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Original Article

The anti-leukemic effects of Obacunone (OCE) by inducing apoptosis via mitochondria-mediated pathway

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Abstract: This study was aimed to investigate the anti-leukemic effects of Obacunone (OCE) in human chronic myeloid leukemia cells and explore its potential mechanisms. The *in vitro* antitumor effects of OCE on K562, U937 and THP-1 cells were evaluated by MTT assay. Cells were treated with OCE at the concentrations of 10, 20 and 40 μM for 24 h. Apoptosis detection was carried out by flow cytometer, and the expressions of apoptosis related proteins were assayed by western blot. The *in vivo* antitumor effects of OCE on K562 cells were further investigated in a xenograft nude mice model at a dose of 40 mg/kg for 21 days. OCE showed significant inhibitory effects on K562, cells with a half maximal inhibitory concentration (IC_{50}) value of 28.9 μM. OCE induced apoptosis in K562 cells by up-regulating the expressions of caspase-3, caspase-9 and Bax ($p < 0.01$), whereas down-regulating expressions of Bcl-2 ($p < 0.01$) with a dose-dependent manner. Expression of EVI-1 was also suppressed by treating with OCE, and OCE-induced apoptosis could be attenuated by transfecting with EVI-1 SiRNA. Furthermore, the *in vivo* experiments also indicated that OCE possessed significant antitumor effects ($p < 0.01$) in xenograft nude mice. Collectively, the results demonstrated that OCE had significant antitumor effects in human chronic myeloid leukemia K562 cells, and the mechanisms were probably related to the induction of apoptosis via mitochondria-mediated pathway. OCE is a potential apoptosis inducer that can be developed into promising antitumor drugs for the treatment of leukemia in the future.

Keywords: Obacunone, anti-leukemic effects, human chronic myeloid leukemia K562 cells, mitochondria-mediated pathway

Introduction

Leukemia is a cancer of the body's bone marrow and the lymphatic system characterized by abnormal proliferation of leukocytes [1, 2]. It is classified into chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) [3]. CML is one of the three most common forms of leukemia, which is an acquired malignant myeloproliferative disorder of hematopoietic stem cells [4]. Leukemia cells can spread to the lymph nodes, spleen, liver, central nervous system and other organs. As a malignant clonal disorder of the hematopoietic stem cells, the incidence and mortality of leukemia are first among all pediatric malignancies [5, 6]. Over the last decade, there has been some progress in developing novel cancer therapies of leukemia. However, no significant improvement was observed in the overall survival rate of leukemia [3].

Natural products have been attracted increasing attention for cancer therapy because of their mechanistic diversity and good availability [7]. They not only have useful therapeutic and pharmacological effects, but also can provide further leads for the development of anticancer drug with favorable antitumor activity and fewer side effects [8]. Limonoids are a unique class of secondary metabolites widely distributed in Citrus species [9]. Until now, more than 62 different limonoids have been found and showed a variety of activities, including anti-tumor, antioxidant, and anti-microbial activities [10]. Obacunone (OCE) (**Figure 1**) is a limonoid commonly present in citrus juices and seeds. It was reported that OCE had neuroprotective effect by inducing heme oxygenase-1 via the p38 MAPK pathway, and it was also an inducer of glutathione S-transferase (GST) enzyme [10, 11]. *In vitro* and animal studies suggest that OCE have obvious anticarcinogenic activities

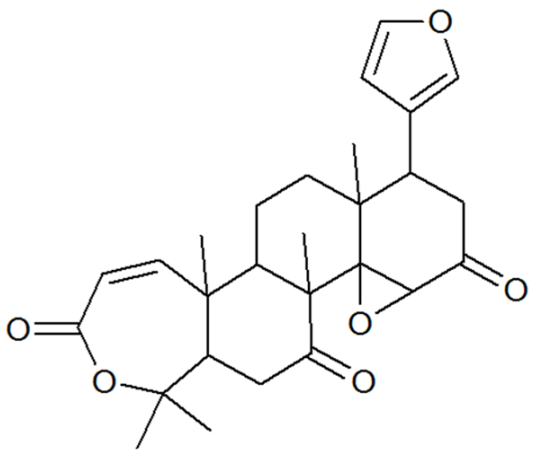

Figure 1. Chemical structure of OCE.

against cancers such as breast adenocarcinoma, pancreatic cancer, prostate cancer and colon cancer, *et al.* by inducing apoptosis and anti-inflammatory effects [12-15]. However, no investigation of OCE on leukemia has been reported in the literature. In this study, the anti-leukemic effects of OCE were investigated on different cell lines, and the mechanisms of anti-leukemic effects were explored in human chronic myeloid leukemia K562 cells.

