

Dual Effects of Silibinin on Human Pancreatic Cancer Cells

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Abstract

Background Silibinin is a flavonoid with antihepatotoxic properties, and exhibits pleiotropic anticancer effects. However, the molecular mechanisms responsible for its anticancer actions in pancreatic cancer cells, and the effects on such cells and normal pancreatic cells, remain unclear. The objective of this study was to determine the effect of silibinin on human pancreatic cancer cells and normal ductal cells.

Methods Human pancreatic cancer cells (MIA PaCa-2 and PANC-1) and normal ductal cells (hTERT-HPNE) were cultured with 0–400 μM silibinin for 48 h. Thereafter, the proliferation, invasion, apoptosis, and signaling pathways of the pancreatic cells were evaluated.

Results Silibinin significantly inhibited the proliferation, invasion, and spheroid formation of human pancreatic cancer cells *in vitro* in a dose-dependent manner ($p < 0.05$). It also induced apoptosis in a dose-dependent manner. Western blot analysis showed that silibinin downregulated extracellular signaling-regulated kinase (ERK) and serine/threonine protein kinase (AKT) in human pancreatic cancer cells. It also upregulated microtubule associated protein 1 light chain 3 β (LC3B) and cleaved caspase-3 *via* c-Jun N-terminal kinases (JNK) signaling. On the other hand, silibinin increased the mRNA and protein levels of c-Jun, Twist-related protein 1, and Snail. It also decreased exogenous *p53* levels, but increased endogenous c-Jun protein levels in human pancreatic cancer cells. However, silibinin did not affect cell viability and endogenous c-Jun levels in pancreatic normal ductal cells. It increased exogenous *p53* levels, but decreased stemness-related gene expression in pancreatic normal ductal cells. Silibinin increased Ki-67 levels in pancreatic cancer cells, but decreased them in pancreatic normal ductal cells.

Conclusion Silibinin not only exerted anticancer effects by inhibiting AKT–ERK and JNK signaling, but also upregulated cancer stemness–related genes in human pancreatic cancer cells. These results suggest that silibinin should be used as a therapeutic agent for human pancreatic cancer with caution.

Introduction

Pancreatic cancer is a highly malignant disease that is characterized by locally advanced, unresectable disease, or metastasis at the time of diagnosis. Despite significant advances in surgery and chemotherapy, the five-year survival rate of patients with pancreatic cancer has not significantly improved beyond $<5\%$ [1–3]. In particular, no curative treatment options exist for patients with advanced cancer. Resistance of pancreatic cancer cells to chemotherapeutic agents is a major problem in oncology [4]. Therefore, new treatment options that can overcome the chemotherapy resistance of pancreatic cancer cells are urgently needed.

Silybum marianum or milk thistle, from which silibinin is extracted, is an annual or biannual plant of the Asteraceae family. Originally native to Southern Europe and Asia, the plant is now found throughout the world. The medicinal part of the plant is its ripe seed [5, 6]. It has been used to treat liver diseases (cirrhosis, jaundice, and hepatitis) and gallbladder disease. It has been claimed to protect the liver against poisons [7]. Silibinin is a hepatoprotective antioxidant that can stabilize and protect the membrane lipids

of hepatocytes [8]. It can inhibit peroxidase and lipoxygenase [9, 10]. Previous studies have shown that silibinin exerts anticancer effects on colon cancer, liver cancer, and breast cancer [11–14]. The role of silibinin in pancreatic cancer cells is as follows. Firstly, silibinin induces apoptosis of pancreatic cancer cells through a multi-signal pathway. Silibinin induces apoptosis by activating c-Jun N-terminal kinases /Stress-activated protein kinases (JNK/SAPK) signaling in SW1990, a human pancreatic cancer cell line [15, 16]. Furthermore, silibinin induces apoptosis by downregulating the glucagon-like peptide-1 receptor and protein kinase A (GLP-1R/PKA) signal pathway in cells of INS-1, a rat insulinoma cell line, with amylin [17]. Secondly, silibinin reduces the tumor volume and inhibits weight loss in nude mice with BxPC-3 and PANC-1(kind of pancreatic ductal adenocarcinoma cell lines) tumor xenografts [18].

The molecular mechanisms involved in the anticancer action of silibinin are complex. Moreover, the targets or molecular mechanisms in human pancreatic cancer cells are the least studied compared with those in other cancers.

Cancer stem cells (CSCs) are cells that possess the ability to self-renew and differentiate into various types of mature cells. Such cells are rare in cancer. However, they play an important role in cancer homeostasis, metastasis, resistance to therapy, and subsequent tumor recurrence [19, 20]. Pancreatic CSCs are associated with poor prognosis, tumor recurrence and metastasis, and epithelial mesenchymal transition [21].

c-Jun protein is an oncogenic transcription factor that is encoded by the *Jun* gene in humans, and undergoes a combination Activator protein 1 (AP-1) early response transcription with c-Fos [22]. It is involved in AKT–ERK and JNK/SAPK signaling [23]. c-Jun is an important signal for cell divisions. The G1 phase of the cell cycle arrests when c-Jun is deficient in cells. This implies that c-Jun regulates cell cycle progression and apoptosis [24]. c-Jun is required for tumor development stages, such as cell proliferation, angiogenesis, and metastasis [25, 26].

Twist-related protein 1 (TWIST1) is a transcription factor encoded by the *TWIST1* gene in humans [27, 28]. *TWIST1* acts as an oncogene in several cancers. It cooperates with N-Myc, and plays an essential role in cancer metastasis, angiogenesis, and epithelial mesenchymal transition [29–32].

Snail is zinc-finger protein that is encoded by the *Snail* gene in humans. It represses E-cadherin for the promotion of epithelial mesenchymal transition during embryonic development [33]. Snail causes the recurrence of human breast cancer by repressing E-cadherin and upregulating epithelial mesenchymal transition [34].

p53 is a known tumor suppressor gene in various organisms. Mutant *p53* is associated with the drug-resistant property of pancreatic cancer [35].

Prior studies have only shown the positive effects of silibinin on human pancreatic cancer cells, and there are no data on the effect of silibinin on human normal pancreatic cells.

The objective of this study was to investigate the molecular mechanism of silibinin and the responses of human pancreatic cells to silibinin treatment.

Methods

1. Cell lines and culture

MIA PaCa-2 and PANC-1 are *p53*-mutated pancreatic cancer cell lines that are derived from human pancreatic carcinoma [18]. hTERT-HPNE is a human pancreatic normal ductal cell line. These pancreatic cancer cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) (GIBCO Invitrogen Inc., USA) containing 4.5 mg/L glucose, 100 mg/L streptomycin, and 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS) (GIBCO Invitrogen Inc.). hTERT-HPNE (ATCC[®] CRL-4023[™], USA) cells were grown in DMEM containing 1.0 mg/L glucose (low glucose), 100 mg/L streptomycin, and 2 mM L-glutamine supplemented with 10% FBS and 10 ng/mL human epidermal growth factor (hEGF) (MERCK, USA). The cells were sub-cultured following enzymatic digestion using trypsin-EDTA solution (GIBCO Invitrogen Inc.), and were maintained under a humidified atmosphere at 37 °C in a 5% CO₂ incubator (Sanyo, Japan).

