

# Notch-1 activation-dependent p53 restoration contributes to resveratrol-induced apoptosis in glioblastoma cells

HONG LIN\*, WEI XIONG\*, XIANG ZHANG, BOLIN LIU, WEI ZHANG,  
YONGQIANG ZHANG, JINXIANG CHENG and HUIYONG HUANG

Department of Neurosurgery, Xijing Institute of Clinical Neuroscience,  
Xijing Hospital, Fourth Military Medical University, 710032 Xi'an, P.R. China

Received April 27, 2011; Accepted June 11, 2011

DOI: 10.3892/or.2011.1380

**Abstract.** Glioblastoma is the most malignant form of adult brain tumor and is associated with a dismal prognosis. Emerging data suggest that Notch signaling participates principally in the formation and malignant progression of glioblastoma. Resveratrol is a terpenoid that exhibits broad pro-apoptotic activity in various types of cancers, including glioblastoma. However, the effects of resveratrol on Notch signaling in glioblastomas have not yet been fully elucidated. We demonstrated that resveratrol strongly suppressed cell growth and induced apoptosis in A172 and T98G glioblastoma cells, which have low active Notch-1 expression and a heterozygous p53 mutation. Our results suggest that resveratrol significantly activates intracellular Notch-1 and restores wild-type p53 expression in a time-dependent manner. Significant de-phosphorylation of Akt, increased Bax expression, decreased Bcl-2 expression and cleavage of caspase-3 were also observed in resveratrol-induced apoptosis in glioblastoma cells. Moreover, simultaneous treatment with resveratrol and a Notch-1 inhibitor (MRK-003) partially attenuated the apoptosis and completely blocked the activation of Notch-1 and the increase in wild-type p53. This suggests that restoration of wild-type p53 expression depends on Notch-1 activation. In addition, the de-phosphorylation of Akt, increased expression of Bax and cleavage of caspase-3 were not fully reversed by MRK-003 treatment, suggesting that p53 restoration is not the only mechanism underlying resveratrol-induced apoptosis. Taken together, we confirmed the anti-proliferative

and pro-apoptotic effects of resveratrol on glioblastoma cells and revealed Notch-1 activation-dependent restoration of p53 as an important causative mechanism.

## Introduction

Glioblastoma multiforme (GBM, WHO IV) is the most common adult intracranial malignancy which is characterized by rapid tumor proliferation and a strong tendency to diffuse and invade surrounding normal brain tissue (1). Despite ongoing improvements in conventional therapeutic regimens, including selective surgical resection and concurrent radiation, the prognosis for glioblastoma patients remains extremely dismal (2). The median survival and median progression-free survival for glioblastoma patients who received radiotherapy alone are 12 and 5 months, respectively (3). Concomitant and adjuvant chemotherapies have been shown to improve the progression and quality of life of glioblastoma patients; however, many glioblastoma patients do not benefit from these clinical interventions due to their minimal responses to anti-cancer agents that promote apoptosis (type I-programmed cell death). Therefore, identification of the genetic characteristics of glioblastomas that contribute to this low response is critical, as is the development of novel therapeutic agents and adjuvants that can effectively induce substantial apoptosis in glioblastoma cells (4).

Emerging evidence suggests that tumor development and progression result from aberrant regulation of oncogenes and cancer-suppressor genes. With respect to genetic alterations, glioblastoma cells are known to exhibit aberrant expression of Notch-1 (5). Since it has been documented that overexpression of Notch-1 is fundamental for promoting malignant progression and maintaining the self-renewal potential in various types of cancers, its potential role in the development and oncobiology of glioblastoma is intriguing. Previous studies have revealed that the Notch-1 protein gradually increases in gliomas according to pathological grade as compared to non-neoplastic brain tissue (6). Notch-1 expression in glioblastomas is lower than that in low-grade gliomas (7). However, its activated fragment, Notch-1 receptor intracellular domain (Notch-1<sup>ICD</sup>) and ligands, Jagged 1 (JAG1) and Delta-like 1 (DLL1), are endogenously expressed in glioblastomas (8). Given that Notch-1 activation depends on cell type and

---

*Correspondence to:* Dr Xiang Zhang, Department of Neurosurgery, Xijing Institute of Clinical Neuroscience, Xijing Hospital, Fourth Military Medical University, 710032 Xi'an, P.R. China  
E-mail: xiangzhang\_fmму@126.com

\*Contributed equally

**Abbreviations:** IRS, immunoreactivity scores; Notch-1<sup>ICD</sup>, Notch-1 receptor intracellular domain

**Key words:** glioblastoma, resveratrol, Notch-1, p53, apoptosis

context, the exact role of Notch-1 signals in the response of glioblastoma cells to pro-apoptotic agents cannot be easily predicted.

