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Original Article

Reactive oxygen species-upregulated miR-135a plays a pivotal role in phenethyl isothiocyanate-induced rat C6 glioma cell apoptosis

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Abstract: Phenethyl isothiocyanate (PEITC), which is a strong reactive oxygen species (ROS) inducer, derived from cruciferous vegetables has shown broad spectrum and remarkable anti-cancer effects in a various cancers including glioblastoma. Yet, microRNAs involving in PEITC exerting anti-cancer effects remain few reports. Accumulating evidence has shown that chemicals-induced ROS regulates the expression of microRNAs including miR-135a, which has been demonstrated to trigger mitochondria-dependent apoptosis of rat C6 glioma cells. Here, we report PEITC-induced ROS could upregulate miR-135a playing a pivotal role in PEITC killing rat C6 glioma cells. Treatment of rat C6 glioma cells with PEITC lead to significantly inhibiting cell viability and inducing apparent apoptotic morphology of cells accompanied with activation of caspase-9 and caspase-3. Moreover, PEITC significantly suppressed the tumorigenicity and migration ability of Rat C6 glioma cells. In addition, PEITC induced cellular GSH depletion and ROS production. Importantly, PEITC was significantly upregulated miR-135a, which was however abolished by GSH (a ROS scavenger). Furthermore, immunobloting analysis showed that PEITC downregulated STAT6, SMAD5 and Bcl-xl, while upregulated Bax and cytochrome C, which were significantly prevented by either miR-135a inhibitor or GSH. MiR-135a inhibitor and GSH also reversed the loss of cell viability by PEITC. Our results demonstrated that ROS-regulated miR-135a played a pivotal role in PEITC triggering rat C6 glioma cells apoptosis, suggesting a potential application of PEITC for the treatment glioblastoma.

Keywords: Phenethyl isothiocyanate, rat C6 glioma cells, apoptosis, ROS, miR-135a, mitochondria

Introduction

Malignant glioma accounts for around 80% of primary central nervous system (CNS) tumors in adults [1]. In the past decades, great advances have been made in the diagnosis, surgery, radiation and chemotherapy, however prognosis of glioma is still very poor with the median survival time of just 12-15 months [2]. Therefore, it is necessary to develop novel and effective modalities for the treatment of glioma.

Many studies have shown that some bioactive compounds in cruciferous vegetables can effectively eliminate malignant cancer cells. Isothiocyanates (ITCs) are enriched in cruciferous vegetables and have been shown to reduce the development of various malignancies [3]. Phenethyl isothiocyanate (PEITC) is one of the most extensively studied ITCs and has shown broad spectrum and remarkable anti-cancer effects [4], including inducing cancer cell apoptosis [5-7], reversing cancer cell chemotherapy drug resistance [8-10] and selectively killing malignant cancer cells but not the corresponding normal cells [8, 11, 12]. One of the mechanisms is through inducing ROS production to trigger activation of multiple signal transduction pathways. In glioma cells, PEITC was reported to inhibit PI3K-MAPK- HIF-1α-VEGF axis and induce apoptosis through the extrinsic (death receptor) pathway, ROS-induced ER stress, and intrinsic (mitochondria) pathway [13]. But the underling molecular mechanism of

PEITC in inhibiting the growth of glioblastoma cells remains elusive.

MicroRNAs (miRNAs) are small noncoding RNA molecules with 19 to 25 nucleotides in length, and play pivotal roles in the initiation and progression of many diseases including human cancers [14]. Accumulating studies have revealed that miRNAs such as miR-27a, miR-491 and miR-135a, play pivotal roles in the response to ROS-inducing anti-tumor agents [15-19]. A previous study showed that miR-135a triggers rat C6 glioma cell apoptosis through mitochondria-dependent pathway [20]. Since PEITC induces ROS in glioma cells [14], we hypothesized that PEITC, through inducing ROS, may inhibit the growth of rat C6 glioma cells through miR-135a-mitochondria-dependent pathway.

In order to further investigate the molecular pharmacology of PEITC for the treatment of glioma, in this study, we explored the anti-cancer effects of PEITC on rat C6 glioma cells and the potential mechanism.

Materials and methods

Chemicals and reagents

PEITC, dimethyl-sulphoxide (DMSO), and 3-(4, 5-dimethylatiazol-2-yl)-2,5-diphenyltrazoliu bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MI, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (New York, NY, USA). Hoechst 33258 Staining, Annexin V-FITC Apoptosis Detection Kit, Caspase-3 and 9 Activity Assay Kit, Caspase Inhibitor Z-VAD-FMK, 0.1% crystal violet, Total Glutathione Assay Kit, Reactive Oxygen Species Assay Kit and RIPA lysis buffer were purchased from Beyotime (Hainan, China). 4% polyoxymethylene, TRIzol reagent, SuperScript III reverse transcriptase, Platinum SYBR Green qPCR SuperMix-UDG and Lipofectamine2000 were purchased from Invitrogen (Grand Island, NY, USA). For Western blotting assays, primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The rat C6 glioma cell line was kindly provided by Dr. Wu (Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China) and cultured in DMEM supplemented with 10% FBS under a humidified 5% CO_2 atmosphere at 37°C in an incubator.

