BioDrugs (2016) 30:129–144
DOI 10.1007/s40259-016-0160-y

ORIGINAL RESEARCH ARTICLE

Combination Therapy with *AKT3* and *PI3KCA* siRNA Enhances the Antitumor Effect of Temozolomide and Carmustine in T98G Glioblastoma Multiforme Cells

Monika Paul-Samojedny[1] · Adam Pudełko[2] · Małgorzata Kowalczyk[1] · Anna Fila-Daniłow[1] · Renata Suchanek-Raif[1] · Paulina Borkowska[1] · Jan Kowalski[1]

Published online: 22 February 2016

Abstract

Background Glioblastoma multiforme (GBM) is the most malignant and invasive human brain tumor, and it is characterized by a poor prognosis and short survival time. Current treatment strategies for GBM, using surgery, chemotherapy and/or radiotherapy, are ineffective. The PI3K/AKT/PTEN signaling pathway is frequently deregulated in this cancer, and it is connected with regulation of the cell cycle, apoptosis, and autophagy.

Objectives The current study was undertaken to examine the effect of small interfering RNA (siRNA) targeting the *AKT3* and *PIK3CA* genes on the susceptibility of T98G cells to temozolomide (TMZ) and carmustine (BCNU).

Methods T98G cells were transfected with *AKT3* or *PI3KCA* siRNA. Transfection efficiency was assessed using flow cytometry and fluorescence microscopy. The influence of *AKT3* and *PI3KCA* siRNA in combination with TMZ and BCNU on T98G cell viability, proliferation, apoptosis, and autophagy was evaluated as well. Alterations in messenger RNA (mRNA) expression of apoptosis-related and autophagy-related genes were analyzed using quantitative reverse transcription polymerase chain reaction (QRT-PCR).

Results Transfection of T98G cells with *AKT3* or *PI3KCA* siRNA and exposure to TMZ and BCNU led to a significant reduction in cell viability, accumulation of subG1-phase cells, and reduction of cells in the S and G2/M phases, as well as induction of apoptosis or necrosis, and regulation of autophagy.

Conclusion The siRNA-induced *AKT3* and *PI3KCA* mRNA knockdown in combination with TMZ and BCNU inhibited proliferation and induced apoptosis and autophagy in T98G cells. Thus, knockdown of these genes in combination with TMZ and BCNU may offer a novel therapeutic strategy to more effectively control the growth of human GBM cells, but further studies are necessary to confirm a positive phenomenon for the treatment of GBM.

Key Points

The combination of temozolomide with *AKT3* or *PI3KCA*-specific small interfering RNA induces autophagy and apoptosis, as well as inhibiting proliferation, of T98G glioblastoma multiforme cells more efficiently than temozolomide alone.

The combination of carmustine with *AKT3* or *PI3KCA*-specific small interfering RNA induces senescence, apoptosis, and autophagy more efficiently than carmustine alone.

1 Introduction

The goal of a cancer treatment strategy is to eliminate or at least reduce the number of tumor cells while having the least influence on healthy host tissues and organs. A desirable reduction of the neoplastic lesion volume can be

✉ Monika Paul-Samojedny
mpaul@sum.edu.pl

[1] Department of Medical Genetics, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia, Jedności 8 Street, 41-200 Sosnowiec, Poland

[2] Department of Clinical Chemistry and Laboratory Diagnostics, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia, Katowice, Poland

achieved by drugs whose mechanism of action is connected with the induction of apoptosis, necroptosis, or autophagic cell death, which represent different types of programmed cell death. Temozolomide (TMZ) and carmustine (BCNU) are the two most common drugs used in the glioblastoma multiforme [GBM; World Health Organization (WHO) grade IV] treatment regimen. TMZ is an oral alkylating drug approved by the US Food and Drug Administration (FDA) for use in the first-line treatment of GBM. This small molecule with high lipophilicity readily penetrates the blood–brain barrier. After spontaneous intracellular conversion into MTIC (monomethyl triazeno imidazole carboxamide), an active agent gains the ability to methylate a number of nucleobases, especially the guanine base. This results in formation of *O*-6-methylguanine in DNA, which mispairs with thymine during the following cycle of DNA replication. Finally, it leads to arrest of the G2/M phase cell cycle or activation of the apoptotic pathway [1]. TMZ associated with radiotherapy increases the average survival rate by 2.5 months. BCNU is also an oral alkylating drug and penetrates the blood–brain barrier. The mechanism of BCNU action is binding of reactive alkyl radicals to the numerous, negatively charged functional groups present in biologically active molecules (such as DNA, RNA, or proteins), thus leading to loss of their activity. The presence of two alkyl radicals allows BCNU to create interstrand crosslinks in DNA and determines its strong antitumor activity. BCNU is known to reduce the DNA replication and DNA transcription that lead to cell death [2]. Few original works showing apoptosis and/or autophagic cell death induction in cancer cells in vitro mediated by TMZ and BCNU are available [3–7]. Since programmed cell death is mediated by an intracellular program and is carried out in a regulated process, apoptosis and autophagic cell death have their own molecular mechanism. In contrast to the apoptosis process, which is generally well described [8, 9], autophagy raises a lot of controversy due to its bidirectional effect on cancer cells [10, 11]. Macroautophagy (here referred to as autophagy) is a physiological process that eukaryotic cells use to digest their own macromolecular cytoplasmic material and recover basic building blocks. In this case, cancer cells can use it to survive unfavorable microenvironment conditions, including those resulting from drug action [12–14]. On the other hand, excessive cellular digestion can cause cell death, and this phenomenon has been called autophagic cell death [15, 16]. Regardless of the type of cell death induction, current treatment strategies for GBM, using chemotherapy and/or radiotherapy, are ineffective and result in poor patient survival [17]. The highly invasive and therapy-resistant character of GBM results in the shortest of all cancer survival times. The median survival is less than 15 months for patients with newly diagnosed cancer, regardless of their treatment methods [17–19]. Signaling through the PI3K/AKT/mTOR pathway in cancer contributes to a tumorigenic phenotype through effects on multiple cellular processes such as apoptosis, proliferation, motility, cell transformation, metabolism, and DNA repair [20]. The consequences of increased PI3K/AKT/mTOR pathway signaling are proposed as a major mechanism of cancer drug resistance [20–22]. As an example, Jin et al. [23] found that Ras-mediated drug resistance is well correlated with resistance to the apoptosis induced by anticancer agents in MCF7 breast cancer cells. Inhibition of the PI3K/Akt pathway enhances the cytotoxicity of paclitaxel, doxorubicin and 5-fluorouracil by reversing Ras-mediated drug resistance. The fundamental role of the PI3K/Akt pathway in conditioning GBM malignancy has been described in depth [24–30].

Thus, the aim of our study was induction of apoptosis and autophagy processes and changes in apoptosis-related and autophagy-related gene expression after knockdown of *AKT3* and *PIK3CA* genes and BCNU or TMZ exposure. The current study was also undertaken to examine the effect of small interfering RNA (siRNA) targeting the *AKT3* and *PIK3CA* genes on the susceptibility of T98G cells to TMZ and BCNU.

2 Methods

2.1 Cell Cultures

The T98G cell line [American Type Culture Collection (ATCC), Manassas, VA, USA] was derived from a 61-year-old male [31]. GBM cells were cultured in a modified Eagle's Minimum Essential Medium (ATCC) supplemented with heat-inactivated 10 % fetal bovine serum (ATCC) and 10 μg/mL gentamicin (Invitrogen). The cell line was maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in air.

This particular cell line was chosen because it is less sensitive to the chemotherapeutics that are currently used in GBM treatment. The T98G cell line shows increased resistance to TMZ [32, 33] as well as to BCNU and etoposide [34], compared with other commercially available cells lines. Thus, it seems reasonable to search for new potential therapeutic methods for glioma therapy by using this particular cell line.

2.2 siRNA Transfection

Transfection of T98G cells with specific siRNA targeting *AKT3* or *PI3KCA* messenger RNA (mRNA) was performed using FlexiTube siRNA Premix (Qiagen, Italy) according

to the manufacturer's protocol, as we have described previously [29]. The average transfection efficiency was 99.2 %.

2.3 Temozolomide and Carmustine Uptake

T98G GBM cells transfected with *AKT3* and *PI3KCA*-specific RNA were treated with TMZ (10 μM) for 48 h or with BCNU (50 μM) for 1 h (48 h after transfection). The concentration and time of the cells' exposure were chosen based on the literature data [35, 36].

2.4 Cell Cycle Analysis

T98G cells were seeded in six-well plates (at a density of 1.6×10^4 cells per well), cultured overnight (for 24 h), and, after *AKT3* and *PI3KCA* gene siRNA silencing, the cells were exposed to TMZ and BCNU, and the cell cycle was analyzed as described previously [29].

2.5 Apoptosis Assay

T98G cells were seeded in six-well plates (at a density of 1.6×10^4 cells per well), cultured overnight (for 24 h), and, after *AKT3* and *PI3KCA* gene siRNA silencing, the cells were treated with TMZ or BCNU. Cells were analyzed using flow cytometry and a Vybrant® DyeCycle™ Violet/SYTOX® AADvanced™ Apoptosis Kit as described previously [29].

2.6 Evaluation of Autophagy Induction

Measurement of the red fluorescence intensity, using LysoTracker Red staining (Invitrogen), indicated an increased number of vesicles with an acidic pH that was characteristic of autophagy in cells that had been transfected with a specific siRNA as well as untransfected T98G cells exposed to BCNU or TMZ with or without addition of an autophagy inductor or inhibitors. (1) *N*-hexanoyl-D-sphingosine (NHDS; 10 μM for 24 h), (2) bafilomycin A1 (BAF; 100 nM for 2 h) and (3) 3-methyladenine (3-MA; 2 mM for 24 h) were used as an autophagy inductor (1) and inhibitors (2 and 3), respectively. NHDS is known to stimulate autophagy by interfering with class I PI3K signaling pathways. 3-MA inhibits autophagy by blocking autophagosome formation via inhibition of class III PI3K signaling. BAF is an inhibitor of the late phase of autophagy and prevents the maturation of autophagic vacuoles by inhibiting the fusion of autophagosomes with lysosomes. The red fluorescence intensity after LysoTracker Red staining was determined using flow cytometry.

2.7 RNA Extraction

Total RNA was isolated from cells cultured by a TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA) according to the manufacturer's protocol. The integrity of total RNA was checked by electrophoresis in 1 % agarose gel stained with ethidium bromide. All RNA extracts were treated with *DNAse I* to avoid genomic DNA contamination, and were assessed qualitatively and quantitatively.

