

Original Article

In vitro studies of phenethyl isothiocyanate against the growth of LN229 human glioma cells

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Received January 28, 2015; Accepted March 23, 2015; Epub April 1, 2015; Published April 15, 2015

Abstract: Phenethyl isothiocyanate (PEITC) is one of the best studied members of isothiocyanates (ITC), a variety of edible cruciferous vegetables including broccoli, watercress, and cabbage, and have generated particular interest because of its remarkable chemopreventive activity. Many literature reports proved that phenethyl isothiocyanate exhibited significant anti-cancer chemopreventive effects including lung, glioma and leukemia cancer. In this study, we explored the inhibitory effects as well as mechanisms of PEITC on human glioma LN229 cells. Results demonstrated that PEITC possesses the potential ability to inhibit proliferation, induce apoptosis and arrest cell cycling against LN229 human glioma cells. Moreover, investigated results showed that PEITC inhibited the expression of superoxide dismutase (SOD) and glutathione (GSH), and caused oxidative stress to tumor cells. Collective results suggested us to believe that PEITC can inhibit the growth of LN229 cells and its mechanism can be related to the fact that PEITC can cause oxidative stress to tumor cells.

Keywords: Phenethyl isothiocyanate, LN229 cells, glioma

Introduction

Glioblastoma is considered to be the most common malignant tumor of adult central nervous system and is hallmarked by high proliferation of tumor cells with increased cellularity and necrosis. Glioma is a highly invasive, rapidly spreading form of brain cancer that is resistant to surgical and medical treatment. Gliomas are a disease can occur in all age groups, especially for elderly population, while a small percentage of patients are genetically predisposed to develop glioblastomas, the disease occurs sporadically with no known underlying cause. Gliomas account for 40-60 percent of primary brain tumors including astrocytoma, glioblastoma, lymphoma, meningioma, schwannoma [1]. Malignant gliomas are notoriously resistant to currently available therapies because these cancer cells can't be induced to undergo apoptosis upon anticancer treatment [2].

Isothiocyanates, plant-derived dietary compounds, are promising chemopreventive agents since they are generally non-toxic substances that interfere with the process of cancer development or carcinogenesis. Several natural and synthetic isothiocyanates have demonstrated cancer-preventive properties in animals treated with chemical carcinogens, including polycyclic-aromatic hydrocarbons and nitrosamines [3-5]. Exposure to ITCs can reduce carcinogen induced tumor formation in a diverse range of organs such as lung, mammary gland, esophagus, liver and intestine [6, 7]. Moreover, Isothiocyanates are chemicals that contain a reactive thiocyanate group (-N=C=S) and are formed by hydrolysis of glucosinolates [8]. These chemicals are absorbed through the intestinal membranes via passive diffusion and bind to plasma protein thiols. Once isothiocyanates enter the cell, glutathione S-transferases convert the isothiocyanates into glutathione conjugates that

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ultimately lead to the depletion of intracellular glutathione. The isothiocyanates that were not converted into glutathione conjugates react with cellular proteins by thiocarbonylation, and when coupled with depletion of intracellular glutathione, signal transduction pathways and apoptosis are activated [9].

Phenethyl isothiocyanate (PEITC) is one of the best studied members of the ITC family and has generated particular interest because of its remarkable chemopreventive activity. PEITC is routinely consumed in the diet as its glucosinolate precursor. PEITC acts to inhibit chemically induced cancers by affecting carcinogen metabolism through alterations of phase I/II enzymes [10-12]. According to Hwang and Lee, the glucosinolate, gluconasturtiin, is hydrolysed to produce PEITC [13]. When the aromatic compound is absorbed and metabolized within the body, it undergoes glutathione conjugation followed by conversion to produce a conjugate of N-acetylcysteine. It is believed that PEITC inhibits carcinogenesis induced by carcinogens such as, 12-dimethylbenz [a] anthracene, benzo [a] pyrene, diethylnitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and methylbenzyl nitrosamine. Accumulating evidence suggests that PEITC can suppress the proliferation of cancer cells in culture by causing cell cycle arrest and/or apoptosis but the exact mechanism of their anti-tumor activity has not yet been fully understood. Therefore, this study is intended to explore the inhibition effect of phenethyl isothiocyanate (PEITC) on LN229 human glioma cells, as well as its potential mechanism to provide more scientific information for future research.

