Gene expression profiling predicts response to temozolomide in malignant gliomas

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Abstract. Malignant gliomas are highly lethal neoplasms that cannot be cured with currently available therapies. Temozolomide (TMZ) is a recently introduced alkylating agent that has yielded significant benefits and become a key agent in the treatment of high-grade gliomas. However, its survival benefit remains unsatisfactory. Understanding the molecular basis of TMZ sensitivity/resistance is necessary for improving the treatment outcome by devising strategies that are able to circumvent primary drug resistance. We therefore combined the *in vitro* TMZ response with microarray gene expression data to identify genes that could potentially be used to predict the response of malignant gliomas to TMZ therapy. We first obtained the individual IC_{50} values for TMZ in seven malignant glioma cell lines (A-172, AM-38, T98G, U-87MG, U-138MG, U-251MG and YH-13) and then identified the genes whose expression correlated most highly with TMZ sensitivity employing a cDNA microarray. We present here a list of the most highly up-regulated and down-regulated genes which may be involved in conferring TMZ sensitivity/resistance in malignant gliomas, although most of the genes have not been implicated as a causal factor in the TMZ response except *MGMT*. We also demonstrated and confirmed the *MGMT* methylation status, quantitative *MGMT* mRNA levels, and MGMT protein expression levels in TMZ resistant glioma cells *in vitro*. Our results are thus consistent with previous studies and suggest that a dominant mechanism conferring sensitivity/resistance to TMZ exists in malignant glioma cells. Although the present study dose have several limitations, our reported candidate genes could represent not only potential molecular markers for TMZ sensitivity/resistance but also chemotherapy targets. Furthermore, the present study could provide a foundation for alternative therapeutic strategies including novel combination treatments that incorporate additional reagents directed at overcoming resistance to TMZ.

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Introduction

Malignant gliomas are the most frequently occurring primary tumors of the central nervous system and constitute one of the most aggressive and lethal malignancies. They continue to present an enormous therapeutic challenge, because multimodality treatments including extensive tumor resection, radiation therapy and chemotherapy have afforded little improvement to the poor prognosis for more than three decades.

In 2004, the European Organisation for Research and Treatment of Cancer (EORTC)/National Cancer Institute of Canada Clinical Trial Group (NCIC) randomized phase III trial on concomitant and adjuvant temozolomide [TMZ; 3-methyl-4-oxo-3,4-dihydroimidazo (5,1-*d*) (1,2,3,5) tetrazine-8-carboxamide, a relatively new alkylating (methylating) agent], in addition to standard postoperative radiotherapy as the first-line treatment for glioblastomas (the most malignant form of glioma), demonstrated an increase in median survival from 12.1 to 14.6 months and an increase in 2-year survival rate from 10 to 26% as compared to postoperative radiotherapy alone (1). TMZ has therefore received much attention and become a current standard chemotherapy agent, notably in the treatment of malignant gliomas, although nitrosourea-based chemotherapy had been widely used previously. More recently, EORTC-NCIC reported final results indicating that the benefits of adjuvant TMZ with radiotherapy for glioblastoma lasted throughout five years of follow-up; the overall survival was 9.8% at five years with TMZ, versus 1.9% with radiotherapy alone (2). Clearly, concomitant radiation therapy with TMZ chemotherapy followed by adjuvant TMZ treatment can yield meaningful results in glioblastoma patients, but their prognosis remains unsatisfactory.

The limited efficacy of chemotherapy can be attributed largely to both inherent and acquired tumor drug resistance mechanisms. In addition to O^6-methylguanine-DNA methyltransferase (MGMT; a protein that removes drug-induced alkylguanine adducts from DNA created by alkylating agents including TMZ), base excision repair (BER) and mismatch repair (MMR) are thought to be involved in the principal mechanisms contributing to TMZ resistance (3-5). Similarly to MGMT, BER plays a crucial role in repairing the cytotoxic methyl DNA adducts, and a high BER activity can confer tumor resistance to TMZ (5). On the other hand, defective DNA MMR can result in tolerance to TMZ regardless of MGMT activity and continuance of DNA replication (4,5). However, some studies have demonstrated resistance to TMZ that was independent of such candidates, suggesting that other major mechanisms were involved in the resistance to TMZ (4,6). Furthermore, tumor chemotherapy resistance may be very complicated, often multifactorial and with many genes involved. The resistance of glioblastomas to TMZ appears to follow a more complex pattern than simple dependence on *MGMT* levels (7,8), although its detailed regulation at the genomic level remains poorly understood.

