



# Silibinin inhibited autophagy and mitochondrial apoptosis in pancreatic carcinoma by activating JNK/SAPK signaling

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## ABSTRACT

**Background:** Previous investigation have indicated Silibinin induces apoptosis and JNK/SAPK in human pancreatic cancer cells. This study aims to evaluate the further mechanism of Silibinin in pancreatic cancer treatment.

**Materials and methods:** Human pancreatic cancer cell lines SW1990 was treated with Silibinin and/or JNK/SAPK inhibitor SP600125 followed by measurement of cell viability, apoptosis, autophagy, ROS and ATP, and western blotting.

**Results:** Silibinin promoted cell viability and promoted cell apoptosis. The expression of ROS and ATP associated with mitochondrial function was also promoted by the treatment of silibinin. Silibinin also promoted autophagy in pancreatic cancer cells. All these biological effects of Silibinin can be reversed by JNK/SAPK inhibitor.

**Conclusions:** The biological effects regulated by Silibinin can be mediated by JNK/SAPK signaling. This provides a solid theoretical basis for the role of Silibinin in the treatment of pancreatic cancer.

## 1. Introduction

Pancreatic carcinoma is called “cancer king” for its aggressive and lethal character, with the mortality for all stages of 94% global wide [1]. Although the incidence is low, pancreatic carcinoma also accounts for the fourth leading cause of cancer associated death. Most kinds of chemotherapy were poorly efficient for pancreatic carcinoma, and largely due to drug resistance [2]. Thus, identification of lead compounds that avoid the resistance mechanism is an important research objective. Silibinin has been identified as a potential anti-pancreatic cancer drugs in cell apoptosis, cell cycle and tumor angiogenesis [3]. Moreover, Silibinin is well tolerated and has essentially no side effects in acute, chronic tests even at large doses [4,5].

The herbal medicine, Silibinin, plays a pivotal role in prevention from malignancies. Silibinin, a flavonoid, can extract from milk thistle plants *Silybum marianum* that is a natural drug to protect the liver [6]. It has been well studied in prostate cancer and under clinical trial for prostate cancer patients [7]. Additionally, it has indicated that Silibinin is strong efficient in skin, colon, lung and hepatocellular carcinoma in both *in vitro* and *in vivo* studies [8–11]. Silibinin has been reported in

colorectal cancer cells that regulated autophagy and apoptosis [12]. However, the limitation of recent studies were that it did not discuss the efficacy of the Silibinin treatment in metabolism and energy effects in pancreatic cancer and the further mechanism for Silibinin effects.

