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Chelidonine Induces Apoptosis via GADD45a-p53 Regulation in Human Pancreatic Cancer Cells

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Abstract

Chelidonium majus has been used as a traditional medicine in China and western countries for various diseases, including inflammation and cancer. However, the anti-cancer effect of chelidonine, a major compound of *C. majus* extracts, on pancreatic cancer remains poorly understood. In this study, we found that treatment with chelidonine inhibited proliferation of BxPC-3 and MIA PaCa-2 human pancreatic cancer cells. Annexin-V/propidium iodide staining assay showed that this growth inhibitory effect of chelidonine was induced through apoptosis. We found that chelidonine treatment upregulated mRNA levels and transcription factor activity in both cell lines. Increases in protein expression levels of p53, GADD45A, p21 and cleaved caspase-3 were also observed, with more distinct changes in MIA PaCa-2 cells compared to the BxPC-3 cells. These results suggest that chelidonine induces pancreatic cancer apoptosis through the p53 and GADD45A pathways. Our findings provide new insights into the use of chelidonine for the treatment of pancreatic cancer.

Keywords

chelidonine, pancreatic cancer, apoptosis, p53, GADD45a

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Introduction

Chelidonine, an isoquinoline alkaloid and a major compound of *Chelidonium majus* L., has been used in traditional Chinese medicine and western phytotherapy for several centuries.[1,2] Chelidonine is known for its broad pharmacological activities that lead to anti-inflammation, anti-viral and anti-cancer effects.[3-5] To be specific, chelidonine treatment induced apoptosis in T98G glioma cells, MCF-7 and SK-BR-3 breast adenocarcinoma, HepG2 hepatoma, HeLa cervical cancer, SW620 colon cancer, head and neck squamous cell carcinoma HNSCC, human gastric carcinoma SGC-7901, and leukemia MT-4 cells, through caspase, cell cycle checkpoints and MAP Kinase pathways.[3,6-12] In colon cancer Caco-2 and leukemia cell line CEM/ADR 5000, metabolic enzyme regulation by chelidonine reversed doxorubicin resistance.[13] Chelidonine was also reported to trigger autophagy, cellular senescence and blocking telomerase activity.[10] However, the cytotoxic effect of chelidonine and its mechanisms on pancreatic cancer have not been elucidated.

Pancreatic cancer is a lethal disease and the 14th most common cancer. The 5-year survival rate of patients with pancreatic cancer is only 9%.[14,15] Gemcitabine has been a standard treatment for pancreatic cancer for over 2 decades. Combination of gemcitabine with other chemotherapy

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regimens such as FOLFIRINOX has been recommended for advanced pancreatic cancer.[16,17] FOLIFIRINOX is composed of folinic acid (leucovorin), fluorouracil (5-FU), irinotecan (Camptosar) and oxaliplatin (Eloxatin), which induce DNA damage as well as block DNA synthesis and repair.[18,19] Two of the most widely used pancreatic cancer cell lines are BxPC-3 and MIA PaCa-2 cells, which showed differences in differentiation status. KRAS, Kirsten ras proto-oncogene, is implied in various malignancies inclucing lung adenocarcinoma, ductoral carcinoma of the pancrease and colorectal cancer.[20-22] BxPC-3 cells have intact KRAS gene and differentiate moderately. MIA PaCa-2 cells, in contrast, show poor differentiation with mutated KRAS genes, and express metastatic and invasive properties, unlike BxPC-3 cells.[23]

The Growth Arrest and DNA Damage-inducible 45 (GADD45) protein, which regulates growth arrest and apoptosis, plays an important role in cellular stress response and acts as a tumor suppressor.[24] Cell cycle regulation by GADD45 activation is coordinated with p53.[25] p53 is important in modulating apoptotic process as it integrates various stress signals into anti-proliferative responses.[26] p53 interacts with the WT1-Egr1 binding site of GADD45 promotor and engages in GADD45 activation. In addition, GADD45 plays a key role in the stabilization of mutated p53 in the process of apoptosis. It stabilizes p53 following DNA damage and affects p53 signaling pathway activation via positive feedback.[27]

In this study, we found that chelidonine triggers apoptotic cell death by inducing the GADD45a-p53 pathway. We used BxPC-3 and MIA PaCa-2 cells because they have different metastatic properties. In addition, chelidonine promoted caspase-3 cleavage by inducing cell cycle arrest with p21 and p53. Taken together, our results show that chelidonine inhibits the growth of pancreatic cancer cells via p53 and GADD45a signaling.

Materials and Methods

Reagents and Chemicals

Antibiotic-antimycotic (100×), 0.25% trypsin-Ethylenediaminetetraacetic acid (EDTA) solution and phosphate-buffered saline (PBS) were procured from Gibco™ (Waltham, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from PAN-Biotech GmbH (Aidenbach, Germany). Muse Annexin V & Dead Cell reagent was obtained from Millipore. Whole cell lysis buffer was procured from iNtRON™ Biotechnology Inc. (Seoul, Korea). Antibodies GADD45A and caspase-3 were supplied from Cell Signaling (Beverly, MA, USA), and antibodies p21, p53 and GAPDH were obtained from Santa Cruz (Dallas, TX, USA).

Cell Lines and Cell Viability Assay

The human pancreatic carcinoma cell lines, BxPC-3 and MIA PaCa-2, were purchased from the American Type Culture Collection (Rockville, MD, USA). They were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) antibiotic–antimycotic at 37°C with 5% (v/v) CO_2. Cells (5×10^3 cells/well) were seeded in 96-well plates and incubated for 24 hours, before exposure to chelidonine for 24 and 48 hours. At the end of each time point, 10 µL of Cell Counting Kit-8 solution (DojinDo, Kumamoto, Japan) was added to each well, and the plates were incubated for 90 minutes at 37°C. To determine cell viability, the absorbance at 450 nm was measured using a microplate reader (Sunrise, Tecan, Switzerland). The assay was performed in triplicates. The half-maximal inhibitory concentration (IC_{50}) was determined by evaluating the cytotoxicity of chelidonine at 24, 48 and 72 hours.

