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Euxanthone exhibits anti-proliferative and anti-invasive activities in hepatocellular carcinoma by inducing pyroptosis: preliminary results

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths. Euxanthone, a xanthone compound extracted from *Polygala caudata*, possesses a variety of pharmacological activities. This work aimed to explore whether euxanthone exhibits anti-cancer activities in HCC.

PATIENTS AND METHODS: Tissue specimen was collected and the mRNA and protein expression of marker proteins were detected by qRT-PCR and Western blot, respectively. The viable cell number was assessed by CCK-8 assay. TUNEL assay was utilized to determine cell death. Cell migration was measured by Wound healing. Cell invasion was measured by transwell assay. Xenograft model was established to evaluate the efficacy of euxanthone *in vivo*.

RESULTS: We demonstrated that HCC tissue and HCC cell line presented with lower expression of caspase-1, IL-1β, and IL-18. Euxanthone markedly suppressed the proliferation of HCC cells and induced cell death. In addition, euxanthone inhibited cell migration and invasion. Meanwhile, our results showed that euxanthone promoted cell death in a caspase-2 dependent manner. In vivo data also showed that euxanthone suppressed tumor growth and promoted pyroptotic cell death.

CONCLUSIONS: We showed that that euxanthone may be a candidate of anticancer agent and provide clinical benefits for patients with HCC.

Key Words:
Euxanthone, Hepatocellular carcinoma, Pyroptosis.

Introduction

Hepatocellular carcinoma (HCC) has the highest incidence rates in Asia and Africa[1-3]. Conventional treatment options for HCC patients include surgery, interventional therapy, radiofrequency ablation, and chemotherapy[4,5]. However, despite advances in the diagnosis and treatment of HCC, the 5-year survival ratio and outcome are still poor[4,6]. The high mortality and poor outcomes are closely associated with distant metastasis[7]. Therefore, it is vital to develop more targeted molecular therapies for HCC. Pyroptosis is a type of proinflammatory programmed cell death[8]. Although it shares biochemical and morphological characteristics of necrosis and apoptosis, it remains a unique process triggered by various stimuli and leads to the release of cytokines that activate proinflammatory immune cell mediators[9]. Caspase 1, the effector protease of the inflammasome[10], is activated during pyroptosis and cleaves the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18. This proinflammatory microenvironment is favorable for tumor initiation and progression, as increased serum levels of proinflammatory ILs such as IL-1b and IL-18 have been observed in several types of cancer. Recently, induction of pyroptosis has been considered as a novel strategy to eradicate cancerous cells. Data from epidemiological researches showed that intake of xanthone compounds contained in plant-derived foods and beverages correlates with lower risk of malignancies[11]. Euxanthone, a flavonoid compound, is isolated from plant *Polygala caudata*, which is commonly found in the Provinces located in the Southwest of China. Use of *Polygala caudata* as a remedy for cough and anxiety was documented in a few ancient medical books[12,13]. Recently, euxanthone has exhibited promising effect against a variety of conditions of neurological systems[12,14,15]. Interestingly, Kuete et al[16] showed that euxanthone presents cytotoxicity against human cancerous cells. Since then, the anti-cancer activities of eu-

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xanthone in ovarian cancer and colorectal cancer have been evidenced[17-19]. Nevertheless, whether euxanthone was able to exhibit anti-neoplastic in HCC remains unclear. The current work aims to investigate the anticancer effect of euxanthone on HCC and its potential mechanisms.

Patients and Methods

Clinical Samples and Immunohistochemical (IHC) Staining

A total of 247 HCC patients who underwent surgery between June 2015 and February 2017 were enrolled in the current paper. HCC tissue and adjacent tissue were sampled and stored in -80°C. Diagnosis and staging of tissue were performed by two independent senior oncologists blindly. Quantitative RT-PCR (qRT-PCR) and Western blot were utilized, respectively, to examine caspase-1 expression at mRNA and protein level in tissues. The Ethics Committee of the First Affiliated Hospital of Bengbu Medical College gave official approval to this study and all patients signed written consent forms.

Cell Culture and Treatments

The Cell Bank of the Chinese Academy of Sciences (Shanghai, China) provided Human HCC cell lines Hep3B, SMMC 7721, and LO2 (the normal human hepatic cell line), which were maintained in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin 100 microg/mL (Sigma-Aldrich, St. Louis, Mo, USA).

Proliferation Assay

CCK-8 assay was executed as previously described[20]. Briefly, following $5x10^3$ cells/well Hep3B and SMMC 7721 cells were inoculated in 96-well plates, the original medium was aspirated and fresh media containing 10% (v/v) CCK-8 reaction solution was added and incubated for 2 hours. The absorbance of cells was measured by a spectrophotometer (Tecan Group Ltd, Männedorf, Germany) at 450 nm.