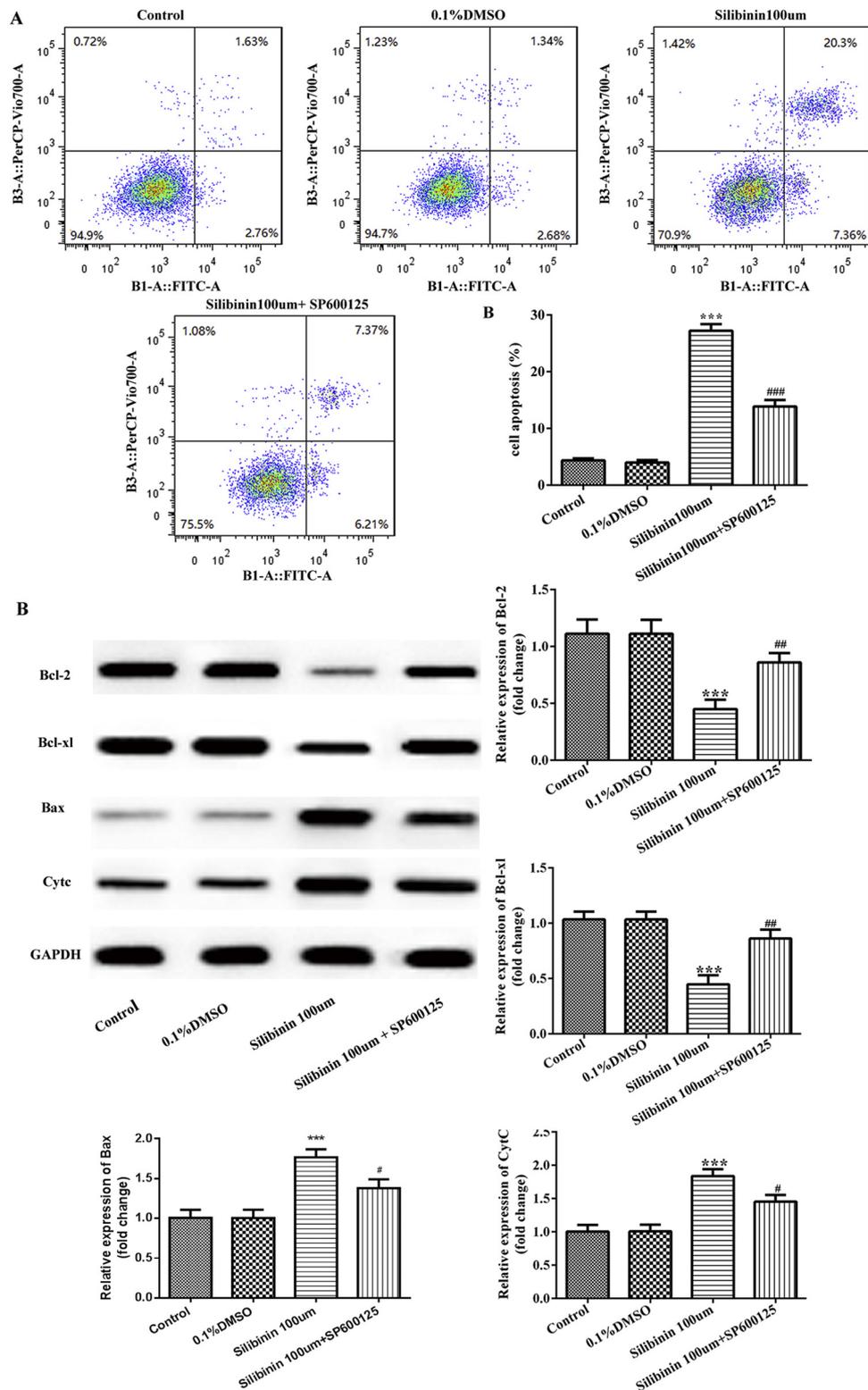
We recently reported that Silibinin induces G1 arrest, apoptosis and JNK/SAPK upregulation in human pancreatic cancer cells [13]. Reactive oxygen species are a molecule that are produced in cells through metabolism of oxygen and function as a destructive molecule at high levels [14]. Cytochrome c (Cyt-c) is discovered concurrent with ROS to mediate apoptosis [15]. ROS participated in autophagic process by inducing mitochondrial redox signals [16]. ROS injury can lead to autophagy in cancer cells. It has been reported that Beclin-1 is upregulated during autophagosome formation, and Bcl-Xl and Bax may be involved in this process [17]. Therefore, we supposed Silibinin induces apoptosis might associated with mitochondrial function and autophagy. Moreover, ROS and oxidative stress induced autophagy can be mediated by FOXO1, p38MAPK, extracellular regulated kinase(ERK) and c-Jun N-terminal kinase [18]. Therefore, JNK/SAPK might also participated in the effects of Silibinin. In this study, we evaluated whether silibinin regulated apoptosis and autophagy mediated by JNK/SAPK

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**Fig. 3.** Silibinin induced apoptotic cell death in pancreatic carcinoma. SW1990 cells were treated with 0.1% DMSO control or 100  $\mu$ M Silibinin for 48 h, or pretreated JNK/SAPK inhibitor (SP600125) for 1 h before 100  $\mu$ M Silibinin treatment. (A) The apoptotic cell population was detected by flow cytometry. (B) The expression levels of apoptotic proteins were detected by western blotting. Data shown are mean  $\pm$  SD of triplicate samples for each treatment. \*\*\*P < 0.001 compared with the control; #P < 0.05, ##P < 0.01 compared with Silibinin 100  $\mu$ M.