2.8 Evaluation of Autophagy-Related and Cell Invasion-Related Genes by QRT-PCR

We performed quantitative reverse transcription polymerase chain reaction (QRT-PCR) for selected genes associated with apoptosis and autophagy: *AIFM2*, *BCL2L1*, *BID*, *BNIP3*, *CASP3*, *CASP8*, *CASP9* (apoptosis), *AMBRA1*, *BECN1*, *MAP1LC3A*, *PIK3C3*, *RB1CC*, *SQSTM1*, *ULK1*, *UVRAG* (autophagy), *DRAM1*, *GSK3β*, *HIF1A*, and *PRKAA1* (both processes). QRT-PCR assays were performed using a CFX96 Real-Time System (BIO-RAD). Real-time fluorescent RT-PCR was performed using specific primers (KiCqStart™ Primers; Sigma Aldrich) and a SensiFAST™ SYBR Hi-ROX One-Step kit (Bioline) according to the manufacturer's protocol. The conditions were as follows: 45 °C for 10 min, 95 °C for 2 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C, and 5 s at 72 °C. RNA for human TBP (Tata Binding Protein) was used as an endogenous control. The copy numbers for each sample were calculated by the C_T-based calibrated standard curve method. Each of 12 replicas' data points for the mRNA copy number is the average of duplicates on the same analyzed plate.

2.9 Statistical Analysis

The data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison tests were used for comparing the analyzed groups. The power of all of the tests was not less than $1 - \beta = 0.8$. The data were analyzed with Statistica software (StatSoft, Inc. 2008), version 10.0 (https://www.statsoft.com). All of the tests were two-sided, and $p < 0.05$ was considered to be statistically significant. The proliferation and apoptotic indexes were determined according to Darzynkiewicz et al. and Henry et al. [37, 38]. Hierarchical clustering of the results based on the Euclidean distance was carried out using GenExEnterprise 5.4.3.703. QRT-PCR analysis was performed in accordance with the mathematical rules that this program uses. In order to identify differentially expressed autophagy-related and apoptosis-related genes, linear regression was performed. Data on the QRT-PCR analysis were also clustered using self-organizing maps (SOMs).

3 Results

3.1 Cell Cycle Changes After siRNA Silencing and Temozolomide or Carmustine Treatment

In order to examine the possible mechanisms of the antiproliferative activity of *AKT3* and *PI3KCA* siRNA in combination with TMZ or BCNU, cell cycle distribution using flow cytometry was performed. The proliferation index (PI), i.e. the percentage of proliferating cells in the S + G2/M cell cycle phases, was determined. The PI was quantified in untransfected T98G cells and after knockdown with adequate 1 nM siRNA and TMZ or BCNU exposition. We found that *AKT3* siRNA in combination with 50 μM BCNU increased the percentage of the cells in the S phase (versus siRNA *PI3KCA* + BCNU and only BCNU as well) and simultaneously increased the percentage of cells in the PI, but these changes were not statistically significant. Simultaneously, siRNA specific for *PI3KCA* in combination with 50 μM BCNU markedly increased the percentage of cells in the subG1 phases compared with *AKT3* siRNA–transfected cells as well as untransfected cells after their exposure to BCNU (Fig. 1a; $p < 0.05$ by one-way ANOVA and post hoc Tukey's multiple comparison test). We also found that *AKT3* and *PI3KCA* siRNA in combination with 10 μM TMZ did not significantly change the cell cycle distribution and their PI as compared with cells exposed only to the drug (Fig. 1a, b, respectively).

3.2 Apoptosis and Necrosis Induction After siRNA Silencing and Temozolomide or Carmustine Treatment

Necrotic and apoptotic cells were detected using flow cytometry and double staining with the above-mentioned apoptosis kit following siRNA silencing and TMZ or BCNU exposition. Knockdown of the *AKT3* and *PI3KCA* genes in combination with BCNU led to induction of apoptosis in 77 % of the cells (siRNA *AKT3* + BCNU) and 77.6 % of the cells (siRNA *PI3KCA* + BCNU), respectively, compared with 8.7 % of the untransfected (control) cells and compared with *AKT3* and *PI3KCA* gene silencing in combination with TMZ treatment (43.6 and 47.4 %, respectively) (Fig. 2a; $p < 0.05$ by one-way ANOVA and post hoc Tukey's multiple comparison test). Moreover, we noticed a general tendency for gene silencing to increase the apoptotic index in cells combined with TMZ or BCNU exposure compared with cells with only drug exposure (Fig. 2b; statistical significance only in the case of siRNA *PI3KCA* + TMZ and siRNA *AKT3* + TMZ).

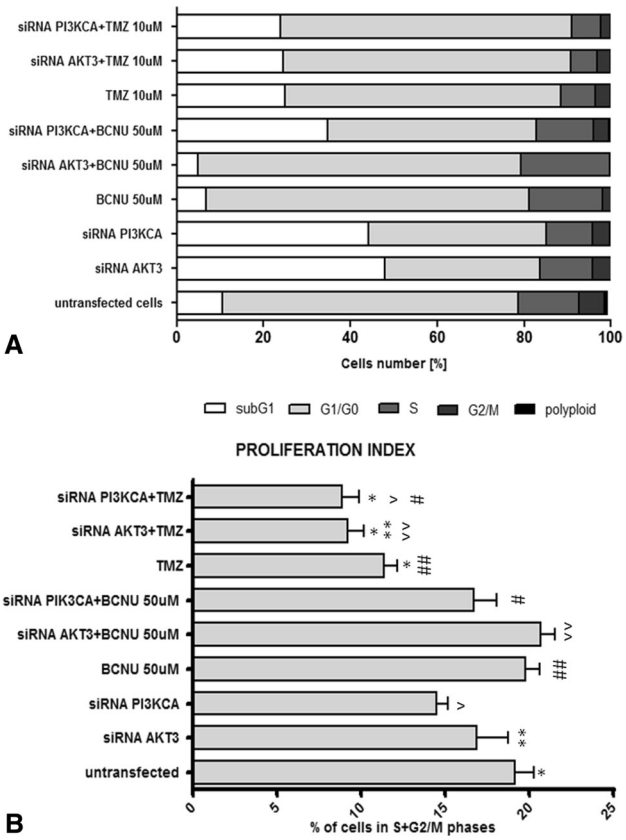

Fig. 1 Cell cycle distribution (**a**) and proliferation index (**b**) in the T98G cell culture that was transfected with *AKT3* and *PI3KCA* small interfering RNA (siRNA) and treated with temozolomide (TMZ) or carmustine (BCNU), respectively. The results are presented as the percentage contribution of the number of cells located in each cell cycle phase, including the subG1 population (**a**) and the number of cells located in the S + G2/M cell cycle phases (**b**). The presented data were obtained from DNA histograms and represent an average of six independent repeats. To estimate statistical significance (**b**), the data were analyzed by one-way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison test, using Statistica software (StatSoft, Inc., 2008), version 10.0 (http://www.statsoft.com) ($p < 0.05$). *Symbols* indicate significant differences: *asterisk* untransfected cells versus TMZ, siRNA PI3KA + TMZ, and siRNA *AKT3* + TMZ; *double asterisk* siRNA *AKT3* versus siRNA *AKT3* + TMZ; *hat symbol* siRNA *PI3KCA* versus siRNA *PI3KCA* + TMZ; *double hat symbol* siRNA *AKT3* + BCNU versus siRNA *AKT3* + TMZ; *number sign* siRNA *PI3KCA* + BCNU versus siRNA *PI3KCA* + TMZ. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

In contrast, the necrosis rates of transfected T98G cells were 35.8 and 30 % after *AKT3* and *PI3KCA* silencing in combination with TMZ, respectively, compared with the necrosis rate of the untransfected (control) cells, which was only 1.8 %, whereas knockdown of *AKT3* or *PI3KCA* genes in combination with BCNU led to necrosis rates of 13.3 and 12.7 %, respectively (Fig. 3a; $p < 0.05$ by one-way ANOVA and post hoc Tukey's multiple comparison test). However, it is noteworthy that combination of gene silencing with TMZ

