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EXPERIMENTAL STUDY

The effect of temozolomide on apoptosis-related gene expression changes in glioblastoma cells

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ABSTRACT

BACKGROUND: Glioblastoma (GB) is the most common and biologically the most aggressive primary brain tumor of the central nervous system (CNS) in adults. Standard treatment for newly diagnosed GB consists of surgical resection, radiotherapy, and chemotherapy with temozolomide (TMZ). Despite numbers of studies, a resistance to chemotherapy is the major obstacle to successful GB treatment.

OBJECTIVES: The aim of our study was to detect the sensitivity of glioblastoma T98G cells to TMZ treatment and subsequently to determine the expression changes of apoptosis-associated genes in glioblastoma cells.

MATERIAL AND METHODS: The human glioblastoma cell line (T98G) was treated with specified concentrations of TMZ during different time periods. Their viability was measured by colorimetric MTT assay and the activation of the apoptotic pathway was determined by measuring the caspase 3/7 activity. Commercial pre-designed microfluidic array was used to quantify expression of human apoptosis-associated genes.

RESULTS: The untreated control of T98G cell line against human brain total RNA standards reported significant changes in several apoptotic genes expression levels. We identified also a deregulation in gene expression levels between the TMZ treated and untreated T98G cells associated with apoptotic pathways. After 48 hours of exposure of T98G cells to TMZ, we observed a significant deregulation of seven genes: *BBC3*, *BCL2L1*, *RIPK1*, *CASP3*, *BIRC2*, *CARD6* and *DAPK1*. These results can contribute to the importance of apoptosis in glioblastoma cells metabolism and effect of TMZ treatment.

CONCLUSIONS: Identification of apoptotic gene panel in T98G cell line could help to improve understanding of brain tumor cells metabolism. Recognizing of the pro-apoptotic and anti-apoptotic genes expression changes could contribute to clarify the sensitivity to TMZ therapy and molecular base in healthy and tumor cells *(Tab. 1, Fig. 2, Ref. 48)*. Text in PDF *www.elis.sk*

KEY WORDS: apoptosis, glioblastoma, temozolomide, gene expression.

Abbreviations: AIC – 5-aminoimidazole-4-carboxamide; APAF1 – apoptotic peptidase activating factor 1; Bcl-2 – B-cell lymphoma 2; BER – base excision repair; CNS – central nervous system; ECACC – European Collection of Authenticated Cell Cultures; GB – glioblastoma; MGMT – O6-methylguanine-DNA methyltransferase; MMR – miss-match repair; MTIC – 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N3-Me-A – N3-methyladenine; N3-Me-Pu – N3-methylpurine; N7-MeG – N7-methylguanine; N7-Me-Pu – N7-methylpurine; O6-Me-G – O6-methylguanine; SCs – stem cells; TMZ – temozolomide; TNF – tumor necrosis factor.

Introduction

The changes in the regulation of cell growth and programmed cell death signalling might result in an uncontrolled proliferation and disorganized growth of tissue cells, followed by tumor formation. Malignant gliomas are one of the most frequent and the most aggressive primary tumors of the central nervous system (CNS) and belong to very serious medical issues nowadays (1, 2). Glioblastoma (GB), WHO grade IV of malignancy, is the most aggressive and the most frequent primary brain tumor of the CNS with account for 60-70% of all gliomas (3). The majority of glioblastomas arise *de novo* as a primary GB, but about one third develops slowly as secondary GB by malignant progression from lower grade astrocytoma (4). The prognosis remains very poor, with the median survival of newly diagnosed GB patients ranged from 9 to 12 months prior to the introduction of the TMZ treatment (5).

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Knowledge of the brain tumors genetic patterns inheritance and molecular pathways alteration is poor and it is difficult to diagnose a patient in the early stages of the disease. The treatment of brain tumors depends on the histological diagnosis. Postoperative radiotherapy and chemotherapy have an improved survival of the patients with high-grade brain tumors (2, 4). For a successful treatment is also very important to eliminate cancer stem cells (SCs), which present the key factor in tumor relapse (6). Stupp et al. defined the standard care and treatment for newly diagnosed GB after the maximal surgical resection as chemotherapy with TMZ combined with radiotherapy, followed by a post-radiation phase of TMZ administration (7). Despite the extensive investigations, a cure for GB is currently not available. Radiotherapy and chemotherapy work predominantly by inducing apoptosis and the resistance to clinical therapy still remains the major obstacle to successful treatment (8, 9).

The addition of TMZ to radiotherapy for newly diagnosed GB brings a clinically meaningful and statistically significant survival benefit and minimal additional toxicity. The median survival for TMZ plus radiotherapy was 14.6 months and 12.1 months with radiotherapy alone (7). Ronning et al. demonstrated, that TMZ added to radiotherapy can prolongs the survival for GB patients to 16.2 months from time of diagnosis (10). The long- term study (>10 years) in 128 patients with the diagnosis of high -grade gliomas set the combination therapy as the gold standard in the treatment of this disease nowadays (11).

A deregulation of the normal mechanism of programmed cell death plays an important role in the pathogenesis and progression of malignant gliomas. Programmed cell death type I, apoptosis, can be activated by multiple pathways that differ among the tissue type and pathological conditions. Better understanding of the apoptotic signalling pathway mechanism in GB may identify target molecules for molecular therapies (12, 13).

In the extrinsic apoptotic pathway, the lethal signal comes from the extracellular environment and is transduced within cells by specific death receptors. The tumor necrosis factor (TNF) family proteins and its corresponding surface death receptors (TNFα or Fas) play important roles in cell death or survival, proliferation, and maturation. Subsequently, the proteins of TNF superfamily activate nuclear factors of kappa light polypeptide gene enhancer in B-cells (NF-κB) superfamily that suppresses apoptosis, cell survival and proliferation. Members of these families represent the main activators of extrinsic apoptotic pathway (14). NF-κB becomes active after releasing from IκB. Constitutively activated NF-κB might be crucial in the development of drug resistance in cancer cells (15, 16).

