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Original research article

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

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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. The PI3K/AKT/PTEN signaling pathway plays a crucial role in GBM development and it is connected with the regulation of apoptosis and autophagy. Akt is involved in various aspects of cancer cell biology such as cell survival, in addition to both apoptosis and autophagy.

The current study was undertaken to examine the effect of the siRNAs that target AKT3 and PI3KCA genes on the apoptosis and autophagy of T98G cells.

Methods: T98G cells were transfected with AKT3 and/or PI3KCA siRNAs. Alterations in the mRNA expression of apoptosis- and autophagy-related genes were analyzed using QRT-PCR. LC3IIA protein-positive cells were identified using flow cytometry with specific antibodies.

Results: Our findings demonstrate for the first time that the siRNAs that target AKT3 and PI3KCA change the expression of the genes that are related to apoptosis and autophagy and change the expression of the LC3IIA protein in T98G cells.

Conclusions: Thus, there is a high probability that the knockdown of these genes induces apoptosis and autophagy in T98G cells, but further studies are necessary in order to clarify and check whether autophagy induction is a positive phenomenon for the treatment of GBM.

Introduction

Glioblastoma multiforme

Glioblastoma multiforme (GBM; WHO grade IV) is the most common and lethal primary brain tumor in adults. GBM is characterized by extensive brain tissue migration and infiltration [1]. These unfavorable features result in poor patient survival due to an insufficient surgical resection. Inefficient adjuvant treatment of the tumor's residual infiltrative component is mediated by the high resistance of glioblastoma to radiation and chemotherapy, mainly through the overexpression of the PI3 K/Akt pathway [2,3], which has an impact on cellular proliferation, growth, survival, motility, differentiation, metabolism, protein synthesis as well as regulating tumor angiogenesis and invasiveness [4]. The highly invasive and therapy-resistant character of GBM results in the shortest survival time of all cancers (the median survival <15 months for patients with newly diagnosed cancer regardless of their treatment methods) [5]. Current treatment strategies for GBM using surgery, chemotherapy and/or radiotherapy are ineffective, but have triggered a great deal of research effort worldwide for new treatment modalities that can be applicable to this cancer.

PI3K/Akt pathway

PI3Ks, which is a family of phosphoinositide 3-kinases, has been divided into three classes (I – described below; II – consists of single catalytic subunits: isoforms PI3KC2α, PI3KC2β and PI3KC2γ) and III – involves a single catalytic subunit Vps34). Class I consists of two subclasses – class IA and IB, respectively. Class IA contains heterodimers that are composed of a p110

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catalytic subunit and a p85 regulatory subunit. The p110 subunit has three isoforms (p110α, p110β, p110γ), which are involved in the regulation of the proliferation, survival, migration, degranulation and vesicular trafficking. It is suggested that only the gene encoding p110α subunit plays a crucial role in tumorigenesis [5,6]. Further, it is reported that the knockdown of *PI3KCA* interferes with PI3K/Akt signaling [7]. Zhou et al. [8] indicate that the knockdown of *PI3KCA* by siRNA may result in a decreased catalytic activity of PI3K. The *PI3KCA* gene was found to be amplified and overexpressed in several types of human cancers and the activating point mutations as well as gene amplification of *PI3KCA* have been reported in human brain tumors including GBM [5]. Mutations or amplification of the *PI3KCA* gene have been reported to constitutively increase PI3K activity in cancer cells. Akt is a major downstream effector of PI3K and impinges on numerous cellular processes as has previously been written. Akt activation has been observed in approximately 80% of human GBM [9], which is well correlated with the fact that RTKs/PI3K/Akt signaling is altered in 88% of GBM [5]. There are three closely related isoforms of Akt (pathologically amplified in human cancers) [10]: Akt1, Akt2 and Akt3. Expression of the last one is more restricted to the neuronal tissue than its other isoforms [11]. The Akt2 and Akt3 are overexpressed in glioma cells and play a pivotal role in the malignancy of gliomas [4]. Akt is involved in a various aspects of cancer cell biology such as cell survival, in addition to both apoptosis and autophagy [12].

Apoptosis and autophagy regulation via *the PI3K/Akt pathway*

Activation of the PI3K/Akt pathway is connected to the phosphorylation of numerous effector proteins and is also related to apoptosis and autophagy. The activation of this pathway leads to the suppression of apoptotic cell death through an Akt direct and indirect manner [13]. Phosphorylation of the Bcl-2 family proapoptotic proteins such as Bad and Bax leads to their inactivation, degradation or changes in their cellular location. Similarly, the phosphorylation of procaspase 9 inhibits its proteolytic maturation and thus stops the subsequent activation of effector caspases. Akt inhibits apoptosis indirectly through its NF-κB activity, which is mediated by the transcription of antiapoptotic proteins. Activated Akt kinase interferes with mitochondrial outer membrane permeabilization, thereby also suppressing cell death in a Bad-independent manner [14]. Furthermore, Akt kinase is responsible for the inhibition of the FOXO family proteins that regulate the transcription of the Bim and FasL pro-apoptotic proteins.

One of the most important Akt effector proteins is mTOR kinase, which intimately linked to PI3K/Akt signaling and to the regulation of protein synthesis, cell growth and survival [15]. mTOR is a major negative regulator of the autophagy process. Activated PI3K/Akt signaling leads to the inhibition of autophagy through TSC1/2 phosphorylation by Akt. Once the mTORC1 kinase activity is inhibited, autophagosome formation occurs [16].

