Arctigenin Inhibits Cholangiocarcinoma Progression by Regulating Cell Migration and Cell Viability via the N-cadherin and Apoptosis Pathway

Sutthiwan Janthamala
Biomedical sciences program, Graduate school, Khon Kaen University
Apinya Jusakul
Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University
Sarinya Kongpetch
Department of Pharmacology, Faculty of Medicine, Khon Kaen University
Phongsaran Kimawaha
Biomedical Sciences Program, Graduate School, Khon Kaen University
Poramate Klanrit
Department of Biochemistry, Faculty of Medicine, Khon Kaen University
Watcharin Loilome
Department of Biochemistry, Faculty of Medicine, Khon Kaen University
Nisana Namwat
Department of Biochemistry, Faculty of Medicine, Khon Kaen University
Anchalee Techasen (anchte@kku.ac.th)
Khon Kaen University https://orcid.org/0000-0002-4230-5641

Research Article

Keywords: N-cadherin, Arctigenin, Cholangiocarcinoma, Epithelial-mesenchymal transition, Metastasis, Apoptosis

Posted Date: May 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-510261/v1

Version of Record: A version of this preprint was published at Naunyn-Schmiedeberg's Archives of Pharmacology on July 20th, 2021. See the published version at https://doi.org/10.1007/s00210-021-

02123-0.

Abstract

Northeast Thailand has the highest incidence of cholangiocarcinoma (CCA) in the world. The lack of promising diagnostic markers and appropriate therapeutic drugs are the main problem for metastatic stage CCA patients who have a poor prognosis. N-cadherin, a cell adhesion molecule, is usually upregulated in cancers and has been proposed as an important mediator in epithelial-mesenchymal transition (EMT), one of the metastasis processes. Additionally, it has been shown that arctigenin, a seed extract from *Arctium lappa*, can inhibit cancer cell progression via suppression of N-cadherin pathway. In this study, we investigated the protein expression of N-cadherin and its correlation with clinicopathological data of CCA patients, as well as the impact of arctigenin on KKU-213A and KKU-100 CCA cell lines and its underlying mechanisms. Immunohistochemistry results demonstrated that high expression of N-cadherin was significantly associated with severe CCA stage (p=0.027), and shorter survival time (p=0.002) of CCA patients. The mean overall survival times between low and high expression of N-cadherin were 31.6 and 14.8 months, respectively. Wound healing assays showed that arctigenin significantly inhibited CCA cell migration by downregulating N-cadherin whereas upregulating E-cadherin expression. Immunocytochemical staining revealed that arctigenin suppressed the expression of N-cadherin in both CCA cell lines. Furthermore, flow cytometry and western blot analysis revealed that arctigenin significantly reduced CCA cell viability and induced apoptosis via the Bax/Bcl-2/Caspase-3 pathway. This research supports the use of N-cadherin as a prognostic marker for CCA and arctigenin as a potential alternative therapy for improving CCA treatment outcomes.

Introduction

Cholangiocarcinoma (CCA) originates from epithelial cells of intrahepatic and extrahepatic bile ducts and is a common form of liver cancer (Khan et al., 2019). CCA is the main public health problem in northeast Thailand, with the highest prevalence worldwide (Kamsa-Ard et al., 2018). The absence of clear symptoms in CCA patients remains a major diagnosis and treatment problem. Most patients are diagnosed with progressive or metastatic stage, with an extremely poor prognosis and not suitable for surgery (Aljiffry et al., 2009, Banales et al., 2020). When the disease reaches an advance stage, palliative treatment is the only option that possible. Many chemotherapy drugs, such as cisplatin and gemcitabine, have been commonly used to treat CCA patients, but response rates are poor and survival times of patients are short (Okusaka et al., 2010). The exploration of appropriate therapeutic agents for CCA is therefore important.

Epithelial-mesenchymal transformation (EMT) is a major point of the tumor metastasis mechanism acting by enhancing cell invasion and migration (Kalluri and Weinberg, 2009). There are a group of proteins that act as EMT biomarkers, such as epithelial marker, E-cadherin and mesenchymal marker, N-cadherin. An alteration of the role of E-cadherin and N-cadherin contributes to tumor metastasis has been was investigated in many cancers (Loh et al., 2019). Techasen et al. reported that low E-cadherin protein expression in CCA tissues was strongly correlated with positive metastasis status, and they proposed that E-cadherin could be used as a CCA metastasis marker (Techasen et al., 2014). Previous studies have

reported that high expression of N-cadherin predicts poor prognosis in patients with colorectal, prostate, and gallbladder cancers (Gravdal et al., 2007, Yi et al., 2014, Yan et al., 2015). It appears that acquiring N-cadherin is a crucial step in epithelial cancer metastasis and disease progression (Kalluri and Weinberg, 2009, Gheldof and Berx, 2013). Therefore, N-cadherin expression is a possible potential biomarker for cancer prognosis.