In the intrinsic apoptotic pathway, death promoting stimuli originate form subcellular compartments and favour mitochondrial membrane permeabilization, therefore mitochondria play a decisive role. The B-cell lymphoma 2 (Bcl-2) family proteins are divided into anti-apoptotic (Bcl-2, Bcl-Xl, Bcl-w, Mcl-1, A1) and pro-apoptotic (Bax, Bak, Bok, Bid, Bad, Bim, Bik, Blk, Hrk, Noxa, Puma) subgroup (13). Anti-apoptotic members stabilize mitochondrial membrane potential and thus prevent the release of cytochrome c and apoptosis inducing factor. On the other hand, pro-apoptotic members lead cell to programmed apoptotic cell death by permeabilization of mitochondrial membrane. The release of cytochrome c into the cytoplasm results in the formation of apoptosome, which moreover contain apoptotic peptidase activating factor 1 (APAF1) and deoxy-ATP. Apoptosome stimulates caspases, the family of apoptosis-related cysteine proteases that initiates cell death and play a critical role in human cell apoptosis (17).

Temozolomide (TMZ) is an orally available, small (194 Da) lipophilic molecule that represents a new class of second generation imidazotetrazine prodrug that undergoes a spontaneous conversion under physiological conditions to the reactive alkylating agent MTIC [5-(3-methyltriazen-1-yl)imidazole-4-carboximide]. TMZ is able to penetrate blood-brain barrier and does not require a hepatic metabolism for activation (18). MTIC degrades and transfers the methyl group to DNA, while the final degradation product AIC (5-aminoimidazole-4-carboximide) is excreted via the kidneys (19). The cytotoxicity of TMZ as an alkylating agent is mediated by methylation of DNA purine bases, at N7 and O6 position of guanine (N7-MeG; O6-MeG) and at N3 position of adenine (N3-MeA), which results in subsequent DNA damage. However, TMZ provides a modest antitumor activity and currently is used to treat malignant gliomas, some GB cells are resistant to the cytotoxicity caused by TMZ. This resistance is related to the implementation of several mechanisms, such as: increased levels

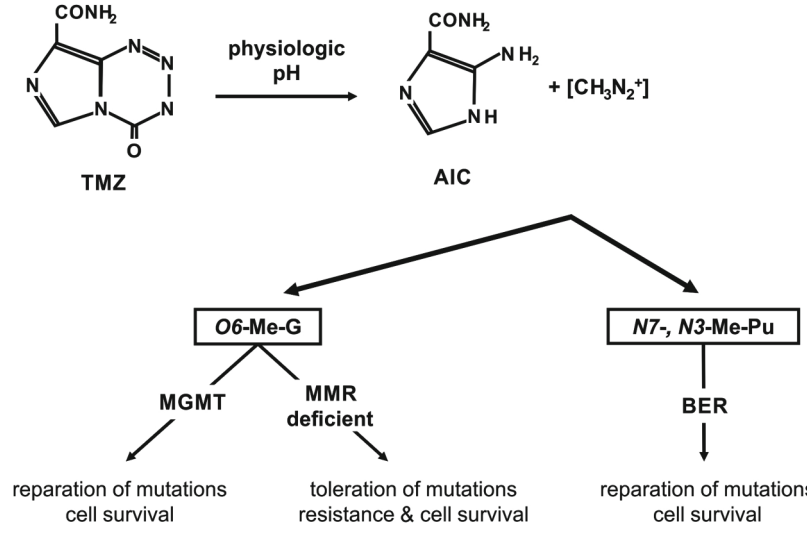

Fig. 1. Structure and activation of temozolomide (TMZ) to the final degradation product 5-aminoimidazole-4-carboximide (AIC). O6-methylguanine-DNA methyltransferase (MGMT), miss-match repair (MMR) and base excision repair (BER) are the key DNA repair mechanisms that provide *O6*-Me-G (*O6*-methylguanine) and N7, *N3*-Me-Pu (*N7, N3*-methylpurine) reparation or toleration in cell after TMZ treatment.

of O6-methylguanine-DNA methyltransferase (MGMT), dysfunction in miss-match repair (MMR) mechanism of DNA, presence of glioblastoma SCs and DNA reparation by base excision repair (BER). Abundant N7-MeG and N3-MeA lesions are rapidly repaired by DNA BER. The most cytotoxic adducts, O6-MeG, can be repaired by enzyme MGMT by removing the methyl group in the O6 position of the substrate guanine (Fig. 1) (19, 20). The level of MGMT expression positively correlates with in vivo and in vitro glioma resistance to TMZ (1, 21, 22).

Cell fate after the treatment with TMZ depends on the preferential activation of repair or cell death pathways. The resistance of GB cells to genotoxic therapies could be explained by their ability to stop growth and survive in quiescent state, or by the involvement of an enhanced DNA damage signalling (23). The competence of a cell to survive or die is theoretically proportional to the doses and duration of TMZ treatment, the DNA damage repair capacity and the proliferation level of the cells and the activating of cell repair proteins. There were several strategies used for enhancing the effect of TMZ treatment, like a modulation of TMZ dosing schedule or a combination treatment with TMZ enhancer (24). In recent years, several molecules have been discovered that, either alone or in combination with TMZ, can modulate apoptosis processes in glial tumor cells. For example, Allicin suppressed the proliferation and colony formation ability of U251 cells in a dose- and time-dependent manner, the activity of caspases was significantly elevated and Fas/FasL expression levels were increased at both the mRNA and protein level (25). Low doses of Piperine (PIP), the other biological compounds, in combination with TMZ inhibited the proliferation of glioma cells by reducing the cell viability, inducing apoptosis and activation of caspase-8/-9/-3, activation of JNK/p38 MAPKs, inhibiting wound healing, and mitochondrial membrane potential depolarization (26). Gjika et al. found out that cold atmospheric plasma (CAP) treatment successfully augmented the effect of a cytotoxic TMZ dose (50 μM) in a glioblastoma cell line U87MG (27). Another treatment combining TMZ, difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, and radiation in GBM cell lines resulted in a consistent higher suppression of proliferation in U251MG and T98G cells and induced a significant higher cell cycle arrest in G2/M phase. Moreover, in T98G cells, the combination treatment increased the activation of caspase-8 (28).