Detection of Apoptosis Using Annexin-V/ Propidium Iodide Staining

To evaluate the apoptotic effects of chelidonine on human pancreatic cancer cells, we performed Annexin-V/propidium iodide (PI) staining using the Annexin V-FITC Apoptosis Detection Kit (Sigma, USA). The cells were treated with various concentrations of chelidonine for 24 and 48 hours, detached using trypsin, and washed twice with Phosphate-Buffered Saline (PBS). The cell suspension in PBS was centrifuged at 200x*g* for 5 minutes, and the supernatant was carefully removed by pipetting. The cell pellet was suspended in 500 µL Annexin-V binding buffer, to which 0.1 µg/mL Annexin V-FITC conjugate and 2 µg/mL PI were added; the cells were incubated for 10 minutes at room temperature with protection from light. The fluorescence of the samples was detected using Guava flow cytometry system (Millipore, Massachusetts, USA) at an excitation wavelength of 488 nm with a 530/30 nm bandpass filter to detect Annexin V and 670 nm high-pass filter to detect PI.

RNA Sequencing

Total RNA was isolated using Trizol reagent (Invitrogen, USA). RNA quality was assessed using Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA). Libraries were prepared from total RNA using the NEBNext Ultra II Directional RNA-Seq Kit (New England BioLabs, Inc., UK). The Poly (A) RNA Selection Kit (Lexogen, Inc., Austria) was used to isolate mRNA. The isolated mRNAs were used for cDNA synthesis and

shearing, according to the manufacturer's instructions. Indexing was carried out with the Illumina indexes 1 to 12 and PCR was used in the enrichment step. Subsequently, the libraries were checked using the Agilent 2100 bioanalyzer (DNA High Sensitivity Kit) to evaluate the mean fragment size. The library quantification kit with a StepOne Real-Time PCR System (Life Technologies, Inc., USA) was utilized for quantification. High-throughput sequencing was performed as paired-end 100 sequencing using HiSeq X10 (Illumina, Inc., USA). For data analysis of RNA sequencing, a quality control of raw sequencing data was carried out via FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter and low-quality reads (<Q20) were removed using FASTX_Trimmer (http://hannonlab.cshl.edu/fastx_toolkit/) and BBMap (https://sourceforge.net/projects/bbmap). Then, the trimmed reads were mapped to the reference genome using TopHat.[28] Gene expression levels were estimated using fragments per kb per million reads (FPKM) values from Cufflinks.[29] The FPKM values were normalized based on the quantile normalization method through EdgeR within R (https://www.r-project.org/). Data mining and graphic visualization were performed using ExDEGA (E-biogen, Inc., Korea).

Gene Ontology-Based Network Analysis

To study the biological functions of the regulated genes through the interaction network, we used STRING database (http://string-db.org/) to examine the biological functions of differentially expressed genes and proteins according to ontology-related interaction networks, including apoptosis signaling. Network generation was optimized based on the obtained expression profiles when possible, and was aimed at producing highly connected networks.

Cloning of Vectors Including Transcription Factor Binding Sequences

All indicated transcription factor binding site-encoding sequences were amplified by PCR. The PCR products were separated and purified from agarose gel and inserted into pGreenFire™-Pathway Reporter Lentivectors containing a minimal CMV promoter, green fluorescence protein (GFP) reporter, and luciferase reporter (SBI System Biosciences, CA, USA). After digestion with restriction enzymes (EcoRI and SpeI; NEB, MA, USA), the double-stranded DNA fragments were ligated to the linearized lentivirus vector by using T4 ligase. The ligated products were transformed into *E. coli* DH5α cells (Enzynomics, Daejeon, Korea) and selected by sequencing confirmation. When a specific transcription factor binds to pGreenFire1™-mCMV-Puro vector including transcription binding sequences, GFP and luciferase are expressed.

Lentivirus Packaging

Human embryonic kidney (HEK) 293T cells were seeded in a 75 cm^2 cell culture flask with DMEM containing 10% FBS without antibiotics. The expression vector and lentivirus packaging vector mixture were transfected into HEK 293T cells with Hillymax (Dojindo, Kumamoto, Japan) and DMEM (Thermo Fisher Scientific, MA, USA). After transfection for 5 hours, the primary media with vectors were replaced with antibiotic-free DMEM containing 1.0 g/L glucose, 5% FBS. The supernatant containing lentivirus was collected at 48 hours after transfection, filtered through a 0.4 μm-pore size syringe filter, and stored at −80°C.

Transcription Factor Network Analysis

Transcription factor expression analysis was performed as described previously.[30] MIA PaCa-2 and BxPC-3 cells were seeded on a black 96-well plate and grown for 24 hours. The cells were transduced using the lentiviral supernatant for 24 hours. After media change, the transduced cells were selected with puromycin for 7 days. The cells were then treated with chelidonine (0.5 μM) for 2 days. Fluorescence was evaluated every 12 hours using a multiple plate reader (TriStar² S LB 942, Berthold Technologies, Bad Wildbad, Germany), excited at 480 nm and detected at 510 nm. Light emission values of treated cells were normalized to that of non-treated cells and the transcription factor activity was evaluated. The regulatory networks of transcription factor activation were schematized using BTNET (http://ibtnet.korea.ac.kr/), as described previously.[31]

Immunoblotting

To prepare total cell lysates, the cells were homogenized in 2 mL of Tris-HCl (20 mM) containing protease inhibitor cocktail (Roche, Basel, Switzerland), placed on ice for 30 minutes, and then centrifuged (10 minutes, 10,000x*g*, 4°C). The protein content in the cell lysates was quantified by the bicinchoninic acid method. Denatured proteins (30 μg) were separated using 12% SDS-polyacrylamide gel electrophoresis and transferred onto a 0.2-μm polyvinylidene fluoride membrane in transfer buffer for 3 hours. The membrane was blocked for 1 hours using 5% (w/v) skimmed milk in Tris-buffered saline with Tween-20 (TBST) and incubated with the following antibodies: p21, p53, GADD45A and GAPDH [all purchased from Santa Cruz (Texas, USA)] and Caspase-3 (Cell signaling, USA). After washing thrice (10 minutes each) with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse or rabbit anti-goat IgG (1:2000 dilution) in TBST containing 5% (w/v) skimmed milk at room temperature for 1 hours. The membrane was rinsed thrice (10 minutes each) with 0.1% (v/v) TBST. An enhanced chemiluminescence