Tumor protein 53 (p53) plays a primary role in controlling carcinogenesis and helps to determine the response of cancer cells. Its tumor-suppressor function has been well documented in a number of epidemiological investigations and experimental studies in past years. The intracellular function of p53 is decreased by mutations or post-translational inactivation. Attenuation of functional p53 enables the translation and activation of many pro-survival genes, such as protein kinase B (PKB/Akt), which promote the development of a malignant phenotype and apoptotic resistance in cancer cells (9). Low levels of functional p53 are characteristic of genetic alterations in several cancer types including glioblastoma (10). Immunohistochemical studies suggest that nearly half of the p53-positive cells in glioblastomas show high levels of p53 in the cytoplasm, where it is inactive. Cytoplasmic retention and inactivation predominantly account for the low levels of functional p53 in glioblastoma cells (11).

Resveratrol (trans-3,4',5-trihydroxystilbene) is a phenolic compound extracted from *Polygonum cuspidatum* and red grape skin. Resveratrol exhibits anti-oxidant and anti-cancer activities and has been used to experimentally treat several types of cancers (12,13). Previous studies have revealed that resveratrol inhibits tumor cell growth, arrests the cell cycle and induces apoptosis in glioblastoma U251MG and C6 cells (14). However, more studies are required to investigate the underlying mechanisms before resveratrol can be used in clinical practice.

The present study confirms resveratrol-induced apoptosis in human glioblastoma A172 and T98G cells. The expression and activation of Notch-1, wild-type p53, p-Akt, Bax and Bcl-2 were analyzed to determine the genetic features of glioblastoma cells and the molecular mechanisms by which resveratrol exerts its anti-cancer activity in this cell type.

## Materials and methods

**Agents.** Resveratrol (Santa Cruz Biotechnology, Santa Cruz, CA) was dissolved in dimethyl sulfoxide (DMSO) (Gibco/Invitrogen, NY, USA) to produce a 100-mM stock solution. Before each experiment, a resveratrol stock solution was dissolved in fresh Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, USA) to obtain 50 and 100  $\mu$ M working solutions. The Notch-1 inhibitor MRK-003 (BioSun Sci & Tech Co., Ltd., Shanghai, China) and a  $\gamma$ -secretase inhibitor were dissolved in DMSO and then added to the culture medium to obtain working solutions (5  $\mu$ M). The final DMSO concentration used in the working solutions did not exceed 1‰ (v/v), so cell growth was not affected.

**Cell lines and cell culture.** Human glioblastoma cell lines (A172 and T98G; cell bank at the Fourth Military Medical University, China) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (BioSun Sci & Tech Co., Ltd.) in a 37°C incubator with a humidified atmosphere of 5% CO<sub>2</sub>-95% O<sub>2</sub>. Primary cultured astrocytes were obtained from a brain tissue fragment from an informed and consenting volunteer with cerebral trauma under the approval of the local

medical research ethics committee. Twenty-four hours before each experiment, cells were transferred to serum-free medium. Working solutions of resveratrol were then added in place of the culture medium.

**MTT assay.** Cell viability was determined by the methyl thiazolyl tetrazolium (MTT) assay. Briefly, A172 and T98G glioblastoma cells and primary-cultured astrocytes were cultured in different 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of  $5 \times 10^3$  cells/well. For the treatment groups, resveratrol was added to the culture medium at final concentrations of 50 and 100  $\mu$ M. In the Notch-1 inhibition experiments, MRK-003 (5  $\mu$ M) was added to the culture medium in combination with resveratrol (50  $\mu$ M). After incubating the cells for 24 or 72 h, MTT (Sigma), dissolved in PBS (0.01 M, pH 7.4), was added to each well at a final concentration of 5 mg/ml. All experiments were performed in triplicate.

**Hoechst 33342 nuclear staining.** Briefly, cells ( $2 \times 10^4$ ) were plated in 6-well plates and treated with resveratrol (50  $\mu$ M) for 24 h. Cells were then washed in PBS (0.01 M, pH 7.4) and fixed in 70% ethanol for 2 h at 4°C. Cell nuclei were stained with Hoechst 33342 (5  $\mu$ g/ml; Sigma). After a final wash in PBS, changes in nuclear morphology were visualized by fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) using excitation wavelengths between 330 and 380 nm.

**Annexin V/propidium iodide.** To estimate the apoptosis induced by resveratrol, we performed flow cytometric analyses as previously described (15). Briefly, cells ( $1 \times 10^5$ ) were plated in a cell culture flask and were treated with resveratrol (50  $\mu$ M) for 24 h. For the Notch-1 inhibition experiments, MRK-003 (5  $\mu$ M) was added to the culture medium in combination with resveratrol (50  $\mu$ M).