We focused on gene expression profiling in different types of healthy and tumor tissues in our previous studies (29–31). Genomic characterization of GB cells can rapidly expand the knowledge of the molecular basis of GB, lead to more precise classification system and is a prerequisite for achieving individualized clinical care of glioma patients (32). We hypothesized that the characterization of the expression changes in apoptotic pathway associated genes in GB cells after TMT treatment may contribute to identification the resistance mechanism after TMZ treatment and can help to improve treatment outcome.

The aim of our study was to detect the sensitivity of glioblastoma T98G cells to TMZ treatment and subsequently to determine the expression changes of apoptosis-associated genes in glioblastoma cells.

Material and methods

Cell culture and drug treatment

The human glioblastoma cell line (T98G) (ECACC, United Kingdom) was cultivated in 89 % (v/v) Dulbecco's modified Eagle's medium (PAA, Austria) supplemented with 10 % (v/v) fetal bovine serum (Gibco, USA) and 1 % penicillin/streptomycin (PAA, Austria). Cell line was incubated in humidified atmosphere with 5 % CO_2 at 37 °C. Medium was renewed every third day.

Chemotherapeutic agent temozolomide [3-methyl-4-oxo-3,4-dihydroimidazo (5,1-*d*) (1,2,3,5) terazine-8-carboxamide] (TMZ) was provided as Temodal by Schering-Plough, s.r.o (Czech Republic). The final TMZ concentrations and treatment time period was used according to our preliminary experiments, the final TMZ treatment was with 2-fold increasing concentrations in range 31.25–1000 μmol/l for 24, 48 and 72 hours.

Cell viability assay

The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay was used to assess the viability of the T98G cells after TMZ treatment. The cells in culture medium were seeded in wells ($5.0x10^3$ per well) of 96-well microtiter plates. On the second day, the medium was changed to culture medium supplemented with TMZ of varied concentration and the incubation

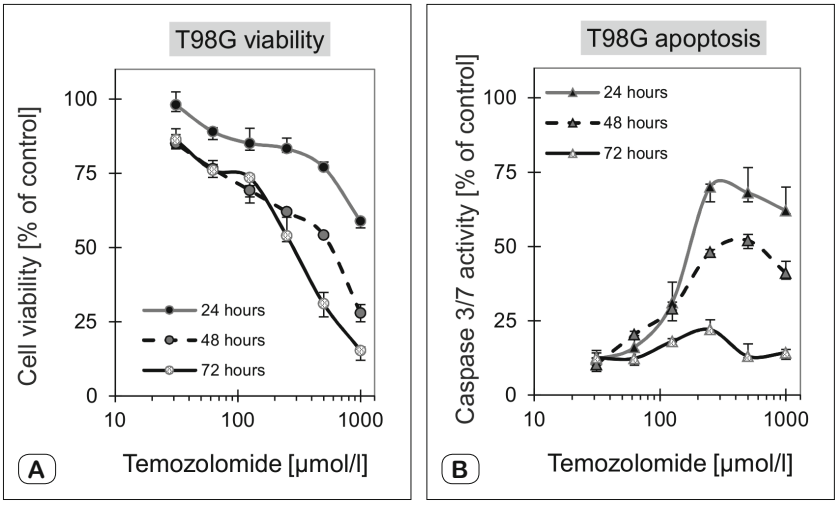

Fig. 2. (A) Viability of the cell line T98G after different hours of TMZ treatment. Results are presented as a median with range of triplicates. IC_{50} 24h = ND; IC_{50} 48h = 583 μmol/l; IC_{50} 72h = 320 μmol/l. (B) Caspase activity 3/7 of T98G cell line after different hours of TMZ treatment. Results are calculated towards to control (T98G w/o TMZ) and are presented as a median with range of triplicates.

continued for the next two days. Subsequently, cells were rinsed once with DPBS w/o Mg^{+} and Ca^{2+} ions (Gibco^{TM}, USA) and further incubated in culture medium supplemented with 0.5 mg/ml MTT in humidified atmosphere for 6 hours. During a subsequent incubation for 16 hours in medium containing SDS [5% (w/v)], the precipitated formazan, the amount of which is proportional to the number of live cells, was solubilized. The absorbance of the formazan containing solution was measured at 540 nm using a microplate reader (Synergy H4, BioTek, USA). The percentage of cell viability was calculated relative to the untreated intact control cells and IC_{50} value was determined. The results are presented as the mean±SD (Fig. 2A) and were measured in triplicates.

Apoptosis assay

Apoptosis was determined by measuring the caspase 3/7 activity (Caspase-Glo 3/7 Assay, Promega, USA) according to the manufacturer's protocol. Cells were seeded at a density of 5.000 cells/well in 96-well white-walled plates. The next day, cells were treated with TMZ and cleaved caspase 3/7 activity was assessed 24, 48, and 72 hours after TMZ influence. Caspase-Glo reagent was distributed to the wells, carefully mixed, and left to incubate in the dark for 90 minutes at room temperature. Luminiscent signals were measured using a Synergy H4 microplate reader, then normalized toward the untreated cells, and denoted as percentage of caspase-3/7 activity. The results are presented as the median (Fig. 2B) and were measured in triplicates.