Therefore, it is very important to discover the exact mechanisms that determine the PI3K/Akt signaling activity and to understand how the inhibition of this pathway influences GBM cell death. The current study was undertaken to examine the effect of the siRNAs that target the *AKT3* and *PI3KCA* genes on the apoptosis- and autophagy-related genes expression of T98G cells.

Materials and methods

Cell cultures

The T98G cell line, which was derived from a 61-year-old male [17] and purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), was cultured in a modified Eagle's minimum Essential Medium (ATCC) that was supplemented with heat-inactivated 10% fetal bovine serum (ATCC) and 10 μg/ml gentamicin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

siRNA transfection

T98G cells were seeded at 1.6×10^4 cells per well in six-well plates and incubated for 24 h. Next, the T98G cell line was transfected with the specific siRNAs that target *AKT3* (without affecting *AKT1* and *AKT2* mRNAs level) or *PI3KCA* mRNA using the FlexiTube siRNA Premix (Qiagen, Italy) according to the manufacturer's protocol. The following target sequences were used: 5′ AACTGTTGGCTTTGGATTAAA 3′ (for *AKT3*) and 5′ CTGAGTCAGTATAAGTATATA 3′ (for *PI3KCA*). Optimum transfection conditions and transfection efficiency were established as has previously been described [18]. AllStars Negative Control siRNA was also tested (Qiagen, Italy) as we previously described [18]. After transfection, but prior to performing assays, the cells were washed with PBS, trypsinized and centrifuged (125 × *g*/5 min) at 4 °C (listed below).

RNA extraction

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

The evaluation of the transcriptional activity of apoptosis and autophagy-related genes by QRT-PCR

QRT-PCR assays were performed using an ABI Prism7700 (Applied Biosystems, Foster City, USA). Real-time fluorescent RT-PCR was performed using a KiCqStart Primers (Sigma Aldrich) and SensiFast SYBR Hi-ROX One-Step Kit (Bioline) according to the manufacturer's protocol under the following conditions: 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 Tata Binding Protein (TBP) was used as an endogenous control. The copy numbers for each sample were calculated using the C_T-based calibrated standard curve method. Each data point is the mean of triplicate measurements. Twelve replicates were used for each gene analysis.

The evaluation of LC3IIA protein expression using flow cytometry

LC3IIA protein-positive cells were identified using direct labeling with a specific rabbit anti-LC3IIA antibody (isotype IgG_A) and flow cytometry. In order to confirm that the observed effects are autophagy-specific, an inducer (N-hexanoyl-D-sphingosine, 10 μM for 24 h) and an inhibitor (bafilomycin A1, 100 nM for 2 h), were used. After *AKT3* and *PI3KCA* knockdown, cells were harvested, washed twice in PBS and fixed in PBS with 4% paraformaldehyde. Then, cells were washed twice in PBS with 1% BSA, permeabilized with 0.1% saponin/1% BSA/PBS for 45 min and incubated overnight at 4 °C with 10 μg/ml of an anti-LC3IIA polyclonal antibody (antibody concentration: 1 μg/μl) conjugated with FITC (Bioss) in 1% BSA/PBS. An isotype-matched monoclonal antibody (isotype control) was used to determine non-specific binding. Subsequently, cells were washed and analyzed using a FACSAria II (BD Biosciences) equipped with Diva Software. Four biological replicates were used in the flow cytometry analysis for the LC3IIA protein measurement. When autophagy is activated, the LC3-I protein that is localized in the cytoplasm is cleaved, lipidated

and inserted as LC3-II into the phagophore membranes [19]. Then, the LC3-II protein as a part of autophagolysosome, which is a digestive vacuole, is degraded by acidic lysosomal hydrolases. LC3IIA protein expression was measured because it is an autophagosome-associated protein and is used as an autophagic flux marker [20]. In fact, commercially available kits to detect the autophagy process, which are based on the quantitative measuring of autophagosome acidification (such as LysoTracker or kits using acridine orange) as part of the whole multistep phenomenon, are nonspecific and susceptible to misinterpretations due to potential blockade of the initial steps of autophagy, thus resulting in an accumulation of autophagosomes and false autophagy induction results [21]. Thus, we decided to measure the LC3IIA protein level, which in connection with specific autophagy inductors and inhibitors gives more accurate results and reflects the autophagic flux as the complete mileage of this process [20].

Statistical analysis

Data that was generated by the RT-QPCR analysis are presented as mean ± SD. The U Mann–Whitney test or Kruskal–Wallis ANOVA was used to compare two or more groups. The power of all of the tests was not less than $\beta = 0.8$. Data were analyzed using Statistica software (StatSoft, Inc. 2008), version 9.0 (www.statsoft.com). All of the tests were two-sided and $p < 0.05$ was considered to be statistically significant. 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- and apoptosis-related genes, linear regression was performed. Data of QRT-PCR analysis were also clustered using SOMs.

Results

We performed QRT-PCR for selected genes that are associated with apoptosis and autophagy: *AIFM2*, *BAD*, *BCL2L1*, *BID*, *BNIP3*, *CASP3*, *CASP8*, *CASP9* (apoptosis), *AMBRA1*, *BECN1*, *MAP1LC3A*, *PIK3C3*, *RB1CC, SQSTM1*, *ULK1*, *UVRAG* (autophagy), *DRAM1*, *GSK3β*, *HIF1*, *PRKAA1* (both pathways). These genes are among the most biologically important for the regulation of the processes that are mentioned. The gene expression levels were compared between untreated cells and cells that had been treated with the siRNA that is specific for *AKT3* or *PI3KCA* gene.

