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ORIGINAL ARTICLE

Combination of all-*trans* retinoic acid and interferon-gamma upregulated $p27^{kip1}$ and down regulated CDK2 to cause cell cycle arrest leading to differentiation and apoptosis in human glioblastoma LN18 (PTEN-proficient) and U87MG (PTEN-deficient) cells

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Abstract
Purpose Deletion or mutation of phosphatase and tensin homolog located on chromosome ten (PTEN) occurs in as high as 80% glioblastoma. All-*trans* retinoic acid (ATRA) induces differentiation in cancer cells. Interferon-gamma (IFN-γ) induces apoptosis in many cancers including glioblastoma. We used the combination of ATRA and IFN-γ to control growth of human glioblastoma LN18 (PTEN-proficient) and U87MG (PTEN-deficient) cells and explored any advantage of having PTEN in the cells.
Methods LN18 and U87MG cells were treated with ATRA (1 μM) for 7 days and then IFN-γ (5 ng/ml) for 1 day. Methylene blue staining indicated astrocytic differentiation. Wright staining and ApopTag assay showed characteristic features of apoptosis. Western blotting demonstrated the levels of specific proteins.
Results ATRA and IFN-γ alone and in combination could induce apoptosis in LN18 cells; while ATRA alone induced differentiation only, IFN-γ alone induced apoptosis, and ATRA plus IFN-γ increased apoptosis in U87MG cells. The variation in induction of apoptosis by ATRA alone might be attributed to difference in PTEN expression in the two cell lines. Compared with control cells, IFN-γ alone and ATRA plus IFN-γ increased PTEN expression in LN18 cells while there was no PTEN expression or induction in U87MG cells after treatments with ATRA alone and ATRA plus IFN-γ. Apoptosis in both cell lines was associated with increases in Bax:Bcl-2 ratio, mitochondrial release of cytochrome c into the cytosol, and calpain and caspase-3 activities. Treatments elevated $p27^{kip1}$ and decreased CDK2 levels in both cell lines, indicating cell cycle arrest at G_1/S phase.
Conclusions The combination of ATRA and IFN-γ could control the growth of both PTEN-proficient and PTEN-deficient glioblastoma cells by arresting cell division and inducing differentiation and apoptosis. Thus, our study indicated that the growth of both PTEN-proficient and PTEN-deficient glioblastoma cells could effectively be controlled by treatment with the combination of ATRA and IFN-γ.

Keywords Apoptosis · ATRA · Cell cycle arrest · Glioblastoma · IFN-γ · PTEN

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Introduction

Glioblastoma is the deadliest and most prevalent brain tumor in humans [15]. The average survival time of glioblastoma patients is less than 12 months even after treatment with the currently available therapeutic regimens [26]. Current treatment strategies include mass-reductive surgery, radiotherapy, and chemotherapy. Because cytotoxic synthetic agents possess such limitations as inability to cross the blood–brain-barrier and severe side effects, they so far showed little promise in extending the patients' survival [26, 27, 29]. So, some investigators switched to using cytostatic medicines for treatment of glioblastoma [11, 19]. The cytostatic medicines, such as retinoids, can cause cell death through promoting differentiation, inhibiting angiogenesis, impairing tumor invasion, and blocking growth factor pathways. Retinoids belong to a class of natural agents that specifically target the cellular receptors and

signaling proteins to induce differentiation and inhibit growth of cancer cells. For example, all-*trans* retinoic acid (ATRA) could induce differentiation and suppress growth of leukemia [2, 8], breast cancer [24], prostate cancer [20], and ovarian cancer [25] cells. The natural biomolecule IFN-γ is known to induce apoptosis and the combination of ATRA and IFN-γ can result in increase in apoptosis in glioblastoma cells [9]. Apoptosis in glioblastoma cells by IFN-γ has been associated with upregulation of pro-apoptotic Bax and down regulation of anti-apoptotic Bcl-2 [22] and increase in caspase-3 activity [9].

Phosphatase and tensin homolog located on chromosome ten (PTEN), which encodes a cytoplasmic enzyme with both protein and lipid phosphatase activity, is frequently mutated or deleted from chromosome 10q23 in malignant glioblastoma [17]. As a tumor suppressor, PTEN is capable of controlling cell proliferation that is abnormally high in many types of solid tumors such as breast cancer, prostate cancer, melanoma, and brain tumors due to loss of normal function of PTEN [6, 7, 17]. Notably, the mutation and deletion of PTEN accounted for as high as 80% of human glioblastomas [7, 17]. The transfection of wild-type PTEN cDNA into glioblastoma cell lines harboring mutated or deleted PTEN could induce cell cycle arrest at G_1 phase and elicit astrocytic differentiation [1]. It has been indicated that ATRA can play an important role in increasing the PTEN expression for reducing the cell growth rate and causing the cell cycle arrest at the G_1 phase [16]. Other reports show that PTEN expression causes cell cycle arrest at G_0/G_1 phase, which is most closely associated with an increase in protein level of the cyclin-dependent protein kinase (CDK) inhibitor $p27^{kip1}$ [3, 12, 14].

Although previous studies have shown that treatment of glioblastoma cells with ATRA induce differentiation and growth arrest [1, 30], there is no specific report yet showing the differential effects of ATRA in glioblastoma cell lines having proficient and deficient PTEN expression. We designed the current investigation to examine the amounts of apoptosis in two human glioblastoma cell lines, LN18 (PTEN-proficient) and U87MG (PTEN-deficient), after treatments with ATRA and IFN-γ alone and in combination. Our data show that the most effective treatment option is the combination of ATRA and IFN-γ for increasing the amounts of apoptosis in these two glioblastoma cell lines due to increase in expression of $p27^{kip1}$ and decrease in expression of CDK2.

Materials and methods

Materials

Human glioblastoma LN18 and U87MG cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). We obtained primary IgG antibodies against CDK2, $p27^{kip1}$, and PTEN from Cell Signaling Technologies (Danvers, MA, USA); glial fibrillary acidic protein (GFAP) from Chemicon International (Temecula, CA, USA); cytochrome c from BD Biosciences (San Jose, CA, USA); Bax, Bcl-2, inhibitor of casapse-3-activated DNase (ICAD) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and α-spectrin from Affiniti (Exeter, UK). Both ATRA and IFN-γ were purchased from Sigma Chemical (St Louis, MO, USA).

Cell culture

Human glioblastoma LN18 and U87MG cell lines were separately grown in DMEM and RPMI 1640, respectively, both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cells were seeded in 75-cm^2 flasks and incubated at 37°C in a fully humidified atmosphere with 5% CO_2. Once the cells were 80% confluent, they were starved in DMEM or RPMI with 5% FBS for 24 h and maintained in this low FBS condition during all treatments. Cells were treated with 1 μM ATRA consecutively for 7 days, with change of medium every 2 days. Then, we added 5 ng/ml IFN-γ to the medium for treatment of cells for the next 24 h. After the treatments, cells were processed for determinations of differentiation, viability, apoptosis, and specific proteins by Western blotting.