**Western blotting.** Western blot analyses were performed as previously described (15). Equivalent amounts (25  $\mu$ g) of protein lysates were separated in each cell line. The following primary antibodies were used: anti-Notch-1 (diluted 1:300, mouse monoclonal), anti-Akt-1 (diluted 1:1000, mouse monoclonal), anti-p-Akt-1 (Ser 473, diluted 1:300, rabbit polyclonal), anti-p53 (C-11, diluted 1:300, mouse monoclonal), anti-Bax (diluted 1:600, rabbit polyclonal), anti-Bcl-2 (diluted 1:600, rabbit polyclonal), anti-cleaved caspase-3 p11 (diluted 1:200, goat polyclonal) and anti- $\beta$ -actin (diluted 1:1000, mouse monoclonal). The following secondary antibodies were used: HRP-conjugated anti-goat IgG (diluted 1:2000), HRP-conjugated anti-rabbit IgG (diluted 1:2000) and HRP-conjugated anti-mouse IgG (diluted 1:2000). All antibodies were purchased from Santa Cruz Biotechnology.

**Immunocytochemistry.** Immunocytochemistry and evaluation of immunoreactivity scores (IRS) for nuclear p53 were performed as previously described (16). A172 and T98G cells were treated with or without resveratrol (50  $\mu$ M) for 24 h and cultured on glass slides. The primary antibody was anti-p53 mouse monoclonal antibody (1:50), and the secondary antibody was biotinylated goat anti-mouse IgG (1:50). Both antibodies were purchased from Santa Cruz Biotechnology.

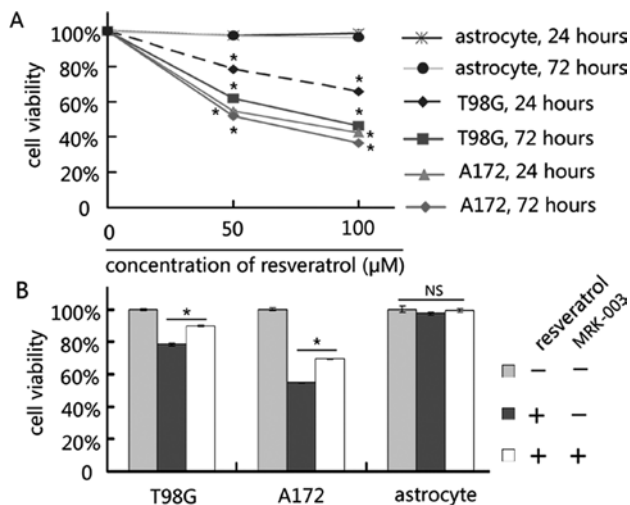


Figure 1. Effects of resveratrol on glioblastoma and astrocyte cell viabilities. (A) Resveratrol inhibited cell viability in glioblastoma A172 and T98G cells in a concentration-dependent manner but rarely affected cell viability in astrocytes. (B) The Notch-1 inhibitor MRK-003 (5  $\mu$ M) significantly attenuated the resveratrol (50  $\mu$ M)-induced inhibitory effect on glioblastoma A172 and T98G cell viabilities.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard error of the mean (SEM) of separate experiments. All data were tested for significance by one-way analysis of variance (ANOVA) followed by Fisher's post hoc test using SPSS 13.0 software (IBM SPSS, Chicago, IL, USA).  $p < 0.05$  was considered statistically significant.

## Results

**Resveratrol suppresses the cell viability of glioblastoma cells but does not affect primary cultured astrocytes.** The cell viability and proliferative capacity of glioblastoma A172 and T98G cells were significantly decreased by resveratrol treatment in a concentration-dependent manner. The inhibitory effect was more pronounced in A172 cells than in T98G cells. Exposure to resveratrol (100  $\mu$ M) for 24 h decreased A172 and T98G cell viabilities to  $42.3 \pm 0.4$  and  $65.8 \pm 0.6\%$  compared to the vehicle-treated controls, respectively (Fig. 1A). Resveratrol treatment did not affect cell viability in primary cultured astrocytes. Simultaneous inhibition of Notch-1 activation via MRK-003 treatment partially rescued the resveratrol-induced inhibition of glioblastoma cell growth. While exposure to resveratrol (50  $\mu$ M, 24 h) treatment alone reduced A172 and T98G cell viabilities to  $54.7 \pm 0.8$  and  $78.4 \pm 0.6\%$ , respectively, treatment with MRK-003 (5  $\mu$ M) significantly attenuated the resveratrol (50  $\mu$ M)-induced inhibitory effect to just  $69 \pm 0.5$  and  $90 \pm 0.4\%$ , respectively (Fig. 1B). Simultaneous MRK-003 (5  $\mu$ M) and resveratrol (50  $\mu$ M) treatment for 24 h did not affect astrocyte cell growth.

**Resveratrol induces apoptosis in glioblastoma cells.** After treatment with resveratrol (50  $\mu$ M) for 24 h, glioblastoma cells experienced apoptotic morphological alterations. Many glioblastoma A172 cells had disrupted cytoskeletons, which were round and contracted, and the residual viable cells displayed shortened and narrowed synapses (Fig. 2A).