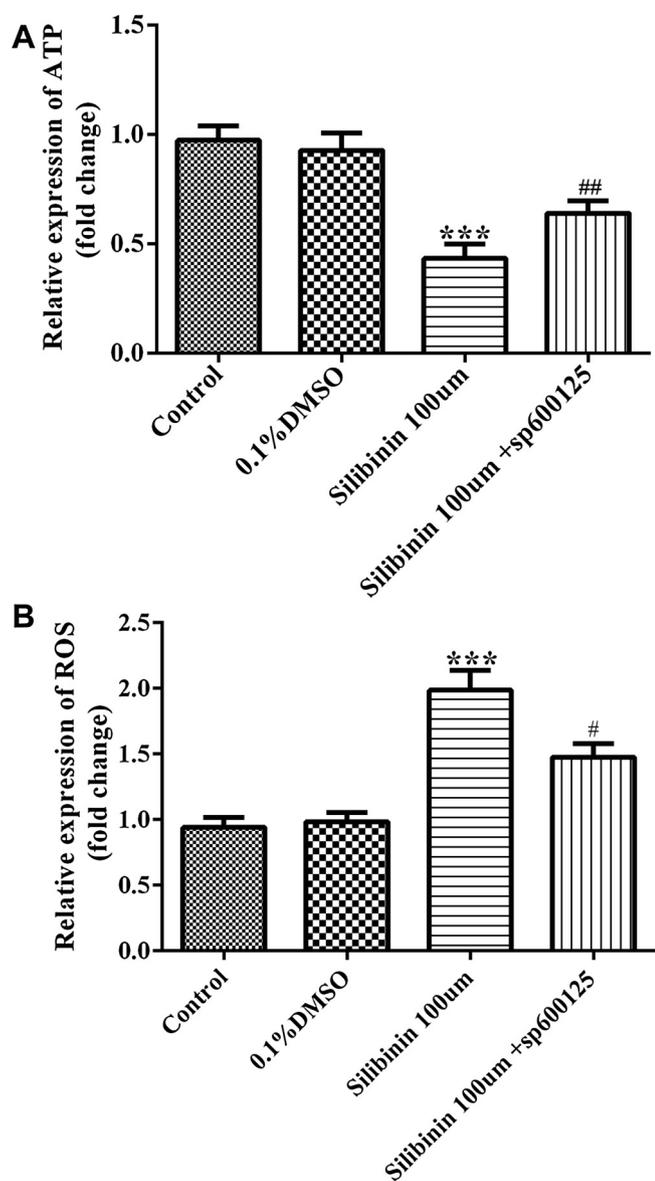


Fig. 4. Silibinin induced mitochondrial injury in pancreatic carcinoma. SW1990 cells were treated with 0.1% DMSO control or 100  $\mu$ M Silibinin for 48 h, or pretreated JNK/SAPK inhibitor (SP600125) for 1 h before 100  $\mu$ M Silibinin treatment. (A) Relative expression of intracellular ATP in cells. (B) Relative expression of intracellular reactive oxygen species (ROS) in cells. Data shown are mean  $\pm$  SD of triplicate samples for each treatment. \*\*\* $P$  < 0.001 compared with the control; # $P$  < 0.05, ## $P$  < 0.01 compared with Silibinin 100  $\mu$ M.

apoptosis. Each assay performed three independent experiments.

#### 2.5. ATP concentration

The ATP concentration was quantified by fluorometric tests of ATP using a ATP Assay kit (Cat. MAK190-1KT, sigma, Merck KGaA, Germany) according to manufacturer's instructions. Results are cumulative from three independent experiments.

