The Effect of PTEN on Proliferation and Drug-, and Radiosensitivity in Malignant Glioma Cells

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Abstract. *Background: Deletions or mutations of the phosphatase and tensin homolog (PTEN) are frequently observed in malignant glioma and are responsible for progression of the disease. Since the molecule is a promising target for gene therapy, the effects of PTEN on glioma proliferation in combination with the anti-neoplastic agent, temozolomide, and ionizing radiation were investigated. Materials and Methods: An adenoviral vector encoding PTEN was used. After infection, changes in proliferation, the cell cycle, as well as drug- and radiosensitivity were investigated. Results: Expression of PTEN led to a 1.21-fold prolongation of the doubling time of the cells. It reduced G_1 and increased G_2/M populations. Forced PTEN expression conferred sensitivity to temozolomide and/or ionizing radiation. Conclusion: In addition to counteracting cell proliferation, expression of PTEN presented advantages in the chemo- and radiosensitivity of glioma cells. Methods for up-regulation of PTEN may have a role in increasing the efficacy of current adjuvant therapies.*

The malignant form of glioma is a highly invasive intracranial tumor and occurs at a frequency of one quarter of all primary brain tumors. Glioma rarely metastasizes to tissues other than the central nervous system. However, it infiltrates the surrounding tissues and can induce local recurrence, leading to death after surgery. Although recent therapies such as surgery, radiotherapy, chemotherapy and immunotherapy have been developed, these have not dramatically improved the prognosis of the patients who still have a low median survival of less than 1 year (1). To resolve this problem, better local therapies are required. A strategy that has gained recent attention is to increase gene expression involved in the inhibition of cell proliferation by transducing the tumor suppressor gene by topical gene delivery (2).

In glioma, various aberrations of gene or protein expression that are closely related to intracellular proliferation signals have been reported. These include EGFR, AKT, p53 and PTEN (3-5). *PTEN* (phosphatase and the tensin homolog deleted on chromosome 10), also known as MMAC1 and TEP1, is a tumor suppressor gene on the long arm of chromosome 10 and it has been found to be absent from or mutated in many malignant diseases, including uterine cancer, breast cancer, malignant melanoma, prostatic cancer, and small cell lung carcinoma (6), in addition to glioma. Deletion or mutation of the *PTEN* gene and a decrease in the amount of its product is a key factor underlying tumor proliferation and the prognosis of the patient. PTEN acts on lipid phosphatase activity and inhibits the phosphatidylinositol-3'-kinase (PI3K)/AKT pathway through dephosphorylation of phosphatidylinositol-(3,4,5)-tri phosphate (PIP3). AKT has a role in cell proliferation, progress of the cell cycle, survival, apoptosis, metastasis, and cell migration, which are suppressed by PTEN through PIP3 dephosphorylation. Additionally, AKT activity is also related to the prognosis of

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glioma (7-10). In the most malignant form of glioma, two-thirds of the tumors lack a detectable level of PTEN expression (11). Furthermore, the lack of PTEN protein is substantially involved in glioma tumorigenesis (12).

Recent advancement of methods of *in vivo* gene transfer has enabled engineered expression of the target molecule into the local area of intracranial tissue (13). We paid attention to *PTEN* as a candidate for further application, since PTEN is one of the key factors that directly regulate tumor growth, progression of the disease, and patient outcome. Many glioma patients are currently treated both with antineoplastic alkylating agents, such as 4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo[4.3.0]nona-2,7,9-triene-9-carboxamide, or temozolomide, and ionizing radiation. As the first step of treatment with PTEN, we attempted to evaluate the effect of forced expression of *PTEN* on the sensitivity of cells to the combination of temozolomide and ionizing radiation.

Materials and Methods

Cell lines and drugs. Glioma cell lines, KNS42 (14), C6 (15), 9L (16), and RT2 (17) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum with 5% CO_2. Other cell lines, U118MG, U138MG U373MG, T98G and A172, were purchased from the ATCC, MD, USA, and cultivated in the same medium. Temozolomide (18) was kindly provided by Merck & Co., Inc (N.J., USA).

Construction of PTEN expression vector and infection of human glioma cells. The construction and production of the recombinant adenoviral vector encoding PTEN (Ad-PTEN) was described previously (19). As a control, adenoviral vector encoding a green fluorescent protein (Ad-GFP) was also used (19). Cells were directly infected with these viruses at various multiplicities of infection (MOIs) (6).

