Study on the Anti-tumor Effect of Resveratrol Enhanced Photodynamic Therapy

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Abstract: Resveratrol, as a broad-spectrum anticancer agent, has significant inhibitory effects on many tumor cells such as murine liver cancer, human liver cancer, gastric cancer, breast cancer, ovarian cancer and leukemia. In this study, resveratrol was used as an inhibitor of ABCG2 to influence the efflux cells of protoporphyrin IX (PpIX) by inhibiting BCRP/ABCG2, and to investigate the effect of resveratrol on enhancing phototherapy (PDT) of human glioma cells treated with 5-aminolevulinic acid (5-ALA). Firstly, four kinds of malignant glioma cells U87MG, U251, A172 and T98G were treated with 5-ALA (1 mmol/L) . Resveratrol at different concentrations (0.01~1.0μmol/L) as a modulator was incubated with glioma cells. The levels of PpIX, BCRP/ABCG2mRNA and ABCG2 protein in vivo and vitro and the effects of PDT in vitro were evaluated. The results showed that resveratrol decreased the expression of BCRP/ABCG2mRNA and thus decreased the expression of BCRP/ABCG2 protein on the cell membrane. Then, resveratrol at or above the concentration of 0.1μmol/L inhibited PpIX efflux in malignant brain glioma cells, and increased PpIX level in all cells with the increase of concentration. Resveratrol can not only inhibit the efflux of PpIX in malignant glioma cells, but also enhance the effect of PDT by increasing the content of PpIX in the cells.

Keywords: Resveratrol; Photodynamic therapy; Tumor therapy

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1 Introduction

Gliomas are the most common primary malignant tumors of the brain resulting from the carcinogenesis of brain and spinal glial cells [1]. Incidence rate accounted for 35.2%~61.0% of intracranial tumors, derived from glial cells, with high incidence rate, high relapse rate, high mortality rate and low cure rate [2-3]. At present, the treatment of glioma includes surgery, radiotherapy, chemotherapy, targeted therapy and so on [4-6]. In recent years, photodynamic therapy (PDT), as a new technique for the treatment of tumors, plays an important role in the comprehensive treatment of tumors [7-11]. Photodynamic therapy (PDT) is a new method for the treatment of tumor diseases by irradiating the tumor site with a specific wavelength, which can activate the photosensitive drug that selectively accumulates in the tumor tissue, and then the photosensitive drug can transfer the energy to the surrounding oxygen to form the active oxygen free radical (ROS), which can react with the nearby biomacromolecules to produce cytotoxicity and kill the tumor cell [12-16]. Compared with conventional tumor therapies, PDT has the advantage of being able to treat accurately and effectively and with little side effect [17]. 5-aminolevulinic acid (5-ALA) and its derivatives have been developed as a new generation of photosensitizers. In
tumor cells, the precursors product of 5-ALA were PEPT1 and PEPT2 peptide converters, which were then converted to protoporphyrin IX (PpIX)\textsuperscript{[18]}. PpIX can be synthesized and accumulated in various tumor cells, so 5-ALA is also used clinically to treat brain, neck and skin tumors\textsuperscript{[19]}.

Resveratrol (Res) is a natural polyphenolic compound. It can be used to treat cancer by inhibiting three stages of cancer (initiation, proliferation and development). To achieve anti-initiation activity by anti-oxidation, anti-mutation and inducing second-stage drug enzymes. Through anti-inflammation, destroying the activity of cyclooxygenase and inhibiting the activity of DNA polymerase, the effect of DNA synthesis was reduced, thus the effect of inhibiting the proliferation of cancer cells was achieved\textsuperscript{[21-22]}. Induction of differentiation of human promyelocytic leukemia cells to inhibit tumor development\textsuperscript{[23-24]}. In addition, resveratrol has a good ability to penetrate the blood-brain barrier and is a promising natural antitumor drug\textsuperscript{[25]}.

Therefore, the relationship between resveratrol through PpIX transformants mediated by ABCG2 receptor and 5-ALA combined with PDT was evaluated by four malignant glioma cell lines. In order to confirm that resveratrol can enhance the therapeutic effect of 5-ALA combined with PDT on malignant brain tumors by inhibiting the overexpression of ABCG2.

2 Materials

2.1 Test Materials

U87MG, U251, A172 and T98G4 tumor cell lines were purchased from American Type Culture Collection (ATCC); resveratrol (AstraZeneca, UK).