Materials and methods

Cell culture

Human glioma LN229 cell lines were obtained from the Shanghai Cell Bank of the Chinese Academy of Medical Science (Shanghai, China). The LN229 cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Gibco Inc., Billings, MT, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Beiyotime, Shanghai, China) in an incubator containing 5% CO₂ at 37°C.

Detection of cell proliferation with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell proliferation was determined by an MTT assay. For this, the LN229 cells were seeded on a 96-well plate at a density of 5×10³ cells/well under 5% CO₂ at a temperature condition of 37°C for 24 h. After the cells were cultured, PEITC was added with the final concentration of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75 μM respectively into the cell samples and PBS was added into the negative control group and incubated for 72 h. After incubation, the supernatant was discarded and 200 μL plasma-free medium and 50 μL MTT solution were added into the cell samples, respectively and cultured for another 4 h. The absorbance at 490 nm was determined using an automatic Enzyme Labeling instrument (Beijing Putian Instrument Ltd., Beijing, China). A cell proliferation curve was generated. Experiments were repeated for three times to get the mean value.

Apoptosis

The human glioma LN229 cells were cultured on a 6-well plate at a density of 2×10⁵ cells/well under the conditions of 37°C, 5% CO₂ for 24 h. Then, added with 10 μM and 20 μM BITC into the cell samples respectively; added PBS into the negative control group. 24 h later, after being digested by trypsin, 100 μL of cell suspension was added with Annexin-V-FITC and 10 μL PI and incubated for 10 min in dark environment. Cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell cycle distribution

The human glioma LN229 cells were seeded on a 6-well plate at a density of 2×10⁵ cells/well under the conditions of 37°C, 5% CO₂ for 24 h. Then, 2 μM and 5 μM PEITC were added into the cell samples respectively and only PBS into the negative control group. After 24 h, the sample was collected and precooled with 70% cold ethanol over night at 4°C. After washing with phosphate-buffered saline (PBS), RNAase A (125 U/ml; Molecular Probes, Eugene, OR, USA) and propidium iodide (50 μg/ml; Molecular Probes) were added, and cells were incubated in the dark at 4°C for 30 min. Cells were analyzed using a FACS Calibur flow cytometer (BD

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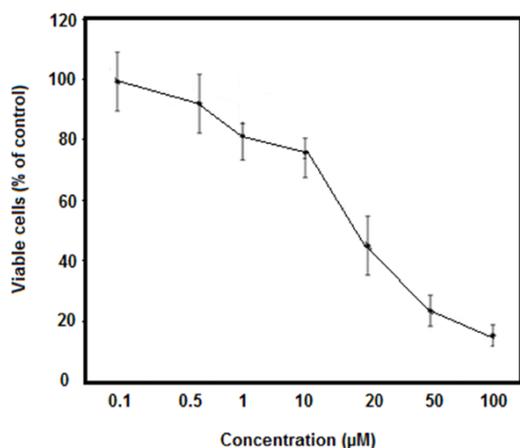


Figure 1. The inhibitory effect of PEITC on the LN229 cell proliferation. The data was presented as mean \pm SD, bars indicate SD, n=6.

Biosciences, Franklin Lakes, NJ, USA), and cell cycle distribution (in the G0/G1, S and G2/M phases) was calculated using the ModFit LT 3.2 software (BD Biosciences).

ROS expression

The human glioma LN229 cells were seeded on a 6-well plate at a density of 2×10^5 cells/well under the conditions of 37°C, 5% CO₂ for 24 h. Then, added 2 µM and 5 µM PEITC into the cell samples respectively and added PBS into the negative control group. 24 h later, after being digested with trypsin, 100 µl cell suspension were added with 5 µL intracellular reactive oxygen detection probe DCFH-DA and incubated at room temperature under dark conditions for 30 min. Then, the cells were analyzed using a FACS Calibur flow cytometer.

GSH expression

The human glioma LN229 cells were seeded on a 6-well plate at a density of 2×10^5 cells/well under the conditions of 37°C, 5% CO₂ for 24 h. Then, added 2 µM and 5 µM PEITC into the cell samples respectively and added PBS into the negative control group. 24 h later, after being digested with trypsin, the cells were lysed and then, the enzyme-labeled meter were used to detect the intracellular GSH expression as per the operation procedures.

SOD activity

The human glioma LN229 cells were seeded on a 6-well plate at a density of 2×10^5 cells/well

under the conditions of 37°C, 5% CO₂ for 24 h. Then, added 2 µM and 5 µM PEITC into the cell samples respectively and added PBS into the negative control group. After 24 h, the medium was replaced with the fresh plasma-free medium and cultured for another 24 h. Then the supernatant was extracted and used the enzyme-labeled meter to detect the intracellular SOD activity as per the operation procedures.

