Apoptosis Induced by ESC-3 Through Notch Signaling Pathway and Mitochondrial Membrane Potential ($\Delta \Psi_m$) in Liver Cancer

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Abstract
In previous studies, our findings demonstrated that ESC-3, which extracted from Crocodylus siamensis bile, could obviously inhibit proliferation and induce apoptosis in human cholangiocarcinoma cells and ovarian cancer. However, the efficient and molecular mechanism of ESC-3 kept unknown in liver cancer. The purpose of the study is to unveil the underlying mechanism and build xenograft models to confirm the efficient of ESC-3 in potential therapeutic. MTT assay was applied to evaluate the effects of ESC-3 on the proliferation of liver cancer cells and human normal liver cancer cells (L02). Flow cytometry assays were performed to explore the distribution of the cell cycle. Western blot analysis, qPCR assay and immunohistochemistry (IHC) were performed to determine the expression levels of apoptosis-related or pathway-related protein isolated from cells and tumors. Xenograft models were employed to confirm the efficient of ESC-3 on inhibiting the growth of liver tumors furtherly. Significant inhibitory effects of ESC-3 were observed in liver cancer, while there is no remarkable change in the proliferation of human normal liver cancer cells after treatment with ESC-3 compared with control group. Our findings elucidated that ESC-3 might cause S-phase cycle arrest through cyclinA1 and CDK2. In addition, we demonstrated that apoptosis induced by ESC-3 was closely contacted with Notch signaling pathway and mitochondrial membrane potential ($\Delta \Psi_m$) in liver cancer. Importantly, ESC-3 could markedly inhibit the growth of transplantation tumors without toxic to BALB/c nude mice in xenograft models. Our study elucidated that ESC-3 could induce apoptosis through Notch signaling pathway and mitochondrial membrane potential ($\Delta \Psi_m$), therefore ESC-3 could be a potential therapeutic in liver cancer.

Key words: ESC-3, Liver Cancer, Notch Pathway, Mitochondrial Membrane Potential ($\Delta \Psi_m$), Xenograft Models.

1. Introduction
Liver cancer is one of the common gynecologic malignant tumors in human beings, ranking fifth in the occurrence rate of male and seventh in the occurrence rate of female. According to the mortality rate of
malignant tumors, liver cancer ranked third following lung cancer and gastric cancer [1, 2]. Ever-increasing evidence indicated that Hepatitis B was one of the main causes of liver cancer while the number of patients with liver cancer has been increasing every year [3]. Surgical resection is still the major method to enhance the survival rate of patients, but the majority of patients with surgical resection appearing recurrence and metastasis, needed to use systemic chemotherapy as adjuvant therapy [4, 5]. Chemotherapy, a kind of systemic treatment therapy, plays a vital role in curing the primary lesions and tumor metastasis. The limited clinical applications of chemotherapy have been attributed to various factors, including poor selectivity, the killing of normal cells, toxic side effects and easy to produce tolerance [6, 7]. Therefore, it is now an important and urgent challenge to search for natural drugs in the pharmaceutical industry.

In traditional Chinese medicines (TCM), animal bile and bile extract have been applying to basic medical treatment for thousands of years [8]. With progressive research on traditional medicine, researchers found the physiological active substance used in detoxification and inflammation were tauroursodeoxycholic acid, ursodeoxycholic acid, and deoxycholic acid [9, 10]. In previous studies, crocodile choline (CCL), an active and natural compound extracted from animal bile, was found to have anti-cancer properties in human non-small cell lung cancer cells in vitro and in vivo [11]. ESC-3 (Fig. 1A), the major natural and effective component isolated and purified from CCL by Sephadex LH-20 and RP-18 reversed-phase column from Crocodylus siamensis bile, could significantly inhibit proliferation and induce apoptosis in human cholangiocarcinoma cells, non-small cell lung cancer and human gastric cancer. Song, W. found that apoptosis could be induced by ESC-3 obviously in human cholangiocarcinoma cell lines (QBC939, Sk-ChA-1 and MZ-ChA-1), meanwhile the localization of prohibitin protein was changed dramatically in the process of apoptosis [12, 13]. However, it remains ambiguous whether ESC-3 could inhibit the proliferation of liver cancer cells and the growth of xenograft tumorigenesis in vivo. In this study, we firstly evaluated the anticancer effects of ESC-3 and elucidated the apoptosis mechanism in liver cancer cell lines. Besides BALB/c nude mice models also were built to affirm the potential and the effectiveness of ESC-3 as a candidate for liver cancer therapy.