Cytotoxicity assay and apoptosis assay

For cytotoxicity assay, rat C6 glioma cells were seeded on 96-well plates at the density of 5×10^3 cells per well. After treated with series concentrations of PEITC for 12 h, 24 h and 48 h, the cytotoxic effect of PEITC against rat C6 glioma cells were evaluated by methyl thiazolyl tetrazolium (MTT) assay according to the manufacturer's instruction. Apoptosis was evaluated by the apoptotic morphology, Hoechst 33258 Staining, flow cytometry, and Caspases' activity. Apoptotic morphology was observed by microscope (Nikon, Japan). For Hoechst 33258 staining assay, cells with condensed and fragmented nuclei were considered as the apoptotic cells and detected by fluorescent microscopy. For flow-cytometry analysis, cells were collected and stained with Annexin V-FITC Apoptosis Detection Kit and analyzed by a flow cytometer (Beckman Coulter, Brea, CA, USA). Caspases' activity was determined by Caspase-3 and 9 Activity Assay Kit.

Foci-formation assay

For foci-formation assay, 400 viable cells were placed in 6-well plate in triplicate for 24 h, then treated with 0, 15, 20 and 25 μM of PEITC and continually maintained in complete medium for 14 days. Foci were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet. The stained colonies were washed by PBS for 3 times and the foci-formation was detected by microscope. The stained colonies were further dissolved in DMSO and quantified by spectrophotometer at 540 nm.

Migration assay

Rat C6 glioma cells were cultured in 6-well plate. After approaching almost 100% confluence, the cells were scratched with a 20 μl tips, followed by washing with PBS and treatment with 0, 15, 20 and 25 μM of PEITC for 24 h. The scratched areas were photographed with a microscope at 0 h and 24 h.

Cellular GSH measurement

Rat C6 glioma cells were treated with 0, 15, 20 and 25 μM of PEITC for 24 h and with 20 μM of PEITC for 0, 1, 3, 5 h. Glutathione (GSH) levels were determined by using Glutathione Qu-

antification Kit. 5, 5-Dithiobis (2-nitrobenzoic acid) (DTNB) and GSH reacted to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in the sample was determined by reading at 412 nm absorbance with a multi-well plate reader (BioTek, USA).

Cellular ROS detection

Rat C6 glioma cells were treated with 0, 15, 20 and 25 μM of PEITC for 24 h and with 20 μM of PEITC for 0, 1, 3, 5 h. The intracellular ROS was detected by Reactive Oxygen Species Assay Kit based on 2',7'-dichlorofluoresceindiacetate (DCFH-DA). DCFH-DA diffuses into cells and is hydrolyzed into non-fluorescent 2',7'-dichlorofluorescin (DCFH). The ROS, including H_2O_2, superoxide and OH-, oxidizes non-fluorescent intra-cellular DCFH into highly fluorescent dichlorofluorescin (DCF), whose intensity of fluorescence is directly proportional to the level of intracellular ROS. DCF fluorescence was detected using fluorescence microscope and photographs were taken by inverted fluorescence microscope (Nikon, Japan). The intensity of fluorescence was measured with a spectrofluorimeter (BioTek, USA) at excitation/emission wavelengths of λ=488/525 nm.

Quantitative real-time PCR for miR-135a

Total RNAs were isolated by TRIzol reagent. The concentration of RNA was determined by measuring the absorbance at 260 nm. Reverse transcription was performed using SuperScript III reverse transcriptase. Q-PCR was performed with Platinum SYBR Green qPCR SuperMix-UDG and detected with the LightCycler 480 instrument (Roche, Basel, Switzerland). Comparative CT method ($2^{-\Delta\Delta CT}$) was adopted to evaluate the relative expression.

Oligonucleotide transfection

MiR-135a inhibitor and miR-135a inhibitor negative control were designed and synthesized by RiboBio (Guangzhou, China). Cells were grown to 70-90% confluence in 6-well plates before transfection. Cells were transfected with these oligonucleotides by using Lipofectamine2000 as recommended by the manufacturer's instructions.

Western blot analysis

Cells were lysed in the ice-cold RIPA lysis buffer and the protein concentration of the lysates was determined by BCA method. Proteins samples were separated by electrophoresis on SDS-PAGE gel and electro-transferred onto a Hybond ECL transfer membrane (Amersham Pharmacia, Piscataway, NJ, USA). After blocking with 5% dry milk, the membrane was incubated with primary antibodies for STAT6, SMAD5, Bcl-xl, Bax, Cyto-C, or β-actin followed by exposure to horseradish peroxidase (HRP)-conjugated secondary anti-mouse or rabbit antibodies. To visualize protein bands, chemiluminescence (ECL) system (BIO-RAD, USA) was used.

Statistical analyses

Statistical analyses of the data were conducted using GraphPad Prism 5 software. All data were expressed as mean ± s.d. (n=3) except special indication. Differences were considered to be statistically significant at $P<0.05$.

Results

PEITC reduced cell viability of rat C6 glioma cells

To evaluate the effects of PEITC on the proliferation of rat C6 glioma cells, cells were plated in 96-well plate at a density of 5×10^3 and then treated with various concentrations (0, 5, 10, 15, 20, 25, 30, 35 μM) of PEITC for 12 h, 24 h and 48 h. Cell viability was assessed by MTT assay. PEITC obviously reduced the cell viability of rat C6 glioma cells in a dose- and time-dependent manner (**Figure 1**). The IC50 values of PEITC for 12 h, 24 h and 48 h were 27.61, 24.37, 14.61 μM, respectively. In the following experiments, we choose the concentrations of 15, 20 and 25 μM of PEITC.