Quantitative Real-Time PCR (qRT-PCR)

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) were utilized to extract total RNA from cells and tumor tissues and reverse transcribe RNA into cDNA, respectively. The primers specific for caspase-1 and GAPDH were synthesized with reference to the previously disclosed sequences[21] (Sangong, Shanghai, China). The primer sequences were as the following: Caspase-1: forward 5'-ACACGTCTTGCCCTCATTATCT-3', reverse 5'-ATAACCTTGGGCTTGTCTTTCA-3'; IL-1β: forward 5'-ATGATGGCTTATTACAGTGGCAA-3', reverse 5'-GTCGGAGATTCGTAGCTGGA-3';IL-18: forward 5'-TCTTCATTGACCAAGGAAATCGG-3', reverse 5'-TCCGGGGTGCATTATCTCTAC-3'. The PCR reaction was conducted using SYBR GREEN master-mix (Solarbio Co., Beijing, China), and the relative expression of caspase-1 was calculated by the comparative ΔCt method (ABPrism software, Applied Biosystems, Foster City, CA, USA).

Western Blotting

An equal amount of proteins extracted from cells and tissues per sample were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking the membrane with 5% non-fat milk, membranes were incubated overnight with the specific primary antibodies at 4°C, and then incubated for 1 h with the corresponding secondary antibody. The band intensities were analyzed and protein expression was quantified using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA).

TUNEL Assay

TUNEL assay (Wanlei Bio Ltd., Shenyang, China) was performed to examine DNA fragmentation in HCC cells. TUNEL ratio was calculated by analyzing at least 300 cells in five random areas (1:100, light microscope).

Transwell Invasion Assay

Cell invasion was evaluated by the transwell assay. Briefly, the Hep3B and SMMC 7721 cells were cultured in fresh serum-free Dulbecco's Modified Eagle Medium (DMEM) overnight, and then plated in the upper chambers of transwell (Boyden chamber with 8.0 μm pore, Corning, Corning, NY, USA) at the density of $1x10^5$ cells in 200 μl. The lower compartment was then filled with 800 μl media as a chemoattractant. Following treatment, the non-invasive cells in the upper compartment were removed using a cotton swap, and the invasive cells were fixed with 4% paraformaldehyde for 15 min and subsequently stained

with 0.2% crystal violet. Five random fields were observed under an inverted microscope at 400x magnification.

In Vivo Study

The People's Hospital of Wuhai approved all animal experiments in the current study and provided male BALB/c nude mice (8-week old). Huh7 cells (5×10^6 cells) were subcutaneously injected into mice on their left flanks. When the tumor volume reached 100 mm^3, mice were divided into 3 groups (6 mice/group) at random and received intraperitoneal (IP) injections as following: (i) vehicle (0.9% sodium chloride plus 1% dimethyl sulfoxide) (DMSO); (ii) Euxanthone (20 mg/kg/d, dissolved in vehicle); and (iii) Euxanthone (40 mg/kg/d, dissolved in vehicle). The body weight of the mice and tumor volumes were measured every three days. The level of marker protein expression was determined by IHC as previously described[22]. TUNEL assay was utilized to assess cell death. The mRNA and protein levels of caspase-1, IL-1β, and IL-18 were evaluated, respectively, using qRT-PCR, Western blot and IHC.

Statistical Analysis

Statistical analysis was conducted using SPSS software 16.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Dunnett's t-test was applied for multiple comparisons. $p < 0.05$ was considered as statistically significance.

Results

Aberrant Low Expression of caspase-1, IL-1 and IL-18 in HCC Tissue and Cell Lines

Evidence[23] has showed that deficiency of pyroptotic cell death is a feature of human malignancy. Compared with adjacent non-cancerous tissue, low mRNA expressions of caspase-1, IL-1β and IL-18 were marked in HCC tissue (Figure 1A). As shown in Figure 1B, the protein expressions of pro-caspase-1, pro-IL-1β and pro-IL-18 were not markedly altered in HCC tissue. In contrast, the protein levels of cleaved caspase-1, mature IL-1β and mature IL-18, were remarkably lower in HCC tissue compared with matched non-cancerous tissue (Figure 1B). Caspase-1, IL-1β and IL-18 mRNA expressions were also examined in normal hepatic cell line LO2 and HCC cell lines Hep3B and SMMC7721. As shown in Figure 1C, the mRNA expression of caspase-1 was significantly lower in both HCC cell lines compared with LO2 cells. Similarly, the mRNA expressions of IL-1β and IL-18 were also markedly lower in Hep3B and SMMC7721 cells relative to LO2 cells. Correspondingly, the protein level of cleaved caspase-1 was significantly lower in Hep3B and SMMC7721 cells, although the expression of pro-caspase-1 presented no difference between cell lines (Figure 1D). In addition, Western blot indicated that mature IL-1β and IL-18 levels were also markedly lower in HCC cell lines compared with LO2 cells. These results showed that the dysfunction of caspase-1 dependent inflammatory signaling pathway was involved in the pathogenesis of HCC.