Natural plant therapeutic properties are currently being used to suppress cancer cell metastasis by the EMT process (Wang and Jiang, 2012, Avila-Carrasco et al., 2019, Jiang et al., 2020, Wang et al., 2020). Curcumin, the main phytochemical in turmeric, can reverse TGF-β-1-induced EMT in gastric cancer cells by upregulating E-cadherin and downregulating N-cadherin expression (Cheng et al., 2018). Resveratrol, a grape extract, has been shown to completely inhibit cancer cell motility, and reverted the N-to E-cadherin switch in colon cancer and CCA cells (Thongchot et al., 2018, Yuan et al., 2019). Gedunin, a compound extracted from *Azadirachta indica*, blocked pancreatic cancer cell metastasis by downregulating the expression of mesenchymal markers, N-cadherin and vimentin, while upregulating the expression of epithelial marker, E-cadherin (Subramani et al., 2017). Moreover, Paeoniflorin, a compound derived from plants in the *Paeonia lactiflora* Pallas, inhibit glioblastoma cell by suppression of TGF-β signaling pathway via decreasing the expression of snail, N-cadherin and vimentin (Wang et al., 2018).

Arctigenin is a member of the *Asteraceae* family and has pharmacological properties such as anti-oxidant, anti-inflammatory, anti-cancer, and anti-metastasis (He et al., 2018). Arctigenin can inhibit cell invasion and migration in hepatocellular carcinoma, colorectal, and lung cancers via suppression of EMT process (Han et al., 2016, Xu et al., 2017, Lu et al., 2019). In addition, Li et al. discovered that arctigenin inhibits cell proliferation and causes apoptosis in colon cancer cells (Li et al., 2016). However, there is no information on the effect of arctigenin on CCA cell progression.

The aims of this study were to investigate the expression of N-cadherin protein in CCA tissues, to determine whether there are any associations with a patient's clinicopathological data, as well as to investigate whether arctigenin could be used as a new therapeutic for inhibiting CCA progression focusing on the switching of N- to E-cadherin pathway. Additionally, the mechanism of arctigenin's action was also examined.

Methods

Human CCA tissues

This study was approved by the Human Ethics Committee of Khon Kaen University, based on the ethics of human specimen experimentation of the National Research Council of Thailand (HE631304). The paraffin-embedded CCA tissues (n = 81) were collected from Srinagarind hospital and were kept in the biospecimen bank of Cholangiocarcinoma Research Institute (CARI), Khon Kaen University, Thailand.

Cell lines and cell culture

The CCA cell lines, KKU-213A (JCRB1557) (Sripa et al., 2020) and KKU-100 (JCRB1568) (Sripa et al., 2005) were developed by Prof. Banchob Sripa at Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. Both cell lines were deposited to the Japanese Collection of Research Bioresources (JCRB), Osaka, Japan for all complete identification of characteristics. In our study, cell lines were purchased from JCRB cultured in Ham's F-12 containing 10% fetal bovine serum and penicillin/streptomycin (50U/ml and 50 µg/ml) and incubated in a humidified incubator of 5% CO_2 at 37°C.

Chemicals

Arctigenin (Sigma-Aldrich, USA) (purity ≥ 95%) stock solution of 50 mM was prepared by dissolving arctigenin in dimethyl sulfoxide (DMSO) and stored at − 80°C. Stock solution was diluted with culture medium to prepare the working solutions which were at the desired concentrations. The final concentration of DMSO was 0.4% (v/v) in all treatment conditions.

Immunohistochemical staining for N-cadherin expression

Formalin-fixed paraffin-embedded tissue of 81 CCA patients were deparaffinized in xylene and dehydrated in graded ethanol series. For antigen retrieval, section slides were heated in sodium citrate in microwave oven for 10 min. The endogenous peroxidase activity in the tissues were blocked by incubating the slide in 0.3% hydrogen peroxide (H_2O_2) solution in PBS for 30 min. Subsequently, 10% skim milk in PBS was added for 30 min to block non-specific substances and all slides were incubated with mouse anti-N-cadherin monoclonal antibody (mAb, dilution 1:100; BD Transduction Laboratories™, USA) in humid chamber at 4°C overnight. After washing, slides were incubated with horseradish peroxidase-conjugated Envision™ secondary antibody (DAKO, Denmark) for 1 h 30 min. DAB peroxidase (HRP) substrate kit (Vector Laboratories, CA), which stained the antigen brown, and hematoxylin counterstaining was used to visualize the reaction. The slides were then dehydrated and mounted, stained sections were investigated under a light microscope.

Immunohistochemical scoring for N-cadherin expression

The Histoscore (H-score), a semi-quantitative method was applied for immunohistochemical staining as described by Detre et al (Detre et al., 1995). The evaluations were recorded as percentages of positively stained cancer cells in each of the four intensity categories, which were denoted as 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). The total number of cells in each field and the number of cells stained at each intensity were counted. The average percentage positive was calculated, and the following formula was applied.

H-score = (% of cells stained at intensity category 1 x 1) + (% of cells stained at intensity category 2 x 2) + (% of cells stained at intensity category 3 x 3).

The range of scores were from 0 to 300. The expression level of protein was classified as low or high according to the mean value of the H-score.

Cell migration assay

Cell migration was determined by using a wound healing assay. The KKU-213A and KKU-100 cells were seeded in 24 well-plate (10^5 cells/well) and incubated to complete 100% cell confluence. After treatment with arctigenin at concentrations of 100 and 200 μM, wounds were made by pipette tip scratching and monitored by microscopy at 18 h for KKU-213A and 24 h for KKU-100.

