Original Article Guggulsterone-induced apoptosis in cholangiocarcinoma cells through ROS/JNK signaling pathway

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Abstract: Cholangiocarcinoma (CCA), the most common biliary tract malignancy, is arising from the bile duct epithelium with the global significantly increased morbidity and mortality. Here, we showed the effect of guggulsterone, a steroid found in the resin of the guggul plant, on human HuCC-T1 and RBE CCA cells. Exposure to various concentrations of guggulsterone for multiple action time resulted in significant apoptosis in the CCA cells via activating both extrinsic and intrinsic pathways. Furthermore, we demonstrated that the apoptosis of CCA cells was induced by Reactive oxygen species (ROS) mediated JNK signaling pathway. Consistently, inhibition of JNK activity, overexpression of JBD, its binding protein or reduction of ROS by overexpression of catalase, all decreased apoptotic cells. Our results also revealed that guggulsterone-induced apoptosis was coupled with endoplasmic reticulum stress (ERS) in CHOP-dependent pathway. Downregulation of CHOP instead of other ERS markers could inhibit CCA cell apoptosis. Taken together, our results showed that guggulsterone could induce apoptosis of human CCA cells through ROS/ JNK signaling pathway, indicating that guggulsterone could be important for the clinical therapy of CCA.

Keywords: Guggulsterone, apoptosis, CCA, JNK, ERS

Introduction

Cholangiocarcinoma (CCA) is the most common biliary tract malignancy, arising from liver to small intestine biliary showing the feature of cholangiocyte differentiation. CCA can be divided into intrahepatic, perihilar, and distal one based on the anatomical location [1]. CCA has an aggressive natural course with a rate of 43.1% in the last 5 years after diagnosis [2]. With the global increase of morbidity and mortality, CCA becomes increasingly important although it's a relatively rare cancer [3, 4]. So far, there are no curative and advanced medical therapies for CCA. Traditional systemic chemotherapy with two drugs, gemcitabine and cisplatin has become the major approach for patients with metastatic disease. However, Effective response to combination chemotherapy in CCA patients is limited, and the 5-year survival remains low [5]. Therefore, development of novel therapies is of great importance and high urgency in this devastating disease.

Currently, induction of apoptosis in targeting therapy against tumor cells plays essential role in development of cancer drug research [6, 7]. Guggulsterone, a steroid in the resin of the guggul plant, has anti-inflammatory, anti-oxidation, lipid-lowering and anti-tumor effects via its pharmacological activity. Previous studies have reported that guggulsterone could induce apoptosis in colon cancer cells through targeted inhibition of STAT3 expression via targeting beta-Catenin signaling in human breast cancer cells [8, 9]. Cheon also found guggulsterone could block NF-kappaB signaling pathway by targeting IKK complex in IEC and attenuate DSS-induced acute murine colitis [10]. However, the effect of guggulsterone on CCA and the relation of guggulsterone to cell apoptosis of CCA remain to unveil.

Reactive oxygen species (ROS) are byproducts of cellular metabolism. Excessive accumulation of ROS could lead to oxidative damage to lipids, proteins and DNA via affecting various cellular signaling pathways including the MAPK signal transduction [11, 12]. JNK, a stress-activated protein kinase (SAPK) of the MAPK family, plays vital roles in apoptosis, autophagy and some other cellular events [13]. ROS/JNK signaling pathway has been used to investigate in cancer cell apoptosis and proved to be a promising drug target in cancer therapy [14, 15]. ERS is essential in maintenance of cell growth and differentiation and homeostasis [16]. Thomas indicated ERS could induce cells arresting within G1 phase [17]. Additionally, ERS could sense diverse and constantly physiological inputs, serving as a checkpoint that allows cells to acquire time to re-establish cellular homeostasis [18]. Prolonged and irreversible ERS induces apoptosis of the damaged cells through breaking the balance of cellular homeostasis [19, 20].