Methylene blue staining to examine astrocytic differentiation

Cells were cultured in monolayer in plates of 10-cm diameter in the absence and presence of 1 μM ATRA for 7 days. At the end of the treatment, the culture medium was aspirated and cells were washed two times with ice-cold phosphate-buffered saline (PBS), pH 7.4, in the culture plate. For in situ methylene blue staining of the cells, each plate was placed on ice and 5 ml of ice-cold 50% (v/v) ethanol was added to fix the cells for 5 min; ethanol was aspirated followed by the addition of 5 ml of ice-cold 0.2% (v/v) methylene blue solution (prepared in 50% ethanol). Cells were stained with methylene blue for 30 s and washed twice with ice-cold water. The plates were dried in air. Cells were examined under the light microscope at 400× magnification for identification of astrocytic differentiation.

Cell viability assay

After treatment with ATRA and IFN-γ alone and in combination, Trypan Blue dye exclusion test [5, 22] was performed to evaluate the cell viability. Viable cells maintained membrane integrity and did not take up Trypan Blue. Cells with compromised cell membranes took up

Trypan Blue, and were counted as dead. At least 600 cells were counted in four different fields and the number of viable cells was calculated as percentage of the total cell population.

Wright staining for morphological features of apoptosis

Cells from all treatments were harvested and washed in PBS, pH 7.4, and sedimented onto the microscopic slides using the Centra CL2 centrifuge (IEC, Needham Heights, MA, USA) at 1,000 rpm for 5 min. Cells were fixed for Wright staining, as we reported previously [5]. Cellular morphology was examined under light microscopy to assess amount of apoptosis. Cells were considered apoptotic with the characteristic reduction in cell volume and condensation of the chromatin and/or the presence of cell membrane blebbing. About 600 cells were counted in each treatment and the percentage of apoptotic cells was calculated.

ApopTag peroxidase assay for detection of apoptotic DNA fragmentation

For detection of DNA fragmentation as a biochemical marker of apoptosis, glioblastoma cells on the microscopic slides were subjected to the TdT-mediated dUTP nick-end labeling (TUNEL) using the ApopTag peroxidase assay kit (Intergen, Purchase, NY, USA). In this TUNEL staining, we used 3,3′-diaminobenzidine (DAB) as a peroxidase substrate. Cells with DNA fragmentation produced a brown product from oxidative polymerization and cyclization of DAB in the course of the ApopTag peroxidase assay. ApopTag-positive cells were brown in a pale green background and were considered as apoptotic cells. Experiments were conducted in triplicate and percentage of ApopTag-positive cells was determined by counting the brown cells from randomly selected fields under the light microscope [4, 5].

Protein extraction and Western blotting

After the treatments, cells were lysed in a buffer composed of 50 mM Tris–HCl, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM EGTA for extraction of cellular proteins. Concentration of total proteins was determined colorimetrically using Coomassie-Plus protein assay reagent (Pierce, Rockford, IL, USA). The samples were mixed with an equal volume of 2× loading buffer [125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM 1,4-dithio-DL-threitol (DTT), and 0.02% bromophenol blue], boiled for 5 min, and loaded (40 μg/lane) onto the 4–20% gradient gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the gels were blotted to Immunobilon-P nylon membrane. The blots were blocked with 5% non-fat milk, 0.1% Tween, Tris–HCl, pH 7.8, for 2 h at room temperature. Then, the blots were incubated with a specific primary IgG antibody for 2 h at room temperature or overnight at cold room followed by alkaline horseradish peroxidase-conjugated secondary IgG antibody for 1 h. Blots were developed using the enhanced chemiluminescence (ECL) or ECL-Plus reagents (Amersham Pharmacia, Buckinghamshire, UK). The ECL autoradiograms were scanned on a PowerLook Scanner (Umax Technologies, Fremont, CA, USA) using Photoshop software (Adobe Systems, Seattle, WA, USA) and optical density (OD) of each band was determined using Quantity One software (Bio-Rad, Hercules, CA, USA).

Analysis of mitochondrial release of cytochrome c into the cytosol

Cells from each treatment were harvested, washed once with ice-cold PBS, and gently lysed for 1 min in 50 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris–HCl, pH 6.8, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM benzamidine, and 0.1 mM PMSF) [21]. Lysates were centrifuged at 12,000*g* in cold (4°C) for 3 min to obtain pellet (the mitochondrial fraction) and supernatant (the cytosolic fraction without mitochondria). Pellet and supernatant were analyzed by Western blotting using cytochrome c antibody.

Statistical analysis

Results were analyzed using StatView software (Abacus Concepts, Berkeley, CA, USA) and compared using one-way analysis of variance (ANOVA) with Fisher's post hoc test. Data were presented as mean ± standard deviation (SD) of separate experiments ($n \geq 3$). Significant difference from control value was indicated by * ($P < 0.05$) or ** ($P < 0.001$).

Results

Levels of PTEN expression in human glioblastoma LN18 and U87MG cell lines

Because PTEN is an acclaimed tumor suppressor, induction of PTEN expression by a therapeutic agent helps suppress survival signaling in malignant cells leading to cell death. We examined any changes in levels of PTEN expression in LN18 and U87MG cells after treatments with 1 μM ATRA, 5 ng/ml IFN-γ, and 1 μM ATRA plus 5 ng/ml IFN-γ (Fig. 1). Treatments with IFN-γ alone and ATRA plus IFN-γ induced PTEN expression in LN18 cells (Fig. 1a). Almost

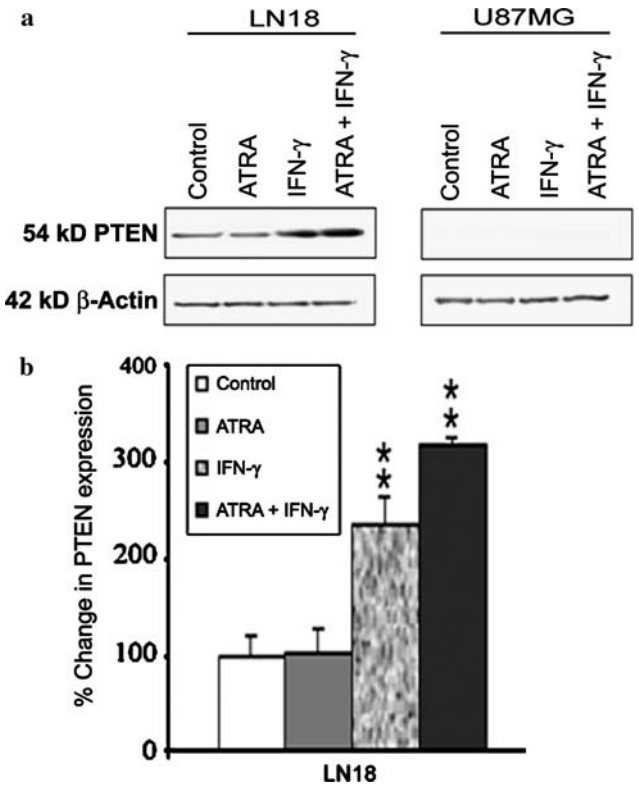


Fig. 1 Western blotting to examine the levels of PTEN in human glioblastoma LN18 and U87MG cells. Treatments: control, 1 μM ATRA (7 days), 5 ng/ml IFN-γ (1 day), and 1 μM ATRA (6 days) + 5 ng/ml IFN-γ (1 day). **a** Representative Western blots showed the levels of PTEN and β-actin following treatments. **b** Densitometric analysis of the levels of PTEN in LN18 cells following treatments