With the recent development of high-density oligonucleotide DNA array technology, global gene expression profiles can be analyzed simultaneously (9). In addition to the identification of new classes of human malignancies, this powerful method has become widely applied in attempts to increase our understanding of chemotherapy resistance and to provide further information about the underlying tumor biology that can be used to guide therapeutic strategies (5,10). Thus, in the present study, to gain new insights into the mechanisms of TMZ sensitivity/resistance in malignant gliomas and to identify new marker and target genes involved in malignant glioma pathogenesis and new therapeutic strategies for TMZ, we attempted to identify specific gene expression signatures associated with pre-existing sensitivity/resistance to TMZ employing the cDNA microarray technique.

Materials and methods

Tumor cell lines, cell culture and TMZ sensitivity determination. Human malignant glioma U-87MG and U-138MG cells were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA) and A-172, AM-38, T98G, U-251MG and YH-13 cells were purchased from Health Science Research Resources Bank (Sennann, Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL) in a standard humidified incubator at 37˚C under a 5% CO_2, 95% air atmosphere.

The sensitivities of the seven glioma cell lines to TMZ were evaluated from the concentration required for 50% growth inhibition (IC_{50}; also known as GI_{50}) in comparison with untreated controls. Briefly, cells were plated at $1x10^4$ or $2x10^4$ cells per well in 24-well, flat-bottomed plates and incubated with medium for 24 h. The cells were subsequently washed twice with medium and incubated further with fresh medium (control) or medium containing 0.1-1,000 μM of TMZ. After exposure to the various concentrations of TMZ for 72 h, cells were detached by trypsinization and the numbers counted. The experiments were repeated at least four times at each concentration.

RNA preparation and hybridization to GeneChip array. The cell lines were subjected to an assessment of their gene expression profile using a high-density oligonucleotide array (GeneChip array; Affymetrix, Santa Clara, CA, USA) as described previously (11). Briefly, the extracted total RNA was purified with an RNeasy mini kit (Qiagen, Valencia, CA), quantified by spectrophotometry and analyzed by gel electrophoresis. Double-stranded cDNA was generated from the total RNA (5 μg) using a One-Cycle cDNA synthesis kit (Affymetrix). Biotinylated cRNA from cDNA was synthesized in an *in vitro* transcription reaction employing an IVT labeling kit (Affymetrix). The biotinylated cRNA (10 μg) was then fragmented and hybridized to a DNA oligonucleotide expression array (Affymetrix Human Genome U133A 2.0 Array) containing greater than 22,277 probe sets for approximately 14,500 human genes (some genes are represented on the array by multiple probe sets). The hybridized probe array was washed and stained with a streptavidin-phycoerythrin conjugate (Molecular Probe, Eugene, OR, USA) using a Fluidics Station 450 (Affymetrix), following the manufacturer's instructions.

Identification of discriminatory genes. The probe array was then scanned with a confocal laser scanner (GeneChip Scanner 3000; Affymetrix) and analyzed with Affymetrix GeneChip Operating Software 1.1 to calculate the signal intensity of the gene expression levels. All of the genes represented on the GeneChip were globally normalized and scaled to signal intensity (gene expression value) as described previously (12). To avoid contributions from artificial sources of variation in the experimentally measured expression patterns, each cell line was grown in three independent cultures, and the entire process was carried out independently on mRNA extracted from each culture. The expression array analysis for each cell line was then run in triplicate.

To identify discriminative genes for TMZ sensitivity/resistance, Pearson's correlation was performed to evaluate the association between the IC_{50} (sensitivity) of TMZ and gene expression level of each gene (22,277 probe sets for approximately 14,500 human genes), and Spearman's rank correlation coefficient was also calculated to confirm Pearson's correlation coefficient (Microsoft Office Excel 2007).

MGMT status. The correlations among the *MGMT* methylation status, quantitative levels of *MGMT* mRNA and MGMT protein expression were assessed in seven glioma cell lines, since one of the most prominent resistance mechanisms to TMZ has been widely considered to be the action of the DNA repair enzyme MGMT (7,13-15).

i) Detection of MGMT methylation. Promoter hypermethylation of the *MGMT* gene was determined by the methylation-specific polymerase chain reaction (MS-PCR) (16). The PCR was performed with primers specific for either methylated or modified unmethylated DNA, as described previously (17-19).