PEITC induced mitochondria-dependent apoptosis of rat C6 glioma cells

We next asked whether PEITC reduced cell viability of rat C6 glioma cells by inducing apoptosis. Firstly, cells were treated with various concentrations of PEITC (0, 15, 20, 25 μM) for 24 h and cell morphology was assessed. The results showed that PEITC dose-dependently induced apoptotic morphological changes of rat C6 glioma cells as demonstrated by smaller, round

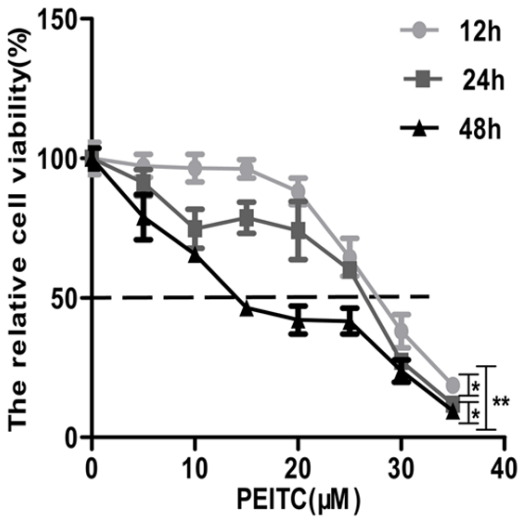

Figure 1. PEITC reduced cell viability of rat C6 glioma cells. Cells were treated with the indicated concentrations of PEITC for 12 h, 24 h and 48 h. Cell viability was determined by MTT assay. *P<0.05, **P<0.01.

and blunt cells compared to untreated control induced (**Figure 2A**). Secondly, Hoechst 33258 staining showed there was increased number of cells stained blue in cells treated with PEITC compared control (**Figure 2B**). Moreover, Annexin V/PI flow-cytometry assay showed PEITC triggered rat C6 glioma cell apoptosis in a dose-dependent manner (**Figure 2C**). Caspases are in charge of apoptotic signaling and caspase-9 and caspase-3 are directly activated in mitochondrial pathway [20]. Caspase activity assay demonstrated that both caspase-9 (**Figure 2D**) and caspase-3 (**Figure 2E**) were significantly activated by PEITC in a dose-dependent manner in rat C6 glioma cells treated with different concentrations of PEITC for 24 h. Finally, we treated rat C6 glioma cells with 15, 20, 25 μM of PEITC or 25 μM PEITC in combination with 10 μM caspases inhibitor Z-VAD-FMK for 24 h and performed cell viability by MTT assay. We found that Z-VAD-FMK significantly prevented the loss of cell viability by 25 μM PEITC (**Figure 2F**). These data implied that PEITC was capable of triggering mitochondria-dependent apoptosis pathway in rat C6 glioma cells.

PEITC suppressed tumorigenicity and migration ability of rat C6 glioma cells

To explore whether PEITC suppresses tumorigenicity and migration of rat C6 glioma cells in vitro, we treated rat C6 glioma cells with 15, 20, or 25 μM of PEITC and performed colony formation and migration assays. The results showed PEITC treated groups displayed significantly fewer and smaller foci of rat C6 glioma cells in a dose-dependent manner as compared with control (**Figure 3A**). **Figure 3B** showed that PEITC significantly and dose-dependently suppressed the migration ability of rat C6 glioma cells. These results indicate a significant anticancer effect of PEITC in rat C6 glioma cells in vitro.

PEITC depleted cellular GSH and promoted cellular ROS of Rat C6 glioma cells

Previous studies have shown that GSH depletion and ROS production are key early events for PEITC in activating mitochondria-dependent apoptosis pathway [7, 21]. We thus measured the cellular GSH and ROS in rat C6 glioma cells after treatment with PEITC. Cellular GSH in rat C6 glioma cells were dramatically depleted in a dose- and time-dependent manner after exposure to PEITC (**Figure 4A** and **4B**). Conversely, fluorescent microscopy analysis showed that PEITC increased positive DCFH-DA staining of rat C6 glioma cells and the rate of stained cells was increased with the increase of the concentration of PEITC (**Figure 4C** and **4D**). Moreover, the rate of stained cells was increased with the elongation of the treatment time of 20 μM PEITC (**Figure 4E** and **4F**), indication of induction of ROS by PEITC. These results suggest that GSH and ROS play an important role in PEITC triggered-apoptosis in rat C6 glioma cells.