Whether guggulsterone has an antitumor effect on CCA cells, the molecular mechanism are still unknown. In this study, we focus on the functional mechanism of guggulsterone-induced CCA apoptosis with the CCA cell model and demonstrate association of apoptosis and ERS. Here, we show that guggulsterone could induce apoptosis of human CCA cells through activating ROS/JNK signaling pathway and guggulsterone-induced CCA cell apoptosis is associated with ER stress in CHOP-dependent pathway. These results suggest that guggulsterone has the potential ability for the further therapy of CCA.

Materials and methods

Cell culture and regents

HuCC-T1 and RBE cells were obtained from Shanghai Bioleaf Biotech Co., Ltd and maintained under standard culture conditions with culture medium and fetal bovine serum (FBS) purchased from GIBCO at 37°C and 5% CO₂. Guggulsterone was brought from Steraloids. Hydroethidine and 6-carboxy-2',7'-dichlorodihydrofluorescein (DCF) diacetate (H2 DCFDA) were from (Beyotime) to measure ROS with flow cytometer (FACSCalibur, BD, San Jose, CA, USA). The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase p38 MAPK inhibitor SB202190, and JNK inhibitor SP600125 were from Calbiochem, The broad-spectrum caspase inhibitor (z-VAD-fmk) was obtained from Millipore (Billerica, MA, USA). Caspase-8 specific inhibitor (z-IETD-fmk) and caspase-9 specific inhibitor (z-LEHD-fmk) were purchased from BioVision (Mountain View, CA, USA).

Apoptosis analysis

HuCC-T1 and RBE cells were seeded at a concentration of 2×10^5 per well, after treated with different concentrations of guggulsterone or DMSO for various time, or some other treatments, like transfection, apoptotic cells were analyzed by flow cytometer (FACSCalibur, BD, San Jose, CA, USA) with Annexin V-PE and 7-AAD double staining (BD Biosciences, San Diego, CA, USA).

Caspase activity assay

Following HuCC-T1 or RBE cells treated with various concentrations of guggulsterone for 24 h, analysis of caspase-3, -8, -9 activities were measured with Caspase Activity Kit (Beyotime biotechnologies, Jiangsu, China) at 405 nm according to the supplied protocol by using MR7000 microplate reader.

RT-qPCR

For each reaction, cDNA for reverse-transcription PCR was synthesized from 10-500 ng total RNA by GoScript Reverse Transcription System (Promega) according to the supplied protocol using oligo dT. QPCR was performed with SYBR Green SuperMix (Promega). QPCR data was analyzed with GAPDH as an endogenous loading control. Primers for eIF2 α , BIP, GRP78, CHOP and DR-5 were purchased from Sangon Biotech (Shanghai) Co.,Ltd.

Western blot

For western blots, samples were separated on SDS-PAGE gels and then transferred to PVDF membranes (Millipore). After blocking with milk, membranes were processed following the ECL Western blotting protocol (GE Healthcare). These antibodies were used in western blots: antibodies against caspase-3, caspase-8, caspase-9, DR5, Bid, phospho-JNK, phospho-p38, phospho-ERK1/2, GAPDH were all purchased from Cell Signaling Technology (Beverly, MA,





Figure 1. Evidence that guggulsterone induces apoptosis in human CCA cell lines. A. HuCC-T1 or RBE cells treated with guggulsterone were stained with annexin V-FITC/7-AAD and analyzed by flow cytometry. The chart illustrates apoptosis proportion from three separate experiments. B-D. Cells were treated with various concentrations of guggulsterone for 24 h or incubated with guggulsterone (20μ M) for different hours. The expressions of caspase-3, -8, -9, DR5 and Bid were determined by western blot. E. Caspase activity assay of cells treated with various concentrations of guggulsterone for 24 h after 2 h pre-treatment with caspase inhibitors, z-IETD-fmk (10μ M), z-LEHD-fmk (40μ M) or z-VAD-fmk (20μ M). Then cells were stained with annexin V-FITC/7-AAD and analyzed by flow cytometry. Results are expressed as the mean \pm S.D. from three independent experiments. *P<0.05 versus control, #P<0.05 versus guggulsterone independently treatment. The statistical differences were calculated by one-way ANOVA analysis of variance with Dunnett's test.