2. Materials and Methods

2.1. Reagents
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 2,7-Dichloroﬂuorescin diacetate (DCFH-DA), acridine orange (AO), ethidium bromide (EB), propidium iodide (PI), and proteinase K were purchased from Sigma-Aldrich Co. (St. Louis, MO, United States). The 40 mg/ml of ESC-3 was dissolved by dimethyl sulfoxide as the stock solution at -20°C. Gallbladders of Crocodylus siamensis were acquired from the Sriracha Tiger Zoo Thailand Co. Ltd.

2.2. Cell culture and MTT assay
The human liver cancer cell lines (HepG2, SMMC-7721 and Huh-7) and hepatocyte cell line L02 were cultured in Roswell Park Memorial Institute (RPMI) 1,640 medium supplemented with 100 μg/ml streptomycin, 100U/ml penicillin and 10 % heat-inactivated fetal calf serum. The inhibition rates of cells were detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, twelve hours after seeding into 96-well plates, liver cancer cell lines were treated with ESC-3 at different concentrations (10, 20, 40 and 80 μg/ml). Then absorption value of each well was recorded at 570 nm using a microplate reader (POLAR star Omega, Germany).

2.3. Morphological changes
After collected and washed with phosphate-buffered saline (PBS) triple, the cells were stained with AO/EB and observed using Nikon TE2000 (Japan).

2.4. Colony-forming assay
After seeded into 60 mm plates for 12 h, the cell were treated with ESC-3 (10, 20, 40 μg/ml) for 48 h. Then media containing ESC-3 was removed and the cells were treated with serum-free medium for 14 days. Fixed with methanol for 10 min and tinted with Giemsa stain for 10 min, the number of colonies was counted manually.

2.5. Flow cytometry assay
Cell cycle analysis: after treated with drug for 48 h, the HepG2 cells were gathered and washed with the PBS triple. The liver cancer cells were fixed in cold 650 mL/L ethanol on ice at 4 °C for 30 min and were resuspended in 100 μg/mL RNase A for 30min after centrifugation. Then the HepG2 cells were dyed with 50 μg/mL propidium iodide (PI) at 4 °C for 40 min in dark environments. Cells were analyzed by FC500 at 488 nm, then the data were analyzed with CellFit software. Cell apoptotic assay was conducted as the previous study [12].
2.6. Western blot analysis and qRT-PCR Assay

Quantitative real-time PCR was performed with two-step reverse transcription PCR as previous study [14]. After treatment with ESC-3, the HepG2 cells were collected and distracted in RIPA buffer for 40 min. Equivalent amounts of protein samples were separated by 12% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with the specific primary antibodies [Bax (1:1000), Bcl-2 (1:1000), PARP (1:1000), CDK1 (1:1000) and CyclinB1 (1:1000) were purchased from ProteinTech Group, Inc; Wnt2 (1:800), β-catenin (1:1000), VEGF (1:1000) and survivin were purchased from Sangon Biotech Co., Ltd. Antibodies (1:1000) against β-action were purchased from Sigma-Aldrich] and probed with horseradish peroxidase conjugated secondary antibodies (Catalogue numbers: A0545; Dilution ratio: 1:60000, Sigma-Aldrich, Rabbit). The signal was finally detected with the ECL system (Pierce Co., USA).

2.7. Xenograft Models

Female nude mice (BALB/c, 18±2 g) were purchased from SLRC Laboratory Animal Co., Ltd. The BALB/c nude mice were maintained on standard water ad libitum and pellet diet throughout the experiments. Additionally, the animals were kept in the condition of 60-65% humidity and a constant temperature (21-22˚C) with a 12h-light/12h-dark cycle. The experimental processes were implemented abiding by the guidelines for the humane treatment of animals set by the Laboratory Animal Center. Briefly, about 1×10⁶ HepG2 cells were injected into nude mice. The body weight and tumor size was recorded every three days.

2.8. Hematoxylin/Eosin staining and Immunohistochemistry (IHC)

Hematoxylin/Eosin staining were performed as the previous study [11]. Immunohistochemistry assay: 4 μm sections were taken on gelatin coated slides from paraffin-embedded tumor tissue, which were dewaxed by immersing in xylene for 10 min after dehydration in ethanol (100%, 100%, 95%, 95%, 80%, 70% and 50%). Dewaxed sections were boiled in 0.01 M Na-citrate (pH 6.0) for 30 min at 100 °C and blocked with blocking solution (5% normal goat serum in PBS) for 30 min in advance. Afterwards, dewaxed sections were incubated with the specific primary antibodies at 4 °C overnight, followed by treating with secondary antibody conjugated with HRP. At last, we used an inverted fluorescence microscope collecting and analyzing images.