Materials and methods

Chemicals and reagents

Obacunone (purity ≥ 98%) was purchased from Qingyun Biology (Nanjing, China). MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against β-actin, caspase-3, caspase-9, Bad, Bcl-2, Bax, P53, ectopic viral integration site 1 (EVI-1), Wilms' tumor suppressor (WT1), transforming growth factor-β_1 (TGF-β_1) and EVI-1 siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated secondary antibodies were obtained from Beyotime Biotechnology (Shanghai, China).

Cell culture

Human chronic myeloid leukemia K562 cell line, human histiocytic lymphocyte U937 cell line, and human acute monocytic leukemia THP-1 cell line were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Cells (5 × 10^4/mL, 0.2 mL) were seeded into each well of a 96-well plate and the cells were incubated for 24 h at 37°C. Then cells were treated with indicated concentrations of OCE. A Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) was used to measure the cell proliferation according to the manufacturer's instructions. A Microplate Reader (Bio-Rad) was used to read the absorbance (OD) at 450 nm.

Cell growth inhibition rate = 1-(A_1/A_0) × 100%

Where A_1 was the OD value of experimental group, A_0 was the OD value of control group.

Flow cytometric assay

Apoptosis detection was carried out by Annexin V-FITC/PI double staining as described previously [16]. Briefly, K562 cells (1 × 10^5 cells/mL, 2 mL) were seeded in 6-well plates and treated with OCE for 48 h. The cells were harvested, washed twice with ice-cold PBS and re-suspended in binding buffer. After incubation with Annexin V-FITC/PI solution in the dark for 15 min at room temperature, the samples were analyzed by using BD FACS Verse flow cytometer (BD Biosciences, San Jose, CA, USA).

Animals

Male BALB/c athymic nude mice (20 ± 1 g) were purchased from Shandong Laboratory animal center (Ji'nan, China). Mice were housed in an environment of 21 ± 2°C and 60 ± 10% humidity under a 12 h-light/12 h-dark cycle with free access to feed and water. All the animal experiments were approved by the Animal Ethics Committee of Linyi Cancer Hospital (Linyi, China).

K562 xenograft mouse model

K562 xenograft mouse model was established according to previously reported [17]. K562 cells (1 × 10^6/200 μL) were injected subcutaneously into the left flank of the nude mice. When the tumors reached a volume of 100 mm^3, the mice were randomly divided into

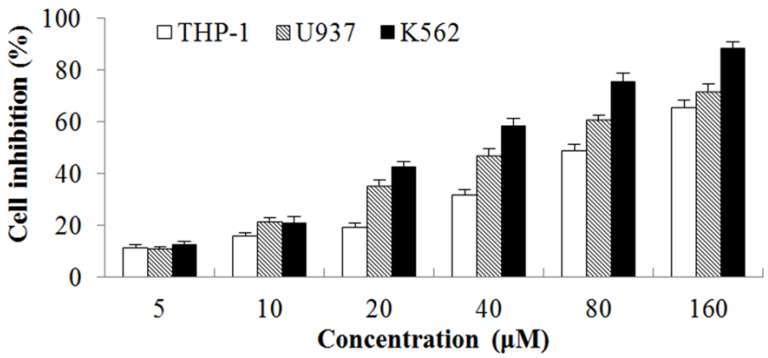

Figure 2. The cytotoxic effects of OCE on K562, U937 and THP-1 cells.