Hierarchical clustering of the QRT-PCR results based on the Euclidean distance was carried out using GenExEnterprise 5.4.3.703 (Fig. 1).

The results that were obtained allowed us to distinguish two groups of genes whose protein products are involved in the process of autophagy (A) and are also involved in the processes of autophagy and/or apoptosis (B) that changed after the knockdown of the *AKT3* or *PI3KCA* genes. Genes with similar expression pattern (measured on the basis of mRNAs levels) were used in cluster analysis. Following clustering untransfected cells were clustered as a separate group, likewise cells transfected with AKT3 siRNA were clustered together with cells transfected with PI3KCA siRNA (Fig. 1).

To identify differentially expressed autophagy- and apoptosis-related genes after the knockdown of the *AKT3* or *PI3KCA* gene, linear regression was performed (Figs. 2 and 3). Regression analysis determines the possible delimitation lines of regression, which set the area of prognosis, taking into account both statistical and biological criteria. The regression model is indexed with biologically meaningful gene-specific parameters. It is generally accepted that a biological criterion is defined as at least a twofold increase or decrease in the mean value of gene expression.

We found that the changes in autophagy- and apoptosis-related genes after *AKT3* knockdown are manifested mainly in: (1) increased *CASP-3* and *ULK-1* (∼2-fold) gene expression as well as (2) decreased *AMBRA1* (∼4.4-fold), *PIK3C3* (∼4-fold), *RB1CC1* (∼3.5-fold), *UVRAG* (∼3.4-fold), *PRKAA1* (∼3-fold), *GSK3B* (∼2.8-fold), *DRAM1* (∼2.2-fold), *BCL2L1* (∼2.2-fold), *BECN1* (∼2-fold), *SQSTM1* (∼2.0-fold), *CASP8* (∼1.8-fold), *MAP1LC3* (∼1.6-fold), *AIFM2* (∼1.5-fold), *BNIP3* (∼1.4-fold) and *Cadherin E* (∼1-fold) gene expression (Fig. 2; Table 1).

Silencing of the *PI3KCA* gene is connected to changes in autophagy- and apoptosis-related genes and are mostly manifested in: (1) increased *ULK1* and *CASP3* (2.0-fold and 1.3-fold, respectively) gene expression, as well as (2) decreased *GSK3B* (∼1.9-fold), PIK3C3 (∼3.5-fold), UVRAG (∼3.4-fold), RB1CC1 (∼3.0-fold), PRKAA1 (∼2.9-fold), DRAM1 (∼2.7-fold), AMBRA1 (∼2.55-fold), AIFM2 (∼2.35-fold), CASP8 (∼2.2-fold), BNIP3 (∼2.2-fold), BCL2L1 (∼2-fold), MAP1LC3 (∼1.8-fold), BECN1 (∼1.8-fold), SQSTM1 (∼1.5-fold) gene expression (Fig. 3; Table 1).

SOM (Self-Organizing Map) analysis was also performed in order to provide a comprehensive, quantitative, yet lucid picture of the gene expression changes in cells with a knockdown of *AKT3* or *PI3KCA* genes. Variance in this group is a measure of the degree of dissimilarity among the gene expressions that are clustered together. We noticed that the genes that are involved in the processes of autophagy and apoptosis can be divided into four categories, which include genes that have similar expression changes after *AKT3* or *PI3KCA* knockdown compared to untransfected cells (Fig. 4).

The SOM is an unsupervised neural network algorithm that can cluster the data of gene expression analysis into biologically meaningful groups. The first group consists of *SQSTM1* and *Bid* genes; the second of *GSK3B*, *ULK1*, *BECN1*, *UVRAG*, *RB1CC1* and *DRAM* genes, the third of *PRKAA1*, *AIFM2*, *BCL2L1*, *PIK3C3*, *AMBRA1* and *CASP*3 and the fourth of *CASP8*, *CASP9*, *BAD* and *MAP1LC3* genes. Genes were grouped by a similar mRNA level of genes that reflected their expression pattern.

To test our hypothesis that the knockdown of *AKT3* or *PI3KCA* gene causes the induction of autophagy, we assessed the LC3IIA protein level and tested it with LysoTracker Red (a deep red-fluorescent dye for labeling and tracking acidic organelles in live cells) that 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 indicate that *AKT3* and *PI3KCA* genes silencing is associated with an increased LC3IIA protein expression and an increased intensity of red fluorescence, which indicates an increased number of vesicles with an acidic pH characteristic for autophagy (Fig. 5; $p < 0.05$).

Discussion

One of the main issues in cancer therapy is to completely reduce the tumor cell mass, e.g., through the induction of apoptosis (type I programmed cell death). Glioblastomas are resistant to therapies that induce apoptosis [22], but several studies have indicated that GBM cells seem to be less resistant to therapies that induce cell death with features of autophagy (type II programmed cell death) [23]. Our goal was to analyze how the knockdown of *AKT3* and *PI3KCA* genes influences the transcriptional activity of autophagy- and apoptosis-related genes in GBM T98G cells. It is well known that various anticancer therapies induce autophagy in different cancer cell types, but whether autophagy in response to therapies is pro-death or pro-survival remains controversial [24]. These antithetic roles of autophagy in cancer cells make it extremely hard to correctly interpret the results that are obtained. Autophagy seems to prevent cell transformation by playing a potential

