# Gene expression profiles predicting the response to IFN-β and a combination of temozolomide and IFN-β in malignant gliomas

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**Abstract.** Temozolomide (TMZ) is an alkylating agent that has yielded significant benefits and is a current standard agent in the treatment of malignant gliomas. However, its survival benefit remains unsatisfactory. Recently, a synergistic antitumor effect between TMZ and interferon-β (IFN-β) was reported in malignant glioma cells. The Japan Clinical Oncology Group (JCOG) brain tumor study group has recently began a randomized phase II study to evaluate the clinical effectiveness of combination therapy with TMZ and IFN-β in glioblastomas. However, it is not sufficient just to evaluate the mechanisms and establish an experimental basis for rational clinical therapy with IFN-β and TMZ. The precise mechanisms governing the direct effects of IFN-β and a combination of IFN-β and TMZ in gliomas are not yet fully understood. To gain insight into the mechanisms of sensitivity/resistance involving IFN-β and combination therapy with IFN-β and TMZ, and further to identify new marker(s) that could be used clinically to predict the response to such therapy and new target gene(s) for therapies related to malignant glioma pathogenesis, we evaluated the gene expression profiles of human malignant glioma cell lines employing a high-density oligonucleotide DNA array, GeneChip. We present a list of the most highly upregulated and downregulated genes which may be involved in conferring a response to IFN-β and synergistic effect between IFN-β and TMZ in malignant gliomas. Although the present study has several limitations, our reported candidate genes could represent not only potential molecular markers but also chemotherapy targets for improving the treatment outcome by devising strategies that are able to circumvent primary drug resistance in malignant gliomas.

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## Introduction

Malignant gliomas, especially glioblastomas, 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, radiotherapy and chemotherapy have afforded little improvement to the poor prognosis for more than three decades.

Recently, the European Organisation for Research and Treatment of Cancer (EORTC)/National Cancer Institute of Canada Clinical Trial Group (NCIC) reported final results indicating that the benefits of concomitant and adjuvant temozolomide (TMZ) in addition to standard postoperative radiotherapy as the first-line treatment for glioblastomas lasted throughout 5 years of follow-up; the overall survival was 9.8% at 5 years with TMZ versus 1.9% with radiotherapy alone (1). Concomitant radiotherapy with TMZ followed by adjuvant TMZ chemotherapy has thus become a current standard treatment for malignant gliomas, especially glioblastomas. However, the prognosis remains unsatisfactory, and the establishment of a more effective regimen of TMZ therapy is needed. The limited efficacy of chemotherapy can be attributed largely to both inherent and acquired tumor drug resistance mechanisms. Among the factors that may contribute to the TMZ resistance, $O^6$-methylguanine-DNA methyltransferase (MGMT, a protein that removes drug-induced alkylguanine adducts from DNA created by TMZ) is thought to be involved in its principal mechanisms (1,2). In addition, we more recently reported a list of the most highly upregulated and downregulated genes which may be involved in conferring TMZ sensitivity/resistance in malignant gliomas employing a cDNA microarray, and suggested that an elevated level of the *MGMT* gene is one of the most robust predictors of the TMZ response in malignant glioma cells *in vitro* (3).

Human interferon-β (IFN-β), which belongs to the type I IFNs, was first discovered on the basis of its antiviral activities. Subsequently, it was shown to exhibit pleiotropic biological activities including an immunomodulatory activity, antiangiogenetic activity and direct antitumor effects: e.g., growth inhibition, apoptosis, etc. (4-6). In addition to such multiple

functions of IFN-β against human neoplasias, it can act as a drug sensitizer enhancing the toxicity when given in combination with nitrosoureas (alkylating agents) in the treatment of malignant gliomas (7). It is therefore widely employed either alone or in combination with other antitumor agents such as nitrosoureas in the treatment of gliomas, particularly in Japan (4,7-9). Recently, a synergistic antitumor effect between TMZ and IFN-β was reported in malignant glioma cells *in vitro* (10,11). Natsume *et al* suggested that a sensitizing effect between IFN-β and TMZ in TMZ-resistant glioma cells was possibly due to an attenuation of MGMT expression via induction of the protein p53 (10). Furthermore, we demonstrated that a significant synergistic antitumor effect and down-regulated quantitative *MGMT* mRNA levels were observed when TMZ was combined with IFN-β at clinically relevant concentrations, as compared to treatment with TMZ or IFN-β alone in TMZ-resistant glioma cells, and that significant amounts of endogenous IFN-β protein were detected in the TMZ-treated cells by ELISA (12).

These findings indicated that favorable therapeutic interactions might take place between IFN-β and TMZ, and that combination therapy with IFN-β and TMZ might further improve the clinical outcome in malignant gliomas as compared to TMZ chemotherapy alone. Currently, a clinical study is being conducted in order to evaluate the clinical effectiveness of a combination of IFN-β and TMZ in glioblastomas: integrated Japanese multicenter clinical trial: a phase II study of interferon-β and temozolomide for glioma in combination with radiotherapy (INTEGRA Study) (13). However, it is not sufficient just to evaluate the mechanisms and establish an experimental basis for rational clinical therapy with IFN-β and TMZ. The precise mechanisms governing the direct effects of IFN-β and a combination of IFN-β and TMZ in gliomas are not yet fully understood.

Sensitivity/resistance to tumor chemotherapy may be very complex and often multifactorial with numerous genes involved. With the recent development of high-density oligonucleotide DNA array technology, the global gene expression profile can be analyzed simultaneously (14). In addition to the identification of new classes of human malignancies, this powerful method has become widely applied in an attempt to increase our understanding of chemotherapy sensitivity/resistance and to provide additional information on the underlying tumor biology that can be used to guide therapeutic strategies (15,16). As mentioned above, we reported previously a list of genes that may confer TMZ sensitivity/resistance in malignant gliomas employing a cDNA microarray. Furthermore, to gain new insight into the mechanisms of sensitivity/resistance involving IFN-β and combination therapy with IFN-β and TMZ in malignant gliomas, and to identify new marker(s) that could predict the response to therapy and new target gene(s) for the therapy related to malignant glioma pathogenesis, we evaluated the gene expression profiles of human malignant glioma cell lines employing the GeneChip high-density oligonucleotide DNA array technology.

## Materials and methods

*Tumor cell lines*. Human malignant glioma A-172, AM-38, T98G, U-251MG and YH-13 cells were purchased from Health Science Research Resources Bank (Sennan, Osaka, Japan) and U-87MG and U-138MG cells were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA). 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.

*Cell culture growth studies with IFN-β and a combination of TMZ and IFN-β.*

*Growth inhibitory effect of IFN-β*. The sensitivities of the seven glioma cell lines to IFN-β were evaluated from the concentration required for 75% growth inhibition ($IC_{75}$) in comparison with untreated controls. Briefly, cells were plated at $2x10^4$ 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 containing 0.1-1,000 IU/ml of IFN-β or medium (control). After exposure to the various concentrations of IFN-β for 72 h, cells were detached by trypsinization and the numbers counted. The experiments were repeated at least four times at each concentration.

