RESEARCH ARTICLE

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Apoptotic and Anti-metastatic Effects of *Atractylodes lancea* (Thunb.) DC. in a Hamster Model of Cholangiocarcinoma

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Abstract

Objectives: Cholangiocarcinoma (CCA) is a highly aggressive tumor with a greater risk of distant metastasis. The promising anti-CCA activity and safety profile of *Atractylodes lancea* (AL) have previously been reported in a series of *in vitro*, *in vivo* and clinical studies. The present study investigated the effect of AL extract on apoptosis and metastasis signaling pathways in the *Opisthorchis viverrini*/dimethylnitrosamine (OV/DMN)-induced CCA hamster model. **Materials and Methods:** Hamster liver tissues were obtained from the four groups (n = 5 per group), i.e., (i) 5-FU treated CCA (40 µg/mL); (ii) CCA; (iii) AL-treated CCA (5,000 mg/kg), and (iv) normal hamsters. Total RNA was isolated, and the expression levels of apoptosis-related and metastasis-related genes were determined by qRT-PCR analysis. **Results:** The expression levels of *p16,* c*aspase-3*, *caspase-8*, *caspase-9*, *Apaf-1*, *p53* and *Eef1a1* were downregulated, while that of the remaining genes were upregulated in CCA hamsters compared with normal hamsters. AL treatment increased the expression of *p16, caspase-9*, *caspase-3*, *Apaf-1*, *p53* and *E-cadherin* and decreased the expression of c*yclin D1*, *cdk4*, *Bax*, *Akt/PKB*, *Bcl-2*, *Mfge-8*, *Lass4*, *S100A6*, *TGF-β*, *Smad-2*, *Smad-3*, *Smad-4*, *MMP-9*, and *N-cadherin*. The expression of *Eef1a1* was unchanged. **Conclusion:** The anti-CCA activity of AL in OV/DMN-induced CCA hamsters could be due to the induction of cell cycle arrest at the G1 phase and activation of the apoptosis pathway, resulting in cancer cell death. The activation of the apoptosis pathway mainly involved the intrinsic pathway (activation of *caspase-3* and *caspase-9* through *p53* and *Mfge-8* modulation and downregulation of anti-apoptotic genes *Akt* and *Bcl-2*). In addition, AL could also inhibit the canonical *TGF-β* signaling pathway, *MMP-9* and *N-cadherin* to suppress tumor metastasis.

Keywords: Herbal medicine - apoptosis signaling - cell cycle - metastasis

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Introduction

Cholangiocarcinoma (CCA) is the bile duct cancer with abnormal differentiation of cholangiocytes (Bray et a., 2018). CCA is classified into intrahepatic cholangiocarcinoma (iCCA), perihillar CCA (pCCA), and distal CCA (dCCA). A high incidence of CCA is reported in Thailand, which is closely related to an endemic *Opisthorchis viverini* infection (Srivatanakul et al., 2001; Squadroni et al., 2017). Although the current incidence rate has decreased, the patient's survival rate is low (Chung et al., 2022). The combination of gemcitabine and cisplatin is the first-line treatment for patients with advanced-stage CCA as it has proved more effective than a single drug, gemcitabine, or 5-fluorouracil (5-FU) alone. However, late diagnosis and resistance of CCA to conventional chemotherapy are the major obstacles to CCA control (Eckmann et al., 2011; Bridgewater et al., 2014; Ramirez-Merino ET AL., 2013). Research and development of alternative drugs with novel mechanisms of action that are effective and safe for CCA treatment and control are urgently needed.

Several medicinal herbs have been investigated for their potential anticancer activities. Among these, *Atractylodes lancea* (Thunb.) DC. (AL) has been reported in a series of non-clinical studies to be a promising candidate for research and development of anti-CCA drug (Na-Bangchang et al., 2017). The plant grows in tropical and subtropical zones of Asia, such as China, Japan, and Thailand. Its dried rhizome is commonly used in Thai traditional medicines ("Khod-Kha-Mao") for the treatment of cancer, inflammation, and microbial infections (Koonrungsrisomboon et al., 2014). In Chinese traditional medicine, AL has been used for the treatment

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of rheumatic diseases, digestive disorders, night blindness, and influenza (Ouyang et al., 2012). The ethanolic extract of AL rhizomes was shown to suppress the growth, and inhibit the invasion and angiogenesis of the CCA cell line (Mahavorasirikul et al., 2010; Koonrungsrisomboon et al., 2014; Mathema et al., 2017). In the *in vivo* studies, AL treatment significantly suppressed tumor size and inhibited lung metastasis in the CCA-xenografted nude mice (Plengsuriyakarn et al., 2012) as well as in the *opisthorchis viverrini*/dimethylnitrosamine (OV/DMN)-induced CCA hamsters (Plengsuriyakarn et al., 2015). Atractylodin and β-eudesmol are the isolated bioactive constituents of AL that were demonstrated to effectively inhibit the growth of CCA cell lines (Kotawng et al., 2018; Mathema et al., 2019; Kotawng et al., 2020). The proteomic analysis demonstrated that this activity was linked to the induction of cell cycle arrest (at G1 phase) and apoptosis, and suppression of cancer cell growth through *PI3-AKT* signaling pathways (Boonmars et al., 2008; Kotawng et al., 2020). The present study further investigated the underlying mechanism of anti-CCA activity of AL in the OV/DMN-induced CCA hamster model (10) on cell apoptosis and metastasis signaling pathways.

