

ERK and PI3K signaling cascades induce Nrf2 activation and regulate cell viability partly through Nrf2 in human glioblastoma cells

ZI-XIANG CONG, HAN-DONG WANG, JIA-WEI WANG, YUAN ZHOU,
HAO PAN, DING-DING ZHANG and LIN ZHU

Department of Neurosurgery, Jinling Hospital, School of Medicine,
Nanjing University, Nanjing, Jiangsu 210002, P.R. China

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Abstract. The ERK and PI3K signaling cascades are aberrantly activated in human glioblastoma cells, resulting in the dysregulation of numerous downstream transcription factors. The dark side of the transcription factor, NF-E2-related factor 2 (Nrf2) in human cancer has been revealed. It has been accepted that high levels of Nrf2 promote tumor progression. In the present study, we investigated the effect of the ERK and PI3K signaling cascades on Nrf2 in human glioblastoma cells. Immunohistochemical staining for Nrf2 in clinical specimens showed that the expression and nuclear localization of Nrf2 were increased in human glioblastoma tissues when compared to peritumoral normal tissues. In addition, we detected decreased nuclear localization of Nrf2 following combined treatment with ERK and PI3K inhibitors in three human glioblastoma cell lines and selected the cell line (U251) most sensitive to the inhibitors for further study. Our data demonstrated that inhibition of ERK and PI3K not only suppressed the nuclear accumulation of Nrf2 protein but also decreased the expression of the Nrf2 protein. In addition, combined inhibition of ERK and PI3K also decreased the mRNA levels of Nrf2 target genes. Finally, we found that Nrf2 overexpression partly reversed the ERK and PI3K inhibitor-induced inhibition of cell viability. Therefore, the ERK and PI3K signaling cascades regulate the expression and activation of Nrf2 and control cell viability partly through Nrf2 in U251 human glioblastoma cells. Thus, targeting the ERK and PI3K signaling cascades for Nrf2 activation may provide new methods for the treatment of glioblastoma.

Introduction

Glioblastoma (WHO grade IV glioma) is the most common and lethal primary intracranial tumor in adults. Despite the use of multimodal treatments, glioblastoma remains incurable and most patients succumb to the disease within 2 years of diagnosis. Glioblastoma is characterized by uncontrolled proliferation, diffuse infiltration, excessive angiogenesis and poor response to treatment, which is mainly due to aberrant signaling networks in the tumor (1,2). The extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) signaling cascades play key roles in the aberrant signaling networks (3). The aberrant activation of the ERK and PI3K signaling cascades is related with oncogene activation and tumor-suppressor gene losses. For example, amplification of the epidermal growth factor receptor (EGFR) gene in glioblastoma leads to hyperactivate the ERK and PI3K signaling cascades (4). Gene alterations of phosphatase and tensin homolog (PTEN) result in constitutive activation of the PI3K signaling cascade (5).

The transcription factor NF-E2-related factor 2 (Nrf2), a member of the Cap 'n' collar family of basic region-leucine zipper transcription factors, controls adaptive defense responses by coordinated upregulation of many cytoprotective genes. In response to cellular stresses, Nrf2 escapes Kelch-like ECH-associated protein 1 (Keap1)-mediated repression, translocates from the cytoplasm to the nucleus and upregulates antioxidant responsive element (ARE)-dependent gene expression (6,7). The Nrf2-ARE downstream genes encode antioxidants, phase II detoxifying enzymes and drug transporters (8,9). These downstream cytoprotective proteins, such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO-1), can maintain cellular homeostasis and promote cell survival under stress. Beyond immediate stress response, Nrf2 is also involved in inflammation, metabolism, apoptosis, proliferation and differentiation (10-14). Nrf2 activation is associated with preventing many types of human diseases such as cancer, ischemia, hyperoxia, inflammatory diseases, neurodegenerative diseases, diabetes and obesity (11,15-21). Unfortunately, the protective effects of Nrf2 cannot discriminate normal cells and cancer cells. The dark side

Correspondence to: Professor Han-Dong Wang, Department of Neurosurgery, Jinling Hospital, School of Medicine, Nanjing University, 305 East Zhongshan Road, Nanjing, Jiangsu 210002, P.R. China
E-mail: hdwang_nj@yahoo.com.cn

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of Nrf2 in cancer promotion has been revealed. It has been verified that Nrf2 is upregulated in various types of cancers, including lung, head and neck, skin and breast cancers (22-25). High levels of Nrf2 promote cancer cell survival, contribute to chemoresistance and may be associated with poor prognosis (26-28).

The mechanisms involved in regulating Nrf2 in cancer are still not well known. Apart from Keap1, the Nrf2-ARE pathway is also regulated by distinct upstream kinases such as ERK and PI3K (29). The ERK and PI3K pathways are hyperactivated in human glioblastoma, which may induce the expression and activation of Nrf2. In the present study, we elucidated the effects of the ERK and PI3K signaling cascades on Nrf2 in human glioblastoma cells.

Materials and methods

Agents. The MEK inhibitor PD98059 was purchased from Cell Signaling Technology (Beverly, MA, USA). The PI3K inhibitor LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA). PD98059 and LY294002 were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. In all experiments, cells were treated with or without PD98059 (50 μ M) and LY292004 (10 μ M). Untreated cells were treated identically as the treated cells, except for PD98059 and LY292004.

Cell culture. The SHG44, U87 and U251 human glioblastoma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Rockford, IL, USA) containing 10% fetal bovine serum (Sijiqing, Hangzhou, China) and 1% penicillin/streptomycin (HyClone) in a humidified incubator with 5% CO₂ at 37°C.