*Combined effect of TMZ and IFN-β*. To assess the antitumor effect of a combination of TMZ and IFN-β, we investigated the following incubation conditions: 10 $\mu$M TMZ, 10 IU/ml IFN-β, or 10 $\mu$M TMZ and 10 IU/ml IFN-β. These conditions represent the relevant concentrations of TMZ and IFN-β (12). Briefly, cells were plated at $2x10^4$ 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 10 $\mu$M TMZ, 10 IU/ml IFN-β, or 10 $\mu$M TMZ and 10 IU/ml IFN-β for 72 h. After exposure to the various conditions for 72 h, cells were detached by trypsinization and the numbers counted. The experiments were repeated six 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 (17). 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 $\mu$g) 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 $\mu$g) 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 ~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 for IFN-β sensitivity*. 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 intensities 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 (18). 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 IFN-β sensitivity/resistance, Pearson's correlation was performed to evaluate the association between the $IC_{75}$ (sensitivity) of IFN-β and gene expression level of each gene, and Spearman's rank correlation coefficient was also calculated to confirm Pearson's correlation coefficient (Microsoft Office Excel 2007).

*Determination of 2'5'OAS activity*. 2'5'-Oligoadenylate synthetase (2'5'OAS) was first discovered in IFN-treated cells. Several reports have demonstrated a relation between 2'5'OAS level and the effect of IFN on chronic hepatitis C, signifying that measurement of the 2'5'OAS activity could be useful for evaluating IFN treatment, and an increase in level of 2'5'OAS may reflect the activation of the cellular IFN system (19-21). However, scarce data are available concerning the role of 2'5'OAS in malignant glioma cells. Therefore, to investigate whether or not IFN-β could induce antiviral proteins, 2'5'OAS in malignant glioma cells, the 2'5'OAS activity was determined at 24 h after incubation with IFN-β.

The 2'5'OAS activity levels in the seven malignant glioma cell lines were estimated by enzyme assay at 24 h after IFN-β treatment, using an Eiken 2-5A RIA kit (Eiken Immunochemical Laboratory, Tokyo, Japan). Briefly, pelleted cell samples ($1x10^6$ cells) were first lysed and incubated with polyI:C-agarose to be adsorbed and activated. Next, a mixture of $^{125}$I-labeled 2'5'OA, the anti-2'5'OA serum, and the second antibody was added. Following incubation, the mixture was centrifugally separated, and the supernatant was removed by suction to perform the B/F separation. Finally, the radioactivity of the precipitate was measured with a well-type scintillation counter. The binding rate with respect to the initially added quantity of $^{125}$I-labeled 2'5'OA was calculated, and the amount of 2'5'OA produced by the 2'5'OAS in the sample was obtained from the standard curve which was prepared at the same time. Enzyme activity was expressed in pmol/dl.

*Identification of discriminatory genes for the synergistic effect between TMZ and IFN-β*. Studies were undertaken to identify discriminative genes whose expression was related to a synergistic antitumor effect of combination therapy with TMZ and IFN-β in the seven malignant glioma cell lines. First, the cell lines were divided into two groups based on the presence or absence of a synergistic effect between TMZ and IFN-β in the cell cultures (see section on *Cell culture growth studies with IFN-β and a combination of TMZ and IFN-β*), confirmed with the Kruskal-Wallis H test and Mann-Whitney U test. The gene expression level of each gene was then compared and the difference calculated between two groups using the Student's t-test.

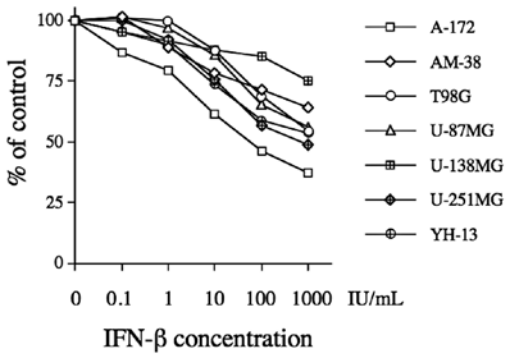


Figure 1. Antitumor effects of IFN-β in seven human malignant glioma-derived cell lines. At 72 h after addition of IFN-β (final concentration, 0-1,000 IU/ml) to the culture medium, the number of viable cells was counted. The number was expressed as a percentage of the untreated control.

Table I. $IC_{75}$ of IFN-β for seven malignant glioma cell lines.

| Cell line | $IC_{75}$ (IU/ml) |
|---|---|
| A-172 | 1.1 |
| AM-38 | 41.7 |
| T98G | 35.1 |
| U-87MG | 29.5 |
| U-138MG | n.r. |
| U-251MG | 9.2 |
| YH-13 | 8.6 |

$IC_{75}$, mean, represents the IFN-β concentration inducing a 75% decrease in cell growth as compared to the control. n.r., not reached.

## Results

We first obtained the individual $IC_{75}$ values of IFN-β (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 IFN-β sensitivity/resistance employing the GeneChip array and Pearson's correlation coefficient. Furthermore, to investigate whether or not IFN-β could induce antiviral proteins, 2'5'OAS in malignant glioma cell lines, the 2'5'OAS activity was determined at 24 h after incubation with IFN-β.

We next identified genes whose expression was related to a synergistic antitumor effect of combination therapy with TMZ and IFN-β in the malignant glioma cells. The cell lines were divided into two groups based on the presence or absence of a synergistic effect between TMZ and IFN-β, and genes were then identified whose expression levels were statistically different between the two groups.

*Antitumor effect and $IC_{75}$ of IFN-β in glioma cell lines*. To evaluate the antitumor effects of IFN-β in malignant glioma cells, we treated the seven malignant glioma cell lines with 0-1,000 IU/ml of IFN-β for 72 h, and assessed the numbers of viable cells. As shown in Fig. 1, cell growth inhibitory effects of IFN-β on all the tumor cell lines were observed in a dose-dependent manner. The $IC_{75}$ of IFN-β for three of the

Table II. Negative and positive differentially expressed genes associated with IFN-β sensitivity.

| Symbol<br>Location | Gene title | Correlation | P-value |
|---|---|---|---|
| PTEN<br>(PTENP1)<br>10q23.3 | Phosphatase and tensin homolog<br>(Phosphatase and tensin homolog pseudogene 1) | 0.878 | <0.0001 |
| ZNF643<br>1p34.2 | Zinc finger protein 643 | 0.866 | <0.0001 |
| NUP50<br>22q13.31 | Nucleoporin 50 kDa | 0.864 | <0.0001 |
| RAB4A<br>(SPHAR)<br>1q42-q43 | RAB4A, member of the Ras oncogene family<br>(S-phase response; cyclin related) | -0.915 | <0.0001 |
| RHOD<br>11q14.3 | Ras homolog gene family, member D | -0.913 | <0.0001 |
| SDF2<br>17q11.2 | Stromal cell-derived factor 2 | -0.898 | <0.0001 |
| HOXD11<br>2q31.1 | Homeobox D11 | -0.881 | <0.0001 |
| EIF2AK1<br>7p22 | Eukaryotic translation initiation factor 2-α kinase 1 | -0.880 | <0.0001 |

