

## Ellagic acid inhibits proliferation and induces apoptosis in human glioblastoma cells<sup>1</sup>

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### ABSTRACT

**PURPOSE:** To investigate the anticancer activity of ellagic acid (EA) in U251 human glioblastoma cells and its possible molecular mechanism.

**METHODS:** The cells were treated with EA at various concentrations for different time periods. Cell viability and cell proliferation were detected by cell counting kit-8(CCK-8) assay and live/dead assay respectively. Cell apoptosis were measured with Annexin V-FITC/PI double staining method by flow cytometry and Mitochondrial membrane potential assay separately. Cell cycle was measured with PI staining method by flow cytometry. The expressions of Bcl-2, Survivin, XIAP, Caspase-3, Bax, JNK, p-JNK, ERK1/2, p-ERK1/2, p38, p-p38, DR4, DR5, CHOP and GRP78-related proteins were detected by western blot after EA treatment.

**RESULTS:** Cell viability and proliferation of glioblastoma cells treated with EA were significantly lower than the control group. EA caused robust apoptosis of the glioblastoma cells compared to the control group. EA significantly decreased the proportion at G0/G1 phases of cell cycling accompanied by increased populations at S phase in U251 cell lines. And the expressions of anti-apoptotic proteins were dramatically down-regulated.

**CONCLUSION:** Ellagic acid potentially up-regulated DR4, DR5 and MAP kinases (JNK, ERK1/2 and p38). EA also caused significant increase in the expressions of CHOP and GRP78. Our findings suggest that EA would be beneficial for the treatment of glioblastoma.

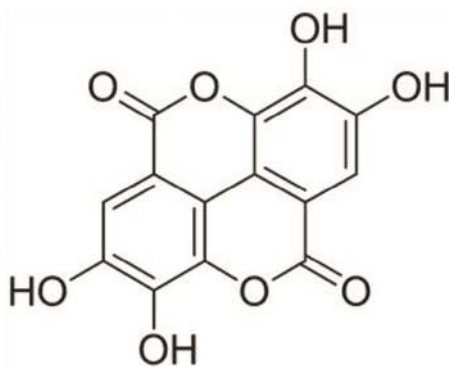
**Key words:** Glioblastoma. Ellagic Acid. Cell Proliferation. Apoptosis. Cell Cycle.

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## Introduction

Gliomas account for more than 50% of primary malignant tumors of the central nervous system with high mortality rates and high incidence rates<sup>1</sup>. World Health Organization (WHO) classifies gliomas to four grades (I-IV) with increasing degree of malignancy<sup>2</sup>. Grade IV, glioma-glioblastoma multiforme (GBM) is the most aggressive type of primary brain tumors with the median survival time of patients less than 14 months even with multimodal therapy comprising the combination of surgery, radiation therapy and chemotherapy<sup>3,4</sup>. The major reason is that the diffuse invasion of tumor cells invading the surrounding brain shelters themselves from surgery and radiation. The malignant glioma seems to proliferate indefinitely. Consequently, development of novel biologicals in brain tumor therapy is crucial.

Recently, the use of phytochemicals as anti-cancer agent has gained high importance. Many plant-derived compounds as curcumin<sup>5</sup>, gingerol<sup>6</sup> and silibinin<sup>7</sup>, have been reported to restrain tumor growth in a series of experiments. Ellagic acid (EA) is a natural phenol present in many fruits and vegetables as cranberries, strawberries, blackberries, raspberries, grapes, and walnuts *et al.*<sup>8</sup>. As a dimeric form of gallic acid, it comprises of a fused four-ring structure with four hydroxyl groups and two lactone rings representing the hydrophilic part (Figure 1). EA has been found to possess various pharmacological properties as anticarcinogenic, antioxidant, antifibrosis, and chemopreventive properties<sup>9-11</sup>. Numerous scientific reports have evaluated the anticancer property of EA both in vivo and in vitro models for various kinds of cancer such as thyroid, breast, prostate, urinary bladder and blood cancer<sup>12-15</sup>. In spite of these findings, the inhibition of glioblastoma growth by EA has been rarely reported. Here in the present study we investigated the influence of EA on the proliferation and apoptosis of human glioblastoma cells and its possible mechanism.