2. Flow cytometry analysis

Apoptosis assay

MIA PaCa-2, PANC-1, and hTERT-HPNE cells were seeded in six-well cell culture plates at a density of 1×10^5 cells/well. After overnight culture, the cells were treated with various concentrations (0–400 μ M) of silibinin for 24 h; then, the cells were harvested using 0.005 M trypsin-EDTA, and washed thrice with PBS. The cells were then stained with Annexin V Apoptosis Detection Kit I (51-66211E; BD Pharmingen[™], USA). The stained cells were acquired and analyzed using Canto II (BD Pharmingen[™]) and FlowJo software (Tree Star Inc., USA).

Ki-67 Analysis

Cells were stained with Ghost dye (BV510; #59863, CST, USA) and washed thrice with PBS. For intracellular staining, cells were stained using a FoxP3/transcription factor staining buffer set (00-5523; eBioscience, USA) and APC-conjugated anti-human Ki-67 (350514; Biolegend, USA).

3. Western blot analysis

Proteins were extracted from silibinin (200 μ M/mL)-treated pancreatic cells (the cells were cultured to 70% confluence in 60 mm dishes) using a radioimmunoprecipitation assay buffer (Sigma R0278, Sigma-Aldrich Co. LLC, USA) containing a protease inhibitor (#p8340, Sigma-Aldrich) and a phosphatase inhibitor (#p2850, Sigma-Aldrich). The proteins were separated using 10% SDS–polyacrylamide gel electrophoresis, and blotted onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). These

membranes were blocked with 5% (w/v) skim milk in TBS-T (20 mM Tris, pH 7.6, 136 mM NaCl containing 0.1% (v/v) Tween-20) at 25°C for 1 h. After washing thrice with TBS-T, the membranes were incubated overnight with diluted primary antibodies (all antibodies were from Cell Signaling Technology Inc., USA; antibody:TBS-T = 1:1,000) at 4 °C. After washing thrice with TBS-T for 10 min each, the membranes were incubated with either anti-rabbit or anti-mouse horseradish peroxidase–conjugated secondary antibodies. ECL SuperSignal chemiluminescent substrate (Millipore Corporation, Billerica, MA, USA) was used to develop the membrane. Protein bands were then visualized using a LAS 3000 Imaging System (FujiFilm, R&D Systems, Minneapolis, MN, USA).

4. Spheroid formation assay

Cells were seeded in six-well plates at a density of 1×10^3 cells/well, and cultured in F-12 DMEM (GIBCO Invitrogen Inc.) containing 10 ng/mL human recombinant basic fibroblast growth factor (bFGF) (R&D Systems) and 10 ng/mL hEGF (R&D Systems) with $1 \times N_2$ supplement (GIBCO Invitrogen Inc.). The cells were incubated at 37 °C under a humidified atmosphere containing 5% CO₂. Spheroids were confirmed after 14 days.

5. Invasion assay

Cell invasion was carried out overnight using a transwell filter chamber (8.0- μ m pores) coated with 1% gelatin/DMEM, followed by drying at RT. Pancreatic cancer cells were harvested, washed once with the growth culture medium, and seeded on the upper chamber at 2×10^5 cells in 120 μ L 0.2% BSA medium. Then, 400 μ L 0.2% BSA medium containing 20 μ g/mL human plasma fibronectin (Calbiochem, La Jolla, CA, USA) was loaded into the lower chamber. The transwell apparatus was incubated at 37 °C for 24 h. Cells that invaded the bottom surface of the upper chamber were fixed with 70% ethanol and stained with Diff-Quik solution (Sysmex, Kobe, Japan), according to the manufacturer's protocol. Non-invasive cells on the top surface were wiped off with cotton balls and stained. Cells on the bottom surface were counted in five selected fields (each 0.5 mm²), using a hemacytometer under a light microscope at $\times 400$ magnification. The results were expressed as means \pm SE of the number of cells per field from three individual experiments.

6. RNA isolation

Total RNA was extracted using TRIzol (Takara, Japan). Briefly, 1 mL TRIzol solution was added into each well, and the suspensions were transferred to 1.5 mL tubes. After adding 200 μ L chloroform (Sigma-Aldrich) and vortex mixing for 15 s, the mixtures were centrifuged at 4°C and 6,000 \times g (grams) for 20 min. The supernatants were then collected, mixed with equal amounts of isopropyl alcohol (MERCK), and centrifuged at 4°C and 6,000 \times g for 20 min. The pellets were washed with 1 mL 70% ethyl alcohol (MERCK) and centrifuged at 4°C and 6,000 \times g for 5 min. After removing the remaining ethyl alcohol, the RNA pellets were air-dried at RT. They were then resuspended in 50 μ L diethyl pyrocarbonate water.

7. Real-time polymerase chain reaction (PCR)

Total RNAs were converted to cDNAs using a reverse transcription system (Promega Corporation, WI 53711–5399, USA). Real-time PCR was performed with an Applied Biosystems StepOnePlus™ (Thermo Fisher Scientific, MA, USA) Real-Time PCR system, according to the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH: HS02786624-g1) was used as a control, and the $2^{-\Delta\Delta CT}$ values were calculated for stem markers (TWIST1: HS 01675818_s1, c-Jun: HS01103582_s1, and Snail: HS00195591_m1). The probes used were Thermo Fisher Taqman® Assay probes (Thermo Fisher Scientific).

8. Wild-type *p53* gene transfection

Cells were seeded in six-well plates and incubated at 37°C overnight. The cells were then transfected with pcDNA3/wild-type *p53* (plasmid #69003, Addgene, MA 02472, USA) expression plasmid vector, using FuGENE®6 (Cat.#E2693, Promega Corporation) reagent, according to the manufacturer's protocol.

9. Statistical analysis

The two-tailed paired t-test was used to assess statistical significance appropriately. The statistical significance of differences between data sets was determined using the paired t-test and one-way ANOVA test. All the reported *p* values are two-sided; $p \leq 0.05$ was considered statistically significant. The Statistical Package for the IBM SPSS Statistics 23 software (SPSS Statistics Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

1. Silibinin induces apoptosis of pancreatic cancer cell lines

Cells were stained with AnnexinV-FITC and propidium iodide (PI), and analyzed using flow cytometry. The binding of fluorescently labeled Annexin V to externalized phosphatidylserine was also determined using flow cytometric analysis to quantify early apoptotic cells. PI uptake was measured to assess cells in the late stage of apoptosis, or cells that sustained direct plasma membrane damage. Figure 1 shows the flow cytometric plots obtained with AnnexinV-FITC PI assay after 24 h exposure to different concentrations (0–400 μ M) of silibinin. After cell exposure to silibinin, the number of early and late apoptotic or directly damaged MIA PaCa-2 and PANC-1 cells significantly ($p < 0.001$) increased. However, silibinin did not affect the cell viability of hTERT-HPNE cells ($p < 0.001$) (Fig. 1).