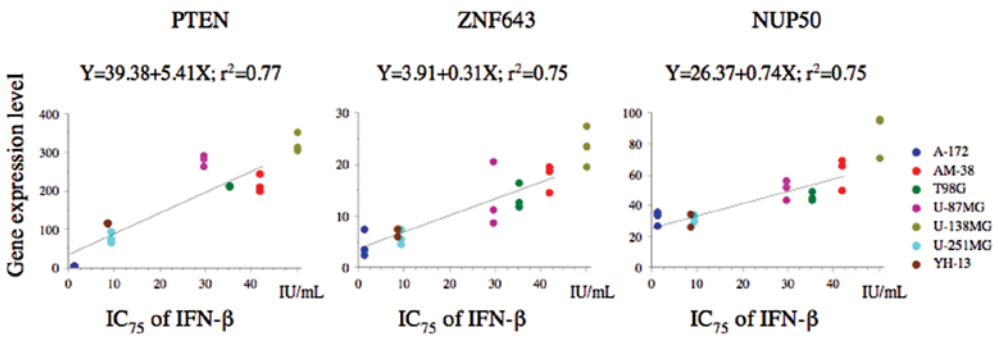


Figure 2. Positive-correlated genes (*PTEN*, *ZNF643* and *NUP50*) associated with IFN-β sensitivity/resistance ($IC_{75}$ of IFN-β) in malignant glioma cell lines.

malignant glioma cell lines, A-172, U-251MG and YH-13, was <10 IU/ml and that for three other malignant glioma cell lines, AM-38, T98MG and U-87MG, was >10 IU/ml. U-138MG was found to be resistant to IFN-β, since the U-138MG cells did not reach their $IC_{75}$ with 0.1-1,000 IU/ml of IFN-β treatment. The $IC_{75}$ values of IFN-β for each cell line are listed in Table I.

*Identification of discriminatory genes for IFN-β sensitivity.* From the total of 22,277 probe sets, no expressed gene was omitted, leaving 16,913 probe sets for subsequent analysis. Several upregulated and downregulated genes in the seven malignant glioma cell lines relative to their IFN-β sensitivity/resistance were observed. First, the highly significant statistically negative- and positive-correlated genes associated with IFN-β sensitivity/resistance were selected from six cell lines (excluding U-138MG, because its did not reach its $IC_{75}$ with 0.1-1,000 IU/ml of IFN-β treatment). We then picked out the genes that were compatible with the signal intensity of the gene expression level in U-138MG, as listed in Tables II and III. Pearson's correlation coefficients (r) and the corresponding P-values for these correlations are also shown in Tables II and III (comprising positive and negative higher ranks of Pearson's correlation coefficient, of >0.85 and <-0.85, and parametric P<0.00001). The gene-specific information was retrieved from Entrez Gene: gene-centered information at NCBI (22).

The genes that were picked out, except for *PTEN* (phosphatase and tensin homolog) and *EIF2AK1* (eukaryotic translation initiation factor 2-α kinase 1), have not, to our knowledge, been previously suspected to play a role in the prediction of IFN-β sensitivity/resistance, chemotherapy resistance, prognosis, tumor progression, or antiproliferation. The *PTEN* and *EIF2AK1* expression levels showed significant negative and positive correlations with IFN-β sensitivity/resistance, respectively (Figs. 2 and 3).

Table III. Up- and downregulated genes among the IFN-β sensitivity-associated genes.

| Symbol | Function | Process |
|---|---|---|
| PTEN | [a]PDZ domain binding | [a]Cell proliferation |
| | Enzyme binding | Inositol phosphate dephosphorylation |
| | Inositol-1,3,4,5-tetrakisphosphate | Negative regulation of cell migration |
| | 3-phosphatase activity | Negative regulation of cell proliferation |
| | Phosphatidylinositol-3,4,5-triphosphate | Negative regulation of focal adhesion assembly |
| | 3-phosphatase activity | Negative regulation of protein kinase B signaling |
| | Phosphatidylinositol-3,4-bisphosphate | cascade |
| | 3-phosphatase activity | Phosphoinositide dephosphorylation |
| | Phosphatidylinosotol-3-phosphatase activity | Protein amino acid dephosphorylation |
| | Protein binding | Regulation of cyclin-dependent protein kinase activity |
| | Protein serine/threonine phosphatase activity | Regulation of protein stability |
| | Protein tyrosine phosphatase activity | |
| ZNF643 | | |
| NUP50 | Protein binding | Intracellular transport |
| | | mRNA transport |
| | | Protein transport |
| | | Transmembrane transport |
| RAB4A | [a]GDP binding | Regulation of endocytosis |
| | GTP binding | Small GTPase-mediated signal transduction |
| | GTPase activity | |
| | Protein binding | |
| RHOD | GTP binding | Rho protein signal transduction |
| | GTPase activity | Small GTPase mediated signal transduction |
| | Nucleotide binding | |
| SDF2 | Dolichyl-phosphate-mannose-protein mannosyltransferase activity | Protein amino acid glycosylation |
| HOXD11 | Sequence-specific DNA binding | Anterior/posterior pattern formation |
| | Transcription factor activity | Branching involved in ureteric bud morphogenesis |
| | | Cartilage development involved in endochondral bone morphogenesis |
| | | Developmental growth |
| | | Dorsal/ventral pattern formation |
| | | Embryonic digit morphogenesis |
| | | Induction of an organ |
| | | Metanephros development |
| | | Pattern specification process |
| | | Positive regulation of cell development |
| | | Positive regulation of chondrocyte differentiation |
| | | Proximal/distal pattern formation |
| | | Regulation of transcription, DNA-dependent |
| | | Skeletal system development |
| EIF2AK1 | [a]Eukaryotic translation initiation factor 2α kinase activity | Negative regulation of cell proliferation |
| | | Negative regulation of hemoglobin biosynthetic process |
| | | Negative regulation of translation |
| | | Negative regulation of translation initiation by iron |
| | | Protein amino acid autophosphorylation |
| | | Response to external stimuli |
| | | Response to stress |

[a]Extracts from Entrez Gene.

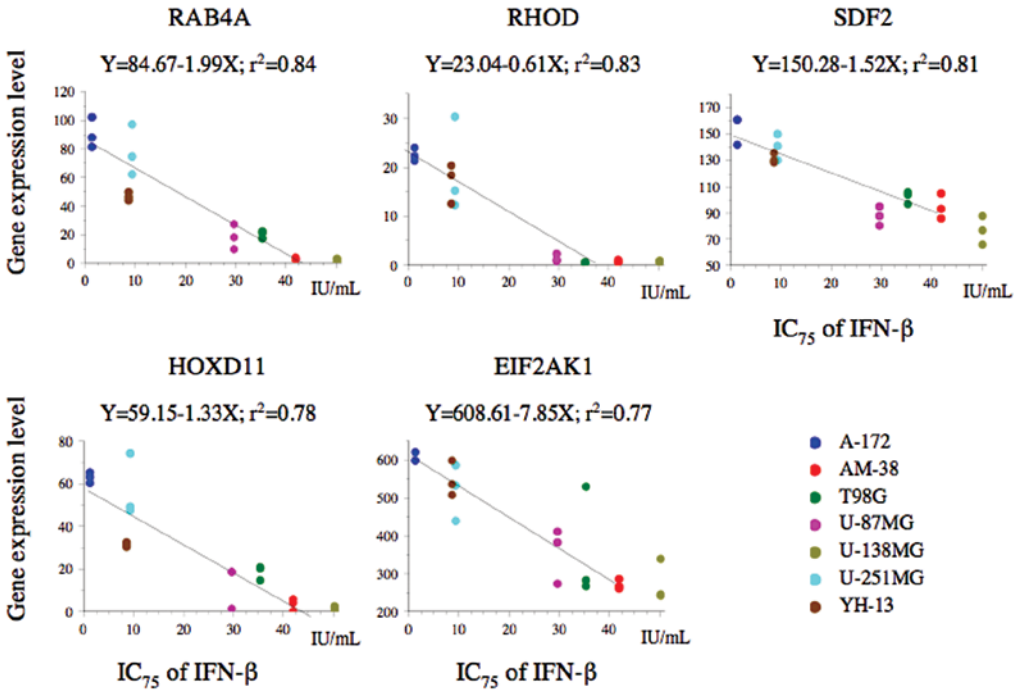


Figure 3. Negative-correlation genes (*RAB4A*, *RHOD*, *SDF2*, *HOXD11* and *EIF2AK1*) associated with IFN-β sensitivity/resistance ($IC_{75}$ of IFN-β) in malignant glioma cell lines.

