EFLDO sensitizes liver cancer cells to TNFSF10-induced apoptosis in a p53-dependent manner

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Abstract. Ent-3α-formylabieta-8(14),13(15)-dien-16,12β-olide (EFLDO) is a compound extracted from *Euphorbia lunulata* Bge exhibiting anti-proliferative activity *in vitro*. In the present study, EFLDO was identified to sensitize HepG2 cells to tumor necrosis factor (TNF) superfamily member 10 (TNFSF10)-induced apoptosis. Liver cancer cells were resistant to TNFSF10; however, EFLDO increased TNFSF10-induced cancer cell viability inhibition and cell apoptosis induction as assessed by MTT assay and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide assay, respectively. The western blotting results suggested that treatment with EFLDO increased TNFSF10-induced upregulation of the protein expression levels of pro-apoptotic proteins, including BCL2 associated agonist of cell death, BCL2 associated X, apoptosis regulator, caspase-3 (CASP3) and CASP8. Furthermore, treatment with EFLDO increased TNFSF10-mediated downregulation of the protein expression level of the anti-apoptotic protein BCL2 apoptosis regulator. Notably, the increase in the activity of CASP3 was consistent with the western blotting results. Treatment with EFLDO sensitized liver cancer cells to TNFSF10, and apoptosis was induced via the upregulation of TNF receptor superfamily member 10a (TNFRSF10A) and TNFRSF10B in a tumor protein p53 (p53)-dependent manner, as detected by reverse transcription-quantitative polymerase chain reaction and western blot analyses. In addition, p53 was identified to be necessary for EFLDO-induced sensitivity to TNFSF10, as assessed by western blotting and Annexin V-FITC assay. Collectively, the present results suggested a novel mechanism underlying EFLDO function in liver cancer. Treatment with EFLDO was able to increase the antitumor effect of TNFSF10 in liver cancer cells in a p53-dependent manner.

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Introduction

Liver cancer is the fifth most common malignant tumor, and exhibits the second highest mortality rate among malignant tumors (1-3). Liver cancer has a high incidence rate, and accounts for ~55% of the cancer incidence worldwide (4). Additionally, the majority of patients with liver cancer at an advanced stage have a poor prognosis (5). Therefore, it is necessary to identify novel drugs to overcome the chemotherapeutic resistance of liver cancer cells and to develop novel treatments for patients with liver cancer.

Tumor necrosis factor (TNF) superfamily member 10 (TNFSF10) belongs to the TNF family, and is able to induce cell apoptosis in various types of tumor cells; however, its pro-apoptotic activity has not been detected in normal healthy cells (6-9). Cell apoptosis may be initiated by the interaction between TNFSF10 and TNF receptor superfamily member 10a (TNFRSF10A)/TNFRSF10B (10,11). Subsequently, caspase-8 (CASP8) is recruited to the death-inducing signaling complex and may cause the activation of CASP3, inducing cell apoptosis (12-14). TNFSF10 resistance may be acquired via multiple molecular mechanisms, including the differential expression of death receptors and the increased expression of anti-apoptotic factors (15). Notably, treatments combining TNFSF10 with various anticancer drugs have been demonstrated to improve the anticancerogenic effects of a number of compounds (16).

Due to the antiproliferative activity exhibited by ent-3α-form ylabieta-8(14),13(15)-dien-16,12β-olide (EFLDO) in MCF-7 cells and NCI-H460 cells (17), EFLDO has attracted the interest of various research studies (18). The aim of the present study was to investigate the antitumor activity of EFLDO and the potential molecular mechanisms underlying EFLDO function in promoting the inhibitory effects of TNFSF10 in liver cancer cells.

Materials and methods

Cell culture. HepG2 cells (19) and non-cancerous normal liver LO2 cells were purchased from The American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% (v/v) fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin (HyClone; GE Healthcare Life Sciences) in an incubator with 5% CO_2 at 37˚C.

Drug treatments. Non-cancerous normal liver LO2 cells were treated with EFLDO (3 μM) and/or TNFSF10 (25 ng/ml, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and cultured in an incubator with 5% CO_2 at 37˚C for 24 h. EFLDO was extracted from *Euphorbia lunulata* Bge as previously described (17). Hepatoblastoma cells were treated with EFLDO (3 μM), TNFSF10 (25 ng/ml), caspase inhibitor z-DEVD-FMK (working concentration, 40 μM; R&D Systems, Inc., Minneapolis, MN, USA; cat. no. FMK004) and/or the p53 inhibitor pifithrin-α (PFT-α; working concentrations, 20 and 40 μM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and cultured in an incubator with 5% CO_2 at 37˚C for 24 h prior to further experimentation.