Materials and Methods

Hamster CCA tissue specimens

Hamster liver tissues were obtained from the previous study (Plengsuriyakarn et al., 2015). Approval of the study protocol was obtained from the Ethics Committee for Animal Research, Thammasat University, Thailand (Approval number 013/2556). Syrian male hamsters were divided into four groups (n = 5 *per* group), i.e., (i) 5-FU treated CCA (40 μg/mL); (ii) CCA; (iii) AL-treated CCA (5,000 mg/kg), and (iv) normal hamsters. CCA was induced by the initial feeding of each hamster with 50 OV metacercariae, followed by drinking water containing 12.5 *ppm* dimethylnitrosamine (DMN) for eight weeks. The development of CCA was measured and confirmed by ultrasonography. Body weight and water consumption were recorded daily for 30 days. All hamster livers were collected for RNA isolation.

Quantitative reverse transcription PCR (qRT-PCR) analysis

Total RNA was isolated from individual liver tissue using Trizol reagent (Invitrogen, California, USA). The purified RNA 1000 ng was used for reverse transcription to generate cDNA with SuperScript® III First-Strand Synthesis System (Invitrogen, California, USA) according to the manufacturer's instructions. The qRT-PCR was performed using iTaq™ Universal SYBR® Green Supermix (Invitrogen, California, USA) in the presence of cDNA templates and specific primers, as shown in Table 1. The specific primers for apoptosis-related and metastasis-related genes were selected from previous studies (Kano et al., 2001; Shimizu et al., 2006; Boonmars et al., 2008; Boonmars et al., 2009; Nagini et al., 2009; Zivec et al., 2011; Wu et al., 2014; Boueroy et al., 2016) or designed using National Center for Biotechnology Information (NCBI).

The qRT-PCR was performed using the following conditions: predenaturation at 95 °C for 5 min, followed by 50 cycles of 95 °C for 30 s, 52-65 °C for 45 s (based on the target genes), and 72 °C for 1 min (Real-time thermocycler, Bio-rad, California, USA). The relative mRNA expression was normalized against the RPL-18 mRNA level using a comparative Ct method. The data are expressed as the percentage of gene transcription in the AL-treated hamsters to that of normal hamsters (100%). Each sample was analyzed in duplicate.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA). Quantitative data are summarized as median (range) values. The significant difference in quantitative data between the groups was analyzed using the Kruskal Wallis test followed by the Mann-Whitney test. Statistical significance was set at $\alpha = 0.05$.

Results

Effects of AL on the expression of cell cycle-associated genes

The expression levels of cell cycle-associated genes -- *p16, cyclin D1*, and *cdk4* in normal, untreated CCA, AL-treated CCA and 5-FU-treated CCA hamsters are presented in Figure 1 (a,b, c). The expression level of *p16* was downregulated, while that of *cyclin D1* and *cdk4* were upregulated in CCA hamsters compared with normal hamsters (0.20-, 2.01- and 3.63-fold, respectively). AL treatment resulted in an increased expression of *p16* and decreased expression of *cyclin D1* and *cdk4* compared with untreated CCA hamsters (2.33-, 0.42- and 0.043-fold, respectively). Similarly, 5-FU treatment resulted in an increased expression of *p16* and decreased expression of *cyclin D1* and *cdk4* compared with untreated CCA hamsters (3.05-, 0.15- and 0.37-fold, respectively).

Effects of AL on the expression of apoptosis-associated genes

The expression levels of apoptosis-associated genes-- *caspase-3*, *caspase-8*, *caspase-9*, *Apaf-1*, *p53*, *Bax*, *Akt*, *Bcl-2*, *Mfge-8*, *Eef1a1*, *Lass-4*, and *S100A6* in normal, untreated CCA, AL-treated CCA and 5-FU-treated CCA hamsters are presented in Figure 2 (a-l). The expression levels of *caspase-3*, *caspase-8*, *caspase-9*, *Apaf-1*, *p53* and *Eef1a1* were downregulated in CCA hamsters compared with normal hamsters (0.159-, 0.86-, 0.049-, 0.039-, 0.14-, and 0.44-fold, respectively). Upregulation of the expression levels was found with Bax, *Akt*, *Bcl-2*, Mf*ge-8*, *Lass-4*, and *S100A6* (1.14-, 1.49-, 357-, 2.19-, 5.36-, and 3.55-fold, respectively). AL treatment resulted in an increased expression of *caspase-3*, *caspase-8*, *caspase-9, Apaf-1*, and *p53* (14.76-, 4.60-, 2,140-, 93.60-, and 93.86-fold, respectively). Similarly, 5-FU treatment also resulted in an increased expression of these apoptosis-related genes (4.54-, 1.39-, 1,738-, 17.69-, and 27.78-fold, respectively). On the other hand, AL treatment decreased expression levels of *Bax*, *Akt*, *Bcl-2*, *Mfge-8*, *Lass-4*, and

