCM4, KIF20B, UBE2C, E2F7, TOP2A, TPX2	0		6.68	0
Deubiquitination	1	1	CDK1	0		6.5	0
DNA replication	11	11	RFC1, RFC4, CDT1, CDK2, PRIM1, RFC3, MCM10, POLA2, MCM4, CDC6, RFC2	0		5.93	0.000001
Chromatin organization	1	1	SETD8	0		4.87	0.000013
DNA damage response	12	11	CDC25C, CCNB1, DDB2, H2AFX, FANCD2, RAD51, CDK2, BRCA1, CCNB2, CDC25A, CCNE2	1	GADD45B	4.5	0.000031
DNA IR-damage and cellular response *via* ATR	13	13	RFWD3, RAD51, FANCI, CLSPN, BRCA2, EXO1, PLK1, BRCA1, CDC25C, FANCD2, CDC45, RMI1, FOXM1	0		4.2	0.000062
Fluoropyrimidine activity	8	7	UPP1, TK1, DHFR, TYMS, RRM1, XRCC3, RRM2	1	ABCC3	4.1	0.00008
Lung fibrosis	11	5	CXCL2, SERPINA1, IL6, CSF3, IL1B	6	FGF1, CTGF, CCL2, EDN1, PDGFB, MMP2	4.07	0.000086
Photodynamic therapy-induced NF-kB survival signaling	8	7	IL1A, IL1B, IL6, IL8, CXCL2, BIRC5, BCL2A1	1	MMP2	4	0.000099
Class A/1 (Rhodopsin-like receptors)	6	3	PTGFR, IL8, NMUR2	3	EDN1, GPR39, OXTR	3.93	0.000118
Complement cascade	1	1	C5	0		3.87	0.000136
Neddylation	1	1	BIRC5	0		3.86	0.000138

Table V. *Continued*

Table V. *Continued*

Pathway	#Total	#Up	Up list	#Down	Down list	Significance	*p*-Value
Immunoregulatory interactions between a lymphoid and a non-lymphoid cell	2	2	SELL, OSCAR	0		3.69	0.000202
DNA repair pathways full network	15	14	RFC1, RFC2, RFC3, RFC4, EXO1, DDB2, HMGB1, BRCA2, FANCD2, FANCI, FANCG, BRCA1, RAD51C, MGMT	1	FANCF	3.63	0.000234
Cytokines and inflammatory response	7	7	IL1A, IL1B, CSF3, CXCL2, IL6, IL11, HLA-DRB1	0		3.63	0.000237
Regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding proteins (IGFBPs)	1	0		1	IGFBP4	3.56	0.000273

the expression of genes related to the MMR pathway was also examined in this study. In GBM-8401/TMZR cells, the up-regulation of genes associated with the MMR pathway, such as *EXO1, RFC1, RFC2, RFC3* and *RFC4*, may consequently lead to the up-regulation of 5'-MMR activity that mediates the restoration of alkylation-induced O6-MeG DNA lesions, due to the pharmacological effect of TMZ, in GBM-8401/TMZR cells. The up-regulated expression levels of several MMR-associated genes (*EXO1* was up-regulated by 3.63-fold, while *RFC1, RFC2, RFC3* and *RFC4* expression were up-regulated by 2.04-, 2.44- 2.02-, 2.84-fold respectively) in GBM-8401/TMZR cells suggested that the MMR pathway may be over activated for the cells to adapt to TMZ-induced genotoxicity.

Discussion

Establishment of a new chemoresistant subline from parental cells is a common practice in laboratories investigating the mechanism of resistance to chemotherapeutics or screen for new therapeutics to overcome resistance (23, 24). The IC_{50}s to TMZ of resistant GBM cell lines have been summarized in a previous report and are in the range of 250-1000 μM (6). Additionally, the experiences with establishing chemoresistant cell lines, such as selecting parental cells, treating intervals, and concentrations have been summarized previously (25). In this study, we selected GBM-8401 cells as a parental cell line representative of GBM cells derived from an Asian patient. The treating concentrations of TMZ were increased gradually during a long period of time (Figure 1A) and resulted in a stable, maintainable, and culturable subline with significantly increased TMZ-tolerance (Figure 1B), and was considered as a typical TMZ-resistant cell line. Additionally, our transcriptomic comparison data indicated the changes in gene expression (Tables II and III) in the newly established subline that were associated with the RB pathway (Figure 4) and DNA repair pathways (Figures 6 and 7). These results may facilitate further studies on TMZ-resistant GBM cells.