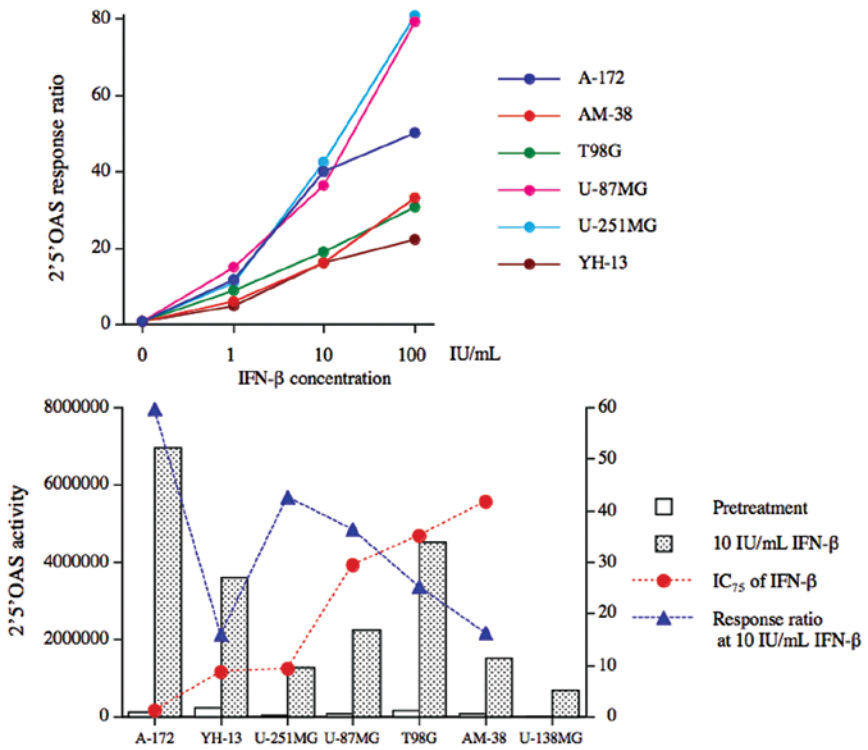


Figure 4. Upper graph: Changes in 2'5'-oligoadenylate synthetase (2'5'OAS) response ratio (measured value/measured value of control) in malignant glioma-derived cell lines at 24 h after addition of IFN-β (final concentration, 0-100 IU/ml) to the culture medium. Lower graph: 2'5'OAS activity in malignant glioma-derived cell lines at pretreatment and 24 h after addition of 10 IU/ml IFN-β to the culture medium. Red circles indicate the $IC_{75}$ of IFN-β for each glioma cell line. Blue triangles indicate the 2'5'OAS response ratio of each glioma cell line at 24 h after addition of 10 IU/ml IFN-β to the culture medium.

*IFN-β induction of 2'5'OAS activity in malignant glioma cells*. 2'5'OAS activities were detected in all the cell lines in the absence of any treatment, although the levels of 2'5'OAS activity varied widely, from 4,450 pmol/dl in U-138MG to 224,000 pmol/dl in YH-13 (Fig. 4). The 2'5'OAS activity level before IFN-β treatment in the U-138MG cell line, which did not reach its $IC_{75}$ with 0.1-1,000 IU/ml of IFN-β, was very low as compared to the other six cell lines (A-172, 117,000;

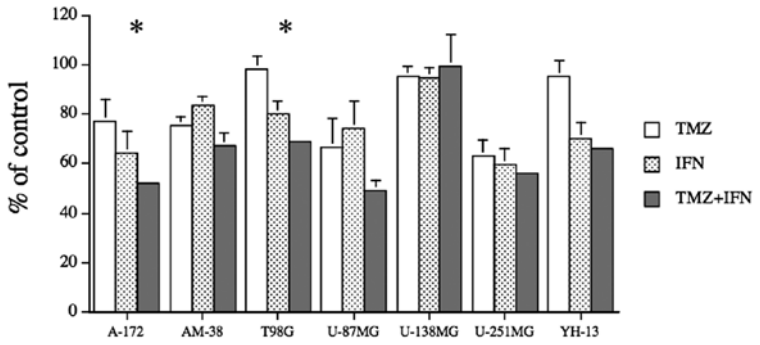


Figure 5. Cell growth inhibitory effect of 10 $\mu$M TMZ, 10 IU/ml IFN-β, or a combination of 10 $\mu$M TMZ and 10 IU/ml IFN-β for 72 h in seven glioma cell lines. The combination of TMZ and IFN-β revealed an additive cell growth inhibitory effect in the A-172 and T98G cell lines.

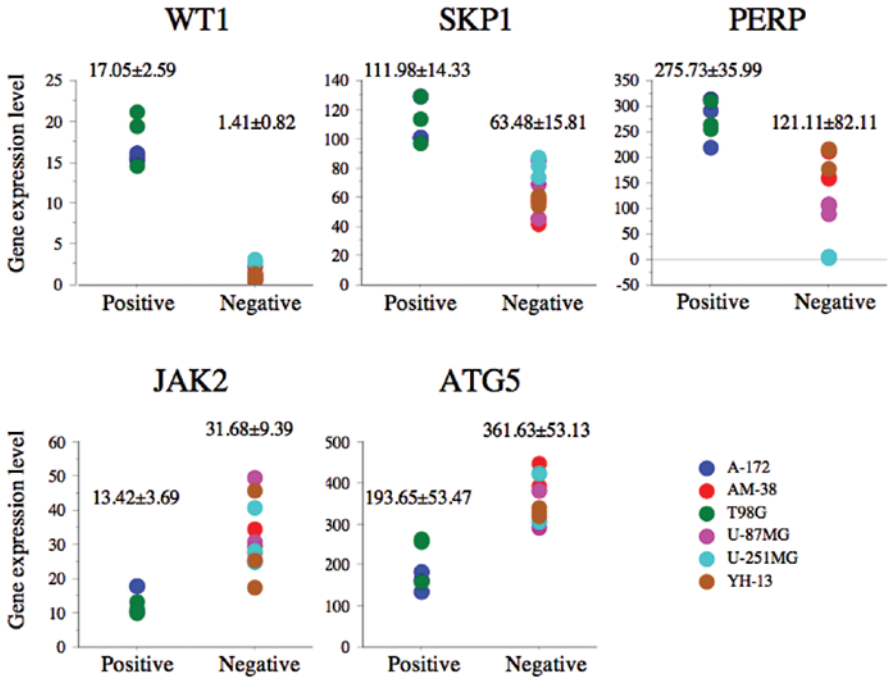


Figure 6. Highly significant statistical correlations of genes associated with the synergistic effect of TMZ and IFN-β.