Euxanthone Suppresses Cell Proliferation, Promotes Cell Death, Inhibits Cell Migration and Invasion

The effect of euxanthone on cell proliferation was also examined. Euxanthone at 5 and 10 μM was able to decrease the number of viable cells in Hep3B cells at 24 and 48 hours (Figure 2A). Similarly, euxanthone at 5 and 10 μM also promoted loss of cell viability in SMMC7721 cells (Figure 2A). Next, the cell death was examined by TUNEL assay following 24 hours' treatment with euxanthone. As shown in Figure 2B, a dose-dependent increase was observed in both Hep3B and SMMC7721 cell lines. Wound healing assay was conducted to investigate the effect of euxanthone on cell migration. Euxanthone at 5 and 10 μM was able to decrease cell migration to 60% and 40% in Hep3B cells, respectively (Figure 2C). In SMMC7721 cells, the anti-migratory activity of euxanthone was found to be more profound (Figure 2C). Furthermore, euxanthone at 5 and 10 μM markedly decreased the invasive cell number in a dose-dependent fashion (Figure 2D). Collectively, this evidence showed that euxanthone suppresses cell proliferation, promoted cell death, inhibited cell migration and invasion.

Euxanthone Promotes HCC Cell Pyroptosis

To validate the function of pyroptosis in the anti-cancer activities of euxanthone in HCC, we examined the level of molecules involved in the caspase-1 signaling pathway. The expression of NLRP3 (nucleotide-binding domain and leucine-rich repeat-containing (NLR) pyrin domain 3), which is an upstream molecule of caspase-1, was examined. The mRNA expression of NL-