Cell proliferation assay

Cell proliferation was determined by using an MTT colorimetric assay. The cells were seeded in 96 well-plate (2,000 cells / well) and were incubated for 24 h. Cells were treated with a series concentration of arctigenin and were incubated at 37°C in 5% CO_2 incubator. After 48 h treatment, the cells were wash with PBS and incubated with MTT reagent at 37°C for 2 h. Subsequently, the MTT reagent was removed and DMSO was added to dissolve formazan crystals. The optical density was measured at an absorbance of 540 nm using a microplate reader. Data was presented as mean ± SD from two independent experiments. Arctigenin concentrations that produce 50% cell viability (the half maximal inhibitory concentration; IC_{50}) were calculated from curves constructed by plotting cell survival (%) versus arctigenin concentration (μM). The values were represented as the percentage of cell viability comparing with the control.

Flow cytometric assay for apoptosis evaluation

The apoptotic-cell distribution was determined using the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen™, USA) according to the manufacturer's protocol. Briefly, after 48-h of arctigenin treatment at concentrations of 50, 100 and 200 μM, the cells were trypsinized with trypsin and washed with cold PBS. After PBS was removed, and cells were resuspended with 100 μl of 1x annexin-bingding buffer, 2.5 μl of Alexa Fluor® 488 annexin V and 1 μl of 100 μg/ml PI. The stained cells were analyzed by flow cytometry and measured the fluorescence emission at 530 nm and 575 nm using 488 nm excitation.

Western blot analysis

Protein was extracted from cell pellets by using a RIPA cell lysis buffer and the protein concentration was determined by using a Pierce™ BCA Protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The protein solutions were boiled at 95 °C for 5 min on hot plate and cooled on ice. An appropriate amount of protein was loaded onto an SDS-PAGE (4% stacking gel and 8% separating gel) and was transferred onto a PVDF membrane (Merck, Billerica, MA, USA). The transferred membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS) at room temperature for 1 h before being incubated with primary antibody overnight at 4°C. The antibodies used in this study included, mouse anti-N-cadherin monoclonal antibody (mAb, dilution 1:250; BD Transduction Laboratories™, USA), mouse anti-E-cadherin monoclonal antibody (mAb, dilution 1:1000; BD Transduction Laboratories™, USA), rabbit anti-caspase-3 monoclonal antibody (mAb, dilution 1:1000; Abcam, UK), rabbit anti-BCL-2 polyclonal antibody (dilution 1:1000; Proteintech, USA), rabbit anti-BAX polyclonal antibody (dilution 1:1000; Proteintech, USA), and mouse anti-β actin monoclonal antibody (mAb, dilution 1:20,000;Sigma-Aldrich, USA). After incubation, the membranes were rinsed with TTBS, incubated with peroxidase-labelled secondary

antibodies for 1 h, and rinsed with TTBS. Finally, the membranes were exposed to an Amersham™ ECL™ Prime Western Blot Detection Reagent (GE Healthcare, Buckinghamshire, UK) for chemiluminescence detection using an Amersham Imager™ 600.

Immunocytochemistry analysis

CCA cells were seeded on slide chambers and treated with or without arctigenin for 48 h. After incubation, cells were fixed for 30 min in 4% paraformaldehyde in PBS. Fixed cells were incubated in 3% (w/v) bovine serum albumin in PBS for non-specific binding blocking. Cells were incubated overnight at 4°C with the primary antibodies specific for N-cadherin (mAb, dilution 1:100; BD Transduction Laboratories™, USA). After washing, cells were incubated with secondary antibodies conjugated with Alexa flour 555 (Invitrogen™, USA) for 1 h. The preparations were mounted with mounting solution containing a 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Slides were visualized under a fluorescence microscope on an Inverted Microscope EVOS™ Imaging System.

Statistical analysis

IBM SPSS V.23.0 statistical package (SPSS Inc., Chicago, IL) was used for statistical analysis. Data were represented as mean ± SD. The associations between N-cadherin expression and clinicopathological parameters of CCA patients was calculated by chi-square test. The log-rank analysis was used to compare survival distributions and the Kaplan-Meier method was plotted for survival analysis. The quantitative bar chart for functional analysis and the dose-response curve for IC_{50} was calculated and created by GraphPad Prism version 8.0 for windows (GraphPad software, San Diao, CA). The statistical significance between different groups was detected by one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

N-cadherin expression in CCA and its correlation with clinicopathological data

The characteristics of all CCA patients are summarized in Table 1. The mean age of patients was 60 years (range 38–83 years), with 52 males and 29 females. Most patients had non-papillary histology type, and stage III and stage IV CCA at the time of diagnosis. The representative IHC staining of N-cadherin in 81 CCA tissue samples is shown in Fig. 1a. N-cadherin was predominantly localized in cytoplasmic of all CCA tissues. The expression of N-cadherin was lower in normal bile ducts compared to tumor cells. Using a mean value of H-score of the IHC result as the cut-off point, N-cadherin expression was found to be elevated in 43.2% (35/81) of CCA tissues. As shown in Table 2, high expression of N-cadherin was significantly associated with severe CCA stage ($p = 0.027$). In addition, high expression of N-cadherin was significantly associated with shorter survival time of CCA patients as shown in Fig. 1b ($p = 0.002$). The mean overall survival times between low and high expression of N-cadherin were 31.6 and 14.8 months, respectively.