Table 1. Sequences of Primers for Investigation of the Expression Levels of Genes Involved in Cell Cycle, Apoptosis, and Metastasis

No.	Gene	Primer sequences	Annealing temp.
1	*Akt*	Forward: 5'-GGTGATCCTGGTGAAGGAGA-3'	65 °C
		Reverse: 5'-GCGTACTCCATGACAAAGCA-3'	
2	*Apaf-1*	Forward: 5'-ATCCTGGTGCTTTGCCTCTA-3'	60 °C
		Reverse: 5'-TACACCCCCTGAAAAGCAAC-3'	
3	*Bax*	Forward: 5'-AGCTGCAGAGGATGATTGCT-3'	52 °C
		Reverse: 5'-CTCTCGGAGGAAGTCCAGTG-3'	
4	*Bcl-2*	Forward: 5'-TGCACCTGACGCCCTTCAC-3'	60 °C
		Reverse: 5'-AGACAGCCAGGAGAAATCAAACAG-3'	
5	*Caspase-3*	Forward: 5'-TTCGAGCCACCGAGGAGATA-3'	56 °C
		Reverse: 5'-TTGGGGACATCATCCACACG-3'	
6	*Caspase-8*	Forward: 5'-TGTGCCGAGGTCAACAAGAG-3'	55 °C
		Reverse: 5'-AGTTTGGGCACGTTCTTCCT-3'	
7	*Caspase-9*	Forward: 5'-CGAAGGCGATAGTTTGGCTCCT-3'	60 °C
		Reverse: 5'-GGGACTGCAGGTCTTCAGAG-3'	
8	*CDK4*	Forward: 5'-CACCCTCGTGTTTGAGCATA-3'	61 °C
		Reverse: 5'-GTTTTCTGGTTTCAGGTCTCGG-3'	
9	*CyclinD1*	Forward: 5'-AGCAGAAGTGCGAAGAGGAGG-3'	64 °C
		Reverse: 5'-GGCAGTCAAGGGAATGGTCTC-3'	
10	*E-cadherin*	Forward: 5'-GTTAAGGTTCTGGAGATGAGATTGG-3'	54 °C
		Reverse: 5'-CATCTTTCCCCTCCGAGACA-3'	
11	*Lass4*	Forward: 5'-AACTCGTAGAATGTTCGGAGGT -3'	60 °C
		Reverse: 5'-GAGCGAGTATATGAAGGGGTG -3'	
12	*Mfge-8*	Forward: 5'-CTGAAGCCCGTCTAGGTCAT-3'	60 °C
		Reverse: 5'-GAGGGACAACCACAACGAGA-3'	
13	*MMP-9*	Forward: 5'-AGTTTGGTGTCGCGGAGCAC-3'	55 °C
		Reverse: 5'-TACATGAGCGCTTCCGGCAC-3'	
14	*N-cadherin*	Forward: 5'-GTTAAGGTTCTGGAGATGAGATTGG-3'	61 °C
		Reverse: 5'-AGGCCATAAGTGGGATTGCC-3'	
15	*p16*	Forward: 5'-GCAACACCCAAGTAGCCAGAC-3'	61 °C
		Reverse: 5'-CGCCAGAGTTTCCAAGAAGCC-	
16	*p53*	Forward: 5'-AAGGCGATAGTTTGGCTCCT-3'	52 °C
		Reverse: 5'-CTGGGGTCTTCCAGTGTGAT-3'	
17	*S100A6*	Forward: 5'-AAGAAGGAGCTGAAGGAGCTGA-3'	55 °C
		Reverse: 5'-CTGCTGGACCTGGCGTTG -3'	
18	*Smad-2*	Forward: 5'-CAGCTTCTCTGAACAAACCAGG-3'	56 °C
		Reverse: 5'-TACTCTGTGGCTCAATTCCTGCTG-3'	
19	*Smad-3*	Forward: 5'-CCAGCCATGTCGTCCATCCTGC-3'	60 °C
		Reverse: 5'-CCCTTCCGATGGGACACCTGCA-3'	
20	*Smad-4*	Forward: 5'-AGAAACTGGAGAGTTTGATT-3'	56 °C
		Reverse: 5'-CTTCAGATTATAAACAGGGT-3'	
21	*TGF-Beta*	Forward: 5'-GAGATGTTGGTTGTGTTGGGC-3'	61 °C
		Reverse: 5'- AACCACCCAGTAAAAACGACA-3'	
22	*RPL-18*	Forward: 5'-GTTTATGAGTCGCACTAACCG-3'	
		Reverse: 5'-TGTTCTCTCGGCCAGGAA-3'	