Detection of PTEN proteins by immunoblotting. Expression of PTEN of glioma cell lines was determined by immunoblotting. Seventy two hours after infection, cells were lysed by CHAPS buffer (Cell Signaling Technology Japan, Tokyo, Japan), and after adjustment of the amounts of protein, an equal volume of SDS buffer, containing Tris-pH 6.8, 2% SDS, glycerol, β-mercaptoethanol and bromophenol blue, was added. After boiling of the sample, 10 μg of the specimens were loaded on 7.5% SDS-PAGE gels (1.5 M Tris-HCl, 10% SDS, 30% *bis*-acrylamide, 10% ammonium persulfate, 0.1% TEMED) and transferred to PVDF membranes (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with Block Ace (DS Pharma Biomedical, Osaka, Japan) and reacted with anti-PTEN rabbit polyclonal antibody (Cell signaling Technology) at a 1000-fold dilution. Specimens were further reacted with anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) and the signals were detected by ECL (GE Healthcare, Little Chalfont, UK). The same membranes were reacted with anti-β-actin antibody to demonstrate the loading of the samples (20).

Cell growth and doubling time in vitro. Cell growth was determined by measurement of the absorption of 0.01% methylene blue after fixation with 5% glutaraldehyde. Ten thousand cells were plated into a 35-mm dish and numbers of exponentially growing cells were evaluated. The doubling time was calculated by assessing their growth curve.

Cell cycle analysis. Cell cycles were measured by a flow cytometer (FACScan, Beckton Dickinson and Company, NJ, USA). Cells (1×10^5) were dispersed with trypsin, suspended in phosphate-buffered saline (PBS), fixed with 75% ethanol and stained with 0.03 μM of PI with 180 units of RNaseA for 30 minutes. Populations in each cell cycle phase were analyzed by the Cell Quest™ pro (Version 5.2.1; Beckton Dickinson and Company) (21, 22).

Assay of cell sensitivity to antineoplastic agent. A total of 1×10^4 wild-type cells, or cells transfected with Ad-GFP or Ad-PTEN at an MOI of 10 were plated in 96-well plates and exposed to different doses (3.9 μM-2 mM) of temozolomide for 72 hours. These cells were they fixed with 5% glutaraldehyde and after several washings with PBS, stained with 0.01% methylene blue. Dye was eluted by 0.4 N hydrochloric acid after washing of the plates. The absorbance was measured at the wavelength of 595 nm on a microplate reader (Bio-rad, CA, USA) and IC_{50} (50% inhibitory concentration) values were determined (23-25).

Combination assay with ionizing radiation, temozolomide and Ad-PTEN infection. After KNS42 cells were infected with Ad-PTEN (MOI 10), the exponentially growing cells were irradiated with a 120-kV X-ray source (MBR1520; Hitachi Medicotechnology, Tokyo, Japan). After treatment, the cells were dispersed with trypsin-EDTA and diffrent numbers of single cells were seeded in the culture plates. Subsequently, these cells were incubated at 37˚C for 3 days, with or without 300 μM of temozolomide. The cells were fixed with 5% glutaraldehyde and stained with 0.01% methylene blue. The absorbance of the plates was measured by a microplate reader and the number of cells was determined.

Results

PTEN expression in 9 glioma cell lines. To investigate PTEN expression among a wide range of glioma cells, immunoblotting for PTEN measurement in human T98G, U373MG, U118MG, KNS42, A172, U138MG, and rat C6, 9L, RT2 cell lines was performed (Figure 1). PTEN expression was undetected by this method except in the T98G cell line and the results demonstrated that 8 out of 9 cell lines lacked a sufficient amount of protein expression.

Forced expression of PTEN by infection of adenoviral vector encoding human PTEN (Ad-PTEN). All the human cell lines expressed PTEN after infection with an adenoviral vector encoding human PTEN (Ad-PTEN). Since the cytopathic effect was minimal in KNS42 cells, the following experiments were performed on this cell line. In this cell line, PTEN expression significantly increased dose-dependently after Ad-PTEN infection. While PTEN expression was up-regulated by Ad-PTEN infection (Figure 2A), there was no increase in protein in the control cells infected with adenovirus encoding GFP (Figure 2B).