Table 1
The characteristics of all CCA patients

Variables	Number (%)
Sex	
Male	52 (64.2%)
Female	29 (35.8%)
Age (year)	
Less than 60	37 (45.7%)
60 or greater	44 (54.3%)
Histology	
Papillary	31 (38.3%)
Non-papillary	50 (61.7%)
Stage	
0-II	16 (19.8%)
III-IV	65 (80.2%)
Lymph node metastasis	
No	41 (50.6%)
Yes	33 (40.7%)
Not applicable	7 (8.7%)
Distance Metastasis	
No	48 (59.3%)
Yes	12 (14.8%)
Not applicable	21 (25.9%)

Table 2
Correlation between N-cadherin and clinicopathological data

Clinicopathological data	N-cadherin expression		*p*-value
	Low (n = 46)	High (n = 35)	
Total (N = 81)			
Gender			
female	18 (39.1%)	11 (31.4%)	0.474
male	28 (60.9%)	24 (68.6%)	
Age			
< 60	23 (50.0%)	14 (40.0%)	0.371
≥ 60	23 (50.0%)	21 (60.0%)	
Histological types			
non-papillary	29 (63.0%)	21 (60.0%)	0.780
papillary	17 (37.0%)	14 (40.0%)	
Stage			
0-II	13 (28.3%)	3 (8.6%)	0.027*
III-IV	33 (71.7%)	32 (91.4%)	
Lymph node metastasis			
No	21 (45.7%)	20 (57.1%)	0.284
Yes	21 (45.7%)	12 (34.3%)	
Not applicable	4 (8.6%)	3 (8.6%)	
Distance Metastasis			
No	22 (47.8%)	26 (74.3%)	0.071
Yes	9 (19.6%)	3 (8.6%)	
Not applicable	15 (32.6%)	6 (17.1%)	
*p-value < 0.05 was considered statistically significant.			

Arctigenin inhibits CCA cells metastasis via reversing of N- to E-cadherin pathway

To further assess the anti-metastatic effect of arctigenin, a wound healing assay was performed to determine whether arctigenin affects the migration ability of KKU-213A and KKU-100 CCA cells. Cell movements were observed 18 and 24 h after arctigenin treatment. In the presence of arctigenin, CCA cell migration was significantly suppressed in a dose-dependent manner whereas control cells migrated toward the scratched site (Fig. 2a and 2b). As compared to the control condition, the percentage of wound area in 100 and 200 μM of arctigenin was significantly different in both CCA cell types (Fig. 2c and 2d).

As N-cadherin has been reported to be associated with the metastasis process, we further determined whether arctigenin effect on N-cadherin expression by western blot analysis. As shown in Fig. 2e, arctigenin treatment significantly decreased the expression of N-cadherin and increased the expression of E-cadherin, an epithelial marker in KKU-213A. Furthermore, immunocytochemistry analysis revealed that arctigenin suppressed N-cadherin expression in KKU-213A and KKU-100 cells compared to controls (Fig. 2f).

Arctigenin inhibits CCA cells proliferation and induces apoptosis via Bax/Bcl-2 and caspase-3 pathway

The effects of arctigenin with a concentration range of 5-250 μM on KKU-213A and KKU-100 cell lines were determined using MTT assay. As shown in Fig. 3, cell proliferation rates of KKU-213A and KKU-100 CCA cell lines were significantly reduced after treatment with arctigenin when compared with the control. After 48 h of treatment, the IC_{50} in KKU-213A cells was 98.16 μM, while in KKU-100 cells was 120.60 μM.

To identify whether arctigenin inhibited CCA cell proliferation by inducing apoptosis. KKU-213A and KKU-100 cells were treated with a series of arctigenin concentrations. Cells were stained with Annexin V-FITC/PI for flow cytometry analysis. As shown in Fig. 4a, KKU-213A cells treated with 50, 100, and 200 μM of arctigenin for 48 h showed increased apoptotic rate by approximately 60.85%, 73.8%, and 89.3%, respectively. For KKU-100 cells, the apoptotic rates were found to increase approximately 4.35%, 14.4%, and 18.00% after treatment with series of arctigenin concentrations, respectively. The results indicated that arctigenin significantly induced CCA apoptosis, as shown in the right quadrants of the flow cytometry graphs. All concentrations showed the capacity to strongly induce CCA apoptosis except for dose 50 μM of arctigenin in KKU-100 (Fig. 4b). In addition, the expression of apoptosis-related proteins in KKU-213A cells was investigated using western blot analysis. As shown in Fig. 4c, the expression of pro-caspase-3 and Bcl-2 was decreased whereas the expression of Bax was increased after arctigenin treatment.