RB signaling is reportedly altered in 78% of patients with GBM (7). The RB protein is a well-established tumor suppressor protein (26), which is associated with the DNA repair mechanism (20) and the prognosis of patients; therefore, it is currently used as a biomarker for determining the most appropriate treatment regimen for GBM in the clinic (27). The RB signaling pathway has also been suggested to represent a potential target for developing anti-GBM therapeutics (27). Our analysis in Figure 4 showed that RB1 protein levels were insignificantly changed in GBM8401/TMZR cells, but E2F2 was up-regulated, leading to an increased E2F2/RB1 ratio compared with that of parental cells. The increase in this ratio implies that the "free form" of E2F2 is released, resulting in the promotion of E2F2-mediated transcription and cell cycle transition. The formation of the RB1 and E2 promoter binding factor (E2F) complex is responsible for numerous biological functions related to the regulation of the cell cycle, which is important for cell proliferation and division, due to the transcriptional regulatory functions of the E2F protein family (20, 28). The up-regulated expression levels of E2F2, accompanied by statistically insignificant changes in the expression levels of RB1, were found to result in the up-regulation of the

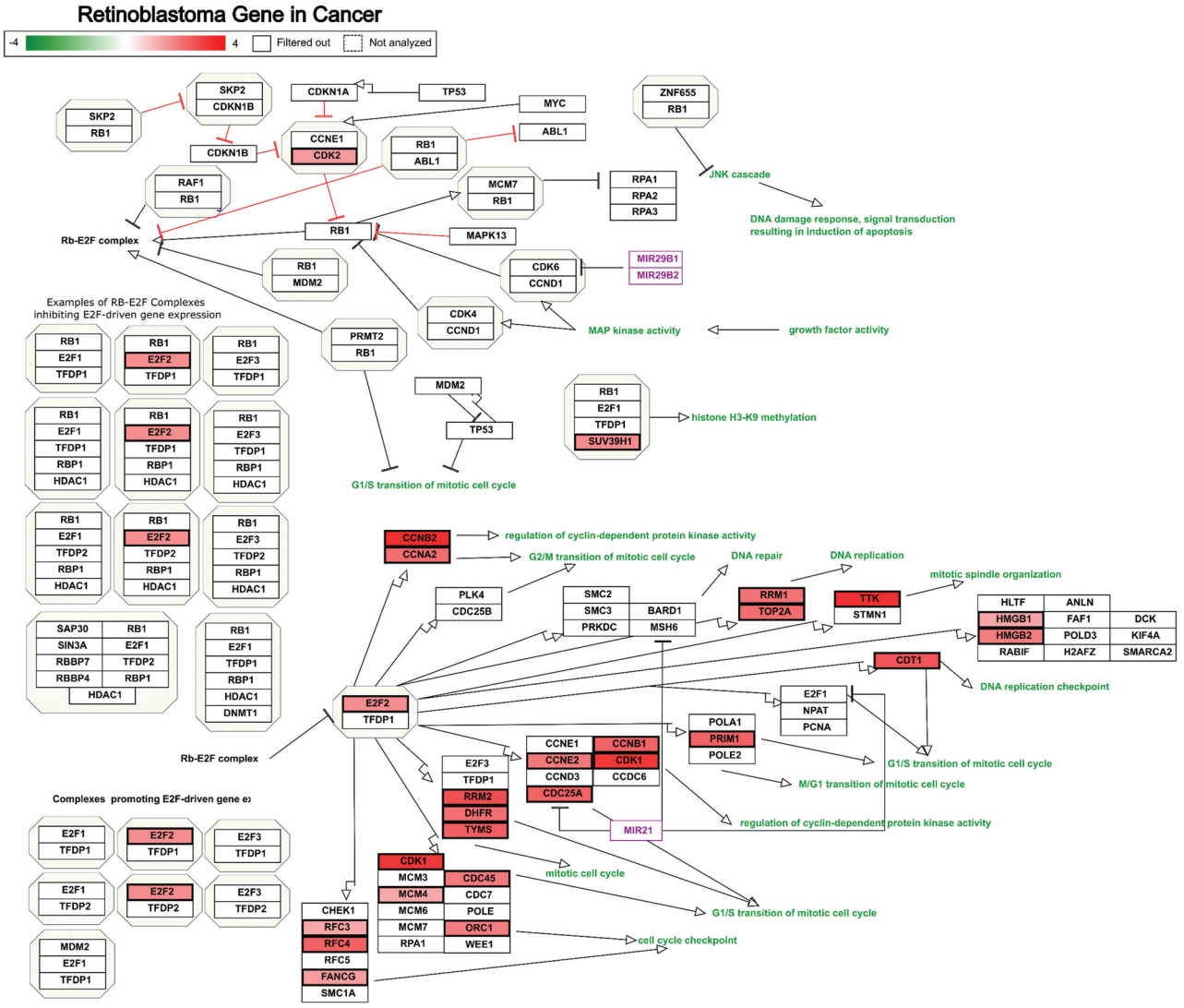