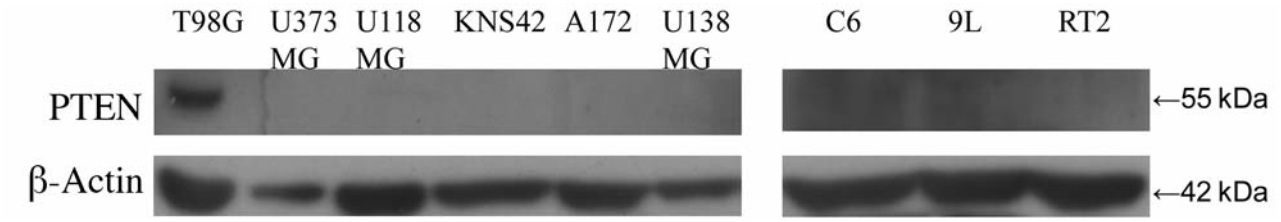

Figure 1. *Expression of PTEN in 6 human and 3 rat glioma cell lines. The expression was compared by immunoblotting. Only T98G human glioma cells expressed detectable levels of PTEN.*

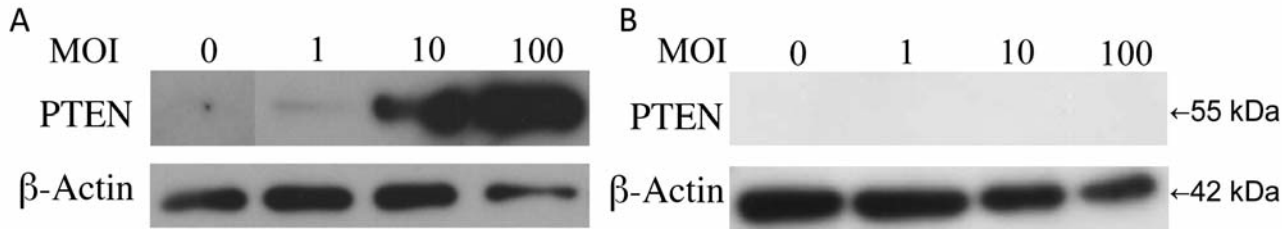

Figure 2. *Expression of PTEN in KNS42 cells after infection with Ad-PTEN. PTEN was expressed in KNS42 cells 72 hours after infection. Levels of expression were dose-dependent. While PTEN was up-regulated by Ad-PTEN infection (A), protein did not increase with infection of cells with the control adenovirus encoding GFP (B).*

Cell proliferation. Since *PTEN* was successfully transduced by Ad-PTEN infection, the effect on cell proliferation was assessed for 4 sequential days after transfection (Figure 3). Ad-PTEN infection at an MOI of 10 significantly inhibited KNS42 cell proliferation. The effect was not observed in the control Ad-GFP-infected cells. The calculated doubling times of KNS42 cells were as follows: 58.5 hours for uninfected cells, 70.9 hours for Ad-PTEN-infected cells, and 57.5 hours for Ad-GFP-infected cells. PTEN transduction significantly prolonged the doubling time of cell proliferation ($p>0.05$).

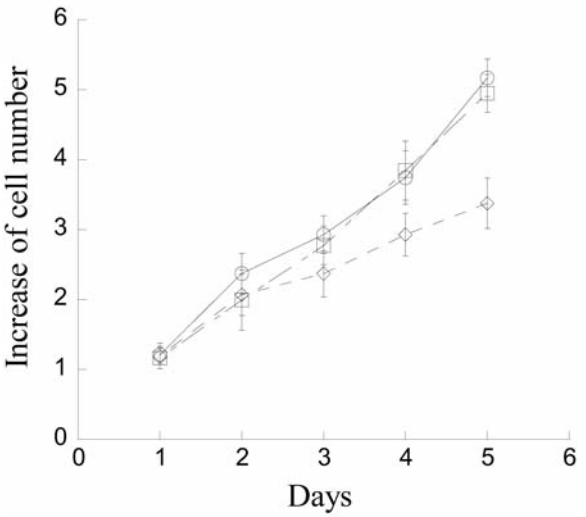

Figure 3. *Effect of PTEN on glioma cell proliferation. After infection of KNS42 cells with Ad-PTEN or Ad-GFP at a multiplicity of infection (MOI) of 10, cells were then plated on a 35-mm dish and the increase in the cell number was determined daily. There was no difference between uninfected cells (○) and Ad-GFP infected cells (□) in cell growth. In contrast, cells infected with Ad-PTEN (◇) revealed a significant disadvantage in terms of their proliferation ($p<0.05$).*