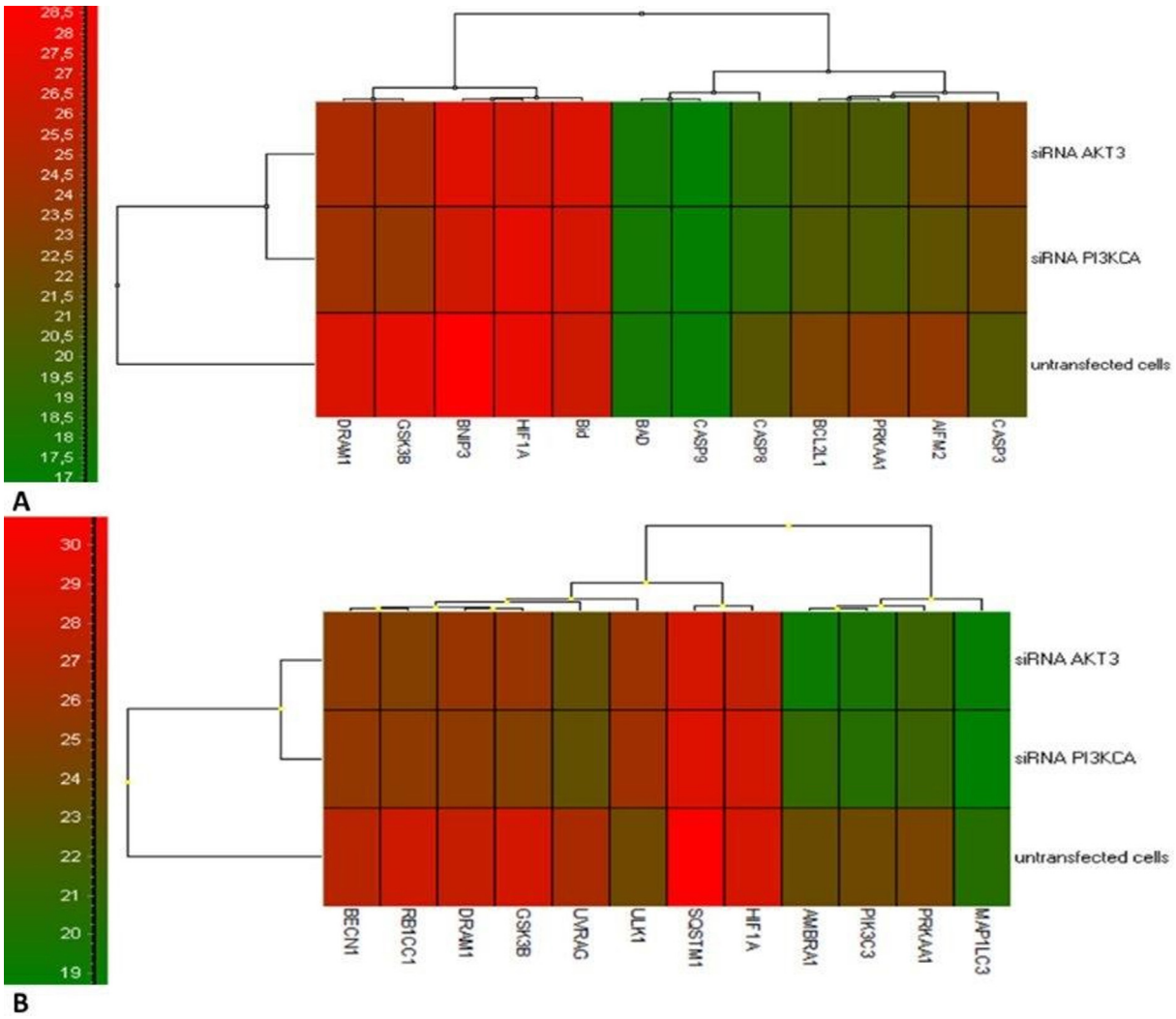

Fig. 1. Hierarchical clustering and heat map of differentially expressed autophagy- (A) and apoptosis- and autophagy-related (B) genes. Data indicating the expression patterns of 21 genes ($p < 0.001$) after the knockdown of *AKT3* or *PI3KCA* genes are shown. Data were clustered using the standard hierarchical method with average linkage using the Pearson correlation to determine the distance function. The normalized expression index for each gene (columns) in each sample (rows) is indicated by a color code (see Expression index bar at left of figure). Colorful rectangles represent genes that were up-regulated (red) or down-regulated (green) in the sample set. Samples with a similar pattern of gene expression are clustered together as is indicated by the dendrogram.

anticancer role. However, the mechanism through which autophagy inhibits tumor development is still unclear.

Autophagy and apoptosis are regulated by the PI3K/Akt/mTOR pathway whose activation allows cells to inhibit apoptotic and autophagic cell death, which may contribute to malignant transformation and tumor growth [25]. Some authors suggest that PI3K-Akt activation suppresses autophagy in mammalian cells [26], but others point out the positive regulation of this process [25]. Other results indicate that the major response to reduced Akt activity in the tumor cells is increased autophagy, whereas classical apoptosis was not the prevailing response [27]. It is postulated that the autophagy that is induced by Akt inhibition can lead to cell death, either as a precursor of apoptosis in apoptosis-sensitive cell lines or as a result of destructive self-digestion. The induction of autophagy through Akt inhibition may be an Achilles' heel that sensitizes cancer cells to manipulations that target this degradative pathway [28]. The effects of early stage autophagy inhibition on cell survival are controversial, whereas blocking autophagy at a late stage has been more consistently shown to cause accelerated cell death under autophagy-inducing conditions [29]. Anticancer drugs that inhibit the PI3K/Akt/mTOR pathway putatively stimulate autophagy, but whether autophagy contributes to the antitumor effect of these drugs or to drug resistance is largely unknown [30].