uniform levels of β-actin expression in both cell lines served as loading controls on the Western blots (Fig. 1a). Quantification showed that treatment with ATRA alone did not alter the level of PTEN expression, while treatment with IFN-γ alone induced PTEN expression and the combination of ATRA and IFN-γ significantly ($P < 0.001$) increased the level of PTEN expression, compared with control LN18 cells (Fig. 1b). We could not detect PTEN expression in control U87MG cells or its induction after the treatments. So, our results confirm that LN18 is a PTEN-proficient cell line while U87MG is a PTEN-deficient cell line.

ATRA treatment induced astrocytic differentiation and increased GFAP expression

Because of its ability for induction of differentiation, ATRA has been widely used as a differentiating agent for the treatment of cancers. We examined the morphological and biochemical markers of astrocytic differentiation (Fig. 2). Methylene blue staining showed the morphological features of astrocytic differentiation in glioblastoma LN18 and U87MG cells after the treatments (Fig. 2a). There were distinct indications of astrocytic differentiation in both LN18 and U87MG cells after treatment with 1 μM ATRA for 7 days. The cells were changed morphologically to long and star-like shapes, which were close enough to normal astrocytes. Astrocytic differentiation produced many well-developed and extended and radially thin processes. Interestingly, 5 ng/ml IFN-γ alone induced astrocytic differentiation to some extent in LN18 cells but not in U87MG cells. However, treatment with ATRA plus IFN-γ induced differentiation and death in both LN18 and U87MG cells. Western blotting showed the overexpression of GFAP in both LN18 and U87MG cells after the treatments with ATRA alone and ATRA plus IFN-γ (Fig. 2b). We used uniform expression of β-actin as the loading control on the Western blots. Overexpression of GFAP is a well-known biochemical marker of astrocytic differentiation because increase in GFAP expression is associated with astrocytic cell shape. Densitometric analysis of the Western blots indicated that compared with control cells, GFAP expression was significantly increased ($P < 0.05$) in both LN18 and U87MG cells following the treatments with ATRA alone and ATRA plus IFN-γ (Fig. 2c).

Decrease in cell viability and increase in apoptosis in glioblastoma cells after treatments

We determined the amounts of residual cell viability and also apoptosis in LN18 and U87MG cells after the treatments (Fig. 3).

Trypan Blue dye exclusion test was used to examine the residual cell viability in both LN18 and U87MG cells after the treatments with ATRA and IFN-γ alone and in combination (Fig. 3a). Treatments with ATRA, IFN-γ, and ATRA plus IFN-γ significantly decreased ($P < 0.001$) residual cell viability to 61, 62, and 53%, respectively, in LN18 cells. In U87MG cells, ATRA alone non-significantly reduced the residual cell viability to 76%, while IFN-γ alone and ATRA plus IFN-γ significantly reduced ($P < 0.05$) the residual cell viability to 64 and 60%, respectively. Comparison between treatments with ATRA alone and ATRA plus IFN-γ showed significant difference ($P < 0.05$) in residual cell viability in U87MG cells. The results indicated that ATRA alone effectively decreased residual cell viability in LN18 cells but not so effectively in U87MG cells, while the combination of ATRA and IFN-γ could effectively decrease the residual cell viability in both cell lines.

We employed Wright staining to detect the morphological characteristics of apoptosis such as cell shrinkage, membrane blebbing, and chromatin condensation, formation of membrane-bound apoptotic bodies in LN18 and U87MG cells after the treatments (Fig. 3b). The percentages of apoptotic characteristics were calculated in both