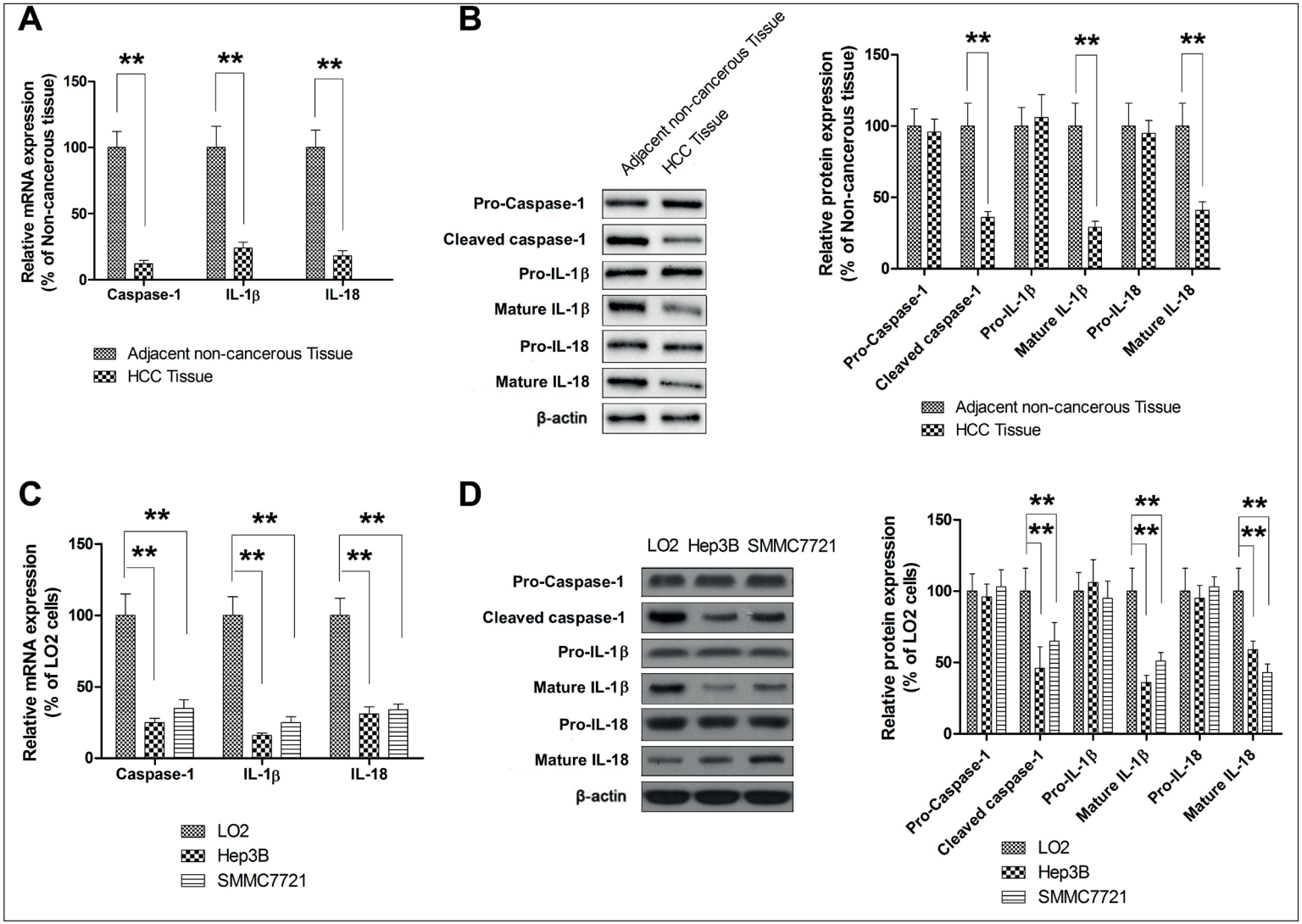

Figure 1. Dysfunction of caspase-1 signaling in HCC tissue and HCC cell lines. ***A.*** The mRNA levels of caspase-1, IL-1β and IL-18 were determined by qRT-PCR in HCC tissue and matched non-cancerous tissue. ***B.*** The protein expression of pro-caspase-1, cleaved caspase-1, pro-IL-1β, mature IL-1β, pro-IL-18 and mature IL-18 in HCC tissue and matched non-cancerous tissue. ***C.*** The mRNA levels of caspase-1, IL-1β and IL-18 in normal hepatic cell line LO2 and HCC cells were detected by qRT-PCR. ***D.*** The protein expression levels of pro-caspase-1, cleaved caspase-1, pro-IL-1β, mature IL-1β, pro-IL-18 and mature IL-18 in normal hepatic cell line LO2 and HCC cells. $^{**}p < 0.01$.

RP3 was remarkably repressed by euxanthone treatment (Figure 3A). Caspase-1 expression was also repressed by euxanthone at mRNA level in dose-dependent fashion in both Hep3B and SMMC7721 cells. IL-1β and IL-18 expression at mRNA level were also examined following euxanthone treatment. As shown in Figure 3A, the mRNA expression of both IL-1β and IL-18 was repressed by euxanthone treatment. Then, the protein expression of these molecules was examined using Western blot. As shown in Figure 3B, NLRP3 expression was significantly elevated by treatment with euxanthone in both Hep3B and SMMC7721 cells lines. Correspondingly, cleaved caspase-1, mature IL-1β, and mature IL-18 expressions were all elevated in Hep3B and SMMC7721 cells treated with euxanthone. These results showed that euxanthone was able to activate caspase-1 dependent pyroptosis signaling pathway.