2. Silibinin decreases phosphorylation of ERK-SAPK/JNK signaling proteins in human pancreatic cancer cell lines

Silibinin can modulate the phosphorylation of mitogen-activated protein kinase (MAPK) cascade signaling proteins in human pancreatic cancer cells [9]. The effect of silibinin on the MAPK signal pathway, which is essential for cell survival and growth during development and carcinogenesis, was examined. Silibinin treatment significantly decreased the expression levels of phosphorylated ERK 1/2

and phosphorylated p42/44 MAPK (Figs. 2A and 2B, and 2D and 2E, respectively). As crosstalk between signal transduction pathways is common, we examined whether silibinin prevented the phosphorylation of p-ERK or SAPK/JNK in human pancreatic cancer cells. Silibinin treatment decreased the phosphorylation levels of AKT and SAPK/JNK. However, it did not alter the levels of total ERK 1/2, SAPK/JNK, p42/44-MAPK, and AKT (Figs. 2A and 2B, and 2D and 2E, respectively). Furthermore, silibinin did not affect phosphorylation, or the total levels of AKT and SAPK/JNK in hTERT-HPNE cells (Figs. 2C and 2F).

3. Silibinin increases autophagy and apoptosis signaling in pancreatic cancer cell lines

Silibinin-induced apoptosis is associated with the modulation of apoptotic regulatory proteins in human pancreatic cancer cells [13–15]. The expression levels of cleaved caspase-3 and LC3B significantly increased in MIA PaCa-2 and PANC-1 cells following treatment with 200 μ M silibinin (Figs. 3A and 3B, and 3D and 3E, respectively). However, silibinin did not affect the levels of cleaved caspase-3 and LC3B in hTERT-HPNE cells (Figs. 3C and 3F).

4. Silibinin decreases spheroid formation in pancreatic cancer cell lines

Cancer cells can be cultured in suspension to form spheres in a serum-free replacement medium. To test whether human pancreatic cancer cell lines could form spheres, MIA PaCa-2 and PANC-1 cells were cultured in a suspension culture system. Silibinin decreased the number of colonies after 14 days of incubation. We also observed a smaller number of colonies in silibinin-treated cultures relative to that in the control, for each cell line ($p < 0.05$). On average, silibinin exposure decreased the colony size of each cell line (Fig. 4B).

5. Silibinin decreases invasion and migration of pancreatic cancer cell lines

To determine whether silibinin influenced the biological activity of the human pancreatic cancer cells used, we performed an invasion assay. After measuring six random squares of a 0.5 mm \times 0.5 mm microscope field, we found that the numbers of invading MIA PaCa-2 cells after treatment with 100 and 200 μ M/mL silibinin were 239 ± 3.5 and 240 ± 15.1 , respectively, whereas that for the negative control was 286 ± 7.3 ($p = 0.007$) (Fig. 4C). The numbers of invading PANC-1 cells after treatment with 100 and 200 μ M/mL silibinin were 66 ± 4.24 and 35 ± 4.24 , respectively, whereas that for the negative control was 112 ± 2.12 ($p = 0.001$) (Fig. 4C). Furthermore, silibinin treatment of MIA PaCa-2 and PANC-1 cells decreased migration, compared to that in negative control cells (Fig. 4A).

6. Silibinin increases expression of stemness-related signal pathway in pancreatic cancer cell lines

Silibinin increased the expression levels of TWIST1, Snail, and c-Jun mRNAs in pancreatic cancer cells, compared to those in the control (Figs. 5A and 5B) ($p < 0.05$). However, it decreased the expression levels of TWIST1, Snail, and c-Jun in pancreatic normal ductal cells (hTERT-HPNE) (Fig. 5C), compared with those in the control ($p < 0.05$). The TWIST1, Snail, and c-Jun mRNA expression levels were significantly higher in silibinin-treated cancer cells than in the negative control cells ($p < 0.05$) (Fig. 5D). However, the

TWIST1, Snail, and c-Jun mRNA expression levels in silibinin-treated normal cells were lower than those in the negative control cells ($p < 0.05$) (Fig. 5D).

7. Silibinin decreases wild-type p53 protein levels and increases c-JUN protein levels in pancreatic cancer cell lines

Silibinin decreased the *p53* protein levels in overexpressed wild-type *p53* protein pancreatic cancer cell lines (Figs. 6A and 6B), however, increased those in overexpressed wild-type *p53* protein pancreatic normal ductal cells (hTERT-HPNE) (Fig. 6C). In addition, silibinin increased c-Jun protein levels in pancreatic cancer cell lines (Figs. 6A and 6B), but decreased those in pancreatic normal ductal cells (hTERT-HPNE) (Fig. 6C).

8. Silibinin increases expression of Ki-67 in pancreatic adenocarcinoma cell lines

Ki-67 is a protein encoded by the *MKI67* gene, and is used as a marker of proliferation. It is overexpressed in the G1, S, G2, and M phases of cell cycles, but is not expressed in the G0 phase [36]. Silibinin increased the expression of Ki-67 by 16.7% and 23.1% in the human pancreatic adenocarcinoma cell lines, MIA PaCa-2 and PANC-1, respectively. On the other hand, silibinin decreased the expression of Ki-67 by 7.3% in the pancreatic normal ductal cell line, hTERT-HPNE ($p = 0.0001$) (Figs. 7A and 7B).

Discussion

The present study demonstrated the following findings: 1) silibinin does not affect cell viability in pancreatic normal ductal cells, but reduces cell viability, migration, invasion, and spheroid formation by downregulating AKT–ERK signaling in pancreatic adenocarcinoma cell lines; 2) silibinin decreases stemness-related signals, including the expression of c-Jun, TWIST1, and Snail, in pancreatic normal ductal cells, but increases those in pancreatic adenocarcinoma cell lines; 3) although silibinin increases wild-type *p53* levels in pancreatic normal ductal cells, silibinin reduces those in pancreatic adenocarcinoma cell lines.

Many studies have shown the tumor suppressive effects of silibinin in human cancer cells [36–38]; however, only a few studies [13, 37–39] have been undertaken to elucidate silibinin-induced apoptosis in human pancreatic cancer cells.