MTT assay. Hepatoblastoma HepG2 cells and non-cancerous normal liver LO2 cells were seeded into a 96-well plate at a density of $3x10^3$ cells/well. Following addition of the indicated drug into the culture medium, the cells were incubated in an incubator with 5% CO_2 at 37˚C for 24 h. Subsequently, the cell viability was measured using the MTT assay at 570 nm. The MTT assay was performed using DMSO (Sigma-Aldrich; Merck KGaA) as the solvent to dissolve the formazan as previously described (20).

Annexin V-fluorescein isothiocyanate (FITC) assay. The Annexin V-FITC assay (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was performed to quantify the percentage of apoptotic cells, according to the manufacturer's protocol. HepG2 cells were incubated for 24 h, collected, washed twice with PBS and incubated in 1X binding buffer (provided in the Annexin V-FITC kit). Subsequently, Annexin V-FITC and propidium iodide (PI) were added to the cells and cells were incubated for 20 min at room temperature in the dark. Following incubation with Annexin V-FITC and PI, the cells were analyzed using a flow cytometer (Becton, Dickinson and Company) and the results were analyzed using the CellQuest Pro software (version 5.1; Becton, Dickinson and Company).

CASP3 Activity assay. CASP3 activity was measured using the CASP3 activity assay kit (Chemicon International; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. HepG2 cells were lysed using lysis buffer provided in the kit on ice for 10 min and centrifuged for 5 min at 12,000 x g at 4˚C. Caspase substrate solution was added to the supernatant and incubated for 2 h at 37˚C, subsequently, the activity was measured using an ELISA plate reader at 405 nm.

Western blot. HepG2 cells were incubated with DMEM for 48 h. Cells at a confluency of 70% were lysed using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). The protein concentration was determined using Bradford Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each lane was loaded with 0.05 μg total protein. Proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked with 5% non-fat milk for 1 h at room temperature, and incubated with the following primary antibodies: Anti-cleaved CASP3 (cat. no. 9661), cleaved CASP8 (cat. no. 9496), p53 (cat. no. 2527) (all 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), GAPDH (cat. no. 59540), TNFRSF10A (cat. no. 32255), TNFRSF10B (cat. no. 166624), BCL2 associated agonist of cell death (BAD; cat. no. 8044), BCL2 associated X, apoptosis regulator apoptosis regulator (BAX; cat. no. 20067), BCL2 apoptosis regulator (BCL2; cat. no. 509) (all 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. 7407) and goat anti-mouse (cat. no. 7076) secondary antibodies (all 1:10,000; Cell Signaling Technology, Inc.) were incubated with the membranes at room temperature for 2 h. Bands were developed with the electrochemiluminescence detection reagent (GE Healthcare, Chicago, IL, USA). GAPDH was used as the loading control and Image J software (version 1.8.0, National Institutes of Health, Bethesda, MD, USA) was used to compare the relative band intensities.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HepG2 cells were incubated with DMEM for 48 h, and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. mRNA was reverse-transcribed to cDNA using the PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. For cDNA synthesis, the RT reactions were incubated at 37˚C for 1 h and at 70˚C for 15 min. The sequences of the PCR primers used were as follows: TNFRSF10A forward: 5'-ACCTTCAAGTTTG TCGTCGTC-3'; TNFRSF10A reverse: 5'-CCAAAGGGCTAT GTTCCCATT-3'; TNFRSF10B forward: 5'-GCCCCACAA CAAAAGAGGTC-3'; TNFRSF10B reverse: 5'-AGGTCATTC CAGTGAGTGCTA-3'; GAPDH forward: 5'-ACAACTTTG GTATCGTGGAAGG-3'; GAPDH reverse: 5'-GCCATCACG CCACAGTTTC-3'. GAPDH gene was used as the reference gene. QuantiTect SYBR® Green RT-PCR kit (cat. no. 204245; Qiagen GmbH, Hilden, Germany) was used to analyze relative gene expression. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec, and a final elongation at 72˚C for 15 sec.

Small interfering (siRNA) transfection. p53 siRNA (cat. no. sc-29435) and control siRNA (cat. no. sc-44238) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). For the siRNA transfection, HepG2 cells were seeded in 6-well plates at $3x10^5$ cells/well and cultured in an incubator at 37˚C overnight. On the following day, 0.67 μg siRNAs were mixed with Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) in Opti-MEM™ (Gibco; Thermo Fisher Scientific, Inc.) and incubated at room temperature for 15 min;