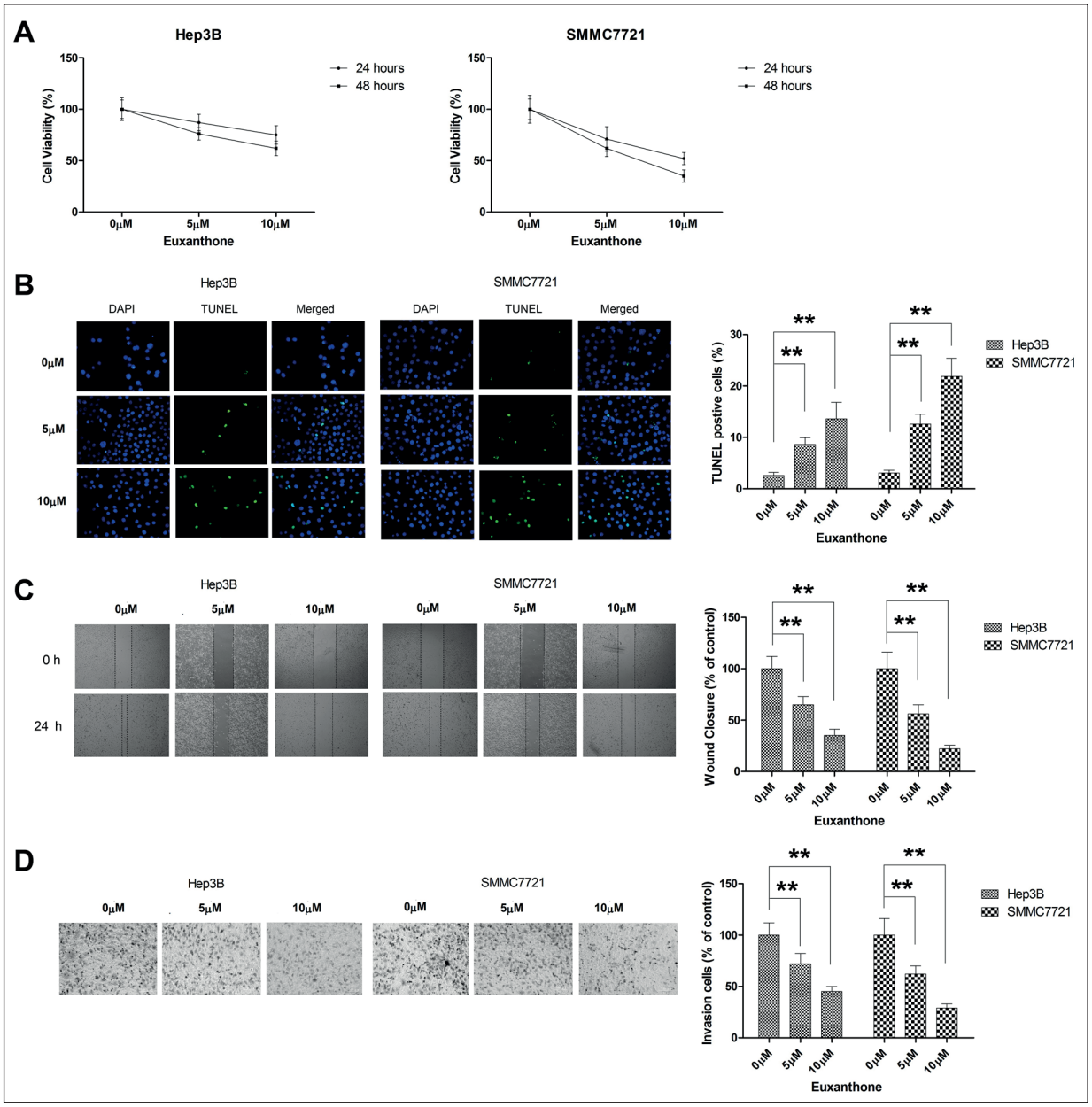

Figure 2. Euxanthone suppresses HCC cell proliferation, migration and invasion, and promotes cell death. ***A.*** Euxanthone exhibits anti-proliferative activities in HCC cells, as determined by CCK-8 assay. ***B.*** Euxanthone promotes cell death. ***C.*** Euxanthone inhibits cell migration. ***D***. Euxanthone inhibits cell invasion. $^{**}p < 0.01$.

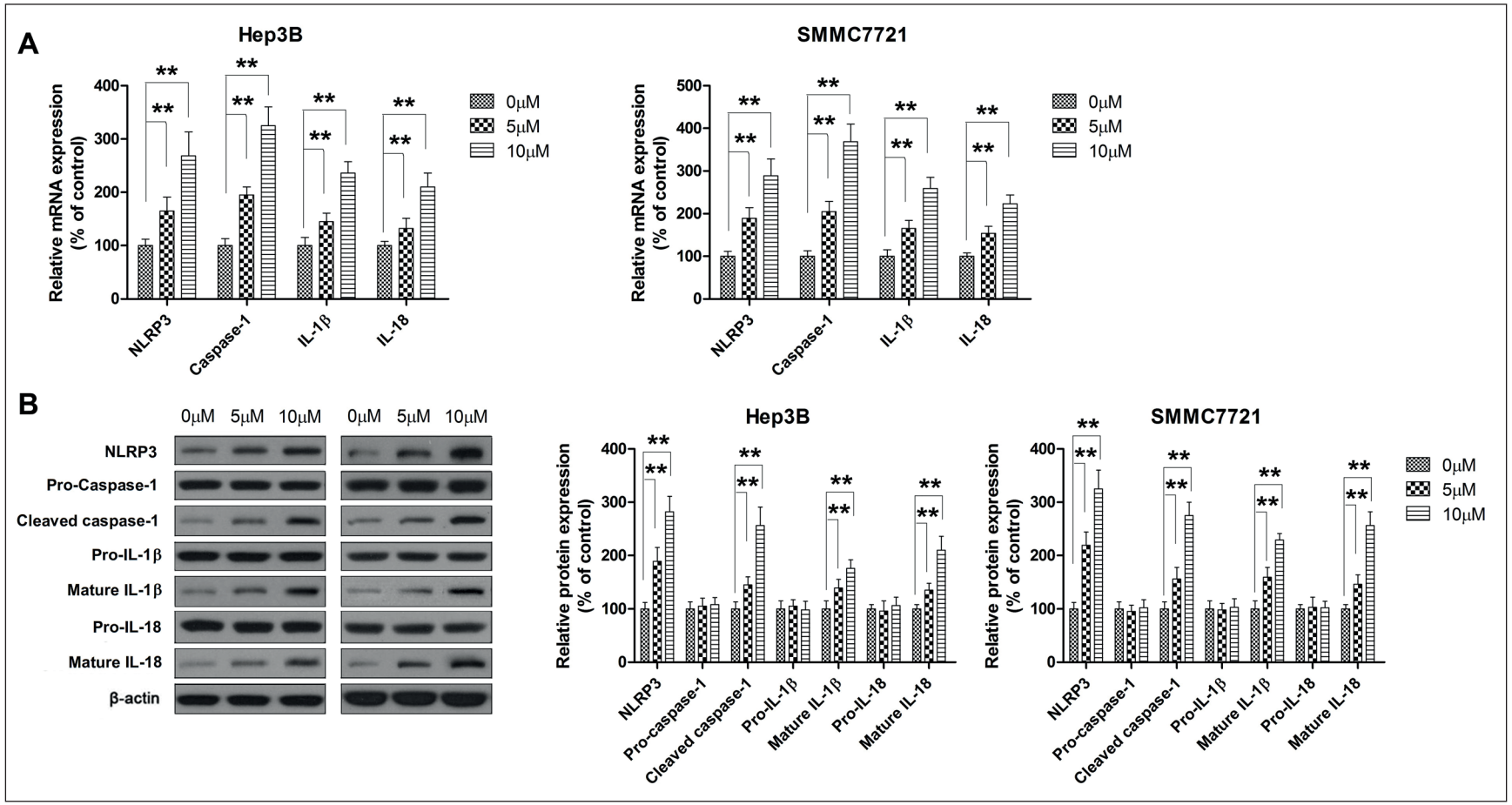

Figure 3. Euxanthone induces pyroptosis in HCC cells. ***A.*** The mRNA expression of NLRP3, caspase-1, IL-1β and IL-18 was detected by qRT-PCR. ***B.*** The protein levels of NLRP3, pro-caspase-1, cleaved caspase-1, pro-IL-1β, mature IL-1β, pro-IL-18 and mature IL-18 were determined by Western blot. $^{**}p < 0.01$.