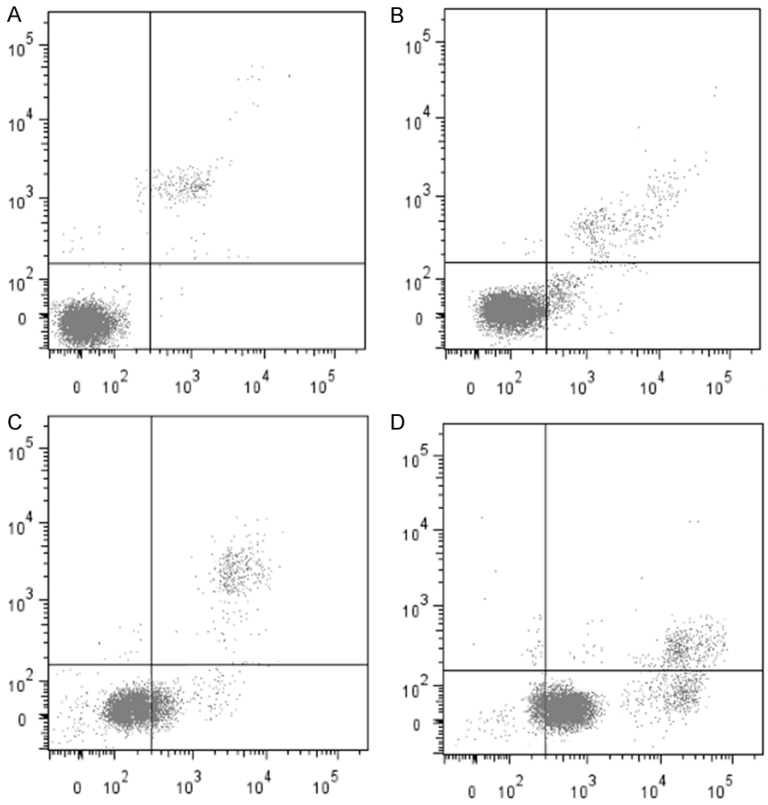

Figure 3. Determination of apoptosis in K562 cells by flow cytometry. A-D. Represents the control, 10, 20 and 40 μM, respectively.

two groups (n = 12): the control group (normal saline) and the OCE group (40 mg/kg). The mice were treated for three weeks by intragastric administration. The tumor volumes (length × width^2 × 0.5) and body weights of the mice were measured every three days.

Western blot analysis

Western blot analysis was carried out as described previously [18]. After treatment with OCE for 48 h, the cells were collected and lysed using RIPA lysis buffer. The protein concentration was determined by using a BCA protein assay kit. Equal amounts of protein (50 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk for 1 h. the membranes were incubated with corresponding primary antibodies at 4°C for 24 h, followed by HRP-conjugated secondary antibodies for 1 h. Protein bands were then visualized using enhanced chemiluminescence reagents.

Statistical analyses

Data are expressed as mean ± standard deviation (SD) of three independent experiments. Statistical analyses were carried out using SPSS version 18.0 for Windows (SPSS, Inc, Chicago, IL, USA). Statistical significance was analyzed using Student's t-test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Growth inhibitory effect of OCE on K562, U937 and THP-1 cells

Cells were exposed to various concentrations (5, 10, 20, 40, 80, 160 μM) of OCE, cell viability was determined by CCK-8 assay. As shown in **Figure 2**, OCE showed significant inhibitory effects on K562, U937 and THP-1 cells under the tested concentrations with a concentration-dependent manner. The half maximal inhibitory concentration (IC_{50}) values of OCE on the three cell lines were 28.9, 48.6, and 87.9 μM, respectively. Since K562 cell exhibited higher response than other cell lines, the K562