A	HuCC-T1 Dose(µM)						В	HuCC-T1 Time(h)					С	HuCC-T1				
	0	1	2	5	10	20		0	0.5	1	2	4	8	SP600/30uM)			+	+
P-JNK1/2		-	-	=	=	=	P-JNK1/2		-	=	=	=	=	Gug(20µM, 24h)	-	+	-	+
P-p38	-	-				_	P-p38		_	_	_	-	-	P-JNK1/2		-	-	
P-ERK1/2	-	=	-	=	=	-	P-ERK1/2		_	-	=	-	-	GAPDH	-	_	_	_
GAPDH	-	-	-	-	-	-	GAPDH	-	-	-	-	-	-					
	RBE Dose(µM)						RBE Time(h)							RBE				
			RI Dos	BE e(µM)						Ti	me(h)					RE	E	
	0	1	Dos 2	BE e(µM) 5	10	20		0	0.5	Ti 1	me(h)	4	8			RB	E	
P-JNK1/2	0	1	2	BE e(µM) 5	10	20	P-JNK1/2	0	0.5	Ti	2	4	8	SP600(30µM) Gug(20µM, 24h)			• •	+ +
P-JNK1/2 P-p38	0	1	2	BE e(µM) 5	10	20	P-JNK1/2 P-p38	0	0.5	1	2	4	8	SP600(30µM) Gug(20µM, 24h) P-JNK1/2	-	RB ÷)E + -	+ +
P-JNK1/2 P-p38 P-ERK1/2	0	1	2	BE e(μM) 5	10	20	P-JNK1/2 P-p38 P-ERK1/2	0	0.5		2	4	8	SP600(30μM) Gug(20μM, 24h) P-JNK1/2 GAPDH		:	: :	: :



Figure 2. Immunoblotting for phospho-JNK1/2 (P-JNK1/2), phospho-p38 MAPK (P-p38), and phospho-ERK1/2 (P-ERK1/2) using lysates from HuCC-T1 or RBE cells treated with DMSO (control) or various concentrations of guggulsterone for 4 h (A) or incubated with guggulsterone (20) for different hours (B). Effect of SP600125 (JNK1/2 inhibitor) and SB202190 (p38 MAPK inhibitor) on guggulsterone-induced JNK1/2 hyperphosphorylation (C) and/or apoptosis (D) in HuCC-T1 or RBE cells. Cells were treated with DMSO (control) or the indicated concentrations of SP600125, SB202190 for 2 h. The cells were then either left untreated (DMSO or inhibitor alone controls) or exposed to guggulsterone (20 μ M) for 24 h in the presence of the inhibitor. The cells were collected and processed for stained with annexin V-FITC/7-AAD and analyzed by flow cytometry. Immunoblotting for JBD and phospho-JNK1/2 in cells transfected with pGA1-JBD or pGA1-Empty as control following a 4 h treatment with DMSO (control) or the indicated concentrations of guggulsterone (E). Cells were incubated with or without guggulsterone for 24 h after transfenction with pGA1-JBD or pGA1-Empty as control (F). Then cells were stained with annexin V-FITC/7-AAD and analyzed by flow cytometry. Results are expressed as the mean ± S.D. from three independent experiments. *P<0.05 versus control, #P<0.05 versus guggulsterone independently treatment. The statistical differences were calculated by one-way ANOVA analysis of variance with Dunnett's test.

USA); antibodies against JBD, catalase, Chop, GRP78, Bip, p-eIF2 α , eIF2 α , Flag were from Abcam (Cambridge, MA, USA).