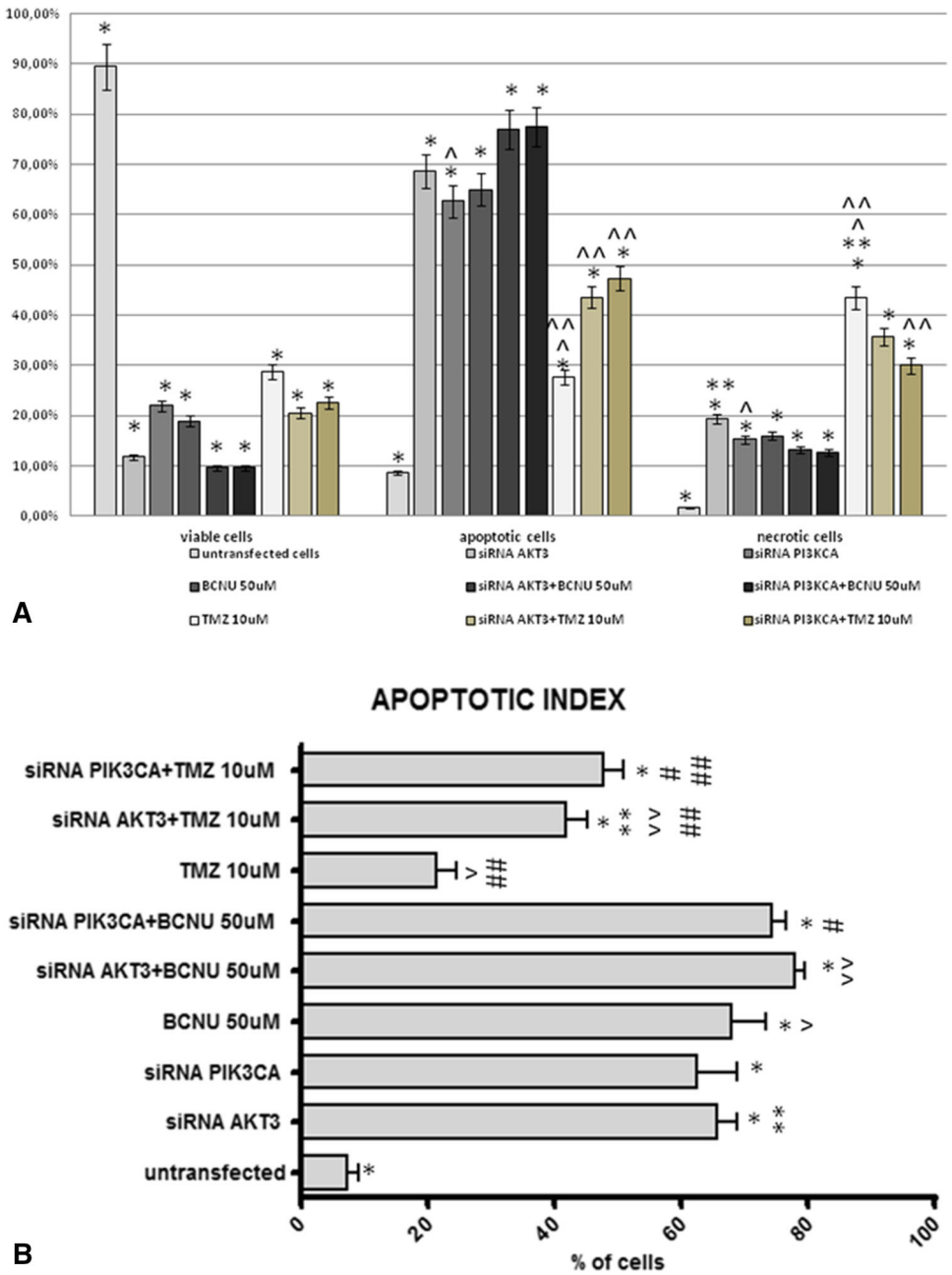

Fig. 2 Influence of knockdown of the *AKT3* and *PI3KCA* genes on induction of apoptosis in T98G cells treated with temozolomide (TMZ) or carmustine (BCNU). The results are presented as a percentage contribution (*Y axis*) of the number of viable cells, apoptotic cells, and necrotic cells in the groups that were analyzed (**a**) and apoptotic index was calculated (**b**). The data are expressed as the means of four separate experiments ($p < 0.05$ by one-way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison test). *Symbols* indicate significant differences: *asterisk* untransfected cells versus siRNA *AKT3*, siRNA *PI3KCA*, BCNU, siRNA *AKT3* + BCNU, siRNA *PI3KCA* + BCNU, siRNA *AKT3* + TMZ, and siRNA PI3KA + TMZ; *double asterisk* siRNA *AKT3* versus siRNA *AKT3* + TMZ; *hat symbol* BCNU versus TMZ; *double hat symbol* siRNA *AKT3* + BCNU versus siRNA *AKT3* + TMZ; *number sign* siRNA *PI3KCA* + BCNU versus siRNA *PI3KCA* + TMZ; *double number sign* TMZ versus siRNA *AKT3* + TMZ and siRNA *PI3KCA* + TMZ. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

or BCNU exposure reduced the percentage of necrotic cells compared with only drug exposure (Fig. 2a; statistical significance only in the case of siRNA *PI3KCA* + TMZ).

3.3 Autophagy in T98G GBM Cells with Downregulated Expression of *AKT3* and *PI3KCA* Genes Exposed to Temozolomide and Carmustine

We performed QRT-PCR for the selected genes associated with apoptosis and autophagy: *AIFM2*, *BCL2L1*, *BID*, *BNIP3*, *CASP3*, *CASP8*, *CASP9* (apoptosis), *AMBRA1*, *BECN1*, *MAP1LC3A*, *PIK3C3*, *RB1CC*, *SQSTM1*, *ULK1*, *UVRAG* (autophagy), *DRAM1*, *GSK3β*, *HIF1A*, and *PRKAA1* (both pathways). The specificity of the reaction was confirmed by agarose gel electrophoresis and a melting temperature curve.

Hierarchical clustering of the results based on the Euclidean distance was carried out using GenExEnterprise 5.4.3.703 (Fig. 3).

The obtained results allowed us to distinguish two groups of genes. The former contained genes whose expression is involved in the process of autophagy, and the latter contained genes whose expression is involved in the

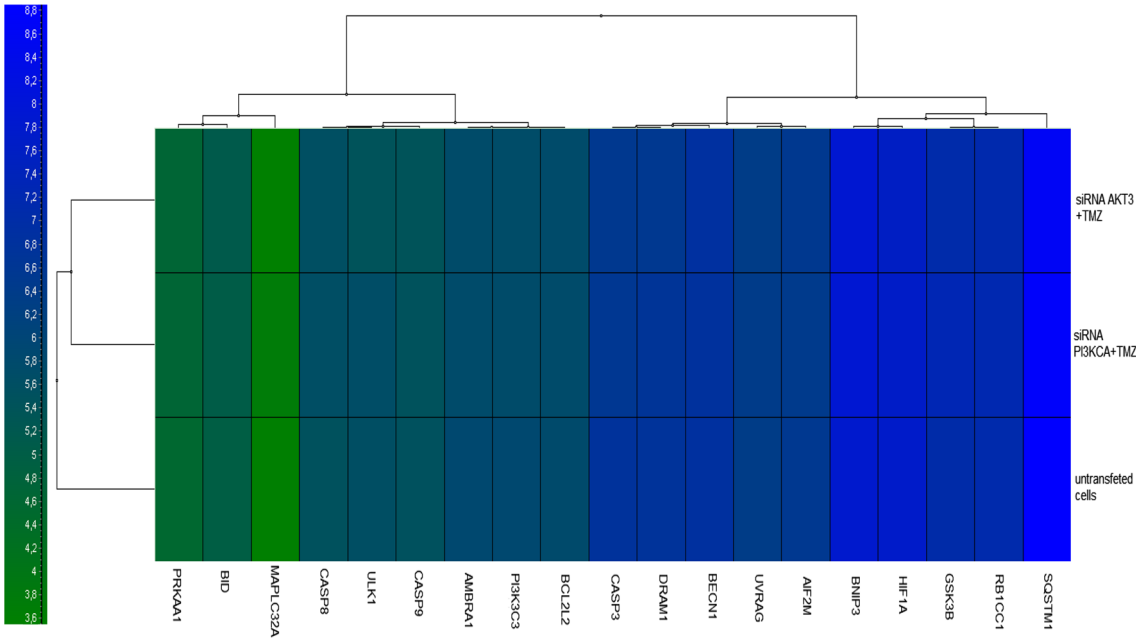

Fig. 3 Hierarchical clustering and heat map of differentially expressed autophagy-related and apoptosis/autophagy–related genes. The presented data indicate the expression pattern of 19 genes ($p < 0.001$) after knockdown of the *AKT3* or *PI3KCA* genes and exposition on temozolomide (TMZ). Data were clustered using the standard hierarchical method with average linkage and using the Pearson correlation to determine the distance function. The normalized expression index for each gene (*rows*) in each sample (*columns*) is indicated by a color code (see *expression index bar* at *left* of figure). *Colored rectangles* represent genes that were upregulated (*green*) or downregulated (*dark blue*) in the sample set. Samples with a similar pattern of gene expression are clustered together, as is indicated by the dendrogram. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

processes of autophagy and apoptosis. When we considered the similar mRNA level of genes as an expression pattern, untransfected cells were clustered as a separate group; likewise, cells that had been transfected with *AKT3* and *PI3KCA* siRNA and treated with TMZ were clustered together (Fig. 3).

We found that changes in the autophagy-related and apoptosis-related genes after *AKT3* knockdown and exposition on TMZ were manifested mainly in (1) increased *BID* (∼1.5-fold), *CASP-8* (∼1.3-fold), *BNIP3* (∼1.2-fold), *PRKAA1* (∼1.3-fold), and *BECN1* (∼1.1-fold) gene expression, as well as (2) decreased *DRAM1* (∼1.7-fold), *HIF1A* (∼1.3-fold), *AMBRA1* (∼1.34-fold), *ULK1* (∼1.5-fold), *PIK3C3* (∼1.4-fold), *SQSTM1* (∼1.6-fold), *CASP3* (∼1.8-fold), and *GSK3B* (∼1.1-fold) gene expression (Fig. 4).

Silencing of the *PI3KCA* gene and exposition on TMZ was connected with changes in the autophagy-related and apoptosis-related genes, mostly manifested in (1) increased *CASP-8* (∼1.4-fold), *ULK1* (∼1.2-fold), *BNIP3* and PRKAA1 (∼1.3-fold), BID (∼1.2-fold), and *GSK3B* (∼1.7-fold) gene expression, as well as (2) decreased *PIK3C3* (∼1.4-fold), *HIF1A* (∼1.1-fold), *AMBRA1* (∼1.3-fold), *BECN1* and *DRAM1* (∼1.1-fold), *SQSTM1* (∼1.2-fold), and *CASP3* (∼1.5-fold) gene expression (Fig. 4).

When we considered a similar mRNA level of genes as an expression pattern for cells treated with BCNU, cells with knockdown of the *PI3KCA* gene treated with BCNU were clustered separately; likewise, untransfected cells and cells transfected with *AKT3* siRNA and treated with BCNU were clustered together (Fig. 5).

We found that changes in autophagy-related and apoptosis-related genes after *AKT3* knockdown and exposition on BCNU were manifested mainly in (1) increased *GSK3B* (∼4.6-fold) and *CASP-8* (∼1.2-fold) gene expression, as well as (2) decreased *PI3K3C3* (∼3.2-fold), *BNIP3* and *AIFM2* (∼2.3-fold), *SQSTM1* and *DRAM1* (∼2.1-fold), *BCL2L1* and *RB1CC1* (∼1.7-fold), *CASP9*, *PRKAA1*, *UVRAG*, *BID* and *AMBRA1* (∼1.5-fold), *ULK1* (∼1.4-fold), *MAPLC3IIA*, *BECN1*, and *HIF1A* (∼1.2-fold) gene expression (Fig. 6).

Silencing of the *PI3KCA* gene and exposition on BCNU was connected with changes in the autophagy-related and apoptosis-related genes, mostly manifested in decreased *ULK1* (∼10.9-fold), *AMBRA1* (∼9-fold), *AIFM2* (7.9-fold), *PI3K3C3* (∼5.7-fold), *SQSTM1* (∼4.8-fold), *HIF1A* (∼4.2-fold), *BNIP3* and *CASP9* (∼4.1-fold), *RB1CC1* and