3. Results and Discussion

3.1. ESC-3 inhibited the proliferation of live cancer cells (HepG2, SMMC-7721 and Huh-7), and suppressed colony-forming ability and elicited the morphological changes in HepG2 cells.

![Fig. 1: ESC-3 inhibited the proliferation of live cancer cells (HepG2, SMMC-7721 and Huh-7), and suppressed colony-forming ability and elicited the morphological changes in HepG2 cells.](image-url)
To evaluate the effects of ESC-3 on the proliferation of liver cancer cell lines and human normal liver cancer cells (LO2) using MTT assay in vitro, liver cancer cell lines were treated with ESC-3 at different concentrations for 24, 48 and 72 h. As shown in Fig. 1B, 1C and 1D, the proliferation of liver cancer cell lines (HepG2, SMMC-7721 and Huh-7) was inhibited significantly, with IC_{50} values of 37.62, 78.93 and 98.82 μg/ml for 48 h, respectively. However, there is no remarkable change in the proliferation of human normal liver cancer cells after treatment with ESC-3 compared control group (Fig. 1E). After treated with ESC-3, the cells represented typical apoptotic features: apoptotic volume decrease, chromatin condensation and dense nuclei as shown in Fig 1F. Based on the quantitative results of cell cloning assays, the colony-forming efficiency of the treated group had a significant dose-dependent reduction (Fig. 1G).

Apoptosis, a normal form of programmed cell death, plays a vital role in the progression of cancer and tissue homeostasis of eukaryotes [15, 16]. It is a highly advisable goal to hold back metastasis of the tumor for cancer control through inducing apoptosis [17]. In this study, we investigated whether ESC-3 exerted anticancer properties on liver cancer cells and interpreted the molecular mechanism. Data demonstrated the ESC-3 inhibited liver cancer cells with IC_{50} value 37.62 μg/ml (HepG2 cells), 78.93 μg/ml (SMMC-7721 cells) and 98.82 μg/ml (Huh-7 cells) for 48 h, respectively. After treated with 40 μg/ml ESC-3, HepG2 cells present the primary indicators of apoptosis[18], which refers to changes in cell morphology: cell shrinkage, dense nuclei, apoptotic body formation et al. Cell cycle control played a vital role in inhibiting tumor growth, while Mork, C. N et al [19] suggested that arresting the cell cycle was a mechanistic method to anticancer therapy.

3.2. ESC-3 caused S-phase cycle arrest

![Fig. 2 ESC-3 could arrest cell cycle at S phase.](image)

To further demonstrate that ESC-3 suppressed the proliferation of HepG2 by disturbing the cell cycle, FCM (flow cytometry) assay was conducted after treatment with ESC-3 (0, 10, 20 and 40 μg/ml) for 48 h. As shown in Fig. 2A and 2B, the proportion of the HepG2 cell line in G0/G1 decreased from 63.21% to 44.21% after treatment with increasing concentration ESC-3, while the proportion of cells in S-phase increased from 20.02% to 41.39% and G2/M remained unchanged. The data of qPCR analysis demonstrated that the mRNA levels of cyclin A1 increased, while the mRNA levels of CDK1 decreased after treatment with ESC-3 (Fig. 2C). We, therefore, demonstrated that ESC-3 might cause S-phase cycle arrest through cyclin A1 and CDK2. Our data revealed that the HepG2 cells were arrested at S-phase through controlling the expression levels of cyclin A1 and CDK2, two kinds of S-transition regulators [20, 21].

3.3. Involvement of the mitochondria-mediated intrinsic pathway in apoptosis induced by ESC-3 in HepG2 cells

The HepG2 cells were analyzed with annexin V and PI to demonstrate the proportion of viable, early apoptotic, late apoptotic or necrotic cells. As shown in Fig. 3A, the percentage of apoptosis and necrosis increased remarkably after treated with ESC-3. With the increasing concentration of ESC-3, the rate of depolarization increased significantly (18.61±0.23%, 20.34±0.18%, 50.34±0.18% for 10 μg/mL, 20 μg/mL and
40 µg/mL respectively) as shown in Fig. 3B. Cytosolic and mitochondrial fractions were prepared and the level of cytochrome c in cytosol and mitochondria were detected by western blotting. As shown in Fig. 3C, the expression levels of cytochrome c increased in the cytosol and decreased in mitochondria after exposure to ESC-3. JC-1, a fluorescent probe for ΔΨm, was used to unearth which emits green fluorescence when the level of ΔΨm is reduced and emits red fluorescence when the level of ΔΨm is high. As shown in Figure 3D, the proportion of green fluorescence increased from 3% to 21% (*p < 0.05) following the increasing in the concentration of ESC-3, which indicates a breakdown of ΔΨm in HepG2 cells.