ROS-induced miR-135a upregulation played a pivotal role in PEITC mediated rat C6 glioma cells apoptosis

It has been reported that miR-135a promotes the death of rat C6 glioma cells through activating mitochondria-dependent apoptosis pathway [20] and could be upregulated by chemicals-induced ROS [18]. The induction of ROS by PEITC suggests that PEITC may trigger rat C6 glioma cell apoptosis through ROS-upregulated miR-135a, which in turn activates mitochondria dependent pathway. To this hypothesis, we treated rat C6 glioma cells with different concentrations (0, 15, 20, 25 μM) of PEITC for 24 h and determined the expression levels of miR-135 by RT-PCR. The results showed that miR-135a levels were obviously upregulated by PEITC in rat C6 glioma cells in a dose-dependent manner (**Figure 5A**). To test whether the upregulation of miR-135a is due to the induction of ROS, we treated rat C6 glioma cells with 20 μM PEITC alone or in combination with 7.5

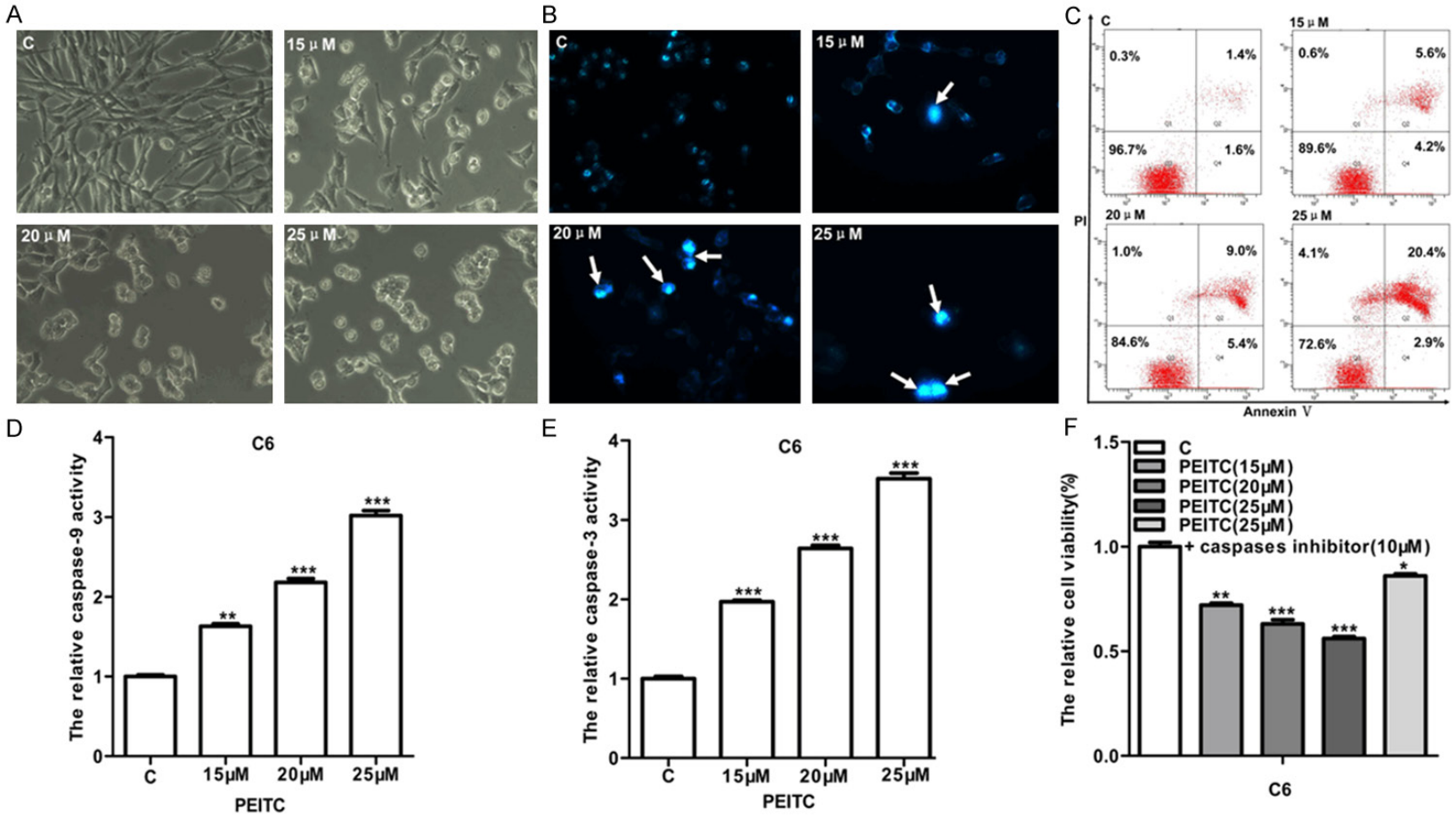

Figure 2. PEITC induced mitochondria-dependent apoptosis in rat C6 glioma cells. Cells were treated with different concentrations (15, 20, 25 μM) of PEITC for 24 h. A. Cells morphological changes were examined under phase contrast microscope at 20×. B. Cells were stained with Hoechst 33258 and detected by fluorescent photomicrographs at 20×. C. Cells were labeled with Annexin V/PI and detected by flow cytometry. Right lower and upper quadrant showed apoptotic cells. D, E. The activity of caspase-9 and caspase-3 were measured. F. Cells were treated with 15, 20, 25 μM of PEITC or 25 μM PEITC in combination with 10 μM caspases inhibitor Z-VAD-FMK for 24 h. Cell viability was assessed by MTT assay. Mean ± SD, n=3, *P<0.05, **P<0.01, ***P<0.001 VS control.