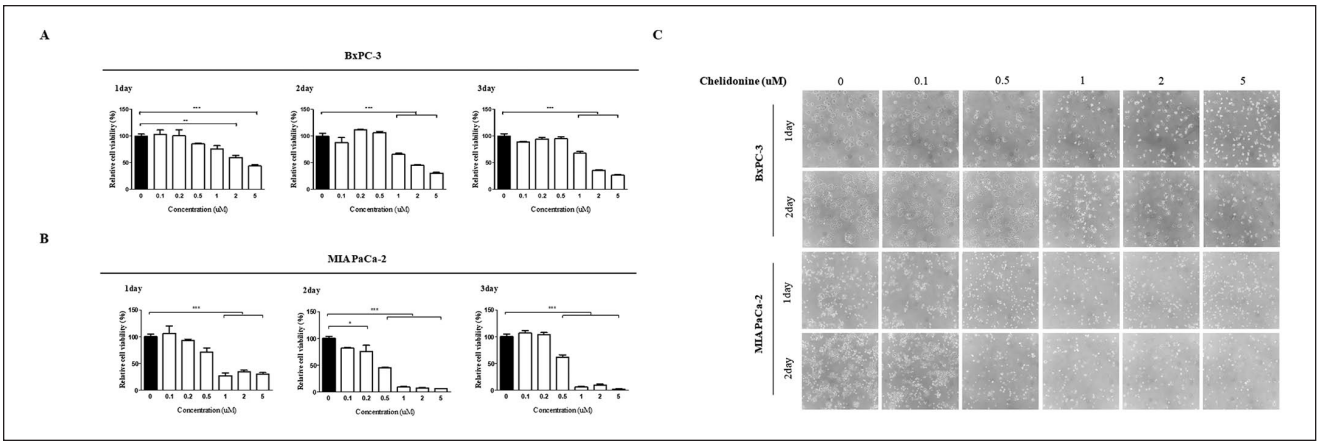


Figure 1. Chelidonine inhibits proliferation in BxPC-3 and MIA PaCa-2 pancreatic cancer cells. Inhibition of the growth of pancreatic cancer cells by chelidonine. (A) BxPC-3 cells and (B) MIA PaCa-2 cells were exposed to 0, 0.1, 0.2, 0.5, 1, 2 and 5 μM chelidonine for 24, 48 and 72 hours. (C) Morphological changes in BxPC-3 and MIA PaCa-2 cells treated with chelidonine for 24 and 48 hours observed under a microscope (40× magnification).

system (Thermo Scientific, Massachusetts, USA) was used to visualize the bands through a ChemiDoc MP system (Bio-Rad, Hercules, CA, USA). Densitometry of the bands was measured using ImageJ software (National Institutes of Health, Maryland, USA). Protein levels were quantitatively analyzed and normalized against GAPDH.

RNA Interference

p53 and GADD45a small interfering RNAs (siRNAs) were purchased from Santa Cruz (USA). After seeding, cells were transfected with sc-29435 (p53 siRNA) and sc-35440 (GADD45a siRNA) using Hillymax (Japan) according to manufacturer's instructions. After 48 hours later for siRNA incubation, the cells were treated with vehicle- or chelidonine for 24 hours and then harvested for immunoblotting analysis.

Statistical Analyses

GraphPad Prism (GraphPad, San Diego, CA, USA) was used to conduct all statistical analyses. All measurements were performed in triplicates, and all values were expressed as means ± standard error of mean (SEM). The results were subjected to an analysis of variance (ANOVA) by Tukey's test to assess the statistical significance of differences between groups. In this study, a $P < .05$ was considered statistically significant.

Results

Chelidonine Inhibits Pancreatic Cancer Cell Growth

To investigate the effects of chelidonine on human pancreatic cancer cell proliferation, BxPC-3 and MIA PaCa-2 cells were treated directly with 0, 0.1, 0.2, 0.5, 1, 2 and 5 μM chelidonine for 24, 48 and 72 hours. As shown in Figures 1A and 1B, chelidonine treatment inhibited cell growth in a dose-dependent manner in both BxPC-3 and MIA PaCa-2 cells. Treatment with 1 μM chelidonine for 24 hours inhibited cancer cell growth, and this effect was more evident in MIA PaCa-2 populations than in BxPC-3 populations. Incubation with chelidonine (1 μM) for 48 hours blocked proliferation in BxPC-3 cells by 50%, whereas 90% of growth inhibition was observed in MIA PaCa-2.

Next, to observe cell death in chelidonine-treated cancer cells, the morphology of pancreatic cancer cells and untreated cells was compared under a light microscope. The morphology of BxPC-3 and MIA PaCa-2 cells changed drastically after treatment with 0.5 and 1 μM chelidonine for 24 hours (Figure 1C). Multiple cells appeared to be shrunken and exhibited a reduced cell volume. The cells began to detach from the surface of the culture plate and appeared buoyant. Such morphological changes from attached cells to round-shaped cells preceded apoptosis. Thus, subsequent analysis was performed with cells treated for 24 hours with 0.5 and 1 μM chelidonine.

Chelidonine Induces Apoptosis in Pancreatic Cancer Cells

The apoptotic effect of chelidonine on BxPC-3 and MIA PaCa-2 pancreatic cancer cells was analyzed using Annexin-V/PI staining and flow cytometry after treatment with 0, 0.5, 1 and 2 μM chelidonine for 24 and 48 hours. The relative proportion of cells undergoing apoptotic process was determined by cells in the early stage of apoptosis (Annexin-V-stained, non-disrupted cells) and cells entering the late stage of apoptosis (disrupted or lysed cells). When cells were treated with 0.5 μM chelidonine for 24 hours, the