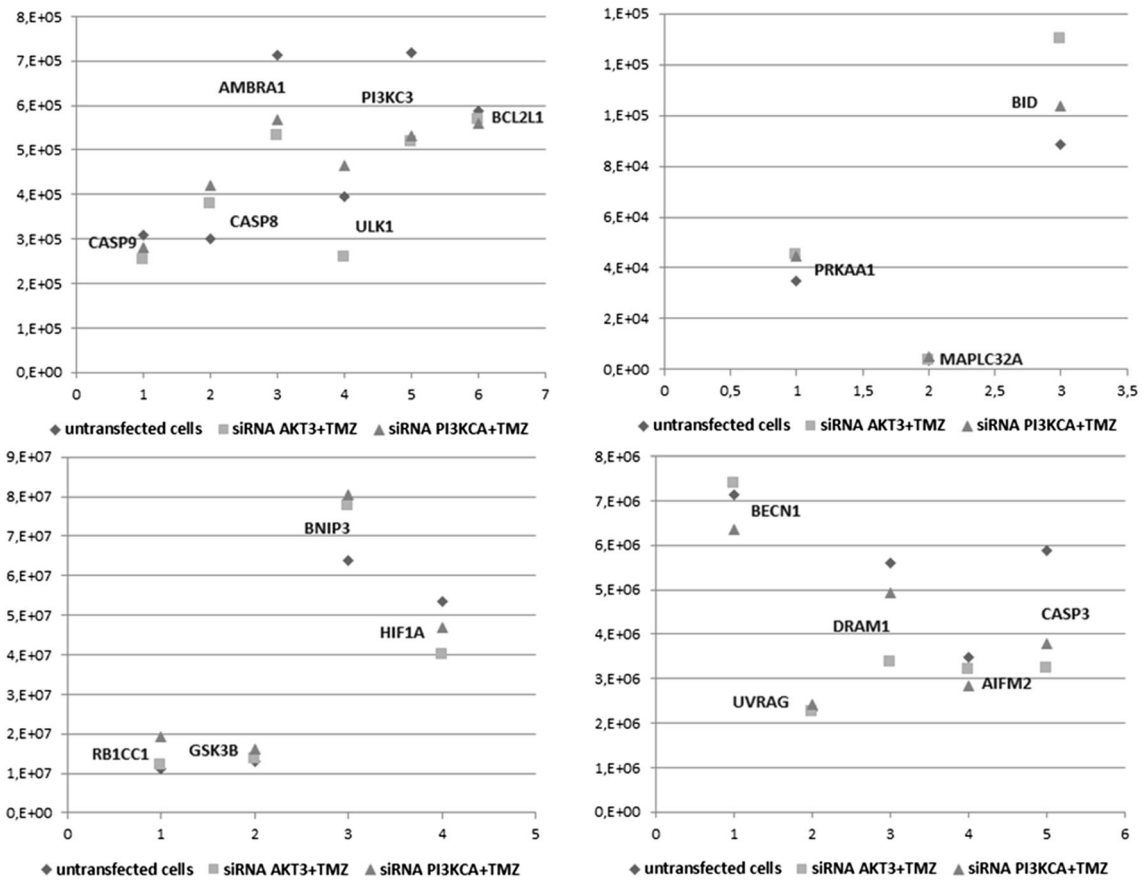

Fig. 4 Comparison of the messenger RNA (mRNA) copy number (*Y axis*) of autophagy-related and apoptosis-related genes differentiating cells transfected with *AKT3* and *PI3KCA* small interfering RNA (siRNA) and treated with temozolomide (TMZ) from untransfected cells. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* - gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

BCL2L1 (∼3.9-fold), *UVRAG* (∼3.7-fold), *DRAM* (∼2.1-fold), *GSK3B* and *BECN1* (∼2-fold), and *PRKAA1* (∼1.5-fold) gene expression (Fig. 6).

To compare the mechanism of action for siRNA specific for the *AKT3* and *PI3KCA* genes in cells exposed to TMZ or BCNU, we analyzed changes in the number of mRNA copies relative to the control (Fig. 7). Our study revealed that knockdown of the *AKT3* gene in cells exposed to BCNU causes a significant increase in the mRNA copy number of *GSK3B* and *CASP8*, while the *PI3KCA* gene silencing in cells exposed to TMZ significantly increased the number of mRNA copies of *RB1CC1*, *GSK3B*, *ULK1*, *CASP8*, *BNIP3*, *BID*, *PRKAA1*, and *MAPLC3IIA*. In contrast, silencing the *AKT3* gene in the same cells caused a significant increase in the number of mRNA copies of *BID*, *GSK3B*, *PRKAA1*, *BNIP3*, and *CASP8* (Fig. 7).

To test our hypothesis that the knockdown of *AKT3* or *PI3KCA* genes in cells exposed to TMZ or BCNU causes the induction of autophagy, we performed LysoTracker Red staining (a deep red-fluorescent dye for labeling and tracking acidic organelles in live cells), which preferentially accumulates in vesicles with an acidic pH and may be used to examine the efficiency of autophagosome/lysosome fusion in live cells. Our results indicated that *AKT3* and *PI3KCA* gene silencing in cells exposed to TMZ or BCNU was associated with an increased intensity of red fluorescence, which indicated an increased number of acidic vesicles characteristic of autophagy (Fig. 8; $*p < 0.05$ by one-way ANOVA and post hoc Tukey's multiple comparison test). These changes were more significant than in untransfected cells.

SOM analysis was also performed in order to provide a comprehensive, quantitative, yet lucid picture of the gene expression changes in cells with knockdown of *AKT3* or *PI3KCA* genes and exposure to TMZ or BCNU. Variance in this group was a measure of the degree of dissimilarity among the gene expressions that were clustered together. We noticed that the genes that are involved in the processes of autophagy and apoptosis could be divided into four categories, which included genes that had similar expression changes after *AKT3* or *PI3KCA* knockdown in cells exposed to TMZ or BCNU compared with untransfected cells (Fig. 9). The SOM is an unsupervised neural network algorithm that can cluster the data of gene expression

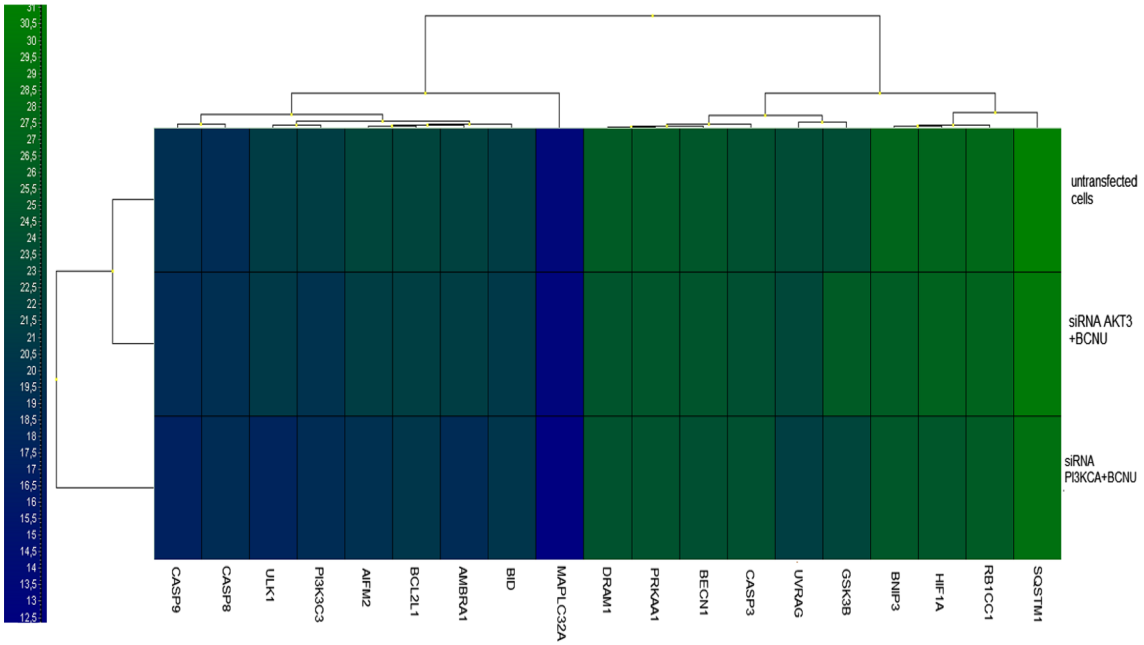

Fig. 5 Hierarchical clustering and heat map of differentially expressed autophagy-related and apoptosis/autophagy-related genes. Data indicating the expression pattern of 19 genes ($p < 0.001$) after knockdown of the *AKT3* or *PI3KCA* genes and exposition on carmustine (BCNU) are shown. Data were clustered using the standard hierarchical method with average linkage and using the Pearson correlation to determine the distance function. The normalized expression index for each gene (*rows*) in each sample (*columns*) is indicated by a color code (see *expression index bar* at *left* of figure). *Colored rectangles* represent genes that were upregulated (*green*) or downregulated (*dark blue*) in the sample set. Samples with a similar pattern of gene expression are clustered together, as is indicated by the dendrogram. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

analysis into biologically meaningful groups. The first group consisted of *MAPLC3IIA*, *CASP8*, and *CASP9* genes (for cells exposed to BCNU) and *RB1CC1*, *GSK3B*, *HIF1A*, *BECN1*, *SQSTM1*, and *BNIP3* genes (for cells exposed to TMZ); the second consisted of *AMBRA1*, *BID*, *ULK1*, *AIFM2*, *BCL2L1*, and *PI3K3C3* genes (for cells exposed to BCNU) and *BCL2L1*, *ULK1*, *AMBRA1*, *PI3K3C3*, *CASP8*, and *CASP9* (for cells exposed to TMZ); the third consisted of *BECN1*, *DRAM1*, *UVRAG*, *GSK3B*, *CASP3*, and *PRKAA1* genes (for cells exposed to BCNU) and *UVRAG*, *DRAM1*, *AIFM2*, and *CASP3* (for cells exposed to TMZ); and the fourth consisted of *SQSTM1*, *RB1CC1*, *BNIP3*, and *HIF1A* genes (for cells exposed to BCNU) and *BID*, *MAPLC3IIA*, and *PRKAA1* (for cells exposed to TMZ). Genes were grouped by a similar mRNA level of genes that reflected their expression pattern.

4 Discussion

TMZ is the first-line treatment in patients with GBM, but 45 % of patients are resistant to this drug. Even after complete resection of a tumor, a recurrence is very often observed. Therefore, more effective therapies with multimodel strategies based on synergistic effects for treatment of central nervous system neoplasms are urgently needed. The present study was focused on the survival as well as apoptotic and autophagy behaviors of the human GBM T98G cell line treated with a combination of *AKT3* and *PI3KCA*-specific siRNA with TMZ or BCNU exposure.

The PI3K/AKT signaling pathway plays a crucial role in the development, progression, and invasiveness of GBM. This pathway is responsible for regulation of cell cycle progression, proliferation, apoptosis, and autophagy. Our previous study showed that knockdown of *AKT3* and/or *PI3KCA* genes in T98G cells led to a significant reduction in cell viability, accumulation of subG1-phase cells, and a reduction of cells in the S and G2/M phases. Additionally, significant differences in the BAX/BCL-2 ratio and an increased percentage of apoptotic cells were found [29]. Thus, the purpose of this study was to evaluate whether knockdown of the *AKT3* and *PI3KCA* genes interfered with the mechanism of action of TMZ or BCNU and sensitized GBM cells to these anticancer drugs.