S100A6 (0.45-, 0.48-, 0.812-, 0.002-, 0.11- and 0.30-fold, respectively). In the 5-FU-treated hamsters, decreased expression levels were found with only *Akt*, *Bcl-2*, *Mfge-8*, *Lass-4*, and *S100A6* genes (0.402-, 0.0016-, 0.14-, 0.45-, and 0.09-fold, respectively), while increased expression was found with Bax (1.31-fold). *Eef1a1* expression was unchanged after AL or 5-FU treatment.

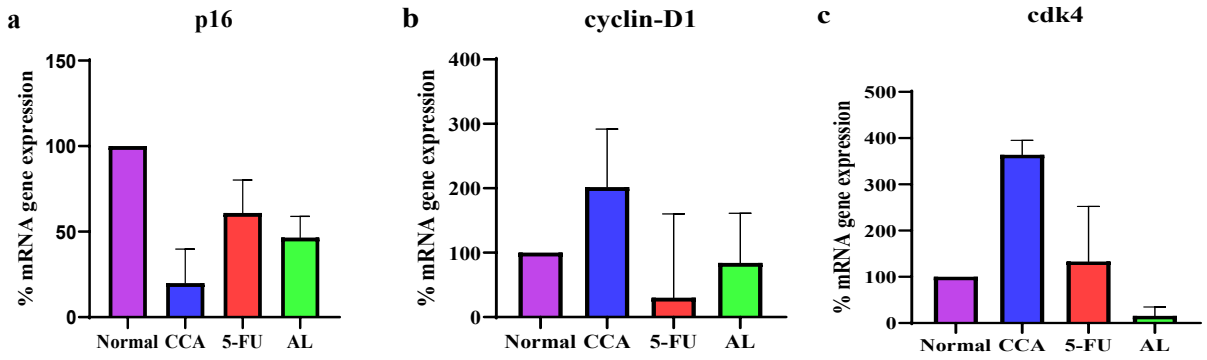

Figure 1. The Expression Levels of Cell Cycle-associated Genes-- *p16*, *cyclin D1*, and *cdk4* in Normal, Untreated CCA, 5-FU-treated CCA, and AL-treated CCA Hamsters. Data are presented as median (range) values from four independent experiments.

Effects of AL on the expression of metastasis-associated genes

The expression levels of metastasis-associated genes -- *TGF-β*, *Smad-2*, *Smad-3*, *Smad-4*, *MMP-9*, *N-cadherin*, and *E-cadherin* in normal, untreated CCA, AL-treated CCA and 5-FU-treated CCA hamsters are presented in Figure 3 (a-g). The expression levels of all genes were upregulated in CCA hamsters compared with normal hamsters (8.90-, 4.32-, 4.27-, 6.99-, 1.22-, 1.70-, and 1.21-fold, respectively). AL treatment resulted in decreased expression levels of all genes except *E-cadherin* (0.005-, 0.514-, 0.81-, 0.29-, 0.46-, 0.02-, and 4.48-fold, respectively). Similarly, the same trend was also found with 5-FU treatment (0.052-, 0.0023-, 0.01-, 0.17-, 0.86-, 0.055-, and 1.37-fold, respectively).