**FIGURE 1** - Chemical structure of EA.

## Methods

### *Cell lines and culture*

The human glioblastoma cell lines U251 MG were purchased from State Key Laboratory of Molecular Biology, Shanghai Institutes for Biological sciences, China Academy of Sciences (Shanghai, China). The Cells were cultured in Dulbecco's Modified Eagle's Medium (Genom company, Hangzhou, China), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator at 37°C containing 5% carbon dioxide. The medium was replaced every 3 days. The cells were checked routinely and trypsinized until they reached about 90% confluency.

### *Antibodies and chemicals*

EA was purchased from Cayman Chemical (Ann Arbor, USA) and dissolved in DMSO prior to usage. DMSO was obtained from MP Biomedicals (OH, USA). Antibodies Bcl-2, caspase-3, Bax, Survivin, CHOP, XIAP (X-linked inhibitor of apoptosis protein), DR4 and DR5 were purchased from Cell Signalling Technology( MA, USA). And JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, p38, phospho-p38, and GRP78 were obtained from Santa Cruz Biotechnology (CA, USA). Unless otherwise stated, all other chemicals and reagents were obtained from Genom company (Hangzhou, China).

### *Cell viability assay*

Influence of EA on the cell viability of human glioblastoma cell lines U251 MG was assessed by the cell counting kit-8 (CCK-8 kit), which was purchased from Dojindo China CO., Ltd (Beijing, China). Put simply, the cells were seeded in a 96-well plate at a density of  $0.8 \times 10^4$  cells/well when cells reached logarithmic growth, and incubated for 24 h until a 70% confluent layer was reached, the cells were exposed to various concentrations of EA (0-200µM) for 24 h, 48 h and 72 h respectively. At each time point, 100µl of fresh medium containing 10µl CCK-8 solution was added to each well and incubated at 37°C for 0.5 h. The absorbance at 450 nm was measured on a Multiskan Spectrum Microplate Spectrophotometer. Each group was repeated in three wells.

*Live/dead assay*

The cells were exposed to EA (0, 50 and 100 $\mu$ M) for 48 h. The LIVE/DEAD cell viability assay (Invitrogen) determines the intracellular esterase activity and cell membrane integrity, to assess cell viability. Live cells retain calcein-AM and produce a green fluorescence through esterase activity, while, the ethidium homodimer binds to nucleic acid of the dead cells and produce red fluorescence. Glioblastoma cells, treated with EA were stained with calcein-AM (5 $\mu$ mol/L) and ethidium homodimer (5 $\mu$ mol/L) and were incubated at 37°C for 30 minutes and analyzed using a Nikon labophot-2 fluorescence microscope.

*Mitochondrial membrane potential assay*

The influence of EA on mitochondrial membrane potential (MMP) was detected with JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical, USA). Culturing cells in 6-well plates at a density of 5x10<sup>5</sup> cells/ml in an incubator overnight at 37°C. Treat the cells with EA of concentration 0, 50, 100 $\mu$ M for 48 h. Add 100  $\mu$ l of the JC-1 Staining Solution per ml of culture medium to each well of the plate. Mix gently. Incubate samples in incubator at 37°C for about 20 minutes. Then immediately analyze the cells with fluorescent microscopy (Olympus, Tokyo, Japan).

*Cell apoptosis and cell cycle distribution analysis*

The influence of EA on apoptosis and cell cycle distribution were detected with the Annexin V-FITC/PI apoptosis kit and cell cycle kit separately according to the manufacturer's instructions from MultiSciences Biotech (Hangzhou, China). U251 cells were exposed to EA for 48h. At the end of the treatment period, about 1x10<sup>6</sup> cells were trypsinized, collected by centrifugation at 900 rpm for 5 minutes and washed with cold PBS. Then corresponding reagents and solution were added and incubated according to manufacturer's instructions respectively. At the end of incubation, cell apoptosis and cell cycle distribution were analyzed on a flow cytometer (BD Biosciences, USA).

*Western blot analysis*

Cell proteins were extracted with RIPA lysis buffer and determined by the standard BCA method (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of protein (40 $\mu$ g) were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked in TBS containing 0.1% Tween-20 and 5% powdered milk, and probed with primary

antibody. Primary antibody directed against Bcl-2, Survivin, XIAP, caspase-3, Bax, JNK, P-JNK, ERK1/2, P-ERK1/2, p38, p-p38, DR4, DR5, CHOP, GRP78, GAPDH and  $\beta$ -actin were used at a dilution according to their specifications. Blots were visualized by LI-COR Odyssey Infrared imaging System with Alex Fluor 680/790 labeled goat anti-rabbit and goat anti-mouse IgG (LI-COR Biosciences, USA) used as second antibody.