Plasmid construction and transfection

All plasmids were constructed by restrictionenzyme double digestion, followed ligation and sequenced for confirmation. PGA1-JBD and pGA1-Catalase are plasmids based on the pGA1 backbone with insertion of the coding region for JBD and Catalase, forming flag-tag fused proteins. Plasmid and siRNA transfection was performed with Lipofectamine 2000 (Invitrogen) according to the supplier's protocol. Oligonucleotides of small interfering RNA were ordered from Sangon Biotech (Shanghai) Co.,Ltd. The following siRNAs were listed: eIF2α siRNA 5'-CGGUCAAAAUUCGAGCAGAdTdT-3', GRP78 siRNA 5'-GGAGCGCAUUGAUAC-UAGATT-3', BIP siRNA 5'-GGAGCGCAUUGAUACU-AGUdTdT-3', CHOP siRNA 5'-AAGAACCAGCAGA-GGUCACAA-3'.

Statistical analyses

Results are performed as Mean \pm SD from at least three independent experiments. The statistical differences were calculated by one-way ANOVA analysis of variance with Dunnett's test or unpaired Student's t-test. Tests were twotailed and statistical significance was defined as P<0.05.

Results

Guggulsterone induces apoptosis of human CCA cells

To investigate the effect of guggulsterone on CCA cells, we performed apoptosis assay analyzed by flow cytometry double stained with 7AAD and Annexin V in human HuCC-T1 and RBE CCA cell lines treated various concertation of guggulsterone (1, 2, 5, 10 and 20 µM). As shown in Figure 1, apoptotic cells were significantly increased with guggulsterone treatment in a dose-dependent manner in both HuCC-T1 and RBE CCA cells (Figure 1A). Additionally, apoptosis was confirmed by western blot to detect caspase-dependent pathway markers including caspase3, caspase8 and caspase9. Expectedly, the expression levels of caspase3, caspase8 and caspase9 in CCA cells indeed increased markedly in response to a gradual

concentration of guggulsterone (Figure 1B). Besides, western blot revealed that exposure to guggulsterone (20 µM) also increased the levels of caspase3, caspase8 and caspase9 in a time-dependent manner (3, 6, 12 and 24 h) in CCA cells (Figure 1C). DR5, associated with the extrinsic pathway and tBid, involved in intrinsic pathway both increased their protein levels, while Bid, another intrinsic pathway marker showed slight decrease under various concertation of guggulsterone (1, 2, 5, 10 and 20 μ M) in CCA cells (Figure 1D). Moreover, caspase activities of caspase-3, -8, -9 were also detected in both HuCC-T1 and RBE CCA cells, which showed enhancing with guggulsterone treatment in a dose-dependent manner for 24 h (Figure 1E). To further confirm these findings, we investigated the roles of caspases using z-VAD-fmk, z-IETD-fmk and z-LEHD-fmk. As expected, apoptosis analysis by flow cytometry with 7AAD and Annexin V double staining showed a moderate inhibitory role of either z-IETD-fmk or z-LEHD-fmk in the guggulsteroneinduced apoptosis while z-VAD-fmk had a more potent inhibitory effect when HuCC-T1 cells incubated with or without guggulsterone (20 µM) for 24 h after 2 h pre-treatment with caspase inhibitors (Figure 1G).

Guggulsterone regulated CCA cell apoptosis via activating JNK pathway

To elucidate the molecular function of guggulsterone-induced apoptosis in CCA cells, we checked the protein levels of phospho-JNK1/2, phospho-ERK1/2 and phospho-p38 by western blot with incubation of guggulsterone. As shown in Figure 2A and 2B in HuCC-T1 and RBE CCA cells, guggulsterone markedly activated phosphorylation of JNK1/2, p38 and ERK1/2 in a dose (1, 2, 5, 10 and 20 µM)-and time (0.5, 1, 2, 4 and 8 h)-dependent manner (Figure 2A, 2B). Next, we investigated the level of phospho-JNK1/2 in the presence of SP600125, a JNK1/2 inhibitor and SB202190, a p38 MAPK inhibitor with guggulsterone (20 µM) treatment. Western blot analysis demonstrated that SP600125 could suppress the upregulated expression of phospho-JNK1/2 in CCA cells (Figure 2C). Additionally, apoptosis was further performed by flow cytometry with 7AAD and Annexin V double staining to show an efficient inhibitory role of SP600125 while SB202190 had no potent inhibitory effect in both CCA cells