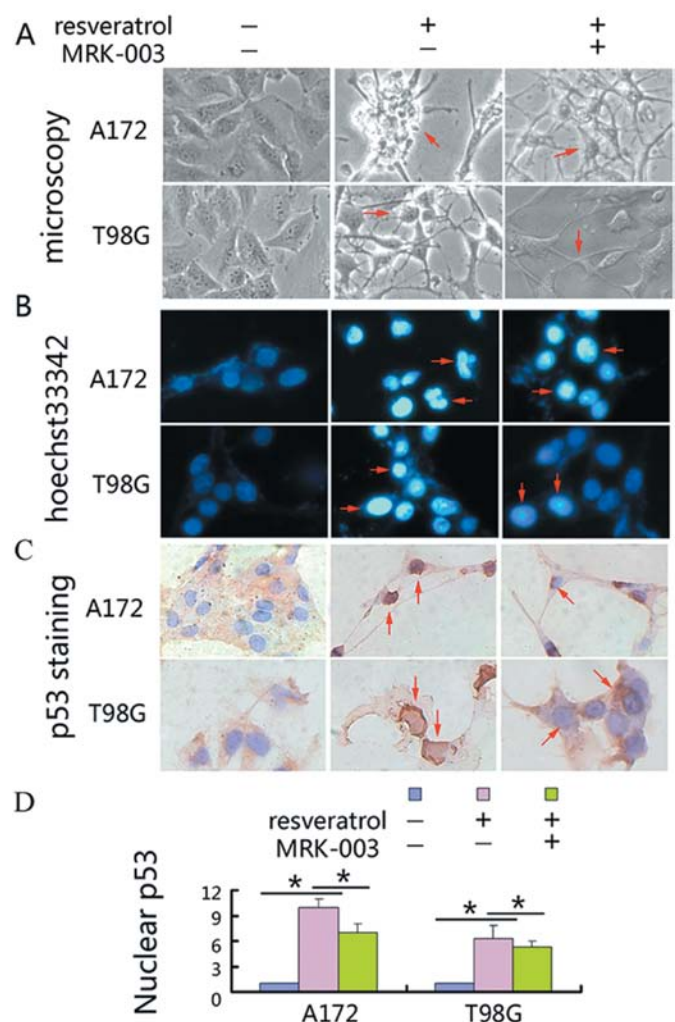


Figure 2. (A and B) Effects of resveratrol (50  $\mu$ M, 24 h) on cellular and nuclear structures in glioblastoma cells. (A) Resveratrol shortened and narrowed neurites and induced contraction and condensation in glioblastoma A172 and T98G cells. Co-treatment with the Notch-1 inhibitor MRK-003 (5  $\mu$ M) decreased the number of apoptotic glioblastoma cells and ameliorated their morphological alterations. (B) Resveratrol treatment induced condensed and bud-forming apoptotic nuclei and increased the dipter in glioblastoma cells. MRK-003 (5  $\mu$ M) lessened the morphologic alterations of the apoptotic nuclei. (C and D) Effects of resveratrol (50  $\mu$ M, 24 h) on p53 expression and nuclear location. (C) Resveratrol significantly increased p53 expression and nuclear localization in glioblastoma A172 and T98G cells. The Notch-1 inhibitor MRK-003 (5  $\mu$ M) partially inhibited these effects. (D) Immunoreactivity scores for nuclear p53 in glioblastoma cells treated with or without resveratrol.

Morphological alterations in the T98G cells were similar to those of the A172 cells; however, the affected population was smaller and the degree was not as severe. Hoechst 33342 nuclear staining revealed that resveratrol exposure significantly condensed the nucleus in both glioblastoma cell lines (Fig. 2B).

Flow cytometric analyses were performed using an Annexin V-FITC/PI staining kit to analyze the apoptosis induced by resveratrol. Treatment with resveratrol (50  $\mu$ M) for 24 h induced apoptosis in  $34 \pm 3.4$  and  $21 \pm 2.8\%$  of the A172 and T98G cells, respectively. In the Notch-1 inhibition experiments, treatment with both resveratrol (50  $\mu$ M) and MRK-003 (5  $\mu$ M) induced apoptosis in only  $17 \pm 1.6$  and  $12 \pm 2.4\%$  of the A172 and T98G cells, respectively (Fig. 3A).