Discussion

Epithelial-mesenchymal transition (EMT) is an important step in the process of tumor metastasis through transforming epithelial cells to mesenchymal phenotypes (Steinestel et al., 2014). The loss of epithelial markers such as E-cadherin while increasing mesenchymal markers such as N-cadherin is the most remarkable occurrence in cancer cells that develop metastatic properties (Suresh and Nathawat, 2014). Techasen et al. reported that low E-cadherin protein expression in CCA tissues was significantly associated with positive metastasis status. They suggested that E-cadherin might act as a central modulator of tumor cell phenotype and can also serve as a potential metastasis marker in CCA (Techasen et al., 2014). Aside from epithelial markers, mesenchymal markers should also be identified. N-cadherin is a member of the calcium-dependent adhesion molecule family of classical cadherins, which specifically mediate cell-cell adhesion. It is typically absent in normal epithelial cells, and the aberrant expression of N-cadherin in epithelial cancer cells is a well-documented characteristic of epithelial malignancies (Tomita et al., 2000, Nakajima et al., 2004, Lascombe et al., 2006, Choi et al., 2013). Several studies have found a correlation between high N-cadherin protein expression and cancer progression in a variety of cancers (Ning et al., 2013, Okubo et al., 2017, Abufaraj et al., 2018). Increasing N-cadherin expression was associated with late stage and lymph node metastasis in patients with breast and prostate cancers (Ning et al., 2013, Drivalos et al., 2016). According to previous reports, patients with elevated N-cadherin expression in breast and lung cancers have a lower survival rate than patients with low N-cadherin expression (Hui et al., 2013, Bock et al., 2014). Similarly, increased N-cadherin immunoreactivity was related to a severe CCA stage and a shorter patient survival time in our study. These findings suggest the N-cadherin could be used as the prognostic and targeted marker for CCA treatment.

Accumulating evidence suggests that without curative operation, the effectiveness of standard chemotherapy for CCA treatment is not satisfactory (Okusaka et al., 2010). The development of effective therapeutic strategies in CCA is of considerable therapeutic benefit. Natural products are major sources for development of novel anti-cancer drugs due to their potentially low toxicity and potential effectiveness. Arctigenin is a bioactive compound which is isolated from *Arctium lappa* L., a popular medicinal herb usually used for anti-influenza treatment in Asia. Arctigenin has attracted the attention of researchers due to promising therapeutic effects including anti-inflammation, anti-infection, and anti-cancer (Gao et al., 2018). Therapeutic effects of arctigenin have been demonstrated in various cancers *in vitro* and *in vivo*, especially the role in EMT modulation (Yao et al., 2011, Wang et al., 2014, Wang et al., 2015, Lu et al., 2019). In CT26 colorectal cancer, arctigenin significantly inhibited lung metastasis through increasing the expression of E-cadherin and decreasing the expressions of N-cadherin. In addition, arctigein induced apoptosis in CT26 cells through the intrinsic apoptotic pathway (Han et al., 2016).

Results from our study demonstrated that arctigenin significantly suppressed CCA cell migration in a dose-dependent manner. Arctigenin repressed the expression of N-cadherin and induced a dramatic increase in the expression of E-cadherin in CCA cell lines. Similar results have also been observed in hepatocellular carcinoma, colorectal, and lung cancers (Han et al., 2016, Xu et al., 2017, Lu et al., 2019). This data supported that arctigenin exhibited an anti-EMT effect on CCA related to the regulation of N-cadherin/E-cadherin switching. Therefore, arctigenin could be used as anti-metastasis agent in CCA.

In addition, our study found that arctigenin plays a role in anti-proliferation and induced apoptosis in CCA cell lines. We performed cell proliferation assays after treatment with arctigenin. Our results show that arctigenin significantly inhibited the CCA cell viability up to 50%. This result was consistent with previous study which reported that arctigenin had an anti-proliferation role in breast cancer cells. MCF-7 human breast cancer cells were treated with 200 μM arctigenin and their viability was found to decrease by up to 50% (Maxwell et al., 2018). In addition, we performed flow cytometry to analyze whether the anti-proliferation effect of arctigenin was mediated via the apoptosis pathway. After being treated with arctigenin, CCA cells showed a significant increase in cellular apoptosis. Apoptosis is controlled by two major pathways including intrinsic and extrinsic pathways. The intrinsic pathway control by the Bcl-2 family proteins (Cavalcante et al., 2019). The extrinsic pathway is induced by activation of the death cell receptor. The activation of the death cell receptor triggers the development of a death-inducing signaling complex, which includes the initiator caspase. There is cross-talking between two apoptotic pathways that have common apoptotic markers including Bid and caspase-3 (Wali et al., 2013). Our results showed that arctigenin treatment reduced Bcl-2 and pro-caspase-3 expression, whereas it induced Bax expression. A Similar results were found by Li et al. who showed that arctigenin induces apoptosis in HT-29 colon cancer cells by caspase-3 activation (Li et al., 2016). Moreover, a significant increase in apoptosis marker as represented by the ratio of Bax to Bcl-2 protein was observed in prostate cancer by arctigenin treatments compared to control (Wang et al., 2017). Consequently, arctigenin is thought to cause CCA apoptosis through the Bax, Bcl-2, and caspase-3 pathways. As a result, arctigenin could be used to improve CCA treatment outcomes as a natural product selection.

Conclusion

These studies showed for the first time that high N-cadherin expression was correlated with severe CCA stage and shorter patient survival times of CCA patient, indicating that N-cadherin could be used as a prognostic or targeted marker for CCA. Results also highlight the use of arctigenin, a seed extract from *Arctium lappa*, to inhibit CCA progression. Our findings revealed that arctigenin inhibited metastasis in CCA cells via reversing of N- to E-cadherin switching. In addition, arctigenin suppressed cell viability and induced apoptosis through Bax/Bcl-2/caspase-3 pathway. This study suggests that arctigenin could be used as an alternative therapeutic agent to improve CCA treatment outcomes. Taken together, our findings suggest that arctigenin could be used as a new natural compound, either alone or in combination with chemotherapy drugs, to improve CCA treatment. However, a combined analysis of arctigenin and chemotherapy is needed to confirm the efficacy in both the in vitro and preclinical settings.