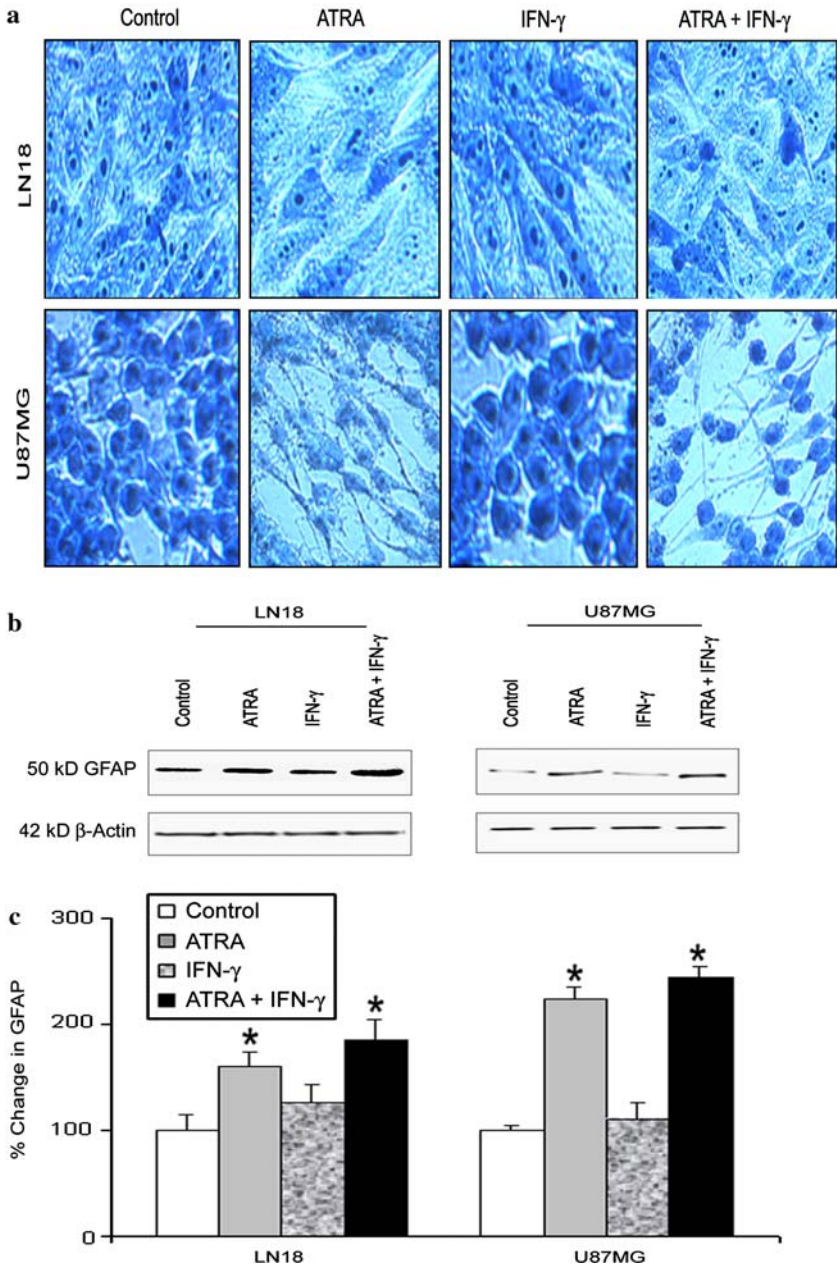
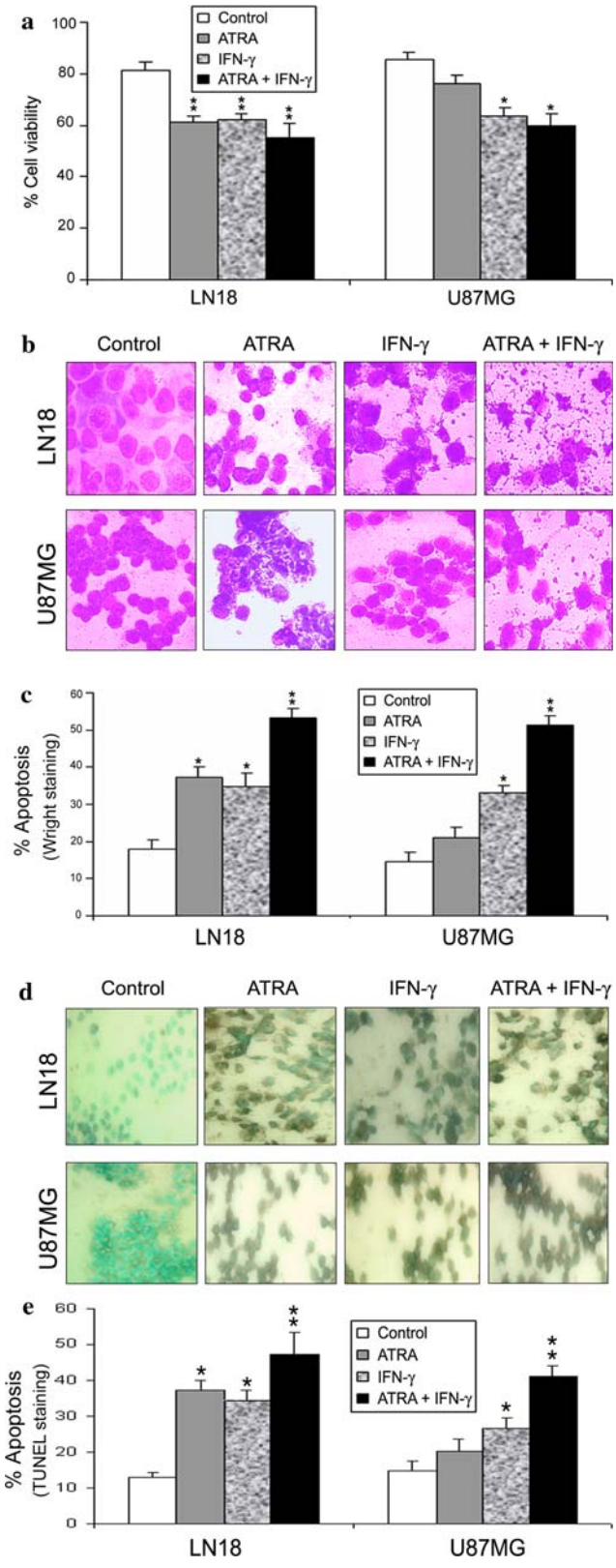


Fig. 2 Morphological features of astrocytic differentiation in LN18 and U87MG cells. Treatments: control, 1 μM ATRA (7 days), 5 ng/ml IFN-γ (1 day), and 1 μM ATRA (6 days) + 5 ng/ml IFN-γ (1 day). **a** Methylene blue staining to detect the morphological features of astrocytic differentiation. **b** Western blotting to examine the levels of expression of GFAP and internal control β-actin following treatments. **c** Densitometric analysis to determine percent changes in expression of GFAP following treatments

cell lines following the treatments (Fig. 3c). Compared with control, ATRA alone and IFN-γ alone induced significant ($P < 0.05$) amounts of apoptosis while the combination of ATRA and IFN-γ greatly increased ($P < 0.001$) apoptosis in LN18 cells. Compared with control, IFN-γ alone induced significant amount ($P < 0.05$) and ATRA plus IFN-γ very significant amount ($P < 0.001$) of apoptosis in U87MG cells. Thus, both cell lines were highly amenable to apoptosis when treated with ATRA plus IFN-γ.

Moreover, we performed TUNEL staining using ApopTag assay kit to identify the DNA fragmentation (brown color) in apoptotic cells (Fig. 3d). Control cells showed little or no brown color, indicating almost absence of apoptotic cells. Compared with control, ATRA alone and IFN-γ alone induced significant amounts ($P < 0.05$) of apoptosis and combination of ATRA and IFN-γ further ($P < 0.001$) increased the amount of apoptosis in LN18 cells (Fig. 3e). Compared with control, ATRA alone could not produce significant amount of apoptosis, IFN-γ alone induced significant amount ($P < 0.05$) of apoptosis and combination of ATRA and IFN-γ caused the most significant amount ($P < 0.001$) of apoptosis in U87MG cells.

It should be noted that treatment with combination of ATRA and IFN-γ produced no added effect on viability loss (or induced cell death), which was assessed considering cell membrane integrity loss. The process of apoptosis that is triggered requires cell membrane integrity at early stages. Combination therapy triggered apoptosis maintaining cell



◀ **Fig. 3** Decrease in cell viability and increase in apoptosis in LN18 and U87MG cells following different treatments. Treatments: control, 1 μM ATRA (7 days), 5 ng/ml IFN-γ (1 day), and 1 μM ATRA (6 days) + 5 ng/ml IFN-γ (1 day). **a** Determination of amounts of residual cell viability based on Trypan Blue dye exclusion test. **b** Wright staining to examine morphological features of apoptosis. **c** Determination of amounts of apoptosis based on Wright staining. **d** TUNEL staining with the use of ApopTag assay kit detected DNA fragmentation in apoptotic cells. **e** Determination of amounts of apoptosis based on TUNEL staining

membrane integrity at early stages and actually showed some synergy in the amount of apoptosis, which was assayed considering even some cells with membrane integrity.