In addition, a limitation of these previous studies is that they focused less on the signal pathway of cell death in pancreatic cancer than that in other cancers, and only the positive effects of silibinin on human pancreatic cancer cells were reported. Furthermore, no studies have been conducted on the effects of silibinin on human pancreatic normal ductal cells. Our study has shown the effect of silibinin on pancreatic cancer cell lines as well as pancreatic normal ductal cell lines. The results of our study show that the effect of silibinin is broader than that indicated by other studies; it is a potent anticancer agent, but also has the potential to cause cancer recurrence. A recent study suggested that silibinin could induce cell death possibly *via* a bcl-2-sensitive apoptotic pathway in a human pancreatic adenocarcinoma cell

line (SW1990) [13]. In the present study as well, 0–400 $\mu\text{M}/\text{mL}$ silibinin induced the apoptotic cell death of pancreatic adenocarcinoma cell lines (MIA PaCa-2 and PANC-1), based on flow cytometer analysis (Fig. 1). Western blot analysis showed that silibinin downregulated the levels of AKT–ERK and SAPK/JNK in human pancreatic adenocarcinoma cells (Fig. 2). ERK 1/2-SAPK/JNK signaling is known to regulate cellular activities, including gene expression, mitosis, movement, metabolism, and programmed death [40]. Therefore, the results of the present study demonstrate that silibinin causes cell death through the p-AKT and p-SAPK/JN–ERK pathway (Fig. 2). The present study has also demonstrated the additional anti-cancer effects of silibinin; it reduced cell migration, invasion, and spheroid formation by downregulating p-AKT and p-SAPK/JNK–ERK signaling in pancreatic adenocarcinoma cell lines. However, silibinin did not downregulate p-AKT and p-SAPK/JNK–ERK signaling in hTERT–HPNE cells (Fig. 4). Our data show that cell death was induced by a decrease in c-Jun-related AKT–ERK and JNK/SAPK signaling, but this mechanism does not explain the increase in c-Jun levels. This study did not reveal the mechanism for the increase in c-Jun levels. However, we speculate that the increase in c-Jun levels involves a molecular mechanism other than AKT–ERK and JNK/SAPK signaling, because this mechanism is complex [41]. A previous study has shown that CSCs can self-renew and differentiate into mature cells [42]. They also play important roles in cancer homeostasis, metastasis, resistance to therapy, and subsequent tumor recurrence [43]. Pancreatic CSCs are highly resistant to a majority of chemotherapeutic agents [44].

It is unknown whether silibinin affects pancreatic cancer stemness. The present study demonstrated that after exposure to silibinin, the expression levels of cancer stemness–related genes increase in human pancreatic cancer cells and other organ cancer cells, such as HepG2 (hepatocarcinoma cell line) and HCT116 (colon adenocarcinoma cell line); conversely, they decrease in human normal pancreatic ductal cells (hTERT-HPNE) and normal kidney cells (293T) (Fig. 5). These results suggest that the effects of silibinin on pancreatic cancer cells and pancreatic normal ductal cells differ.

p53 is a tumor suppressor protein encoded by the *Tp53* gene. *p53* is an important cell-cycle inhibitor that prevents cancer in multicellular organisms. A mutation in the *p53* protein cannot kill damaged cells. However, other healthy cells can break down such damaged cells. If a mutation of the *p53* gene does not allow the *p53* protein to function properly, cells with the damaged DNA will progress to cell division. These damaged cells can continue to divide. They will not be able to recognize the damaged DNA, resulting in more mutations. Such mutations cause cancer [45–49]. *p53* protein overexpression is a common genetic alteration, and occurs very early in the development of pancreatic cancer [50–51]. The results of the present study indicate that silibinin-induced increases in stemness-related signals may be mediated by mutations in the *p53* protein. In normal pancreatic ductal cells (hTERT-HPNE) that overexpress wild-type *p53*, silibinin increases the *p53* protein levels. However, it decreases the *p53* protein levels in *p53*-mutated cells such as MIA PaCa-2 and PANC-1 cells that overexpress wild-type *p53* (Fig. 6). These results suggest that mutations in *p53* may influence the effect of silibinin. However, the present study did not demonstrate that silibinin increases the expression of stemness-related genes, such as those of c-Jun, TWIST1, and Snail, in wild-type *p53* pancreatic adenocarcinoma cells. Further studies are necessary to investigate the relationship between wild-type *p53* pancreatic adenocarcinoma cells (such as SW1990) and silibinin.

Silibinin not only exerts anticancer effects by inhibiting AKT–ERK and JNK, but also increases the expression of cancer stemness–related genes in human pancreatic adenocarcinoma cells. However, silibinin does not have an adverse effect on pancreatic normal ductal cells.

Conclusions

In conclusion, pancreatic cancer cells susceptible to silibinin undergo cell death; on the other hand, if pancreatic cancer cells become resistant to silibinin and survive, it is possible that they will engage in activity similar to that of CSCs. These results suggest that when silibinin is used as a therapeutic agent for human pancreatic cancer, caution should be exercised.

List Of Abbreviations

1. ERK: Extracellular signaling-regulated kinase
2. AKT: serin/threonine protein kinase
3. LC3B: microtubule associated protein 1 light chain 3 β
4. JNK: c-Jun N-terminal kinases
5. JNK/SAPK: c-Jun N-terminal kinases/Stress-activated protein kinases
6. GLP-1R/SKA: glucagon-like peptide-1 receptor and protein kinase A
7. CSCs: Cancer stem cells
8. AP-1: Activator protein 1
9. TWIST1: Twist-related protein 1
10. DMEM: Dulbecco's Modified Eagle's medium
11. FBS: fetal bovine serum
12. hEGF: human epidermal growth factor
13. PBS: phosphate buffered saline
14. SDS-polyacrylamide: Sodium dodecyl sulphate-polyacrylamide
15. PVDF: Polyvinylidene difluoride
16. TBS-T: tris-buffered saline-Tween 20
17. ECL: electrogenerated chemiluminescence
18. h-bFGF: human recombinant basic fibroblast growth factor
19. BSA: bovine serum albumin
20. RT: room temperature
21. PCR: polymerase chain reaction
22. cDNA: Complementary DNA
23. CTs: del/del threshold cycles
24. ANOVA test: analysis of variance

- 25. PI: propidium iodide
- 26. MAPK: mitogen-activated protein kinase
- 27. Fig: figure

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

All authors agree with the content and submission of the manuscript.

Availability of supporting data

Not applicable

Competing interests

Not applicable

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Authors' contributions

-Chang-Hwan Park: study concept and design; analysis and interpretation of data; drafting and finalizing the manuscript; study supervision (correspondence author)

-Su-Mi Lee: Carrying out the experiment; Analysis and interpretation of data; drafting the manuscript (first author)

-Gil-Woo Lee: FACS analysis; drafting the manuscript (first author)

-SeonYoung Park, Hosouk Joung, Eun-Ae Cho, Hyun-Soo Kim, Sung-Kyu Choi and Jong-Sun Rew: Analysis and interpretation of data; discussion and data review.