#### 2.6. Measurement of ROS

The intracellular ROS production was detected according to ROS assay kit (Beijing Solarbio Science & Technology Co., Ltd) according to the manual. Briefly, cells ( $5 \times 10^3$  cells per well) were seeded in 96 well plates overnight and treated with silibinin and/or JNK/SAPK inhibitor

for 48 h. Then, 10  $\mu$ M DCFH-DA was added to cells and cultured for 20 min at 37  $^{\circ}$ C, and the fluorescence was measured using excitation/emission wavelengths of 488/525 nm. Each assay performed three independent experiments.

#### 2.7. Cell immunofluorescence

Cells ( $1.5 \times 10^4$  cells per well) were grown on coverslips in 12 well plate. After attached to the plate, cells were treated with Silibinin and/or SP600125. Subsequently, cells were fixed with 4% paraformaldehyde for 15 min and then treated with 0.5% Triton X-100. 20 min later, cells were incubated with rabbit LC3II antibody (1:200, Cell Signaling Technology) at room temperature. 1.5 h later, cells were incubated with anti-rabbit IgG antibody (1:1000, Cell Signaling Technology) at room temperature for 1 h. Cells were then treated with HOECHST to assess nuclear morphology at room temperature for 2 min. Cells were evaluated using Olympus confocal microscope at  $\times 40$  magnification.

#### 2.8. Statistical analysis

All experiments were performed at least in biological triplicate. Data are presented as mean  $\pm$  SD. Statistical analysis was calculated by GraphPad Prism 6.0 using a one-way ANOVA followed by Turkey's post hoc test for multiple comparisons.  $P \leq 0.05$  is considered as statistically significant.

### 3. Results

#### 3.1. Silibinin inhibits cell viability in SW1990 cells

Following the treatment of Silibinin in SW1990 cells at 24, 48, 72 h, cell viability was detected (Fig. 1). The results showed that Silibinin inhibited the proliferation of SW1990 cells. In this investigation, no differences were found between 0.1% (v/v) DMSO and the negative control. Cell proliferation was a significant decrease in SW1990 cells after 48 h treatment with 100  $\mu$ M Silibinin. That is, Silibinin is sensitive to pancreatic cancer cells.