Inhibition of Pyroptosis Compromises the Anti-Cancer Activities of Euxanthone in HCC Cells

To further examine the role of pyroptosis in the anti-cancer activities of euxanthone, a caspase-1 inhibitor, Ac-YVAD-cmk, was used. As shown in Figure 4A, pretreatment with Ac-YVAD-cmk significantly attenuated the anti-proliferative activities of euxanthone in HCC cells. Then, the cell death was detected by TUNEL assay. Pretreatment with Ac-YVAD-cmk significantly attenuated euxanthone-induced cell death (Figure 4B). In addition, pretreatment with Ac-YVAD-cmk also reversed the suppressing effect

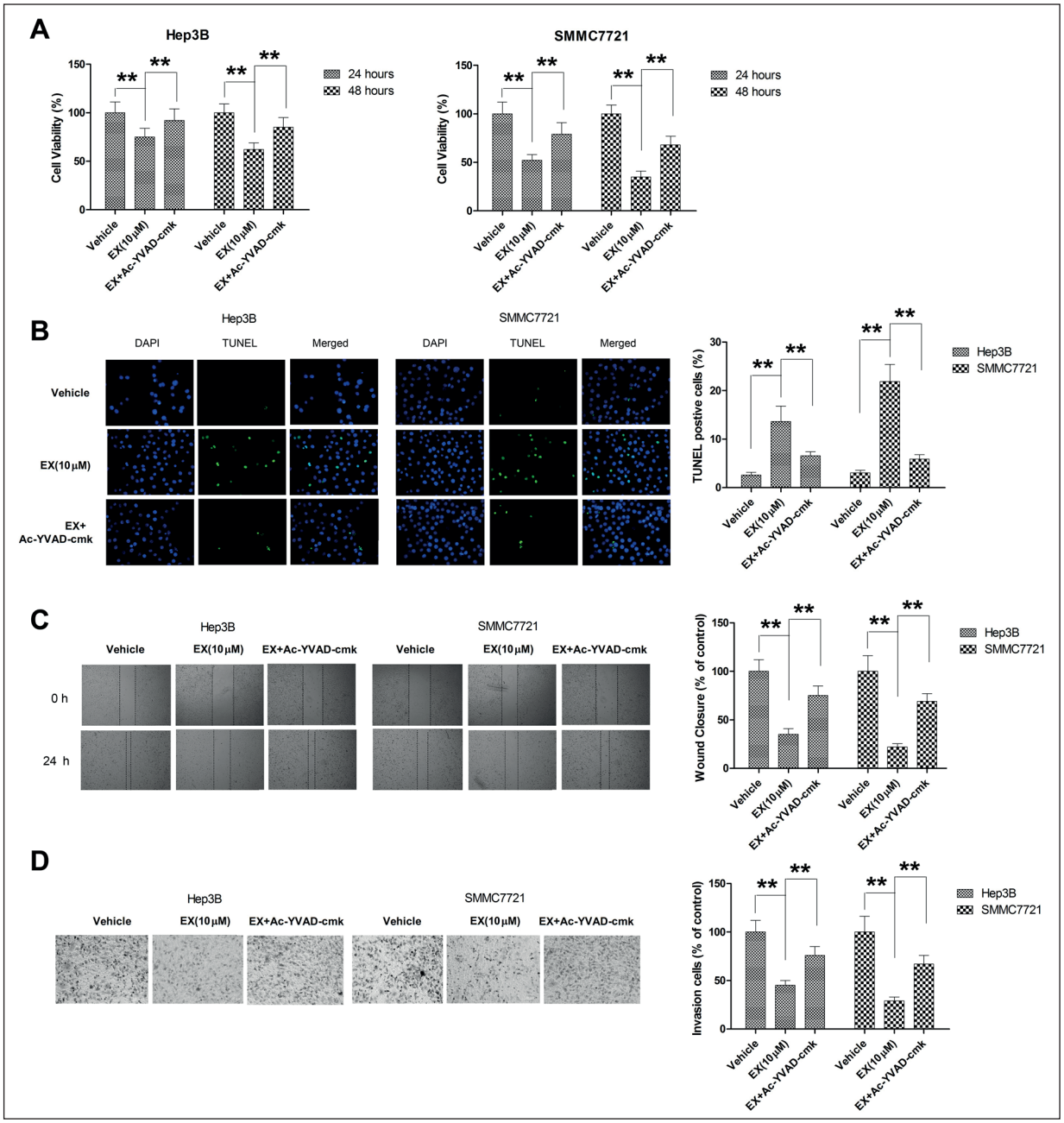

Figure 4. Caspase-1 inhibitor (Ac-YVAD-cmk) attenuates the anti-cancer activities of euxanthone in HCC cells. HCC cells were pretreated with Ac-YVAD-cmk (100 μM) for 8 hours before incubated with euxanthone for 24 hours. ***A.*** Cell viability was determined by CCK-8 assay. ***B.*** Cell death was detected by TUNEL assay. ***C.*** Cell migration was detected by Wound dealing assay. ***D.*** Cell invasion was detected by transwell assay. $^{**}p < 0.01$.

of euxanthone on cell migration and invasion (Figure 4C and Figure 4D). Taken together, these findings revealed that pyroptosis induction by euxanthone positively contributed to its anti-cancer activities.