Transient transfection. The method for construction of the recombinant plasmids and transfection efficiency has been described in our previous study (30). Human Nrf2 cDNA was cloned into the pEGFP-N1 vector (pEGFP-Nrf2), and an empty vector was used as the negative control (pEGFP). Nrf2 shRNA was cloned into the pGPH1/GFP/Neo vector (sh-Nrf2), and a random sequence was used as the negative control (sh-control). Cells (10⁶/well) were seeded into 6-well plates and allowed to attach for 24 h. The cells were then transfected with the various plasmids (pEGFP, pEGFP-Nrf2, sh-control or sh-Nrf2) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfected cells were cultured for at least 24 h after transfection. Transfection efficiency, monitored by GFP fluorescence, was ~80%.

Clinical specimens. Sixteen patients with primary glioblastoma who underwent maximal safe surgical resection at Jinling Hospital (Nanjing, China) were selected for the present study. All the patients received no chemotherapy or radiotherapy before surgery. The study was approved by the local ethics committee. Informed consent was obtained for the use of brain tissue. Sixteen tumor tissues were collected from the main bulk of the tumor tissues. Eleven peritumoral normal tissues were collected from the border zones between the tumors and the normal brain tissues. All specimens were fixed in 10% neutral formalin and were then embedded in paraffin.

All cases were reviewed independently by two pathologists. The diagnosis of glioblastoma was confirmed according to the current World Health Organization (WHO) classification of central nervous system tumors.

Immunohistochemistry and evaluation of immunostaining. For immunohistochemistry, sections (4- μ m) from 10% buffered formalin-fixed, paraffin-embedded tumor tissues were cut and placed onto slides. All the sections were deparaffinized in xylene and rehydrated through a series of graded alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and methanol. The sections were autoclaved in 10 mmol/l citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval, and then cooled at room temperature. The sections were blocked with 10% normal calf serum in PBS for 30 min, and incubated with the primary antibody against Nrf2 (1:50; Abcam, Cambridge, MA, USA) overnight at 4°C. The sections were then incubated with the secondary antibody (Dako, UK) for 30 min at room temperature. The peroxidase reaction was developed using 3,3'-diaminobenzidine as chromogen. Nuclei were counterstained with hematoxylin. Serial sections of the samples were also subjected to hematoxylin and eosin staining. The specificity of the immunohistochemical reaction was evaluated by replacement of the primary antibody with non-specific rabbit IgG. Serial sections of the individual samples were also subjected to hematoxylin and eosin staining.

The scoring of nuclear Nrf2 expression was carried out semi-quantitatively by assessing the staining intensity and the percentage of nuclear-positive cells. The staining intensity was scored as: 0 for no staining, 1 for weak staining, 2 for moderate staining and 3 for strong staining. The percentage of nuclear-positive cells was scored as: 0 when 0-1% of the tumor cells were positive, 1 when 2-10% of the tumor cells were positive, 2 when 11-50% of the tumor cells were positive and 3 when 51-100% of the tumor cells were positive. The percentage and intensity scores were added to obtain the total score (range, 0-6). The Nrf2 staining was divided into four levels: negative (score 0), weak (score 1-2), moderate (score 3-4) and strong (score 5-6). The nuclear-positive cells were identified, counted and analyzed by two pathologists blinded to the experimental design.

Western blot analysis. To prepare the total proteins, cells were lysed in cold RIPA lysis buffer (Beyotime, Jiangsu, China) containing 1 mM phenylmethylsulphonyl fluoride and centrifuged at 13,000 x g for 5 min at 4°C to remove debris. To prepare the cytoplasmic and nuclear proteins, cells were lysed using a nuclear and cytoplasmic protein extraction kit (Beyotime) according to the manufacturer's instructions. Protein concentrations were determined using the BCA assay, and the protein extracts were heat denatured in Laemmli sample loading buffer. Equal amounts (50 μ g/lane) of protein were separated by 10% SDS-polyacrylamide gel electrophoresis, and electroblotted onto polyvinylidene-difluoride membranes. The membranes were blocked for 2 h at room temperature with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), and incubated overnight at 4°C with the primary antibodies diluted in blocking buffer: anti-Nrf2 (1:500; Abcam), anti- β -actin (1:1,000; Cell Signaling Technology)

and anti-Lamin B1 (1:1,000; Abcam). The membranes were washed with TBST (3 x 10 min), and incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (1:1,000; Cell Signaling Technology) for 2 h at room temperature. The blotted protein bands were developed using an ECL detection reagent (Amersham Biosciences, Buckinghamshire, UK). Lamin B1 was used as a loading control for nuclear extracts. β -actin was used as a loading control for cytoplasmic and whole cell extracts.

Immunofluorescence. Cells growing on glass coverslips were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and then blocked with Immunol Staining Blocking Buffer (Beyotime). The coverslips were incubated with the primary antibody against Nrf2 (1:100; Abcam) overnight at 4°C. Following washes with PBS, the coverslips were incubated with an FITC-labeled secondary antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1 h at 37°C. Nuclei were then counterstained with DAPI. The specificity of the immunofluorescence reaction was evaluated by replacement of the primary antibody with non-specific rabbit IgG.

Quantitative real-time PCR. Total RNA was isolated from cells with RNAiso Plus (Takara, Dalian, China). The concentration of total RNA was determined by a spectrophotometer ($OD_{260/280} = 1.8-2.2$). Single-stranded cDNA was synthesized from 2 μ g of total RNA using the PrimeScript™ RT Reagent kit (Takara). Quantitative real-time PCR analysis was performed using the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), applying real-time SYBR-Green PCR technology. The reaction mixtures contained 10 μ l of Real-Time PCR Master Mix (Toyobo, Osaka, Japan), 1 μ l of cDNA, 1 μ l of each forward and reverse primer (10 μ M) and 7 μ l of DEPC-treated water. The PCR thermal cycling was carried out with a 5-min initial denaturation step at 95°C, followed by 40 three-step cycles: a denaturation step (95°C for 15 sec), an annealing step (60°C for 30 sec) and an extension step (72°C for 30 sec). The experimental results were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Primer sequences used in this study were as follows: HO-1 forward and reverse primers were 5'-TCTCCGATGGGTCCTTACTC-3' and 5'-GGCATAAAGCCCTACAGCAACT-3'. NQO1 forward and reverse primers were 5'-ATGGTCCG CAGAAGAGC-3' and 5'-GGAAATGATGGGATTGAAGT-3'. β -actin forward and reverse primers were 5'-GCAGAAGGA GATCACTGCCCT-3' and 5'-GCTGATCCACATCTGCTG GAA-3'.