All of these facts underline the complexity of autophagic process. There is a mechanistic overlap between autophagy and apoptosis, and moreover, some evidence indicates that there is cross-talk between them [29]. We found [18] that the silencing of *AKT3* and *PI3KCA* genes is connected with apoptosis induction. The knockdown or inactivation of Akt does not significantly induce apoptosis, but rather increases autophagy markedly [27]. Degtyarev et al. hypothesized that autophagy, which is induced by Akt inhibition, may sensitize tumor cells to agents that target the later steps of this lysosomal degradation process [28].

The results of our study indicate that the knockdown of *AKT3* and *PI3KCA* genes in T98G cells led to the induction of apoptosis and autophagy and it is consistent with other results that have shown that active autophagy appeared to increase the tendency to undergo apoptosis [31]. On the other hand, autophagy may play a role in preventing cells from apoptosis through the sequestration of cytochrome c [32]. The simultaneous activation of both autophagy and apoptosis are observed in many systems [33].

We found an increased *ULK1* (Unc51-like kinase, hATG1) mRNA copy number after the knockdown of *AKT3* and *PI3KCA* genes.

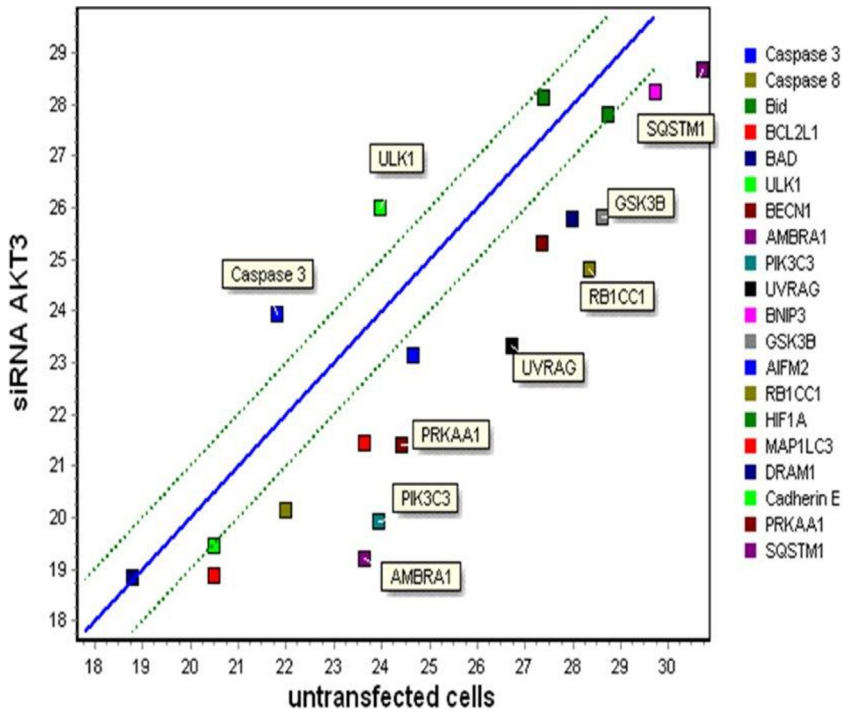

Fig. 2. Transcripts for autophagy- and apoptosis-related genes differentiating cells that had been transfected with *AKT3* siRNA from untransfected cells. mRNA copy numbers were analyzed using the QRT-PCR method. The copy numbers for each sample were calculated using the C_T-based calibrated standard curve method. Each data point is $\log_2$ of the average of mRNA copy number. Twelve biological replicates were used for each gene.

ULK1/2 induces autophagy in several ways [34] and plays a key role in inducing autophagy in response to starvation. ULK1 is negatively regulated by mTORC1 and this kinase is not only a downstream effector of mTORC1, but is also a negative regulator of mTORC1 signaling. Thus, ULK1 is considered to be a key molecule that is involved in the regulation of mTORC1 signaling to the PI3KC3 autophagy machinery.

Because ULK1 is found in a complex with Atg13, RB1CC1 (FIP200) and Atg101 [35], we also analyzed the expression of the *RB1CC1* gene and we found a decreased mRNA copy number in the