Effect of PTEN on the cell cycle of glioma cells. In order to address the reason for the prolongation, the cell cycle of *PTEN*-expressing cells was analyzed (Figure 4). The results demonstrated that PTEN expression altered the cell cycle distribution. In particular, PTEN inhibited transition of G_2/M cells to G_1, resulting in an increased G_2/M phase population at an MOI of 1. Consequently, this effect led to a decrease in the G_0/G_1 phase population of the glioma cells. With Ad-PTEN at an MOI of 10, there was a further decrease in the proportion of cells in the G_0/G_1 cell phase and an increase in the G_2/M population (25.2% and 48.1%; MOI 0: 68.0% and 18.9%; MOI 1: 44.3% and 20.9%; MOI 10: 61.5% and 19.1%, of infections of Ad-GFP).

The effect of PTEN on cytotoxity of temozolomide in glioma cells. Since expression of PTEN inhibited transition from G_2/M to G_1 and obstructed cell proliferation, the influence on cytotoxity caused by temozolomide was investigated. When cells were infected with Ad-PTEN at an MOI of 10 and the exposed to different doses of temozolomide for 72

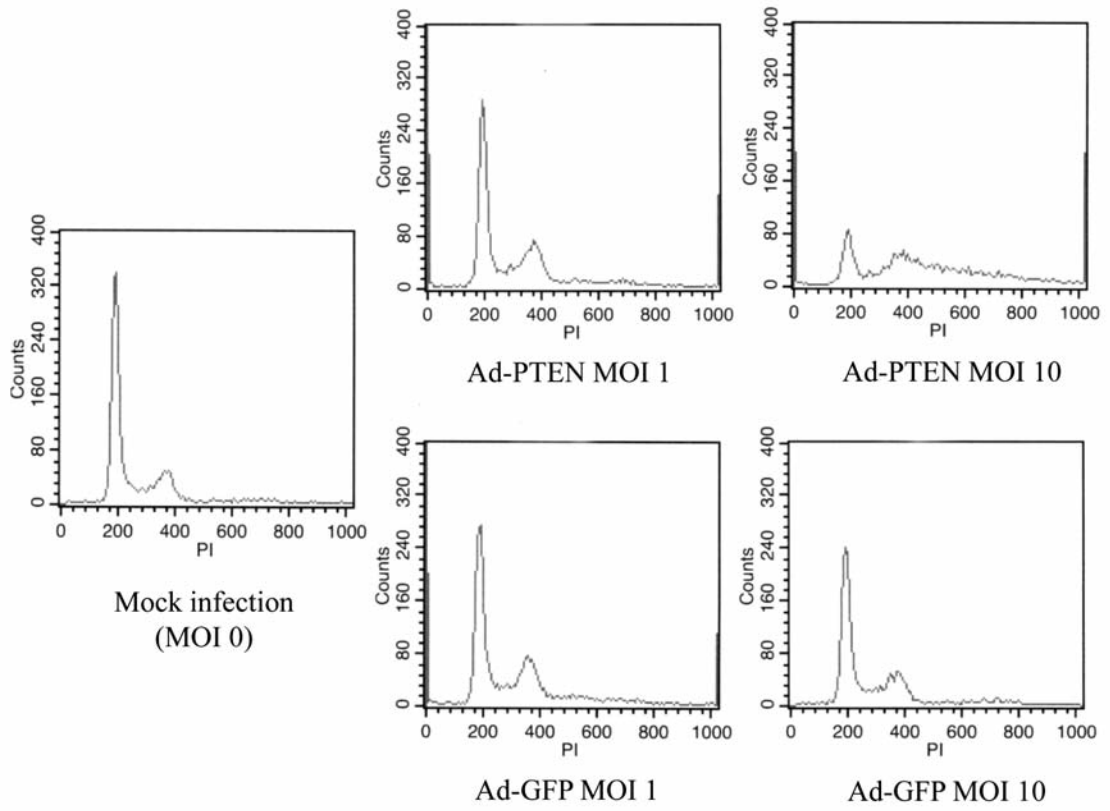

Figure 4. *Effect of adenoviral infection with PTEN and GFP on the cell cycle distribution. KNS42 cells were infected with Ad-PTEN or Ad-GFP at two different multiplicities of infection (MOI). PTEN transduction prolonged the transition of cells from G_2/M to G_1. Accordingly, it redused the G_0/G_1 phase population of cells (MOI=0: 68.0%, MOI=1: 46.2% and MOI=10: 16.7%). The effect was not observed with Ad-GFP infection. Note the increase in the population over diploid in cells transfected with Ad-PTEN at MOI=10. The horizontal axis indicates the amount of DNA, and the vertical axis indicates the cell number.*

hours, cells exhibited increased drug sensitivity (Figure 5). This effect was not observed with control Ad-GFP infection. When IC_{50} values were calculated, the results were 601.3 μM (vehicle alone), 630.6 μM (Ad-GFP) and 387.5 μM (Ad-PTEN).