HuCC-T1 Control pGA1-Caspase Gug(µM) 10 20 10 20 0 1 0 Caspse P-JNK1/2 GAPDH С HuCC-T1 50 Con DGA1-Catalase 40 Apoptosis proportion(%) $\overset{\otimes}{\underset{8}{\times}}$ 10 0 20 2 5 10 Gug(µM) HuCC-T1 1.4 1.2







Figure 3. Percentage of DCF fluorescence in HuCC-T1 or RBE cells following treatment with DMSO or different concentrations of guggulsterone for 4 h (A). Immunoblotting for catalase and phospho-JNK1/2 (B) in HuCC-T1 or RBE cells transfected with pGA1-Catalase or pGA1-Empty following a 4 h treatment with either DMSO (control) or the indicated concentrations of guggulsterone. Cells were incubated with or without guggulsterone for 24 h after transfenction with pGA1-Catalase or pGA1-Empty as control (C). The cells were collected and processed for stained with annexin V-FITC/7-AAD and analyzed by flow cytometry. Fold increase in DCF fluorescence relative to DMSO-treated control in cells transfected with pGA1-Catalase or pGA1-Empty and treated for 2 h with the indicated concentrations of guggulsterone (D). Results are expressed as the mean ± S.D. from three independent experiments. *P<0.05

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versus control, #P<0.05 versus guggulsterone independently treatment. The statistical differences were calculated by one-way ANOVA analysis of variance with Dunnett's test.

(Figure 2D). Consistently, overexpression of the JBD, a binding protein of JNK, could also efficiently suppress the increase protein level of phospho-JNK1/2 analyzed by western blot with guggulsterone treatment in a dose-dependent manner (Figure 2E). In further, overexpression of the JBD could also significantly inhibit apoptosis at increasing concentrations (10, 20 μ M) of guggulsterone after treatment for 24 h in CCA cells (Figure 2F). These results suggest activation of JNK pathway is required in guggulsterone-induced apoptosis in CCA cells.

ROS-mediated JNK1/2 pathway in guggulsterone-regulated CCA cell apoptosis

ROS have been reported to participate in the regulation of apoptosis [21] and also promote the sustained JNK activation by inhibiting MAP kinase phosphatases [22]. DCF fluorescence revealed that guggulsterone markedly increased the generation of ROS at doses of high concentrations (10, 20 µM) in HuCC-T1 cells and (5, 10, 20 µM) in RBE cells for 4 h (Figure 3A). To elucidate this result more clearly, we focused on catalase, which catalyzes the decomposition of hydrogen peroxide to water and oxygen [23]. In line with expectation, overexpression of catalase could significantly suppress the increase protein level of phospho-JNK1/2 analyzed by western blot in a dosedependent manner of guggulsterone treatment in CCA cells (Figure 3B). Consistently, overexpression of catalase could also efficiently suppress apoptosis at the dose of 5, 10, 20 µM guggulsterone in HuCC-T1 cells and 10, 20 µM in RBE cells at 4 h (**Figure 3C**). Furthermore, overexpression of catalase could also significantly inhibit apoptosis at increasing concentrations (5, 10, 20 μ M) of guggulsterone for 24 h after treatment in HuCC-T1 CCA cells (**Figure 3D**). These results indicated ROS contributed much in JNK pathway activation in guggulsterone-induced apoptosis.