2.2 Test Equipment

Magnetic Stirrer, Hangzhou Huier Instrument & Equipment Co., Ltd. Transmission Electron Microscope, Hitachi, Japan (HT7700); Vacuum Freeze Dryer, SP, USA; Standard pH meter PB-10, Beijing Sedoris Instrument System Co., Ltd.

3 Methodology

3.1 Determination of Intracellular and Extracellular PpIX Levels

The level of PpIX in each cell line was determined by FACS. Four glioma cells (U87MG, U251, A172 and T98G) were inoculated in petri dishes, and then added in 8mL Dulbecco's Modified Eagle Medium (containing 10%FBS). The cells were incubated with 1 mmol/L 5-ALA for 6 hours. After washing with PBS, the content of PpIX was determined. The excitation wavelength of PpIX is 407nm. The fluorescence intensity is measured at 655nm (LP) and 675nm (BP). Then cell staining and FACS analysis were performed. The active concentration of cells was calculated by ratio to standard cell line. The ratio of intracellular and extracellular PpIX was measured by microcassette fluorometer counter.

3.2 Expression of ABCG2/BCRP Gene in Cell Membrane

Four kinds of malignant glioma cells were divided into two groups: the experimental group treated with resveratrol and the control group treated with PBS. The expression of ABCG2 gene was detected by real-time PCR after 48 h with 1 μmol/L resveratrol.

3.3 Detection of ABCG2/BCRP Protein Expression

The expression of ABCG2 protein in cell membrane was detected by FACS (antibody: 5D3, titer: 1:1000). Monoclonal antibody 5D3 was used in cells without resveratrol, with an initial antibody titer of 1 : 1000, since the antibody reaction epitope was located on the cell membrane of ABCG2. Four kinds of glioma cells were inoculated into 12-well plates, cultured with fresh DMEM (10%FBS) for 12 hours, then incubated with resveratrol (0.1μmol/L) for 24 hours, then washed with PBS solution for 3 times. After the cells were digested, 50μL cell suspension was centrifuged at for 5 minutes (1000rpm), the supernatant was discarded and added with 20μL cleaning solution, then shaken evenly for 5 minutes, added with 20μL 5D3 antibody for 30 minutes and flow cytometry secondary solution for 30min(Anti-goat, Anti-mouse IgG-FITC, 1 : 1000) ,dead cells were stained by homologous PI. The blue laser wavelength on FACS is 488nm, BP and LP are 530/30nm and 502nm, respectively. Finally, the level of ABCG2/BCRP fluorescence expression in resveratrol and control group was recorded.

3.4 Effect of PDT

The anti-tumor effects of resveratrol-treated U87MG, U251, A172 and T98G4 glioma cells were analyzed. The cells were treated with resveratrol at concentrations of 0μmol/L, 0.1μmol/L, 1μmol/L and 10μmol/L for 48 h, and then incubated with 1 mmol/L 5-ALA for 6 h. The cells were irradiated with 0, 0.6, 1.8 and 3J/cm\textsuperscript{2} diode laser at a wavelength of 405nm. The cells were then incubated with 11~14d until colony formation. The colony formed was fixed with 10% formaldehyde, stained with 0.4% trypan blue, and finally counted (>50 cells per colony).
4 Results and Discussion

As shown in Figure 1, with the increase of resveratrol concentration, the intracellular PpIX concentration of the four malignant glioma cell lines increased while the extracellular PpIX concentration decreased (P<0.05~P<0.01). In addition, each cell line showed a dose-dependent linear relationship, suggesting that resveratrol increased PpIX levels in malignant glioma cells and decreased PpIX levels in extracellular cells. The expression of ABCG2 on the cell membrane is represented by the mean of FITC. Other monoclonal antibodies affect the outer membrane of ABCG2 protein. In the resveratrol group, the level of ABCG2 transporter in the cell membrane was decreased, especially in the U87MG cells (Fig. 2), indicating that more PpIX was efflux. The expression of ABCG2 mRNA in resveratrol (0.1 mol/L) cells was significantly decreased in the four glioma cells (all P < 0.05). As shown in Figure 3.

Then we examined the effect of resveratrol combined with 5-ALA on PDT, with the increase of resveratrol concentration, the number of cell cultures binding 5-ALA decreased significantly, while the increase of energy remained consistent. The photosensitivity of resveratrol increased in a dose-dependent manner (Figure 4). The relationship between cell viability and ABCG2 gene expression on cell membrane is shown in Figure 5. As shown in Figure 5, overexpression of ABCG2 in malignant glioma cells predisposes to drug resistance during 5-ALA-induced PDT.