We found that siRNA that targeted *AKT3* and *PI3KCA* in combination with TMZ greatly decreased the percentage of T98G cells in the S phase and the proliferative index—

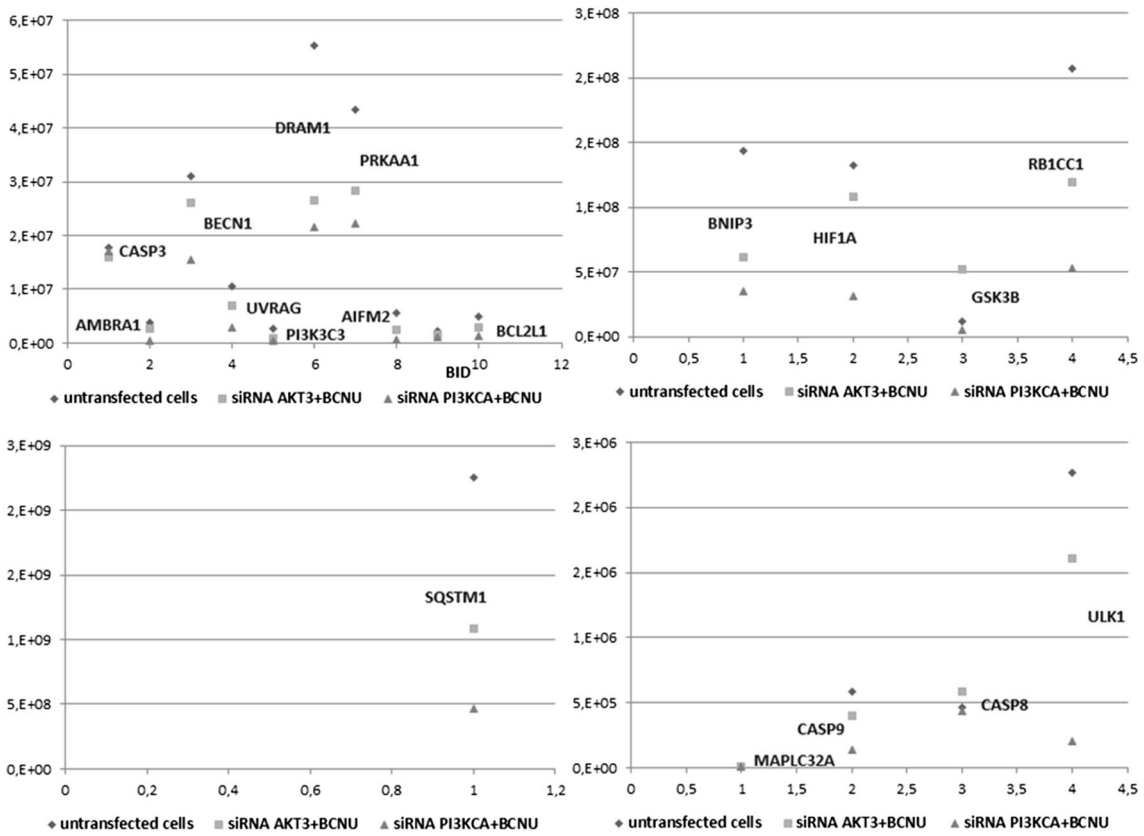

Fig. 6 Comparison of the messenger RNA (mRNA) copy number (*Y axis*) of autophagy-related and apoptosis-related genes differentiating cells transfected with *AKT3* and *PI3KCA* small interfering RNA (siRNA) and treated with carmustine (BCNU) from untransfected cells. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* - gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

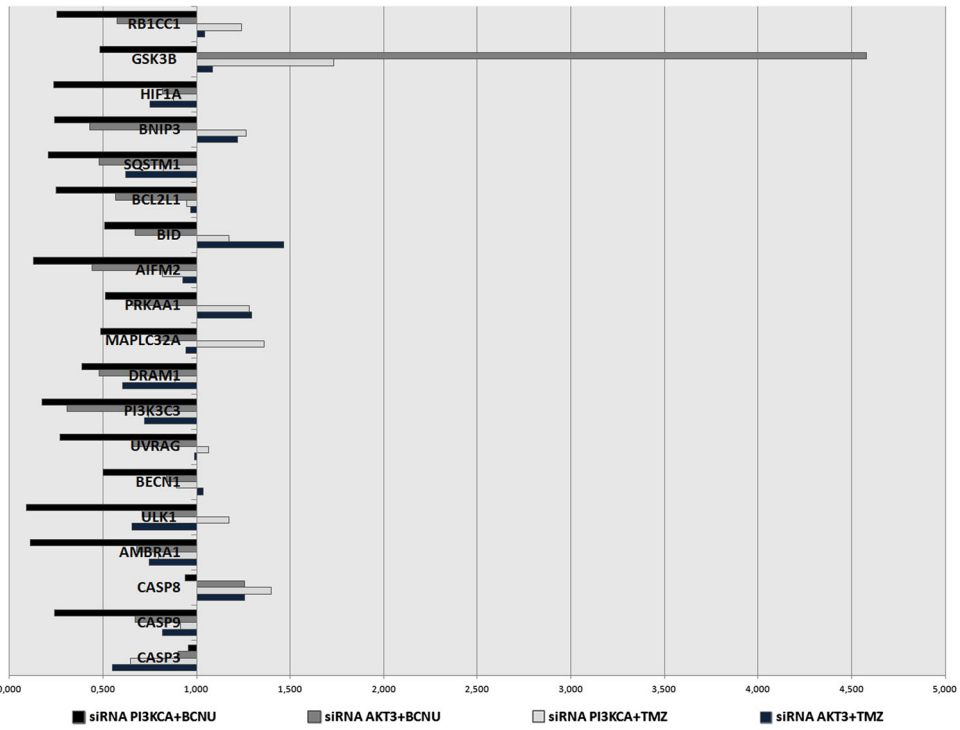

Fig. 7 Changes with autophagy-related and apoptosis-related genes differentiating cells transfected with *AKT3* and *PI3KCA* small interfering RNA (siRNA) and treated with temozolomide (TMZ) or carmustine (BCNU) from untransfected cells. The data are presented as the change in the number of messenger RNA (mRNA) copies relative to the control (*X axis*). *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

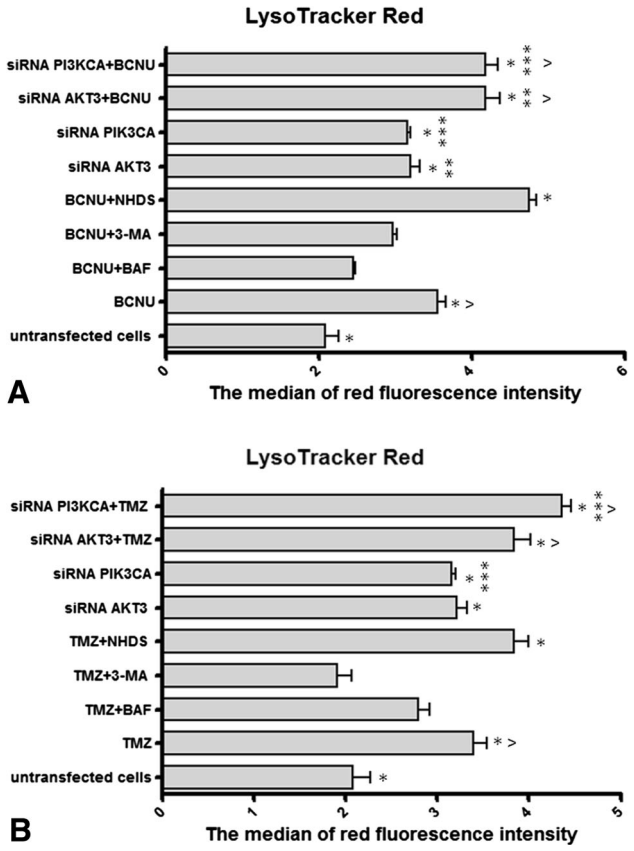

Fig. 8 Comparison of red fluorescence intensity (LysoTracker Red staining) indicating an increased number of acidic vesicles that are characteristic for induction of autophagy. The results concern cells that were transfected with specific small interfering RNA (siRNA) as well as untransfected T98G cells exposed to carmustine (BCNU) (**a**) or temozolomide (TMZ) (**b**) with or without addition of an autophagy inductor [*N*-hexanoyl-D-sphingosine (NHDS)] or inhibitors [3-methyladenine (3-MA) and bafilomycin A1 (BAF)]. The red fluorescence intensity after LysoTracker Red staining was determined using flow cytometry [$p < 0.05$ by one-way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison test]. *Symbols* indicate significant differences: *asterisk* untransfected cells versus siRNA *AKT3*, siRNA *PI3KCA*, BCNU, BCNU + NHDS, siRNA *AKT3* + BCNU, siRNA *PI3KCA* + BCNU, TMZ + NHDS, siRNA *AKT3* + TMZ, and siRNA *PI3KCA* + TMZ; *double asterisk* siRNA *AKT3* versus siRNA *AKT3* + BCNU; *triple asterisk* siRNA *PI3KCA* versus siRNA *PI3KCA* + BCNU and siRNA *PI3KCA* + TMZ; *hat symbol* BCNU versus siRNA *AKT3* + BCNU and siRNA *PI3KCA* + BCNU, or TMZ versus siRNA *AKT3* + TMZ and siRNA *PI3KCA* + TMZ. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

more so than the same siRNA in combination with BCNU. In contrast to our findings, Hirose et al. and Kanzawa [33, 34] found that malignant glioma cells responded to TMZ by undergoing G2/M arrest. These differences may result from the use of other doses of TMZ or exposure times as well as sensitizing T98G cells to this drug. Kanzawa et al. tested TMZ in a concentration of 5–1000 μM for 72 h [34]. In our case, we tested 10 μM of TMZ for 48 h. Carmo et al. described the effect of TMZ on the U-118 glioma cell line and found, like us, that TMZ reduced U-118 proliferation. Those authors suggested the need to use a combined therapy using TMZ and inhibitors of the PI3K/Akt and ERK1/2 MAP kinase signaling pathways [33, 34]. We also showed that knockdown of the *AKT3* gene in combination with BCNU triggered an increase in T98G cells in the S phase. Teicher et al. analyzed exposure of the T98G cells to BCNU (1, 5, 10, 50, 100, and 250 μM) for 1 h, and this resulted in a half-maximal inhibitory concentration (IC_{50}) of about 250 μM [39]. We observed a significant decrease of T98G viability after a 50 μM dose of BCNU, but only in cells transfected with siRNA specific for the *AKT3* or *PI3KCA* genes. We also showed that knockdown of the *AKT3* and *PI3KCA* genes in combination with BCNU was associated with a significantly higher apoptotic index in comparison with transfected cells treated with TMZ. Opel et al. (2008) reported that inhibition of PI3K was an efficient strategy to sensitize glioblastoma cells to the induction of apoptosis [40]. We noticed that *PI3KCA*-specific siRNA in combination with BCNU increased the percentage of T98G cells that underwent apoptosis (subG1 fraction). On the other hand, De Salvo et al. suggested that TMZ-induced apoptosis occurred through Akt/glycogen-synthase-kinase-3β (GSK3β) signaling and was mediated by c-Myc oncoprotein [41]. We found significantly increased *GSK3β* mRNA copy numbers in T98G cells after knockdown of *PI3KCA* and treatment with TMZ, whereas Carmo et al. found a significantly higher percentage of cells in the subG1 phase after incubation of U-118 cells with TMZ. However, their study demonstrated that TMZ induced a low level of apoptosis [42]. Our study clearly showed that knockdown of either *AKT3* or *PI3KCA* genes sensitized T98G cells to apoptosis induction after TMZ exposure. It is noteworthy that in our study, the combination of TMZ with *AKT3* and *PI3KCA* siRNA decreased necrosis in T98G cells in comparison with cells treated only with TMZ. The high percentage of necrosis within cells after *AKT3* and *PI3KCA* knockdown, as well as after treatment with BCNU or TMZ, is a well-described fact. Jakubowicz-Gil et al. observed that TMZ applied to T98G culture for 48 and 72 h at a concentration higher than 100 μM induced necrosis, and the increased number of necrotic cells was accompanied by a decreased number of apoptotic ones [4]. Thus, a very important general observation of our study was that the percentage of apoptotic cells was increased and the percentage of necrotic cells was decreased after combination of TMZ and BCNU with silencing of the analyzed genes.