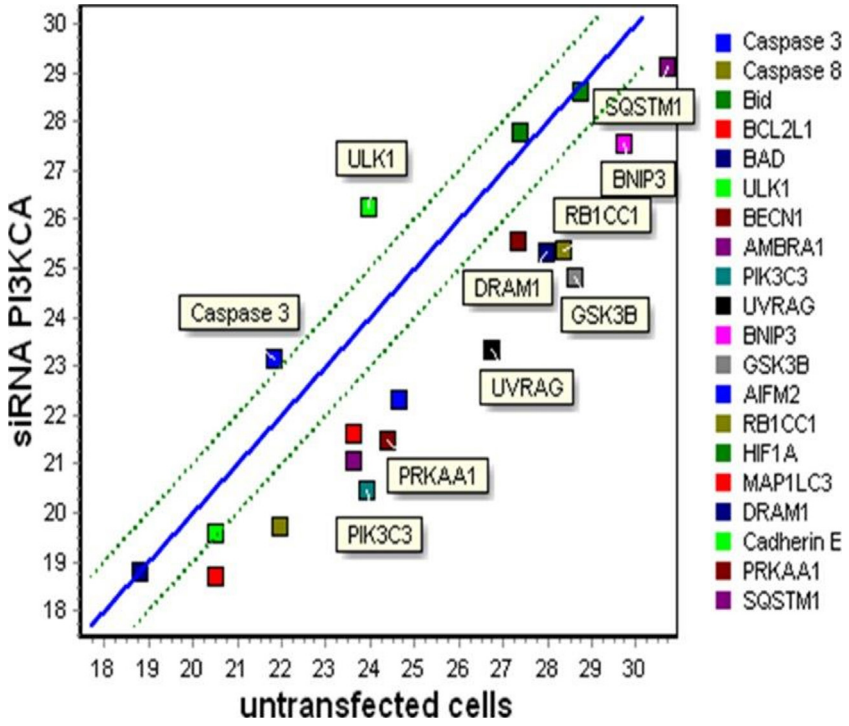

Fig. 3. Transcripts for autophagy- and apoptosis-related genes differentiating cells transfected with *PI3KCA* siRNA from untransfected cells. mRNA copy numbers were analyzed using the QRT-PCR method. The copy numbers for each sample were calculated using the C_T-based calibrated standard curve method. Each data point is $\log_2$ of the average of mRNA copy number. Twelve biological replicates were used for each gene.

Table 1
The list of autophagy- and apoptosis-related genes that were differentially expressed in cells that had been transfected with *AKT3* or *PI3KCA* siRNAs compared to untransfected cells. The potential differentiating genes were selected using regression analysis (Figs. 2 and 3).

Gene symbol	Diff (Y–X)	Gene symbol	Diff (Y–X)	Gene expression (kind of change)
siRNA AKT3				
CASP3	2118	ULK1	2037	↑
AMBRA1	−4413	BECN1	−2034	↓
PIK3C3	−4029	SQSTM1	−2015	
RB1CC1	−3555	CASP8	−1818	
UVRAG	3383	MAP1LC3	−1607	
PRKAA1	−2993	AIFM2	−1529	
GSK3B	−2788	BNIP3	−1486	
DRAM1	−2202	CADHERIN E	−1054	
BCL2L1	−2193			
siRNA PI3KCA				
ULK1	2289	CASP3	1310	↑
GSK3B	−3777	AIFM2	−2358	↓
PIK3C3	−3491	CASP8	−2236	
UVRAG	−3395	BNIP3	−2194	
RB1CC1	−2961	BCL2L1	−2005	
PRKAA1	−2925	MAP1LC3	−1804	
DRAM1	−2677	BECN1	−1800	
AMBRA1	−2550	SQSTM1	−1567	

↑ – increased gene expression; ↓ – decreased gene expression.

transfected cells. RB1CC1 is involved in the regulation of cell growth, proliferation, survival, spreading/migration, which is essential for autophagosome formation and also inhibit the kinase activity of Pyk2 whose overexpression has been shown to induce apoptosis in a number of cell lines [36]. Thus, a decreased *RB1CC1* mRNA copy number might be connected with apoptosis induction.

Decreased *Ambra1* and *GSK3B* mRNA copy numbers in the transfected cells were also observed. The autophagy core complex, which is comprised of BECN1, PIK3C3, PIK3R4, ATG14 and AMBRA1 proteins, plays a crucial role in the autophagy activation process [37]. Ambra1 (a crucial upstream regulator of autophagy initiation [38]) binds to Beclin1 and stabilizes the Beclin 1/Vps34 complex, finally promoting autophagosome formation [39]. Fimia et al. [40] found that reduced levels of Ambra1 in a variety of cell lines led to an increased susceptibility to different apoptotic stimuli. Researchers postulate that apoptosis induction causes the Ambra1 degradation that occurs in a caspase- and calpain-dependent manner and that the cleavage of this protein occurs early during the apoptotic process [40]. GSK3B kinase (*glycogen synthase kinase-3 beta*) is involved in energy metabolism, neuronal cell development and body pattern formation through triggering the degradation of signaling or functional proteins. The activation of PI3KCA induces the phosphorylation of GSK3B at Ser9 and attenuates the interaction of GSK3B with Bcl-2. Interrupted GSK3B and Bcl-2 interaction precludes the phosphorylation of Bcl-2 at Ser70 and is connected with the ubiquitin-mediated degradation of Bcl-2. The increased expression of Bcl-2 interferes with the activation of BECN1 and attenuates autophagy in cancer cells [41].

Our results have also shown a decreased *BECN1* mRNA copy number after the knockdown of these genes. Beclin1 is a major determinant in the initiation of autophagy [42], in the formation of preautophagosomal structures and its interactions with Bcl-2 and Bcl-xL [43] inhibit autophagy [44]. We found an increased *Bax* mRNA copy number [18]. It is known that several proapoptotic Bcl-2 family members, Bax, Bak, Bad and ABT737, promote autophagy and their activity is connected with disrupting the interaction between Beclin1 and Bcl-2/Bcl-xL. It is suggested that the interaction between Bcl-2 and Beclin1 is important for regulating autophagy and apoptosis [33]. We previously found a decreased *Bcl-2* mRNA copy number [18] and these results may suggest a lack of the inhibition of Beclin1-dependent autophagy. In a series of human brain tumors including glial and non-glial neoplasms, the expression of the Beclin1 protein and its mRNA was found to be decreased [45].