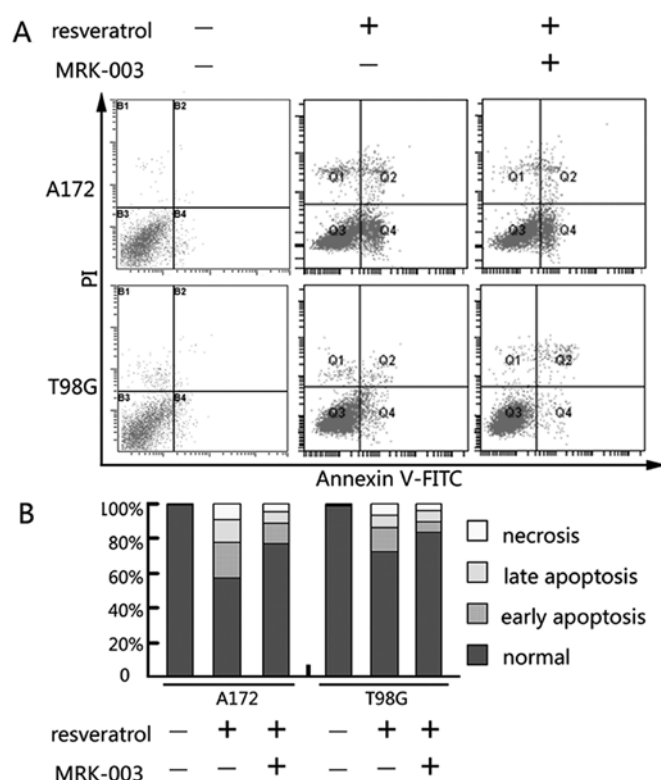


Figure 3. Flow cytometric analysis. (A) Resveratrol (50  $\mu$ M, 24 h) induced pronounced apoptosis in glioblastoma A172 and T98G cells. The Notch-1 inhibitor MRK-003 (5  $\mu$ M) partially rescued this apoptosis. (B) Resveratrol significantly induced early and late apoptosis in both glioblastoma cell lines. Simultaneous MRK-003 (5  $\mu$ M) treatment reduced the number of cells exhibiting early and late apoptosis.

This suggests that inhibition of Notch-1 activation significantly attenuates resveratrol-induced apoptosis (Fig. 3B).

**Resveratrol promotes nuclear translocation of p53 in A172 and T98G cells.** To determine whether p53 plays a functional role in the response to resveratrol exposure, the subcellular distribution of the p53 protein in glioblastoma cells was investigated by immunocytochemistry (Fig. 2C and D). Weak p53 expression was observed mainly in the cytoplasm of the vehicle-treated glioblastoma A172 and T98G cells. After resveratrol treatment (50  $\mu$ M) for 24 h, p53 expression was significantly increased in both glioblastoma cell lines. Data indicated that p53 accumulated in the nucleus of apoptotic glioblastoma cells (IRS =  $10 \pm 1$  in A172 cells; IRS =  $6.33 \pm 1.45$  in T98G cells). These results suggested that p53 function is restored in resveratrol-induced apoptosis. In addition, simultaneous MRK-003 treatment significantly rescued the nuclear accumulation of p53 in both cell types (IRS =  $7 \pm 1$  in A172 cells; IRS =  $5.33 \pm 0.67$  in T98G cells).

**Resveratrol increases active Notch-1 and p53 expression in glioblastoma cells.** To further explore the molecular mechanisms underlying the effect of resveratrol on glioblastoma cells, we quantified changes in the expression levels of active Notch-1 and p53 during treatment. After treatment with resveratrol (50  $\mu$ M) for 24 h, expression of both active Notch-1 and p53 was significantly increased (Fig. 4A).

Western blot analysis revealed that the expression level of the Notch-1 intracellular domain (Notch-1<sup>ICD</sup>, functional form of Notch-1) was 3.2-fold ( $p=0.001$ ) and 2.9-fold ( $p=0.001$ ) higher in the resveratrol-treated A172 and T98G cells, respectively, compared to the vehicle-treated controls (Fig. 4B). Expression of p53 was 1.2-fold ( $p=0.04$ ) and 1.1-fold ( $p=0.03$ ) higher in the resveratrol-treated A172 and T98G cells, respectively, compared to the vehicle-treated controls. In addition, exposure to MRK-003 completely blocked the expression of Notch-1<sup>ICD</sup> and significantly reversed the increase in p53 expression. These results suggest that activation of the Notch-1 pathway and enhanced expression of p53 occur during resveratrol-induced apoptosis in glioblastoma cells.

**Resveratrol decreases Akt-1 phosphorylation and Bcl-2 expression and increases Bax expression in glioblastoma cells.** Akt-1 was significantly de-phosphorylated in the resveratrol-treated glioblastoma cells compared to the vehicle-treated controls (Fig. 4A). As early as 12 h after treatment with resveratrol (50  $\mu$ M), phosphorylated Akt-1 (Ser 473) protein expression began to decrease; it remained low for the rest of the experiment. Moreover, pro-apoptotic Bax protein expression was up-regulated, and the pro-survival Bcl-2 protein expression was down-regulated in resveratrol-induced apoptosis. A colorimetric assay was then performed using BandScan 4.3 software (Glyko, Inc., USA), with protein levels standardized to the  $\beta$ -actin loading control. The p-Akt-1/ $\beta$ -actin ratio was reduced to 17.2 and 35.8% in the A172 and T98G cells, respectively, after treatment with resveratrol (50  $\mu$ M) for 24 h. In addition, resveratrol exposure reduced the Bcl-2/ $\beta$ -actin ratio to 18.7 and 26.4% in the A172 and T98G cells, respectively. Resveratrol increased the Bax/ $\beta$ -actin ratio to 523 and 636% in the A172 and T98G cells, respectively. The p-Akt-1/ $\beta$ -actin, Bcl-2/ $\beta$ -actin and Bax/ $\beta$ -actin ratios of the vehicle-treated control cells were set to 100%.