An increase in Bax:Bcl-2 ratio triggered apoptosis

We employed Western blotting to examine the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 in LN18 and U87MG cells after the treatments (Fig. 4). We revealed that ATRA plus IFN-γ most effectively increased expression of Bax and but decreased expression of Bcl-2 in both LN18 and U87MG cells (Fig. 4a). Densitometric analysis of the Western blots and calculation were performed for an assessment of any increase in Bax:Bcl-2 ratio after the treatments (Fig. 4b). Compared with control, ATRA alone and IFN-γ alone caused significant increase ($P < 0.05$) in Bax:Bcl-2 ratio while ATRA plus IFN-γ caused the most significant increase ($P < 0.001$) in Bax:Bcl-2 ratio in LN18 cells. Compared with control, ATRA alone did not alter the Bax:Bcl-2 ratio but IFN-γ alone and combination of ATRA and IFN-γ very significantly increased ($P < 0.001$) the Bax:Bcl-2 ratio in U87MG cells. A significant increase in the Bax:Bcl-2 ratio after treatment indicated that cells made a full commitment to trigger apoptotic process via mitochondrial pathway.

Treatments induced mitochondrial release of cytochrome c into the cytosol

We analyzed the mitochondrial and cytosolic fractions for assessment of mitochondrial release of cytochrome c into the cytosol by Western blotting (Fig. 5). Mitochondrial release of cytochrome c into the cytosol occurred in LN18 cells after all treatments whereas in U87MG cells, only after treatment with IFN-γ alone or ATRA plus IFN-γ (Fig. 5a). Compared with control, significant disappearance of cytochrome c from mitochondria (Fig. 5b) and significant appearance of cytochrome c into the cytosol (Fig. 5c) happened in LN18 cells after all treatments but in U87MG cells, only after treatments with IFN-γ alone and ATRA plus IFN-γ. The results showed the synergistic effects of the combination of ATRA and IFN-γ in causing almost complete mitochondrial release of cytochrome c into cytosol in both LN18 and U87MG cells. After treatment with combination of ATRA and IFN-γ, very significant increase ($P < 0.001$) in cytosolic cytochrome c in both cell lines indicated activation of proteolytic pathways for mediation of apoptosis.

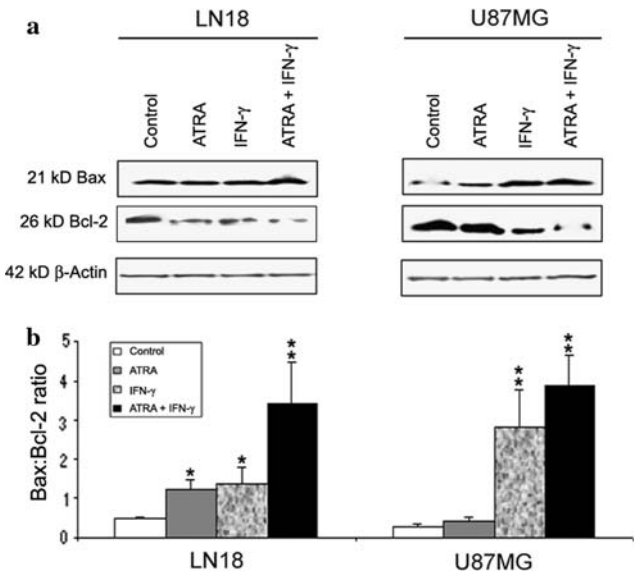


Fig. 4 Western blotting to examine alterations in levels of Bax and Bcl-2 proteins in LN18 and U87MG cells. Treatments: control, 1 μM ATRA (7 days), 5 ng/ml IFN-γ (1 day), and 1 μM ATRA (6 days) + 5 ng/ml IFN-γ (1 day). **a** Representative Western blots show levels of Bax, Bcl-2, and β-actin in LN18 and U87MG cells following treatments. **b** Determination of Bax:Bcl-2 ratio in LN18 and U87MG cells. An increase in Bax:Bcl-2 ratio following a treatment indicated the commitment of cells to apoptosis

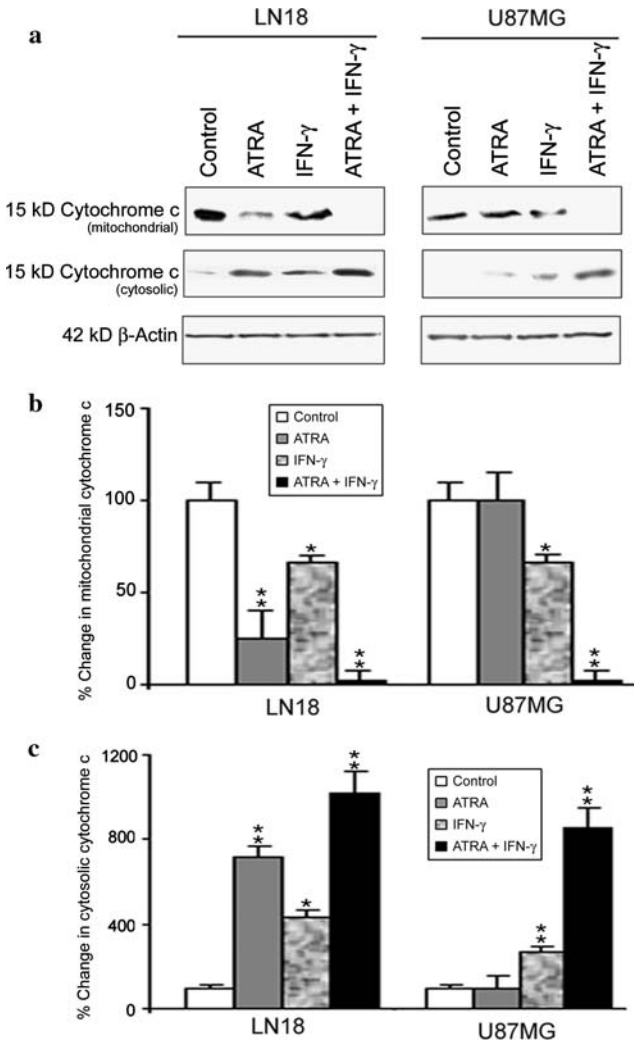


Fig. 5 Western blotting to examine mitochondrial release of cytochrome c into the cytosol in LN18 and U87MG cells. Treatments: control, 1 μM ATRA (7 days), 5 ng/ml IFN-γ (1 day), and 1 μM ATRA (6 days) + 5 ng/ml IFN-γ (1 day). **a** Representative Western blots show levels of mitochondrial cytochrome c and cytosolic cytochrome c following treatments. **b** Densitometric analysis of the Western blots shows the amounts of disappearance of cytochrome c from the mitochondria. **c** Densitometric analysis of the Western blots showed the amounts of mitochondrial release of cytochrome c into the cytosol

Increases in proteolytic activities of calpain and caspase-3

We performed Western blotting to monitor the increases in proteolytic activities of calpain and caspase-3 in both LN18 and U87MG cells after the treatments (Fig. 6). We detected increases in calpain and caspase-3 activities in the cleavage of α-spectrin to calpain-specific 145 kDa spectrin breakdown product (SBDP) and caspase-3-specific 120 kDa SBDP, and also increase in caspase-3 activity in the cleavage of 45 kDa ICAD to 40 kDa ICAD fragment in both LN18 and U87MG cells (Fig. 6a). Densitometric analysis of the Western blots clearly showed significant increases in 145 kDa SBDP (Fig. 6b), 120 kDa SBDP (Fig. 6c), and 40 kDa ICAD (Fig. 6d) in both cell lines after treatment with the combination ATRA and IFN-γ. Our results suggested that increases in proteolytic activities of calpain and caspase-3 played major roles in mediation of apoptosis in both LN18 and U87MG cells after the combination therapy.