Discussion

Tumorigenesis and metastasis generally involve the induction of cell cycle arrest, expression of anti-apoptotic, cytoprotective, and cell proliferation-associated genes. The cell cycle in eukaryotic cells is regulated by the expression and sequential activation of cell cycle-dependent cyclins, CDKs, CDK inhibitors, and down-stream signaling molecules (Lavecchia et al., 2010). Activation of cell cycle arrest may finally lead to cell apoptosis if unrepaired. CCA has also been reported to be associated with the abnormality of cell cycle signaling molecules, e.g., p38 (mitogen activated protein kinase) (Blechacz et al., 2008; Maemura et al., 2014), *p53* (tumor suppressor gene *p53*) (Blechacz et al., 2008; Maemura et al., 2014), and *p21Waf1* (cyclin dependent kinase inhibitor 1) (Blechacz et al., 2008; Zabron et al., 2013). In the present study, upregulation of *cyclin D1* and *cdk4* (2.01- to 3.63-fold) and downregulation of *p16* (0.20-fold) were found in the liver tissues of CCA hamsters. AL, similarly to 5-FU treatment, reversed the expression levels of these genes to the opposite direction, resulting in suppression of the transition of CCA cells from the G1 to S phase of the cell cycle. This suggests that the arrest of the cell cycle at the G1 phase induced by AL was linked with the induction of *p16* expression and suppression of *cdk4* and *cyclin D1* expression. During the G1/S phase transition of the cell cycle, *cyclin D1* binds *CDK4/6* to form a CDK complex, which is controlled by CDK inhibitors such as *p15*, *p16, p18*, and *p19* (Boonmars et al., 2009; Zivcec et al., 2011; Boueroy et al., 2016). Several chemotherapeutic drugs and herbal medicines induce cell cycle arrest at the G1/S phase. 5-FU was reported to induce CCA cell cycle arrest at the G1 phase through activation of *p53* and *p21* (Boonmars et al., 2008; Senggunprai et al., 2016). Berberine and sho-saiko-to isolated from Berberis vulgaris and a mixture recipe of seven herbs (*Bupleuri radix, Pinelliae tuber, Scutellariae radix, Zizyphi fructus, Ginseng radix, Glycyrrhizae radix, and Zingiberis rhizoma*) were also shown to induce cell cycle arrest at the G1 phase in human CCA and hepatocellular carcinoma cells (Yano et al., 1994; He et al., 2012). The observations support the results of our previous studies demonstrating the inducing activity of AL, atractylodin and β-eudesmol on CCA cell cycle arrest at the G1 phase (Kotawng et al., 2018; Narahara et al., 2020).

Cell apoptosis plays an important role in maintaining tissue homeostasis between cell survival and death (Elmore et al., 2007). This program cell death starts with changes in cell nuclear morphology (chromatin condensation and fragmentation), cell shrinking, plasma membrane blebbing, and apoptotic body formation. Cancer cells are known to express abnormally elevated levels of cytoprotective and antiapoptotic enzymes, which assist them to endure cellular stress and abrogate apoptosis, resulting in enhanced survival (Basu et al., 2012). Targeting cell apoptosis is, therefore, one of the promising approaches for cancer chemotherapeutic. Two major signaling pathways are involved in cell apoptosis, i.e., the intrinsic (mitochondrial-mediated), and extrinsic (death receptor) pathways. The intrinsic pathway is activated by DNA damage, ischemia, and oxidative stress while the extrinsic pathway is triggered by death ligand binding to a death receptor. The *Bcl-2*, *Bax*, *cytochrome c*, *caspase-3*, and *caspase-9* are involved in the intrinsic pathway, whereas *CD147*, *TNF-β*, *FASL*, *TRAIL*, *TRAF2*, *caspase-8* and *caspase-3* are involved in the extrinsic pathway (Zeriouh et al., 2007; Wang et al., 2014; Karimian et al., 2016; Yu et al., 2017). Caspases are intracellular cysteine protease enzymes that play major role in apoptotic mechanism and *caspase-3/7* is the final step in both the intrinsic or extrinsic pathways