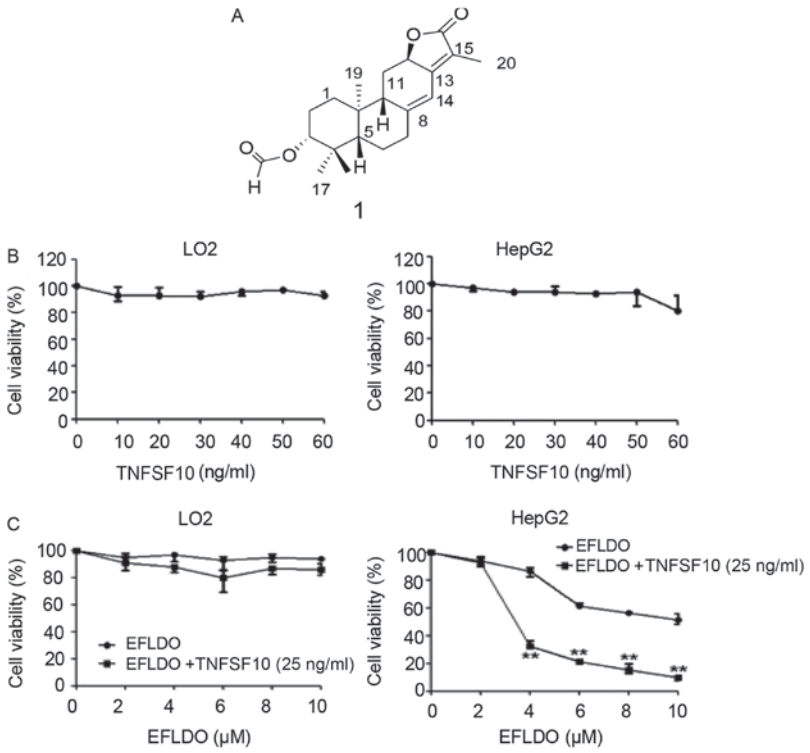

Figure 1. EFLDO increases TNFSF10-induced cell viability inhibition of HepG2 cells. (A) Chemical structure of EFLDO. (B) Non-cancerous normal liver LO2 cells and liver cancer HepG2 cells treated with TNFSF10 and with increasing concentrations of TNFSF10 for 24 h. Cell viability was measured using the MTT assay. (C) LO2 cells and HepG2 cells treated with TNFSF10 (25 ng/ml) and EFLDO for 24 h. Cell viability was assessed using the MTT assay. **P<0.01 vs. respective EFLDO group. EFLDO, Ent-3α-formylabieta-8(14),13(15)-dien-16,12β-olide; TNFSF10, tumor necrosis factor superfamily member 10.

subsequently, the mixtures were added into the culture medium and incubated at 37˚C for 24 h prior to further experimentation.

Statistical analysis. All data were analyzed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation. Comparisons between two groups and multiple groups were performed using Student's t-test and one-way analysis of variance followed by Newman-Keuls test, respectively. P<0.05 was considered to indicate a statistically significant difference. All the experiments were performed ≥3 times.

Results

Synergistic effects of EFLDO and TNFSF10 on HepG2 cell viability. The chemical structure of EFLDO is presented in Fig. 1A. The cytotoxicity of TNFSF10 at various concentrations (10, 20, 30, 40, 50 and 60 ng/ml) on LO2 and HepG2 cells was assessed using the MTT assay. The present MTT results suggested that TNFSF10 exhibited no obvious cytotoxicity in cells at concentrations ≤60 ng/ml (Fig. 1B).

Subsequently, the effects of various concentrations of EFLDO (2, 4, 6, 8 and 10 μM) and EFLDO (2, 4, 6, 8 and 10 μM) + TNFSF10 (25 ng/ml) on cell viability were detected. The present results suggested that the cell viability of HepG2 cells significantly decreased following treatment with EFLDO, in a concentration-dependent manner. Notably, the cell viability of normal liver LO2 cells was unaffected by treatment with EFLDO (Fig. 1C). Additionally, the cell viability of HepG2 cells was significantly decreased following the administration of EFLDO + TNFSF10 compared with administration of EFLDO alone. Furthermore, treatment with EFLDO (3 μM) + TNFSF10 (25 ng/ml) exhibited a significant antiproliferative activity (data not shown). Therefore, 25 ng/ml was selected at the working concentration of TNFSF10 and 3 μM was selected as the working concentration of EFLDO for further experiments.

EFLDO increases TNFSF10-induced cell apoptosis in HepG2 cells. The potential of EFLDO in sensitizing HepG2 cells to TNFSF10-induced apoptosis was investigated. Flow cytometry results suggested that the apoptotic rate of HepG2 cells following treatment with EFLDO + TNFSF10 increased compared with the control, TNFSF10 and EFLDO groups (Fig. 2A). The apoptotic rates for HepG2 cells in the TNFSF10 and EFLDO + TNFSF10 group were 9.57 and 60.7%, respectively (Fig. 2B). The present results suggested that the combination of EFLDO and TNFSF10 increased the effect of TNFSF10 in inducing the apoptosis of HepG2 cells.