3.4. ESC-3 suppressed the increasing volume of tumors without toxic to viscera through mitochondrial membrane potential (ΔΨm)

Xenograft models were built to assess the anti-tumor effects of ESC-3. The experimental processes were implemented abiding by the guidelines for the humane treatment of animals set by the Laboratory Animal Center. The present study used ESC-3(80mg/kg/3days) mixed with corn oil via intraperitoneal injection (control with corn oil only).
The body weight and relative visceral coefficient of BALB/c nude mice show no remarkable changes after treatment with ESC-3 (Fig. 4A and 4B), while there is a significant difference between ESC-3 group and the corn oil group in the volume of tumors. The viscera (tumor, liver and lung) of animals were dyed with H&E staining, as showed in Fig. 4D, internal organs (heart, liver, spleen, lung and kidney) didn’t present an obvious changes in the structure of pathological sections. The nude mice were put to death and tumors was removed (Fig. 4E) and weighted (Fig. 4F), tumors from treated group were lighter and smaller compared with those of the corn oil group (*P<0.05). Furthermore, the proteins and mRNA acquired from tumors were detected through qPCR assay and western blot analysis. ESC-3 decreased the protein levels of Apa1, Survivin, Caspase3, Caspase9, Bcl-2 and VEGF, while increased the protein levels of Bax and P53 (Fig. 4G). And the effect of ESC-3 on Bax, Caspase3 protein is consistent with the expression of mRNA (Fig. 4H and 4I). The Bcl-2 family participates in regulating of apoptosis, including Bcl-2 and Bax, of which the former could arrest the progression of cancer and the latter induce apoptosis [22]. The Mitochondria-mediated intrinsic pathway and the cell death receptor pathway are two kinds of signaling pathways involving the mechanisms of apoptosis mainly, of which the former serves a vital role in the progression of apoptosis through regulating numerous signaling pathway from intracellular space [23, 24]. In this study, the data demonstrated that ΔΨm was collapsed after treatment with ESC-3 and cytochrome c flows from cytoplasm to the mitochondria in HepG2 cells activating the activity of caspase 3 and caspase 9, through which could induce apoptosis and arrest cell cycle [25, 26]. The BALB/c nude mice models were used to affirm the efficient of ESC-3 in inhibiting the growth of liver tumors furtherly. The experimental processes were implemented abiding by the guidelines for the humane treatment of animals set by the Laboratory Animal Center. In this study, we presented evidence demonstrated that ESC-3 could suppressed the increasing volume of tumors and the T/C (%) is 52.8%. The results of the western blot demonstrated the consistency between the expression of proteins in vitro and in vivo. Our findings indicated that apoptosis signaling pathway in HepG2 cells was not in agreement with mechanism in SMMC-7721 xenograft models. Data demonstrated that ESC-3 could suppressed the increasing volume of tumors in SMMC-7721 models and the T/C (%) is 44.7%, but proteins (caspase-3 and caspase-9), which participated in mitochondria-mediated intrinsic pathway, remain stable and that involving notch signaling pathway changed significantly. The protein levels of notch 1 and HES family changed dramatically by ESC-3, of which the former is the typical Notch ligands and the latter is Notch receptors. Our findings also revealed that ESC-3 could down-regulate the expression of PCNA in outer edge and inside of SMMC-7721 tumor, which might impact the growth of tumors [27].Our research demonstrated that ESC-3 could result in the increasing of Bax/Bcl-2 ratio through up-regulation the expression of Bax proteins in liver cancer, which usually could induce apoptosis[35, 36].
3.5. The growth of tumors was suppressed by ESC-3 through Notch signaling pathway in SMMC-7721 xenograft models

To determine the anti-tumor effects and molecular mechanism of ESC-3 in SMMC-7721 xenograft models, the tumor volume and body weight of mice were recorded to confirm the effectiveness and non-toxic of ESC-3 in vivo. Our data indicated that the weight of bodies displayed no obviously changes after treatment with ESC-3, while the volume of tumors was suppressed by ESC-3 (in Fig. 5A and 5C).