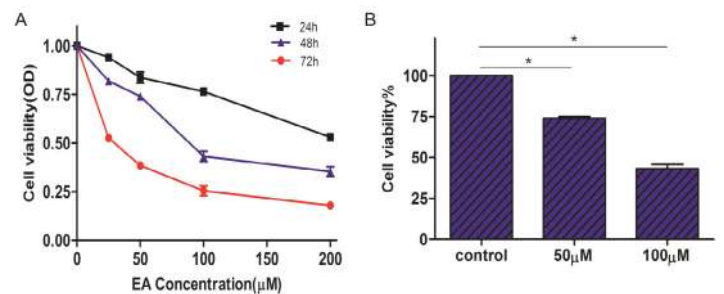
*Statistical analysis*

All experimental results are represented as mean $\pm$ SD. The Student's t-test was used to determine the significances between two mean values, and more than two values are determined by one-way Analysis of variance (ANOVA). Values at p<0.05 were considered statistically significant. The obtained values were analyzed using SPSS software, version 21.0.

**Results**

*EA suppressed the cell viability of glioblastoma cells*

In order to investigate whether EA treatment affects cell viability, U251 cells treated with various concentrations (0-200 $\mu$ M) of EA were tested using CCK-8 at several different time points. EA significantly reduced cell viability in U251 cells in a time and dose-dependent manner. And EA at 100  $\mu$ M was more effective on U251 MG than 50  $\mu$ M. Exposure to 100  $\mu$ M EA reduced the cell viability of U251 MG to 33.24% (Figure 2).

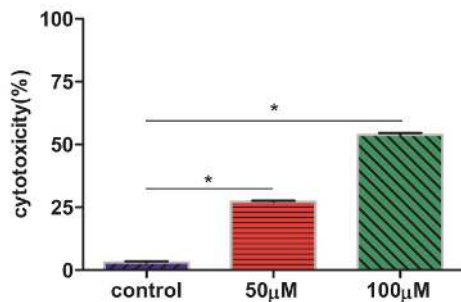


**FIGURE 2** - EA suppresses the cell viability of glioblastoma cells. U251 cells were exposed to culture medium containing various concentrations of EA for different time periods, and then cell viability was measured using the CCK-8 assay. The data were represented by mean $\pm$ SD of three experiments, and each experiment was conducted in triplicate.

*Live/dead assay*

Live/Dead assay for determination of cell viability was performed to assess if EA had cytotoxicity effects. Exposure to 50

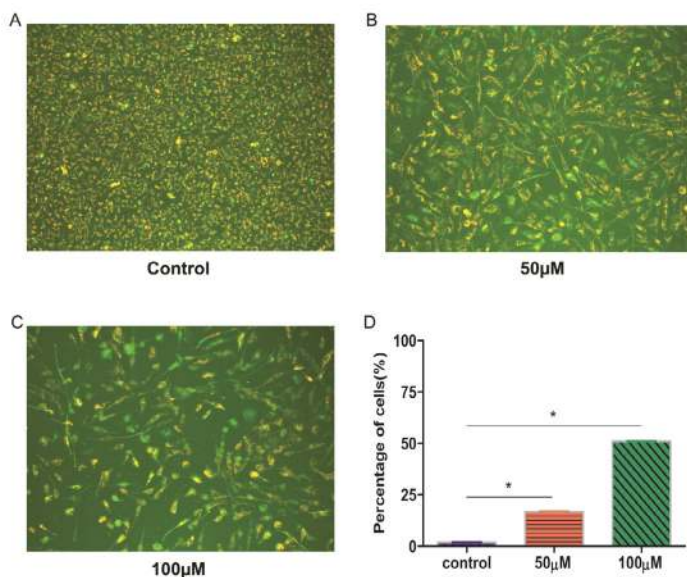
$\mu\text{M}$  EA resulted in a marked increase ( $p < 0.05$ ) in the apoptotic cell counts, the cytotoxicity percentage rose to 27.21 % in U251 MG. As exposure to 100  $\mu\text{M}$  EA, interestingly, caused robust increase in cytotoxicity to 53.79 % in U251 MG ( $p < 0.05$ ) (Figure 3).



**FIGURE 3** - The cytotoxic effect of EA on U251 MG. Values are represented as mean $\pm$ SD, n=3. \* represents statistical significance at  $p < 0.05$  compared against control as determined by compared t test.