AM-38, 92,800; T98G, 179,000; U-87MG, 61,700; U-251MG, 30,300; and YH-13, 224,000 pmol/dl). The 2'5'OAS activity levels at 24 h after 10 IU/ml IFN-β administration increased in a widely varied manner, ranging from 691,000 in U-138MG to 6,970,000 pmol/dl in A-172. The 2'5'OAS activity levels and response ratios (measured value/measured value of control 2'5'OAS) at 24 h in all the cell lines including U-138MG were dose-dependently increased by IFN-β (Fig. 4). However, the 2'5'OAS activity levels in U-138MG at 24 h after 1, 10 and 100 IU/ml IFN-β treatment were 137,000 (response ratio: 30.79), 691,000 (155.28) and 2,190,000 (492.13) pmol/dl, respectively, and these response ratios were very high as compared to the other six cell lines. With the exception of U-138MG, therefore, the 2'5' OAS response ratio appeared to be related to the IFN-β sensitivity/resistance, the $IC_{75}$ of IFN-β, although there was no statistical significance (Pearson's correlation coefficients (r) and corresponding P-values).

*Antitumor effect of a combination of TMZ and IFN-β.* To assess further whether or not a combination of TMZ and IFN-β could produce an additive antitumor effect in the seven malignant glioma cell lines, we employed Kruskal-Wallis one way analysis of variance on ranks to compare the overall differences among the cell growth inhibitory effects of TMZ, IFN-β and TMZ with IFN-β. When the Kruskal-Wallis H test revealed a significant difference between these therapies, non-parametric tests (Mann-Whitney U tests) were performed on the variables. As shown in Fig. 5, the combination of TMZ and IFN-β revealed an additive cell growth inhibitory effect in A-172 (Kruskal-Wallis H test: P=0.014; Mann-Whitney U test: TMZ vs. TMZ + IFN-β, P=0.046 and IFN-β vs. TMZ + IFN-β, P=0.046) and T98G (Kruskal-Wallis H test: P=0.004; Mann-Whitney U test: TMZ vs. TMZ + IFN-β, P=0.046 and IFN-β vs. TMZ + IFN-β, P=0.046).

We divided the seven malignant glioma cell lines into two groups according to the presence (A-172 and T98G) or absence (AM-38, U-87MG, U-251MG, U-138MG and YH-13) of a synergistic effect of combination therapy with TMZ and IFN-β. U-138MG was excluded from the next analysis of data because its did not reach its $IC_{75}$ with 0.1-1,000 IU/ml of IFN-β treatment.

Table IV. Positive differentially expressed genes associated with the synergistic effect of TMZ and IFN-β.

| Symbol | Gene title | P-value |
|---|---|---|
| ASXL1 | Additional sex combs like 1 (*Drosophila*) | 9.64862E-10 |
| SP110 | SP110 nuclear body protein | 3.57677E-09 |
| PRKCI | Protein kinase C, ι | 9.97207E-08 |
| IL15RA | Interleukin 15 receptor, α | 1.7555E-07 |
| IDH3B | Isocitrate dehydrogenase 3 ($NAD^+$) β | 3.57176E-07 |
| SQSTM1 | Sequestosome 1 | 1.00473E-06 |
| FAM20B | Family with sequence similarity 20, member B | 1.34683E-06 |
| RPS4Y1 | Ribosomal protein S4, Y-linked 1 | 1.41684E-06 |
| CYB561 | Cytochrome b-561 | 3.22711E-06 |
| FDPS | Farnesyl diphosphate synthase | 3.25631E-06 |
| NSFL1C | NSFL1 (p97) cofactor (p47) | 3.27798E-06 |
| C20orf29 | Chromosome 20 open reading frame 29 | 3.59118E-06 |
| SARM1 | Sterile α and TIR motif containing 1 | 3.62956E-06 |
| SLMO2 | Slowmo homolog 2 (*Drosophila*) | 4.31792E-06 |
| CANX | Calnexin | 4.6137E-06 |
| CYB561 | Cytochrome b-561 | 5.20632E-06 |
| TRIM13 | Tripartite motif-containing 13 | 5.25434E-06 |
| CRNKL1 | Crooked neck pre-mRNA splicing factor-like 1 (*Drosophila*) | 5.40425E-06 |
| PARVA | Parvin, α | 5.56053E-06 |
| CDC14B | CDC14 cell division cycle 14 homolog B (*S. cerevisiae*) | 5.85645E-06 |
| FBXO38 | F-box protein 38 | 8.40968E-06 |
| DBNDD2 | Dysbindin domain containing 2 | 9.78368E-06 |
| C16orf59 | Chromosome 16 open reading frame 59 | 9.92373E-06 |
| WT1 | Wilms tumor 1 | 1.27384E-05 |
| BAIAP2 | BAI1-associated protein 2 | 1.45021E-05 |
| NFE2L3 | Nuclear factor (erythroid-derived 2)-like 3 | 1.58679E-05 |
| FBXO41 | F-box protein 41 | 1.83297E-05 |
| TRAF3IP3 | TRAF3 interacting protein 3 | 1.89558E-05 |
| DHRS11 | Dehydrogenase/reductase (SDR family) member 11 | 2.06968E-05 |
| HDGF | Hepatoma-derived growth factor | 2.10099E-05 |
| NFYC | Nuclear transcription factor Y, γ | 2.39037E-05 |
| MX2 | Myxovirus (influenza virus) resistance 2 (mouse) | 3.46831E-05 |
| TST | Thiosulfate sulfurtransferase (rhodanese) | 3.69159E-05 |
| C22orf9 | Chromosome 22 open reading frame 9 | 3.72462E-05 |
| MST1R | Macrophage stimulating 1 receptor (c-met-related tyrosine kinase) | 3.81903E-05 |
| CYB561 | Cytochrome b-561 | 3.91764E-05 |
| KIAA0753 | KIAA0753 | 3.93048E-05 |
| SKP1 | S-phase kinase-associated protein 1 | 4.08025E-05 |
| MED8 | Mediator complex subunit 8 | 4.40488E-05 |
| PERP | PERP, TP53 apoptosis effector | 4.53502E-05 |
| F12 | Coagulation factor XII (Hageman factor) | 4.54464E-05 |
| PSMF1 | Proteasome inhibitor subunit 1 (PI31) | 4.59967E-05 |
| HMGN1 | High-mobility group nucleosome binding domain 1 | 4.85703E-05 |
| FECH | Ferrochelatase (protoporphyria) | 5.10453E-05 |
| TGIF2 | TGFB-induced factor homeobox 2 | 5.23765E-05 |
| KDM5D | Lysine (K)-specific demethylase 5D | 5.46928E-05 |
| IL10RB | Interleukin 10 receptor, β | 5.52969E-05 |
| SCLY | Selenocysteine lyase | 5.69998E-05 |
| MEGF9 | Multiple EGF-like-domains 9 | 5.98117E-05 |
| CRKRS | Cdc2-related kinase, arginine/serine-rich | 6.0001E-05 |

Table IV. Continued.

| Symbol | Gene title | P-value |
|---|---|---|
| TSFM | Ts translation elongation factor, mitochondrial | 6.72525E-05 |
| SEPT6 | Septin 6 | 7.24721E-05 |
| CLNS1A | Chloride channel, nucleotide-sensitive, 1A | 7.62169E-05 |
| CCNJL | Cyclin J-like | 9.11443E-05 |
| CDK5RAP1 | CDK5 regulatory subunit-associated protein 1 | 9.4337E-05 |
| GYPC | Glycophorin C (Gerbich blood group) | 9.78694E-05 |
| COL18A1 | Collagen, type XVIII, α 1 | 9.81393E-05 |
| MPHOSPH8 | M-phase phosphoprotein 8 | 9.81774E-05 |

*Identification of discriminatory genes for the synergistic effect of combination therapy with TMZ and IFN-β*. We compared each gene expression level of 16,913 probe sets between the two groups (presence vs. absence of a synergistic antitumor effect) employing the unpaired test. Many upregulated and downregulated genes relative to the synergistic effect of TMZ and IFN-β were observed. The genes exhibiting highly significant statistical correlations associated with the synergistic effect of TMZ and IFN-β are listed in Tables IV and V. Most of the genes in Tables IV and V have not, to our knowledge, been previously suspected to play a role in the prediction of chemotherapy resistance, prognosis, tumor progression, or antiproliferation. However, five genes [namely, Wilms' tumor 1 (*WT1*), S-phase kinase-associated protein 1 (*SKP1*), TP53 apoptosis effector (*PERP*), Janus-activated kinase 2 (*JAK2*) and autophagy-related 5 homolog (*ATG5*)] have previously been reported to be associated with such processes (Table VI, Fig. 6). The gene-specific information was retrieved from Entrez Gene: gene-centered information at NCBI (22).