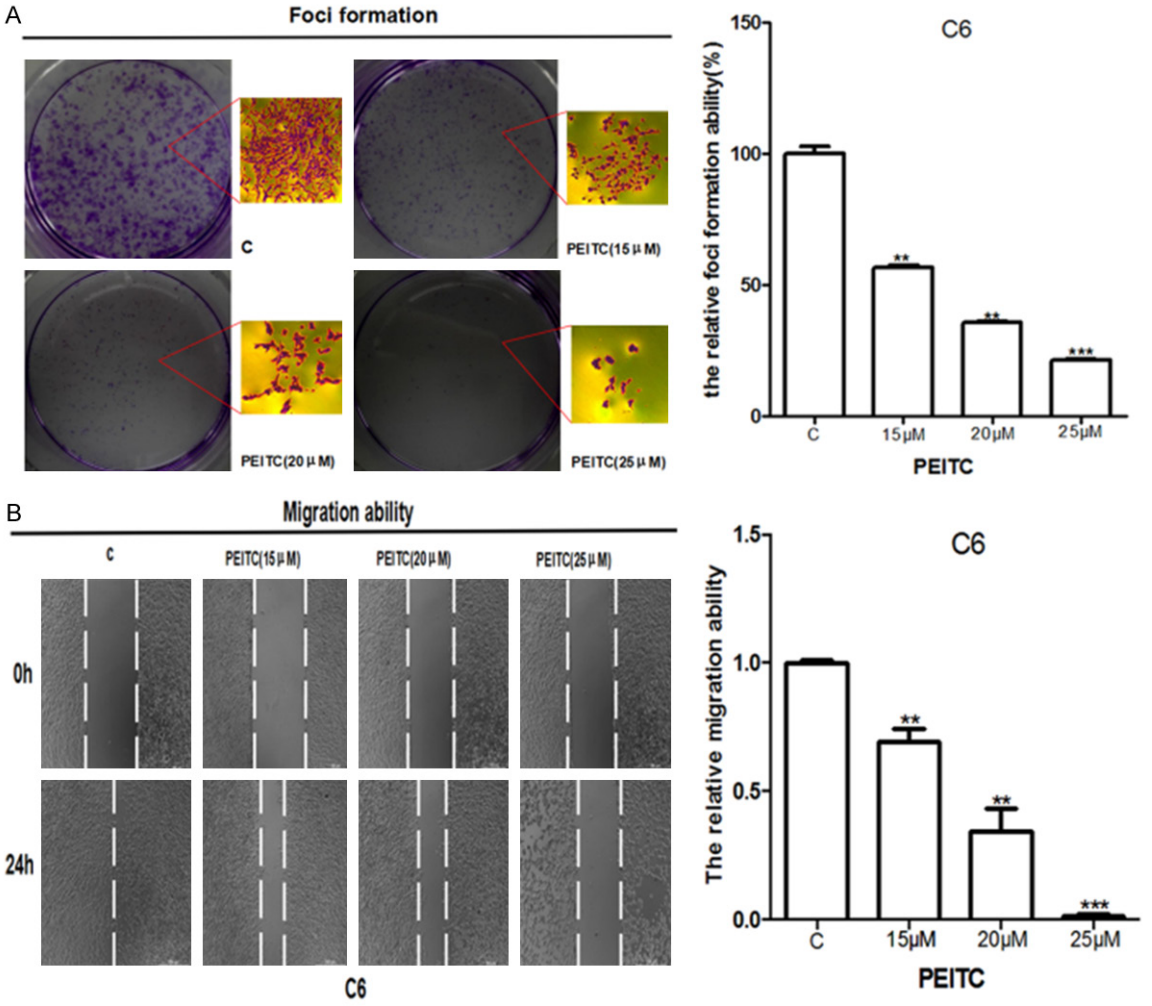

Figure 3. PEITC suppressed tumorigenicity and migration ability of rat C6 glioma cells in vitro. Cells were treated with different concentrations (15, 20, 25 μM) of PEITC. A. Colony formation was determined as described in materials and methods. B. The scratched area was determined as described in material and methods. Mean ± SD, n=3, **P<0.01, ***P<0.001 VS control.

mM GSH, a scavenger of ROS, for 24 h and determined the expression levels of miR-135 by RT-PCR. We found GSH dramatically abolished the induction of miR-135a by PEITC (**Figure 5B**). To support that miR-135a upregulation contributes to PEITC induced apoptosis in rat C6 glioma cells, we treated rat C6 glioma cells with 20 μM PEITC alone or in combination with either 7.5 mM GSH or 50 nM inhibitor for 24 h and determined the activity of caspase 9 and 3. MiR-135a inhibitor partially but significantly prevented, while GSH completely reversed the induction of the activity of caspase 9 and 3 by PEITC (**Figure 5C** and **5D**). STAT6 and SMAD5 are two targets of miR-135a and play critical roles in miR-135a-mitochondria dependent apoptosis pathway through regulating Bcl-xl and Bax [20]. To further support our observation, we treated rat C6 glioma cells with 20 μM PEITC alone or in combination with either 7.5 mM GSH or pre-transfection with 50 nM miR-135a inhibitor and then with 20 μM PEITC for 24 h and determined the protein levels of STAT6, SMAD5, Bcl-xl, Bax and Cytochrome C by immunoblotting. The results showed that PEITC downregulated STAT6, SMAD5 and Bcl-xl, which were partly and completely reversed by miR-135a inhibitor and GSH, respectively. In contrast, PEITC upregulated Bax and Cytochrome C, which were significantly abolished by either miR-135a inhibitor or GSH (**Figure 5E**). Moreover, miR-135a inhibitor and GSH also partly and completely reversed the loss of cell viability by PEITC, respectively (**Figure 5F**). Taken together, these results indicated that ROS-miR-135a-mitochodria pathway plays a pivotal