Euxanthone Suppresses Tumor Growth in Vivo

Xenograft model was utilized to assess whether euxanthone could exhibit anti-cancer activities in *vivo*. As shown in Figure 5A, euxanthone was able to dose-dependently suppress tumor growth. TUNEL assay of tumor tissue showed that euxanthone treatment was associated with an increased proportion of cell death. IHC assay was performed to examine whether euxanthone altered the expression of molecular markers. As shown in Figure 5C, Ki-67 expression in tumor tissue was dose-dependently suppressed by euxanthone, supporting that euxanthone inhibited the tumor growth. In addition, euxanthone treatment correlated with increased expression of caspase-1, IL-1β and IL-18, supporting that euxanthone promoted pyroptosis in tumor tissue. Meanwhile, the body weight of the murine model was examined, showing that euxanthone did not cause a significant loss of body and did not produce general toxicity on murine model.

Discussion

Since naturally occurring compounds could exhibit anti-neoplastic activities without producing toxicity to healthy tissues, a lot of efforts have been made to explore the role of these

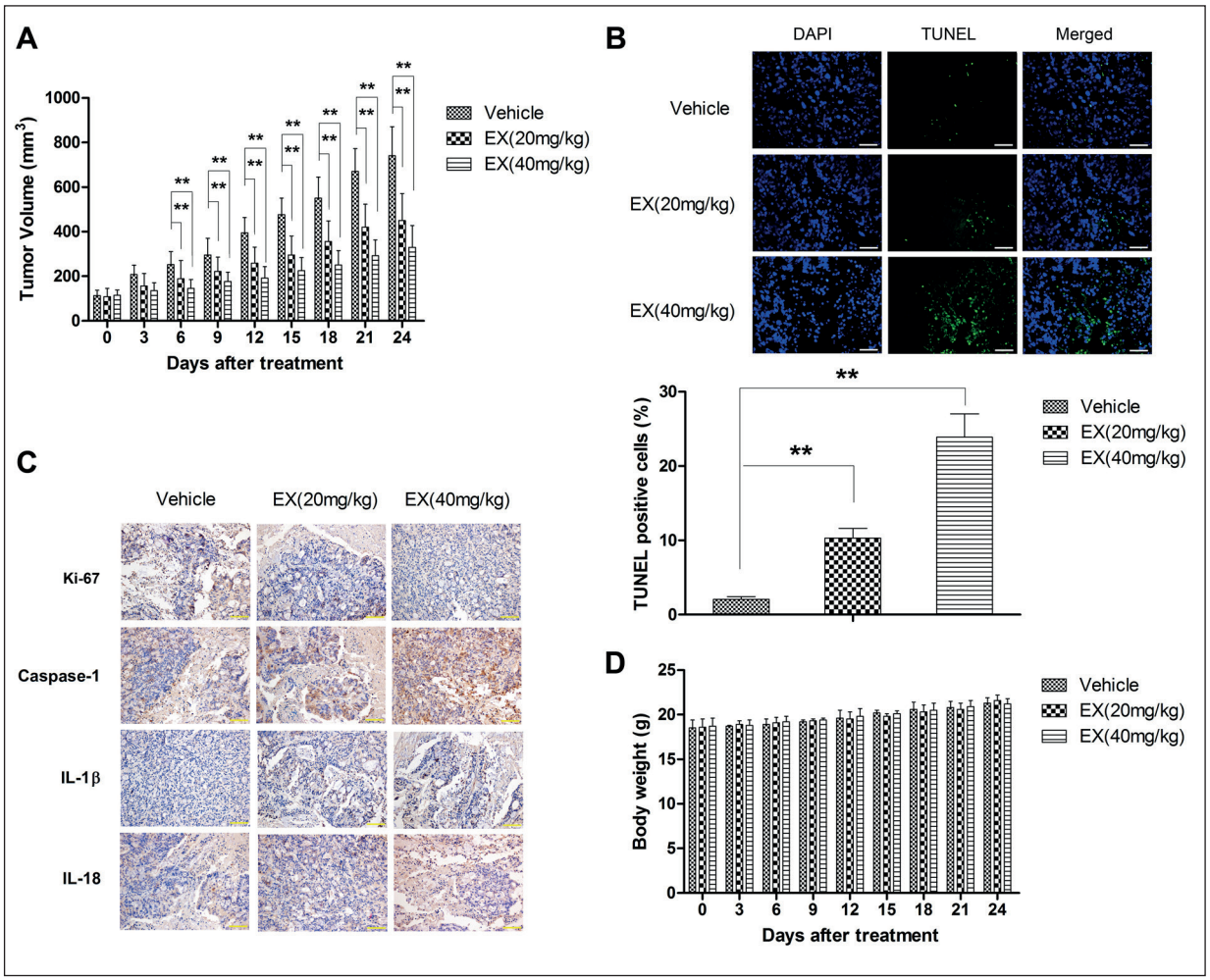

Figure 5. Euxanthone suppresses tumor growth of xenograft model. ***A.*** Tumor volume was documented every three days. ***B.*** TUNEL assay was conducted to determine cell death in tumor tissue. ***C.*** IHC assay was performed to examine the expression levels of caspase-1, IL-1β and IL-18. ***D.*** Body weight was measured every three days. $^{**}p < 0.01$.