EFLDO promotes TNFSF10-induced apoptosis of HepG2 cells in a caspase-dependent manner. Previous studies demonstrated that activation of CASP8 induced by TNFSF10 may activate CASP3 and CASP9 (12-14). To investigate the molecular mechanisms underlying cell apoptosis induced by treatment with EFLDO + TNFSF10, the activity levels of CASP8 and 3 in HepG2 cells treated with TNFSF10 and/or EFLDO were detected. The present results suggested that proteolytic cleavage of proCASP8 and proCASP3 increased following treatment with EFLDO + TNFSF10 compared with the control, TNFSF10 and EFLDO groups

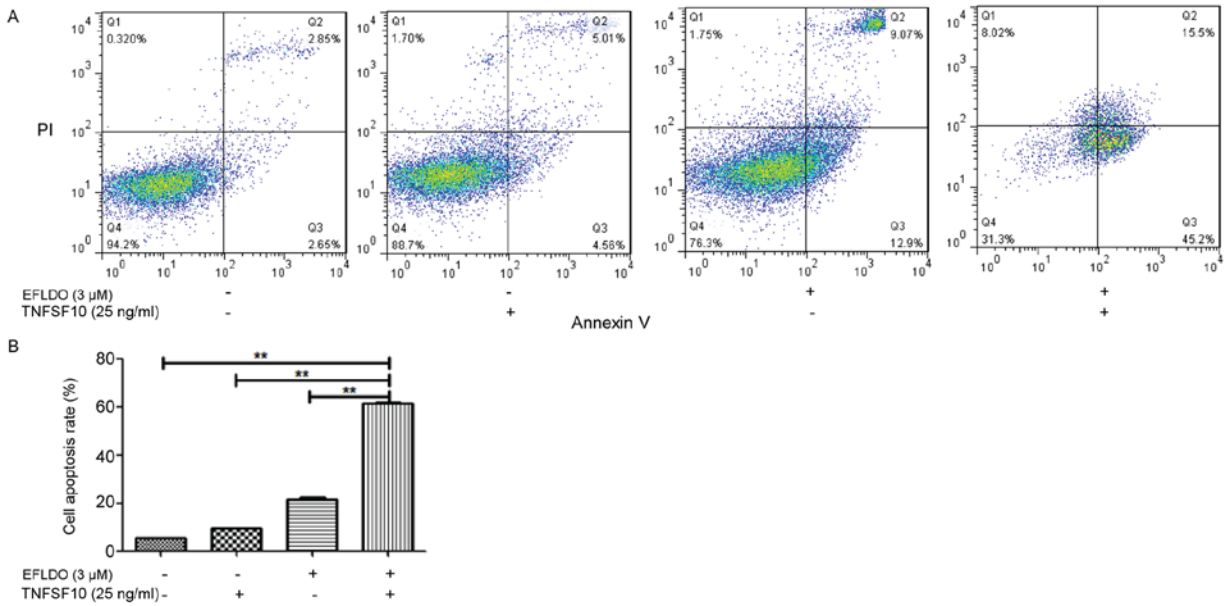

Figure 2. EFLDO increases TNFSF10-induced cell apoptosis. (A) HepG2 cells treated with EFLDO and/or TNFSF10 for 24 h. Flow cytometry was used to analyze the percentage of Annexin V/PI positive cells and the percentage of apoptotic cells was measured. (B) Statistical analysis of apoptotic rates. **P<0.01. EFLDO, Ent-3α-formylabieta-8(14),13(15)-dien-16,12β-olide; TNFSF10, tumor necrosis factor superfamily member 10; PI, propidium iodide.