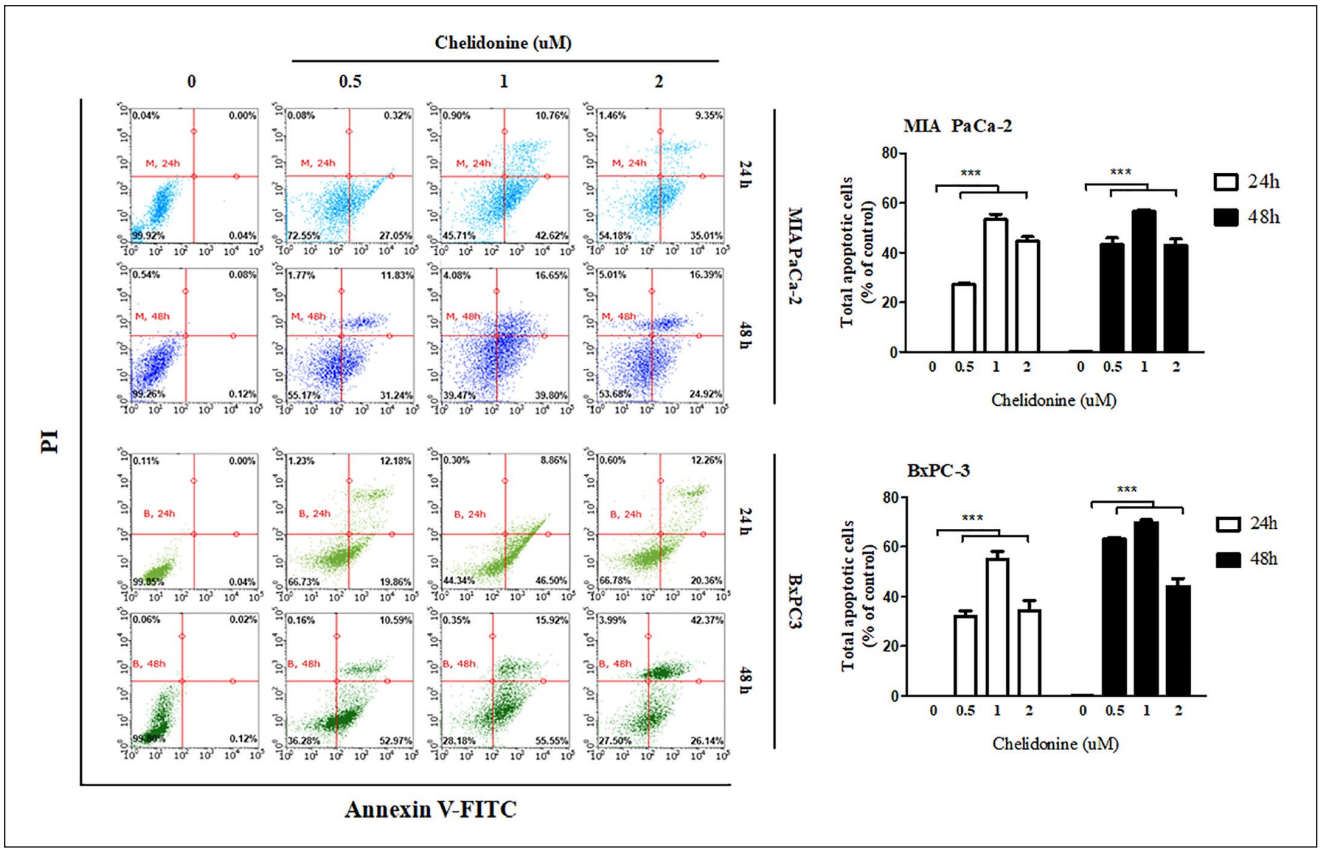


Figure 2. Apoptosis in BxPC-3 and MIA PaCa-2 pancreatic cancer cells exposed to chelidonine. The cells were detected using a Guava flow cytometry system (Millipore) after staining with the Annexin V-FITC Apoptosis Detection Kit. The statistics shown are cell percentages represented mainly by early and late apoptosis which is apparent when the percentage of live cells markedly decreases.

Data represent the mean ± standard error of mean (SEM) of 3 independent experiments.

***$P < .001$ versus vehicle-treated cells.

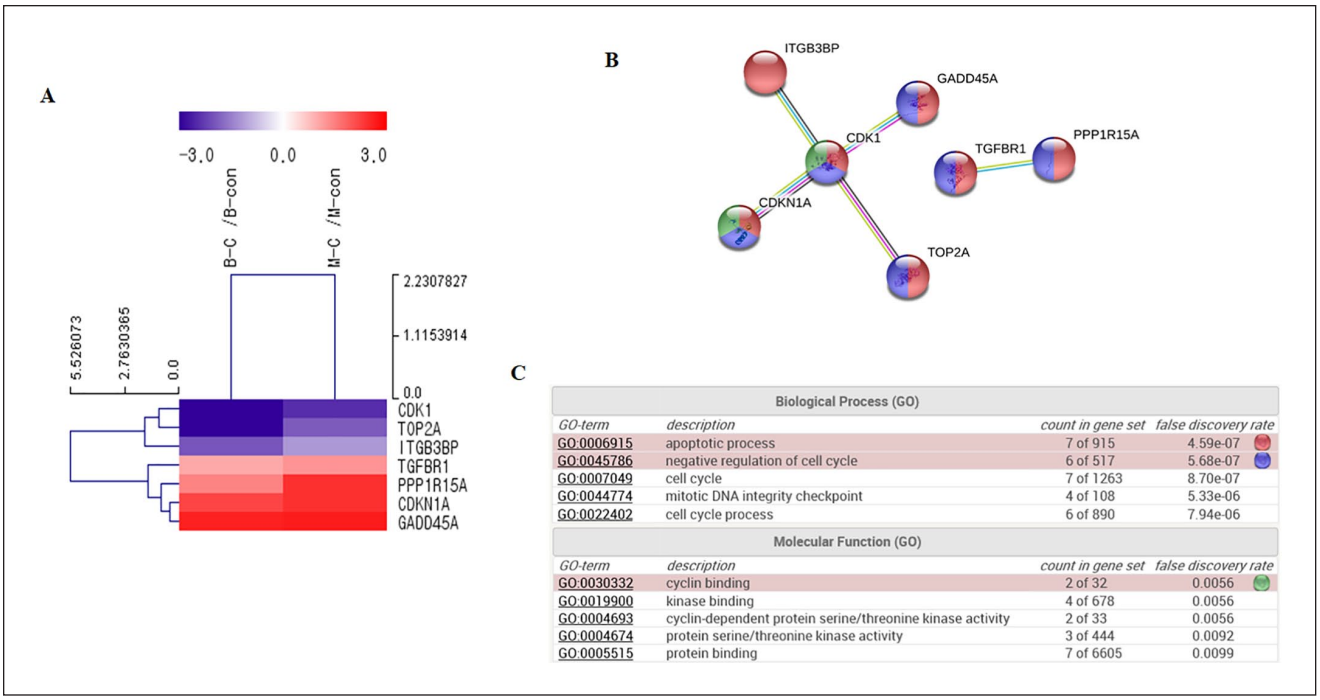
fraction of viable cells stained with Annexin-V changed (99% to 72.5% in MIA PaCa-2 and 99% to 66.7% in BxPC-3) (Figure 2). Moreover, the cells treated with 5 μM chelidonine for 48 hours markedly shifted from the normal state to the apoptotic stage (0.1% to 41% in MIA PaCa-2 cells and 0.1% to 68% in BxPC-3 cells), whereas the viable fraction reduced from 99% to 53% in MIA PaCa-2 cells and 27.5% in BxPC-3 cells. Thus, treatment with 5 μM chelidonine for 24 hours induced apoptosis in MIA PaCa-2 and BxPC-3 cells.