MTT assay. In brief, 24 h after transfection, the transfected and untransfected cells (4,000/well) were treated with or without PD98059 (50 μ M) plus LY294002 (10 μ M) for another 48 h. Afterward, MTT (5 mg/ml, 20 μ l) was added to each well and the plates were incubated for 4 h. The blue formazan product was then dissolved with DMSO, and the optical density (OD) value was measured at 490 nm using an enzyme-labeling instrument.

The viability rate (%) = $(OD_{\text{treatment}} - \text{mean } OD_{\text{blank control}}) / (\text{mean } OD_{\text{negative control}} - \text{mean } OD_{\text{blank control}}) \times 100\%$.

The wells containing only cells and complete medium were used as the negative control. The wells containing only complete medium were used as the blank control.

Statistical analysis. All experiments were repeated at least three times, and the results were analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data are presented as means \pm SEM, and evaluated by the Student's t-test or one-way ANOVA followed by LSD test. P-value <0.05 was considered to indicate a statistically significant result.

Results

Increased expression and nuclear localization of Nrf2 in human glioblastoma tissues. To evaluate the expression and distribution of Nrf2, we performed immunohistochemistry on tumor and peritumoral normal tissue sections. As shown in Fig. 1A, the expression and nuclear localization of Nrf2 in the main bulk of tumor tissues were markedly increased when compared with the expression and the nuclear localization in the peritumoral normal tissues which were located at a distance from the tumor tissues. Fig. 1B shows Nrf2 staining in a sample of tumor and peritumoral normal tissues. Peritumoral normal tissues exhibited weak Nrf2 staining which was predominant in the cytoplasm. In the adjacent tumor tissues, strong Nrf2 staining was found in most tumor cells and was located in both the cytoplasm and nucleus. Semi-quantitative analysis of the nuclear Nrf2 protein is shown in Fig. 1C. In the peritumoral normal tissues, 0 (0/11), 72.72 (8/11), 27.28 (3/11) and 0% (0/11) of samples showed negative, weak, moderate and strong staining, respectively. In the tumor tissues, 0 (0/16), 18.75 (3/16), 56.25 (9/16) and 25% (4/16) of samples showed negative, weak, moderate and strong staining, respectively. These results suggest that expression and nuclear localization of Nrf2 are increased in human glioblastoma tissues.

Combined inhibition of ERK and PI3K decreases the expression and transcriptional activation of Nrf2. It is well known that nuclear accumulation of Nrf2 proteins indicates activation of the Nrf2-ARE pathway. In the present study, we aimed to ascertain whether inhibition of ERK and PI3K decreases nuclear distribution of Nrf2. To this end, SHG44, U87 and U251 cell lines were treated with or without the MEK inhibitor PD08059 plus PI3K inhibitor LY292004 for 3 h. We then performed immunofluorescence to analyze the Nrf2 distribution. Representative images of immunofluorescence staining are shown in Fig. 2. Three hours after treatment with PD98059 plus LY292004, the nuclear staining of Nrf2 was decreased in all three cell lines. In addition, the cytoplasmic staining of Nrf2 was also decreased in the U251 and U87 cells. We found that the U251 cells were more sensitive to the inhibitors than the other cell lines. Thus, we chose the U251 cells to further verify these findings and carry out the subsequent studies.

Next, to further determine whether combined inhibition of ERK and PI3K decreases the expression and activation of Nrf2, we analyzed Nrf2 protein in the nuclear and cytoplasmic fractions of U251 cells by western blot analysis. In addition, we further investigated the effect of each inhibitor on the expression and activation of Nrf2. The U251 cells were harvested after different treatments (untreated, PD98059, LY292004