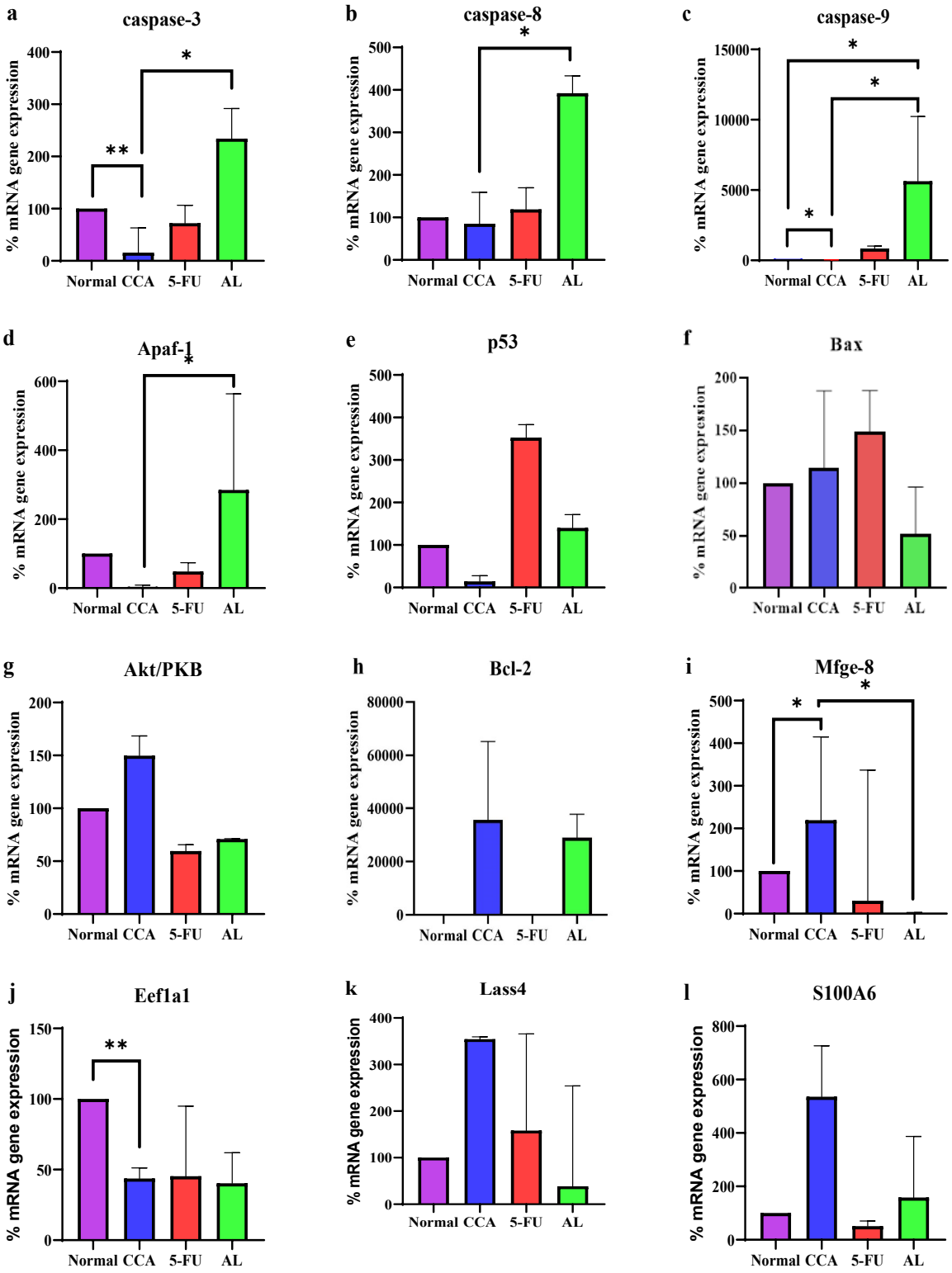

Figure 2. The Expression Levels of Apoptosis-associated genes-- *caspase-3*, *caspase-8*, *caspase-9*, *Apaf-1*, *p53*, *Bax*, *Akt/PKB*, *Bcl-2*, *Mfg-e8*, *Eef1a1*, *Lass-4*, and *S100A6* in normal, untreated CCA, 5-FU-treated CCA, and AL-treated CCA Hamsters. The expression levels of all genes in normal hamsters were normalized to 100%. Data are presented as median (range) values from four independent experiments.* represents p-value < 0.05 and ** represents p-value < 0.01

of apoptosis (Mcllwain et al., 2013). The process that promotes pro-apoptotic factor Bax expression or/and decreases anti-apoptotic factor *Bcl-2* expression results in the release of cytochrome to the cytosol and subsequently, initiation of *caspase-9* and *caspase-3* cascades, leading to cell apoptosis (Mathema et al., 2017). Stimulation of apoptosis pathways by anticancer agents was shown to suppress cancer survival, of which increased expression levels of *Akt/PKB*, *Bcl-2*, *caspase-3*, *caspase-9*, and *p53* were observed (Boonmars et al., 2009; Qin et al., 2016; Narahara et al., 2020). The binding of cytochrome c to Apaf-1 and *caspase-9* leads to the activation of the cell