Briefly, the DNA was extracted using a QIAamp® DNA Mini Kit (Qiagen), and then 1 μg of extracted DNA underwent bisulfite modification using a CpGenome™ DNA Modification Kit (Chemicon, Millipore Corp., Billerica, MA, USA), according to the manufacturer's instructions. Amplified products were electrophoresed on 3% agarose gels, and were visualized with ethidium bromide. Enzymatically methylated human male genomic DNA (CpGenome Universal Methylated DNA; Chemicon, Millipore Corp.) and DNA from normal lymphocytes were included in each set of the PCR as methylated and unmethylated controls, respectively.

ii) Quantitation of MGMT mRNA by real-time quantitative RT-PCR. Quantitation of the *MGMT* gene expression was performed by the real-time quantitative reverse transcription-PCR (RT-PCR) method, as developed recently (20,21). Briefly, the real-time PCR reaction mixture was prepared using a TaqMan Universal Master Mix (Applied Biosystems, CA, USA), 120 nM of each *MGMT* primer, 200 nM probe (5'-CGA GCA GTG GGA GGA GCA ATG AGA-3'), and 2.5 μl of each cDNA sample. The PCR conditions were as follows: 1st stage, 95˚C for 10 min; 2nd stage, 45 cycles at 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec; and 3rd stage, 72˚C for 10 sec, employing a real-time PCR system (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems). *Glyceraldehyde-3-phosphatase dehydrogenase* (*GAPDH*) mRNA expression levels were used as the quantitative internal control. Standard curves for *MGMT* and *GAPDH* mRNA were generated employing 10-fold serially diluted standard plasmid clones inserted by MGMT or GAPDH PCR products as templates, and the amount of each mRNA expression level was calculated from the relevant standard curve. For precise quantification, the *MGMT* mRNA expression level of each sample was normalized using the expression of the *GAPDH* gene.

iii) Western blot analysis of MGMT. To determine the protein levels of MGMT, Western blot analysis was employed as described previously (22,23). Briefly, soluble protein lysates of glioma cells were obtained employing lysis buffer (Medical & Biological Laboratories, Woburn, MA, USA) for 20 min on ice. Portion (5 μg) of these proteins was loaded and separated by 12.5% polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Franklin Lakes, NJ, USA) for 30 min at 10 V with a Bio-Rad transblot (Bio-Rad Laboratories). Non-specific binding was blocked with a wash buffer (PBS/0.05% Tween-20) containing 5% ECL blocking agent (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, UK) for 60 min. The primary antibodies and dilutions used for the immunoblotting were MGMT (1:200) (MT 3.1; Neomarkers, Fremont, CA, USA) for 120 min and ß-actin (1:5000) (AC-15; Sigma-Aldrich, St. Louis, MO, USA) for 60 min. The secondary antibodies employed were biotinylated anti-mouse IgG (H and L) (1:1000) (BA-2000; Vector Laboratories, Burlingame, CA, USA) for 60 min. The immunoblotted complex was visualized with the aid of an ECL Western blotting analysis system (GE Healthcare UK Ltd.).

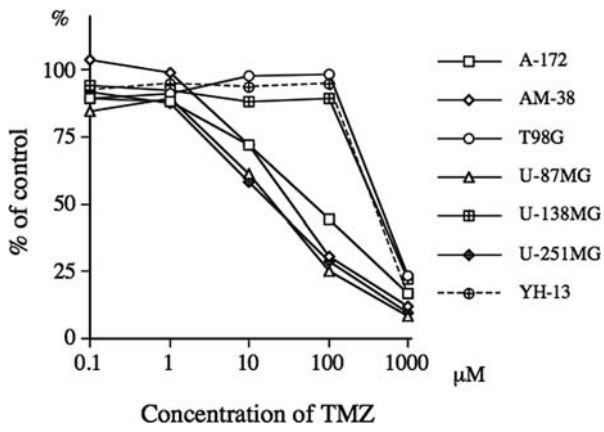

Figure 1. Antitumor effects of TMZ against seven human glioma derived cell lines. At 72 h after addition of TMZ (final concentration, 0-1,000 μM) to the culture medium, the number of viable cells was counted. The number was expressed as a percentage of the untreated control.

Table I. IC_{50} of temozolomide for seven malignant glioma cell lines.

Cell line	IC_{50} (μM)
A-172	52.4
AM-38	40.7
T98G	441.6
U-87MG	23.0
U-138MG	387.3
U-251MG	22.5
YH-13	371.3

The IC_{50} (mean), represents the temozolomide concentration required for a 50% decrease in cell growth compared to the control.

Results

We first obtained the individual IC_{50} values of TMZ (sensitivity/resistance) from the cell growth inhibitory effects of the seven malignant glioma cell lines, and then identified the genes whose expression correlated most highly with TMZ sensitivity/resistance employing the GeneChip array and Pearson's correlation coefficient.