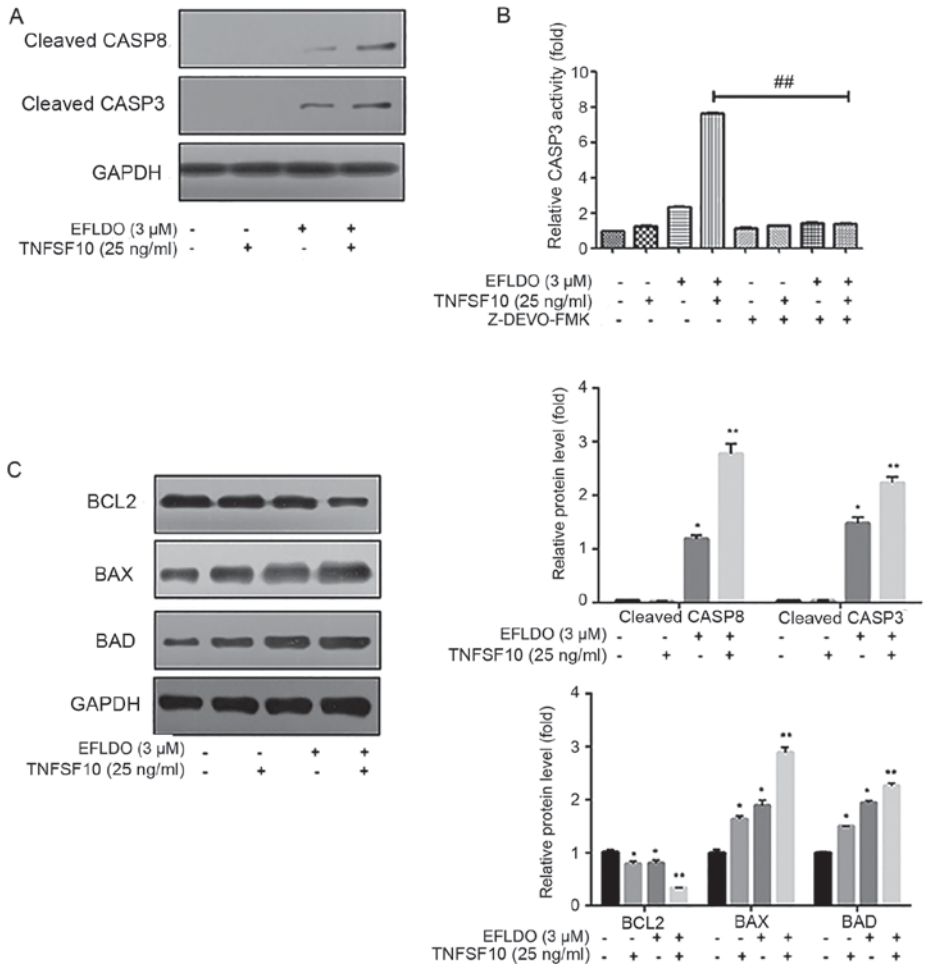

Figure 3. Treatment with EFLDO sensitizes HepG2 cells to TNFSF10-induced apoptosis via the caspase pathway. (A) Protein expression levels of cleaved CASP8 and CASP3 in HepG2 cells following treatment with EFLDO and/or TNFSF10. (B) Activity of CASP3 in liver cancer cells treated with EFLDO, TNFSF10 and/or caspase inhibitor z-DEVD-FMK. Data are presented as fold change relative to the mean CASP3 activity of the control group. $^{\#\#}$P<0.01. (C) Protein expression levels of BLC2, BAX and BAD in HepG2 cells treated with EFLDO and/or TNFSF10. *P<0.05, **P<0.01 vs. respective control group. EFLDO, Ent-3α-formylabieta-8(14),13(15)-dien-16,12β-olide; TNFSF10, tumor necrosis factor superfamily member 10; CASP, caspase; BCL2, BCL2 apoptosis regulator; BAD, BCL2 associated agonist of cell death; BAX, BCL2 associated X, apoptosis regulator.