ERS associates with guggulsterone-induced apoptosis

Whether apoptosis in CCA cells was associated with ERS or not has been carried out. To determine whether guggulsterone-induced apoptosis has correlations with ERS, western blot performed that the markers in protein kinase RNAlike endoplasmic reticulum kinase (PERK) pathway including P-eIF2a, BIP, GRP78 and CHOP except elF2 α showed significantly increase exposure to guggulsterone in a dose-dependent manner (Figure 4A). With guggulsterone (20 μ M) treatment, the JNK1/2 inhibitor could downregulate the expression of CHOP instead of other markers (Figure 4B). Next, we measured the knockdown efficiency of P-eIF2 α , BIP, GRP78 and CHOP analyzed after transfection of siRNAs for 24 h (Figure 5A). After 20 µM guggulsterone treatment, only knockdown of CHOP could downregulate the mRNA level analyzed by QPCR (Figure 5B) and protein level by western blot (Figure 5C). Consistently, downregulation of CHOP instead of GRP78 could also significantly inhibit apoptosis at increasing concentrations (5, 10, 20 μ M) of guggulsterone for 24 h after treatment in CCA cells (Figure 5D,

Figure 4. Immunoblotting for RES associated proapoptotic proteins from HuCC-T1 cells treated with DMSO (control) or various concentrations of guggulsterone for 24 h (A) or treated with DMSO (control) or guggulsterone (20 μ M) for 24 h after 2 h pre-treatment with JNK1/2 inhibitor SP600125 (B).



Figure 5. Quantitative reverse transcription-PCR analysis of DR-5 genes. Cells were transfected with control or p-eIF2a, BIP, GRP78, CHOP siRNA (A) or/and treated with guggulsterone (20μ M) (B). Immunoblotting for DR-5 proteins from HuCC-T1 cells or treated with DMSO (control) or guggulsterone (20μ M) for 24 h after transfection with control or p-eIF2a, BIP, GRP78, CHOP siRNA (C). Cells were treated with DMSO (control) or various concentrations of guggulsterone after transfection with control or GRP78 (D), CHOP (E) siRNA. The cells were collected and processed stained with annexin V-FITC/7-AAD and analyzed by flow cytometry. Results are expressed as the mean ± S.D. from three independent experiments. *P<0.05.

5E). These results indicate that guggulsterone induced apoptosis is associated with CHOP and its downstream pathways in ERS.

Discussion

Despite guggulsterone, as the bioactive constituent responsible for guggul's therapeutic effects, has been used to treat major diseases [24, 25], whether guggulsterone has the antitumor effects on CCA cells and its potential mechanism remain still unclear. In this study, we found that guggulsterone could induce apoptosis in HuCC-T1 and BRE CCA cell lines with both intrinsic and extrinsic pathways activation. In further, guggulsterone-induced apoptosis was regulated via ROS/JNK signaling pathway and associated with ERS in CHOPdependent pathway.

Apoptosis has a closely correlation with many major diseases, excessive apoptosis leading to autophagy and apoptosis deficiency causing loss-control of proliferation, eventually forming cancer [26]. Antitumor insights give rise to rosy prospects for cancer-cell-specific therapy [6, 7]. Here, we demonstrated that guggulsterone could induce apoptosis through activating both intrinsic and extrinsic pathway. This result suggested the induction of apoptosis in CCA cells was sensitive to caspase inhibitors. Expectedly, pre-treatment of caspase inhibitors could prevent guggulsterone-induced apoptosis in CCA cells. Moreover, treatment of guggulsterone in CCA cells could also enhance caspase activities of caspase-3, -8, -9 in a dose-dependent manner. These results indicated that guggulsterone was toxic and lethal to CCA cells through induction of apoptosis.