#### 3.2. Silibinin promoted apoptosis of pancreatic cancer cells via JNK/SAPK signaling

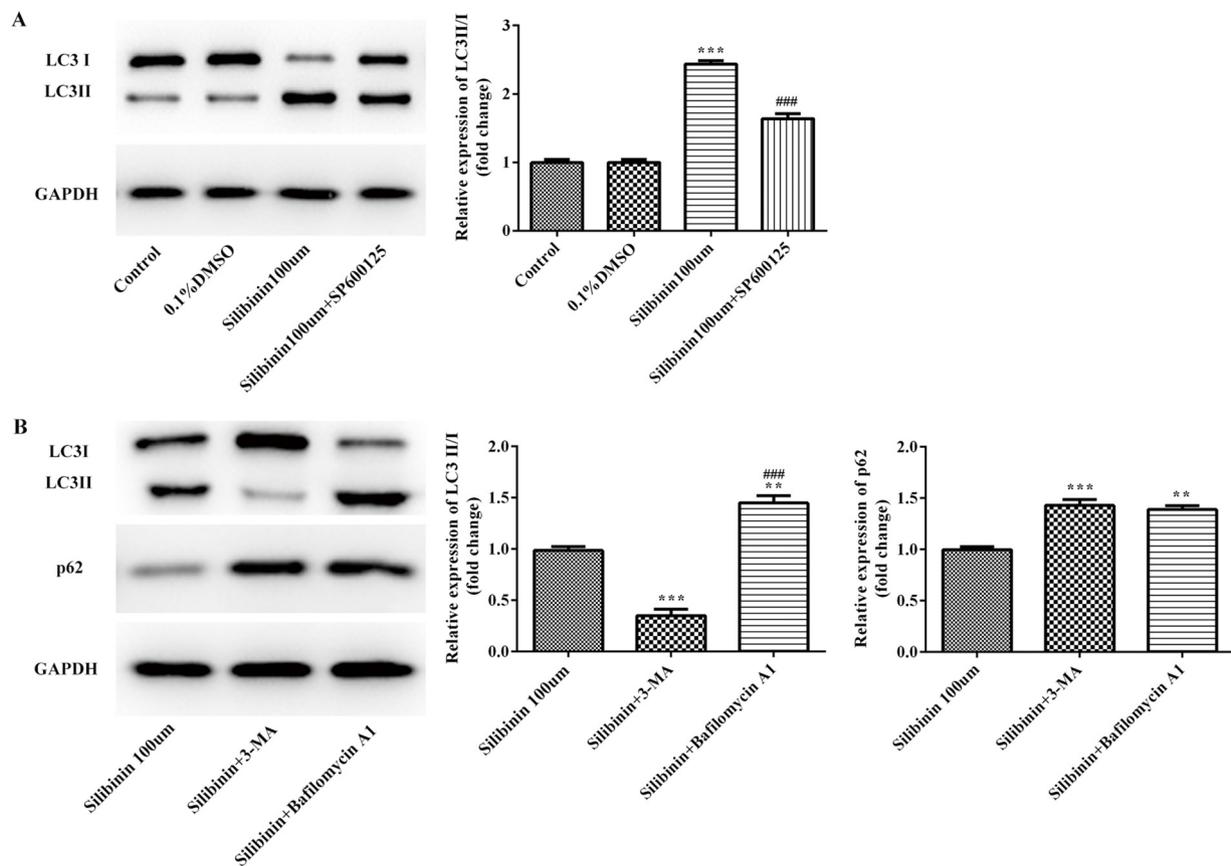
Previous investigation indicated that Silibinin activated JNK/SAPK by phosphorylation [13]. As shown in Fig. 2, the expression of phosphorylation of JNK/SAPK (p-JNK/SAPK) was upregulated after treatment of 100  $\mu$ M Silibinin. Furthermore, 10  $\mu$ M JNK/SAPK inhibitor (SP600125) was used to inhibit the activation of JNK/SAPK.

To evaluate whether inhibition of cell viability was caused by the induction of apoptosis, flow cytometry was performed. In SW1990 cells, cell apoptosis, including early stages and later stages, was significantly increased following the treatment with silibinin in Fig. 3A. Moreover, SP600125 reversed the induction effect of 100  $\mu$ M Silibinin in SW1990 cells.

Silibinin promoted mitochondrial apoptosis via JNK/SAPK signaling

As shown in Fig. 3B, to detect whether Silibinin induced apoptosis was mitochondrial apoptosis, the expression of Bcl-2 signaling family proteins and respiration function protein was detected. The expression of Bcl-2 and Bcl-xl was significantly decreased, whereas the expression of Bax was significantly increased compared with control. Cyt-c, the mitochondrial respiration function protein, was expressed significantly higher after the treatment of Silibinin compared with control. Additionally, JNK/SAPK inhibitor, SP600125 significantly reversed the expression changes induced by Silibinin.