*Mitochondrial membrane potential assay*

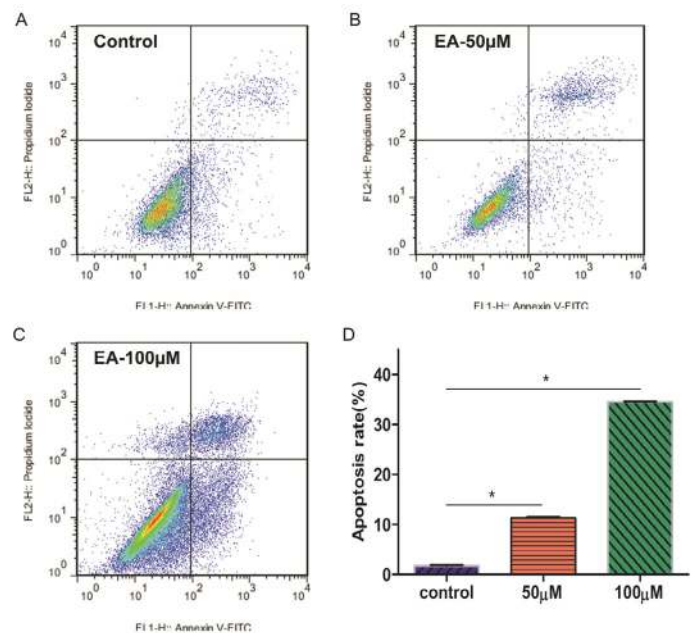
Mitochondrial Membrane Potential Assay Kit provides the most robust assay method for monitoring MMP changes. In normal cells, JC-1 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates. And, in apoptotic and necrotic cells, JC-1 diffuses out of mitochondria. It changes to monomeric form and stains cells in green fluorescence. We observed that EA exposure increased the green fluorescence rate of U251 cells to 16.83% and 50.59% separately with a dose of 50 $\mu\text{M}$  and 100 $\mu\text{M}$  EA than that of the control group ( $p < 0.05$ ) (Figure 4). It indicates that with the increasing of EA concentrations, the number of cells with MMP decrease is increasing.



**FIGURE 4** - EA induces MMP decreased of U251 MG. Values are represented as mean $\pm$ SD, n=3. \* represents statistical significance at  $p < 0.05$  compared against control as determined by compared t test.

*EA induced cell apoptosis of glioblastoma cells*

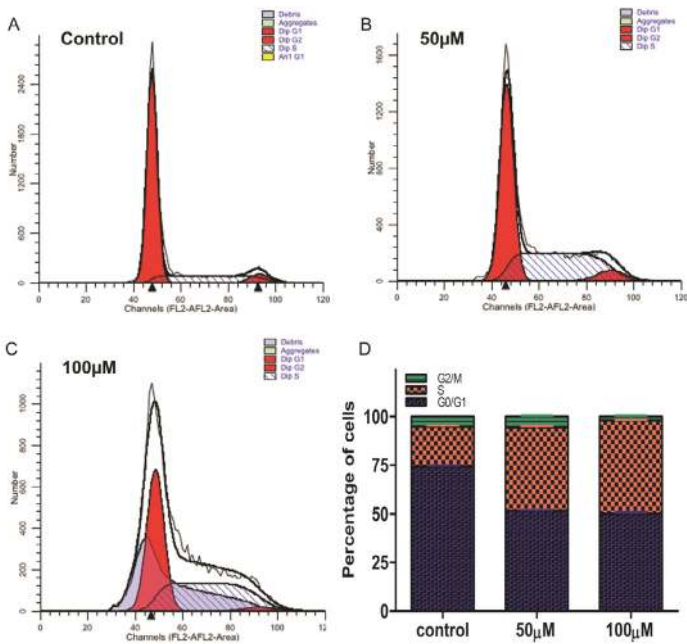
The effect of EA on cell apoptosis was investigated by flow cytometry. The apoptosis rates at 48 h after treatment with and without EA are shown in Figure 5 (A, B, and C). EA exposure increased the apoptosis rate of U251 cells to 11.3% and 34.5% separately with a dose of 50 $\mu\text{M}$  and 100 $\mu\text{M}$  (Figure 5D). The results indicated that EA induced apoptosis in glioblastoma U251 MG.



**FIGURE 5** - EA induces cell apoptosis of glioblastoma cells. The Apoptosis of U251 cells of control group (A), EA concentration 50 $\mu\text{M}$  (B) and EA concentration 100 $\mu\text{M}$  (C) were analyzed by Annexin V-FITC/PI staining at 48 h. And apoptosis rates of U251 cells with or without EA treated were represented (D). Values are represented as mean $\pm$ SD, n=3. \* represents statistical significance at  $p < 0.05$  compared against control as determined by compared t test.