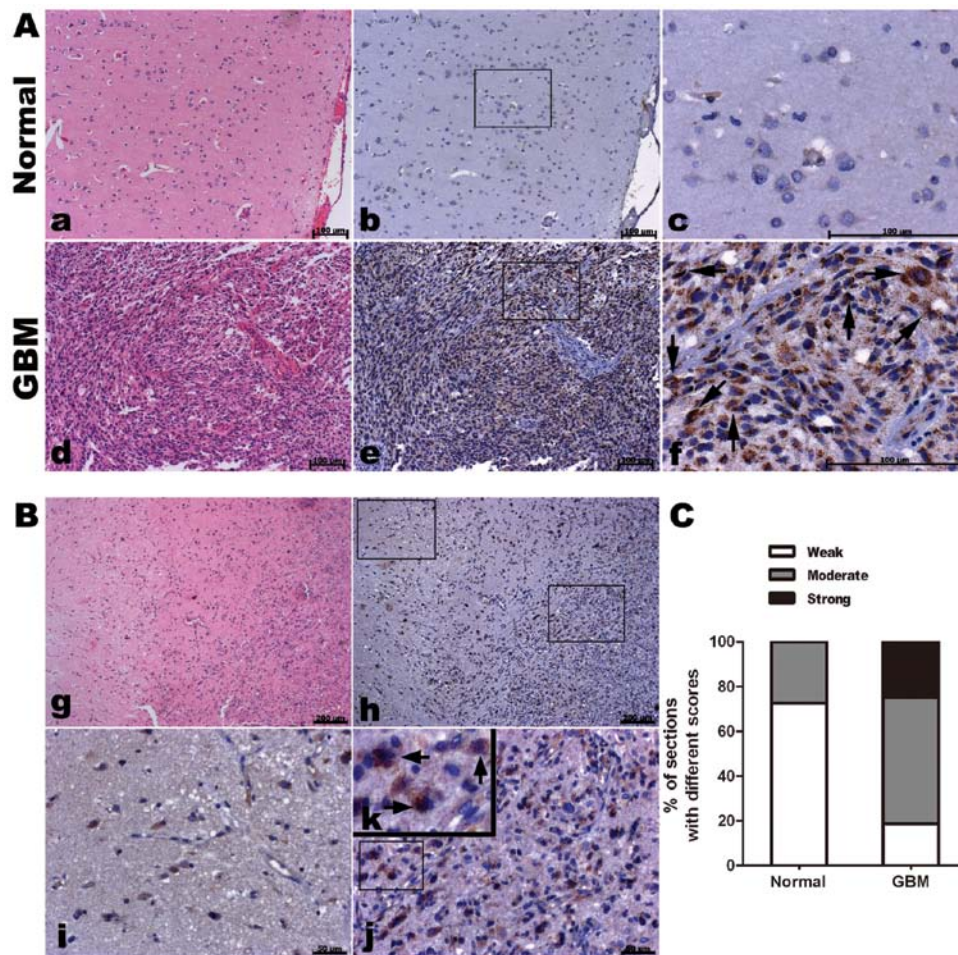


Figure 1. Increased expression and nuclear localization of Nrf2 in human glioblastoma (GBM) tissues. (A) Representative staining of serial sections from the clinical samples of the same patient. Tissue samples were collected during surgery from two sites: normal tissue, from the peritumoral normal tissue which was located at a distance from the tumor tissue; GBM tissue, from the main bulk of the tumor tissue. (a and d) H&E staining. (b and e) Staining with the antibody for Nrf2. (c and f) Magnification of the areas indicated by the black rectangles. (B) Representative staining of serial sections from a sample with peritumoral normal and tumor tissues. (g) H&E staining. (h) Staining with the antibody for Nrf2. (i and j) Magnification of the areas indicated by the black rectangles in peritumoral normal tissues in h. (k) Magnification of the area in the black rectangle in j. Arrows indicate the nuclear-positive cells which exist only in the tumor tissues. Scale bar, 100 μ m in A; 200 μ m in B-g and -h; 50 μ m in B-i and -j. (C) Quantitation of nuclear Nrf2 protein in the peritumoral normal and tumor tissues. The expression of nuclear Nrf2 was divided into four levels: negative, weak, moderate and strong.

or PD98059+LY292004) for 3 h and the cytoplasmic and nuclear fractions were isolated from the cells. Representative immunoblots are shown in Fig. 3. Three hours after treatment with PD98059 plus LY292004, there was a significant decrease in the protein level of nuclear Nrf2 when compared with the protein level in the untreated group. Consistent with the immunofluorescence results, there was also a decrease in the protein level of cytoplasmic Nrf2 following combined inhibition of ERK and PI3K. As expected, inhibiting either ERK or PI3K moderately suppressed nuclear accumulation of Nrf2 protein. However, there was no significant decrease in the expression of cytoplasmic Nrf2 following treatment with either inhibitor alone. In addition, there was no statistical change in the mRNA level of Nrf2 after treatment with both inhibitors (data not shown). Therefore, combined inhibition of ERK and PI3K signaling not only reduced nuclear accumulation of Nrf2 but also suppressed the protein expression of Nrf2 in the U251 cells.

Nrf2 activation plays a cytoprotective role through upregulating the expression of Nrf2-ARE downstream target genes,

therefore, we examined whether inhibition of ERK and PI3K suppresses the expression of Nrf2 target genes. The U251 cells were treated with PD98059 plus LY292004 for 3 h, and then real-time PCR analysis was performed. As shown in Fig. 4, the mRNA levels of Nrf2-ARE downstream target genes (HO-1 and NQO-1) were decreased in the PD98059+LY292004 group in comparison with the DMSO group. Together, these results imply that the ERK and PI3K signaling cascades are closely associated with the expression and activation of Nrf2 in human glioblastoma cells.

ERK and PI3K signaling cascades regulate cell viability partly through Nrf2. It is well known that the ERK and PI3K signaling cascades play central roles in controlling cancer cell viability. There are numerous downstream proteins involved in cell viability. Thus, we evaluated whether the ERK and PI3K signaling cascades regulate cell viability through Nrf2. Here, first we assessed the effect of Nrf2 on U251 cell viability. The U251 cells were transfected with different plasmids (sh-control, sh-Nrf2, pEGFP and pEGFP-Nrf2). Following