Declarations

Author contributions

SJ, AJ, SK, and AT contribute to the concept and design of the research. SJ performed experiments and procedures. SJ and PhK performed data analysis. AJ, SK, PoK, WL, NN and AT provided procedures and laboratory techniques. SJ and AT prepared the initial manuscript and figures. AT provided project

leadership. All authors read and approved the manuscript, and all data were generated in-house and that no paper mill was used.

Acknowledgements

We would like to acknowledge Prof. Ross H Andrews for editing the MS via Publication Clinic KKU, Thailand.

Funding

This research was supported by the Program Management Unit for Human Resources & Institutional Development, Research and Innovation (grant number B05F630053) and grant from Khon Kaen University to A.T. (KKU63)

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

Ethics approval

All human specimens and the protocols in this study were approved by the Human Ethics Committee of Khon Kaen University, based on the ethics of human specimen experimentation of the National Research Council of Thailand (HE631304) and informed consents were obtained from each subject before surgery.

Consent for publication

Not applicable.

Conflict to interest

The authors declare no potential conflicts of interest.

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Figures

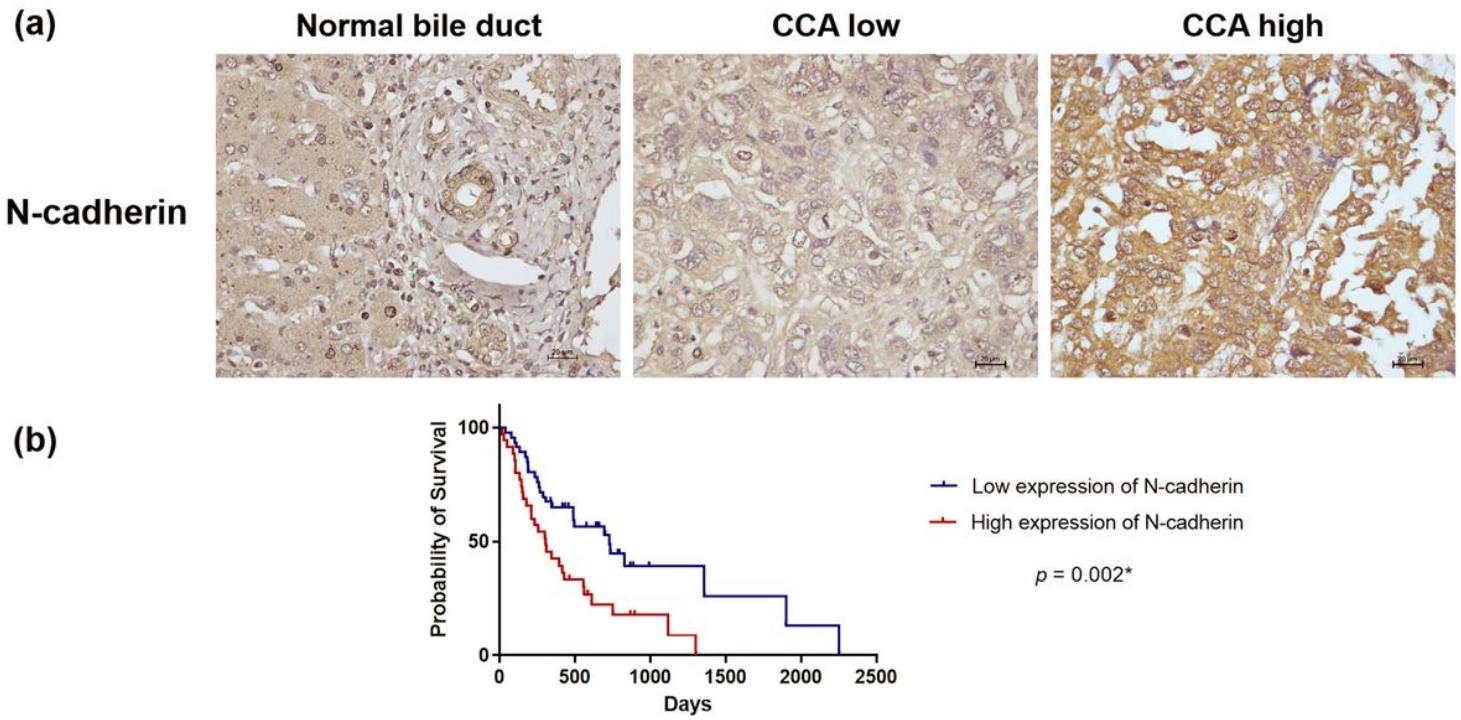

Figure 1

Immunohistochemical staining for N-cadherin protein expression in human CCA tissues. (a) expression of N-cadherin in normal bile duct, cancerous area with low and high levels. An original magnification is ×40 for all figures, (b) Kaplan-Meier analysis for overall survival in CCA is shown.