Caspase-3 activity

The human glioma LN229 cells were seeded on a 6-well plate at a density of 2×10^5 cells/well under the conditions of 37°C, 5% CO₂ for 24 h. Then, added 2 µM and 5 µM PEITC into the cell samples respectively and added PBS into the negative control group. 24 h later, after being digested with trypsin, the cells were lysed and centrifuged at 4°C and extracted the supernatant to obtain the total protein of tissues. Electrophoresis were performed using 12% SDS polyacrylamide gel and then, transferred the protein samples to a PVDF membrane and then used 5% skimmed milk to seal the samples and rest for overnight at 4°C followed by the addition of first antibody (1:500) and β -actin (1:5000) into the samples, respectively. Then, incubated the samples at 4°C for overnight. IgG (1:2000) were used to label with horseradish peroxidase to incubate the samples at room temperature for 1 h and then finally the images were developed.

Statistical methods

All data are expressed as the mean \pm standard deviation and analyzed with an SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare the data by considering $*P < 0.05$ as the standard of statistically significant. Each experiment was repeated for three times.

Results

Effect of PEITC on proliferation of human glioma LN229 cells

The MTT assay was performed to observe the proliferation effect of PEITC on in-vitro growth of human glioma LN229 cells. The treatment of PEITC on human glioma LN229 cells for 72 h showed that the cell proliferation was inhibited and exhibited the dose-dependent manner with IC₅₀ value equal to 1.25 µM (**Figure 1**). The inhibition rate was less when treated with 10 µM/L

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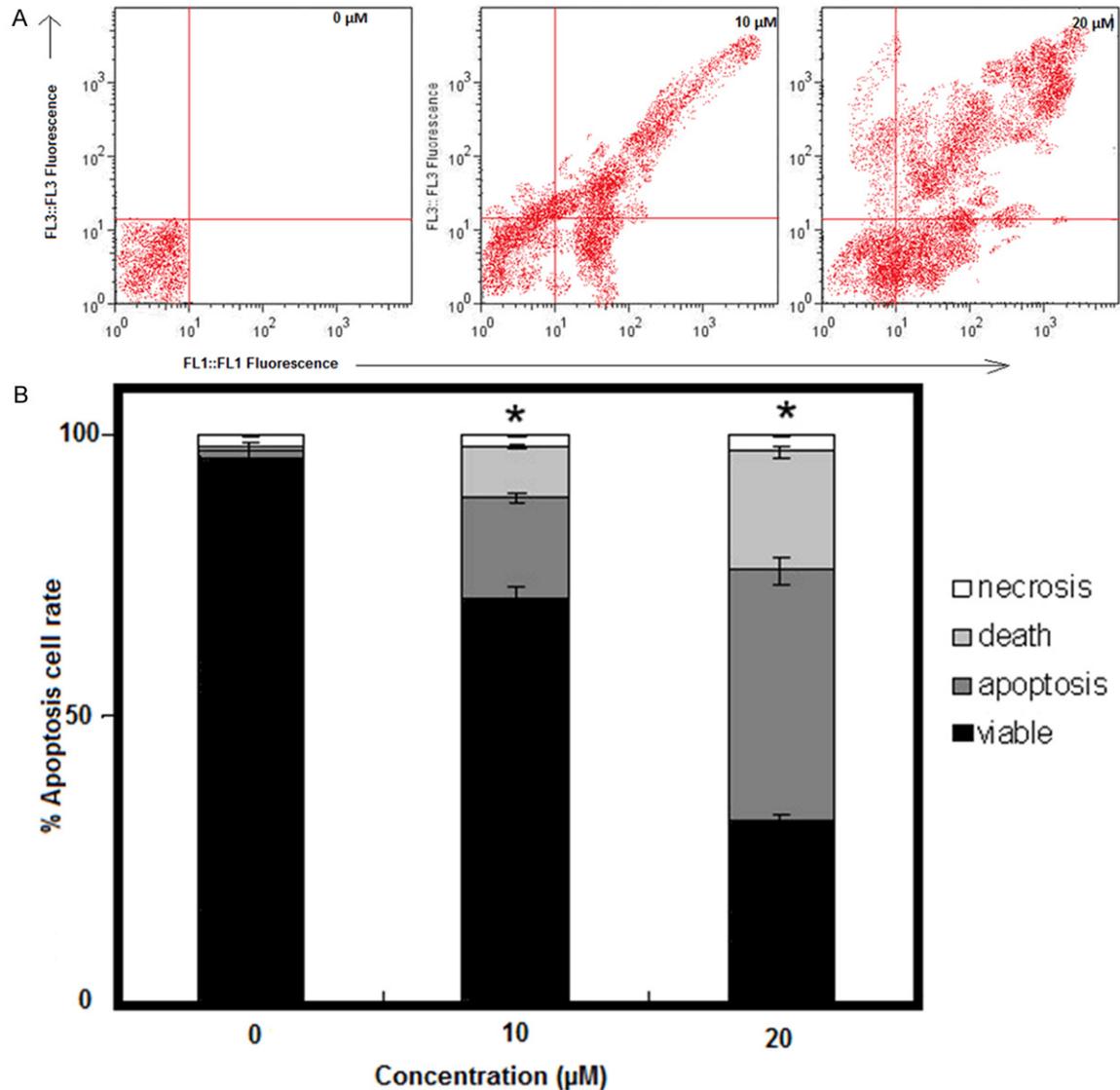