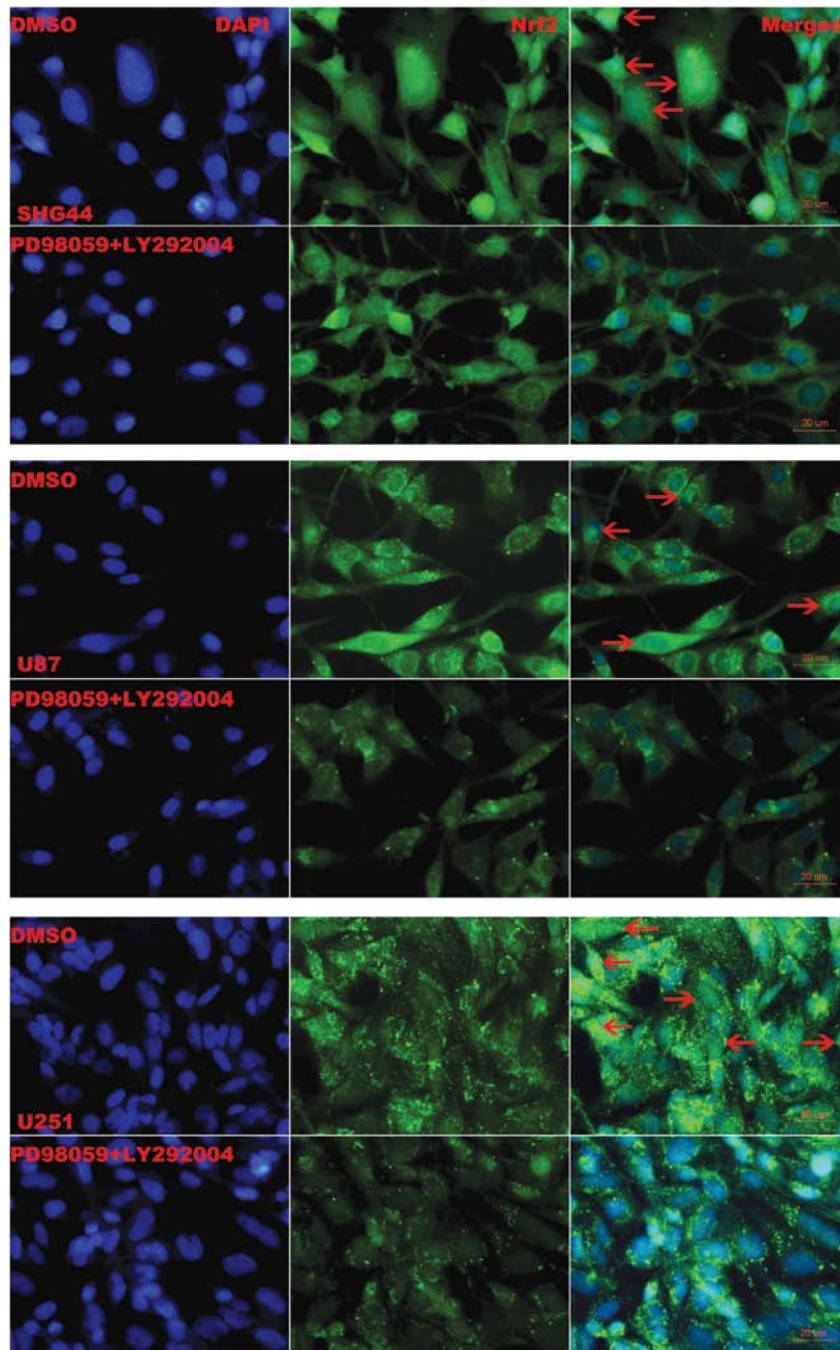


Figure 2. Inhibition of ERK and PI3K decreases nuclear location of Nrf2 in the human glioblastoma cell lines. SHG44, U87 and U251 cells were treated with or without PD98059 (50 μ M) plus LY292004 (10 μ M) for 3 h. Representative photomicrographs of Nrf2 immunofluorescence staining are shown. Arrows indicate the nuclear-positive cells which were reduced in the PD98059+LY292004 groups. Scale bar, 20 μ m.

transfection, cells were incubated for 48 h, and cell viability was detected by MTT analysis. As shown in Fig. 5A, down-regulation of Nrf2 protein suppressed U251 cell viability; the viability rate decreased from 96.02 (the sh-control group) to 86.99% (the sh-Nrf2 group). On the contrary, upregulation of Nrf2 protein promoted U251 cell growth; the relative growth rate increased from 96.90 (the pEGFP group) to 105.90% (the pEGFP-Nrf2 group). There was no difference between the two control groups (the sh-control group compared with the pEGFP group). In addition, the transfection effect of the four plasmids was further verified by western blot analysis (Fig. 5B).

Next, we investigated whether the overexpression of Nrf2 protein could partly reverse the MEK and PI3K inhibitor-induced inhibition of cancer cell viability. The U251 cells were treated with PD98059 plus LY294002 for 48 h. The morphological changes in the U251 cells in the different groups were observed by microscopy. As shown in Fig. 6A, we observed that many cells lost normal shape and detached from the culture plates (the PD98059+LY292004 group and the PD98059+LY292004+pEGFP group). However, fewer abnormal cells were noted in the PD98059+LY292004+pEGFP-Nrf2 group than that in the

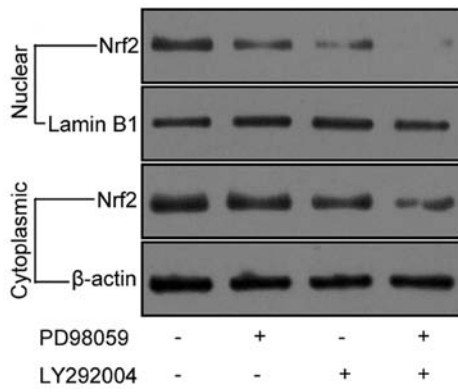


Figure 3. Inhibition of ERK and PI3K suppresses the expression of Nrf2 in the nuclear and cytoplasmic fractions. The U251 cells were treated with PD98059 (50 μ M) plus LY292004 (10 μ M) for 3 h. Representative immunoblots showing Nrf2 expression in cytoplasmic and nuclear fractions. Lamin B1 served as a loading control for nuclear extracts. β -actin served as a loading control for cytoplasmic extracts.