RNA extraction and reverse transcription

Total RNA from treated and untreated T98G cells was extracted with TRI Reagent (MRC, USA) following manufacturer's protocol. The RNA isolation quality was checked by NanoDrop (Thermo Scientific, USA). As RNA control for brain expression were used two commercially available human RNA standards, (i) Human Brain Total RNA (Clontech, Canada) and (ii) FirstChoice Human Brain Reference Total RNA (Ambion, USA). Five micrograms of total RNA were reverse transcribed in a total volume of 14 µl using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to manufacturer's protocol.

The microfluidic study

The regulation of gene expression was studied using TaqMan® Human Apoptosis Array (Applied Biosystems, USA) based on the qRT-PCR reaction. The predesigned 384-well microfluidic card contained assay for 93 human apoptotic genes and three genes of endogenous control (18S, ACTB, GAPDH). The reaction mixtures with 100 ng cDNA template and equal volume of TaqMan Gene Expression Master Mix were loaded into microfluidic card, distributed into the wells by centrifugation (450 x g, 1 min, 2x) sealed and loaded into the ViiA7 Real-Time PCR System (Applied Biosystems, USA). The standard amplification protocol consisted of a ramp of 50 °C for 2 min and a hot start of 94.5 °C for 10 min, followed by 40 cycles of 15 sec at 95.0 °C and 60 sec at 60.0 °C. Cell line samples and human brain total RNA standards were measured as triplicates. Data transformation was corrected for the signal from the three endogenous controls used as the reference genes. The relative changes in gene expression between non-treated T98G cells and human brain total RNA standards (RNA^{CTRL}) or treated cells ($T98G^{TMZ}$) and non-treated T98G cells were analysed using the $2^{-\Delta\Delta Ct}$ method. Differentially expressed genes were selected from the normalized data.

Statistical analysis

Representative curve results of cell viability and caspase 3/7 activity are presented as the median with range (Fig. 2), measured in triplicates. The raw Ct data from the genes of interest and housekeepers were performed using the Qiagen RT^2 Profiler PCR Array Data Analysis Template (v3.5, 2010) Microsoft excel spreadsheet. The changes between all three groups were assessed by Kruskal--Wallis (nonparametric) test and the differences between the pairs of groups were assessed by Dunn post-hoc test. Fold changes of the genes were calculated as the mean values of $2^{-\Delta\Delta Ct}$ relative to the control group and were converted to log2 fold changes for use in Table 1. The transcription of particular gene was considered to be significant, when the average fold change was less than −2.0 for downregulated genes or more than 2.0 for upregulated genes, and statistically significant, when the corresponding p values were * < 0.05, ** < 0.01 or *** < 0.001.

Results

Sensitivity of the T98G cell line to TMZ treatment

The colorimetric MTT assay was used to detect the sensitivity of T98G cells. The effect of TMZ was tested on cell line T98G after 24, 48 and 72 hours in concentration-dependent manners. The IC_{50} values of T98G cell line after TMZ treatment were 583 µmol/l at 48 hours and 320 µmol/l at 72 hours. Cell survival with TMZ treatment during the 24 hours incubation was higher than 50 %, therefore IC_{50} value was not detected (ND). The viability of the T98G cells refers to their relative resistance to TMZ (Fig. 2A). The concentration of TMZ (58.3 µmol/l), 10 times lower than IC_{50} value for 48 hours, was used for studying the expression of apoptosis-associated genes in T98G cells after TMZ treatment.

The presence of apoptotic process was evaluated by Caspase-Glo 3/7. We recorded the highest luminiscent signal after 24 hours of culturing cells with TMZ. In contrast, during 72 hours of incubation, we detected a signal loss even at high concentrations of TMZ (Fig. 2B).

Expression of apoptosis-associated genes in intact T98G cell line against human brain total RNA standards

The expression patterns of the apoptotic genes were significantly different and showed an altered regulation of apoptotic pathways in the intact T98G cell line compared to reference human brain total RNA standards (Tab. 1).

Bcl-2 family regulated pathway. The significantly higher expression was observed in the expression of the *BCL10* (FC = 3.78) and *BCL3* (FC = 3.63) gene. On the other hand, a significant decrease was observed in the expression of *BOK* (FC = –111.7) and *BNIP3L* (FC = –17.7), *BCL2* (FC = –35.7) and *BCL2L2*

Tab. 1. List of deregulated apoptotic genes and their fold changes in glioblastoma cell line T98G against commercial RNA control of healthy brain tissue (RNA^{CTRL}) and T98G cell line treated with TMZ ($T98G^{TMZ}$) against control cells cultivated in standard conditions (T98G). Genes identified as deregulated (≤ –2 or ≥ 2) are presented in bold with a corresponding p value * < 0.05, ** < 0.01 or * < 0.001.**