Next, we provided convincing evidence that guggulsterone-induced apoptosis was regulated via activating JNK signaling pathway. Treatment of guggulsterone on CCA cells resulted in upregulation of JNK1/2 normalized to GAPDH analyzed by western blot. Consistently, downregulation of phosphor-JNK1/2 could inhibit apoptosis of CCA cells with guggulsterone treatment. Published papers have concluded that excessive generation of ROS could break the balance of cellular homeostasis via affecting multiple signaling pathways [12] and JNK played significant roles in many cellular events as well [27]. Our work revealed that guggulsterone could induce the generation of ROS at high concentrations in both HuCC-T1 and BRE CCA cell lines. Moreover, overexpression of catalase also suppressed the level of phospho-JNK1/2 and inhibited apoptosis in CCA cells. From these data, we summarized that exposure to guggulsterone resulted in activation ROS-mediated JNK signaling pathway, further inducing apoptosis in CCA cells.

Interestingly, previous papers including ours demonstrated that guggulsterone could upregulate the death receptor DR5 [28] and enhancing DR5 expression also contributed to ERSinduced apoptosis [29]. These findings suggest that guggulsterone-induced apoptosis might be coupled with ERS. In compliance with our hypothesis, the markers of ERS indeed showed upregulation in CCA cell with various concentrations of guggulsterone treatment. Interestingly, knockdown of CHOP with siRNA instead of other markers showed significant decrease of DR-5 in both mRNA and protein level, leading to reduction of apoptotic cell numbers with guggulsterone incubation. What's the upstream of CHOP pathway in guggulsterone-induced CCA cell apoptosis left to be investigated by others.

In conclusion, our work was the first performance with the mechanism of guggulsteroneinduced apoptosis in CCA cells. The apoptosis induction of CCA cells with guggulsterone treatment was regulated trough ROS-mediated JNK signaling pathway and associated with CHOPdependent pathway in ERS. These results provide evidence for therapy of CCA with guggulsterone by induction of apoptosis.

Disclosure of conflict of interest

None.

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References

- Ghouri YA, Mian I and Blechacz B. Cancer review: Cholangiocarcinoma. J Carcinog 2015; 14: 1.
- [2] Nagino M, Ebata T, Yokoyama Y, Igami T, Sugawara G, Takahashi Y and Nimura Y. Evolution of surgical treatment for perihilar cholangiocarcinoma: a single-center 34-year review of 574 consecutive resections. Ann Surg 2013; 258: 129-140.
- [3] Razumilava N and Gores GJ. Cholangiocarcinoma. Lancet 2014; 383: 2168-2179.
- [4] Wang H, Li C, Jian Z, Ou Y and Ou J. TGF-beta1 reduces mir-29a expression to promote tumorigenicity and metastasis of cholangiocarcinoma by targeting HDAC4. PLoS One 2015; 10: e0136703.
- [5] Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, Madhusudan S, Iveson T, Hughes S, Pereira SP, Roughton M,

Bridgewater J; ABC-02 Trial Investigators. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. N Engl J Med 2010; 362: 1273-1281.