Figure 2. The apoptosis-induced effect of PEITC on the LN229 Cell Line. A. The flow cytometry results in apoptosis effect-induced of LN229 cells. B. The apoptosis effect of PEITC on the LN229 cell line. Bars indicate SD. n=3. * $P < 0.05$ compared with control group.

PEITC and 20 μM/L PEITC for 24 h and found to be non-toxic and low-toxic, respectively. Hence, we decided to use 10 μM/L and 20 μM/L concentration for cell cycle analysis, the ROS, SOD and GSH expression/activity and the caspase-3 activity, in order to eliminate the interference of cell growth inhibition and apoptosis for these experiments.

Effects of PEITC on apoptosis of human glioma LN229 cells

The flow cytometry results in apoptosis effect induced by LN229 cells and the apoptosis

effect of PEITC on the LN229 cell lines are shown in **Figure 2**. The Annexin-V-FITC and PI double staining test after treating the cells with PEITC for 24 h showed that the apoptosis rate caused by 10 μM and 20 μM PEITC on the LN229 cells were found to be 30.3% and 64.9% respectively, which was 5% significantly higher than that of the control group ($P < 0.05$).

Effects of PEITC on cell cycle arrest of human glioma LN229 cells

The flow cytometry results in cell cycle arrest of LN229 cells and the cell cycle arrest of PEITC