The above-mentioned observations are also consistent with our findings concerning changes in the mRNA copy numbers of genes associated with apoptosis compared

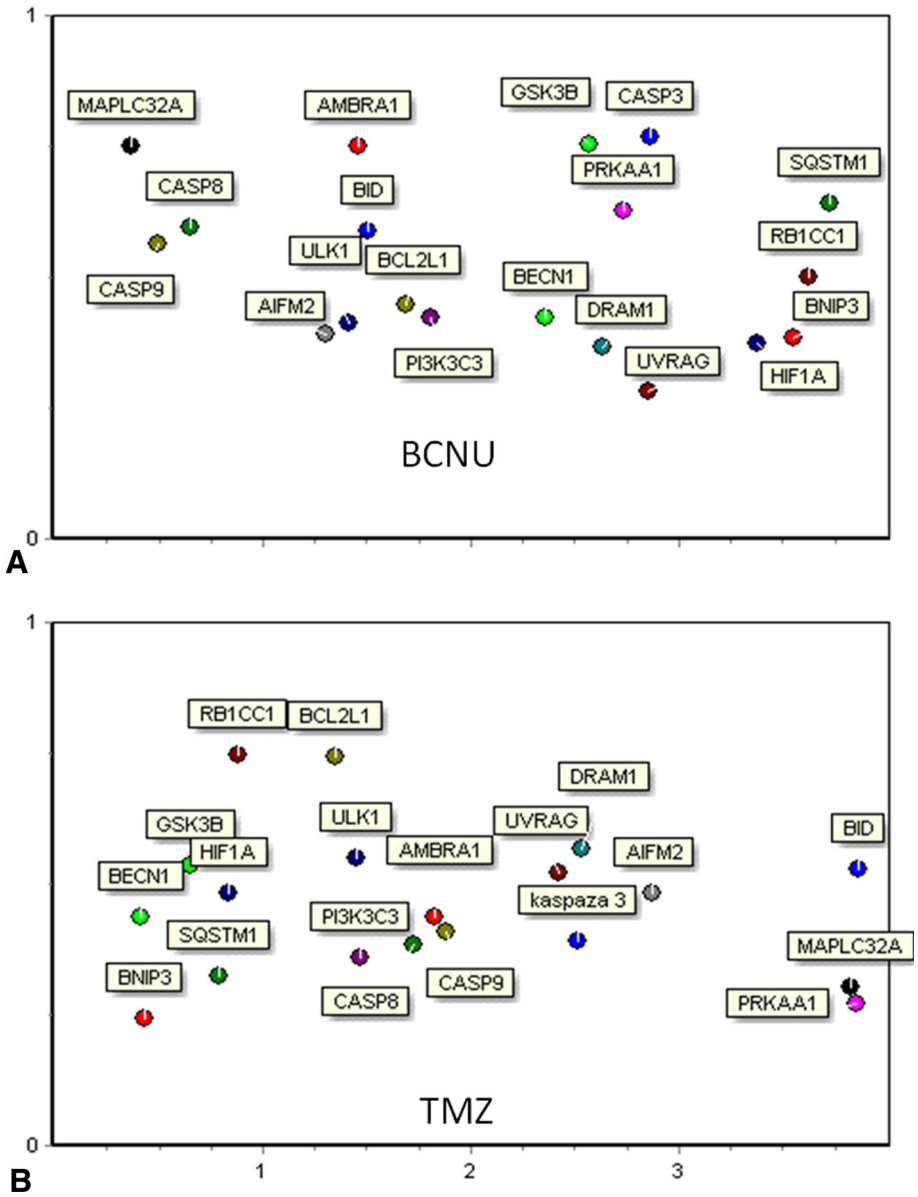

Fig. 9 Kohonen map [self-organizing map (SOM) analysis result] illustrating the gene expression patterns after *AKT3* or *PI3KCA* knockdown in T98G cells exposed to carmustine (BCNU) (**a**) or temozolomide (TMZ) (**b**) compared with untransfected cells. SOM clusters this gene expression data set into four groups that show similar gene expression profiles and also contain functionally related genes. The algorithm places genes with similar, but not identical, profiles in neighboring groups, thus creating a smooth transition of related profiles over the whole matrix. SOM uses Euclidean distance to measure the similarities between data items. Map units that are close on the grid represent more similar expression profiles, and units farther away represent progressively more different profiles. *AKT3* gene encoding isoform 3 of protein kinase B, *PI3KCA* gene encoding p110α catalytic subunit of phosphoinositide 3-kinase

with untransfected (control) cells. We found increased expression of *BNIP3*, *BID*, and *CASP8* genes and decreased expression of *BCL2L1*, *AIFM2*, *CASP3*, *CASP9*, and *HIF1A* genes in *PI3KCA* siRNA-transfected cells treated with TMZ. On the other hand, our study also revealed that knockdown of the *AKT3* gene in cells exposed to BCNU determined a significant increase in the mRNA copy number of *CASP8* and decreases in *BNIP3*, *AIFM2*, *BCL2L1*, *CASP9*, and *BID*. We found decreased expression of the *BNIP3*, *BCL2L1*, *AIFM2*, and *CASP3* genes in *PI3KCA* siRNA-transfected cells treated with BCNU.

BNIP3 is known to induce autophagy and plays a central role in As_2O_3-induced autophagic cell death in malignant glioma cells [43]. BNIP3 is localized to the nucleus in the majority of GBM and fails to induce cell death. It is known that BNIP3 binds to the promoter of the apoptosis-inducing factor (AIF) gene and represses its expression. It can explain the decreased mRNA copy number of the *AIFM2* gene in cells transfected with *AKT3* and *PI3KCA* siRNA and exposed to tested drugs.

We also revealed increased expression of the caspase 8 encoding gene (*CASP8*), which may suggest activation of extrinsic apoptosis as in the case of exposition of BCNU. We also postulated the activation of intrinsic apoptosis, because we found increased expression of the *BID* gene. BID is a mediator of the mitochondrial damage induced by CASP8. This caspase cleaves the BID protein, and the COOH-terminal part translocates to the mitochondria and triggers the release of cytochrome c.

Our results also showed a decreased *BCL2L1* mRNA copy number in T98G cells after knockdown of the *AKT3* or *PI3KCA* gene and exposure to BCNU. The proteins encoded by this gene are located at the outer mitochondrial membrane and have been shown to regulate the outer mitochondrial membrane channel opening. BCL2L1 is a potent inhibitor of cell death. It inhibits the activation of caspases. This protein appears to regulate cell death by blocking the voltage-dependent anion channel (VDAC) by binding to it and preventing the release of the caspase activator, CYC1, from the mitochondrial membrane. BCL2L1 also acts as a regulator of the G2 checkpoint and progression to cytokinesis during mitosis [44].

We also analyzed how the knockdown of the *AKT3* or *PI3KCA* gene in combination with TMZ or BCNU affected autophagy. The role of autophagy in cancer cells is controversial, and some studies have indicated that the specific function of autophagy depends on certain circumstances, i.e. cell types, cellular context, and the nature of the treatment, and this process has been implicated both as a mechanism of cell death and as a cytoprotective phenomenon [45]. Many anticancer agents (i.e. tamoxifen, rapamycin, arsenic trioxide, histone deacetylase inhibitors, ionizing radiation, vitamin D analogs, etoposide, and TMZ) have been reported to induce autophagy, and this kind of cell death may be an important mechanism for killing tumor cells [46–48]. The effect of autophagy inhibition on cell survival is controversial, but blocking autophagy at a late stage has been shown to cause accelerated cell death under autophagy-inducing conditions [38, 49–52]. Some results have indicated that activation of the PI3K-Akt pathway suppresses autophagy in mammalian cells [53]. However, other studies have pointed out that the PI3K-Akt pathway positively regulates autophagy and suppresses [54] or induces apoptosis [55].

Our study also revealed a significant increase of red fluorescence intensity after LysoTracker Red staining in cells with knockdown of the *AKT3* or *PI3KCA* gene and exposure to TMZ or BCNU compared with cells treated with these drugs without prior transfection. Bearing in mind the foregoing differences in the mRNA copy numbers of genes connected with the autophagy process, the last results suggest the induction of autophagy in T98G cells.

Our previous results showed that the knockdown of *AKT3* and *PI3KCA* genes induced the autophagy process in T98G cells [56]. Thus, we were very interested to evaluate whether the knockdown of the examined genes would also sensitize GBM cells to TMZ and BCNU by affecting the autophagy process. Autophagy may be induced by Akt inhibition, either as a precursor of apoptosis in apoptosis-sensitive cell lines or as a result of destructive self-digestion. It is known that activators of apoptosis can induce autophagy, whereas factors that negatively regulate apoptosis also inhibit autophagy induction [57]. We used a specific recommended autophagy inductor and inhibitors, which would give more accurate results concerning autophagy as a multistep process. The obtained results indicated that the combined therapy sensitized T98G to TMZ and BCNU by also affecting the autophagy process, but these results need to be confirmed and expanded upon by other authors.

There is evidence that autophagy and apoptosis can cooperate, antagonize, or assist each other. Nodes of crosstalk between apoptosis and autophagy include the Beclin 1–BCL-2 interaction, caspase-mediated Beclin 1 cleavage and UVRAG–BAX interaction. Thus, we also analyzed the changes in the mRNA copy numbers of genes associated with autophagy and genes associated with both autophagy and apoptosis processes in comparison with untransfected (control) cells. We found increased expression of *ULK1*, *MAPLC3IIA*, *RB1CC1*, *PRKAA1*, *UVRAG*, and *GSK3B* genes and decreased expression of *BECN1*, *AMBRA1*, *PI3K3C3*, *SQSTM1*, *DRAM1*, and *HIF1A* genes in *PI3KCA* siRNA-transfected cells treated with TMZ. On the other hand, our study also revealed that knockdown of the *AKT3* gene in cells exposed to BCNU determined a significant increase in the mRNA copy number of *GSK3B* and decreases in *PI3K3C3*, *SQSTM1*, *DRAM1*, *RB1CC1*, *PRKAA1*, *UVRAG*, *AMBRA1*, *ULK1*, *MAPLC3IIA*, *BECN1*, and *HIF1A*. We found decreased expression of the *ULK1*, *RB1CC1*, *UVRAG*, *GSK3B*, *BECN1*, *AMBRA1*, *PI3K3C3*, *SQSTM1*, and *HIF1A* genes in *PI3KCA* siRNA-transfected cells treated with BCNU.