IC_{50} of TMZ in glioma cell lines. To evaluate the antitumor effects of TMZ in malignant glioma cells, we treated the seven malignant glioma cell lines with 0-1,000 μM of TMZ for 72 h, and assessed the numbers of viable cells. As shown in Fig. 1, cell growth inhibitory effects of TMZ on all the tumor cell lines were observed in a dose-dependent manner. The IC_{50} of TMZ for four malignant glioma cells (A-172, AM-38, U-87MG and U-251MG) was <100 μM, although T98G, U-138MG and YH-13 cells were found to be resistant to TMZ (IC_{50} >350 μM). The IC_{50} values of TMZ for each cell line are listed in Table I.

Table II. The 15 negative and 15 positive differentially expressed genes associated with TMZ sensitivity.

Symbol	Gene title	Correlation	P-value
ATXN10	Ataxin 10	0.965	<0.0001
EIF4ENIF1	Eukaryotic translation initiation factor 4E nuclear import factor 1	0.934	<0.0001
MGMT	O^6-methylguanine-DNA methyltransferase	0.924	<0.0001
LPAR1	Lysophosphatidic acid receptor 1	0.905	<0.0001
LAMB1	Laminin, ß1	0.898	<0.0001
MDK	Midkine (neurite growth-promoting factor 2)	0.898	<0.0001
SECTM1	Secreted and transmembrane 1	0.897	<0.0001
GTPBP1	GTP binding protein 1	0.897	<0.0001
UGDH	UDP-glucose dehydrogenase	0.891	<0.0001
ZNF354A	Zinc finger protein 354A	0.889	<0.0001
CASP1	Caspase 1, apoptosis-related cysteine peptidase	0.888	<0.0001
VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor	0.882	<0.0001
RBM9	RNA binding motif protein 9	0.881	<0.0001
RYBP	RING1 and YY1 binding protein	0.881	<0.0001
HMG2L1	High-mobility group protein 2-like 1	0.877	<0.0001
FAM57A	Family with sequence similarity 57, member A	-0.885	<0.0001
ZNF365	Zinc finger protein 365	-0.870	<0.0001
HIST3H2A	Histone 3, H2a	-0.854	<0.0001
ECD	Ecdysoneless homolog	-0.835	<0.0001
CYFIP2	Cytoplasmic FMR1 interacting protein 2	-0.831	<0.0001
LHFPL2	Lipoma HMGIC fusion partner-like 2	-0.829	<0.0001
WDR37	WD repeat domain 37	-0.815	<0.0001
MCM6	Minichromosome maintenance deficient 6	-0.813	<0.0001
PDE4DIP	Phosphodiesterase 4D interacting protein	-0.813	<0.0001
WIPF2	WAS/WASL interacting protein family, member 2	-0.811	<0.0001
PARD3	par-3 partitioning defective 3 homolog	-0.811	<0.0001
PCMT1	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	-0.811	<0.0001
MLXIP	MLX interacting protein	-0.807	<0.0001
PIP4K2A	Phosphatidylinositol-5-phosphate 4-kinase, type II, α	-0.806	<0.0001
CCDC109B	Coiled-coil domain containing 109B	-0.800	<0.0001

Identification of discriminatory genes. From the total of 22,277 probe sets, no expressed gene was omitted, leaving 16,913 probe sets for subsequent analysis. Several up-regulated and down-regulated genes in the seven malignant glioma cell lines relative to their TMZ sensitivity were observed. The highly significant statistically negative and positive correlation of genes associated with TMZ sensitivity are indicated in Tables II and III. Pearson's correlation coefficient (r) and the corresponding P-values for these correlations are also shown in Tables II and III (the positive and negative higher rank of Pearson's correlation coefficient, and a parametric P<0.00001, are listed in Tables II and III). The gene-specific information was retrieved from Entrez gene: gene-centered information at NCBI (24).

Most of the genes indicated in Table II have not, to our knowledge, been previously suspected to play a role in the prediction of TMZ sensitivity/resistance, chemotherapy sensitivity, or prognosis. However, the *MGMT* expression level was significantly positively correlated with TMZ resistance (Pearson's correlation coefficient and the corresponding P-values were 0.924 and <0.0001, respectively), and confirmed the capacity of *MGMT* to predict the sensitivity/resistance of TMZ in malignant glioma cell lines. Further four genes [namely, midkine (*MDK*), caspase 1 (*CASP1*), vitamin D receptor (*VDR*), and protein-L-isoaspartate O-methyltransferase (*PCMT1*)], for which the results were significantly correlated in our analyses (with *MDK*, *CASP1* and *VDR* being positively correlated to TMZ resistance, and PCMT1 negatively correlated to TMZ resistance) (Fig. 2), have previously been reported to be associated with chemotherapy resistance, prognosis, tumor progression, or antiproliferation, in malignant gliomas (25-29).