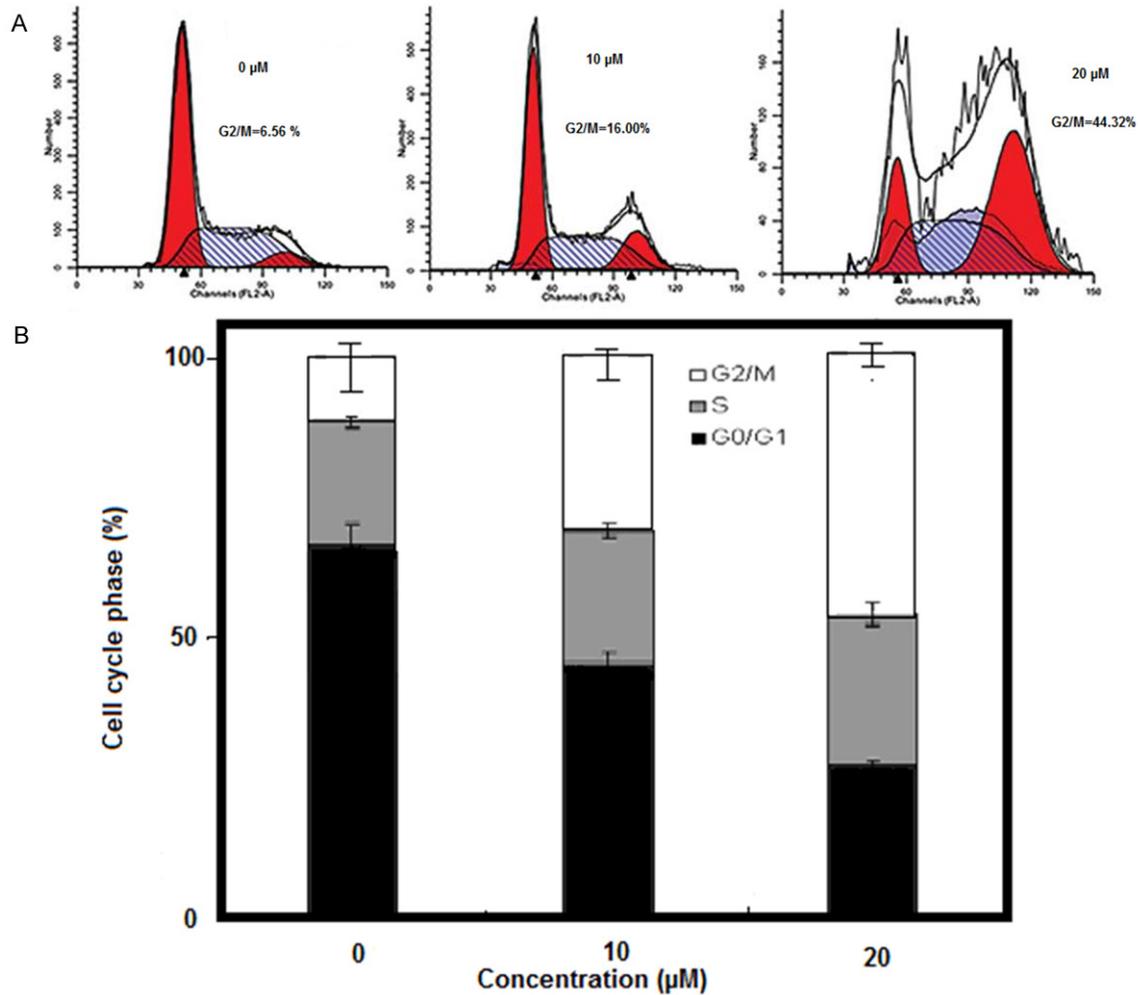


Figure 3. The effect of PEITC on the cell cycle of LN229 Cell Line. A. The flow cytometry results in cell cycle effect of LN229 cells. B. The cell cycle effect of PEITC on the LN229 cell line. Bars indicate SD. n=3. *P < 0.05 compared with control group.

on the LN229 cell lines are shown in **Figure 3**. The measurement results of the flow cytometer showed that after treating the cells with 10 μM and 20 μM BITC for 24 h, the cell cycle distribution of the tumor was changed ($P < 0.05$). Specifically, the proportion of phase G2/M was found to be increased whereas the proportion of phase G0/G1 was decreased ($P < 0.05$) and suggested that PEITC can arrest the cell cycle of LN229 cells at phase G2/M.

Effects of PEITC on ROS expression of human glioma LN229 cells

The flow cytometry results in ROS expression of LN229 cells and the ROS expression of PEITC on the LN229 cell lines are shown in **Figure 4**. The results of the flow cytometer showed that after treating the LN229 cells with 10 μM and

20 μM PEITC for 24 h, the ROS expression of the tumor cells was significantly increased and specifically, the ROS expression of the 10 μM group and 20 μM group was 4-fold and 6-fold higher than that of the control group, respectively ($P < 0.05$) (**Figure 4**).

Effects of PEITC on GSH expression and SOD expression of human glioma LN229 cells

The effect of PEITC on expression of GSH in the LN229 cell lines and the effect of PEITC on activity of SOD in the LN229 cell lines are shown in **Figure 5**. The results showed that after treating the LN229 cells with 10 μM and 20 μM PEITC for 24 h, the GSH expression and SOD expression of the tumor cells were found to be significantly decreased. The GSH expression of the 10 μM group and 20 μM group was