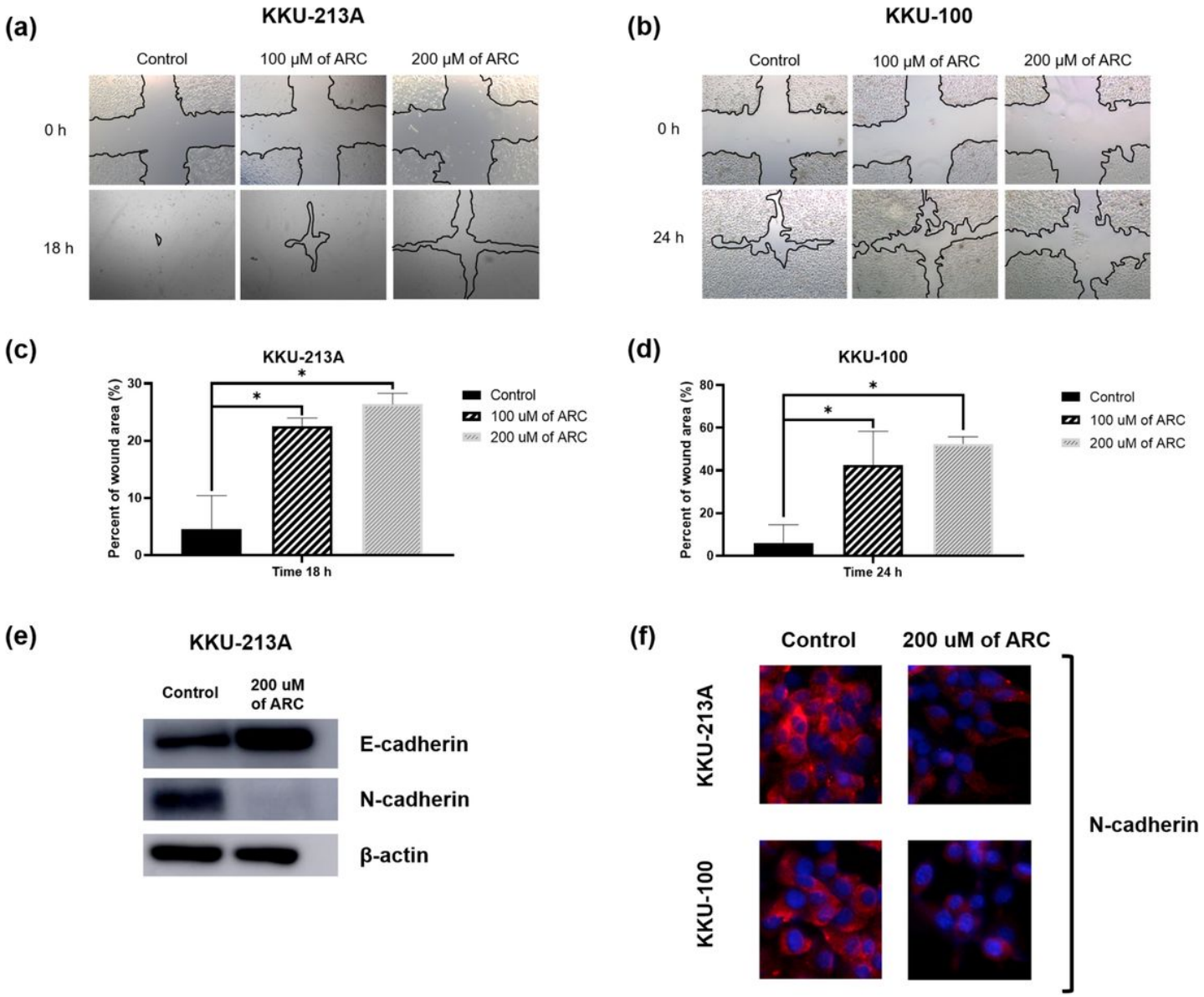

Figure 2

Arctigenin inhibited CCA cell migration via reversing of N- to E-cadherin switching. KKU-213A and KKU-100 cells were treated with arctigenin (ARC). The cells were photographed under a microscope (a) 18 h for KKU-213, and (b) 24 h for KKU-100 cells after scratching. Percent of wound area was investigated in (c) KKU-213A cell, and (d) KKU-100 cell. Western blot analysis was performed to assess the expression levels of E-cadherin, and N-cadherin after ARC treatment in (e) KKU-213A cells. Immunocytochemistry analysis was performed to assess the expression levels of N-cadherin after ARC treatment in (f) KKU-213A and KKU-100 cells. Statistical significance; *p < 0.05