- [6] Bai L and Wang S. Targeting apoptosis pathways for new cancer therapeutics. Annu Rev Med 2014; 65: 139-155.
- [7] Wong RS. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 2011; 30: 87.
- [8] Jiang G, Xiao X, Zeng Y, Nagabhushanam K, Majeed M and Xiao D. Targeting beta-catenin signaling to induce apoptosis in human breast cancer cells by z-guggulsterone and Gugulipid extract of Ayurvedic medicine plant Commiphora mukul. BMC Complement Altern Med 2013; 13: 203.
- [9] Kim ES, Hong SY, Lee HK, Kim SW, An MJ, Kim TI, Lee KR, Kim WH and Cheon JH. Guggulsterone inhibits angiogenesis by blocking STAT3 and VEGF expression in colon cancer cells. Oncol Rep 2008; 20: 1321-1327.
- [10] Cheon JH, Kim JS, Kim JM, Kim N, Jung HC and Song IS. Plant sterol guggulsterone inhibits nuclear factor-kappaB signaling in intestinal epithelial cells by blocking IkappaB kinase and ameliorates acute murine colitis. Inflamm Bowel Dis 2006; 12: 1152-1161.
- [11] Shi Y, Nikulenkov F, Zawacka-Pankau J, Li H, Gabdoulline R, Xu J, Eriksson S, Hedstrom E, Issaeva N, Kel A, Arner ES and Selivanova G. ROS-dependent activation of JNK converts p53 into an efficient inhibitor of oncogenes leading to robust apoptosis. Cell Death Differ 2014; 21: 612-623.
- [12] Simon HU, Haj-Yehia A and Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 2000; 5: 415-418.
- [13] Tsujimoto Y and Shimizu S. Another way to die: autophagic programmed cell death. Cell Death Differ 2005; 12 Suppl 2: 1528-1534.
- [14] Cao XH, Wang AH, Wang CL, Mao DZ, Lu MF, Cui YQ and Jiao RZ. Surfactin induces apoptosis in human breast cancer MCF-7 cells through a ROS/JNK-mediated mitochondrial/ caspase pathway. Chem Biol Interact 2010; 183: 357-362.
- [15] Trachootham D, Alexandre J and Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov 2009; 8: 579-591.
- [16] Dai LM, Huang C, Chen L, Shan G and Li ZY. Altered expression of microRNAs in the response to ER stress. Science Bulletin 2015; 60: 202-209.
- [17] Thomas SE, Malzer E, Ordóñez A, Dalton LE, van 't Wout EF, Liniker E, Crowther DC, Lomas DA, Marciniak SJ. p53 and translation attenuation regulate distinct cell cycle checkpoints

during endoplasmic reticulum (ER) stress. J Biol Chem 2013; 288: 7606-7617.

- [18] Rutkowski DT and Hegde RS. Regulation of basal cellular physiology by the homeostatic unfolded protein response. J Cell Biol 2010; 189: 783-794.
- [19] Healy SJ, Gorman AM, Mousavi-Shafaei P, Gupta S and Samali A. Targeting the endoplasmic reticulum-stress response as an anticancer strategy. Eur J Pharmacol 2009; 625: 234-246.
- [20] Ye J and Koumenis C. ATF4, an ER stress and hypoxia-inducible transcription factor and its potential role in hypoxia tolerance and tumorigenesis. Curr Mol Med 2009; 9: 411-416.
- [21] Sauer H, Wartenberg M and Hescheler J. Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell Physiol Biochem 2001; 11: 173-186.
- [22] Kamata H, Honda S, Maeda S, Chang L, Hirata H and Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 2005; 120: 649-661.
- [23] Chelikani P, Fita I and Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci 2004; 61: 192-208.
- [24] Deng R. Therapeutic effects of guggul and its constituent guggulsterone: cardiovascular benefits. Cardiovasc Drug Rev 2007; 25: 375-390.
- [25] Shishodia S and Aggarwal BB. Guggulsterone inhibits NF-kappaB and IkappaBalpha kinase activation, suppresses expression of antiapoptotic gene products, and enhances apoptosis. J Biol Chem 2004; 279: 47148-47158.
- [26] Zhu Q, Zhang X, Zhang L, Li W, Wu H, Yuan X, Mao F, Wang M, Zhu W, Qian H and Xu W. The IL-6-STAT3 axis mediates a reciprocal crosstalk between cancer-derived mesenchymal stem cells and neutrophils to synergistically prompt gastric cancer progression. Cell Death Dis 2014; 5: e1295.
- [27] Goss VL, Cross JV, Ma K, Qian Y, Mola PW and Templeton DJ. SAPK/JNK regulates cdc2/cyclin B kinase through phosphorylation and inhibition of cdc25c. Cell Signal 2003; 15: 709-718.
- [28] Moon DO, Park SY, Choi YH, Ahn JS and Kim GY. Guggulsterone sensitizes hepatoma cells to TRAIL-induced apoptosis through the induction of CHOP-dependent DR5: involvement of ROS-dependent ER-stress. Biochem Pharmacol 2011; 82: 1641-1650.
- [29] Yamaguchi H and Wang HG. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. J Biol Chem 2004; 279: 45495-45502.