**Resveratrol induces cleavage and activation of caspase-3 in glioblastoma cells.** Cleavage and activation of caspase-3 is one of the most important mechanisms of apoptosis. An increase in the active 11-kDa caspase-3 fragment was noted in the resveratrol-treated glioblastoma cells (Fig. 4A). Western blot analysis indicated that in response to resveratrol treatment, pro-caspase-3 became cleaved and produced active fragments within 24 h. The colorimetric assay indicated that the cleaved caspase-3/ $\beta$ -actin ratio increased by 460 and 338% in the A172 and T98G cells, respectively. The cleaved caspase-3/ $\beta$ -actin ratio of the vehicle-treated control cells was set to 100%.

## Discussion

Our results confirmed the therapeutic potential of resveratrol in glioblastoma A172 and T98G cells. Treatment with resveratrol (50-100  $\mu$ M) significantly inhibited cell viability and induced apoptosis in both glioblastoma cell lines within 24 h. Resveratrol treatment also resulted in an increased expression of active Notch-1 in both cell lines. Furthermore, increased expression and nuclear translocation of wild-type p53, de-phosphorylation of Akt-1, up-regulated expression of the pro-apoptotic Bax protein and down-regulated expression of the pro-survival Bcl-2 protein were also demonstrated. In

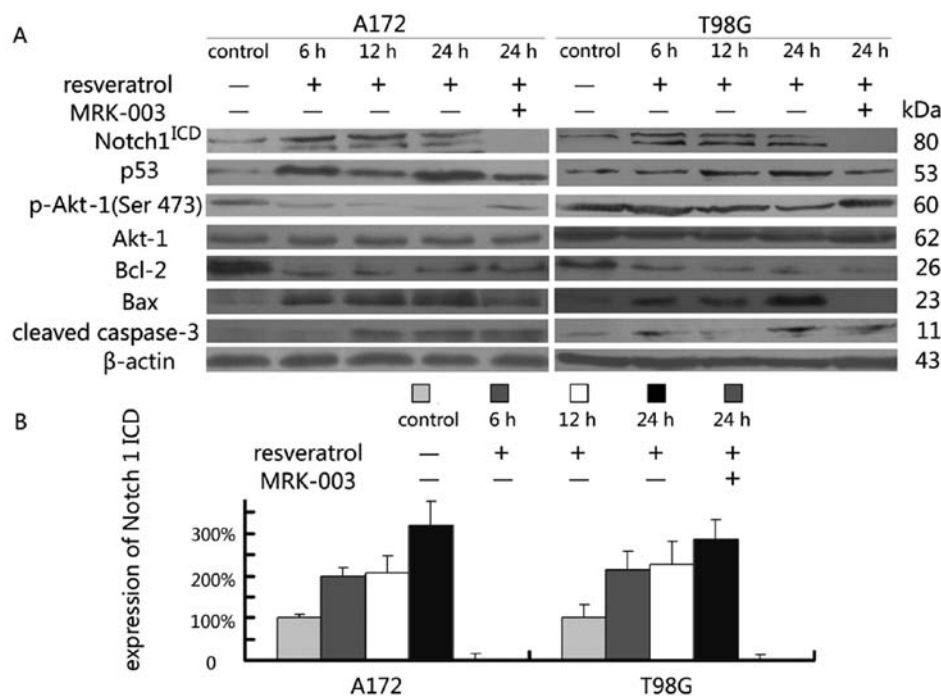


Figure 4. Effects of resveratrol (50  $\mu$ M, 24 h) on protein expression profiles in glioblastoma A172 and T98G cells. (A) Western blot analysis. (B) Relative expression of the Notch-1 intracellular domain (Notch-1<sup>ICD</sup>) in glioblastoma cells treated with or without resveratrol.

addition, MRK-003 significantly blocked Notch-1 activation and partially reversed resveratrol-induced apoptosis. Taken together, these data suggest that Notch-1 activation and restoration of functional p53 play critical roles in the effect of resveratrol on glioblastoma.

The polyphenolic resveratrol is a natural product which has attracted much interest for its use as a therapy for multiple cancer types as it has promising growth-inhibitory potency in cancer cells and rarely harms the surrounding normal parenchyma. Previous studies have suggested that, at therapeutic doses, resveratrol has minimal cytotoxic effects and is protective in neurons, human lens epithelial cells, human endothelium and mouse fibroblast 3T3 cells (14). Our study also found that resveratrol did not affect the cell viability of primary cultured astrocytes, which are substantially distributed in normal brain parenchyma. However, despite the anti-cancer properties and biologic safety of resveratrol, its functional mechanisms are still not fully understood.