*EA affected the cell cycle progression of glioblastoma cells*

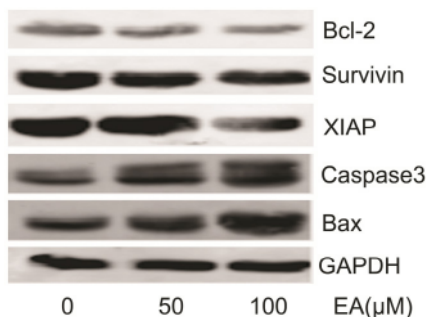
In order to examine the possible mechanism of anti-proliferation and pro-apoptosis activity of EA, the cell cycle distribution of U251 MG was evaluated by flow cytometry in presence and absence of EA. As shown in Figure 6, cultivating U251 cells with EA (50 $\mu\text{M}$ , 100 $\mu\text{M}$ ) for 48 h resulted in 23.14% and 24.65 decreases separately in the percentage of cells in the G0/G1 phase compared with the control group, which was accompanied by a concomitant increase in the percentage of cells in the S phase. It suggested that EA induces cell cycle arrest at S phase in glioblastoma cells.



**FIGURE 6** - EA affects cell cycle progression of U251 MG. U251 cells were treated with or without 50-100 μM EA for 48 h, the DNA content was analyzed by flow cytometry (A, B and C). And the percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle were calculated. The Results are expressed as mean±SD from three independent experiments.

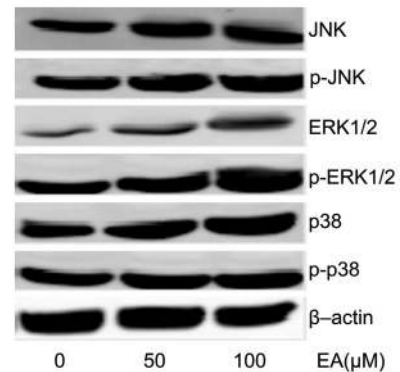
*EA affected cell apoptotic and survival signaling in glioblastoma cells.*

Changes in the expression of proteins related to cell apoptotic and survival signaling were analyzed by western blot in order to determine the mechanism of EA-induced inhibitory effects. We studied the effects of EA on the activation of Caspases. EA at 50 and 100 μM significantly ( $p < 0.05$ ) up-regulated the expression levels of Caspase-3 in a dose-dependent manner in the U251 glioma cell lines. Further, EA significantly ( $p < 0.05$ ) inhibited the expressions of Bcl-2, Survivin and XIAP, whereas up-regulated the pro-apoptotic protein, Bax (Figure 7).



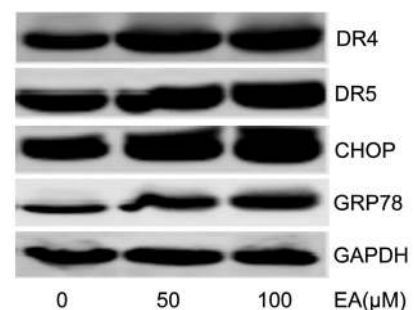
**FIGURE 7** - EA regulates the expression of apoptotic pathway proteins. EA exposure caused marked up-regulations in the expressions of caspases3 and further up-regulated Bax, while the expressions of the anti-apoptotic proteins (Bcl-2, Survivin and XIAP) were down-regulated effectively.

We investigated whether the activation of MAP kinases-ERK1/2, p38 and JNK exert their roles in EA-induced apoptosis. Incubation with EA caused significant ( $p < 0.05$ ) up-regulations in the expression patterns of ERK1/2, JNK and p38. EA caused multifold elevations in the phosphorylated forms of ERK1/2 and JNK, resulting in activation of ERK1/2 and JNK. Though considerable increase in p-p38 levels were observed, the elevations were non-significant. Further, EA caused marked up-regulation of p-ERK1/2 than p-JNK levels, suggesting that ERK is more involved in this signal pathway (Figure 8).



**FIGURE 8** - EA up-regulated MAPK in glioblastoma cell lines. EA exposure caused significant up-regulations in the expressions of JNK, ERK1/2 and p-38. Marked elevations in the phosphorylated forms of JNK, ERK1/2 and p-38 suggest that EA enhanced the activation of these kinases.