Figure 4. *Role of the retinoblastoma gene in temozolomide resistance in glioblastoma multiforme. Pathway analysis with gene annotation of differentially expressed genes in GBM-8401/TMZR cells compared with GBM-8401 cells. Red color, up-regulated genes; green color, down-regulated genes. GBM-8401/TMZR cells: GBM-8401 cells that are resistant to TMZ; TMZ: temozolomide.*

expression of E2F target genes and subsequently acceleration of the cell cycle, and facilitation of DNA replication and DNA repair. The upregulated expression of CCNB2, cyclin A2 and CDK2 (Figure 4) may be due to the up-regulation of E2F2 and the cross-talk with the RB signaling pathway. The results of the signaling pathway enrichment analysis revealed that the DEGs in GBM-8401/TMZR cells were involved in processes such as the 'promotion of cyclin-dependent protein kinase activity', 'G_1/S transition of mitotic cell cycle' and 'increased cell survival and growth'.

There is currently controversy regarding the roles of other cancer-related genes in TMZ-resistant GBM. For example, the results on the expression of the tumor suppressor gene *TP53* and the activation of the p53 pathway in TMZ-resistant GBM cells have not been consistent in previous studies (21, 29-31). Emerging evidence has indicated that p53 may not play a major role in TMZ resistance (6). However, MGMT expression was found to be an important indicator of TMZ sensitivity, due to its involvement in the DNA repair pathways as response to DNA damages (6, 22). In our analysis of the DDR pathway, a number of up-regulated gene products were found having direct relationship within both of the two main branches of the DDR pathway (ATM-TP53-CHEK2 and ATR-CHEK1, Figure 5). According to the