compounds in cancer prevention and treatment. The medicinal plant *Polygala caudata* has been applied in traditional Chinese medicine for centuries[13]. Since last decade, the pharmacological activities of one of the major active ingredients of *Polygala caudate*, euxanthone (2,7-dihydroxyxanthone), have been discovered. For instance, neuritogenic effect[12,14,24], regulation effect on protein kinase C[25], vasodilatory effects[13,26,27], and selective cytotoxic effects[16]. Recently, euxanthone has been found to promote protective autophagy in ovarian cancer cells by modulating pSTAT3/Bcl-2 signaling[19]. The modulating role of euxanthone on the glycolytic process has also been evidenced by the same group in ovarian cancer cells[18]. Moreover, Wang et al[17] have identified that euxanthone exerts anti-cancer activities via targeting cancerous inhibitor of PP2A (CIP2A) in colorectal cancer. For the first time, we showed that euxanthone exhibited anti-cancer activities against HCC. Moreover, our findings showed that pyroptosis induction at least partly mediated its anti-cancer activities against HCC. Pyroptosis is another type of programmed cell death, which involves the caspase-1 dependent inflammatory response of cells[21]. Since it was firstly documented in 1992 by Zychlinsky' research group, pyroptosis has been found to play a role in a number of pathological conditions, including infection, myocardial infraction, stroke and malignancies[28]. In terms of human malignancies, the role of pyroptosis seems to be controversial. Given that pyroptosis is associated with activation of inflammation signaling, it is reasonable to believe that this inflammatory environment will favor the process of tumorigenesis since accumulating evidence has showed that chronic inflammatory response positively contributes to the development and progression of human malignancies via providing key factors involved in the regulation of tumor microenvironment, such as growth factors and angiogenetic factors[29]. On the contrary, it has been proposed[30] that pyroptosis plays a negative regulatory role in cancer biology. Hu et al[31] reported that caspase-1 was able to directly regulate the proliferation and survival of colonic epithelial cell. In addition, their research showed that colon epithelial cells with caspase-1 knockdown exhibited enhanced proliferation at the first stage of tumor genesis and speeded tumor growth compared with wild-type in colitis-associated colorectal cancer model[32]. In prostate cancer cells, it has also been found that upregulation of caspase-1 by cytochrome P450 inhibitor impaired cell proliferation and migration[33]. Meanwhile, caspase-1 deficiency has also been evidenced in tumor tissue of non-small cell lung carcinoma[23]. In line with these previous studies, our findings indicated that the expression of caspase-1 and downstream molecules IL-1β and IL-18 were significantly lower at mRNA level in HCC tissue compared with matched non-cancerous tissue. Moreover, the expression of activated caspase-1, mature IL-1β and IL-18 proteins was also significantly lower in HCC tissue. Our data provided further evidence supporting the anti-cancer role of pyroptosis in HCC. As a programmed cell death, pyroptosis in human cancer cells occurs via caspase-1 in the canonical pathway, and via caspase-4/-5 in the noncanonical pathway[34,35]. Either canonical pathway or noncanonical pathway needs two steps to complete pyroptosis. These two pathways share the same first step, which needs the priming of inflammasomes, the large pro-caspase-1 activating multiprotein oligomers. Meanwhile, the production of pro-IL-1β and pro-IL-18 will be elevated upon the activation of pattern-recognition receptors[23]. The canonical and noncanonical pathway presents difference in the second step. In the canonical pathway, cleaved caspase-1, which is activated by inflammasomes, promotes the proteolysis of pro-IL-1β and pro-IL-18 and produces active IL-1β and IL-18. These mature and active forms of cytokines will then execute pyroptosis[34]. On the other hand, activated caspase-11 mediates the noncanonical pathway[35]. In our study, we found that euxanthone markedly increased the mRNA expression of NLRP3, caspase-1, and IL-1β and IL-18. In addition, euxanthone was found to markedly activated caspase-1 and promoted maturation of IL-1β and IL-18. These showed that euxanthone stimulated pyroptosis in HCC cells. Furthermore, the anti-proliferative activities of euxanthone on HCC cells were markedly dampened by specific caspase-1 inhibitor (Ac-YVAD-CMK). The suppressing activities of euxanthone on cell migration and invasion were also attenuated when caspase-1 was inhibited. These evidence showed that pyroptosis was involved in the anti-proliferative and anti-invasive activities of euxanthone. Our examination in xenograft tissue also supported that the suppressing activities of euxanthone on tumor growth correlated with increased expression of caspase-1, IL-1β and IL-18, further confirming our *in vitro* data.

Conclusions

We showed that euxanthone exhibited anti-proliferative and anti-invasive activities in HCC. Mechanistically, this inhibition was associated with pyroptosis induction. Our findings highlight the potential of euxanthone to be employed in the treatment of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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