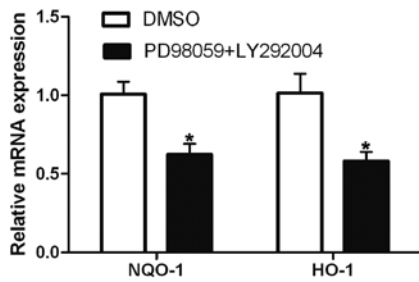


Figure 4. Inhibition of ERK and PI3K suppresses the expression of Nrf2 target genes. The U251 cells were treated with PD98059 (50 μ M) plus LY292004 (10 μ M) for 3 h. The relative mRNA levels of the Nrf2-ARE-dependent genes (HO-1/ β -actin and NQO-1/ β -actin) were analyzed by quantitative real-time PCR. Data are presented as means \pm SEM. * P <0.05 compared to the DMSO group.

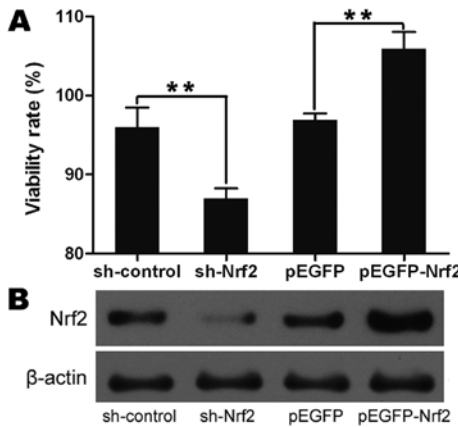


Figure 5. Levels of Nrf2 protein are related with U251 cell viability. (A) Twenty-four hours after transfection, the U251 cells (4,000/well) were incubated for 48 h. Cell viability was analyzed by MTT assay. Data are presented as means \pm SEM. ** P <0.01, the sh-control group compared with the sh-Nrf2 group; ** P <0.01, the pEGFP group compared with the pEGFP-Nrf2 group. (B) Representative immunoblots showing Nrf2 expression after transfection.

PD98059+LY292004+pEGFP group. Cell viability was further measured by MTT assay. As shown in Fig. 6B, combined inhi-

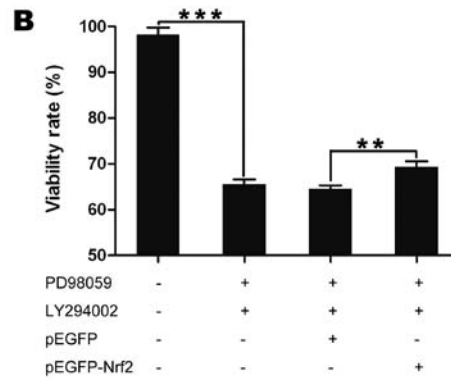
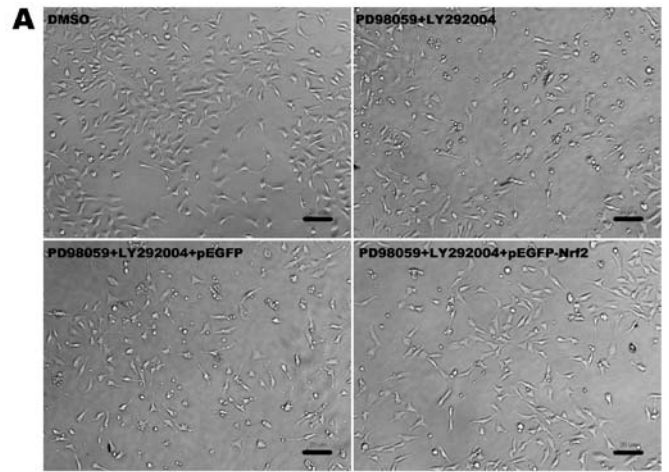


Figure 6. Overexpression of Nrf2 partly reverses ERK and PI3K inhibitor-induced inhibition of cancer cell viability. The untransfected and transfected U251 cells (4,000/well) were treated with or without PD98059 (50 μ M) plus LY294002 (10 μ M) for 48 h. (A) The morphological changes in the U251 cells were observed by microscopy. (B) Cell viability was analyzed by MTT assay. Data are presented in means \pm SEM. *** P <0.001, the untreated group compared with the PD98059+LY292004 group; ** P <0.01, the PD98059+LY292004+pEGFP group compared with the PD98059+LY292004+pEGFP-Nrf2 group.

bition of ERK and PI3K markedly reduced U251 cell viability; the viability rate decreased from 98.20 (the untreated group) to 65.60% (the PD98059+LY292004 group). Upregulation of Nrf2 protein partly reversed the ERK and PI3K inhibitor-induced U251 cell viability; the viability rate increased from 64.57 (the PD98059+LY292004+pEGFP group) to 69.41% (the PD98059+LY292004+pEGFP-Nrf2 group). Thus, overexpression of Nrf2 reversed nearly one-sixth of the total inhibition. There was no difference between the PD98059+LY292004 group and the PD98059+LY292004+pEGFP group. These results suggest that the ERK and PI3K signaling cascades regulate cell viability partly through Nrf2 in the U251 cells.

Discussion

In the present study, we demonstrated that combined inhibition of the ERK and PI3K signaling cascades decreased the expression and activation of Nrf2 and then suppressed cell viability partly through Nrf2 in human glioblastoma cells. First, immunohistochemical staining revealed that the expression and nuclear location of Nrf2 were increased in the glioblastoma tissues when compared with the expression and

nuclear localization in the peritumoral normal tissues. Next, our data demonstrated that inhibition of ERK and PI3K not only suppressed the nuclear accumulation of Nrf2 but also decreased the expression of Nrf2 protein. In addition, combined inhibition of ERK and PI3K also decreased the mRNA levels of Nrf2-ARE downstream target genes. Finally, we provide evidence that the ERK and PI3K signaling cascades regulate cell viability partly through Nrf2.