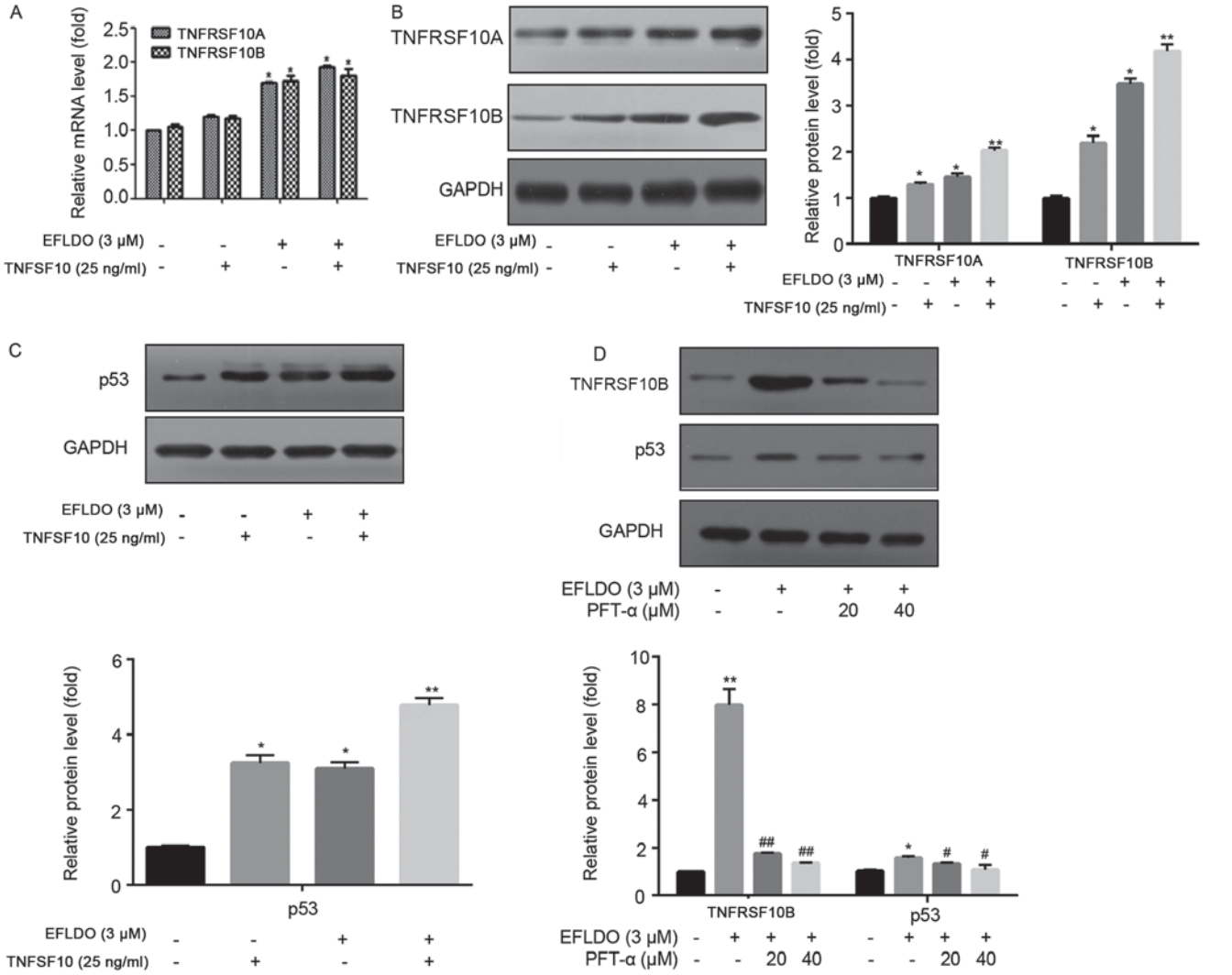

Figure 4. EFLDO treatment sensitizes liver cancer cells to TNFSF10-induced apoptosis by upregulating the protein expression levels of TNFRSF10A and TNFRSF10B in a p53-dependent manner. (A) HepG2 cells were treated with EFLDO and/or TNFSF10 for 24 h. Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the mRNA expression levels of TNFRSF10A and TNFRSF10B. In total, three independent experiments were performed. (B) Western blotting was performed to detect the protein expression levels of TNFRSF10A and TNFRSF10B. (C) Protein expression levels of p53 in HepG2 cells treated with EFLDO and/or TNFSF10. (D) Protein expression levels of TNFRSF10B and p53 in HepG2 cells treated with EFLDO, TNFSF10 and/or PFT-α. *P<0.05, **P<0.01 vs. respective control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. respective EFLDO group. EFLDO, Ent-3α-formylabieta-8(14),13(15)-dien-16,12β-olide; TNFSF10, tumor necrosis factor superfamily member 10; TNFRSF10A, TNF receptor superfamily member 10a; TNFRSF10B, TNF receptor superfamily member 10b.

(Fig. 3A). CASP3 activity in the EFLDO + TNFSF10 group increased to 7.0-fold compared with the control group, which was increased compared with TNFSF10 (0.7-fold) and EFLDO (1.4-fold) groups, and was significantly decreased following treatment with the caspase inhibitor z-DEVD-FMK (Fig. 3B). Subsequently, treatment with EFLDO + TNFSF10 significantly increased the protein expression levels of BCL2 associated agonist of cell death (BAD) and BAX, and decreased the protein expression level of BCL2 in HepG2 cells compared with the control, TNFSF10 and EFLDO groups (Fig. 3C). The present results suggested that EFLDO significantly sensitized HepG2 cells to TNFSF10-induced apoptosis in a caspase-dependent manner.

EFLDO sensitizes HepG2 cells to TNFSF10-induced cell apoptosis by increasing the expression levels of TNFRSF10A and TNFRSF10B. TNFRSF10A and TNFRSF10B (TNFSF10 receptors) were identified to be able to initiate the apoptotic cascade by binding to TNFSF10 (21). The present RT-qPCR and western blotting results suggested that treatment with EFLDO or EFLDO + TNFSF10 increased the mRNA (Fig. 4A) and protein expression levels (Fig. 4B) of TNFRSF10A and TNFRSF10B compared with the control group. A previous study observed that p53 may serve a role in regulating the functions of TNFRSF10A and TNFRSF10B (22). The present western blotting results suggested that, compared with the control group, the protein expression level of p53 was significantly increased following treatment with EFLDO or EFLDO + TNFSF10 (Fig. 4C). The effects of EFLDO on the protein expression levels of p53 and TNFRSF10B were inhibited by treatment with PFT-α (Fig. 4D). The present results suggested that treatment with EFLDO + TNFSF10 increased the protein expression levels of TNFRSF10A and TNFRSF10B by increasing the protein expression level of p53 in HepG2 cells.