Regulated pathway of gene	Gene of Interest	Fold Change		Gene Description
		$T98G/RNA^{CTRL}$ (*F, P, df*)	$T98G^{TMZ}/T98G$ (*F, P, df*)	
Bcl-2 signalling pathway	BBC3	ND	**–2.22**	BCL2 Binding Component 3 (PUMA)
	BCL10	**3.78*** (*7.2, 0.0273, 2*)	1.67	B-cell CLL/lymphoma 10
	BCL2	**–35.7**** (*8.3, 0.014, 2*)	1.47	B-cell CLL/lymhoma 2
	BCL2L1	–1.01	**–6.94*** (*5.9, 0.0491, 2*)	BCL2-like1 (BCLX)
	BCL2L2	**–19.0*** (*7.1, 0.0372, 2*)	1.39	BCL2-like2 (BCLW)
	BCL3	**3.63*** (*7.0, 0.0398, 2*)	–1.69	B-Cell CLL/Lymphoma 3
	BNIP3L	**–17.7*** (*7.2, 0.0174, 2*)	–1.85	BCL2 Interacting Protein 3-like
	BOK	**–111.7*** (*6.5, 0.0390, 2*)	–1.54	BCL2 Related Ovarian Killer (BCL2L9)
TNF receptor pathway	LRDD	**–8.32*** (*6.2, 0.0475, 2*)	–1.16	Leucine-Rich Repeats and Death Domain Containing
	PEA15	**–10.3*** (*7.3, 0.0287, 2*)	1.02	Phosphoprotein Enriched in Astrocytes 15
	RIPK1	1.38	**2.20**	Receptor Interacting Serine/Threonine Kinase 1
	TNFRSF10B	**6.06*** (*6.9, 0.0231, 2*)	1.24	TNF Receptor Superfamily Member 10B
	TNFRSF1A	**5.53*** (*6.9, 0.0354, 2*)	–1.26	TNF Receptor Superfamily Member 1A
Caspases	CASP1	**2.75*** (*7.1, 0.0212, 2*)	–1.68	Caspase-1, Apoptosis-related Cysteine Peptidase
	CASP3	1.78	**2.37**	Caspase-3, Apoptosis-related Cysteine Peptidase
	CASP4	**9.45*** (*7.4, 0.0182, 2*)	1.03	Caspase-4, Apoptosis-related Cysteine Peptidase
	CASP10	**–55,2**** (*8.1, 0.0091, 2*)	1.52	Caspase-10, Apoptosis-related Cysteine Peptidase
	CFLAR	**–11.8*** (*6.3, 0.034, 2*)	1.21	CASP8 and FADD Like Apoptosis Regulator (FLIP)
NF-κB signalling pathway	NFKBIB	**–6.27*** (*7.1, 0.0331, 2*)	1.92	NF-κB Light Polyp. Gene Enhancer Inhibitor. Beta
	REL	**–7.13*** (*7.2, 0.0298, 2*)	1.36	REL Proto-Oncogene. NF-κB Subunit
	RELA	**2.06*** (*6.1, 0.0492, 2*)	–1.40	RELA Proto-Oncogene. NF-κB Subunit
IAP family	BIRC2	**5.25*** (*6.2, 0.0439, 2*)	**5.30**	Baculoviral IAP Repeat-containing 2
CARD family	CARD6	ND	**3.46*** (*6.2, 0.0387, 2*)	Caspase Recruitment Domain Family. Member 6
	PYCARD	**2.43*** (*6.0, 0.0371, 2*)	–1.11	PYD And CARD Domain Containing
Other genes	DAPK1	**–344.2***** (*9.1, 0.0009, 2*)	**–2.08*** (*5.8, 0.0475, 2*)	Death-associated Protein Kinase 1
	DIABLO	**–8.64*** (*7.4, 0.0198, 2*)	–1.34	Direct IAP-Binding Protein with Low PI (SMAC)
	HIP1	**–6.89*** (*7.1, 0.0292, 2*)	–1.78	Huntingtin Interacting Protein 1

ND – genes with not detected fold changes, *F* – the test value, *P* – p value, *df* – degrees of freedom

(FC = –19.0) genes. Higher level of the *BCL10* expression and a decrease in the expression of *BOK* and *BNIP3L* genes, indicated the increased anti-apoptotic properties of the glioblastoma cell line compared to human brain total RNA standards.

TNF receptor pathway. The most pronounced statistically significant increase was found in the expression of *TNFRSF1A* (FC = 5.53), *TNFRSF10B* (FC = 6.06) genes and a decrease in expression of *PEA15* (FC = –10.3) and *LRDD* (FC = –8.32) genes.

Caspases. Our results showed a statistically significant increase in *CASP4* (FC = 9.45) and *CASP1* (FC = 2.75) expression, but it does not seem to play any important causative role in the expression activation of *CASP10* (FC = –55.2) and *CFLAR* (FC = –11.8), which were downregulated.

NF-κB signalling pathway. Genetic analysis revealed a statistically significant upregulation of *RELA* (FC = 2.06), while a statistically significant downregulation of *NFKBIB* (FC = –6.27) and *REL* (FC = –7.13) in NF-κB signalling pathway was detected.

CARD family. In the intact T98G cell line. there was a statistically significant upregulated *PYCARD* gene (FC = 2.43) encoded protein containing PYD and CARD domain.

Changes observed in expression of other genes. There was a statistically significant fold change decrease in the expression of *DAPK1* (–344.2), *HIP1* (FC = –6.89) and *DIABLO* (FC = –8.64) gene.

Expression of apoptosis-associated genes in T98G^{TMZ} cell line against untreated T98G

The expression patterns of apoptotic genes were statistically different for ten apoptosis-associated genes in T98G cell line treated with TMZ for 48 hours compared to the untreated T98G cell line as the reference.

Bcl-2 family regulated pathway. The significant downregulation of the BCL2L1 gene (FC = –6.94) indicated the increased anti-apoptotic properties of GB cell line after TMZ treatment. Expression of the *BBC3* (FC = –2.22) was also decreased but without a significance.

NF-κB signalling pathway. Genetic analysis revealed insignificant differences in the relevant genes.

IAP family. There was a statistically significant increase in the expression of *BIRC2* gene (FC = 5.30). *Changes observed in the expression of other genes.* The following data are summarized in Table 1. There was a statistically significant decrease in the expression of *DAPK1* (FC = –2.08).

Discussion

Glioblastomas, classified as malignity grade IV according to the WHO, are the most common brain tumors in adulthood (3). A resistance to clinically used radiotherapy and chemotherapy is the major obstacle to successful treatment, therefore the purpose of many research groups is to search for new markers and treatment targets. The aim of our study was to determine the sensitivity of T98G cells to TMZ and to evaluate the expression profile of pro-apoptotic and anti-apoptotic genes using the commercially available set of genes included in the microfluidic card TaqMan® Human Apoptosis Array supplied by Applied Biosystems. Cell line T98G is a very well studied model of cancer research usually used in drug screening and molecular GB experimental models. The cells manifest major glioblastoma properties and exhibit the typical expression profile for mesenchymal and growth factor genes (33).