Transcription factor Nrf2 plays a key regulatory role in cytoprotection. However, recent studies highlight the dark role of Nrf2 in cancer promotion. Many groups have found that Nrf2 is overexpressed in many types of cancers. To study the mechanism of Nrf2 dysregulation in cancer cells, many groups have focused on the mutations in Nrf2 or Keap1. Gain-of-function mutations in Nrf2 have been found in lung, esophageal and skin cancers (24,31). Loss-of-function mutations in KEAP1 have been found in lung, breast, gallbladder, liver, stomach, colon and prostate cancers (22,25,26,32-34). These mutations disrupt Nrf2-Keap1 interaction leading to stabilize Nrf2 and activate Nrf2-mediated cytoprotective responses. However, mutations in Nrf2 and Keap1 are rare in tumors (16); therefore, the mutations may not be the main reason leading to the expression and activation of Nrf2. In the present study, we focused on other mechanisms regulating Nrf2 in human glioblastoma cells. It has been suggested that protein kinases [ERK, PI3K, protein kinase C (PKC), glycogen synthase kinase 3- β (GSK3- β)] are also involved in regulating Nrf2 (29). Our data showed that combined inhibition of ERK and PI3K decreased the nuclear protein level of Nrf2 and the mRNA levels of Nrf2 target genes in U251 cells. These findings indicate that inhibition of the ERK and PI3K signaling cascades suppresses activation of the Nrf2-ARE pathway in U251 cells. In addition, we also found that the inhibitors decreased the protein levels of Nrf2 not only in the nucleus but also in the cytoplasm. In support of our findings, Banerjee *et al* (35) reported that activation of H-Ras and ERK induced nuclear translocation of Nrf2, and promoted Nrf2-mediated HO-1 transcription in human renal cancer cells. Chowdhry *et al* (36) demonstrated that the LY294002 PI3K inhibitor decreased the protein levels of Nrf2, HO-1 and NQO-1 in A549 cells. We found that combined inhibition of the ERK and PI3K signaling cascades was more effective in suppressing the expression and nuclear accumulation of Nrf2 than inhibition of either inhibitor alone in the U251 cells. These findings imply that combined inhibition may block the crosstalk between the ERK and PI3K signaling cascades. Experimental evidence suggests that ERK and PI3K may regulate the expression and activation of Nrf2 by a post-transcriptional mechanism. Phosphorylation of Nrf2 increases its stability and subsequent transactivation activity (29,37). However, the specific mechanism of Nrf2 regulation by ERK and PI3K is still unclear and warrants further research.

The ERK and PI3K signaling cascades play central roles in controlling cell viability in glioblastoma cells. There are numerous downstream proteins promoting cell survival, such as topoisomerase II- α , CREB, NF- κ B, c-Fos, mTOR and GSK3 β (3). Our data suggest that Nrf2 is one of these proteins related with glioblastoma cell viability. Upregulated Nrf2 expression was able to reverse one-sixth of the ERK and PI3K inhibitor-induced inhibition of cell viability. Thus, Nrf2 may serve as a key player in the ERK and PI3K-mediated cell viability. It has

been revealed that Nrf2 plays an important role in lung cancer cell proliferation. Nrf2 knockdown inhibits the proliferation of lung cancer cells (22,38). Homma *et al* (28) reported that Nrf2 knockdown induced cell cycle arrest at G1 phase with a reduction in the phosphorylated form of retinoblastoma protein in human lung cancer cell lines. However, Reddy *et al* (13) found that Nrf2 deficiency induced G2/M phase arrest and decreased cell proliferation by impairing GSH-induced redox signaling in primary epithelial culture cells. Recently, Niture *et al* (12) provided initial evidence that Nrf2 controls anti-apoptotic protein Bcl-2 and cellular apoptosis. The present study suggests that high levels of Nrf2 promote cancer cell growth partly through decreased apoptotic cell death. According to these findings, we propose to further study the effect of Nrf2 on glioblastoma cell growth.

In summary, ERK and PI3K signaling cascade-induced Nrf2 activation plays a role in cell viability in U251 human glioblastoma cells. In addition to ERK and PI3K, Nrf2 has many other regulators such as PKC, GSK3- β , P38 and JNK (29). Thus, we hypothesize that Nrf2 is regulated at multiple levels by a coordinated process in cancer cells. Overexpression of Nrf2 in cancer cells provides an advantage for cell growth, drug resistance (39), radioresistance (40,41), migration and invasion (30). Therefore, further study of the molecular regulation of Nrf2 is warranted and may provide feasible methods against cancer promotion.

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References

1. Furnari FB, Fenton T, Bachoo RM, *et al*: Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev* 21: 2683-2710, 2007.
2. Nakada M, Kita D, Watanabe T, *et al*: Aberrant signaling pathways in glioma. *Cancers* 3: 3242-3278, 2011.
3. Wong ML, Kaye AH and Hovens CM: Targeting malignant glioma survival signalling to improve clinical outcomes. *J Clin Neurosci* 14: 301-308, 2007.
4. Golding SE, Morgan RN, Adams BR, Hawkins AJ, Povirk LF and Valerie K: Pro-survival AKT and ERK signaling from EGFR and mutant EGFRvIII enhances DNA double-strand break repair in human glioma cells. *Cancer Biol Ther* 8: 730-738, 2009.
5. Holand K, Salm F and Arcaro A: The phosphoinositide 3-kinase signaling pathway as a therapeutic target in grade IV brain tumors. *Curr Cancer Drug Targets* 11: 894-918, 2011.
6. Li W and Kong AN: Molecular mechanisms of Nrf2-mediated antioxidant response. *Mol Carcinog* 48: 91-104, 2009.
7. Lee JM and Johnson JA: An important role of Nrf2-ARE pathway in the cellular defense mechanism. *J Biochem Mol Biol* 37: 139-143, 2004.
8. Lau A, Villeneuve NF, Sun Z, Wong PK and Zhang DD: Dual roles of Nrf2 in cancer. *Pharmacol Res* 58: 262-270, 2008.
9. Niture SK, Kaspar JW, Shen J and Jaiswal AK: Nrf2 signaling and cell survival. *Toxicol Appl Pharmacol* 244: 37-42, 2010.
10. Rangasamy T, Guo J, Mitzner WA, *et al*: Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med* 202: 47-59, 2005.
11. Chen XL and Kunsch C: Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. *Curr Pharm Des* 10: 879-891, 2004.