## Discussion

It is possible that large-scale gene expression analysis, rather than analysis of single genes or pathways, can yield a more robust predictor of responses to chemotherapy in tumors, since their sensitivity/resistance may be complex with multiple mechanisms and involve many genes (16,23). Moreover, with the variable responses to chemotherapy in tumors, the clinical therapeutic outcome tends to be determined after a relatively long-term treatment, so that early marker(s) of treatment sensitivity/resistance are required. Such marker(s) would help to improve patient care, allowing the rapid selection of effective drug(s) and equally the exclusion of ineffective drug(s), with time-saving consequences, and a more efficient use of resources (24). We therefore evaluated the gene expression profiles employing GeneChip in seven malignant human glioma-derived cell lines in an attempt to identify new marker(s) that could be applied clinically to predict the response to therapy and new target gene(s) for the therapy related to malignant glioma pathogenesis.

One purpose of the present study was to identify molecular marker(s) that reflected therapeutic responses to the direct antitumor effect of IFN-β in malignant glioma cells, since IFN-β is known to be partially effective for treating malignant glioma patients; clinical trials of IFN-β have resulted in partial remission in 50% or more of patients tested (25,26), and the pharmaco-dynamics at the genomic level in humans remain poorly understood. To our knowledge, this is the first report on gene expression profiles to identify marker(s) that could be used clinically to predict malignant gliomas responses to IFN-β. The classification of the IFN family of proteins is based mainly on their sequence, chromosomal location and receptor specification (27). IFNs are classified into three major groups: type I, II and III. Type I IFNs include IFN-α, -β, -ω, -ε and -κ. The human type I IFN family includes 13 IFN-α genes, one IFN-β gene and two IFN-ω genes (27). It is now accepted that most nucleated cells are capable of producing type I IFN, whereas type II IFN production is restricted to immune system cells (5). All members of the type I IFN family transmit signals through a receptor complex composed of two subunits, IFNAR-1 and IFNAR-2. In response to the different types of IFNs, members of the signal transducer and activator of transcription (STAT) family are activated through tyrosine phosphorylation, by the JAK family, which leads to their homo- or hetero-dimerization, nuclear translocation and transcription of IFN-stimulated genes (ISGs). The transcriptional regulation of effector genes downstream to the JAK/STAT or other IFN-regulated pathways contributes to the pleiotropic responses induced by IFNs (27). It is conceivable that numerous direct effects may play a central role in the overall antitumor response, such as downregulation of oncogene expression, induction of differentiation, tumor suppressor genes and programmed cell death, and inhibition of cell cycle progression (5). Furthermore, IFN-β has consistently been proven to be more potent than IFNs-α in inducing antiproliferative effects and apoptosis (5,28).

In the present report, the highly significant statistically positive- and negative-correlation genes associated with IFN-β sensitivity/resistance are listed in Tables II and III. To our knowledge, six of the eight genes have not previously been suspected to be related to IFN-β, chemotherapy resistance, prognosis, tumor progression, or antiproliferation. The other two genes, one upregulated gene, *PTEN*, and one downregulated gene, *EIF2AK1*, have previously been reported to be associated with such processes. Although the underlying mechanism of these genes that confers IFN-β sensitivity/resistance remains to be fully elucidated, the following facts could lend support

Table V. Negative differentially expressed genes associated with the synergistic effect of TMZ and IFN-β.

| Symbol | Gene title | P-value |
|---|---|---|
| BAG5 | BCL2-associated athanogene 5 | 4.18357E-08 |
| HCFC2 | Host cell factor C2 | 1.42298E-07 |
| C6orf120 | Chromosome 6 open reading frame 120 | 3.42941E-07 |
| STK17A | Serine/threonine kinase 17a | 6.6393E-07 |
| MAGED4 | Melanoma antigen family D, 4 | 7.5016E-07 |
| PLAGL1 | Pleiomorphic adenoma gene-like 1 | 8.03872E-07 |
| MYO5A | Myosin VA | 1.3738E-06 |
| C1orf114 | Chromosome 1 open reading frame 114 | 1.81194E-06 |
| MORC4 | MORC family CW-type zinc finger 4 | 2.86267E-06 |
| MARK3 | MAP/microtubule affinity-regulating kinase 3 | 3.74741E-06 |
| NOVA1 | Neuro-oncological ventral antigen 1 | 4.25897E-06 |
| EIF5 | Eukaryotic translation initiation factor 5 | 4.34682E-06 |
| FBXL14 | F-box and leucine-rich repeat protein 14 | 6.93089E-06 |
| VTI1B | Vesicle transport through interaction with t-SNAREs homolog 1B | 7.04442E-06 |
| C14orf1 | Chromosome 14 open reading frame 1 | 8.3473E-06 |
| AGTPBP1 | ATP/GTP binding protein 1 | 1.05056E-05 |
| USE1 | Unconventional SNARE in the ER 1 homolog | 1.14613E-05 |
| SYNJ2BP | Synaptojanin 2 binding protein | 1.21478E-05 |
| DAGLA | Diacylglycerol lipase, α | 1.31789E-05 |
| FAM3C | Family with sequence similarity 3, member C | 1.34942E-05 |
| QDPR | Quinoid dihydropteridine reductase | 1.38781E-05 |
| ZNF410 | Zinc finger protein 410 | 1.43664E-05 |
| CHST2 | Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 | 1.67993E-05 |
| MKLN1 | Muskelin 1, intracellular mediator | 1.81471E-05 |
| SNRPN | Small nuclear ribonucleoprotein polypeptide N | 1.81994E-05 |
| GMFB | Glia maturation factor, β | 1.82028E-05 |
| C14orf2 | Chromosome 14 open reading frame 2 | 1.83431E-05 |
| CD164 | CD164 molecule, sialomucin | 1.87403E-05 |
| DIAPH2 | Diaphanous homolog 2 (*Drosophila*) | 2.09903E-05 |
| JAK2 | Janus kinase 2 | 2.55919E-05 |
| GLRB | Glycine receptor, β | 2.57272E-05 |
| IPW | Imprinted in Prader-Willi syndrome (non-protein coding) | 2.63332E-05 |
| FYN | FYN oncogene related to SRC, FGR, YES | 2.67459E-05 |
| VCAN | Versican | 2.72731E-05 |
| CNIH | Cornichon homolog (*Drosophila*) | 2.731E-05 |
| SRP54 | Signal recognition particle 54 kDa | 2.80296E-05 |
| GOLGA5 | Golgi autoantigen, golgin subfamily a, 5 | 3.09569E-05 |
| CRKRS | Cdc2-related kinase, arginine/serine-rich | 3.19435E-05 |
| MID1 | Midline 1 (Opitz/BBB syndrome) | 3.31528E-05 |
| PRDX4 | Peroxiredoxin 4 | 3.33043E-05 |
| OCRL | Oculocerebrorenal syndrome of Lowe | 3.40594E-05 |
| TNC | Tenascin C | 3.46468E-05 |
| ARMCX3 | Armadillo repeat containing, X-linked 3 | 3.55829E-05 |
| ELOVL4 | Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4 | 3.56104E-05 |
| APLP2 | Amyloid β (A4) precursor-like protein 2 | 3.8049E-05 |
| GEM | GTP binding protein overexpressed in skeletal muscle | 4.15723E-05 |
| RP6-213H19.1 | Serine/threonine protein kinase MST4 | 4.23636E-05 |
| LOC222070 | Hypothetical protein LOC222070 | 4.73949E-05 |
| SFRS9 | Splicing factor, arginine/serine-rich 9 | 4.84891E-05 |
| SPRY4 | Sprouty homolog 4 (*Drosophila*) | 4.86094E-05 |