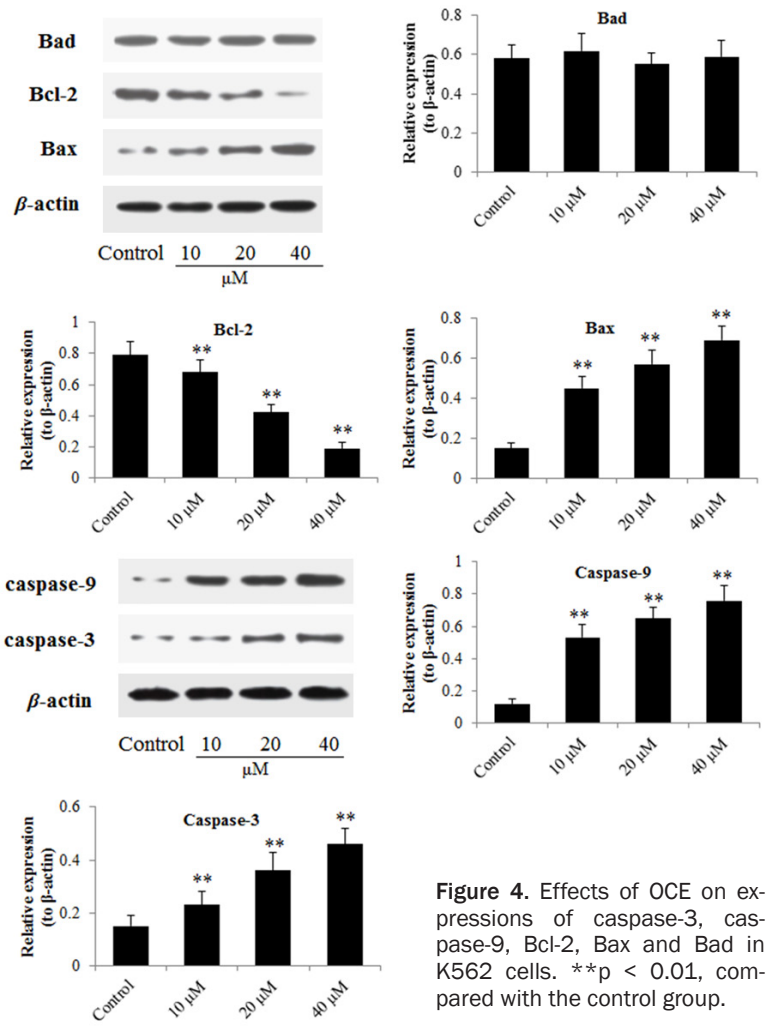

Figure 4. Effects of OCE on expressions of caspase-3, caspase-9, Bcl-2, Bax and Bad in K562 cells. **p < 0.01, compared with the control group.

cell line was selected from three cell lines for further experiments.

OCE induces apoptosis in K562 cells

Annexin V-FITC/PI double staining was performed to investigate whether the reduction of cell viability in K562 cells was caused by apoptosis. The results were shown in **Figure 3**, OCE induced apoptosis in K562 cells in a dose-dependent manner at the concentrations of 10, 20 and 40 μM.

To investigate the potential mechanism of apoptosis induced by OCE, the expression of apoptosis related proteins was analyzed by western blot. As can be seen from **Figure 4**, the expressions of caspase-3 and caspase-9 were significantly up-regulated by the treatment of OCE at 10, 20 and 40 μM ($p < 0.01$). Down-regulation of Bcl-2 and up-regulation of Bax were also observed by treating with OCE at different concentrations ($p < 0.01$). These results indicated that the apoptosis induced by OCE might be involved in a mitochondria pathway.

OCE significantly down-regulated the expression of EVI-1

To further investigate the antitumor mechanism of OCE, the expressions of P53, TGF-β_1, WT1 and EVI-1 were assayed by western blot. As shown in **Figure 5**, OCE only up-regulated the expressions of P53, TGF-β_1, WT1 at the high concentration of 40 μM ($p < 0.05$). Interestingly, by treating with OCE, the expression of EVI-1 was significantly down-regulated at the concentrations of 10, 20 and 40 μM ($p < 0.01$) when compare with the control cells with a concentration-dependent manner.

Down-regulation of EVI-1 is required for the OCE-induced apoptosis

To investigate the important role of EVI-1 in the OCE-induced apoptosis, K562 cells were transfected with EVI-1 siRNA. As shown in **Figure 6**, cells were treated with OCE (40 μM) in the presence or absence of the EVI-1 siRNA. The expression of caspase-3, caspase-9 and Bax were significantly down-regulated ($p < 0.01$), while the expression of Bcl-2 was significantly up-regulated ($p < 0.01$) in EVI-1 siRNA-transfected cells compared with OCE-treated cells (without EVI-1 SiRNA transfected). The results indicated that EVI-1 expression was essential in OCE-induced apoptosis in K562 cells.