Chelidonine Changes the Gene Expression of Human Pancreatic Cancer Cells

To determine the genes potentially involved in the anti-cancer activity of chelidonine, we conducted RNA-sequencing of BxPC-3 and MIA PaCa-2 cancer cells treated with 0.7 μM chelidonine for 24 hours. Out of the 24,424 unique genes tested, 885 genes were expressed in the treated cells, among which 398 were upregulated and 487 were downregulated.

Based on gene expression analysis, we categorized genes that play a crucial role during apoptosis. Using an Excel-based differentially expressed gene analysis (ExDEGA) program, we put together the biological functional features. With the Multiple Experiment Viewer (MeV) tool and hierarchical cluster analyses, the apoptosis-related genes up- and downregulated by 2-fold in chelidonine treated BxPC-3 and MIA PaCa-2 cells were analyzed. The heat maps and hierarchical cluster analysis showed 7 genes affected by chelidonine treatment, among which 4 genes were upregulated and 3 genes were downregulated. As shown in Figure 3, chelidonine triggered apoptosis through cyclin-dependent kinase inhibitor 1A (CDKN1A), which encodes the p21 protein. An increase in GADD45a expression was also found in both BxPC-3 and MIA PaCa-2 cells.

From these results, we analyzed protein-protein interactions and gene ontology using the STRING database. Here, we found correlations between the 7 genes, including the interaction between CDKN1A and GADD45a. Chelidonine treatment modulated the regulation of the apoptotic process



Biological Process (GO)			
GO-term	*description*	*count in gene set*	*false discovery rate*
GO:0006915	apoptotic process	7 of 915	4.59e-07
GO:0045786	negative regulation of cell cycle	6 of 517	5.68e-07
GO:0007049	cell cycle	7 of 1263	8.70e-07
GO:0044774	mitotic DNA integrity checkpoint	4 of 108	5.33e-06
GO:0022402	cell cycle process	6 of 890	7.94e-06

Molecular Function (GO)			
GO-term	*description*	*count in gene set*	*false discovery rate*
GO:0030332	cyclin binding	2 of 32	0.0056
GO:0019900	kinase binding	4 of 678	0.0056
GO:0004693	cyclin-dependent protein serine/threonine kinase activity	2 of 33	0.0056
GO:0004674	protein serine/threonine kinase activity	3 of 444	0.0092
GO:0005515	protein binding	7 of 6605	0.0099

Figure 3. RNA sequencing analysis to identify alteration in gene expression and signal network by chelidonine. (A) Hierarchical gene clustering was generated with the TM4 Microarray Software Suite (MeV) from chelidonine-treated BxPC-3 and MIA PaCa-2 pancreatic cancer cells. The red and blue colors represent genes that were up- and down-regulated, respectively, by more than 2.5-fold. The ratios of gene profiles are presented as a heat map (left panel) and gene expression pattern (right panel). (B) Combined screenshots from the STRING website, showing results obtained upon entering a set of 7 proteins suspected to be involved in the apoptotic process (Gene Ontology (GO) analysis) and cell cycle pathway (KEGG pathway). The insets show the accessory information available for a single protein, a reported enrichment of functional connections among the set of proteins, and statistical enrichments detected in functional subsystems. (C) GO and KEGG pathway analysis on protein–protein interactions. Enriched functions were selected, and the corresponding protein nodes in the network were automatically highlighted.

through the cyclin binding mechanism (GO: 0006915; false discovery rate $P=4.59e{-}07$: Cyclin-dependent kinase 1(CDK1), CDKN1A, GADD45A, Integrin subunit beta 3 binding protein (ITGB3BP), Protein phosphatase 1 regulatory subunit 15A (PPP1R15A), Transforming growth factor beta receptor 1 (TGFBR1), and DNA Topoisomerase II Alpha (TOP2A)).

Chelidonine Treatment Upregulates p53 Transcription Factor Activity

To investigate transcriptional factor regulation by chelidonine in pancreatic cancer cells, we performed transcription network analysis. Transcriptional factor activation can be detected through the lentivirus-included transcription factor binding sequence. As shown in Figure 4, chelidonine treatment upregulated p53 transcriptional activity in both BxPC-3 and MIA PaCa-2 cells during 48 hours. In BxPC-3 cells, the activity of Yin Yang1 (YY1), hepatocyte nuclear factor 1 homeobox A (Hnf1a) and Serum response factor (SRE) was decreased, whereas the transcription factors of BCL11B, Hypoxia-inducible factor 1 (HIF1), Nuclear factor of activated T-cell (NFAT) and ETS translocation variant 4 (ETV4) were activated (Figure 4A). However, in MIA PaCa-2 cells, transcription factors BCL11B and NFAT were downregulated by chelidonine treatment, whereas p53 activity was increased (Figure 4B). Chelidonine treatment induced the activity of NFAT and BCL11B in BxPC-3 cells, but not in MIA PaCa-2 cells. The transcription network analysis results indicated that chelidonine treatment triggered p53 transcriptional factor activation in BxPC-3 cells as well as in MIA PaCa-2 cells.

Chelidonine Treatment Increases Caspase-3 Cleavage with p21, p53, and GADD45a Up-Regulation

To identify the intercellular mechanism of chelidonine in the apoptosis-inducing effect on BxPC-3 and MIA PaCa-2 cells, we assessed the changes in p21, p53 and GADD45A protein expression using western blotting after treatment with 0, 0.5 and 1 μM chelidonine for 24 hours (Figure 5). The cell cycle arrest-related proteins p21 and p53 were increased by chelidonine treatment in both pancreatic cancer cell lines. In