Research in the past two decades has revealed that resveratrol induces apoptosis in cancer cells through dually regulating pro-survival oncogenes and pro-apoptotic cancer-suppressor genes. On the one hand, resveratrol causes loss of mitochondrial membrane potential, release of cytochrome C, and activation of pro-apoptotic proteins such as Bax and caspases; however, it also antagonizes pro-survival proteins such as Bcl-2. Our results are concordant with this research and demonstrate that resveratrol treatment down-regulates Bcl-2 expression and up-regulates Bax expression. In addition, previous studies have also suggested that modulation of Notch signaling is an important mechanism in the anti-cancer effect of resveratrol. Resveratrol affects Notch signaling at the transcriptional and post-transcriptional levels (affecting the proteolytic cleavage-dependent nuclear translocation and activation). It has been

shown that resveratrol induces Notch-2 mRNA expression and promotes profound growth inhibition and apoptosis in medullary thyroid cancer cells (17) and human GI carcinoid BON cells (8). It has also been shown that resveratrol represses Notch-1 activation in human T-cell acute lymphoblastic leukemia MOLT-4 cells (19).

Dysregulated Notch signaling has been shown to participate in the apoptosis-survival switch in certain cancer cells. In glioblastoma, activation of Notch-1 has been suggested to predict poor prognosis in glioblastoma patients (20). Inhibition of Notch-1 signaling by RNA interference (RNAi) or  $\gamma$ -secretase inhibitors (GSIs) experimentally blocks cell growth and induces apoptosis in glioblastoma cells and inhibits repopulation and tumorigenesis in glioblastoma-derived neurospheres. Furthermore, Notch-1 inhibition also leads to enhanced chemosensitivity of glioblastoma cells to temozolomide treatment (21). However, strengthened activation of Notch-1 in glioblastoma A172 and T98G cells was noted during resveratrol-induced apoptosis, evidenced by increased expression of Notch-1<sup>ICD</sup>. To investigate whether this increase in Notch-1 signaling acts as an initiating factor in resveratrol-induced apoptosis or as a self-protective event to antagonize resveratrol stimulation, A172 and T98G cells were treated with resveratrol and the GSI MRK-003 simultaneously. This treatment completely blocked the activation of Notch-1 and partially rescued the apoptosis induced by resveratrol in both A172 and T98G cells, suggesting a critical role for Notch-1 activation in resveratrol-induced apoptosis. Prior studies have demonstrated a close association between Notch-2 activation and apoptosis and found that Notch-1 signaling minimally contributes to apoptosis in glioblastoma cells (6). The present results suggest an alternative mechanism for resveratrol-induced apoptosis in glioblastoma cells. In our preliminary



study, we investigated the activation of Notch-1 signaling in five untreated glioblastoma cell lines. A172 and T98G cells have less active Notch-1 than SHG44, U87MG and U251MG cells (data not shown). Glioblastomas are genetically heterogeneous, and the expression of Notch receptors and ligands differs between individual glioblastoma cell lines. Therefore, it is possible that the contributions of Notch-1 and -2 signaling to cell growth and apoptosis primarily depend on their expression status. Given that A172 cells are more sensitive to resveratrol treatment than T98G cells, we concluded that glioblastoma cells with less active Notch-1 are more susceptible to resveratrol. However, since we measured changes in Notch-1 activation in only two glioblastoma cell lines, the association between resveratrol-induced apoptosis and Notch-1 activation should be confirmed in more glioblastoma cell lines and primary glioblastoma cultures from clinical specimens with less active Notch-1.

Since p53 is one of the major cancer-suppressor genes and its restoration is an important mechanism for various anti-cancer agents, p53 expression and activation were investigated in resveratrol-treated glioblastoma cells. This study suggests that resveratrol significantly increases intracellular p53 content and promotes its nuclear translocation. Our results indicate that the restoration and activation of p53 are important events in resveratrol-induced apoptosis. To investigate the possible association between Notch-1 activation and p53 restoration, p53 expression and activation were compared in glioblastoma cells treated with or without the Notch-1 inhibitor MRK-003 in combination with resveratrol. Simultaneous MRK-003 treatment slightly decreased p53 restoration in both A172 and T98G cells and significantly inhibited the nuclear translocation of p53, especially in A172 cells. These data suggest that Notch-1 activation can augment p53 expression and restore p53 function in glioblastoma A172 and T98G cells. Activation of the Notch-1-p53 signaling pathway appears to be an initiating factor in resveratrol-induced apoptosis, with augmented Bax expression and decreased Bcl-2 expression promoting the activation of the caspase cascade and eventual apoptosis.

The present study suggests that resveratrol induces apoptosis in a sub-population of glioblastoma cells that have mutated p53 and express less active p53 by activating Notch-1. This novel mechanism differs from prior notions that i) induction of Notch-2, not Notch-1, plays a dominant role in the induction of apoptosis in glioblastoma cells by anti-cancer agents; and ii) inhibition but not induction of Notch-1 signaling represents a unique mechanism for inhibiting glioblastoma cell growth and promoting apoptosis. Our results suggest that the activated p53 protein, which was reinstated by Notch-1 activation, plays an important mediator in resveratrol-induced apoptosis in glioblastoma cells by up-regulating Bax expression, down-regulating Bcl-2 expression, de-phosphorylating Akt-1 and activating caspase-3. Taken together, we demonstrated the chemotherapeutic potential of resveratrol in glioblastomas and highlighted the importance of evaluating gene expression profiles prior to selecting a chemotherapeutic strategy. Further investigations are needed to improve our understanding of Notch signaling in glioblastomas and to determine the mechanisms underlying the anti-cancer activity of resveratrol.