![Figure 5](image)

**Fig. 5.** The growth of tumors was inhibited by ESC-3 in SMMC-7721 xenograft models. (A) The growth curve of nude mice in weight in HepG2 xenograft models. (B) Relative visceral coefficient between control and treated group. (C) The curve of mean tumor volume. (D, E) Pathological paraffin sections of tumors. (F) Solid tumors. (G) Mean tumor weight at the end of 21 days.

The viscera of mice was excised, weighted and dyed with H&E stain, the weight and organizational structure of internal organs had no significant changes between the treated and control group (5 Fig. 5B, 5D and 5E). As shown in Fig. 5F and 5G, the growth of tumors was inhibited by ESC-3 and the weight of tumors was lighter in ESC-3-treated group[37]. The apoptosis-associated proteins were measured by western blot and immunohistochemistry. As shown the Fig. 6A and 6B, the value of Bax/Bcl-2 was increased markedly (Fig. 6D), while there is no significant change in mitochondrial pathway related proteins (caspase 3 and cleaved caspase 3; caspase 9 and cleaved caspase 9) between ESC-3 and control group. The effect of ESC-3 on the protein levels of P27, P62, MMP-9, MMP-2 and survivin were analyzed. The date revealed that the expression of MMP-9 and MMP-2 was decreased markedly (Fig. 6E).

The protein expression level of P62 and survivin also decreased with ESC-3 treatment, while there is no significant change in the expression level of P27 between the ESC-3 group and corn oil group. The expression levels of Notch1, notch pathway related-protein, increased significantly, while the protein levels of AIB1 remained steady between ESC-3 and control group. The protein levels of Hes1, downstream proteins of Notch signaling, increased remarkably after treatment with ESC-3, while Hes5 decreased in the expression level (Fig. 6C and 6F). The protein expression levels of PCNA, VEGF and Ki67 decreased obviously (Fig. 6G). Therefore, our findings indicated that ESC-3 might induce apoptosis in SMMC-7721 xenograft models through notch signaling pathway. The Notch signaling plays a vital role in the produce of cells fate determination and development [28]. It was also involved in immune regulation, neural stem cell survival and vascular development [29]. Four kinds of Notch genes have been discovered and appraised in the highly conserved Notch signaling [30]. As Shown in Fig. 7, our findings elucidated that the growth of tumors was inhibited through notch.
Fig. 6. Molecular mechanism: the growth of tumors was suppressed by ESC-3 without toxic to viscera. (A, B and C) The expression levels of proteins involving apoptosis pathway. (D) Immunohistochemistry was conducted to detect the protein levels of Notch1, COX-IV, PCNA and MMP-9. (E, F and G) The quantified results of proteins involving the signaling pathway.

signaling pathway in SMMC-7721 xenograft models, and it is an interesting finding that apoptosis was induced through different signaling pathways in HepG2 cells and SMMC-7721 cells, which was in concordance with the previous studies showing the same drug could induce apoptosis through different signaling pathways in differentiated cancer cells [31, 32]. Perumalsamy et al. found that mitochondrion is an indispensable part of a Notch-activated signaling cascade [33], and increasing evidence elucidated the crosstalk [34].

Collectively, ESC-3 not only could arrest cell cycle at S-phase through Cyclin A1 and CDK2, but also induce apoptosis through Notch signaling pathway and mitochondrial membrane potential (ΔΨm) in liver cancer. However, the exact mechanism by which achieved the crosstalk between notch signaling pathway and...
Mitochondria-mediated intrinsic pathway keep unclear. Regardless, our findings highlight that ESC-3 could be a potential therapeutic in liver cancer.

4. Conclusions

ESC-3 could suppress the increasing volume of tumors significantly without toxic to BALB/c nude mice in xenograft models. In the meantime, our study elucidated that ESC-3 could induce apoptosis through Notch signaling pathway and mitochondrial membrane potential (ΔΨm). In conclusions, our findings highlight that ESC-3 could be a potential therapeutic in liver cancer.

Competing interests

The authors declare that they have no conflict of interest.

Author’s Contributions Acknowledgements

Qi-Rui Fu and Shao-Min Huang performed the experiments and acquired data. Qi-Rui Fu and Shao-Min contributed equally to this work. Qiong-Hua Chen, Jiang-Xing Zhuang designed the research and analyzed the data. Huan Yu took part in some experiments. Qing-Xi Chen and Jin-Kun Zeng revised the data. All authors approve of the version to be published.

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