Table V. Continued.

| Symbol | Gene title | P-value |
|---|---|---|
| ADAM12 | ADAM metallopeptidase domain 12 | 5.20472E-05 |
| C14orf143 | Chromosome 14 open reading frame 143 | 5.31393E-05 |
| EBP | Emopamil binding protein (sterol isomerase) | 6.18726E-05 |
| TMED10 | Transmembrane emp24-like trafficking protein 10 (yeast) | 6.20724E-05 |
| CREM | cAMP responsive element modulator | 6.49662E-05 |
| CDC42BPB | CDC42 binding protein kinase β (DMPK-like) | 6.66371E-05 |
| GJA1 | Gap junction protein, α 1, 43 kDa | 7.17477E-05 |
| ZHX2 | Zinc fingers and homeoboxes 2 | 7.50947E-05 |
| ATG5 | ATG5 autophagy-related 5 homolog (*S. cerevisiae*) | 8.78822E-05 |
| RSU1 | Ras suppressor protein 1 | 9.04917E-05 |
| SLC9A1 | Solute carrier family 9 (sodium/hydrogen exchanger), member 1 | 9.12332E-05 |
| GLRX5 | Glutaredoxin 5 | 9.4899E-05 |
| PTPN12 | Protein tyrosine phosphatase, non-receptor type 12 | 9.82443E-05 |

to our results and suggest alternative therapeutic rationales as part of a new biomolecular approach to the treatment of malignant gliomas.

*PTEN* is one of the most frequent mutated genes in human cancers. The frequency of *PTEN* mutations in glioblastomas is ~30%, varying from 9 to 44% (29). The potential protein targets of PTEN range from membrane-bound receptor tyrosine kinases and cytoplasmic signaling molecules to transcription factors in the nucleus. The phosphoinositol-3-kinase (PI3-kinase)/AKT pathway is regarded as the primary physiological target of PTEN. Phosphatase activity, particularly lipid phosphatase activity, is believed to be the most pertinent property of PTEN as a tumor suppressor (29). Indeed, overexpression of wild-type *PTEN* in tumor cells induces apoptosis and cell cycle arrest, colony formation and cell migration (29-32). In particular, high nuclear PTEN levels have been found to be associated with the G0-G1 phase and lower nuclear PTEN levels with the S phase, implying that nuclear PTEN activity might directly regulate the cell cycle (33). It has been suggested therefore that PTEN acts on chromatin and regulates the transcription of important genes involved in critical cellular processes such as cell cycle control and DNA repair (29). On the other hand, *p53* is the most frequently mutated gene in human tumors; and in terms of overall frequency, *PTEN* is the second most commonly mutated gene in human tumors. However, the mutation spectra of *p53* and *PTEN* in human tumors are different. Mutations of *p53* occur at high frequencies in lung, colon and breast cancers, whereas *PTEN* mutations are found mostly in glioblastomas, endometrial cancers, malignant melanomas and prostate cancers (29). In their role as the autonomic surveillance and defense systems to genomic instability and tumorigenesis, tumor suppressors act in coordination with each other to form regulatory networks (29). The existence of relationships between *PTEN* and *p53* is thus an intriguing and theoretically attractive question. Studies on their spectra of mutations have revealed that *PTEN* and *p53* are usually independent and mutually exclusive (29,34). Furthermore, the p53 protein is maintained at low levels in normal cells and its induction requires stimulation upon cellular stress, whereas PTEN expression levels are steady and high in normal cells and maintenance of its abundance is important for sustaining its function in safeguarding the genome (29). On the other hand, PTEN has been reported as a downstream transcriptional target of p53 in mediating apoptosis (29). It has also been suggested that PTEN may act upstream of p53 to regulate its expression levels and activity (29,35,36). Furthermore, transcription of the *p53* gene can be induced by IFN-α/β in tumors, accompanied by an increase in p53 protein level (37). The above findings indicate that these two guardians of the genome, *PTEN* and *p53*, may act to complicate the antitumor effect of IFN-β in malignant gliomas.

The proteins with important biological functions that are induced by IFNs include two double-stranded RNA-activated enzymes: double-stranded RNA-dependent protein kinase (PKR) and 2'5'OAS (21). When an antiviral pathway is induced by IFNs or activated by double-stranded RNA, the PKR becomes autophoshorylated and catalyzes phosphorylation of the protein synthesis initiation factor EIF2 (eukaryotic translation initiation factor 2), while the 2'5'OAS forms 2'5'OA molecules that activate the latent endoribonuclease, RNase L; the PKR/EIF2 and 2'5'OAS/RNase L pathways, respectively (21,38). These enzymes are also implicated in the regulation of other cellular events, such as gene induction, normal control of cell growth, differentiation and apoptosis (21,38,39).

The other noteworthy gene in the present study that may be involved in conferring IFN-β sensitivity/resistance is *EIF2AK1*; the PKR/EIF2 pathway. PKR, namely eukaryotic translation initiation factor 2-α kinase (EIF2AK), can inhibit overall protein synthesis via phosphorylation of the α subunit of eukaryotic initiation factor 2, EIF2A. Such regulation of translation may potentially play an important role in apoptosis induction (21). To distinct degrees, at least EIF2A, NF-κB, ATF-3 and p53 have been implicated in mediating PKR-induced apoptosis (40). As a consequence of the effects of PKR on translation, transcription and apoptosis, PKR can function to control cell growth and cell differentiation, and its

Table VI. Up- and downregulated genes associated with the synergistic effect of TMZ and IFN-β.