Antitumor effects of OCE on nude mice

The *in vivo* antitumor effects of OCE on K562 cells were further conformed in a xenograft nude mice model. As shown in **Figure 7A**, tumor growth in the mice was significantly inhibited by treating with OCE at a dose of 40 mg/kg during

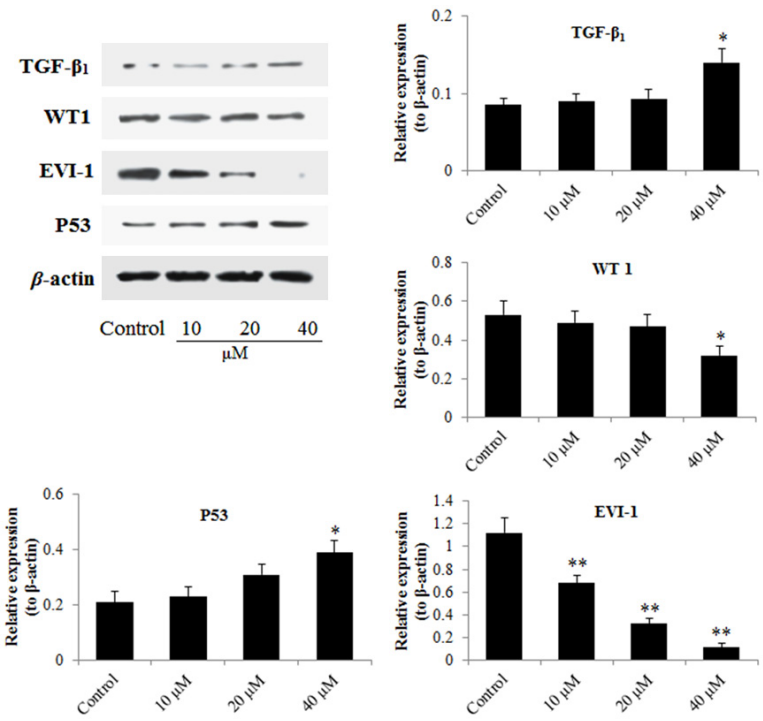

Figure 5. Effects of OCE on expressions of P53, EVI-1, WT1 and TGF-β1 in K562 cells. *p < 0.05 and **p < 0.01, compared with the control group.

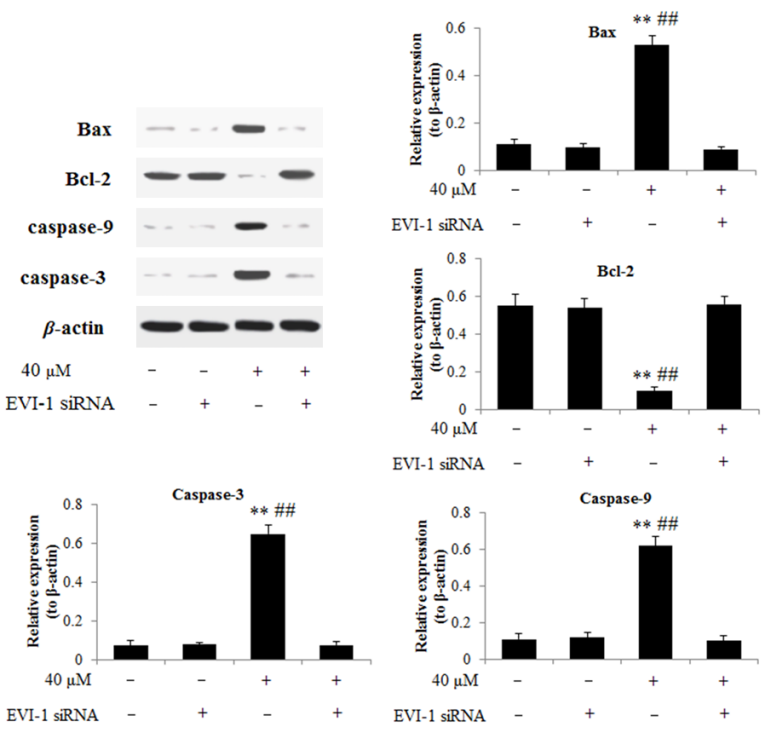

Figure 6. Effects of EVI-1 siRNA on expressions of apoptosis-related proteins in K562 cells. **p < 0.01, compared with the OCE-treated group (40 μM) without EVI-1 SiRNA transfected; ##p < 0.01, compared with EVI-1 SiRNA transfected group.