We also mapped the selected features to chromosomes (Tables III and IV). The chromosomal locus 22q13 was

Table III. Up-regulated genes among the TMZ resistance associated genes.

Symbol	Location	Function	Process
ATXN10	22q13.31	protein binding	cell death. nervous system development
EIF4NIF1	22q11.2	protein binding. protein transporter activity	protein transport
MGMT	10q26	DNA binding DNA-methyltransferase activity metal ion binding methylated-DNA-cysteine S-methyltransferase activity transferase activity zinc ion binding	DNA dealkylation DNA ligation regulation of caspase activity response to DNA damage stimulus
LPAR1	9q31.3	G-protein coupled receptor activity PDZ domain binding lysosphingolipid and lysophosphatidic acid receptor activity receptor activity	G-protein coupled receptor protein signaling pathway activation of MAPK activity activation of phospholipase C activity bleb formation elevation of cytosolic calcium ion concentration positive regulation of 1-κB kinase/NF-κB cascade positive regulation of MAPKKK cascade positive regulation of Rho protein signal transduction signal transduction
LAMB1	7q22	extracellular matrix structural constituent protein binding	cell adhesion. neurite development odontogenesis positive regulation of cell migration positive regulation of epithelial cell proliferation
MDK	11p11.2	growth factor activity heparin binding	adrenal gland development cell differentiation. cell proliferation multicellular organismal development nervous system development response to wounding. signal transduction
SECTM1	17q25	cytokine activity signal transducer activity	immune response. mesoderm development positive regulation of 1-κB kinase/NF-κB cascade
GTPBP1	22q13.1	GTP binding GTPase activity nucleotide binding	immune response signal transduction

Table III. Continued.

Symbol	Location	Function	Process
UGDH	4p15.1	NDA or NADH binding UDP-glucose 6-dehydrogenase activity binding electron carrier activity oxidoreductase activity	UDP-glucose metabolic process UDP-glucuronate biosynthetic process gastrulation with mouth forming second glycosaminoglycan biosynthetic process oxidation reduction
ZNF354A	5q35.3	DNA binding RNA polymerase II transcription factor activity metal ion binding zinc ion binding	regulation of transcription from RNA polymerase II promoter sensory perception of sound transcription
CASP1	11q23	caspase activator activity cysteine-type endopeptidase activity peptidase activity protein binding	positive regulation of 1-κB kinase/NF-κB cascade proteolysis regulation of apoptosis signal transduction
VDR	12q13.11	metal ion binding protein binding sequence-specific DNA binding steroid hormone receptor activity transcription factor activity vitamin D3 receptor activity zinc ion binding	calcium ion transport. cellular calcium ion homeostasis intestinal absorption multicellular organismal development negative regulation of transcriptiom organ morphogenesis regulation of transcription, DNA-dependent signal transduction. skeletal system development
RBM9	22q13.1	RNA binding nucleotide binding transcription corepressor activity transcription factor binding	RNA metabolic process. RNA splicing estrogen receptor signaling pathway mRNA processing negative regulation of transcription regulation of cell proliferation
RYBP	3p13	DNA binding metal ion binding protein binding transcription corepressor activity zinc ion binding	apoptosis multicellular organismal development negative regulation of transcription from RNA polymerase II promoter regulation of transcription, DNA-dependent
HMG2L1	22q13.1	DNA binding	endosome to lysosome transport

Table IV. Down-regulated genes in TMZ resistance associated genes.

Symbol	Location	Function	Process
FAM57A	17p13.3		
ZNF365	10q21.2	protein binding zinc ion binding	
HIST3H2A	1q42.13	DNA binding	nucleosome assembly
ECD	10q22.1	transcription coactivator activity	regulation of glycolysis regulation of transcription, DNA-dependent transcription from RNA polymerase II promoter
CYFIP2	5q33.3	protein binding	apoptosis cell-cell adhesion
LHFPL2	5q14.1		
WDR37	10p15.3		
MCM6	2q21	ATP binding DNA binding DNA helicase activity identical protein binding nucleotide binding single-strand DNA binding	DNA replication DNA replication initiation DNA unwinding during replication cell cycle regulation of transcription, DNA-dependent transcription
PDE4DIP	1q12		
WIPF2	17q21.2	actin binding	
PARD3	10p11.2-p11.21	protein binding	activation of protein kinase C activity by G-protein coupled receptor protein signaling pathway asymmetric cell division axonogenesis cell cycle establishment or maintenance of cell polarity
PCMT1	6q24-q25	identical protein binding methyltransferase activity protein-L-isoaspartate (D-aspartate) O^6-methyltransferase activity transferase activity	protein complex assembly protein amino acid methylation protein modification process protein repair
MLXIP	12q24.31	DNA binding transcription regulator activity	regulation of transcription, DNA-dependent
PIP4K2A	10p12.2	1-phosphatidylinositol-4-phosphate 5-kinase activity 1-phosphatidylinositol-4-phosphate 4-kinase activity ATP binding kinase activity nucleotide binding transferase activity	phosphatidylinositol metabolic process phosphorylation
CCDC109B	4q25		