Changes in $p27^{kip1}$ and CDK2 levels to cause cell cycle arrest

We performed Western blotting to examine changes in levels of $p27^{kip1}$ (an inhibitor of CDK2) and CDK2 (an important kinase for cell cycle transition from G_1 to S phase) in LN18 and U87MG cells after the treatments (Fig. 7). All treatments altered the levels of expression of $p27^{kip1}$ and CDK2 in both cell lines (Fig. 7a). Compared with control, all treatments significantly increased the expression of $p27^{kip1}$ (Fig. 7b) but significantly decreased the expression of CDK2 (Fig. 7c). Combination of ATRA and IFN-γ was the most effective treatment in upregulating $p27^{kip1}$ and down regulating CDK2 in both LN18 and U87MG cells. After the combination therapy, the increased expression of $p27^{kip1}$ very effectively inhibited CDK2 for cell cycle arrest at G_1/S transition leading to induction of apoptosis in both LN18 and U87MG cells.

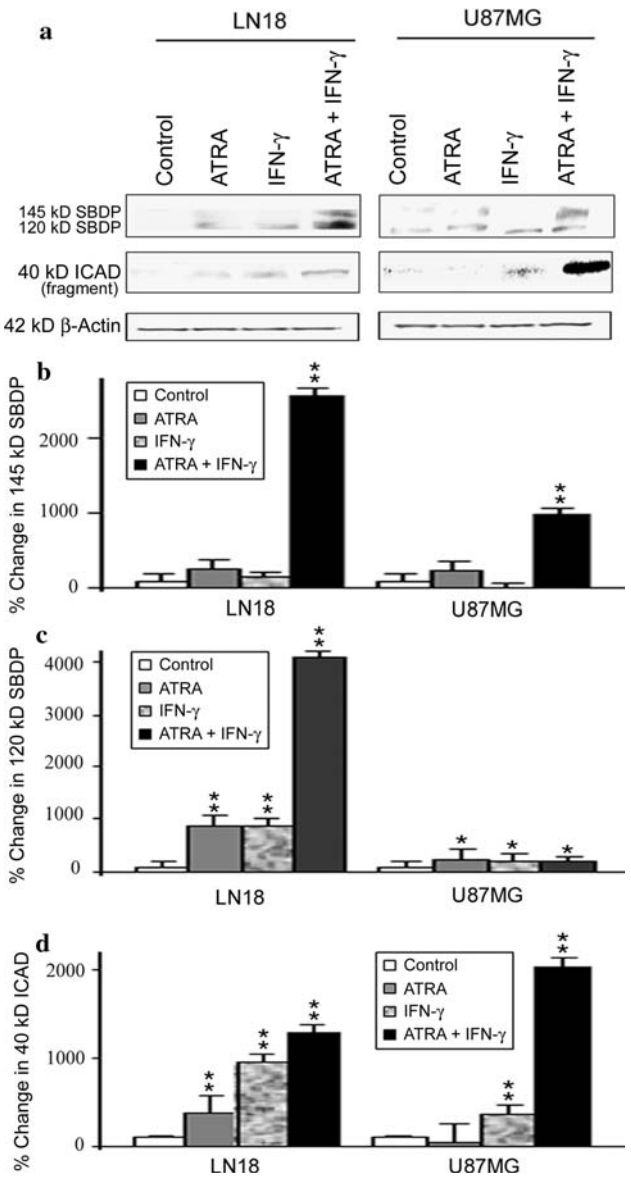


Fig. 6 Western blotting to examine proteolytic activities of calpain and caspase-3 in LN18 and U87MG cells. Treatments: control, 1 μM ATRA (7 days), 5 ng/ml IFN-γ (1 day), and 1 μM ATRA (6 days) + 5 ng/ml IFN-γ (1 day). **a** Representative Western blots show levels of 145 kDa SBDP and 120 kDa SBDP and also 40 kDa ICAD fragment following treatments. **b** Determination of the levels of calpain-specific 145 kDa SBDP after the treatments. **c** Determination of the levels of caspase-3-specific 120 kDa SBDP after the treatments. **d** Determination of the caspase-3 activity in the formation of 40 kDa ICAD fragment after the treatments