21 days compared with the mice in the control group (p < 0.01). **Figure 7B** showed that the body weight of the mice was not significantly affected by treating with OCE, indicating that OCE had no obvious toxicity to the mice.

In addition, by treating with OCE, the expressions of caspase-3, caspase-9 and Bax in the mice were significantly up-regulated, whereas the expressions of Bcl-2 and EVI-1 were down-regulated when compared to the mice in the control group (p < 0.01, **Figure 8**). These results also indicated the *in vivo* antitumor activity of OCE in K562 cells is closely related to the induced apoptosis via mitochondria-mediated pathway.

Discussion

A large amount of studies has revealed that natural products isolated from different plants could provide additional strategies for treatments of different cancers [19]. OCE is a limonoid mainly present in citrus juices and seeds. The present study indicated that OCE could significantly inhibit the growth of human chronic myeloid leukemia K562 cells with a considerable IC_{50} value of 28.9 μM. Therefore, further investigations were carried out to explore the possible mechanisms of the effects.

It's reported that apoptosis is a kind of cell death known as "programmed cell death" that plays a vital role in different kinds of physiological and pathological processes. More importantly, many anticancer agents take their effects mainly through inducing apopto-

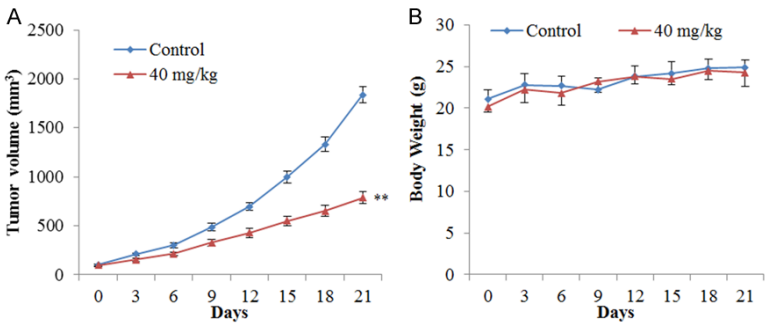

Figure 7. Effects of OCE on tumor volumes (A) and body weight (B) of nude mice. **p < 0.01, compared with control group.