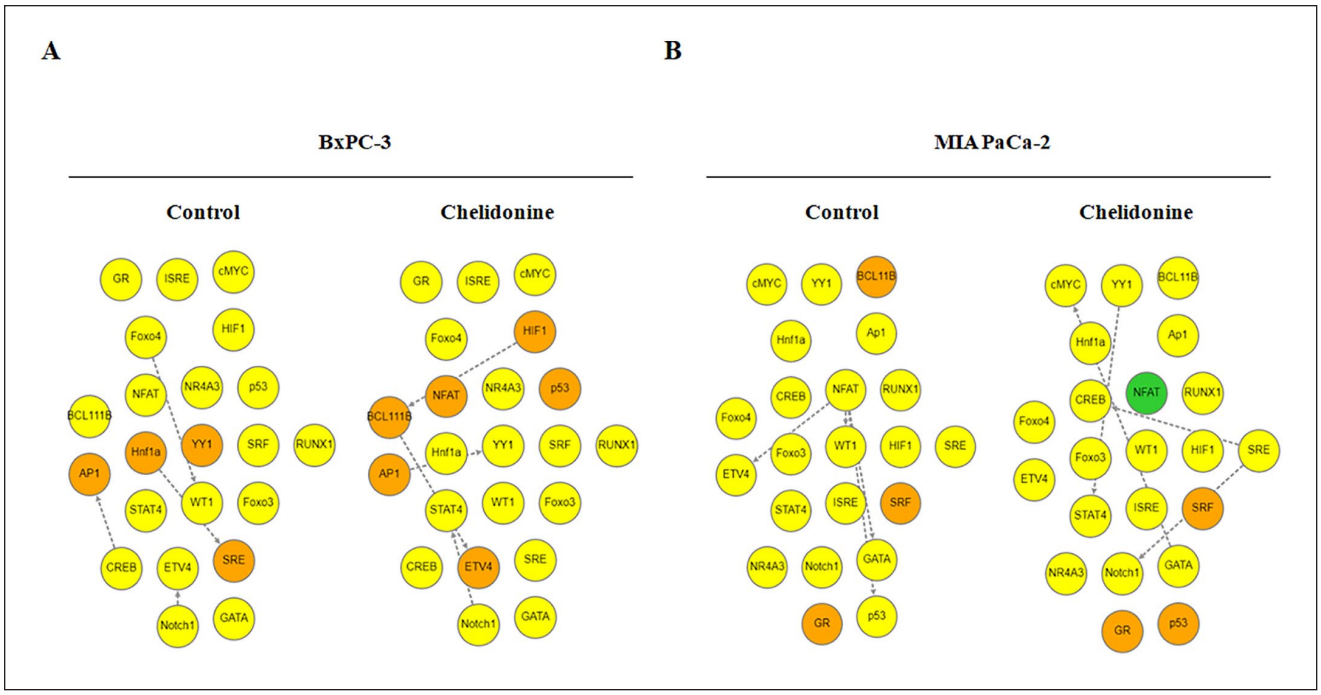


Figure 4. Time-dependent changes in the transcriptional regulatory network in chelidonine-treated pancreatic cancer cells. After chelidonine treatment, (A) BxPC-3 cells and (B) MIA PaCa-2 cells were seeded in a black 96-well plate and the expressions of several transcription factors were investigated by measuring the fluorescence intensity every 12 hours for 2 days in cells transduced with lentiviral vector containing transcription factor binding sites. The transcriptional regulatory network was inferred using BTNET. The color of each circle in A indicates the change in expression level of each transcription factor (red for up-regulation and green for down-regulation) and the arrow between circles indicates edge information (dotted line for inference and solid line for prior knowledge).

particular, 0.5 μM chelidonine treatment induced a more rapid increase (over 2-fold) of p21 and p53 expression in MIA PaCa-2 cells than in BxPC-3 cells. We also analyzed the expression levels of GADD45a protein, which enhances p53 stabilization. Chelidonine upregulated GADD45A expression in BxPC-3 and MIA PaCa-2 cell lines by 5- and 2-fold, respectively. In addition, the expression levels of caspase-3 cleavage significantly increased in a dose-dependent manner in both cell lines, by 10-fold in BxPC-3 and 60-fold in MIA PaCa-2.

Finally, to confirm the p53 and GADD45a mediated apoptotic effect of chelidonine in BxPC-3 and MIA PaCa-2 cells, we inhibited p53 and GADD45a by siRNA transfection before treatment with 0 and 1 μM chelidonine for 48 h (Figure 6). Chelidonine treatment induced cleaved caspase-3 through p53 and GADD45a pathway. However, blocking p53 or GADD45a markedly reduced cleavage of caspase-3 in BxPC-3 and MIA PaCa-2 cells.

Discussion

Defective apoptotic pathway is one of the main causes allowing neoplastic cells to grow abnormally.[32] Thus, induction of apoptosis is important to control cancer cell proliferation for cancer treatment.[33] Especially, as only 15% to 20% of pancreatic cancer patients have resectable disease, apoptosis inducing reagent is the most succeceful non-surgical treatment.[32,33] Therefore, we evaluated the intercellular mechanism of chelidonine-induced apoptosis in BxPC-3 cells and MIA PaCa-2 cells.

In this study, we found that chelidonine inhibited the proliferation of BxPC-3 and MIA PaCa-2 human pancreatic cancer cells in a dose- and time-dependent manner, confirming its apoptotic potential. In addition, flow cytometry analysis revealed that over 50% of BxPC-3 and MIA PaCa-2 cells exhibit early- and late-phase apoptosis after exposure to chelidonine (1 μM) for 24 h (Figure 2). Moreover, through RNA-sequencing analysis, changes over 3-fold in the expression levels of CDK and GADD45A genes were observed in both BxPC-3 and MIA PaCa-2 cells (Figure 3). These changes in expression levels following chelidonine treatment were re-confirmed through the analysis of transcription factor activity in both pancreatic cancer cell lines. During the first 48 h, chelidonine treatment increased the activation of p53 transcription factor in both BxPC-3 and MIA PaCa-2 cells (Figure 4). In addition, the mechanisms of action of chelidonine were determined by western blotting. The chelidonine treatment induced p21, p53 and GADD45a