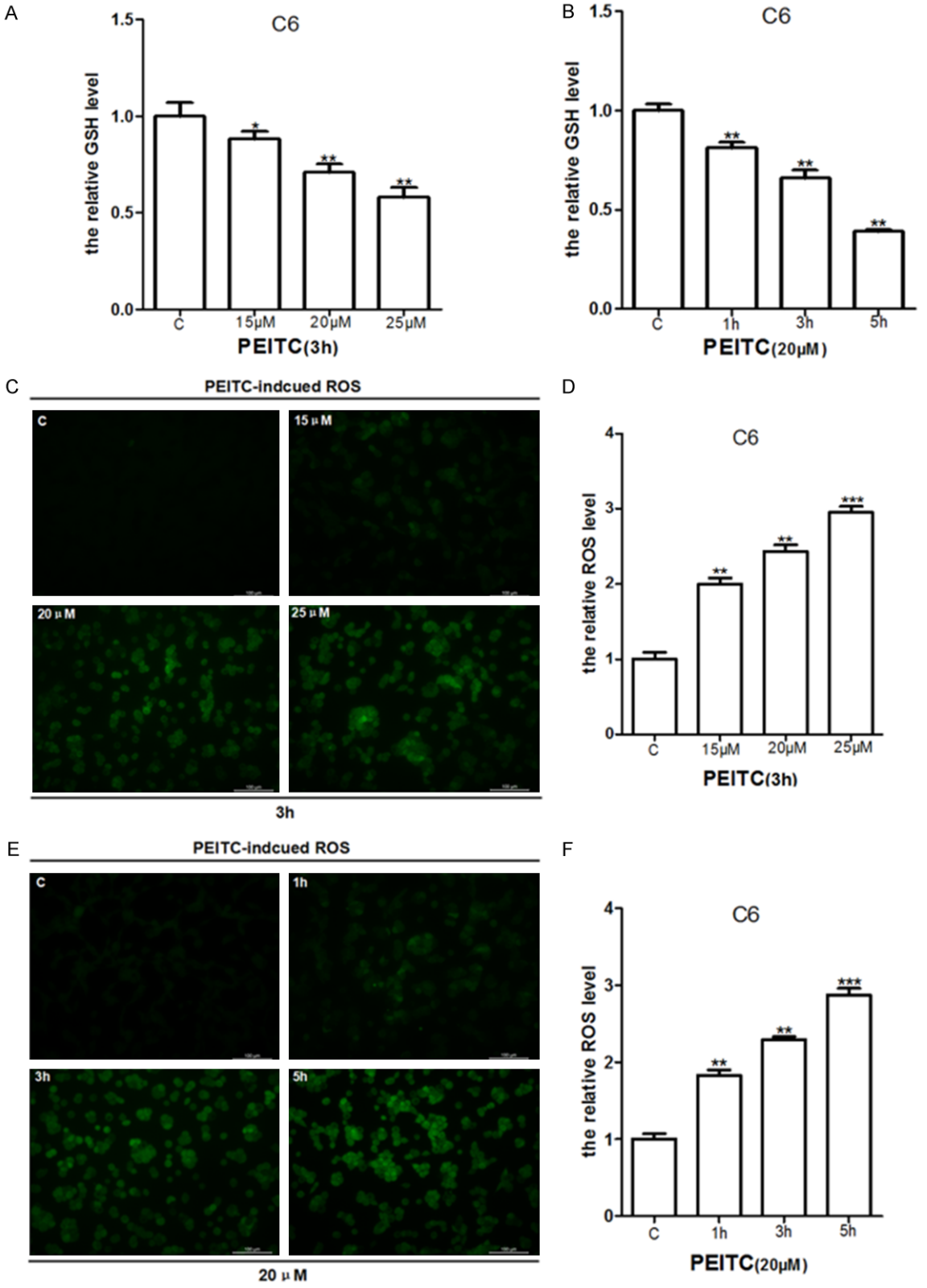

Figure 4. PEITC induced cellular GSH depletion and ROS production of rat C6 glioma cells. Cells were treated with the indicated concentrations of PEITC for different time periods. A, B. Cellular GSH changes were measured by GSH Quantification Kit. C, D. Cellular ROS production was detected using fluorescent microscopy after staining with DCFH-DA probe. E, F. Cellular ROS production was measured by spectrofluorimeter after staining with DCFH-DA probe.