The four malignant glioma cell lines have shown that, with the increase of resveratrol concentration, the intracellular PpIX concentration increased while the extracellular PpIX concentration decreased (P<0.05~P<0.01), even in low-concentration resveratrol group (0.1 mol/L), the difference of the intracellular and extracellular PpIX concentration was statistically significant (P<0.05). As shown in Figure 3.

The four malignant glioma cell lines have shown that, with the increase of resveratrol concentration and irradiation time, the survival rate of four cell lines decreased significantly (* P <0.05;**P<0.01,n=5), the ordinate is the survival fraction, the abscissa is the power of PDT.

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With the prolongation of irradiation time and the increase of resveratrol concentration, the survival rate of four cell lines was significantly reduced (* P<0.05;**P<0.01,n=5). The ordinate is the survival fraction, the abscissa is the power of PDT.

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Fig 1 The changes of intracellular and extracellular PpIX levels in groups with different resveratrol concentrations.

Fig 2 FACS analysis of ABCG2 protein expression in the cell membrane of 4 malignant glioma cell lines.

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Fig 3 The effect of resveratrol on the expression of ABCG2 mRNA in cell membranes of four malignant glioma cells.

Fig 4 The effect of resveratrol on PDT of four malignant glioma cells.
The ABCG2 protein levels in cell membrane was positively correlated with PDT survival fraction of four cell lines. The ordinate is the survival fraction, the abscissa is the expression level of ABCG2 gene in the cell membrane.

**Fig. 5** The ABCG2 protein levels in cell membrane was positively correlated with the PDT survival fraction of four malignant glioma cells.

### 5 Conclusion

At present, dynamic therapy has become a hotspot of cancer treatment because of its advantages of space, time selectivity and low toxicity. When combined with near infrared light, photosensitizer can effectively kill tumor cells, reduce toxic side effects and improve anti-tumor effect. In this study, PpIX was excessive transportation to extracellular regions of malignant brain tumors. After co-culture with 5-ALA, the content of extracellular PpIX in the four cell lines was higher than that in the cells. PpIX from cells co-cultured with 5-ALA appears to be closely associated with ABCG2 gene expression (Figure 5). Resveratrol, as an inhibitor of ABCG2, inhibits PpIX transport in a dose-dependent manner (Figure 1). This phenomenon suggests that resveratrol can enhance the PDD and PDT effects of 5-ALA. Resveratrol has two effects on intracellular photosensitivity and PpIX accumulation: Firstly, resveratrol competitively inhibits ABCG2-mediated PpIX transport. Resveratrol inhibited the expression of ABCG2 gene. The effects of resveratrol on PDT were significantly different (Figure 4). With the increase of resveratrol concentration and photodynamic intensity, the survival rate of tumor cells decreased rapidly. When the photodynamic intensity was 3.0 J/cm2, most glioma cells died. The concentration of resveratrol (referred to as IC50) inhibiting ABCG2 transport was 0.1 mol/L, which was much lower than the concentration of resveratrol used clinically. Therefore, resveratrol combined with 5-ala-PDT can decrease mortality rate and enhance PDT, especially for early brain malignant glioma.

Membrane-associated proteins encoded by ABCG2 are contained in the superfamily of ATP-binding cassette (ABC) transporters, and ABC proteins transport molecules through extracellular membranes and intracellular membranes. Overexpression of ABCG2 is an important form of drug-resistant gene expression in many malignant tumors. It was found that the expression of ABCG2 and the increase of ABCG2 levels in gliomas were related to the degree of pathological malignancy. Although to date, overexpression of ABCG2 has been associated with drug resistance in many malignant tumor cells. But it is not clear whether ABCG2 is resistant to PDT. In this study, the expression of ABCG2 gene was proved to be an important index to reduce the cytotoxicity of 5-ala-PDT cells (Figure 5), and resveratrol could enhance the efficacy of PDT by inhibiting ABCG2. However, the exact mechanism of resveratrol inhibiting ABCG2 has not been clarified.

In conclusion, resveratrol can inhibit the transport of ABCG2 and mRNA expression, thus reducing the loss of PpIX in malignant glioma cells. Therefore, resveratrol or other protein kinase inhibitors can be used to enhance PDD and PDT of 5-ALA. PDT combined with resveratrol may play a further role in the treatment of malignant brain tumors, and further research is needed to achieve this goal.

### References


transporters. ABC PROTEIN TRANSPORTS MOLECULAR MEMBRANE AND ENDOMETRY.