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Figures

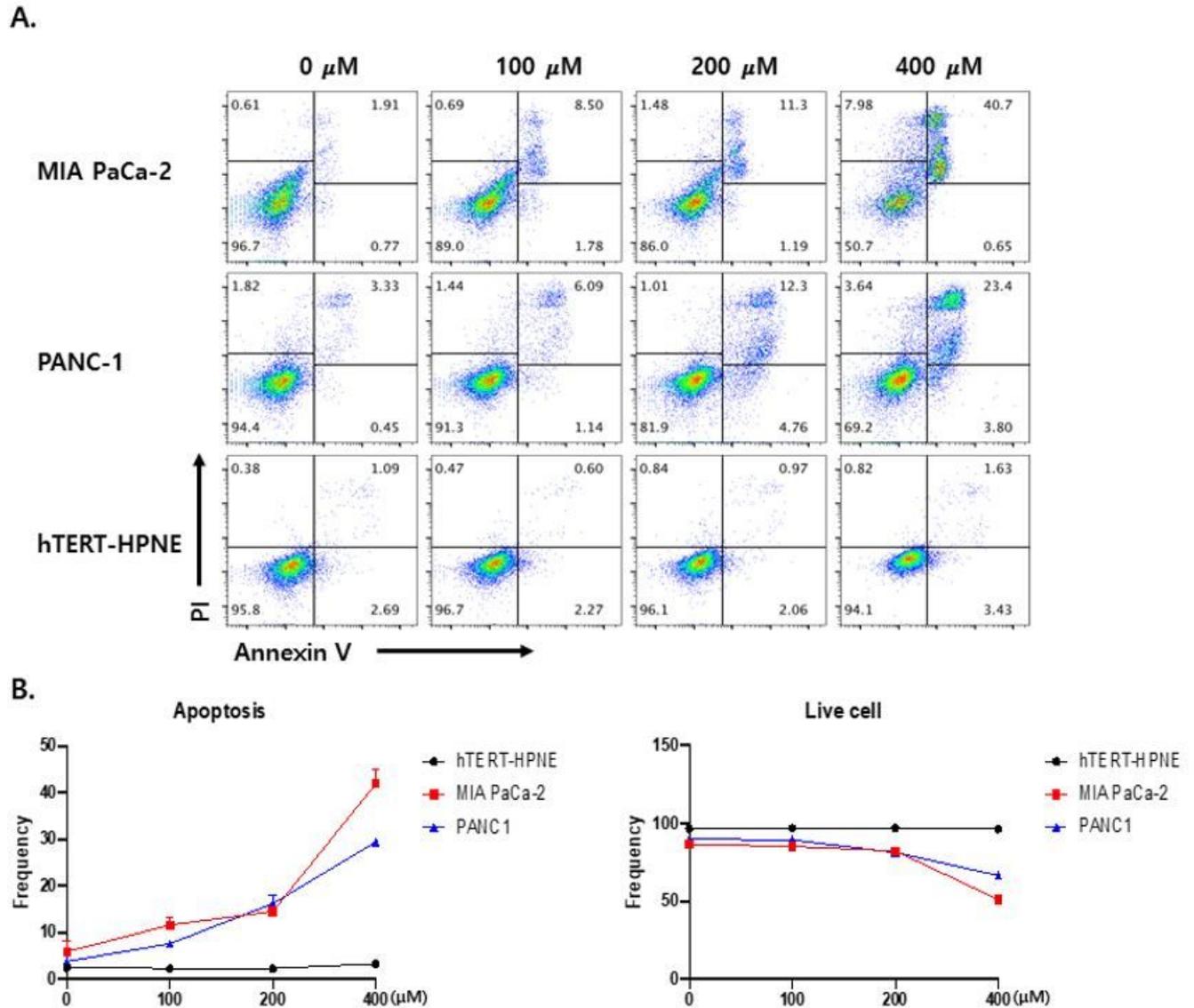


Figure 1

(A) Apoptosis assay of MIA PaCa-2, PANC-1 (human pancreatic adenocarcinoma ductal cell lines), and hTERT-HPNE (human pancreatic normal ductal cell line) after silibinin treatment. Data are representative of three independent experiments. (B) Silibinin induces apoptotic cell death in both MIA PaCa-2 and PANC-1 cells in a dose-dependent manner, but does not affect hTERT-HPNE cells ($p < 0.0001$).

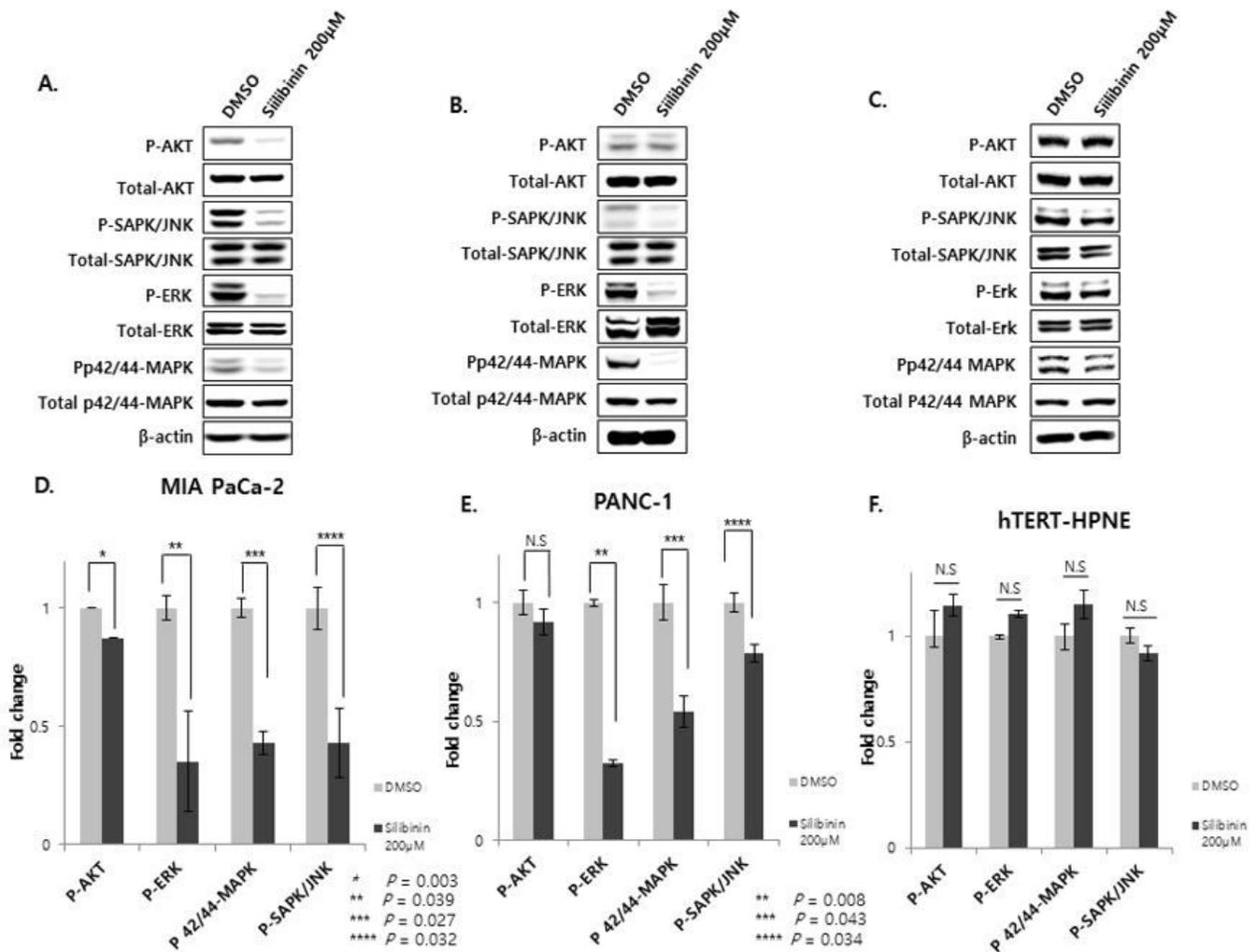


Figure 2

Silibinin decreases the expression of phosphorylated AKT, ERK 1/2, JNK, and p42/44 MAPK in pancreatic cancer cells (MIA PaCa-2 and PANC-1). However, silibinin increases the expression of phosphorylated AKT ($p < 0.01$), ERK 1/2 ($p < 0.05$), SAPK/JNK ($p < 0.05$), and p42/44 MAPK ($p < 0.05$) in normal pancreatic ductal cells (hTERT-HPNE). Figs. A and D: MIA PaCa-2, figs B and E: PANC-1, and figs C and F: hTERT-HPNE cells are incubated with or without silibinin (200 μ M) for 24 h ($p < 0.05$).