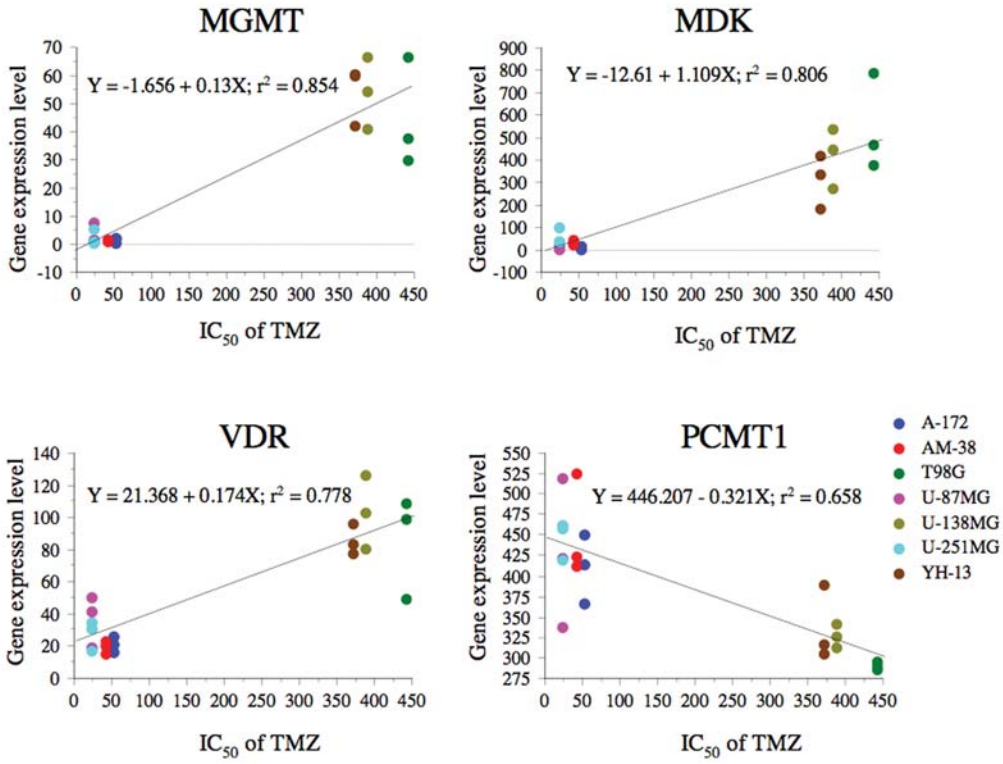

Figure 2. Relationship between gene expression levels (*MGMT*, *MDK*, *VDR*, and *PCMT1*) and TMZ sensitivity (IC_{50} of TMZ) of seven malignant glioma cell lines.

Table V. *MGMT* methylation status, quantitative of *MGMT* mRNA, and MGMT protein expression in seven malignant glioma cell lines.

	A-172	AM-38	T98G	U-87MG	U-138MG	U-251MG	YH-13
MGMT methylation status	M	M	M, U	M	U	M, U	U
Quantitative *MGMT* mRNA (copy/μg RNA)	ND	ND	$5.9x10^3$	ND	$6.3x10^3$	ND	$5.4x10^3$
MGMT protein expression	ND	ND	+	ND	+	ND	+

M, methylated; U, unmethylated; ND, not detected; +, expression.

suspected to be a potential hotspot for aberrant gene expression associated with TMZ resistance in malignant glioma cell lines.