Irradiation and chemotherapy of Ad-PTEN infected cells. Since expression of PTEN significantly lowered the IC_{50} value of temozolomide and increased the sensitivity of cells to the drug, the combined effect of ionizing radiation and temozolomide was also examined. After infection, transduced cells were exposed to 2, 4, and 6 Gy of ionizing radiation and cultured with or without 300 μM of temozolomide (Figure 6). Results were evaluated using self-renewal of clonogenecity of the glioma cells. The numbers of colonies decreased in proportion to the irradiation dose. PTEN-transduction redused the surviving fraction to 2.8% with 6 Gy of ionizing radiation. This effect was further enhanced by treatment with temozolomide.

Discussion

PTEN is a tumor suppressor gene, and deletion or mutation of this gene has been associated with various kinds of malignancies. Cheng et al reported that PTEN expression in PTEN-deficient cells inhibited cell proliferation (26). In the present study, we investigated the effect of PTEN expression in glioma cells and its effect on cell proliferation, the cell cycle, sensitivity to the antineoplastic agent temozolomide, and to ionizing radiation. The results revealed that *PTEN* expression delayed the doubling time for cell proliferation, possibly due to the inhibition of the G_2/M to G_1 transition and PTEN expression increased the sensitivity to temozolomide and ionizing radiation. PTEN expression was barely detectable in any of the human and rat glioma cell lines except for T98G. The results confirmed the findings of a previous study reporting that many glioma cells lacked PTEN expression (11).

KNS42 is one of the representative cell lines established from Japanese glioma patients and its characteristics have been well studied (14). When the cells were infected with

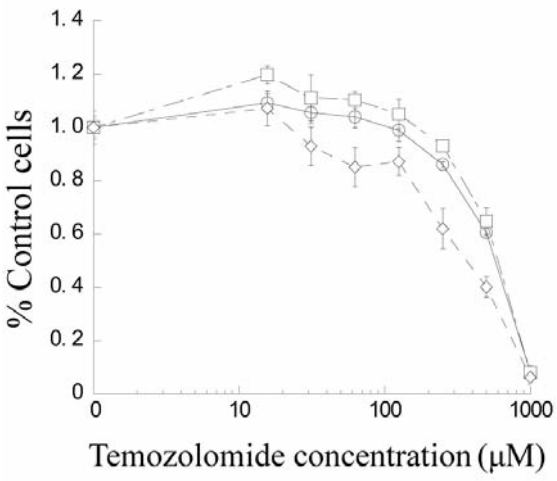

Figure 5. *Effect of forced expression of PTEN on the cytotoxity of temozolomide towards glioma cells. After infection with Ad-PTEN or Ad-GFP, KNS42 cells were exposed to temozolomide for 72 hours and cytotoxicity was measured. Expression of PTEN significantly increased cell sensitivity to the drug ($p<0.05$: 500 μM and 250 μM.) This effect was not observed in cells infected with Ad-GFP. The 50% inhibitory concentration (IC_{50}) decreased from 601.3 μM to 387.5 μM after Ad-PTEN infection. The symbols represent KNS42 cells infected with viral vehicle (○), Ad-GFP (□), and Ad-PTEN (◇) (n=4, bars, SD).*