| Symbol | Function | Process |
|---|---|---|
| WT1 | [a]$C_2H_2$ zinc finger domain binding | [a]Adrenal gland development |
| | Promoter binding | Branching involved in ureteric bud morphogenesis |
| | Protein binding | Glomerular basement membrane development |
| | Sequence-specific DNA binding | Glomerulus development |
| | Specific transcriptional repressor activity | Heart development |
| | Transcription activator activity | Induction of apoptosis |
| | NOT transcription activator activity | Kidney development |
| | Transcription activator activity | Male gonad development |
| | Transcription factor activity | Mesonephros development |
| | Transcription repressor activity | Metanephric S-shaped body morphogenesis |
| | Zinc ion binding | Negative regulation of apoptosis |
| | | Negative regulation of cell growth |
| | | Negative regulation of cell proliferation |
| | | Negative regulation of gene-specific transcription |
| | | Negative regulation of transcription |
| | | Negative regulation of transcription from RNA polymerase II promoter |
| | | Negative regulation of translation |
| | | Positive regulation of gene-specific transcription |
| | | NOT-positive regulation of gene-specific transcription |
| | | Positive regulation of transcription |
| | | Regulation of transcription, DNA-dependent |
| | | Sex determination |
| | | Visceral serous pericardium development |
| SKP1 | [a]Protein binding | SCF-dependent proteasomal ubiquitin-dependent protein catabolic processes |
| | | Positive regulation of the ubiquitin-protein ligase activity involved in the mitotic cell cycle |
| | | Protein ubiquitination |
| PERP | | Apoptosis |
| | | Cell adhesion |
| | | Induction of apoptosis |
| JAK2 | [a]SH2 domain binding | [a]JAK-STAT cascade |
| | Histone binding | Cellular component movement |
| | Histone kinase activity (H3-Y41 specific) | Histone H3-Y41 phosphorylation |
| | Protein binding | Intracellular signaling pathway |
| | Protein kinase activity | Mesoderm development |
| | Protein tyrosine kinase activity | Protein amino acid phosphorylation |
| | Receptor binding | Response to antibiotics |
| ATG5 | | Autophagic vacuole assembly |
| | | Autophagy |
| | | Blood vessel remodeling |
| | | Heart contraction |
| | | Negative regulation of apoptosis |
| | | Negative regulation of protein ubiquitination |
| | | Post-translational protein modification |
| | | Response to drugs |
| | | Vasodilation |
| | | Ventricular cardiac muscle cell development |

[a]Extracts from Entrez Gene.

activity can be controlled by the action of several tumorigenes (40).

The other pathway among the proteins with important biological functions induced by IFNs, as described above, is the 2'5'OAS/RNase L pathway. 2'5'OAS is specifically induced by viral infection or extracellular introduction of IFN and plays an important role in molecular events that lead to the antiviral action provoked by IFN in the host cells (19). Indeed, based on the clinical observation in chronic hepatitis C patients that the serum 2'5'OAS level is generally increased on administration of IFN, the serum level of this enzyme has been used as a clinical marker to predict the responsiveness of the host to IFN (19,21). However, there are few data concerning the detailed role of 2'5'OAS in malignant glioma cells. Therefore, to investigate whether or not IFN-β could induce antiviral proteins, 2'5'OAS in malignant glioma cells, the 2'5'OAS activity was determined at 24 h after incubation with IFN-β. Although the reason remains unclear, the 2'5'OAS level before IFN-β treatment in the U-138MG cell line, which did not reach its $IC_{75}$ with 0.1-1,000 IU/ml of IFN-β, was very low compared to that of the other six cell lines. The levels of 2'5'OAS activity and response ratio (measured value/measured value of control 2'5'OAS) in all the glioma cell lines were dose-dependently increased by IFN-β. Furthermore, the 2'5'OAS response ratio was closely related to IFN-β sensitivity/resistance, the $IC_{75}$ of IFN-β, but not statistically significant (Fig. 4). Although the pretreatment value and response ratio of 2'5'OAS activity appear to be important factors for IFN-β sensitivity/resistance, the present results suggest that measurement of the 2'5'OAS activity might be a useful parameter for assessing the response to IFN-β treatment not only in virus infections but also in malignant gliomas, and could help to resolve the question of the optimal dose and schedule of IFN-β treatment.

On the other hand, we demonstrated a sensitization of glioma cells to the TMZ-induced antitumor effect of IFN-β in the A-172 and T98G cell lines. Our prior studies have shown that T98G, U-138MG and YH-13 had MGMT mRNA and protein expression, whereas A-172, AM-38, U-87MG and U-251 did not have them (3). Further, a synergistic antitumor effect of combination treatment with IFN-β and TMZ was observed in the TMZ-resistant glioma cells, T98G, due possibly to an attenuation of their *MGMT* mRNA levels (12). However, in the present study, other MGMT-positive cell lines, at least YH-13, did not exhibit a synergistic effect between IFN-β and TMZ, whereas the MGMT-negative cell line, A-172, displayed a synergistic effect with a combination of TMZ and IFN-β. Thus, the synergistic effect of TMZ and IFN-β may not be limited to MGMT-negative or -positive cell lines, suggesting that IFN-β might represent a powerful agent for increasing the benefit from TMZ in certain malignant gliomas. Many upregulated and downregulated genes associated with the synergistic effect between TMZ and IFN-β are listed in Tables IV and V. To our knowledge, most of the genes in these tables have not previously been suspected to be related to chemotherapy resistance, prognosis, tumor progression, or antiproliferation, in gliomas. However, five genes, three upregulated genes (*WT1*, *SKP1* and *PERP*) and two downregulated genes (*JAK2* and *ATG5*), have previously been reported to be associated with such processes. In particular, the noteworthy genes that may be involved in conferring the synergistic effect between TMZ and IFN-β are *WT1* and *JAK2*.

Although *WT1*, the Wilms' tumor gene, was originally isolated as a tumor-suppressor gene that was inactivated in a subset of Wilms' tumors, a common pediatric kidney cancer, it was later found to be overexpressed in many types of tumors, including breast carcinomas, acute leukemia and glioblastomas (41,42). It was proposed therefore that the wild-type *WT1* gene may play an oncogenic role rather than a tumor-suppressor role in the tumorigenesis of various types of tumors. Recent studies have indicated that the *WT1* gene plays an important role in the tumorigenesis of malignant gliomas and suggested that *WT1* may represent a new molecular target for the treatment of malignant gliomas expressing WT1 (41,42). JAK2 is one member of a family of four cytoplasmic tyrosine kinases that also includes JAK1, JAK3 and Tyk2 (tyrosine kinase 2) (43). JAK2 can phosphorylate EGFR (epidermal growth factor receptor) to activate the mitogen-activated protein kinase (MAPK) pathway (44). STAT3, which can be activated by JAK2, EGFR and other tyrosine kinases, is a center for many signaling pathways. It plays a crucial role in tumorigenesis, and efforts are ongoing to target it in antitumor drug developments (45,46). The above characterizations of the molecular targets implicated in the sensitivity/resistance of combination therapy with IFN-β and TMZ are considered valuable at least in helping to refine the therapeutic benefits of TMZ for malignant gliomas in the near future.

Finally, several limitations and implications of the present study will be briefly discussed. First, the criticism can always be made that the expression profiles of cell lines do not exactly represent the genetic events that occur in actual tumors. Second, the present study was restricted to the use of only seven malignant glioma-derived cell lines, and because of the heterogeneity of the disease, this was not sufficient to provide a conclusive picture of the mechanism of action. Third, it is possible that if the entire genome were to be analyzed, the number of potential molecular markers for treatment and chemotherapy targets would increase. Fourth, in general, the gene expression correlates of drug sensitivity/resistance tend to be complex, and their biological significance is not easily interpretable. Although larger experiments are needed to confirm our findings, the data do identify genes and pathways that could not only be potential molecular markers but also attractive targets for therapeutic modulation of malignant gliomas. Furthermore, the present study may provide a foundation for treatments that could improve the outcome by devising strategies that are able to circumvent primary drug resistance in malignant gliomas.

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