The IC_{50} value of TMZ was previously determined to be approximately 200 mmol/l, while plasma concentration found in human subjects was approximately 100 μmol/l (18). According to our results, TMZ is able to significantly reduce the number of viable cells, with IC_{50} value 583 μmol/l in glioblastoma cell line T98G under *in vitro* conditions (Fig. 2A). These findings are in alignment with the recent published studies (34, 35).

The results of our study demonstrated several anti-apoptotic properties of T98G cells compared to total RNA from healthy human brain and showed that the expression patterns of the apoptotic genes were significantly different in GB cells (Tab. 1). The high diversity of glial tumor cells is presented also by different MGMT expression status observed in multiple cell lines (U87MG, T98G and H4) whereas in T98G the level was detected as the highest (36). The revelation of biological attributes and molecular phenotypes appears to be still limited and remains one of the most challenging issues in glioblastomas.

Human malignant gliomas present high levels of Bcl-2 protein, which may confer a resistance to apoptosis of glioblastoma cells (37). An overexpression of *BCL2* provides a survival advantage to cancer cells in response to a wide range of apoptotic stimuli through the inhibition of mitochondrial cytochrome c release (38). The potential function of Bcl-2 protein in neuronal cells has been first described by Reed et al (39). Despite these findings, our results showed a decrease in the expression of *BCL2* gene and also a downregulation of *BCL2L2* (Bcl-w) gene in human GB cells. On contrary, downregulation of *BOK* and *BNIP3L* genes, which acts as apoptotic activators and the upregulation of anti-apoptotic *BCL10* gene may serve as a protective factor against the apoptosis in GB cell line T98G.

The extrinsic apoptosis pathway is triggered by the TNF family, which is responsible for the transmission of signals from the extracellular death ligands (40). Our data indicate the alteration of several members of the TNF signalling pathway, but it has been shown in several published studies that GB are resistant to Fas-related apoptosis (41, 42). The extrinsic apoptotic pathway in human GB treatment has not been systematically studied yet. The previous report has showed an inhibition of this pathway in the high-grade gliomas (42). Our experiments show a significant downregulation in the expression of *PEA15* and *LRDD* genes, but an upregulation in *TNFRSF1A* and *TNFRSF10B* genes. The changes in the expression of these genes could affect DISC formation or caspase -8 activation and thus avoid apoptotic cell death in GB cells. Cellular FLIP protein inhibits apoptosis by competing with the caspase -8 for binding to FADD (43). Moreover, a decrease in gene expression of initiator *CASP10* and *CFLAR* followed by an upregulation of associated *CASP1* and *CASP4* should play an important role in the activation of effectors, such as *CASP*6 or *CASP7* genes, which were slightly upregulated. The upregulation of *CASP1* gene should be a positive regulator of the NF-κB cascade and suggests a potentially important role of this

pathway in the development of resistance to TMZ in GB cells. An increase of expression of *CASP1* in T98G cells compared to human brain RNA control results in the upregulation of *NFKB2* and *RELA*, which plays the key role in NF-κB pathway activation. Gliomas are naturally resistant to apoptosis as the consequence to constitutively active NF-κB signalling (19). An inhibition of NF-κB with TMZ co-treatment resulted in altered cell proliferation by decreasing GBM viability, suppressed NF-κB pathway and enhanced apoptosis. Moreover, a decrease in the migration pattern of patient derived GBM cells by modulating actin cytoskeleton pathway was observed (44).

The significance of NF-κB signalling pathway in GB cells survival has been reported in several studies. Active NF-κB stimulates the expression of the genes that maintain cellular proliferation and protects cell from the conditions leading cell to apoptosis. In contrary to these findings, we have not observed any significant changes in expression of genes encoding proteins involved in those pathways after TMZ treatment. The extrinsic apoptosis pathway triggered by the TNF and NF-κB signalling pathway family does not seem to play an important role in TMZ resistance in T98G cells after treatment with TMZ. The involvement of NF-κB signalling pathway in response to DNA damage caused by *O6*-alkylating agents can mediate chemoresistance in GB cells, primarily mediated by its anti-apoptotic activity (14, 15).

HIP1 is involved in several regulation pathways in CNS and may act as a pro-apoptotic protein. A decrease in expression of *HIP1* and genes encoding pro-apototic proteins DAPK1 (mediator of gamma-interferon induced programmed cell death) and DIABLO (IAP-binding protein) may serve as a protective factor against apoptosis in GB cell line T98G (45).

TMZ can induce apoptotic cell death preceded by ER stress followed by activation of the mitochondrial death pathway. Gliomas naturally resist apoptosis – a caspase dependent programmed cell death. Combination of TMZ with quercetin induces apoptosis in GB cells (T98G) through mitochondrial pathway activation. It was correlated at the molecular level with a decreased mitochondrial membrane potential, a release of cytochrome c from the mitochondria and the activation of caspase 3 and 9 (34). Any of TMZ treatments alone had no effect on autophagy induction in T98G cells that was confirmed by an unchanged level of the main autophagy marker beclin 1. Moreover, the activation of caspase 3 blocks beclin 1 activity and thus inhibits the autophagosome formation (34, 45). In the T98G cells treated by TMZ, the presence of caspase 12 followed by induction of apoptosis was shown (34). However, we were not able to observe any statistically significant changes in the expression of these caspase genes. The treatment with TMZ alters in the expression of pro-apoptotic *BAX* and anti-apoptotic *BCL2* genes, both involved in the intrinsic apoptotic pathway (20). The expression of proapototic genes *BBC3* (*PUMA*), *BNIP3L*, *BOK* was decreased after TMZ treatment of T98G cells that can lead to the apoptosis inhibition in GB cells after TMZ treatment.