## Acknowledgements

The authors would like to thank Dr Xuewu Liu of the Department of Biochemistry, Fourth Military Medical University, China for helpful discussions.

## References

1. Khasraw M and Lassman AB: Advances in the treatment of malignant gliomas. *Curr Oncol Rep* 12: 26-33, 2010.
2. Jahraus CD and Friedman AH: Chemopotentiality by ultra-fractionated radiotherapy in glioblastoma resistant to conventional therapy. *Tumori* 96: 771-775, 2010.
3. Stupp R, Mason WP, van den Bent MJ, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352: 987-996, 2005.
4. Quant EC and Wen PY: Novel medical therapeutics in glioblastomas, including targeted molecular therapies, current and future clinical trials. *Neuroimaging Clin N Am* 20: 425-448, 2010.
5. Kanamori M, Kawaguchi T, Nigro JM, *et al*: Contribution of Notch signaling activation to human glioblastoma multiforme. *J Neurosurg* 106: 417-427, 2007.
6. Lino MM, Merlo A and Boulay JL: Notch signaling in glioblastoma: a developmental drug target? *BMC Med* 8: 72, 2010.
7. Margareto J, Leis O, Larrarte E, Idoate MA, Carrasco A and Lafuente JV: Gene expression profiling of human gliomas reveals differences between GBM and LGA related to energy metabolism and Notch signaling pathways. *J Mol Neurosci* 32: 53-63, 2007.
8. Li JL, Sainson RC, Shi W, *et al*: Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo. *Cancer Res* 67: 11244-11253, 2007.
9. Soussi T and Wiman KG: Shaping genetic alterations in human cancer: the p53 mutation paradigm. *Cancer Cell* 12: 303-312, 2007.
10. Zhang WB, Wang Z, Shu F, *et al*: Activation of AMP-activated protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p53 activation and mTORC1 inhibition. *J Biol Chem* 285: 40461-40471, 2010.
11. Berger B, Capper D, Lemke D, *et al*: Defective p53 antiangiogenic signaling in glioblastoma. *Neuro Oncol* 12: 894-907, 2010.
12. Schneider JG, Alosi JA, McDonald DE and McFadden DW: Pterostilbene inhibits lung cancer through induction of apoptosis. *J Surg Res* 161: 18-22, 2010.
13. He X, Wang Y, Zhu J, Orloff M and Eng C: Resveratrol enhances the anti-tumor activity of the mTOR inhibitor rapamycin in multiple breast cancer cell lines mainly by suppressing rapamycin-induced AKT signaling. *Cancer Lett* 301: 168-176, 2011.
14. Zhang W, Fei Z, Zhen HN, Zhang JN and Zhang X: Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells. *J Neurooncol* 81: 231-240, 2007.
15. Lin H, Zhang X, Cheng G, *et al*: Apoptosis induced by ardisinilioside III through BAD dephosphorylation and cleavage in human glioblastoma U251MG cells. *Apoptosis* 13: 247-257, 2008.
16. Lin H, Wang YG, Zhang X, Liu BL, Zhang W and Cheng JX: Prognostic significance of kappaB-Ras1 in gliomas. *Med Oncol*: Feb 8, 2011 (Epub ahead of print).
17. Truong M, Cook MR, Pinchot SN, Kunnimalaiyaan M and Chen H: Resveratrol induces Notch2-mediated apoptosis and suppression of neuroendocrine markers in medullary thyroid cancer. *Ann Surg Oncol* 18: 1506-1511, 2011.
18. Pinchot SN, Jaskula-Sztul R, Ning L, *et al*: Identification and validation of Notch pathway activating compounds through a novel high-throughput screening method. *Cancer* 117: 1386-1398, 2011.
19. Cecchinato V, Chiaramonte R, Nizzardo M, *et al*: Resveratrol-induced apoptosis in human T-cell acute lymphoblastic leukaemia MOLT-4 cells. *Biochem Pharmacol* 74: 1568-1574, 2007.
20. Li J, Cui Y, Gao G, Zhao Z, Zhang H and Wang X: Notch1 is an independent prognostic factor for patients with glioma. *J Surg Oncol*: doi:10.1002/jso.21851, 2011.
21. Gilbert CA, Daou MC, Moser RP and Ross AH: Gamma-secretase inhibitors enhance temozolomide treatment of human gliomas by inhibiting neurosphere repopulation and xenograft recurrence. *Cancer Res* 70: 6870-6879, 2010.