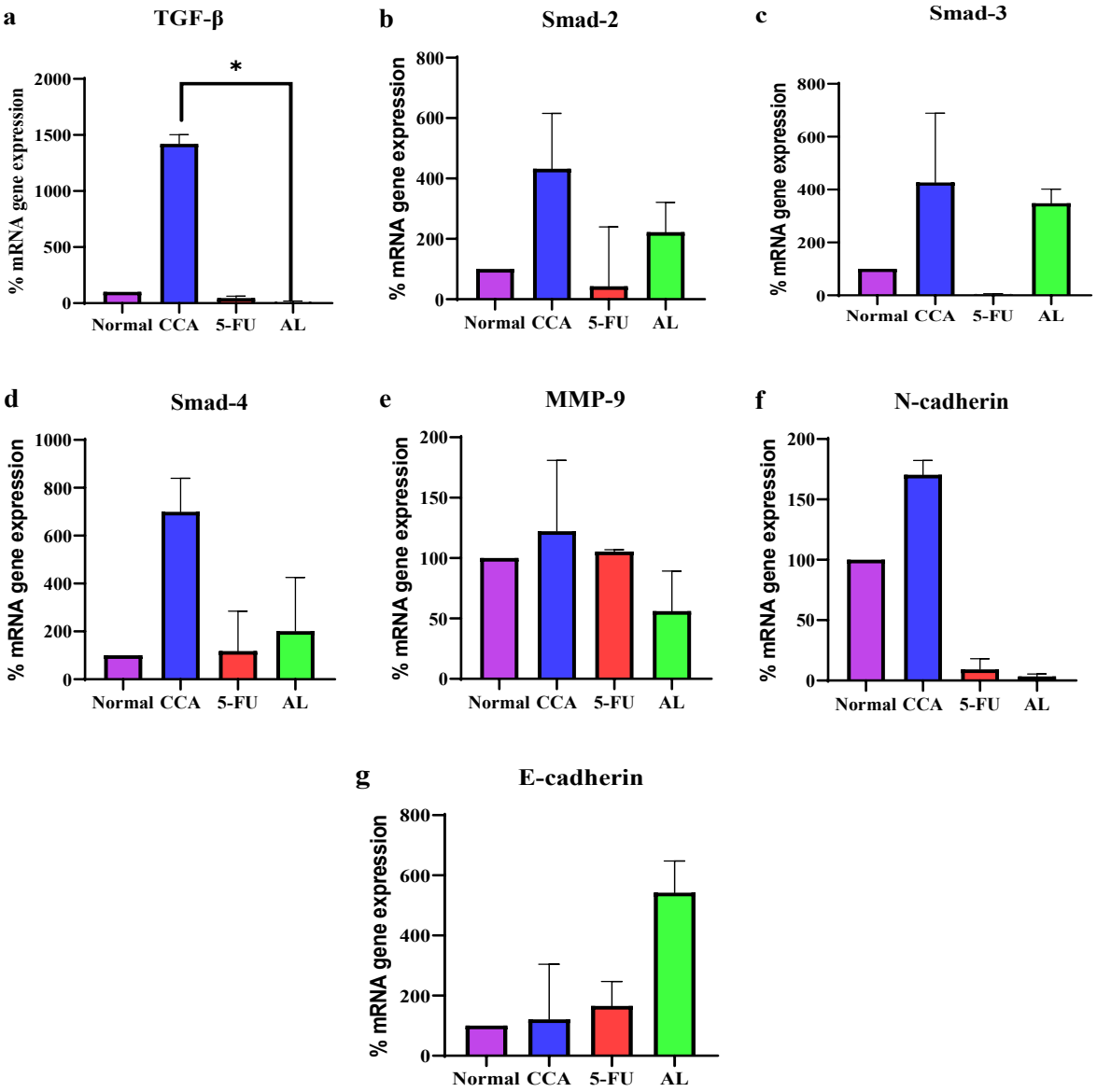

Figure 3. The Expression Level of Metastasis-associated genes -- *TGF-β*, *Smad-2*, *Smad-3*, *Smad-4*, *MMP-9*, *N-cadherin*, and *E-cadherin* in normal, untread CCA, 5-FU-treated CCA, and AL-treated CCA hamsters. The expression levels of all genes in normal hamsters were normalized to 100%. Data are presented as median (range) values from four independent experiments.* represents p-value < 0.05.

death cascade. Moreover, downregulation of *Akt/PKB* and upregulation of *caspase-9* lead to induction of intrinsic apoptotic signaling pathway. The balance between the proapoptotic Bax and anti-apoptotic *Bcl-2* protein regulators is a critical key point to determine cell apoptosis. In this study, downregulation in the expression levels of *caspase-3*, *caspase-8*, *caspase-9*, *Apaf-1*, *p53*, and *Eef1a1* (0.039- to 0.86-fold) were found in the livers of CCA hamsters, while the upregulation of the expression levels were found with *Bax*, *Akt*, *Bcl-2*, *Megf-8*, *Lass-4*, and *S100A6* (1.14- to 357-fold). AL and 5-FU treatment reversed the regulation levels of almost all genes except *Eef1a1* to the opposite direction. The marked changes were found with *caspase-9* (2,104-fold) and *Mfge-8* (0.002-fold). The *Bax/Bcl-2* ratio was markedly increased following 5-FU compared with AL treatment (0.0032, 2.67 and 0.0018 for untreated, 5-FU-treated and AL-treated hamsters, respectively). This suggests that AL treatment induced apoptosis in CCA hamsters by inducing *caspase-3* activity through *p53* modulation, downregulating anti-apoptotic protein, inducing outer membrane pore opening, and releasing cytochrome c. For *caspase-8* in the extrinsic apoptotic pathway, gene upregulation and binding to *caspase-3* lead to the cell death cascase. Previous studies showed that atractylodin and β-eudesmol induced mitochondria-mediated apoptosis (Kotawng et al., 2018; Srijiwangsa et al., 2018; Kotawng et al., 2020; Narahara et al., 2020). Both compounds significantly activated *caspase-3/7* compared to untreated control cells, suggesting the potentiating cytotoxic effect of β-eudesmol on CCA cells in the final stage of apoptosis (Kotawong et al, 2018; Narahara et al., 2020). β-Eudesmol-induced enhacement of chemosensitivity of CCA cells by promoting their apoptosis was shown to be associated with increase of the *Bax/Bcl-2* ratio and caspase activation.