Many human tumor studies analyzed and confirmed the changes in p53 gene expression, which is associated with programmed apoptotic cell death. Alternations in apoptosis pathways play the key role in tumor formation and progression (46, 47). We observed a significant downregulation in pro-apoptotic *BBC3* gene as well, which could play the crucial role in apoptosis inhibition. Ito et al. demonstrated that an overexpression of *PUMA* gene induced the activation of caspases and cytochrome c release and thus induced apoptosis in malignant glioma cells (48).

Such progress in DNA microarray technologies has revolutionized research and enabled progress in gene expression profiling of gliomas with a possibility of individualized clinical care providing to glioma patients in the future (32). Glioblastoma cells have totally defective apoptotic signalling pathways, what results in raising cell proliferation and uncontrolled tumor growth. In our work, we demonstrated that human GB cell line T98G showed changes in several apoptosis associated gene expression profiles compared to human total brain RNA control samples, therefore apoptosis can be inhibited on gene expression level in GB cells. Targeting apoptotic pathways in selected cell line after TMZ treatment may offer a unique opportunity to develop novel therapeutic strategies to improve the treatment of GB tumors and thus may overcome tumor resistance.

Conclusions

Identification of differentially expressed human apoptosis-associated genes in cell line T98G compared to human total brain RNA control might approximate the relevancy of commercial cell lines as a useful experimental model and research tool of the neuronal cells. The gene expression profiles were determined also in untreated cells and in commercially available RNA control of healthy brain tissue, therefore our data relate to the sensitivity to TMZ therapy and molecular base in healthy and tumor cells.

References

1. Richterova R, Jurecekova J, Evinova A et al. Most frequent molecular and immunohistochemical markers present in selected types of brain tumors. Gen Physiol Biophys 2014; 33 (3): 259–279.

2. Ricard D, Idbaih A, Ducray F, Lahutte M, Hoang-Xuan K, Delattre JY. Primary brain tumours in adults. Lancet 2012; 379 (9830): 1984–1996.

3. Wesseling P, Capper D. WHO 2016 Classification of gliomas. Neuropathol Appl Neurobiol 2018; 44 (2): 139–150.

4. Persano L, Rampazzo E, Basso G, Viola G. Glioblastoma cancer stem cells: Role of the microenvironment and therapeutic targeting. Biochem Pharmacol 2013; 85 (5): 612–622.

5. Dresemann G. Temozolomide in Malignant Glioma. Onco Targ Ther 2010; 3: 139–146.

6. Schonberg DL, Lubelski D, Miller TE, Rich JN. Brain tumor stem cells: Molecular characteristics and their impact on therapy. Mol Aspects Med 2014; 39: 82–101.

7. Stupp R, Hegi ME, van den Bent MJ et al. European Organization for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Changing paradigms – An update on the multidisciplinary management of malignant glioma. Oncologist 2006; 11 (2): 165–180.

8. Westhoff MA, Brühl O, Nonnenmacher L, Karpel-Massler G, Debatin KM. Killing me softly – future challenges in apoptosis research. Int J Mol Sci 2014; 15 (3): 3746–3767.

9. Barazzuol L, Jena R, Burnet NG et al. In vitro evaluation of combined temozolomide and radiotherapy using X rays and high-linear energy transfer radiation for glioblastoma. Radiat Res 2012; 177 (5): 651–662.

10. Ronning PA, Helseth E, Meling TR, Johannesen TB. A population-based study on the effect of temozolomide in the treatment of glioblastoma multiforme. Neuro Oncol 2012; 14 (9): 1178–1184.

11. Parisi S, Corsa P, Raguso A et al. Temozolomide and radiotherapy versus radiotherapy alone in high grade gliomas: a very long-term comparative study and literature review. Biomed Res Int 2015; 620643.

12. Thomas S, Quinn BA, Das SK et al. Targeting the Bcl-2 family for cancer therapy. Expert Opin Ther Targets 2013; 17 (1): 61–75.

13. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006; 25 (34): 4798–4811.

14. Tchoghadjian A, Jennewein C, Eckhardt I, Momma S, Branger DF, Fulda S. Smac mimetic promotes glioblastoma cancer stem-like cell differentiation by activating NF-κB. Cell Death Differ 2014; 21 (5): 735–747.

15. Nakanishi Ch, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer 2005; 5 (4): 297–309.

16. Mabuchi S, Ohmichi M, Nishio Y et al. Inhibition of NFkappaB increases the efficacy of cisplatin in in vitro and in vivo ovarian cancer models. J Biol Chem 2004; 279 (22): 23477–23485.

17. Llambi F, Green DR. Apoptosis and oncogenesis: give and take in the BCL-2 family. Curr Opin Genet Dev 2011; 21 (1): 12–20.

18. Shen W, Hu JA, Zheng JS. Mechanism of temozolomide-induced antitumour effects on glioma cells. J Int Med Res 2014; 42 (1): 164–172.

19. Zhang J, Stevens MF, Bradshaw TD. Temozolomide: mechanisms of action, repair and resistance. Curr Mol Pharmacol 2012; 5 (1): 102–114.

20. Miao W, Liu X, Wang H et al. p53 upregulated modulator of apoptosis sensitizes drug-resistant U251 glioblastoma stem cells to temozolomide through enhanced apoptosis. Mol Med Rep 2015; 11 (6): 4165–4173.

21. Karsy M, Neil JA, Guan J, Mark MA, Colman H, Jensen RL. A practical review of prognostic correlations of molecular biomarkers in glioblastoma. Neurosurg Focus 2015; 38 (3): E4.