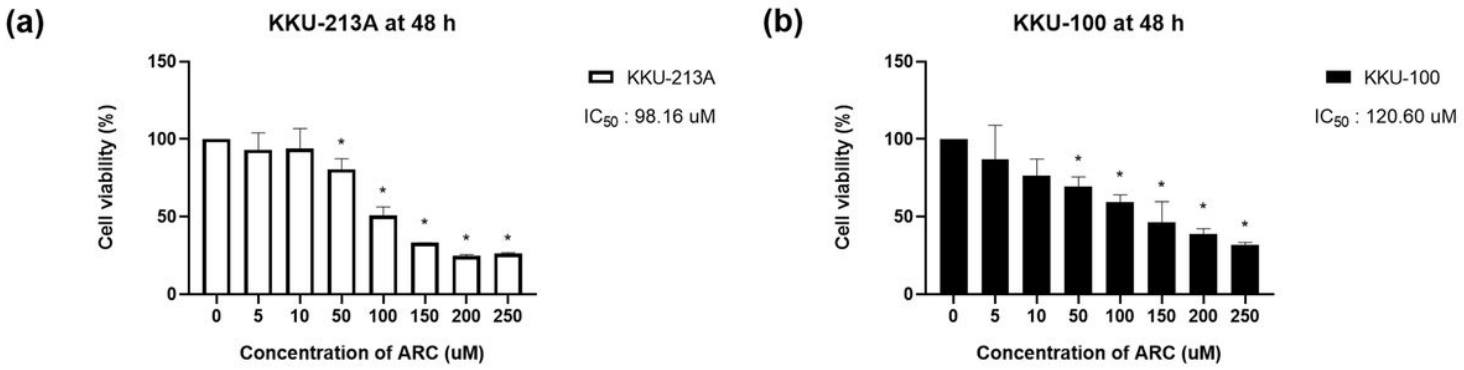

Figure 3

Arctigenin (ARC) suppressed CCA cell viability. Percentage of CCA cell viability for (a) KKU-213A, and (b) KKU-100 after treatment with ARC for 48 h. Statistical significance; *p < 0.05

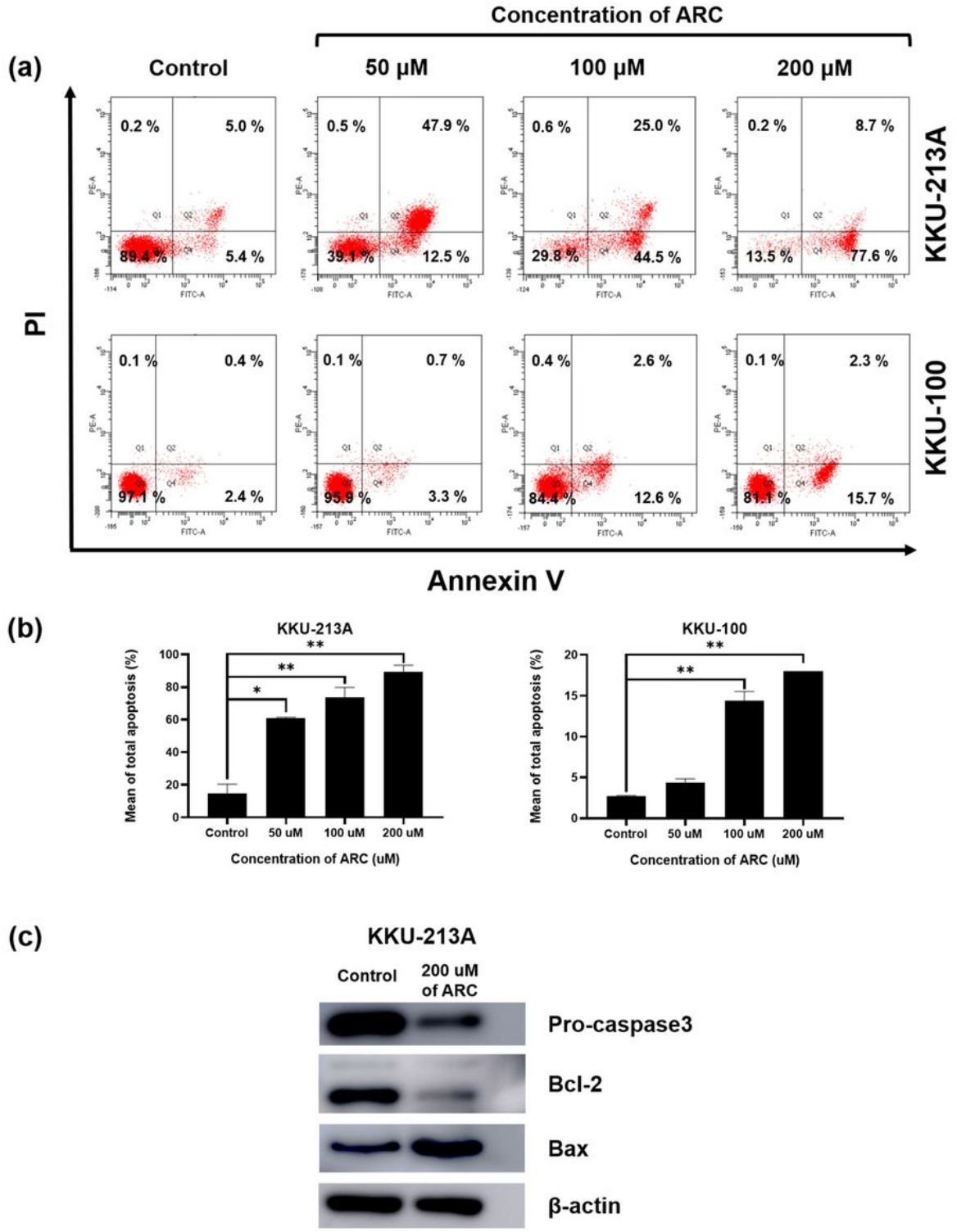

Figure 4

Arctigenin (ARC) induced CCA cell apoptosis through Bax/Bcl-2/caspase-3 pathway. Apoptosis was determined by flow cytometry analysis after ARC treatment for 48 h in (a) KKU-213A and KKU-100 cells, (b) quantitative analysis of apoptosis is shown. Western blot analysis was performed to assess the expression levels of pro-caspase3, Bcl-2 and Bax after ARC treatment in (c) KKU-213A cells. Statistical significance; *p < 0.05, **p < 0.001

Supplementary Files

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