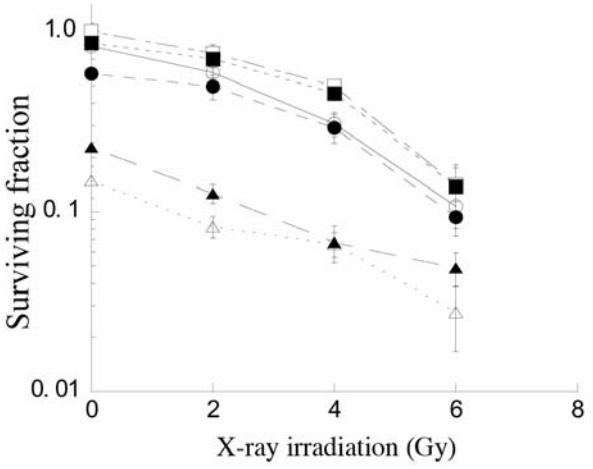

Figure 6. *Combination effect of ionizing radiation and temozolomide on cells with PTEN expression. After infection with vehicle, Ad-PTEN or Ad-GFP, cells were irradiated with 2, 4, and 6 Gy and exposed to 300 M of temozolomide. While an increase in ionizing radiation (2, 4, 6 Gy) or drug concentration (0 or 300 mM) reduced the survival of glioma cells, the combination of both significantly reduced the number of cells in the surviving fraction ($p<0.05$: at 2, 4, 6 Gy). The symbols represent vehicle only (□), temozolomide (■), Ad-GFP (○), Ad-GFP and temozolomide (●), Ad-PTEN (△), Ad-PTEN and temozolomide (▲). (n=3, bars, SD).*

Ad-PTEN and the expression levels of the products were compared, PTEN protein expression increased in proportion to MOI. PTEN expression delayed cell growth approximately by 18%. This result was consistent with the findings of a previous report (27), and the ability of PTEN to inhibit cell proliferation of glioma cells was confirmed. According to our cell cycle experiment, this effect was attributable to inhibition of the transition of cells from G_2/M to G_1. This property of PTEN offers a great advantage in the treatment of glioma patients. Since glioma rarely metastasizes to distant tissue and local recurrence of residual tumor cells determines the prognosis of the patients, topical *PTEN* gene delivery that notably inhibits the proliferation of the glioma cell itself might effectively prolong patient survival.

Alkylating agents, such as nitrosourea and temozolomide, have been utilized in adjuvant chemotherapy of malignant brain tumors. Due to the isolated circumstances of brain parenchyma, agents need to cross the blood–brain barrier in order to reach the tumor tissue. Moreover, drugs need to act on the non-proliferative or quiescent phase of the malignant cells, as it has been found that only approximately 30% of cells actively proliferate in an *in vivo* glioma (28). Temozolomide has been developed recently to serve as such an agent and its effect on brain tumor in combination with ionizing radiation has been reported (29). In the present study, transduction of *PTEN* significantly enhanced the toxicity and reduced the IC_{50} value of temozolomide. Although temozolomide acts on tumor cells in the non-proliferating phase, the agent also alkylates and cross-links the DNA of the accumulated G_2 phase cells induced by PTEN expression and this might account for the synergic effect on glioma cells. In addition, it enhanced the effect of ionizing radiation. Ionizing radiation acts on the G_1, S and G_2 phases of the cells, but it especially effective on the G_1 phase of cells, resulting in their G_1 arrest (30). Since, cells are sensitive to ionizing radiation at the G_1 phase and PTEN arrested glioma cell growth in the G_2/M phase, temozolomide provides considerable efficacy in the combination therapy. The dose of 2 Gy used in the study is comparable to the standard dose used in fractionated irradiation. Topical *PTEN* delivery might lead to a greater therapeutic effect if the patients are treated with the combination of temozolomide and ionizing radiation.

As we found in this investigation PTEN expression was lacking from the KN242 cell line. If expression of PTEN leads to an increase in sensitivity to temozolomide and ionizing radiation, as a next step, we need to compare the effect of the combination using gliomas with or without native PTEN protein expression. Furthermore, in contrast to our results, another group demonstrated that PTEN caused G_1 arrest (6). It is important to address the various factors causing cell cycle differences in the glioma cell and, accordingly, further studies are needed.

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