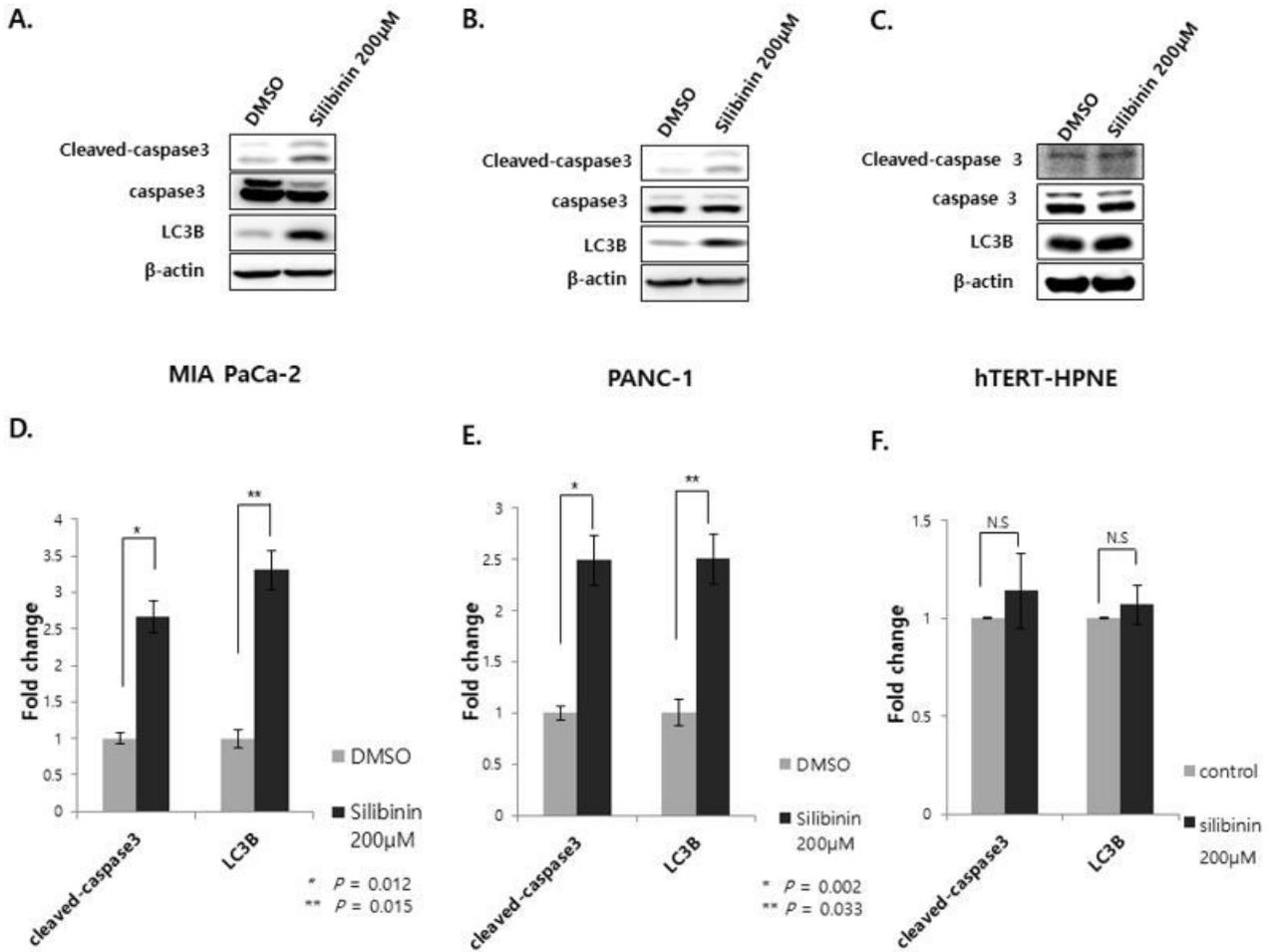


Figure 3

Effect of silibinin on apoptosis-related protein expression. Increases in the expression of cleaved caspase-3 and LC3B are detected in pancreatic cancer cells figs A and D: MIA PaCa-2 and figs. B and E: PANC-1, and figs. C and F: hTERT-HPNE cells following treatment with silibinin (200 µM) for 24 h ($p < 0.05$).