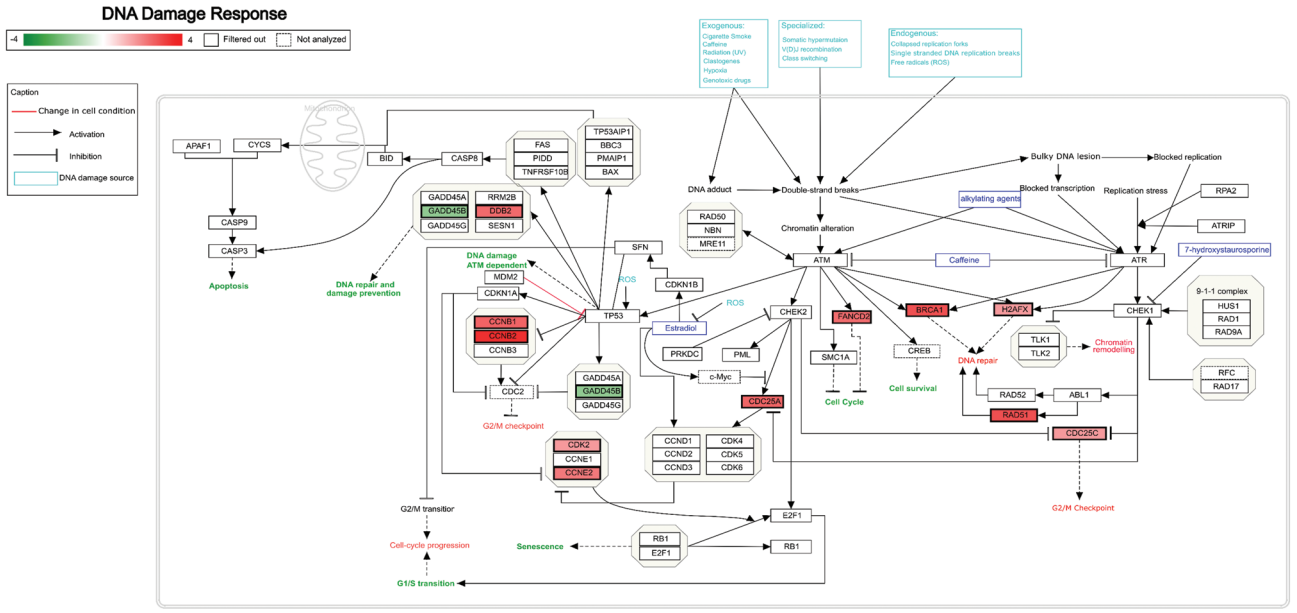

Figure 5. *Role of the DDR pathways in temozolomide resistance in glioblastoma multiforme. Pathway analysis with gene annotation of differentially expressed genes associated with DDR pathways in GBM-8401/TMZR cells compared with GBM-8401 cells. Red color, up-regulated genes; green color, down-regulated genes. GBM-8401/TMZR cells: GBM-8401 cells that are resistant to TMZ; TMZ: temozolomide; DDR: DNA damage response.*

pathway, the up-regulation of BRCA1, H2AFX and RAD51 may eventually promote the DNA repair mechanism; and FANCD2, CDC25C, CDK2, CCNE2 are related to the regulation of cell cycle. The results of this analysis suggested that the DDR pathway in GBM-8401/TMZR cells may be manipulated towards activating the DNA repair systems and mediating the progression of cell cycle.

DNA repair is a complex mechanism, involving several different pathways, such as MGMT, MMR, nucleotide excision repair, base excision repair (BER) and the Fanconi anemia (FA) pathway, which work independently or in a collaborative manner to maintain genomic integrity (2, 20, 21). The MGMT pathway, which removes a methyl group from O6-MeG, has been shown to have a crucial role in DNA repair (21, 30). Meanwhile, MMR and BER act as indirect mechanisms for DNA repair in situations when minor damage to DNA has occurred, usually in response to a DNA alkylating agent (30).

As presented in Figure 6, the significant upregulation of the expression of MGMT, and other genes associated with MMR, BER and FA pathways reveal the involvement of these DNA repair systems in GBM-8401/TMZR cells. Previous studies investigating the pharmacological mechanism of TMZ have reported that MGMT activity and activation of other DNA repair pathways played crucial roles in mediating the sensitivity of GBM cells to TMZ (6, 16, 22). As an alkylating agent, TMZ methylates nucleobases (guanine and adenine), and has been demonstrated to synergistically induce cytotoxicity with concomitant radiation therapy. Following TMZ treatment, the presence of MeG (O6-MeG or N7-MeG) or N3-methyladenine has been found to elicit DNA damage by interfering with DNA replication, thereby inhibiting cell viability and inducing cell death. In fact, O6-MeG formation has been discovered to be a major mechanism of TMZ-induced cytotoxicity. The expression of MGMT and the activation of other DNA repair systems are natural defense mechanisms of the cell that are activated in response to the detection of methylated nucleobases. The expression levels of MGMT have been previously found to be up-regulated in both primary and adaptive TMZ resistance (21, 22). Notably, the up-regulation of MGMT expression has been previously associated with TMZ resistance in numerous GBM cell lines (15). The enzymatic activity of MGMT has been found to mediate TMZ sensitivity in GBM cells by removing the methyl group from O6-MeG, ultimately repairing TMZ-induced DNA damage by DNA repair pathways (21, 22). Upon successful repair of the DNA lesions, TMZ-induced cytotoxicity was found to be suppressed. Not only MGMT expression, but also other DNA repair mechanisms, especially MMR are involved in TMZ-induced cytotoxicity (6, 21, 22). Thus, DNA repair mechanisms play pivotal roles in both the efficacy of the anticancer activity of TMZ and the resistance of the cell to TMZ. The results of the present study further