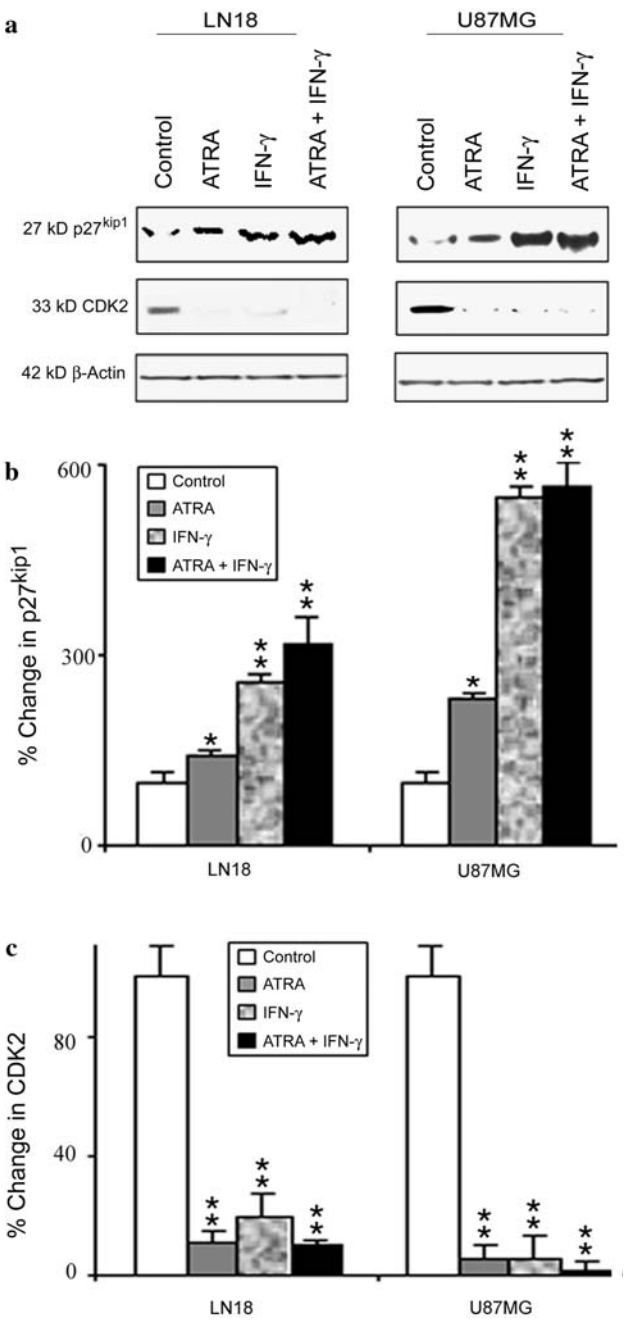


Fig. 7 Western blotting to examine the cell cycle regulatory proteins $p27^{kip1}$ and CDK2 in LN18 and U87MG cells. Treatments: control, 1 μM ATRA (7 days), 5 ng/ml IFN-γ (1 day), and 1 μM ATRA (6 days) + 5 ng/ml IFN-γ (1 day). **a** Representative Western blots show levels of $p27^{kip1}$ and CDK2. **b** Densitometric analysis of the Western blots showed upregulation of the G_1/S transition inhibitor $p27^{kip1}$. **c** Densitometric analysis of the Western blots showed down-regulation of the G_1/S transition key player CDK2

Discussion

A decrease or deficiency in PTEN expression in cancer cells can affect the therapeutic outcome. Many solid tumors such as breast cancer, thyroid cancer, melanoma, and glioblastoma harbor mutation or deletion of PTEN [7, 17], posing a real challenge to therapeutic designs for controlling the malignant growth of these tumors. Inactivation of PTEN gene plays a critical role in progression of many glioblastomas, which are not quite easily amenable to apoptosis after single agent treatment. Therapeutic agents that induce PTEN expression are quite helpful in controlling

malignant growth. However, a successful therapeutic strategy should control malignant growth of both PTEN-proficient and PTEN-deficient glioblastoma cells. In this investigation, we used human glioblastoma LN18 (PTEN-proficient) and U87MG (PTEN-deficient) cells and demonstrated that combination of ATRA and IFN-γ very effectively induced differentiation and apoptosis in these cell lines due to increase in expression of $p27^{kip1}$ and decrease in expression of CDK2 (Figs. 1–7).

Previously, ATRA has been shown to cause differentiation in many cancers including glioblastoma [9]. Moreover, combination of ATRA and IFN-γ controlled growth and induced immune response in glioblastoma cells [9]. Suppression of cell survival and signaling could be responsible for induction of apoptotic changes in glioblastoma cells following treatment with the combination of ATRA and IFN-γ [31]. In this investigation, we demonstrated that combination of ATRA and IFN-γ induced a level of PTEN expression in LN18 cells (Fig. 1) so as to make the cells rapidly succumb to apoptosis. We found that ATRA alone induced morphological features of astrocytic differentiation with overexpression of GFAP in both LN18 and U87MG cells (Fig. 2). The fact that low-dose ATRA induced apoptosis in LN18 cells but not in U87MG cells (Fig. 3) again reinforced the importance of PTEN expression to therapeutic advantage. Because ATRA alone could only induce differentiation without apoptosis in U87MG cells, this indicated that single agent chemotherapeutic approach might face similar difficulty in inducing death in PTEN-deficient glioblastoma cells. It is encouraging enough that combination of ATRA and IFN-γ very effectively induce apoptosis not only in PTEN-proficient but also in PTEN-deficient glioblastoma cells (Fig. 3).

Then, we examined the molecular mechanisms that could be accounted for induction of significant amounts of apoptosis in LN18 and U87MG cells following treatment with the combination of ATRA and IFN-γ. The levels of expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 were altered so as to significantly increase the Bax:Bcl-2 ratio making the LN18 and U87MG commit apoptosis after treatment with the combination of ATRA and IFN-γ (Fig. 4). There has been a previous report that ATRA can work synergistically to cause an increase in Bax expression in glioblastoma cells [30]. Our current data indicated that the treatments with ATRA and IFN-γ alone and in combination could significantly increase the Bax:Bcl-2 ratio in LN18 cells (Fig. 4). On the other hand, treatments with IFN-γ alone and ATRA plus INF-γ caused significant increases in Bax:Bcl-2 ratio in U87MG cells (Fig. 4). Increase in Bax:Bcl-2 ratio provides an indication that mitochondrial release of cytochrome c into the cytosol can lead to apoptosis [5, 32]. Our results clearly showed that treatment with the combination of ATRA and IFN-γ most effectively induced mitochondrial release of cytochrome c into the cytosol in both LN18 and U87MG cells (Fig. 5). Treatment with ATRA alone did induce significant amount of mitochondrial release of cytochrome c into the cytosol only in LN18 cells but not in U87MG cells, indicating that ATRA alone was capable of inducing apoptosis in PTEN-proficient but not in PTEN-deficient glioblastoma cells. Cytosolic cytochrome c leads to activation of proteolytic cascades [5]. Increases in proteolytic activities of calpain and caspase-3 could be demonstrated in the cleavage of α-spectrin and formation of ICAD fragment [13, 18, 28]. We confirmed that combination of ATRA and IFN-γ could produce significant amounts of calpain-specific 145 kDa SBDP and also caspase-3-specific 120 kDa SBDP as well as 40 kDa ICAD fragment in LN18 and U87MG cells (Fig. 6). These results demonstrated that apoptosis occurred in both LN18 and U87MG cells due to increases in proteolytic activities of calpain and caspase-3.

It is well-known that CDK2 plays an important role for the G_1 to S transition in cell cycle, whereas $p27^{kip1}$ is an endogenous inhibitor of cell cycle as it binds to CDK/cyclin complexes for preventing the G_1 to S transition [3, 12]. We evaluated the expression of $p27^{kip1}$ and CDK2 after the treatments and discovered that all treatments increased the expression of $p27^{kip1}$ and decreased expression of CDK2 in both LN18 and U87MG cells (Fig. 7). Notably, other investigators also reported that PTEN had similar effects for arresting the cell cycle in glioblastoma cells [3, 10, 23].

In conclusion, this study demonstrated that the combination of ATRA and IFN-γ could work synergistically to cause cell cycle arrest and thereby induce differentiation and apoptosis in both PTEN-proficient and PTEN-deficient glioblastoma cells. Therefore, the synergistic effect of this combination therapy per se could be further explored for growth inhibition of heterogeneous populations of human glioblastoma cells.