22. Park CK, Kim JE, Kim JY et al. The changes in MGMT promoter methylation status in initial and recurrent glioblastomas. Transl Oncol 2012; 5 (5): 393–397.

23. Annovazzi L, Caldera V, Mellai M et al. The DNA damage/repair cascade in glioblastoma cell lines after chemotherapeutic agent treatment. Int J Oncol 2015; 46 (6): 2299–2308.

24. Jiapaer S, Furuta T, Tanaka S, Kitabayashi T, Nakada M. Potential Strategies Overcoming the Temozolomide Resistance for Glioblastoma. Neurol Med Chir (Tokyo) 2018; 58 (10): 405–421.

25. Li C, Jing H, Ma G, Liang P. Allicin induces apoptosis through activation of both intrinsic and extrinsic pathways in glioma cells. Mol Med Rep 2018; 17 (4): 5976–5981.

26. Jeong S, Jung S, Park GS, Shin J, Oh JW. Piperine synergistically enhances the effect of temozolomide against temozolomide-resistant human glioma cell lines. Bioengineered 2020; 11 (1): 791–800.

27. Gjika E, Pal-Ghosh S, Kirschner ME et al. Combination therapy of cold atmospheric plasma (CAP) with temozolomide in the treatment of U87MG glioblastoma cells. Sci Rep 2020; 10 (1): 16495.

28. Alexiou GA, Vartholomatos E, I Tsamis K et al. Combination treatment for glioblastoma with temozolomide, DFMO and radiation. J BUON 2019; 24 (1): 397–404.

29. Blahovcova E, Richterova R, Kolarovski B, Dobrota D, Racay P, Hatok J. Apoptosis-related gene expression in tumor tissue samples obtained from patients diagnosed with glioblastoma multiforme. Int J Mol Med 2015; 36 (6): 1677–1684.

30. Zubor P, Hatok J, Moricova P et al. Gene expression profiling of histologically normal brest tissue in females with human epidermal growth factor receptor 2-positive breast cancer. Mol Med Rep 2015; 11 (2): 1421–1427.

31. Zubor P, Hatok J, Galo S et al. Anti-apoptotic and pro-apoptotic gene expression evaluated from eutopic endometrium in the proliferative phase of the menstrual cycle among women with endometriosis and healthy controls. Eur J Obstet Gynecol Reprod Biol 2009; 145 (2): 172–176.

32. Vitucci M, Hayes DN, Miller CR. Gene expression profiling of gliomas: merging genomic and histopathological classification for personalised therapy. Br J Cancer 2011; 104 (4): 545–553.

33. Kiseleva, LN, Kartashev, AV, Vartanyan, NL, Pinevich AA, Samoilovich MP. Characterization of New Human Glioblastoma Cell Lines. Cell Tiss Biol 2018; 12: 1–6.

34. Jakubowicz-Gil J, Langner E, Bądziul D, Wertel I, Rzeski W. Apoptosis induction in human glioblastoma multiforme T98G cells upon temozolomide and quercetin treatment. Tumour Biol 2013; 34 (4): 2367–2378.

35. Palumbo S, Pirtoli L, Tini P et al. Different involvement of autophagy in human malignant glioma cell lines undergoing irradiation and temozolomide combined treatments. J Cell Bioch 2012; 113 (7): 2308–2318.

36. Chu CW, Yang MC, Chou Ch et al. GSK3β-mediated Ser156 phosphorylation modulates a BH3-like domain in BCL2L12 during TMZ-induced apoptosis and autophagy in glioma cells. Int J Mol Med 2018; 42 (2): 905–918.

37. Tagscherer KE, Fassl A, Campos B et al. Apoptosis-based treatment of glioblastomas with ABT-737, a novel small molecule inhibitor of Bcl-2 family proteins. Oncogene 2008; 27 (52): 6646–6656.

38. Wong RSY. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 2011; 30: 87.

39. Reed JC, Meister L, Tanaka S et al. Differential expression of bcl2 proto-oncogene in neuroblastoma and others human tumor cell lines of neural origin. Cancer Res 1991; 51 (24): 6529–6538.

40. Gaur U, Aggarwal BB. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. Biochem Pharmacol 2003; 66 (8): 1403–1408.

41. Tirapelli LF, Bolini PH, Tirapelli DP et al. Caspase-3 and Bcl-2 expression in glioblastoma: An immunohistochemical study. Arq Neuropsiquiatr 2010; 68 (4): 603–607.

42. Ashley DM, Riffkin CD, Muscat AM et al. Caspase 8 is absent or low in many ex vivo gliomas. Cancer 2005; 104 (7): 1487–1496.

43. Son JK, Varadarajan S, Bratton SB. TRAIL-activated stress kinases suppress apoptosis through transcriptional upregulation of MCL-1. Cell Death Differ 2010; 17 (8): 1288–1301.

44. Avci NG, Ebrahimzadeh-Pustchi S, Akay YM et al. NF-κB inhibitor with Temozolomide results in significant apoptosis in glioblastoma via the NF-κB(p65) and actin cytoskeleton regulatory pathways. Sci Rep 2020; 10 (1): 13352.

45. Luo S, Rubinsztein DC. Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ 2010; 17 (2): 268–277.

46. Wei B, Wang I, Du Ch et al. Identification of differentially expressed genes regulated by transcription factors in glioblastomas by bioinformatics analysis. Mol Med Rep 2015; 11 (4): 2548–2554.

47. Stancheva G, Goranova T, Laleva M et al. IDH1/IDH2 but not TP53 mutations predict prognosis in Bulgarian glioblastoma patients. Biomed Res Int 2014, ID 654727.

48. Ito H, Kanzawa T, Miyoshi T et al. Therapeutic efficacy of PUMA for malignant glioma cells regardless of p53 status. Human Gene Ther 2005; 16 (6): 685–698.

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