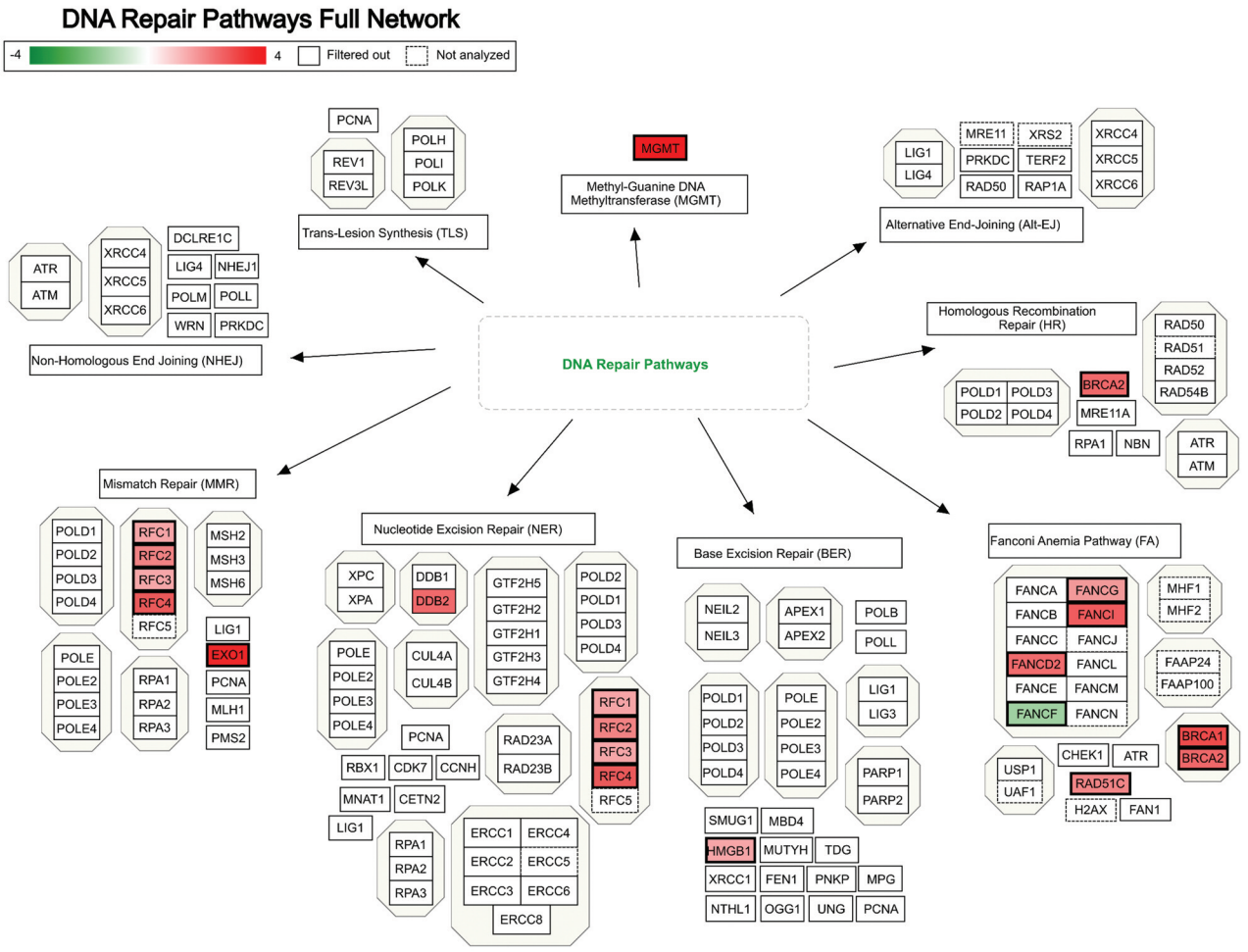

Figure 6. *Role of the DNA repair pathways in temozolomide resistance in glioblastoma multiforme. Pathway analysis with gene annotation of differentially expressed genes associated with DNA repair pathways in GBM-8401/TMZR cells compared with GBM-8401 cells. Red color, up-regulated genes; green color, down-regulated genes. GBM-8401/TMZR cells: GBM-8401 cells that are resistant to TMZ; TMZ: temozolomide.*

suggested the central role of MGMT in the resistance mechanism of GBM cells to TMZ, and are also consistent with the findings of recent reviews (1, 22, 30, 32).