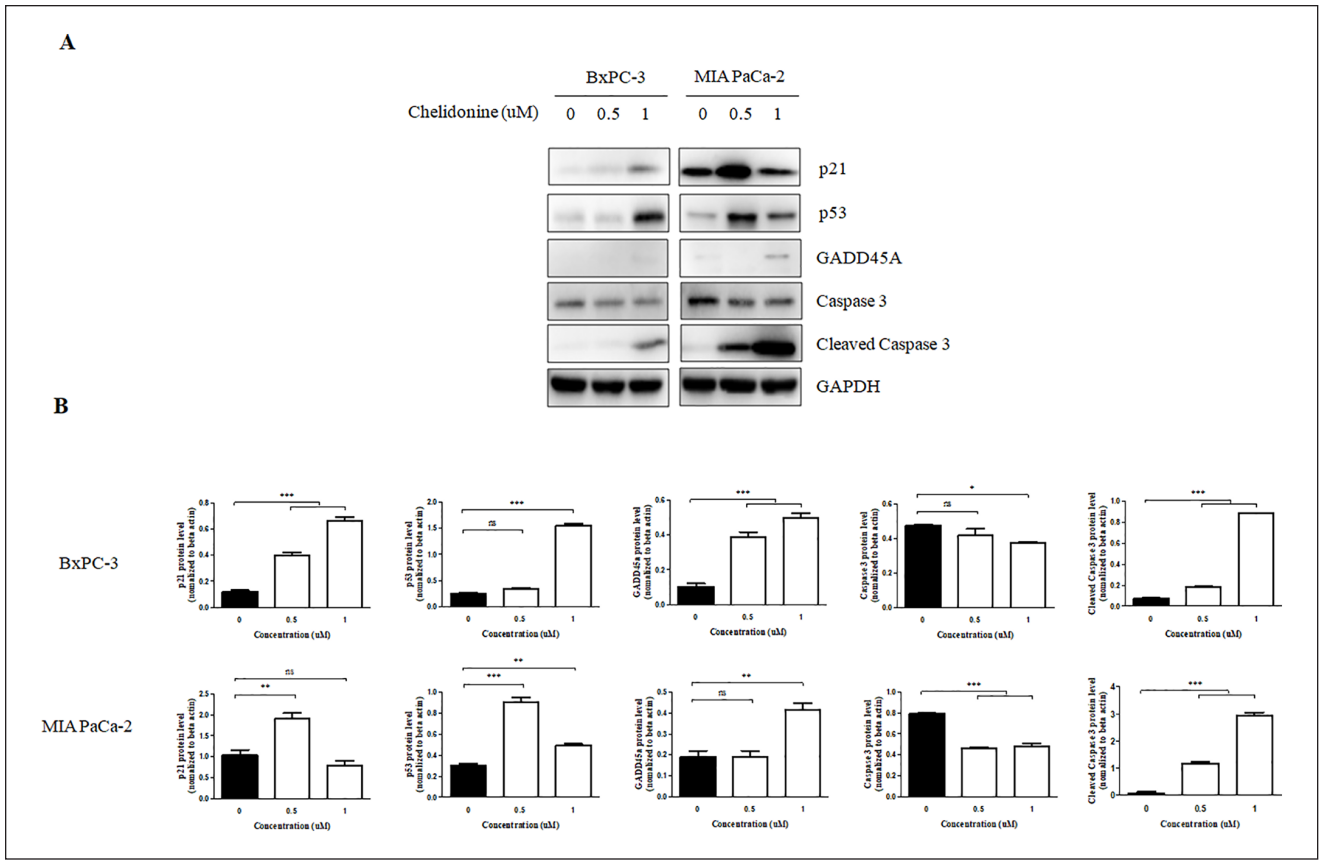


Figure 5. Chelidonine induces caspase-3 cleavage with p53 pathway activation by upregulating GADD45a and p21 signaling in pancreatic cancer cells. (A) Treatment of chelidonine (0, 0.5, and 1 μM) for 24 hours increases p21, p53, GADD45A, and cleaved caspase-3 protein expression in BxPC-3 and MIA PaCa-2 cells. (B) The band density was quantified and normalized to β-actin. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ versus non-treated cells.

expression, as well as caspase-3 cleavage (Figure 5). Finally, anti-cancer effect of chelidonine mediated by p53 and GADD45a was re-confirmed by loss of function study using siRNA against p53 and GADD45a (Figure 6). As p53 and GADD45a play a role in cell cycle arrest and cell death, downregulation of those genes triggered cells to have anti-apoptotic and chemoresistance-related characteristics.[34] The reduced apoptosis vulnerability by p53 and GADD45a blocking may decrease caspase-3 expression.

CDKN1A encodes the p21 protein.[6,8] The p21 protein inhibits cyclin-dependent kinase 2 (CDK2) and proliferating cell nuclear antigen (PCNA), and is implicated in p53-dependent G1/S cell cycle arrest.[6] Subsequently, CDK1 regulates G2/M arrest.[35,36] In the present study, chelidonine treatment resulted in increased CDKN1A and GADD45A gene expression levels, while CDK1 gene levels were decreased (Figure 3A and 3B).

The regulation of caspase-3 cleavage is dependent on p21-mediated cell cycle arrest and apoptosis downregulation.[37,38] In contrast to p21, p53 triggers apoptosis through caspase-3 cleavage by mitochondrial cytochrome c release, which is regulated by the Bax and Bcl-2 pathways.[39,40] As shown in Figure 4, chelidonine treatment upregulated p53 in both BxPC-3 and MIA PaCa-2 cells. However, B-cell lymphoma 11B (BCL11B) and nuclear factor of activated T cells (NFAT) were upregulated in BxPC-3 cells, while decreased in MIA PaCa-2. A previous study demonstrated that BCL11B, a member of the BCL family, acts as a tumor suppressor through the inhibition of mouse double minute 2 homolog (MDM2) expression in a p53-dependent manner.[41,42] In addition, in patients who have lymphoblastic leukemia, the BCL11B mutation was detected with mutated KRAS, NRAS, phosphatase and tensin homolog (PTEN), and Notch homolog 1 (NOTCH1).[41] However, Grabarczyk *et al*[43] reported that BCL11B inhibition leads to malignant T cell apoptosis. NFAT is involved in immune response, calcium signaling, cell proliferation, survival, migration and invasion.[44,45] Niitsu *et al*[44,46] demonstrated that KRAS mutation induced tumor proliferation through the downregulation of the calcineurin-NFAT signaling pathway. Transcription factor NFAT upregulates the MDM2 oncogene, which regulates p53 expression, and promotes pancreatic cancer by inflammation, induced via the NFAT-Signal Transducer and Transcription3 (STAT3) pathway.[47,48] Although the roles of BCL11B and NFAT in