ULK1/2 induces autophagy in several ways [58–61] and plays a key role in inducing autophagy in response to starvation [62]. Klionsky et al. (2008) suggested that decreased expression of SQSTM1 may constitute evidence of an active process of autophagy [63]. The autophagy core complex, comprising BECN1, PIK3C3, PIK3R4, ATG14, and Ambra1 proteins, plays a crucial role in the autophagy activation process [64]. Ambra1 binds to Beclin 1 and stabilizes the Beclin 1/Vps34 complex, finally promoting autophagosome formation [65]. Fimia et al. suggested that reduced levels of Ambra1 in a variety of cell lines lead to increased susceptibility to different apoptotic stimuli. Researchers have postulated that apoptosis induction causes Ambra1 degradation, which occurs in a caspase- and calpain-dependent manner, and that cleavage of this protein occurs early during the apoptotic process [66]. *BECN1* plays a role in two fundamentally important cell biological pathways—autophagy and apoptosis. It is a major determinant in the initiation of autophagy [67], which is involved in the formation of preautophagosomal structures, and its interactions with Bcl-2 and Bcl-xL [68] inhibit autophagy [69–71].

We also found increased expression of the *PRKAA1* gene in cells with knockdown of the *PI3KCA* gene and treatment with TMZ. The protein encoded by this gene belongs to the ser/thr protein kinase family, and it is a catalytic subunit of 5′-adenosine monophosphate–activated protein kinase (AMPK). AMPK acts as a key regulator of cell growth and proliferation by phosphorylating TSC2, RPTOR, and ATG1/ULK1. AMPK promotes autophagy [72]. Our study revealed decreased expression of the *DRAM1* gene. It is a lysosomal modulator of autophagy, which plays a central role in TP53-mediated apoptosis. TP53 induces autophagy via transcriptional activation of *DRAM1*. Downregulation of the *DRAM1* gene in T98G cells may be connected with mutation of the *TP53* gene in this cell line [73, 74]. Downregulation of *DRAM1* expression may be a good phenomenon, because high levels of DRAM1 have been associated with shorter overall survival in GBM patients [75].

An increased mRNA copy number of the *GSK3B* gene was also observed after knockdown of the *AKT3* and *PI3KCA* genes in combination with TMZ. GSK3B is involved in energy metabolism, neuronal cell development, and body pattern formation through triggering of the degradation of signaling or functional proteins. PIK3CA induces phosphorylation of GSK3B at Ser9 and attenuates the interaction of GSK3B with BCL2, and it is connected with the ubiquitin-mediated degradation of BCL2. It may also be connected with the induction of autophagy, because the increased expression of BCL2 interferes with the activation of BECN1 and attenuates autophagy in cancer cells [76, 77].

We also observed decreased expression of the *HIF1A* gene in *PI3KCA* siRNA-transfected cells treated with TMZ. It is known that HIF-1A is necessary for proliferation, growth, and angiogenesis [78], and overexpression of HIF-1A has been observed in many different human cancer cases, including brain tumors [79].

In our studies, the results interestingly showed that knockdown of the analyzed genes in combination with BCNU was connected with downregulation of *UVRAG* (UV radiation resistance-associated gene) expression. It is known that UVRAG or overexpression of a dominant-negative form of UVRAG decreases the autophagy level and triggers uncontrolled cell proliferation [80]. Downregulated *UVRAG* expression might be connected with autophagy induction and decreased GBM cell proliferation, which we have described previously [56]. Downregulation of the *UVRAG* mRNA copy number might be connected with induction of apoptosis through interactions with Bax. However, UVRAG forms two different complexes (UVRAG–Beclin1 and UVRAG–Bax), which regulate the balance between apoptosis and autophagy, and, in this complex, UVRAG is proposed to function as a positive regulator of autophagy and a negative regulator of apoptosis [81].

We also analyzed the expression of the *RB1CC1* gene, and we found a decreased mRNA copy number in transfected cells exposed to BCNU. RB1CC1 is involved in the regulation of cell growth, proliferation, survival, and spreading/migration, which is essential for autophagosome formation and also inhibits the kinase activity of Pyk2, whose overexpression has been shown to induce apoptosis in a number of cell lines [82]. Thus, a decreased *RB1CC1* mRNA copy number might be connected with induction of apoptosis.

5 Conclusion

In the present study, we demonstrated for the first time that TMZ in combination with *AKT3* or *PI3KCA*-specific siRNA-induced autophagy and apoptosis, and reduced necrosis, as well as inhibiting the proliferation of T98G GBM cells more efficiently than TMZ alone. We also revealed that BCNU in combination with *AKT3* or *PI3KCA*-specific siRNA was suggested to induce apoptosis and autophagy in T98G cells more efficiently than BCNU alone. We showed that knockdown of the *AKT3* and *PI3KCA* genes had some beneficial aspects as a part of combined therapy with routinely used drugs. Because this is only a preliminary study, the precise mechanism of the mentioned combination needs to be clarified in further studies.

Compliance with Ethical Standards

Funding This work was supported by a grant from the Medical University of Silesia. The University had no further role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

Conflicts of interest Monika Paul-Samojedny, Adam Pudełko, Malgorzata Kowalczyk, Anna Fila-Daniłow, Renata Suchanek-Raif, Paulina Borkowska, and Jan Kowalski have no conflicts of interest that are directly relevant to the content of this study.

References

1. Barciszewska AM, Gurda D, Głodowicz P, Nowak S, Naskręt-Barciszewska MZ. A new epigenetic mechanism of temozolomide action in glioma cells. PLoS One. 2015;10(8):e0136669. doi:10.1371/journal.pone.0136669 **(eCollection 2015)**.
2. Nagpal S. The role of BCNU polymer wafers (Gliadel) in the treatment of malignant glioma. Neurosurg Clin N Am. 2012;23(2):289–95, ix. doi:10.1016/j.nec.2012.01.004.
3. Zou Y, Wang Q, Li B, Xie B, Wang W. Temozolomide induces autophagy via ATM–AMPK–ULK1 pathways in glioma. Mol Med Rep. 2014;1:411–6. doi:10.3892/mmr.2014.2151.
4. 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):2367–78. doi:10.1007/s13277-013-0785-0.
5. Knizhnik AV, Roos WP, Nikolova T, Quiros S, Tomaszowski KH, Christmann M, Kaina B. Survival and death strategies in glioma cells: autophagy, senescence and apoptosis triggered by a single type of temozolomide-induced DNA damage. PLoS One. 2013;8(1):e55665. doi:10.1371/journal.pone.0055665.
6. Chen MY, Clark AJ, Chan DC, Ware JL, Holt SE, Chidambaram A, Fillmore HL, Broaddus WC. Wilms' tumor 1 silencing decreases the viability and chemoresistance of glioblastoma cells in vitro: a potential role for IGF-1R de-repression. J Neurooncol. 2011;103(1):87–102. doi:10.1007/s11060-010-0374-7.
7. Zhu Y, Zhao L, Liu L, Gao P, Tian W, Wang X, Jin H, Xu H, Chen Q. Beclin 1 cleavage by caspase-3 inactivates autophagy and promotes apoptosis. Protein Cell. 2010;1:468–77. doi:10.1007/s13238-010-0048-4.
8. Goldar S, Khaniani MS, Derakhshan SM, Baradaran B. Molecular mechanisms of apoptosis and roles in cancer development and treatment. Asian Pac J Cancer Prev. 2015;16(6):2129–44.
9. Ryter SW, Mizumura K, Choi AM. The impact of autophagy on cell death modalities. Int J Cell Biol. 2014:2014:502676. doi:10.1155/2014/502676.
10. Zhi X, Zhong Q. Autophagy in cancer. F1000 Prime Rep. 2015;7:18. doi:10.12703/P7-18.
11. Hönscheid P, Datta K, Muders MH. Autophagy: detection, regulation and its role in cancer and therapy response. Int J Radiat Biol. 2014;90(8):628–35. doi:10.3109/09553002.2014.907932.
12. Ryter SW, Cloonan SM, Choi AM. Autophagy: a critical regulator of cellular metabolism and homeostasis. Mol Cells. 2013;36(1):7–16. doi:10.1007/s10059-013-0140-8.
13. Polewska J. Autophagy—molecular mechanism, apoptosis and cancer. Postepy Hig Med Dosw (Online). 2012;66:921–36. doi:10.5604/17322693.1021109.
14. Thorburn A, Thamm DH, Gustafson DL. Autophagy and cancer therapy. Mol Pharmacol. 2014;85(6):830–8. doi:10.1124/mol.114.091850.
15. Zhang J. Teaching the basics of autophagy and mitophagy to redox biologists—mechanisms and experimental approaches. Redox Biol. 2015;4C:242–59. doi:10.1016/j.redox.2015.01.003.
16. Liu Y, Levine B. Autosis and autophagic cell death: the dark side of autophagy. Cell Death Differ. 2015;22(3):367–76. doi:10.1038/cdd.2014.143.
17. Tanaka S, Louis DN, Curry WT, Batchelor TT, Dietrich J. Diagnostic and therapeutic avenues for glioblastoma: no longer a dead end? Nat Rev Clin Oncol. 2013;10:14–26.
18. Wen PY, Lee EQ, Reardon DA, Ligon KL, Alfred Yung WK. Current clinical development of PI3K pathway inhibitors in glioblastoma. Neuro Oncol. 2012;14(7):819–29. doi:10.1093/neuonc/nos117.
19. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005;352:987–96.
20. Burris HA. Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/mTOR pathway. Cancer Chemother Pharmacol. 2013;71:829–42.
21. Sami A, Karsy M. Targeting the PI3K/AKT/mTOR signaling pathway in glioblastoma: novel therapeutic agents and advances in understanding. Tumour Biol. 2013;34:1991–2002.
22. Hafsi S, Pezzino FM, Candido S, Ligresti G, Spandidos DA, Soua Z, et al. Gene alterations in the PI3K/PTEN/AKT pathway as a mechanism of drug-resistance (review). Int J Oncol. 2012;40:639–44.
23. Jin W, Wu L, Liang K, Liu B, Lu Y, Fan Z. Roles of the PI-3K and MEK pathways in Ras-mediated chemoresistance in breast cancer cells. Br J Cancer. 2003;89(1):185–91.
24. Krześlak A. Akt kinase: a key regulator of metabolism and progression of tumors. Postepy Hig Med Dosw. 2010;64:490–503.
25. Mure H, Matsuzaki K, Kitazato KT, Mizobuchi Y, Kuwayama K, Kageji T, et al. Akt2 and Akt3 play a pivotal role in malignant gliomas. Neuro Oncol. 2010;12:221–32.
26. Matheny RW, Adamo ML. Current perspectives on Akt Akt-ivation and Akt-ions. Exp Biol Med (Maywood). 2009;234:1264–70.
27. Young CD, Anderson SM. Sugar and fat—that's where it's at: metabolic changes in tumors. Breast Cancer Res. 2008;10:202.
28. Lefranc F, Facchini V, Kiss R. Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. Oncologist. 2007;12:1395–403.
29. Paul-Samojedny M, Suchanek R, Borkowska P, Pudełko A, Owczarek A, Kowalczyk M, Machnik G, Fila-Daniłow A, Kowalski J. Knockdown of AKT3 (PKBγ) and PI3KCA suppresses cell viability and proliferation and induces the apoptosis of glioblastoma multiforme T98G cells. Biomed Res Int. 2014;2014:768181. doi:10.1155/2014/768181.
30. Paul-Samojedny M, Pudełko A, Suchanek-Raif R, Kowalczyk M, Fila-Daniłow A, Borkowska P, Kowalski J. Knockdown of the AKT3 (PKBγ), PI3KCA, and VEGFR2 genes by RNA interference suppresses glioblastoma multiforme T98G cells invasiveness in vitro. Tumour Biol. 2014;36(5):3263–77. doi:10.1007/s13277-014-2955-0.
31. Stein GH. T98G: an anchorage-independent human tumor cell line that exhibits stationary phase G1 arrest in vitro. J Cell Physiol. 1979;99:43–54.
32. Nanegrungsunk D, Onchan W, Chattipakorn N, Chattipakorn SC. Current evidence of temozolomide and bevacizumab in treatment of gliomas. Neurol Res. 2015;37(2):167–83. doi:10.1179/1743132814Y.0000000423.
33. Hirose Y, Katayama M, Mirzoeva OK, Berger MS, Piper RO. Akt activation suppress Chk2-mediated, methylating agent-induced G2 arrest and protects from temozolomide-induced mitotic catastrophe and cellular senescence. Cancer Res. 2005;65:4861–9.
34. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ. 2004;11:448–57.
35. Markiewicz-Żukowska R, Borawska MH, Fiedorowicz A, Naliwajko SK, Sawicka D, Car H. Propolis changes the anticancer activity of temozolomide in U87MG human glioblastoma cell line. BMC Complement Altern Med. 2013;13:50. doi:10.1186/1472-6882-13-50.
36. Khoshyomn S, Nathan D, Manske GC, Osler TM, Penar PL. Synergistic effect of genistein and BCNU on growth inhibition and cytotoxicity of glioblastoma cells. J Neurooncol. 2002;57(3):193–200.
37. Darzynkiewicz Z, Robinson JP, Crissman HA, editors. Flow cytometry. Methods in cell biology, 41, 42. Academic Press, Inc., San Diego; 1994.
38. Henery S, George T, Hall B, Basiji D, Ortyn W, Morrissey P. Quantitative image based apoptotic index measurement using multispectral imaging flow cytometry: a comparison with standard photometric methods. Apoptosis. 2008;13(8):1054–63.
39. Teicher BA, Menon K, Avarez E, Galbreath E, Shih C, Faul M. Antiangiogenic and antitumor effects of protein kinase Cb inhibitor in human T98G glioblastoma multiforme xenografts. Clin Cancer Res. 2001;7(3):634–40.
40. Opel D, Westhoff MA, Bender A, Braun V, Debatin KM, Fulda S. Phosphatidylinositol 3-kinase inhibition broadly sensitizes glioblastoma cells to death receptor- and drug-induced apoptosis. Cancer Res. 2008;68(15):6271–80. doi:10.1158/0008-5472.CAN-07-6769.