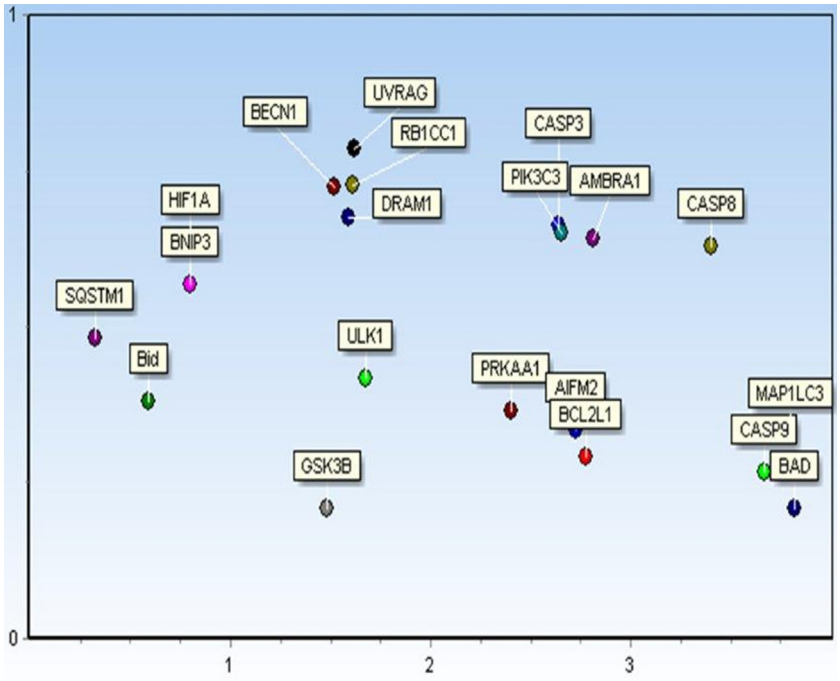

Fig. 4. Kohonen Map (SOM analysis result) illustrating the gene expression patterns after *AKT3* or *PI3KCA* knockdown compared to untransfected cells. SOM clusters this gene expression data set into four groups that show similar gene expression profiles and also contains functionally related genes. The algorithm places genes with similar, but not identical, profiles in neighboring groups 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.