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References

1. Adachi J, Ohbayashi K, Suzuki T, Sasaki T (1999) Cell cycle arrest and astrocytic differentiation resulting from PTEN expression in glioma cells. J Neurosurg 91:822–830
2. Asou N (2007) All-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. Intern Med 46:91–93
3. Brandts CH, Bilanges B, Hare G, McCormick F, Stokoe D (2005) Phosphorylation-independent stabilization of $p27^{kip1}$ by the phosphoinositide-3-kinase pathway in glioblastoma cells. J Biol Chem 280:2012–2019
4. Das A, Banik NL, Patel SJ, Ray SK (2004) Dexamethasone protected human glioblastoma U87MG cells from temozolomide

induced apoptosis by maintaining Bax:Bcl-2 ratio and preventing proteolytic activities. Mol Cancer 3(1):36
5. Das A, Banik NL, Ray SK (2006) Mechanism of apoptosis with the involvement of calpain and caspase cascades in human malignant neuroblastoma SH-SY5Y cells exposed to flavonoids. Int J Cancer 119:2575–2585
6. Eng C (2003) PTEN: one gene, many syndromes. Hum Mutat 22:183–198
7. Fax X, Aalto Y, Sanko SG, Knuutila S, Klatzmann D, Castresana JS (2002) Genetic profile, PTEN mutation and therapeutic role of PTEN in glioblastomas. Int J Oncol 21:1141–1150
8. Garzon R, Pichiorri F, Palumbo F, Visentini M, Aqueilan R, Cimmino A, Wang H, Sun H, Volinia S, Alder H, Calin GA, Liu CG, Andreeff M, Croce CM (2007) MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene 29:1–10
9. Haque A, Das A, Hajiaghamohseni LM,Younger A, Banik NL (2007) Induction of apoptosis and immune response by all-*trans* retinoic acid plus interferon-gamma in human malignant glioblastoma T98G and U87MG cells. Cancer Immunol Immunother 56:615–625
10. Hwang PH, Yi HK, Kim DS, Nam SY, Kim JS, Lee DY (2001) Suppression of tumorigenicity and metastasis in B16F10 cells by PTEN/MMAC1/TEP1 gene. Cancer Lett 172:83–91
11. Jendrossek V, Belka C, Bamberg M (2003) Novel chemotherapeutic agents for the treatment of glioblastoma multiforme. Expert Opin Investig Drugs 12:1899–1924
12. Jonason JH, Gavrilova N, Wu M, Zhang H, Sun H (2007) Regulation of SCF (SKP2) ubiquitin E3 ligase assembly and $p27^{kip1}$ proteolysis by the PTEN pathway and cyclin D1. Cell Cycle 6:951–961
13. Karmakar S, Banik NL, Patel SJ, Ray SK (2007) Garlic compounds induced calpain and intrinsic caspase cascade for apoptosis in human malignant neuroblastoma SH-SY5Y cells. Apoptosis 12:671–684
14. Kim JS, Lee C, Bonifant CL, Ressom H, Waldman T (2007) Activation of p53-dependent growth suppression in human cells by mutations in PTEN or PIK3CA. Mol Cell Biol 27:662–677
15. Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK (2002) The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol 61:215–225
16. Lee YR, Yu HN, Noh EM, Kim JS, Song EK, Han MK, Kim BS, Lee SH, Park J (2007) Peroxisome proliferator-activated receptor gamma and retinoic acid receptor synergistically upregulate the tumor suppressor PTEN in human promyeloid leukemia cells. Int J Hematol 85:231–237
17. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943–1947
18. Nath R, Raser KJ, Stafford D, Hajimohammadreza I, Posner A, Allen H, Talanian RV, Yuen P, Gilbertsen RB, Wang KK (1996) Non-erythroid α-spectrin breakdown by calpain and interleukin-1β-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. Biochem J 319:683–690
19. Parney IF, Chang SM (2003) Current chemotherapy for glioblastoma. Cancer J 9:149–156
20. Pasquali D, Chieffi P, Deery WJ, Nicoletti G, Bellastella A, Sinisi AA (2005) Differential effects of all-*trans* retinoic acid (RA) on Erk1/2 phosphorylation and cAMP accumulation in normal and malignant human prostate epithelial cells: Erk1/2 inhibition restores RA-induced decrease of cell growth in malignant prostate cells. Eur J Endocrinol 152:663–669
21. Pique M, Barragan M, Dalmau M, Bellosillo B, Pons G, Gil J (2000) Aspirin induces apoptosis through mitochondrial cytochrome c release. FEBS Lett 480:193–196
22. Ray SK, Wilford GG, Crosby CV, Hogan EL, Banik NL (1999) Diverse stimuli induce calpain overexpression and apoptosis in C6 glioma cells. Brain Res 829:18–27
23. Shingu T, Yamada K, Hara N, Moritake K, Osago H, Terashima M, Uemura T, Yamasaki T, Tsuchiya M (2003) Growth inhibition of human malignant glioma cells induced by the PI3K-specific inhibitor. J Neurosurg 98:154–161
24. Simeone AM, Tari AM (2004) How retinoids regulate breast cancer cell proliferation and apoptosis. Cell Mol Life Sci 61:1475–1484
25. Soprano KJ, Purev E, Vuocolo S, Soprano DR (2006) Rb2/p130 and protein phosphatase 2A: key mediators of ovarian carcinoma cell growth suppression by all-*trans* retinoic acid. Oncogene 25:5315–5325
26. Stewart LA (2002) Chemotherapy in adult high-grade glioma: a systemic review and meta-analysis of individual patient data from 12 random trials. Lancet 359:1011–1018
27. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO; European Organisation for Research, Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group (2005) Radiodynamic therapy plus concomitant and adjuvant temozolomide for glioblastoma. N Eng J Med 352:987–996
28. Wang KK, Posmantur R, Nath R, McGinnis K, Whitton M, Talanian RV, Glantz SB, Morrow1 JS (1998) Simultaneous degradation of αII- and βII-spectrin by caspase-3 (CPP32) in apoptotic cells. J Biol Chem 273:22490–22497
29. Wong ML, Kaye AH, Hovens AM (2007) Targeting malignant glioma survival signaling to improve clinical outcomes. J Clin Neurosci 14:301–308
30. Zang C, Wachter M, Liu H, Posch MG, Fenner MH, Stadelmann C, von Deimling A, Possinger K, Black KL, Koeffler HP, Elstner E (2003) Ligands for PPARγ and RAR cause induction of growth inhibition and apoptosis in human glioblastomas. J Neurooncol 65:107–118
31. Zhang R, Banik NL, Ray SK (2007) Combination of all-*trans* retinoic acid and interferon-gamma suppressed PI3K/Akt survival pathway in glioblastoma T98G cells whereas NF-κB survival signaling in glioblastoma U87MG cells for induction of apoptosis. Neurochem Res (in press)
32. Zheng A, Castren K, Saily M, Savolainen ER, Koistinen P, Vahakangas K (1999) p53 status of newly established acute myeloid leukaemia cell lines. Br J Cancer 79:407–415