41. De Salvo M, Maresca G, D'Agnano I, Marchese R, Stigliano A, Gagliassi R, Brunetti E, Raza GH, De Paula U, Bucci B. Temozolomide induced c-Myc-mediated apoptosis via Akt signalling in MGMT expressing glioblastoma cells. Int J Radiat Biol. 2011;87(5):518–33. doi:10.3109/09553002.2011.556173.
42. Carmo A, Carvalheiro H, Crespo I, Nunes I, Lopes MC. Effect of temozolomide on the U-118 glioma cell line. Oncol Lett. 2011;2(6):1165–70.
43. Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, Kondo S. Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. Oncogene. 2005;24:980–91.
44. Varna M, Ratajczak P, Bertheau P, Janin A. BCL2L1 (BCL2-like 1). Atlas Genet Cytogenet Oncol Haematol. 2010;14(9):866.
45. Scarlatti F, Granata R, Meijer AJ, Codogno P. Does autophagy have a license to kill mammalian cells? Cell Death Differ. 2009;16:12–20. doi:10.1038/cdd.2008.101.
46. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. Nat Rev Cancer. 2005;5:726–34.
47. Hoyer-Hansen M, Bastholm L, Mathiasen IS, Elling F, Jaattela M. Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. Cell Death Differ. 2005;12:1297–309.
48. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol. 2004;6:1221–8.
49. Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Métivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 2005;25:1025–40.
50. Gonzalez-Polo RA, Boya P, Pauleau AL, Jalil A, Larochette N, Souquere S, Eskelinen EL, Pierron G, Saftig P, Kroemer G. The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death. J Cell Sci. 2005;118:3091–102.
51. Kroemer G, Jaattela M. Lysosomes and autophagy in cell death control. Nat Rev Cancer. 2005;5:886–97.
52. Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, Baehrecke EH, Lenardo M. Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci USA. 2006;103:4952–7.
53. Roy B, Pattanaik AK, Das J, Bhutia SK, Behera B, Singh P, Maiti TK. Role of PI3K/Akt/mTOR and MEK/ERK pathway in concanavalin A induced autophagy in HeLa cells. Chem Biol Interact. 2014;210:96–102. doi:10.1016/j.cbi.2014.01.003.
54. Ren Y, Huang F, Liu Y, Yang Y, Jiang Q, Xu C. Autophagy inhibition through PI3K/Akt increases apoptosis by sodium selenite in NB4 cells. BMB Rep. 2009;42(9):599–604.
55. Chen S, Rehman SK, Zhang W, Wen A, Yao L, Zhang J. Autophagy is a therapeutic target in anticancer drug resistance. Biochim Biophys Acta. 2010;1806(2):220–9. doi:10.1016/j.bbcan.2010.07.003.
56. Paul-Samojedny M, Pudełko A, Kowalczyk M, Fila-Daniłow A, Suchanek-Raif R, Borkowska P, Kowalski J. Knockdown of AKT3 and PI3KCA by RNA interference changes the expression of the genes that are related to apoptosis and autophagy in T98G glioblastoma multiforme cells. Pharmacol Rep. 2015;67(6):1115–23. doi:10.1016/j.pharep.2015.04.012.
57. Su M, Mei Y, Sinha S. Role of the crosstalk between autophagy and apoptosis in cancer. J Oncol. 2013;2013:102735. doi:10.1155/2013/102735.
58. Alers S, Löffler AS, Wesselborg S, Gao P, Tian W, Wang X, Jin H, Xu H, Chen Q. Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. Mol Cell Biol. 2012;32:2–11. doi:10.1128/MCB.06159-11.
59. Chang YY, Neufeld TP. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. Mol. Biol. Cell. 2009;20:2004–14. doi:10.1091/mbc.E08-12-1250.
60. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol. 2010;22:124–31. doi:10.1016/j.ceb.2009.11.014.
61. He CK, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43:67–93. doi:10.1146/annurev-genet-102808-114910.
62. Jung CH, Seo M, Otto NM, Kim DH. ULK1 inhibits the kinase activity of mTORC1 and cell proliferation. Autophagy. 2011;7(10):1212–21. doi:10.4161/auto.7.10.16660.
63. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy. 2008;4(2):151–75.
64. Sinha S, Levine B. The autophagy effector Beclin 1: a novel BH3-only protein. Oncogene. 2008;27(Suppl 1):S137–48. doi:10.1038/onc.2009.51.
65. Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P, Piacentini M, Chowdhury K, Cecconi F. Ambra1 regulates autophagy and development of the nervous system. Nature. 2007;447:1121–5.
66. Fimia GM, Corazzari M, Antonioli M, Piacentini M. Ambra1 at the crossroad between autophagy and cell death. Oncogene. 2013;32:3311–8. doi:10.1038/onc.2012.455.
67. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ. 2011;18:571–80. doi:10.1038/cdd.2010.191.
68. Oberstein A, Jeffrey PD, Shi Y. Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. J Biol Chem. 2007;282:13123–32.
69. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. Nat Cell Biol. 2004;6:1221–8.
70. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122:927–39.
71. Pattingre S, Levine B. Bcl-2 inhibition of autophagy: a new route to cancer? Cancer Res. 2006;66:2885–8.
72. Krishan S, Richardson DR, Sahni S. Gene of the month: AMP kinase (PRKAA1). J Clin Pathol. 2014;67(9):758–63.
73. Mah LY, O'Prey J, Baudot AD, Hoekstra A, Ryan KM. DRAM-1 encodes multiple isoforms that regulate autophagy. Autophagy. 2012;8(1):18–28. doi:10.4161/auto.8.1.18077.
74. Guan JJ, Zhang XD, Sun W, Qi L, Wu JC, Qin ZH. DRAM1 regulates apoptosis through increasing protein levels and lysosomal localization of BAX. Cell Death Dis. 2015;6:e1624. doi:10.1038/cddis.2014.546.
75. Galavotti S, Bartesaghi S, Faccenda D, Shaked-Rabi M, Sanzone S, McEvoy A, Dinsdale D, Condorelli F, Brandner S, Campanella M, Grose R, Jones C, Salomoni P. The autophagy-associated factors DRAM1 and p62 regulate cell migration and invasion in glioblastoma stem cells. Oncogene. 2013;32(6):699–712. doi:10.1038/onc.2012.111.
76. Wang Q, Qian J, Wang J, Luo C, Chen J, Hu G, Lu Y. Knockdown of RLIP76 expression by RNA interference inhibits invasion, induces cell cycle arrest, and increases chemosensitivity to the anticancer drug temozolomide in glioma cells. J Neurooncol. 2013;112(1):73–82. doi:10.1007/s11060-013-1045-2.
77. Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P. Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science. 1996;272:1023–6.

78. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature. 1998;394:485–90.
79. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res. 1999;59:5830–5.
80. Zhao Z, Ni D, Ghozalli I, Pirooz SD, Ma B, Liang C. UVRAG: at the crossroad of autophagy and genomic stability. Autophagy. 2012;8:1392–3. doi:10.4161/auto.21035.
81. Yin X, Cao L, Peng Y, Tan Y, Xie M, Kang R, Livesey KM, Tang D. A critical role for UVRAG in apoptosis. Autophagy. 2011;7:1242–4. doi:10.4161/auto.7.10.16507.
82. Hara T, Mizushima N. Role of ULK-FIP200 complex in mammalian autophagy: FIP200, a counterpart of yeast Atg17? Autophagy. 2009;5:85–7.