The DDR pathway induces different biological processes, such as DNA repair, cell cycle arrest or apoptotic cell death, depending on the type and intensity of damage (16). The normal functioning of the MMR system in correcting errors of mispairing between nucleotides in the DNA molecule is an essential mechanism to ensure genomic integrity (33). Forms of DNA damage, such as damage due to single base pair mismatches and minor misalignments in the DNA molecule, are identified and corrected by the MMR system (21, 33). To correct mismatches in the DNA, the MMR system acts through several steps: Recognition, excision, re-synthesis and ligation (Figure 7). Briefly, MMR targets newly synthesized DNA strands and removes inappropriate nucleotides. On the 5' side of the recognized mismatch, the exonuclease activity of EXO1 facilitates the elimination of the incorrect DNA fragment. Then, proliferating cell nuclear antigen and RFCs fill the gap with the correct nucleotides (33). Finally, DNA ligase I is involved in the ligation step by sealing the DNA to complete the repair process of the MMR system. TMZ treatment induces DNA damage, as the presence of O6-MeG instead of guanine results in the mispairing of C with T instead of C with G in the newly synthesized DNA strand. Thus, O6-MeG is also recognized in newly synthesized strands and repaired by the MMR system, thereby attenuating TMZ-induced cytotoxicity. Previous studies have reported that TMZ resistance was enhanced by MMR deficiency (14, 22). In the present study, analysis of the gene expression profile of GBM-8401/TMZR cells revealed that the expression levels of EXO1 were