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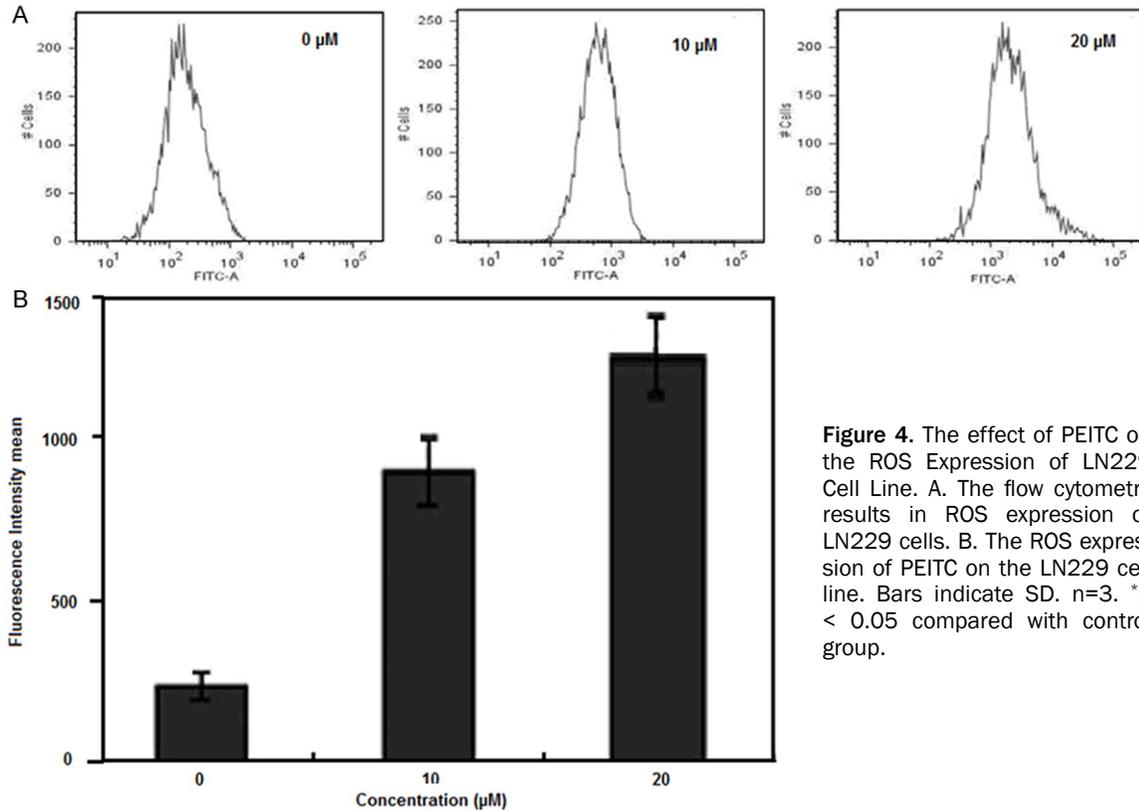


Figure 4. The effect of PEITC on the ROS Expression of LN229 Cell Line. A. The flow cytometry results in ROS expression of LN229 cells. B. The ROS expression of PEITC on the LN229 cell line. Bars indicate SD. n=3. * $P < 0.05$ compared with control group.

69.3% and 42.4% lower than that of the control group, respectively ($P < 0.05$) whereas the SOD expression of 10 μM group and 20 μM group was found to be 60.7% and 20.6% lower than that of the control group, re-spectively ($P < 0.05$).

Effects of PEITC on caspase-3 activity of human glioma LN229 cells

Figure 6 showed the Western blot experimental result with increased cleaved caspase-3 expression in LN229 cell lines with different concentrations of PEITC treatment with β-actin as an internal control of loading. The result showed that after treating the LN229 cells with 10 μM and 20 μM PEITC for 24 h, the caspase-3 activity of the tumor cells was significantly found to be decreased ($P < 0.05$).

Discussion

Malignant gliomas rank among the most lethal cancers. Gliomas display a striking cellular heterogeneity with a hierarchy of differentiation states. Treatment of glioma includes surgery, radiation and chemotherapy. However, the median survival rate of glioma patients is only

915 months [14, 15]. Furthermore, the therapeutic effects of such treatments are limited, particularly for high grade gliomas, where the incidence and mortality rate remain high with a postoperative median survival time of less than one year [16]. Difficulty in completely removing tumors and the recurrence of cancer after treatment remain a significant barrier to long-term survival. To overcome these challenges, focus on the development of novel therapies for the treatment of glioma is required. Studies have shown that glioma is in part caused by deregulation of the Wnt signaling pathway and this deregulation is crucial in regulating processes, such as the initiation, proliferation and development of glioma cells [17-19]. Inhibition of activated abnormal tumor signaling pathways may be an effective therapy with which to kill tumor cells, suppress cell proliferation and induce cellular differentiation. This study observed that PEITC has an inhibition effect on the proliferation of LN229 cells and it can induce the apoptosis and also arrest the cell cycle of the human glioma LN229 cells, indicating that PEITC has the potential anti-tumor effect.