Meanwhile, The high ATP levels was significantly decreased following treatment of Silibinin compared with control (Fig. 4A). In addition, High ROS levels initiated apoptosis by some anticancer drugs. In the present study, silibinin increased the ROS generation in SW1990



**Fig. 5.** Silibinin induced autophagy flux in pancreatic carcinoma. SW1990 cells were treated with 0.1% DMSO control or 100  $\mu$ M Silibinin or for 48 h, or pretreated JNK/SAPK specific inhibitor (SP600125) for 1 h before 100  $\mu$ M Silibinin treatment. (A) The expression of LC3II was determined by cell immunofluorescence. (B) The expression levels of autophagic proteins were detected by western blotting. Data shown are mean  $\pm$  SD of triplicate samples for each treatment. \*\*\* $P$  < 0.001 compared with the control; # $P$  < 0.05 compared with Silibinin 100  $\mu$ M.

cells, whereas SP600125 alleviates the effect of Silibinin (Fig. 4B). These results indicated that Silibinin promoted cell apoptosis mediated by a mitochondrial apoptosis pathway and JNK/SAPK signaling may serve a critical role in the induction of SW1990 cells apoptosis.

### 3.3. Silibinin promoted autophagy via JNK/SAPK signaling

Lots of anti-cancer drugs can induce both apoptosis and autophagy. We found that Silibinin induced conversion of LC3I to LC3II detected by immunoblotting (Fig. 5A). To estimate whether LC3 conversion influenced by autophagy induction or lysosome fusion, a LC3 conversion inhibitor, 3-MA, and a lysosome inhibitor, bafilomycin A1, were used. Results showed that 3-MA significantly inhibit Silibinin induced LC3 conversion, whereas bafilomycin A1 could enhance the effects of Silibinin (Fig. 5B). In addition, the substrate of autophagy, p62/SQSTM1, was significantly decreased following the treatment of Silibinin, whereas co-treated with 3-MA or bafilomycin A1 enhanced the expression of p62/SQSTM1 (Fig. 5B). In SW1990 cells, increase of fluorescence intensity of GFP-LC3B as compared to control revealed that Silibinin induced autophagosome formation, which further confirmed the immunoblotting results (Fig. 6A). The expression of autophagic protein Atg5 and Beclin-1 was increased after treatment with Silibinin (Fig. 6B). Furthermore, SP600125 was significantly reversed the effects of Silibinin on the autophagy flux. Therefore, Silibinin increased autophagy flux and can be mediated by JNK/SAPK signaling.