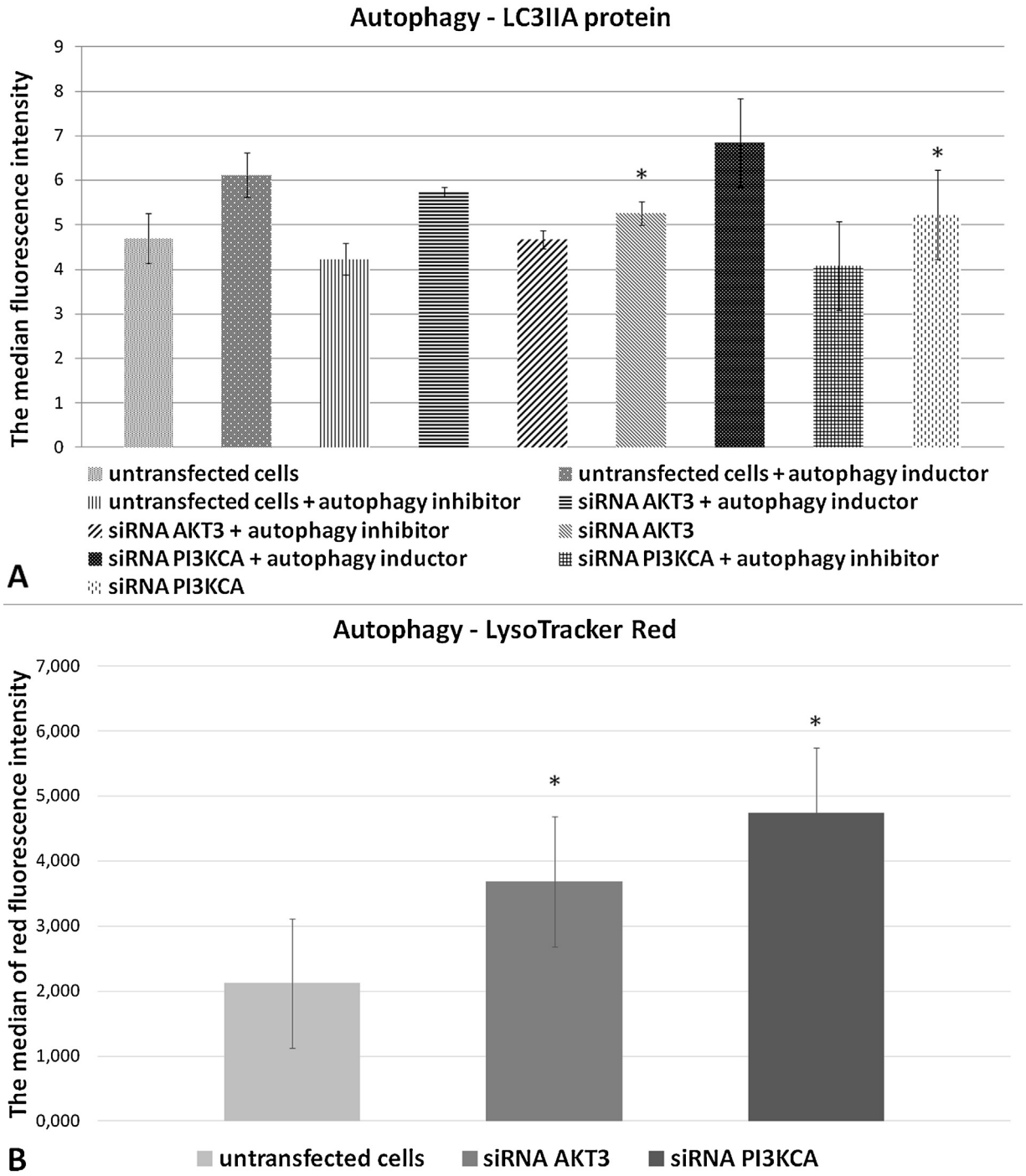

Fig. 5. A comparison of the LC3IIA protein expression level (A) and red fluorescence intensity (B) indicated an increased number of vesicles with an acidic pH characteristic for autophagy in cells that had been transfected with a specific siRNAs as well untransfected T98G cells with or without the addition of an autophagy inducer or inhibitor. Tracking the conversion of LC3-I to LC3-II is indicative of autophagic activity. N-hexanoyl-D-sphingosine (10 μM for 24 h) and bafilomycin A1 (100 nM for 2 h), were used as an autophagy inductor and as an inhibitor, respectively. N-hexanoyl-D-sphingosine is known to stimulate autophagy by interfering with the class I PI3K signaling pathways. Bafilomycin A1 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 LC3IIA protein expression level and red fluorescence intensity after LysoTracker Red staining was determined using flow cytometry. The asterisk (*) shows significant difference at level of $p < 0.05$.

In our studies, the results interestingly show that the knockdown of the analyzed genes is connected with the down-regulation of *PI3KC3* and *UVRAG* expression. PI3KC3 (*phosphatidylinositol 3-kinase catalytic subunit type 3*) is a component of the second key complex of autophagy, which produces an autophagy-specific pool of PI3P [46]. It is known, that UVRAG (*UV radiation resistance-associated gene*) or the overexpression of a dominant-negative form of UVRAG decreases the autophagy level and triggers uncontrolled cell proliferation [47]. Down-regulated *UVRAG* expression might be connected with autophagy induction and decreased GBM cell proliferation, which we described previously [18]. Down-regulation of the *UVRAG* mRNA copy number might be connected with apoptosis induction 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 [48].

In a present study, our results also revealed that siRNAs that targets *AKT3* and *PI3KCA* decrease the *BNIP3* mRNAs level. The *BNIP3* (Bcl-2/adenovirus E1B 19 kDa interacting protein 3) is a member of the pro-cell death Bcl-2 family, but its proapoptotic activity is questionable. BNIP3 is known to induce autophagy and plays a central role in As_2O_3-induced autophagic cell death in malignant glioma cells [49]. BNIP3 is localized to the nucleus in the majority of GBM and fails to induce cell death. BNIP3 binds to the promoter of *AIF* gene, represses its expression and decreases temozolomide (TMZ)-induced apoptosis in glioma cells [50]. We postulated that a decreased *BNIP3* mRNA copy number in GBM transfected cells may be connected with apoptosis induction. We also found an increased *CASP3* mRNA copy number. Caspase-3 cleavage of Beclin1 may regulate the switch of autophagy toward apoptosis [33].

The autophagosome-associated protein microtubule-associated protein 1 light chain 3 (LC3) was also used as a marker of autophagy. Two forms of LC3 protein are known–type I (cytosolic) and type II (membrane-bound). During autophagy, LC3 type I is converted to LC3 type II and is localized in the autophagosomal membranes. We analyzed LC3IIA protein level in a set with the autophagy inhibitor bafilomycin A1 (an inhibitor of Hţ-ATPase) and the autophagy inductor N-hexanoyl-D-sphingosine. Bafilomycin A1 attenuates the acidification of vacuoles, thus resulting in the inhibition of autophagosomes and lysosomes fusion during the late stage [52]. Bafilomycin A1 inhibits autophagy and is connected with apoptosis induction in the same malignant glioma cell types that are treated with TMZ [53].

We found an increased expression level of the LC3IIA protein in the transfected cells. This result may indicate increased autophagy because the amount of LC3-II is closely related to the number of autophagosomes and serves as a good indicator of autophagosome formation [30]. Our findings demonstrate for the first time that the siRNAs that target *AKT3* and *PI3KCA* increased the expression level of the LC3IIA protein in T98G cells. However, owing to the dynamic nature of autophagy, an increased level of LC3 does not necessarily mean increased an autophagy flow [30].

The cross-talk between autophagy and apoptosis is highly complex [54]. Despite the fact that autophagy and apoptosis are two independent processes, some parallelism can be observed, e.g., the activators of apoptosis can induce autophagy, whereas the factors that negatively regulate apoptosis also inhibit autophagy induction. A key factor in this context is the anti-apoptotic factor Bcl-2 [40].

We found autophagy and apoptosis induction after the knockdown of *AKT3* and *PI3KCA* genes. There is also evidence that the simultaneous activation of these processes is observed in many systems. Our results are difficult to interpret unambiguously due to dual role of autophagy–tumor-promoting and tumor-suppressing [30]. The autophagy can both stimulate and prevent cancer depending on the cell types and cellular context [17]. Moreover, some findings suggest that the inhibition of autophagy at different stages may yield different outcomes [51]. Thus, further studies are necessary to clarify what the exact mechanism of autophagy and apoptosis induction in GBM cells after the knockdown of *AKT3* and *PI3KCA* genes is and whether autophagy induction is a positive phenomenon for the treatment of GBM.

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Conflict of interest

The authors declare that they have no conflict of interest.

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