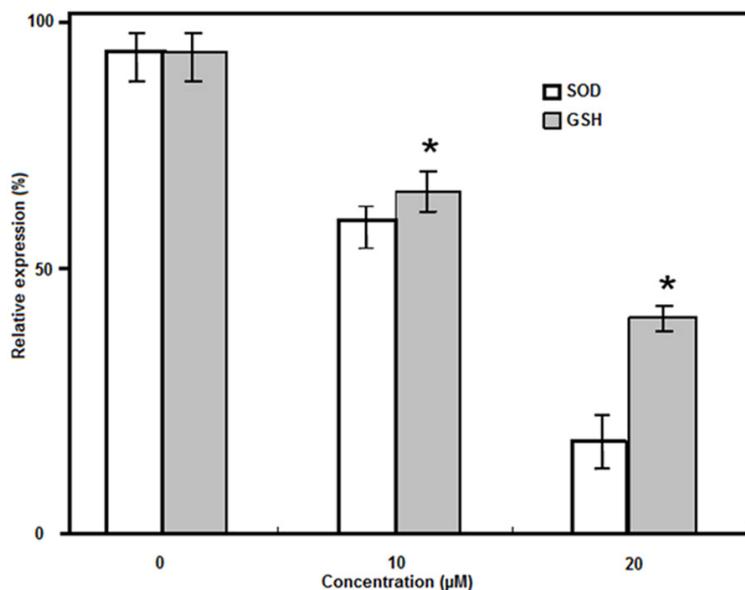


Figure 5. The effect of PEITC on GSH expression and SOD activity of LN229 cells.

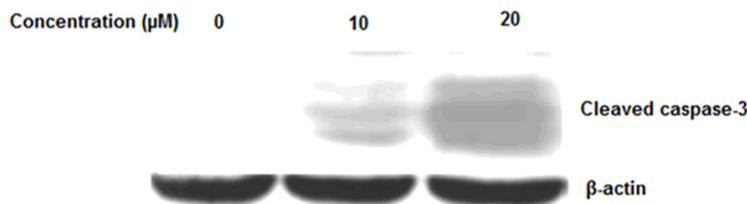


Figure 6. Western blot analysis.

Apoptosis is an important mechanism by which most current anticancer agents induce cancer cell death [20]. The specific apoptotic pathway activated by an anticancer drug is not only important to predict its efficacy but also to gain insight for improved design of therapeutic trials. Thus, we investigated whether PEITC induced apoptotic cell death events. This study showed that PEITC can arrest the cell cycle at phase G2/M, so that cells cannot enter phase G0/G1 and phase S to conduct DNA synthesis and repair. As a result, the tumor cells cannot enter the next cycle, and thereby, the proliferation of tumor cells can be inhibited and the cell apoptotic program is initiated.

Recent studies suggest that in contrast to the view of PEITC as an antioxidant, this could increase ROS generation and induce apoptosis in cancer cell lines [21-23]. However, whether PEITC has a selective effect against cancer cells and whether the intrinsic redox status

could affect the fate of cellular response to ROS-modulating agents remain unclear. Recently it has been suggested that oxidative stress has a pivotal role as a common mediator of apoptosis [24-26]. Both SOD and GSH two elements play an important role in the biochemical defense system of human body, able to remove free radical in the body. This study showed that PEITC can reduce SOD expression and GSH expression, which is helpful for strengthening the anti-tumor effect. Furthermore, it was observed that PEITC can raise ROS expression in the tumor cells and suggesting that PEITC can activate caspase-3 activity by affecting the cell cycle and inhibiting the SOD activity and the GSH expression, so as to accelerate the apoptosis of human glioma LN229 cells. Its mechanism can be related to the fact that PEITC can cause oxidative stress to tumor cells.

In conclusion, PEITC, served as a potential antitumor agent that can induce apoptosis through oxidative stress. However, further studies are required to investigate the complete mechanism of PEITC for cell cycle arrest and its impact on intracellular signal transduction, with an idea to provide an experimental basis for clinical trial in medicinal use.

Acknowledgements

This work was supported by research grants from the Second Affiliated Hospital, Taian Central Hospital, Liaocheng People's Hospital, Taishan Coal Sanatorium of Shandong Province and Qilu Hospital of Qingdao Branch.

Disclosure of conflict of interest

None.