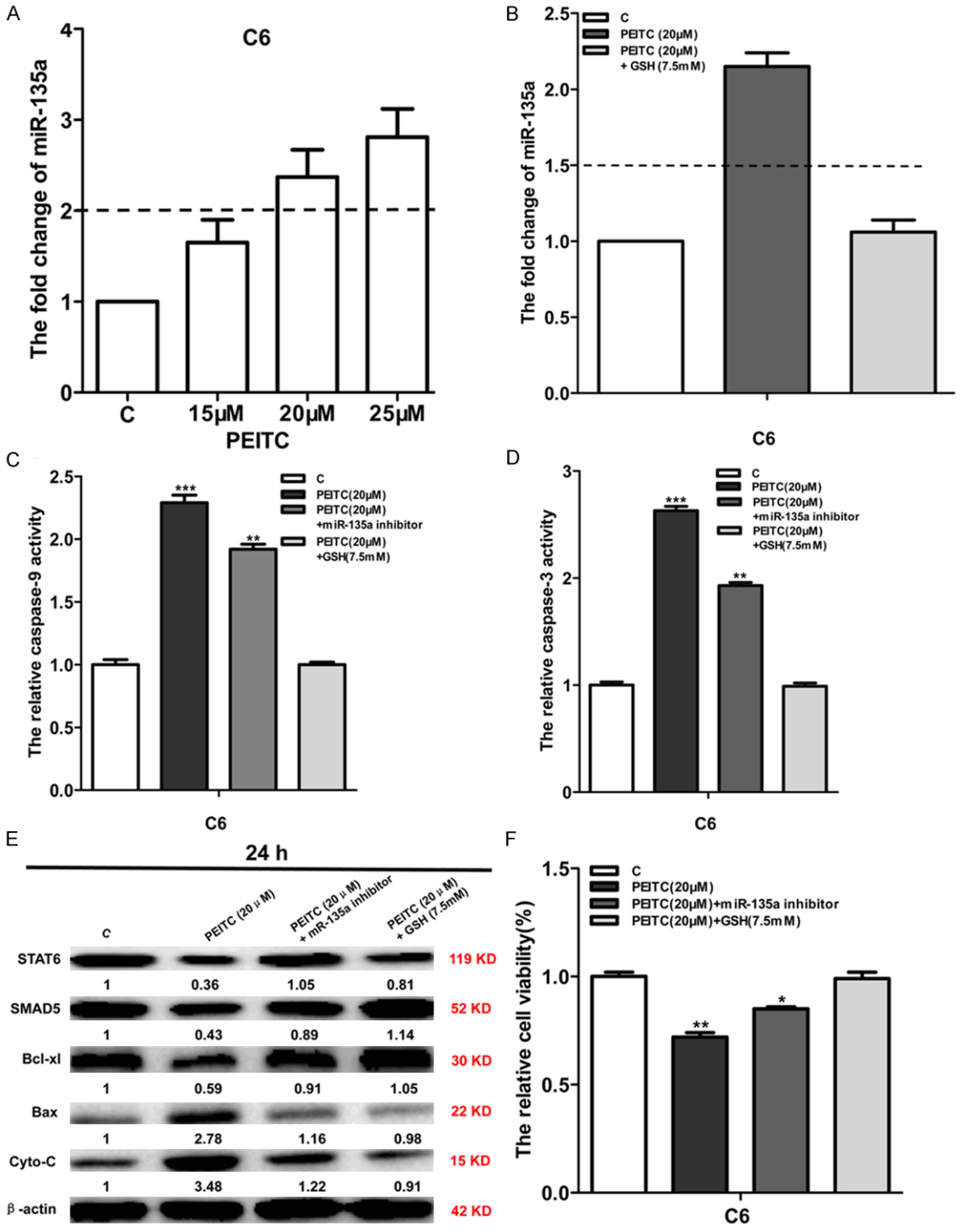

Figure 5. ROS-miR-135a-mitochondria dependent pathways played a pivotal role in PEITC-induced rat C6 glioma cell apoptosis. (A) Cells were treated with different concentrations (0, 15, 20, 25 μM) of PEITC for 24 h. The relative quantities levels of miR-135a were detected by RT-PCR. (B) Rat C6 glioma cells were treated with 20 μM PEITC alone or in combination with 7.5 mM GSH, a scavenger of ROS, for 24 h. The relative quantities of miR-135 were determined by RT-PCR. C-F. Rat C6 glioma cells were treated with 20 μM PEITC alone or in combination with either 7.5 mM GSH or pre-transfection with 50 nM miR-135a inhibitor then with 20 μM PEITC for 24 h, followed by the detection of the activity of caspase 9 (C) and caspase 3 (D). The protein levels of STAT6, SMAD5, Bcl-xl, Bax and Cytochrome C were determined by immunoblotting with β-actin as loading control (E). Cell viability was assessed by MTT assay (F), Mean ± SD, n=3, fold change >2 regarded as significant difference and *P<0.05, **P<0.01, ***P<0.001 VS control.