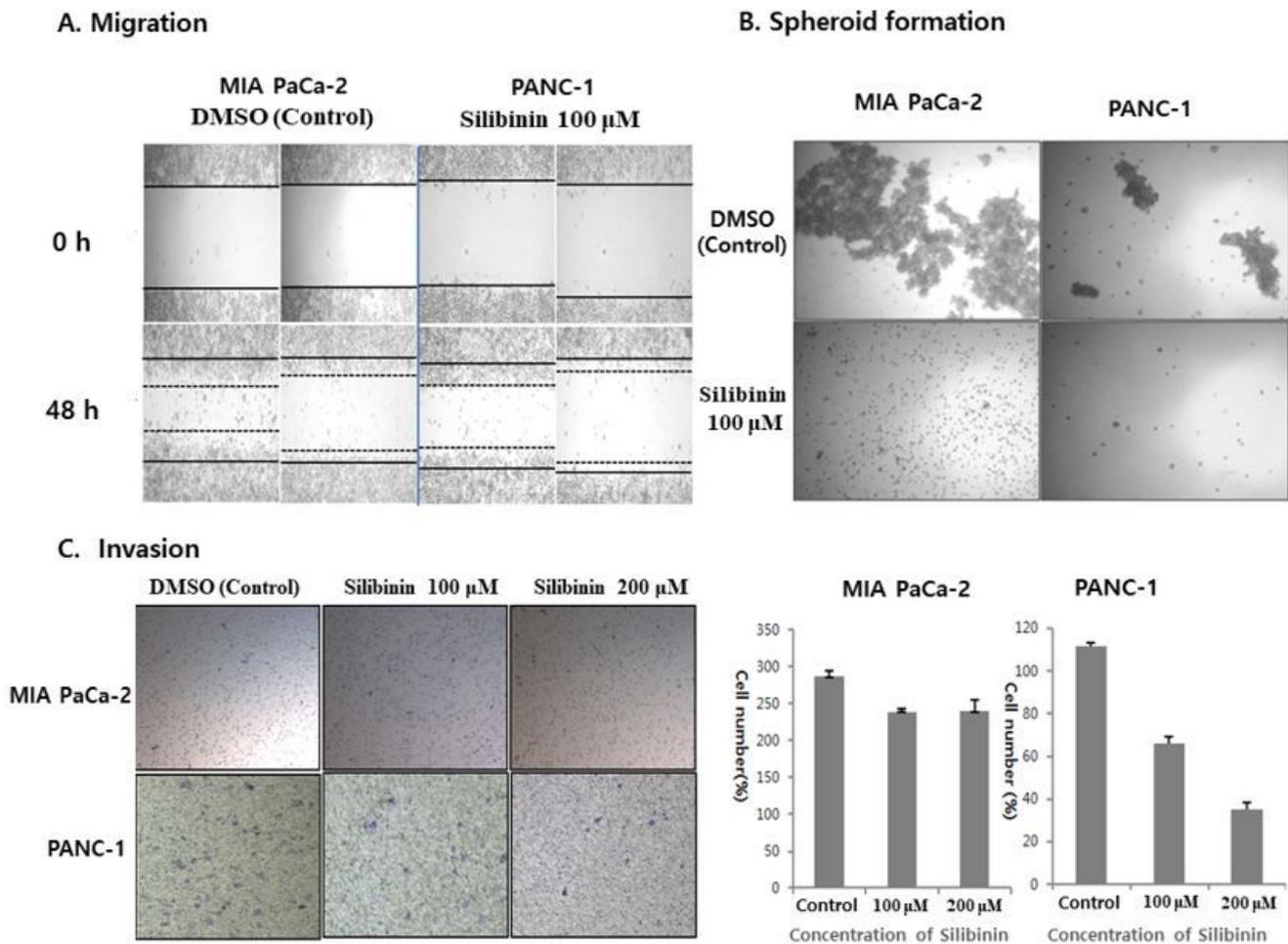
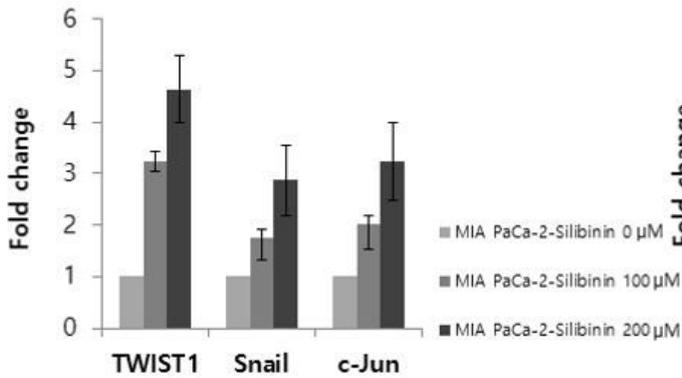
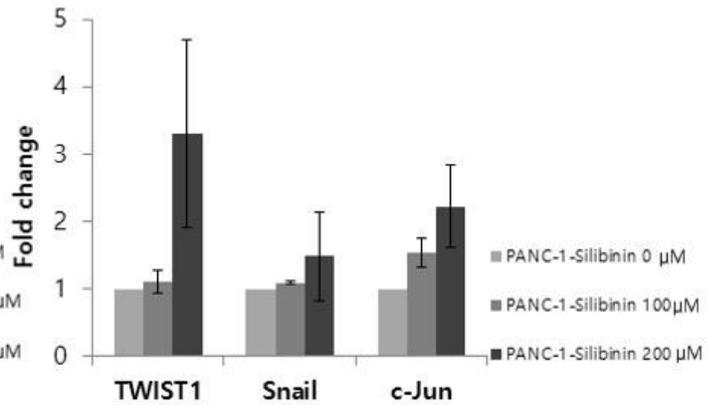
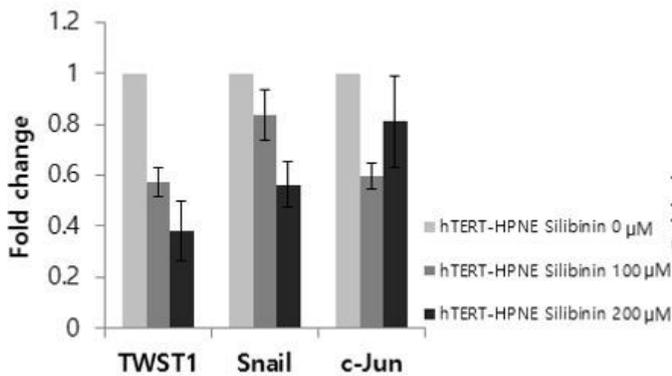
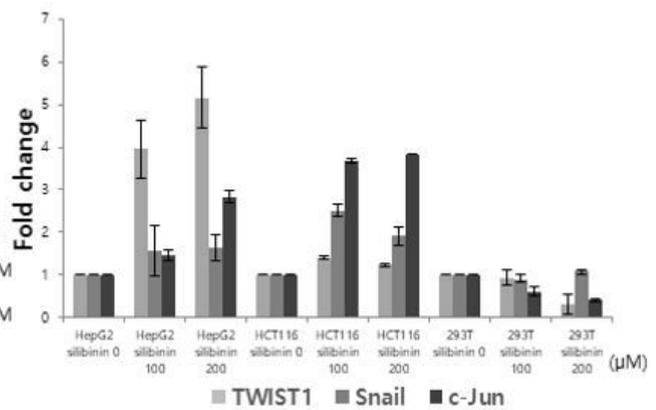


Figure 4

Silibinin decreases migration, spheroid formation, and invasion. Fig. A: Silibinin decreases migration in MIA PaCa-2 and PANC-1 cells, compared to that in the negative control. Fig. B: Silibinin decreases spheroid formation in MIA PaCa-2 and PANC-1 cells. Silibinin decreases the number of colonies after 14 days of incubation ($p < 0.05$). On average, silibinin exposure decreases the colony size in each cell line. Fig. C: Effects of silibinin on cell invasion. The numbers of invading MIA PaCa-2 cells after treatment with 100 and 200 μ M silibinin are 239 ± 3.5 and 240 ± 15.1 , respectively, whereas that of the negative control are 286 ± 7.3 , as measured for six random squares of a $0.5 \text{ mm} \times 0.5 \text{ mm}$ microscope field ($p = 0.007$). The numbers of invading PANC-1 cells treated with 100 and 200 μ M silibinin are 66 ± 4.24 and 35 ± 4.24 , respectively, whereas that of the negative control are 112 ± 2.12 , as measured for six random squares of a $0.5 \text{ m} \times 0.5 \text{ mm}$ microscope field ($p = 0.001$).

A. MIA PaCa-2**B. PANC-1****C. hTERT-HPNE****D. Other cell lines****Figure 5**

Expression levels of TWIST1, Snail, and c-Jun mRNA in pancreatic cancer cells: Fig. A: MIA PaCa-2, B: PANC-1, fig. C: human pancreatic normal ductal cell line (hTERT-HPNE), and fig. D: liver cancer cell line (HepG2), colon cancer cell line (HCT116), and normal kidney cell line (293T). The results show that TWIST1, Snail, and c-Jun mRNA expression levels are significantly higher in silibinin-treated cancer cells than in negative control cells. However, they are lower in silibinin-treated normal cells than in negative control cells ($p < 0.05$).