Correlations of TMZ sensitivity with MGMT methylation status, quantitative MGMT mRNA expression and MGMT protein expression. As shown in Fig. 2, a significant correlation ($P<0.0001$, $r^2=0.854$) was noted between TMZ sensitivity and the *MGMT* gene expression detected with the high-density oligonucleotide microarray (HG-U133A 2.0, Affymetrix). The MGMT status of the seven malignant glioma cell lines was also determined by the *MGMT* methylation status, quantitative *MGMT* mRNA expression, and MGMT protein expression. Methylation of the CpG islands in promoter regions is a major factor in gene regulation and is correlated with transcriptional silencing. To examine the *MGMT* methylation status, DNA from the present seven malignant glioma cell lines was isolated and investigated by performing the MS-PCR. Furthermore, in order to confirm whether or not MGMT expression might be responsible for part of the resistance mechanism to TMZ in glioma cells, we tested the MGMT expression at the mRNA and protein levels.

Parts of the results obtained have recently been reported elsewhere (22,23). Promoter hypermethylation of *MGMT* was demonstrated in A-172, AM-38 and U-87MG cells (TMZ sensitive cell lines) but not in U-138MG and YH-13 cells (TMZ resistant cell lines). The other two cell lines,

T98G and U-251MG, appeared to contain methylated and unmethylated DNA at this locus. Furthermore, MGMT expression as determined from the *MGMT* mRNA expression levels using the real-time quantitative RT-PCR and Western blot analysis was not detected in the TMZ sensitive cell lines, A-172, AM-38, U-87MG and U-251MG. In contrast, such expression was detected in the TMZ-resistant cell lines, T98G, U-138MG and YH-13. The absolute values of *MGMT* mRNA normalized to the level of GAPDH in T98G, U-138MG and YH-13 cells were $5.9x10^3$, $6.3x10^3$ and $5.4x10^3$ copy/μg RNA, respectively (Table V). These findings suggested that the resistance of T98G, U-138MG and YH-13 cells to TMZ is probably related to MGMT.

Discussion

It is possible that large-scale gene expression analysis, rather than an analysis of a single gene or pathway, can yield a more robust predictor of malignant glioma responses to TMZ, since it would involve the analysis of genes associated with multiple mechanisms of resistance (5,8). In this study, we evaluated the gene expression profiles employing GeneChip in seven malignant human glioma-derived cell lines in an attempt to identify marker(s) that could be used clinically to predict the response of malignant gliomas to TMZ therapy. Since the gene expression profiles were determined in untreated cells, our data relate to the sensitivity/resistance to TMZ therapy rather than to the molecular consequences of therapy (30).

Resistance to TMZ therapy is thought to arise when the methylated DNA bases are repaired by MGMT (13) or BER (31), or when the MMR pathway is deficient (32), or the damaged DNA is tolerated (4,5). Notably, glioblastoma patients with promoter hypermethylation of the *MGMT* gene that silences its expression have been shown to benefit from TMZ therapy (2,33-35). In the present study, as expected, our results indicated that an elevated expression of the *MGMT* gene is one of the most robust predictors of the TMZ response in malignant glioma cells. We also demonstrated and confirmed the MGMT protein expression levels and quantitative *MGMT* mRNA levels in TMZ resistant, T98G, U-138MG and YH-13 cells. Furthermore, the *MGMT* promoter methylation observed in nearly half of the seven malignant glioma cell lines analyzed in this study corresponded with the observed expression of MGMT protein, *MGMT* mRNA and TMZ sensitivity/resistance. Our findings are thus consistent with previous studies and suggest that a dominant mechanism conferring sensitivity/resistance to TMZ exists in malignant glioma cells (2,13,14,20,34,35). On the other hand, a differential gene expression was not clearly identified for BER and MMR genes in the present study. Although BER can repair N-methylated lesions generated by alkylating agents such as TMZ (5,36), our results suggest that this pathway is likely to have a minimal effect on TMZ resistance in malignant glioma cell lines. Moreover, our data corroborate the finding of Maxwell *et al* that MMR deficiency does not appear to be responsible for mediating TMZ resistance in malignant gliomas (4).