12. Niture SK and Jaiswal AK: Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *J Biol Chem* 287: 9873-9886, 2012.
13. Reddy NM, Kleeberger SR, Bream JH, *et al*: Genetic disruption of the Nrf2 compromises cell-cycle progression by impairing GSH-induced redox signaling. *Oncogene* 27: 5821-5832, 2008.
14. Zhao F, Wu T, Lau A, *et al*: Nrf2 promotes neuronal cell differentiation. *Free Radic Biol Med* 47: 867-879, 2009.
15. Cho HY, Reddy SP, Yamamoto M and Kleeberger SR: The transcription factor NRF2 protects against pulmonary fibrosis. *FASEB J* 18: 1258-1260, 2004.
16. Sporn MB and Liby KT: NRF2 and cancer: the good, the bad and the importance of context. *Nat Rev Cancer* 12: 564-571, 2012.
17. Shah ZA, Li RC, Thimmulappa RK, *et al*: Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. *Neuroscience* 147: 53-59, 2007.
18. Cho HY, Jedlicka AE, Reddy SP, *et al*: Role of NRF2 in protection against hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 26: 175-182, 2002.
19. Calkins MJ, Johnson DA, Townsend JA, *et al*: The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. *Antioxid Redox Signal* 11: 497-508, 2009.
20. He M, Siow RC, Sugden D, Gao L, Cheng X and Mann GE: Induction of HO-1 and redox signaling in endothelial cells by advanced glycation end products: a role for Nrf2 in vascular protection in diabetes. *Nutr Metab Cardiovasc Dis* 21: 277-285, 2011.
21. Shin S, Wakabayashi J, Yates MS, *et al*: Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide. *Eur J Pharmacol* 620: 138-144, 2009.
22. Ohta T, Iijima K, Miyamoto M, *et al*: Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res* 68: 1303-1309, 2008.
23. Stacy DR, Ely K, Massion PP, *et al*: Increased expression of nuclear factor E2 p45-related factor 2 (NRF2) in head and neck squamous cell carcinomas. *Head Neck* 28: 813-818, 2006.
24. Kim YR, Oh JE, Kim MS, *et al*: Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. *J Pathol* 220: 446-451, 2010.
25. Nioi P and Nguyen T: A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. *Biochem Biophys Res Commun* 362: 816-821, 2007.
26. Solis LM, Behrens C, Dong W, *et al*: Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. *Clin Cancer Res* 16: 3743-3753, 2010.
27. Kweon MH, Adhami VM, Lee JS and Mukhtar H: Constitutive overexpression of Nrf2-dependent heme oxygenase-1 in A549 cells contributes to resistance to apoptosis induced by epigallocatechin 3-gallate. *J Biol Chem* 281: 33761-33772, 2006.
28. Homma S, Ishii Y, Morishima Y, *et al*: Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clin Cancer Res* 15: 3423-3432, 2009.
29. Baird L and Dinkova-Kostova AT: The cytoprotective role of the Keap1-Nrf2 pathway. *Arch Toxicol* 85: 241-272, 2011.
30. Pan H, Wang H, Zhu L, Mao L, Qiao L and Su X: The role of Nrf2 in migration and invasion of human glioma cell U251. *World Neurosurg*: Nov 7, 2011 (Epub ahead of print).
31. Shibata T, Ohta T, Tong KI, *et al*: Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci USA* 105: 13568-13573, 2008.
32. Yoo NJ, Kim HR, Kim YR, An CH and Lee SH: Somatic mutations of the KEAP1 gene in common solid cancers. *Histopathology* 60: 943-952, 2012.
33. Shibata T, Kokubu A, Gotoh M, *et al*: Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* 135: 1358-1368, 2008.
34. Padmanabhan B, Tong KI, Ohta T, *et al*: Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell* 21: 689-700, 2006.
35. Banerjee P, Basu A, Datta D, Gasser M, Waaga-Gasser AM and Pal S: The heme oxygenase-1 protein is overexpressed in human renal cancer cells following activation of the Ras-Raf-ERK pathway and mediates anti-apoptotic signal. *J Biol Chem* 286: 33580-33590, 2011.
36. Chowdhry S, Zhang Y, McMahon M, Sutherland C, Cuadrado A and Hayes JD: Nrf2 is controlled by two distinct β -TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene*: Sep 10, 2012 (Epub ahead of print).
37. Nguyen T, Sherratt PJ, Huang HC, Yang CS and Pickett CB: Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem* 278: 4536-4541, 2003.
38. Singh A, Boldin-Adamsky S, Thimmulappa RK, *et al*: RNAi-mediated silencing of nuclear factor erythroid-2-related factor 2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. *Cancer Res* 68: 7975-7984, 2008.
39. Wang XJ, Sun Z, Villeneuve NF, *et al*: Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis* 29: 1235-1243, 2008.
40. Singh A, Bodas M, Wakabayashi N, Bunz F and Biswal S: Gain of Nrf2 function in non-small-cell lung cancer cells confers radioresistance. *Antioxid Redox Signal* 13: 1627-1637, 2010.
41. McDonald JT, Kim K, Norris AJ, *et al*: Ionizing radiation activates the Nrf2 antioxidant response. *Cancer Res* 70: 8886-8895, 2010.