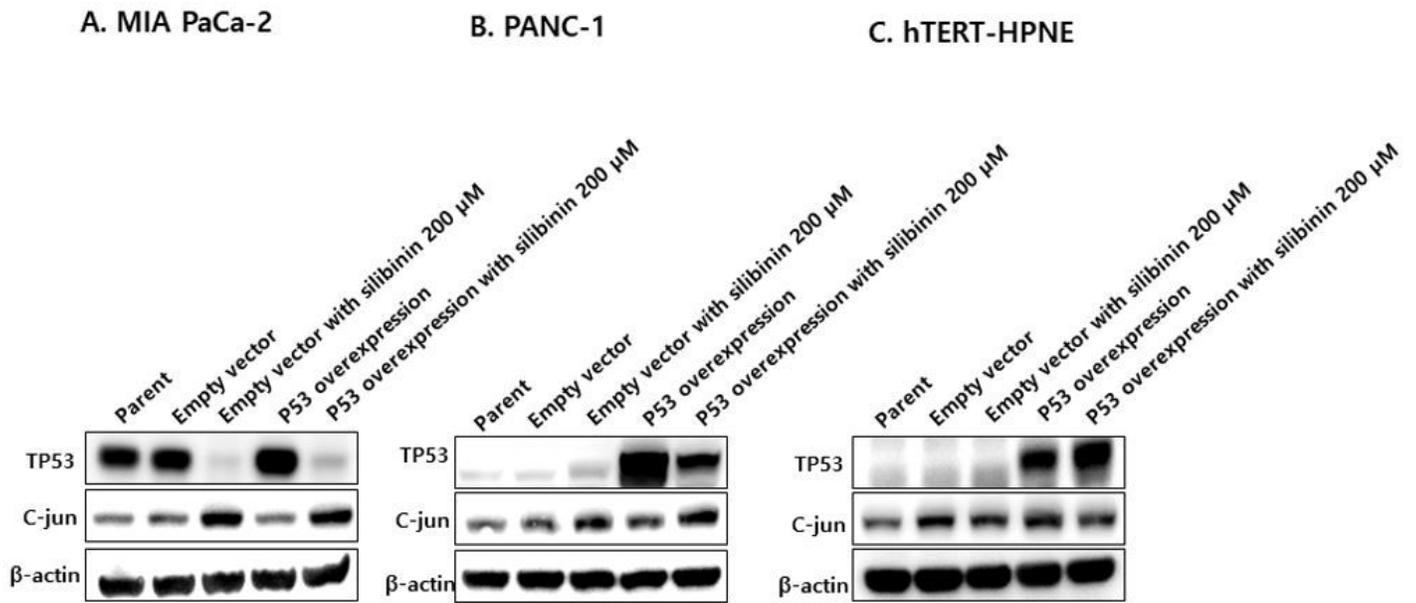


Figure 6

Silibinin reduces the exogenous wild-type p53 expression in fig. A: MIA PaCa-2 and fig. B: PANC-1 cells, and increases endogenous c-Jun expression. However, silibinin does not affect p53 expression and c-Jun levels in fig. C: hTERT-HPNE cells. The expression levels are determined using western blotting.

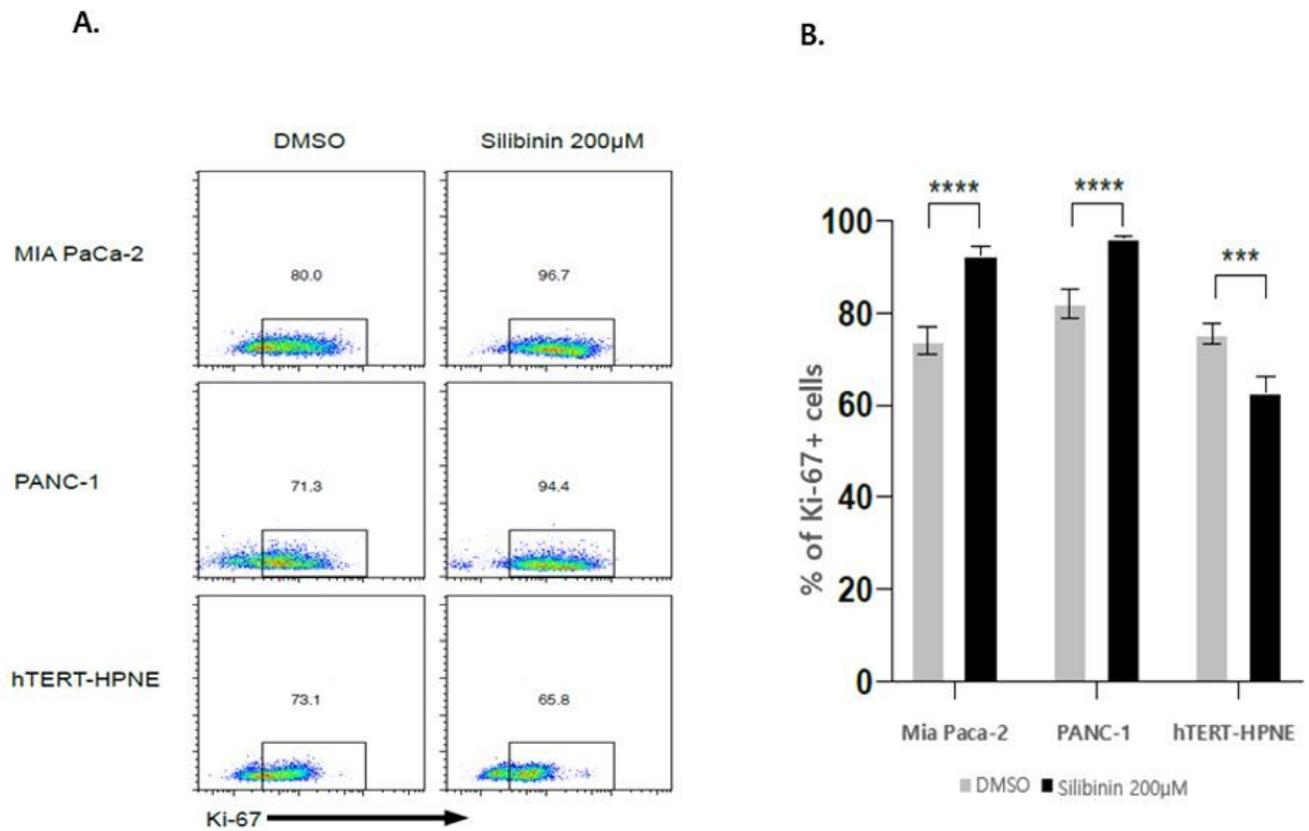


Figure 7

Figs. A and B Proliferation analysis of cells surviving silibinin treatment. Ki-67 staining shows extensive proliferation in MIA PaCa-2 and PANC-1 cells; conversely, hTERT-HPNE cells shows a slight decrease in proliferation after silibinin treatment, followed by a one-day resting period ($p < 0.0001$).