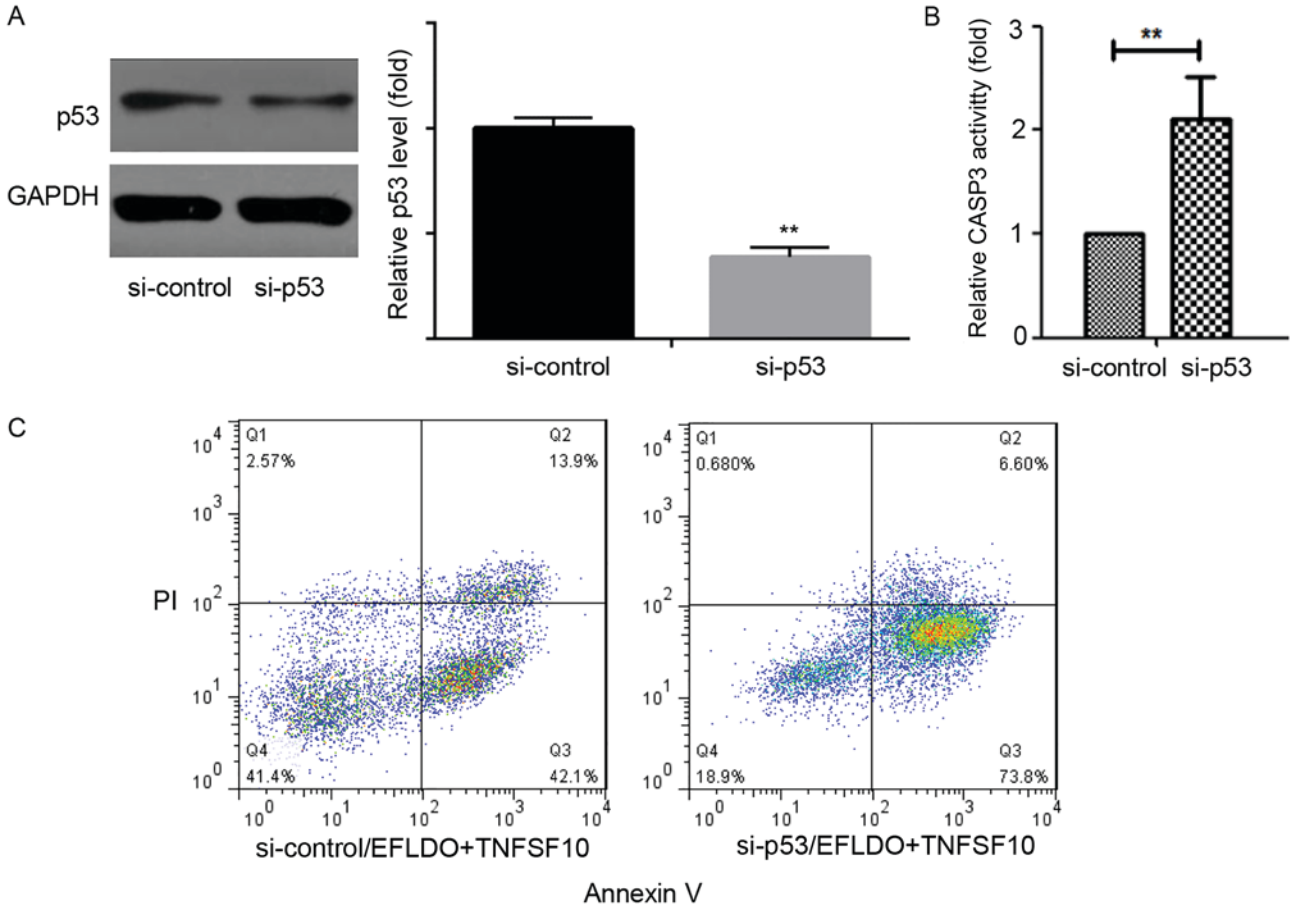

Figure 5. EFLDO increases TNFSF10-induced cell apoptosis in a p53-dependent manner in HepG2 cells. (A) p53 was silenced using siRNA technology in HepG2 cells. (B) Activity of CASP3 was detected in HepG2 cells transfected with si-p53 or si-control and treated with the combination of EFLDO and TNFSF10 for 24 h. Data are presented as a fold change relative to the mean CASP3 activity of the control group. (C) HepG2 cells were transfected with si-p53 and treated with the combination of EFLDO and TNFSF10. Following 24 h, cells were harvested for flow cytometry analysis. **P<0.01 vs. si-control. EFLDO, Ent-3α-formylabieta-8(14),13(15)-dien-16,12β-olide; TNFSF10, tumor necrosis factor superfamily member 10; PI, propidium iodide; FITC, fluorescein isothiocyanate; si-, small interfering RNA; CASP, caspase.