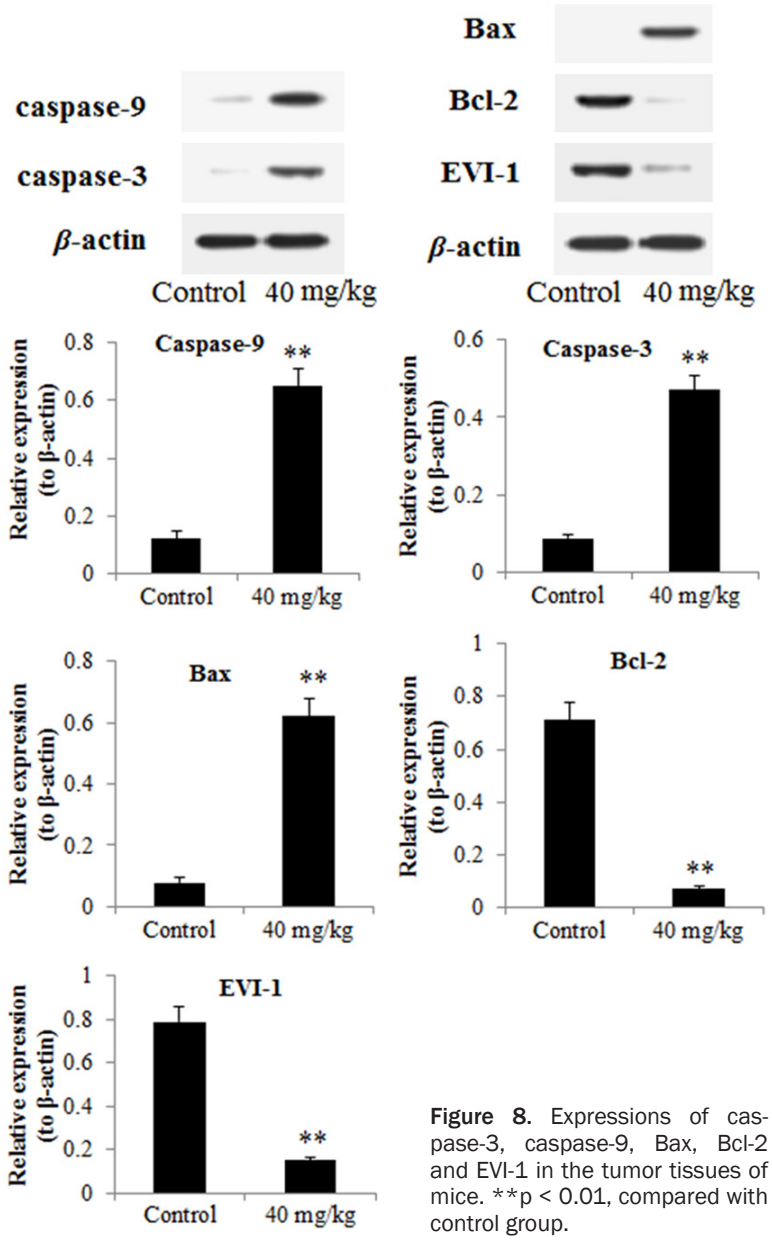

Figure 8. Expressions of caspase-3, caspase-9, Bax, Bcl-2 and EVI-1 in the tumor tissues of mice. **p < 0.01, compared with control group.

sis in the cancer cell [4, 20]. In this dtudy, Annexin V-FITC/PI double staining was carried out, and confirmed that OCE induced apoptosis in K562 cells with a concentration-dependent manner.

Bcl-2 family members are crucial mediators of apoptosis via mitochondrial pathway [7, 16]. In this family, anti-apoptotic members (such as Bcl-2) prevent or delay cell death, whereas the pro-apoptotic member (such as Bax) promotes cell apoptosis. An increase in the ratio of Bax/Bcl-2 could decrease the cellular resistance to apoptotic stimuli and lead to apoptosis [21]. In addition, studies have also indicated that the caspase family plays a vital important role in the induction of apoptosis [22]. In this study, OCE treatment significantly up-regulated expressions of caspase-3, caspase-9, and Bax whereas down-regulated expression of Bcl-2, indicating that the apoptosis induced by OCE might be involved in the mitochondria pathway.

It is reported that p53 mediates apoptosis through a pathway involving bax/cytochrome c/caspase-9 activation, followed by the activation of caspase-3, -6, and -7 cascades [23]. Researches have also proved that aberrant expression of ectopic viral integration site 1 (EVI-1) and the Wilms' tumor suppressor (WT1) genes play critical roles in the pathogenesis of hematologic malignancies, and they have been shown to be involved in cell apoptosis [24, 25]. Furthermore, EVI-1

was found to interfere with transforming growth factor-β (TGF-β) signaling and antagonizes the growth-inhibitory effects of TGF-β [26]. In this study, to further investigate the antitumor mechanism of OCE, the expressions of P53, TGF-β1, WT1 and EVI-1 were evaluated. The results showed that OCE treatment significantly down-regulated expression of EVI-1 in K562 cells. To investigate the role of EVI-1 in the OCE-induced apoptosis, K562 cells were transfected with EVI-1 siRNA, and the results revealed that EVI-1 expression was essential in OCE-induced apoptosis. Furthermore, the *in vivo* antitumor effects of OCE on K562 cells was further conformed in a xenograft nude mice model, and the results also demonstrated that the effects were via inducing apoptosis in mitochondria pathway.

Conclusion

Collectively, the results in this study demonstrated that OCE had significant antitumor effects in human chronic myeloid leukemia K562 cells, and the mechanisms were probably related to the induction of apoptosis via mitochondria-mediated pathway. Therefore, OCE is a potential apoptosis inducer that can be developed into promising antitumor drugs for the treatment of leukemia in the future.

Disclosure of conflict of interest

None.

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