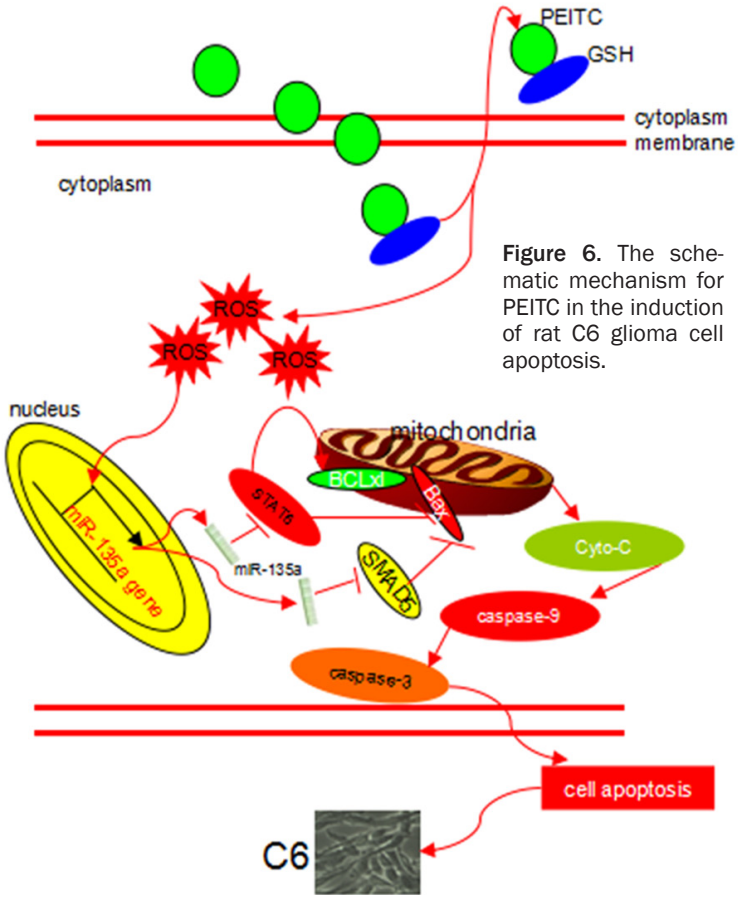

Figure 6. The schematic mechanism for PEITC in the induction of rat C6 glioma cell apoptosis.

role in PEITC triggered rat C6 glioma cells apoptosis.

Discussion

In the present study, we found that PEITC induced apoptosis while suppressed colony formation and migration ability of rat C6 glioma cells. Mechanistically, our findings demonstrated that PEITC exerted its anti-tumor functions through ROS-miR-135a-mitochondria dependent apoptosis pathways. These results suggest that PEITC is an effective agent for the treatment of glioma.

Various studies have demonstrated that PEITC has broad-spectrum antitumor effects mainly through inducing ROS production to activate mitochondria-dependent apoptosis pathways in cancer cells [21]. It was proposed that PEITC enters into cells through the passive diffusion and binds GSH (an important anti-ROS element in cells) leading to GSH depletion with the help of transporters such as MRP1, MRP2 and BCRP and accordingly ROS production [4]. The accumulation of ROS may result in mitochondria damage thereby activation of caspases-dependent apoptosis pathway. Herein, we found PEITC induced GSH depletion and ROS production, accompanied with apoptosis in Rat C6 glioma cells.

In recent years, various studies have revealed that miRNAs play critical roles for anti-cancer agents in eliminating cancer cells. For example, miR-124 and miR-506 were reported to augment responses to chemotherapy through regulating the double-strand DNA damage repair genes such as RAD51 and PARP1 in breast cancer, osteosarcoma and ovarian cancer cells [22, 23]. MiR-27a was reported to play pivotal roles in ROS-inducing agents such as Curcumin and Celastrol in triggering cancer cell apoptosis through targeting ZBTB10, thereby down-regulating Sp1, Sp3 and Sp4 in colon and bladder cancer cells [15, 16]. MiR-491 was reported involved in Arsenic trioxide (As2O3) in attenuating the invasion potential of human liver cancer cells through the demethylation-activated effect and targeting MMP2 [19]. Herein, we found PEITC-induced miR-135a upregulation played a key role in PEITC-triggered rat C6 glioma cell apoptosis. Wu et al. previously found that miR-135a stimulates rat C6 glioma cell apoptosis through targeting STAT6 and SMAD5 [20]. We revealed that miR-135a was involved in PEITC-induced rat C6 glioma cell apoptosis. Most importantly, our study revealed that the PEITC-induced ROS may be a key factor in the upregulation of miR-135a by PEITC. Many studies have shown that chemical-induced ROS increase the expression of microRNAs in malignant cancer cells. For example, TRAIL in combination with Tanshinone I induces ROS, accompanied with upregulation of miR-135a-3p in prostate cancer [18]; As2O3 which is a strong ROS inducer could restore miR-491 in human liver cancer cells [19, 24]. As for the mechanisms of ROS in reactivating microRNAs in cancer cells, it was reported that

ROS could regulate DNA methyltransferases (DNMTs) to restore hyper-methylation silenced tumor suppressors [25]. MiR-135a functions as tumor suppressor in malignant glioma cells and is negatively correlated with the glioma grading [20]. It is thus possible that miR-135a may be silenced by hyper-methylation and PEITC-induced ROS upregulation of miR-135a in rat C6 glioma cells may depend on demethylation effect. Further studies are needed to test this hypothesis in the future.

In conclusion, our studies demonstrated that PEITC has potent anti-cancer effect through the inhibition of cell viability, induction of cell apoptosis and suppression of tumorigenicity and migration in rat C6 glioma cells and the possible signaling pathways are summarized (**Figure 6**). PEITC eliminating rat C6 glioma cells could be at least in part through depleting GSH and accordingly ROS production leading to restoring miR-135a expression, which could target STAT6 and SMAD5 leading to Bcl-xl downregulation and Bax and Cytochrome C upregulation and activating caspase-9 and caspase-3 dependent apoptosis pathway. Taken together, our findings reveal ROS-miR-135a-mitcohondria dependent apoptosis pathways play a pivotal role in PEITC performing the anti-cancer effects on rat C6 glioma cells, providing a potential treatment way for glioblastoma.

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Disclosure of conflict of interest

None.

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