EFLDO increases TNFSF10-induced cell apoptosis in a p53-dependent manner in HepG2 cells. To investigate the role of p53 on the effects of EFLDO in increasing TNFSF10-induced cell apoptosis, p53 was silenced by transfecting a siRNA targeting p53 (si-p53) in HepG2 cells (Fig. 5A). Knockdown of p53 significantly increased the activity of CASP3 in liver cancer cells (Fig. 5B). si-p53 significantly increased the apoptosis of HepG2 cells following treatment of TNFSF10 + EFLDO (Fig. 5C). The present results suggested that inhibition of p53 may increase EFLDO-induced TNFSF10 sensitivity in HepG2 cells.

Discussion

Previous studies in liver cancer demonstrated that the upregulation of antiapoptotic genes, and the increase in the protein expression levels of dysadherin and various cytokines, may serve roles in the negative outcome of certain therapies and in cancer metastasis (23-25). Combinatorial treatments were identified to exhibit an increased effectiveness compared with single-agent chemotherapy in various types of cancer, including liver cancer (26). Nevertheless, the efficacy of chemotherapy to treat cancer may be decreased by the occurrence of drug resistance. Therefore, the identification of novel therapies is required to improve the treatment of patients with liver cancer.

Due to its strong antiproliferative activity in MCF-7 and NCI-H460 cells (17), EFLDO has attracted the interest of various research studies (18). However, the effects of the combination of EFLDO with additional anticancer drugs in liver cancer remain unknown.

The present results suggested that TNFSF10 did not induce cytotoxicity. By contrast, the combination of EFLDO and TNFSF10 presented a synergistic effect in suppressing cell viability of HepG2 cells; however, this combinatorial treatment did not affect the viability of non-cancerous normal liver LO2 cells. Subsequently, EFLDO was identified to sensitize HepG2 cells to TNFSF10-induced apoptosis. The present results suggested that apoptotic rates of HepG2 cells increased significantly following the cotreatment with TNFSF10 and EFLDO compared with single-treatment with TNSFS10 or EFLDO, indicating a potential synergistic effect between TNFSF10 and EFLDO in inducing apoptosis in liver cancer cells.

Furthermore, the activities of CASP8 and 3 following treatment with TNFSF10 and/or EFLDO were detected to investigate the molecular mechanisms underlying cell apoptosis induced by the combined treatment with EFLDO and TNFSF10. The present results suggested that liver cancer

cell apoptosis was induced by EFLDO + TNFSF10 via the activation of CASP3 and CASP8. Treatment with EFLDO and TNSFS10 induced the upregulation of pro-apoptotic molecules, including BAD and BAX, and the downregulation of the anti-apoptotic factor BCL2. The present results suggested that EFLDO may promote TNFSF10-induced liver cancer cell apoptosis in a caspase-dependent manner.

p53 was previously identified to serve a role in regulating the function of TNFRSF10A and TNFRSF10B (22). p53 is able to inhibit cell proliferation by inducing cell apoptosis and cell cycle arrest in response to cellular stresses (27-29). The present results suggested that the synergistic effects between EFLDO and TNFSF10 in increasing the protein expression level of TNFRSF10B may be due to the upregulation of p53, since the protein expression level of TNFRSF10B was decreased following treatment with the p53 inhibitor PFT-α. In addition, knockdown of p53 increased the activity of CASP3 and increased cell apoptosis in HepG2 cells, suggesting that p53 may regulate the synergistic effects between EFLDO and TNFSF10 in liver cancer cells. However, treatment with EFLDO and TNFSF10 led to an increase in the expression level of p53, in contrast with the results of p53 knockdown. p53 may be involved in various pathways regulating HepG2 cells, and further studies are required to confirm the present results.

In conclusion, combinatorial treatment with TNFSF10 and EFLDO induced cell apoptosis in HepG2 cells by promoting the caspase pathway, suggesting a potential therapeutic effect of the combination of EFLDO and TNFSF10 in treating liver cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YQ acquired the data, analyzed and interpreted the data, drafted the manuscript and revised it critically. ZL designed and conceived the study and revised the manuscript. XW, JZ and CL performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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