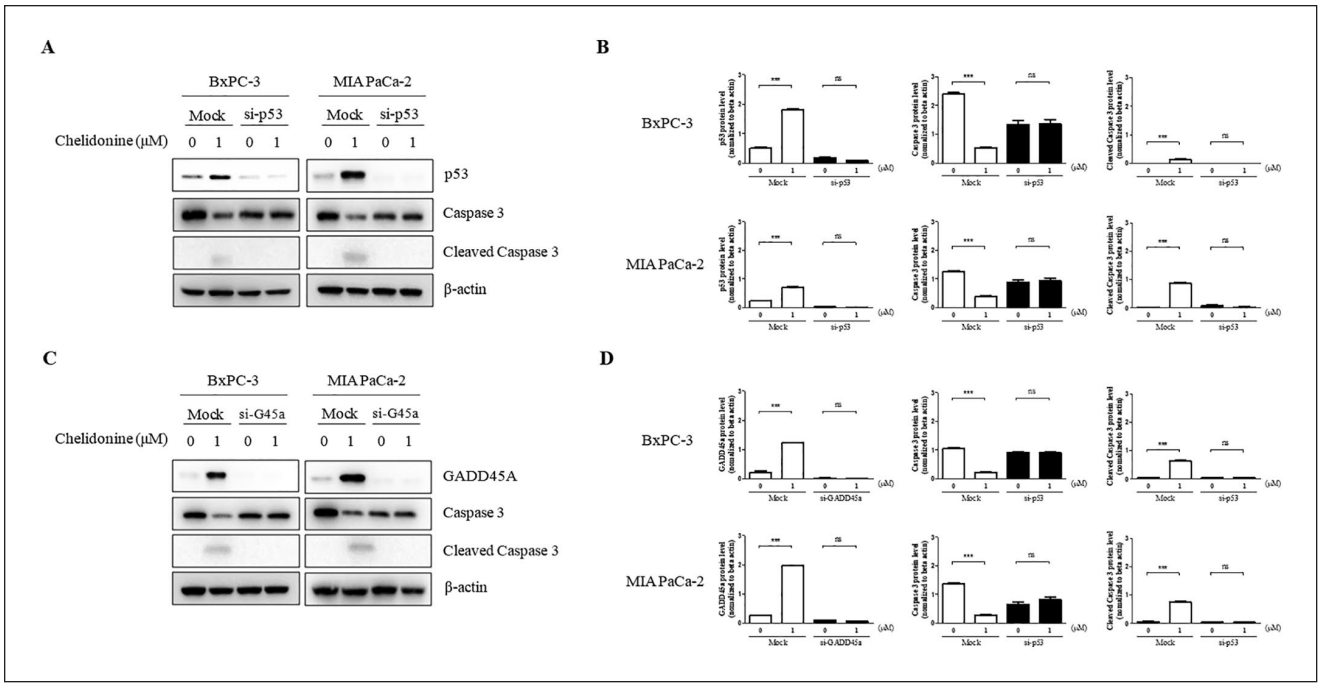


Figure 6. Inhibition of p53 and GADD45a blocked caspase-3 cleavage by chelidonine treatment in pancreatic cancer cells. After gene expression inhibition with (A) si-p53 or (B) si-GADD45a, the protein expressional changes were analyzed by western blotting on chelidonine (0 and 1 μM) treated BxPC-3 and MIA PaCa-2 cells for 48 hours. (C), (D) The band density was quantified and normalized to β-actin.
***$P < .001$ versus non-treated cells.

pancreatic cancer remain to be explored, it can be deduced that the KRAS gene mutation in MIA PaCa-2 cells may affect the activity of both transcription factors, in contrast to BxPC-3 cells which do not display KRAS gene mutation.

In human pancreatic cancers, mutations of Ras genes such as HRAS, KRAS and NRAS are frequently confirmed.[49] Furthermore, these KRAS mutations induce p53 mutation and suppress p53 expression for cancer maintenance.[20,50] In the case of pancreatic cancer cells, MIA PaCa-2 has KRAS mutation, which induces apoptosis through the GADD45a and p38 MAPK pathway. In contrast, BxPC-3 cells carry wild type Ras which suppresses GADD45a expression. Moreover, Qian *et al*,[51-53] investigated that chelidonine inhibited NRAS signaling through STK19 kinase inhibition. KRAS mutation and the NRAS-inhibiting effect of chelidonine in addition to KRAS mutation, may explain the higher expression levels of GADD45a and p53 in MIA PaCa-2 compared to those in BxPC-3. Subsequently, this difference may have led to more sensitive cell death of MIA PaCa-2 than BxPC-3. In a previous study, chelidonine also induced anti-proliferative effect in HeLa cells via p38-p53 and PI3K/AKT signaling pathway.[12] However, HeLa cells exhibit wild type of RAS and p53 gene, while both MIA PaCa-2 and BxPC-3 genome include p53 mutation, but KRAS is mutated in MIA PaCa-2 cells additionally.[54] These investigations may suggest the applicability of chelidonine to RAS and p53 mutated cancer cell lines.

In the present study, chelidonine treatment increased p53 expression (Figures 4 and 5). The role of p53 regulation in inhibiting cell growth is especially important in pancreatic cancer cells, since Protein tyrosine phosphatase N (PTPN)-p53 is related to the Hippo pathway, which regulates tissue regeneration and organ size through Yap in pancreatic cancer.

In cancer, GADD45 acts as a central player in tumorigenesis, due to its functions in DNA repair, senescence, and cell cycle control.[27] In response to DNA damage, GADD45a regulates p53 by stabilization during post-transcriptional modification.[55] As shown in Figures 3 and 5, treatment of BxPC-3 and MIA PaCa-2 cells with chelidonine upregulated both mRNA and protein levels of GADD45a, 3-fold and 2-fold, respectively. Moreover, with GADD45a increase, the p53 protein levels were also upregulated over 3-fold in chelidonine-treated BxPC-3 and MIA PaCa-2 cells (Figure 5).

These results demonstrate that chelidonine induced G2/M cell cycle arrest by downregulating CDK1, S cell cycle arrest by upregulating p21 and p53 with GADD45a increase, and finally triggered apoptotic cell death with caspase-3 cleavage in BxPC-3 and MIA PaCa-2 cells. Our findings describe the intercellular molecular mechanisms of apoptosis by chelidonine, which may provide a theoretical basis for the application of chelidonine derivatives in pancreatic cancer treatment.

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Authors' Contributions

Jang IS designed the research; Jang HJ and Hong EM performed the research; Yang JH, Jo E, Lee S, Lee S, Choi JS, Yoo HS, and Kang H contributed new reagents/analytical tools and analyzed the data; Jang HJ wrote the paper, and Choi JS, Yoo HS and Kang H revised the paper; all authors have read and approved the final version of this paper.

Declaration of Conflicting Interests

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