To investigate the influence of EA on the expressions of DR5 and CHOP at the protein level, western blot was performed. EA also induced the expressions of DR5, DR4 and CHOP at the protein levels also as observed in western blot analysis (Figure 9). However, the up-regulation of DR5 and CHOP were more pronounced than DR4. Furthermore, the ER stress marker proteins as-GRP78 induces the expression of CHOP. EA caused marked increase in the expression of GRP78 that could have possibly induced the expression of CHOP, indicating the involvement of ER stress in EA-induced apoptosis (Figure 9).



**FIGURE 9** - EA induced up-regulated expressions of DR5 and CHOP. EA caused up-regulations in the expressions of DR5 and CHOP at protein level and also caused enhanced expressions of death receptor 4 and ER stress marker protein GRP78.

## Discussion

In spite of continuous efforts to improve the current conventional cancer treatments, the long-term outcomes following surgery, chemotherapy and/or radiotherapy are still not optimal. Further, the lack of selectivity of these approaches towards cancer cells leads to significant side-effects and toxicity. Glioblastoma multiforme (GBM) is a highly aggressive major primary brain tumor in adults<sup>16</sup> and is a challenge to treat GBM<sup>17</sup>. Thus it becomes vital to identify novel agents that can inhibit its proliferation.

EA, a naturally occurring dietary polyphenolic compound, is present in several plants and fruits. EA is a dimeric derivative of gallic acid, which occurs in fruits and nuts either in the free form, as EA-glycosides, or as bound ETs<sup>18</sup>. EA has antioxidant, antifibrotic, and anticarcinogenic properties *et al.*<sup>19</sup>. It has been shown to reduce the incidence of a variety of carcinogen-induced tumors<sup>20</sup>. The anticarcinogenic effect of EA was shown in several types of cancers<sup>21-25</sup>. In our study we observed, EA at 25-200µM strikingly reduced the cell viability of U251 cells, suggesting the anti-proliferative efficacy. EA significantly down-regulated the expressions of anti-apoptotic proteins, Bcl-2 and Survivin. Apart from that, it enhanced the expressions of Caspase-3 and pro-apoptotic protein, Bax as well. The modulations in the expression patterns observed was dose-dependent, with 200 µM EA had a maximum influence on expressions.

Also, over expression of other anti-apoptotic proteins, including cellular FLICE-like inhibitory protein (c-FLIP)<sup>26</sup>, and inhibitor of apoptosis proteins (IAPs) as XIAP were also reported in cells inducing apoptosis. We also found EA induced down-regulation of XIAP also could have contributed to the activation of the Caspase cascade leading to apoptosis.

It has been shown that the induction of DR5 is also mediated through activation of CHOP<sup>27</sup>. CHOP is a major endoplasmic reticulum (ER) stress-regulated protein that is involved in ER stress-induced apoptosis<sup>28</sup>. EA enhanced the expression of CHOP at the protein level in a dose-dependent way. CHOP expression is principally regulated at the transcriptional level and is also one of the most induced genes during ER stress<sup>29</sup>. Additionally, CHOP expression is enhanced during conditions of stress as when cells were deprived of essential nutrients, and on exposure to anticancer drugs<sup>30,31</sup>. The results of our study suggest that EA possibly induced ER stress that leads to induction of GRP78 and CHOP expressions.

Recent research works have suggested that MAPKs-p38 MAPK and ERK1/2, regulate the expression of DR4 and DR5<sup>32,33</sup>. Also, stress activated protein, JNK regulates mitochondrial apoptotic pathway<sup>34</sup>. EA induced activation and elevated expressions of JNK, ERK1/2 and p38 suggest the involvement

of the MAPK signalling cascades in EA-induced apoptosis. Our results indicate that EA could have contributed to stress and further caused activation of JNK, suggesting the involvement of ROS-JNK/ERK signalling in cell death. Collectively the results of our study suggest that EA potentiates glioblastoma cells apoptosis by up-regulating the death receptors and through the activation of CHOP. EA could be possibly employed in chemotherapy for gliomas.

## Conclusions

We demonstrated that EA inhibited the proliferation and induced apoptosis of human glioblastoma cell lines. The cell apoptosis and cell cycle arrest caused by EA could be involved in mechanism of anticancer effects of EA on these human brain malignant tumor cells. However, epigenetic alternations and in-vivo experiments were absent in this research. In addition, the evidences of EA negatively regulating the invasiveness and growth of glioblastoma cells in vitro experiments will provide more convincing basis to support that EA may be a candidate to treat malignant brain tumors in the future.

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