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References

- [1] Laws ER, Thapar K. Brain tumors. *CA Cancer J Clin* 1993; 43: 263-271.
- [2] Wang WX, Ji YH. Scorpion venom induces glioma cell apoptosis in vitro and inhibits glioma tumor growth in vivo. *J Euro Oncol* 2005; 73: 1-7.
- [3] Chung FL. Chemoprevention of lung cancer by isothiocyanates and their conjugates in A/J mouse. *Exp Lung Res* 2001; 27: 319-330.
- [4] Conaway CC, Wang CX, Pittman B, Yang YM, Schwartz JE, Tian D, McIntee EJ, Hecht SS, Chung FL. Phenethyl isothiocyanate and sulforaphane and their N-acetylcysteine conjugates inhibit malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice. *Cancer Res* 2005; 65: 8548-8557.
- [5] Sticha KR, Kenney PM, Boysen G, Liang H, Su X, Wang M, Upadhyaya P, Hecht SS. Effects of benzyl isothiocyanate and phenethyl isothiocyanate on DNA adduct formation by a mixture of benzo (a) pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mouse lung. *Carcinogenesis* 2002; 23: 1433-1439.
- [6] Zhang Y, Talalay P. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. *Cancer Res* 1994; 54: 1976s-1981s.
- [7] Verhoeven DT, Verhagen H, Goldbohm RA, Van Poppel G. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. *Chem Biol Interact* 1997; 103: 79-126.
- [8] Kassie F, Knasmuller S. Genotoxic effects of allyl isothiocyanate (AITC) and phenethyl isothiocyanate (PEITC). *Chem Biol Interact* 2000; 127: 163-180.
- [9] Satyan KS, Swamy N, Dizon DS, Singh R, Granai CO, Brard L. Phenethyl isothiocyanate (PEITC) inhibits growth of ovarian cancer cells by inducing apoptosis: Role of caspase and MAPK activation. *Gynecol Oncol* 2006; 103: 261-279.
- [10] Conaway CC, Yang YM, Chung FL. Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. *Curr Drug Metab* 2002; 3: 233-255.
- [11] Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 2001; 56: 5-51.
- [12] Hecht SS. Inhibition of carcinogenesis by isothiocyanates. *Drug Metab Rev* 2000; 32: 395-411.
- [13] Hwang ES, Lee HJ. Effects of Phenethyl isothiocyanate and its metabolite on cell-cycle arrest and apoptosis in LNCaP human prostate cancer cells. *Int J Food Sci Nutr* 2010; 61: 324-336.
- [14] Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med* 2008; 359: 492507.
- [15] Louis DN. Molecular pathology of malignant gliomas. *Annu Rev Pathol* 2006; 1: 97117.
- [16] Claus EB, Black PM. Survival rates and patterns of care for patients diagnosed with supratentorial lowgrade gliomas: data from the SEER program, 19732001. *Cancer* 2006; 106: 13581363.
- [17] Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* 2013; 13: 1126.
- [18] Cerchia L, Martinez Montero JC, Monfared P. Signal transduction alterations in glioma: implications for diagnosis and therapy. *J Signal Transduct* 2012; 2012: 704247.
- [19] Mellinshoff IK, Lassman AB, Wen PY. Signal transduction inhibitors and antiangiogenic therapies for malignant glioma. *Glia* 2011; 59: 12051212.
- [20] Kim R, Emi M, Tanabe K, Uchida Y, Arihiro K. The role of apoptotic or nonapoptotic cell death in determining cellular response to anti-cancer treatment. *Eur J Surg Oncol* 2006; 32: 269-277.
- [21] Yu R, Mandlekar S, Harvey KJ, Ucker DS, Kong AN. Chemopreventive isothiocyanates induce apoptosis and caspase-3-like activity. *Cancer Res* 1998; 58: 402-408.
- [22] Zhang Y, Tang L, Gonzalez V. Selected isothiocyanates rapidly induce growth inhibition of cancer cells. *Mol Cancer Ther* 2003; 2: 1045-1052.
- [23] Wu SJ, Ng LT, Lin CC. Effects of antioxidants and caspase-3 inhibitor on the Phenethyl isothiocyanate-induced apoptotic signaling pathways in human PLC/PRF/5 cells. *Eur J Pharmacol* 2005; 518: 96-106.
- [24] Lee DH, Rhee JG, Lee YJ. Reactive oxygen species up-regulate p53 and Puma; a possible mechanism for apoptosis during combined treatment with TRAIL and wogonin. *Br J Pharmacol* 2009; 157: 1189-1202.
- [25] Hori YS, Kuno A, Hosoda R, Horio Y. Regulation of FOXOs and p53 by SIRT1 modulators under oxidative stress. *PLoS One* 2013; 8: e73875.
- [26] Zhong YJ, Liu SP, Firestone RA, Hong YP, Li Y. Anticancer effects of AcPhe Lys PABC doxorubicin via mitochondria centered apoptosis involving reactive oxidative stress and the ERK1/2 signaling pathway in MGC803 cells. *Oncol Rep* 2013; 30: 1681-1686.