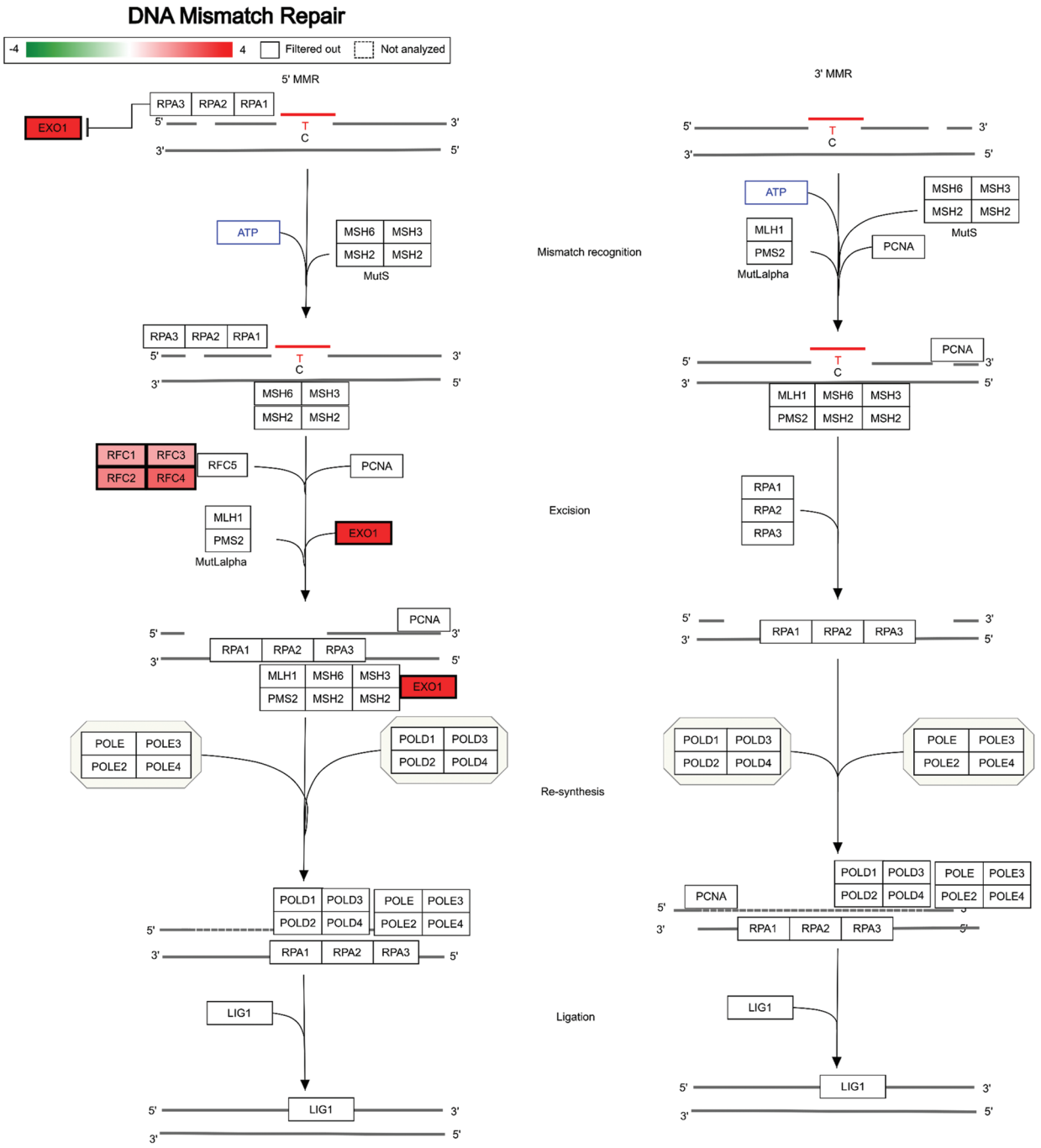

Figure 7. *Role of the DNA MMR pathway in temozolomide resistance in glioblastoma multiforme. Pathway analysis with gene annotation of differentially expressed genes associated with the DNA MMR pathway in GBM-8401/TMZR cells compared with GBM-8401 cells. Red color, up-regulated genes; green color, down-regulated genes. GBM-8401/TMZR cells: GBM-8401 cells that are resistant to TMZ; TMZ: temozolomide; MMR: mismatch repair.*

significantly up-regulated, suggesting that the MMR system may be activated to deal with the recognized DNA damage. Previous studies have also found that the susceptibility of GBM cells to TMZ was dependent on both MGMT expression and the MMR state (5, 6, 30). The present results suggested that following TMZ exposure, the MMR machinery may be activated to adapt with the higher rate of DNA damage, and TMZ resistance in GBM-8401/TMZR

cells may be associated with the activation of the MMR mechanism and avoiding O6-MeG-induced apoptosis, which is consistent with the results of previous studies (8, 34).

As previously discussed, TMZ methylates DNA, leading to the activation of DNA repair pathways (14, 15, 21). This event may promote apoptosis and thereby induce cell death. Patients with GBM with lower expression levels of MGMT have been found to have a higher sensitivity to TMZ, which lead to an improved survival outcome (35). In addition to the involvement of the DDR response, other mechanisms have been reported to be possibly associated with TMZ resistance, such as drug reflux, changes in the tumor microenvironment, the interplay between apoptosis-autophagy, post-translational mRNA modifications and receptor tyrosine kinase activity (30).

In conclusion, the present study established a novel TMZ-resistant subline of GBM cells, which was developed from the commonly used GBM cell line, GBM-8401, by long-term exposure to TMZ. The transcriptomic profile of the new subline (GBM-8401/TMZR) indicated that it exhibited adaptive alterations to the cell cycle regulatory RB pathway and other DNA repair pathways, such as MGMT and MMR. These results may provide a novel insight into the genotype of newly TMZ-resistant GBM cells at the transcriptional level and a novel cell-based GBM model for further biomedical investigations into the underlying mechanisms of TMZ-resistant GBM.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

H.A. HA, J.S. YANG, F.J. TSAI, M.J. HOUR and Y.J. CHIU contributed to the study design. F.J. TSAI, C.W. LI, Y.D. CHENG, J. Li and Y.J. CHIU conducted the experiments. C.W. LI, Y.D. CHENG and J.Li analyzed the data. H.A. HA, J.S. YANG, M.J. HOUR and Y.J. CHIU wrote and revised the paper. All Authors read and approved the final manuscript.

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Supplementary Information

Supplementary data are available at http://tiny.cc/svvvtz

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