The Milk fat globule-EGF factor 8 (*Mfge-8*) is a glycoprotein initially identified as a component of milk fat globules secreted from mammary epithelial cells. It is an opsonin that mediates the clearance of dying cells via integrin-expressing phagocytes (Hanayama et al., 2002; Wu et al., 2017). Recent studies have shown that *Mfge-8* is involved in the tumorigenesis, progression, and enhancement of tumor resistance to apoptosis of various types of cancer, including CCA, breast cancer, ovarian cancer, and colorectal cancer (Li et al., 2013; Jia et al., 2017; Wu et al., 2017; Yang et al., 2011). Human *Mfge-8* consists of an N-terminal EGF-like domain that contains an integrin-binding RGD motif and two repeated C-terminal discoidin/F5/8C domains that have been shown to be responsible for phosphatidylserine binding (Aziz et al., 2011). In addition, *Mfge-8* also inhibits the *caspase-3* process, leading to the promotion of cell survival and apoptosis resistance in melanoma (Jinushi et al., 2008). The expression of *Mfge-8* was shown to be significantly upregulated in the livers of OV/DMN-induced CCA hamsters and CCA patients compared with non-CCA tissues (Wu et al., 2017). Our study also showed that *Mfge-8* expression was upregulated in the CCA hamsters compared with normal hamsters. Following treatment with AL, however, the expression of *Mfge-8* was downregulated. Altogether, the results of the present study may suggest that AL treatment induced apoptosis by inhibiting *Mfge-8* and thereby, inducing *caspase-3* expression. The underlying molecular mechanism of the link between tumorigenesis and downregulation of *Mfge-8* expression needs to be clarified.

Metastasis is one of the most critical characteristics of cancers indicating poor prognosis and death in cancer patients. The process reflects the ability of cancer cells to break away from the primary tumor and enter the bloodstream or lymphatic system. The key steps include degradation of tumor extracellular matrix, cell invasion, and cell migration (Tan et al., 2002). Inhibitors of these metastasis-associated processes would, therefore, provide a significant impact on cancer chemoprevention and chemotherapy. In the present study, all metastasis-associated genes except *E-cadherin*, i.e., *TGF-β*, Sm*ad-2*, *Smad-3*, *Smad-4*, *MMP-9*, and *N-cadtherin* were upregulated in CCA (1.21- to 8.90-fold). AL and 5-FU treatment resulted in the decrease in the expression of all of these upregulated genes. The marked change was found with *TGF-β* (8.90-fold), which was suppressed by AL and 5-FU treatment (0.005-fold and 0.052-fold, respectively). Altogether, the results suggest the antimetastasis activity of AL by inhibition of canonical *TGF-β* signaling pathway, cell invasion (through downregulation of *MMP-9*), and cell migration (through upregulation of *E-cadherin* and down-regulation of *N-cadherin*). This explains significant suppressive effect of AL on tumor invasion, angiogenesis, and lung metastasis observed in the CCA-xenograft model in the previous study (Plengsuriyakarn et al., 2012). In the canonical *TGF-β* signaling pathway, the binding of *TGF-β* to the receptors induces *Smad-2/-3* phosphorylation and promotes *Smad-2/-3-Smad-4* translocation into the nucleus (Nakao et al., 1997). Recent studies have shown that excessive expression of *TGF-β* induces upregulation of metastasis-associated genes, which trigger the expression of mesenchymal genes (*E-cadherin and N-cadherin*) and *matrix-metalloproteinase* (*MMP*) (Xie et al., 2018). Searching for a new anticancer drug by targeting *TGF-β*/*Smad-4* signaling pathway was proposed to inhibit cancer metastasis.

In conclusion, the anti-CCA activity of AL observed in OV/DMN-induced CCA hamsters (Plengsuriyakarn et al., 2015) could be through the induction of cell cycle arrest at the G1 phase and activation of the apoptosis pathway, resulting in cancer cell death. The activation of the apoptosis pathway mainly involved the intrinsic pathway (activation of *caspase-3* and *caspase-9* through *p53* and *Mfge-8* modulation and downregulation of anti-apoptotic genes *Akt* and *Bcl-2*). In addition, AL could also inhibit the canonical *TGF-β* signaling pathway, *MMP-9* and *N-cadherin* to suppress tumor metastasis.

Author Contribution Statement

MT and KN were involved in the design of the experimental study. PS and MT performed the experiments. PS. MT, TP, KB and KN performed data analysis. PS drafted the manuscript. KN and MT revised the manuscript. All authors reviewed and approved the final manuscript for submission. All meet the ICMJE criteria for authorship.

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Approval of Scientific Body

The article is part of an approved thesis of Mr. Paradon Sonsomnuek.

Ethics Approval

Approval of the study protocol was obtained from the Ethics Committee for Animal Research, Thammasat University, Thailand (Approval number 013/2556).

Availability of Data

The datasets generated during and/or analyzed during this study are the corresponding author on reasonable request.

Conflict of Interest

The authors declare no competing interests.

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