The Affymetrix Human Genome U133A 2.0 Array also contains the well-known multi-drug resistance candidate genes and other genes involved in drug resistance mechanisms including drug-metabolism and detoxification (37,38). However, we did not identify any of those genes to be strongly associated with the TMZ response in malignant gliomas. Ma *et al* found that tumor cell-acquired resistance to TMZ was associated with an increased MGMT activity and alterations of apoptosis-controlling proteins, i.e., reduced Bad, Bax and Bcl-Xs, in a human glioma cell line (39). However, again, none of these genes were identified in our microarray analysis. On the other hand, highly significant statistically positive- and negative-correlation of genes concerned with TMZ sensitivity/resistance were found as shown in Tables II and III. To our knowledge, 25 of these 30 genes have not been reported previously to be associated with chemotherapy resistance, prognosis, tumor progression, or antiproliferation, in malignant gliomas. The other five genes, four up-regulated genes (*MGMT*, *MDK*, *CASP1* and *VDR*) and one down-regulated (*PCMT1*) gene, that have been reported previously to have such associations (chemotherapy resistance, *MGMT* and *MDK*; prognosis, *MGMT* and *MDK*; tumor progression, *MDK* and *PCMT1*; antiproliferation, *MDK* and *VDR*; and other associations, *CASP1*) (25-29), were differentially expressed in association with TMZ sensitivity/resistance in the glioma-derived cell lines. Among the genes positively correlated to TMZ resistance in our analysis, one of the most noteworthy is *MGMT*, as mentioned above; MGMT expression provides a resistance measure that can potentially be applied to current TMZ-based treatment strategies for malignant glioma patients. Depletion of MGMT by the substrate analogue inhibitor O^6-benzylguanine or by interferon-ß (which is known to exhibit pleiotropic biological activities including antitumor effects) has yielded encouraging results and suggests a therapeutic potential for chemotherapy in combination with such a resistance modulator for the treatment of malignant gliomas (5,13,23,40,41). However, although a phase II clinical trial has indicated that O^6-benzylguanine is able to restore TMZ sensitivity in patients with TMZ-resistant anaplastic gliomas, there appeared to be no significant restoration of TMZ sensitivity in patients with TMZ resistant glioblastoma (42).

Following the introduction of the current standard treatment involving concomitant radiation plus TMZ followed by adjuvant TMZ, two significant papers on the analysis of glioblastoma-derived gene expression profiles to TMZ resistance were published. Bredel *et al* highlighted the involvement of a cellular pathway of nuclear factor-κB (NF-κB, which is activated in response to DNA damage caused by O^6-alkylating agents), especially the zinc finger protein tumor necrosis factor-α-induced protein 3 (TNFAIP3, a potent inhibitor of NF-κB signaling), mediating resistance to O^6-alkylating agents in glioblastoma cells (8). Excessive and prolonged activation of NF-κB has been suggested as a principal mechanism of tumor chemoresistance, which is primarily mediated by its antiapoptotic activity (43,44), and some evidence has also indicated a link between the NF-κB pathway and resistance of glioblastoma cells to O^6-alkylating agents (45). The consistent up-regulated genes of this pathway in our study (*LPAR1*, *SECTM1* and *CASP1*, a positive regulator of the 1-κB kinase/NF-κB cascade) suggest a potentially important role for his pathway in the development

of resistance to TMZ in glioblastoma cells. Murat *et al* (46), in the other significant paper, reported an analysis of glioblastoma-derived gene expression profiles from patients treated in two prospective clinical trials (1,47). They suggested that the homeobox gene (*HOX*), which comprises *Prominin-1* (CD133; CD133 positivity has been postulated to be a glioma stem-cell marker), and epidermal growth factor receptor gene (*EGFR*) expression were independent prognostic factors in a multivariate analysis adjusted for *MGMT* methylation status and age, although epigenetic inactivation of the *MGMT* gene promoter remained the most prominent predictive factor.

On the other hand, many new antitumor drugs are being developed that target-specific molecular or genetic lesions, and although targeted therapies alone do not often demonstrate sufficient antitumor effects, it seems likely that when combined with standard chemotherapies, the responses could be higher and more durable (5). Thus, our reported genes (especially *MDK*, *CASP1*, *VDR* and *PCMT1*) and pathway (the NF-κB pathway) may represent not only potential molecular markers for TMZ sensitivity/resistance but also attractive targets for therapeutic modulation of glioblastomas. Furthermore, the present study could provide a foundation for alternative therapeutic strategies including novel combination treatments that incorporate additional reagents directed at overcoming resistance to TMZ.

Finally, several limitations of the present study should be briefly mentioned. First, it can always be viewed that the expression profiles of cell lines which have undergone many passages do not exactly represent the genetic events that occur in actual tumors (48). Second, the present study was restricted to the use of only seven glioma cell lines. Third, it is possible that if the entire genome were to be analyzed, the number of potential molecular markers for TMZ sensitivity/resistance and chemotherapy targets would increase (9). Fourth, in general, the gene expression correlation of drug sensitivity/resistance are complex, and their biological significance is not readily interpretable (9). Further experiments are therefore needed to test how the candidate genes affect the TMZ response in malignant glioma cells *in vitro*, and it is also important to determine whether modulation of the proteins encoded by these genes can enhance the treatment efficacy in patients with TMZ resistant malignant gliomas.

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