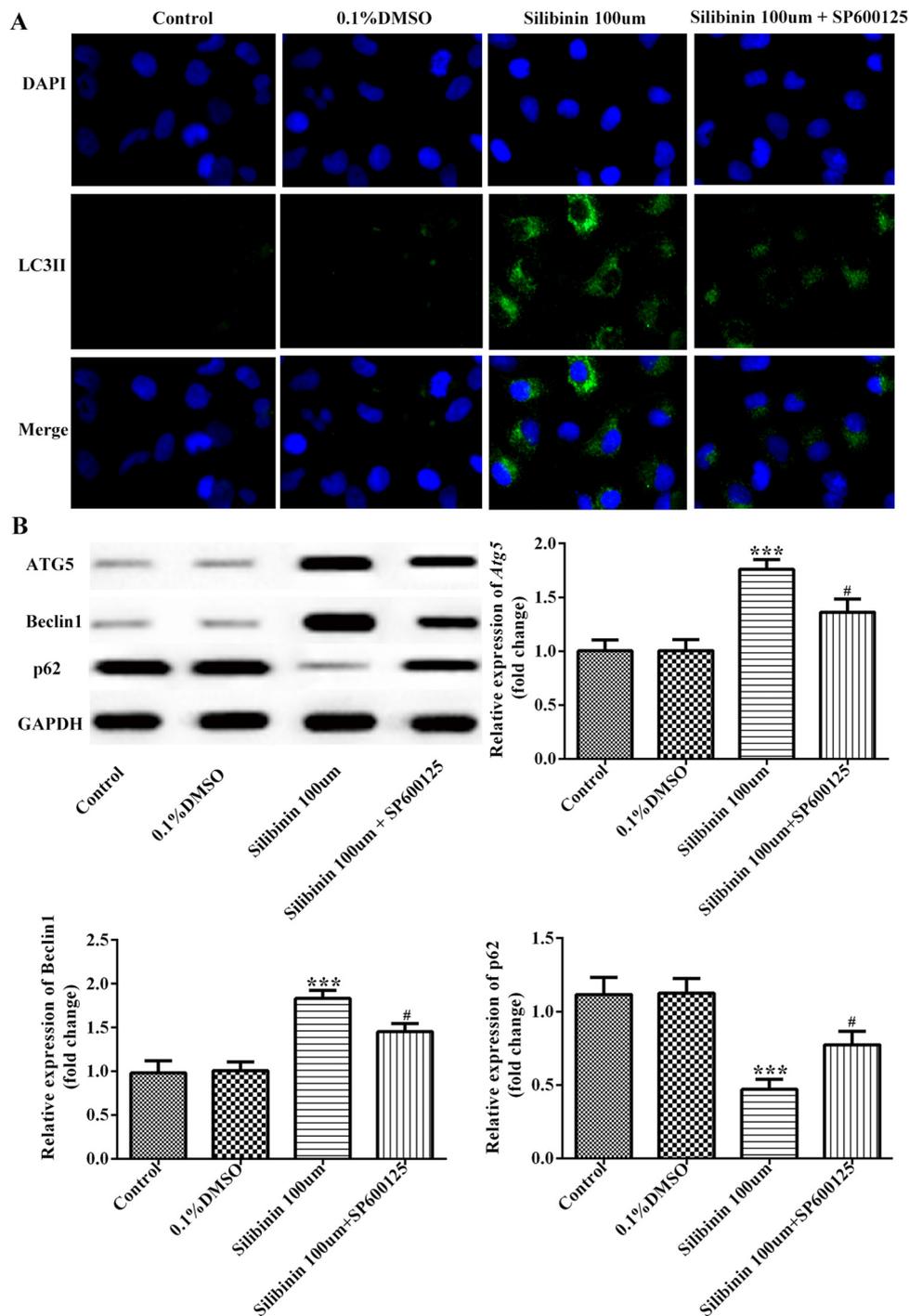
Bcl-2 signaling expression was usually associated with the expression of Beclin-1, while Bcl-2 is an anti-apoptosis protein. 3-MA significantly inhibited Silibinin induced apoptosis (Fig. 7A). Moreover, inhibition of Bcl-2 and Bcl-xl expression by Silibinin in SW1990 cells

was reversed by 3-MA, whereas elevation of Bax and CytC was significantly inhibited (Fig. 7B). The results showed that inhibiting autophagy promotes cell apoptosis in SW1990 cells.

## 4. Discussion

According to our previous investigation, we found that Silibinin promoted apoptosis and JNK/SAPK signaling in pancreatic cancer cells, and exerted an potential. The present study used a JNK/SAPK inhibitor SP600125 to investigate that Silibinin promoted cell apoptosis was mediated by JNK/SAPK signaling. In addition, Silibinin promoted cell autophagy in pancreatic cancer cells and also mediated by JNK/SAPK signaling.

Silibinin possess as an apoptotic inducer, autophagy modulator, cell cycle inhibitor to exert anti-cancer effects [22]. The present study indicated that Silibinin markedly suppressed the viability of pancreatic cancer cell lines, which is consistent with our previous research. Apoptosis is a pivotal effects in cancer initiation, progression and metastasis. The reported intrinsic apoptotic pathway is characterized by permeabilization of mitochondrial outer membrane, which is regulated by Bcl2 family members. Silibinin downregulated the anti-apoptotic protein, Bcl-2 and Bcl-xl, whereas upregulated the pro-apoptotic protein, Bax and Bim, and these proteins reside in mitochondrial outer membrane or cytosol, which promotes the release of certain factors by mitochondria and promotes apoptosis. Moreover, Cytc, part of the mitochondrial electron transport chain, participates in electron transfer and is an fundamental part of the energy production process. Early in 1996, report showing that Cytc plays an important role in the cell death using a cell free apoptotic system [23]. Bax is an important molecule to

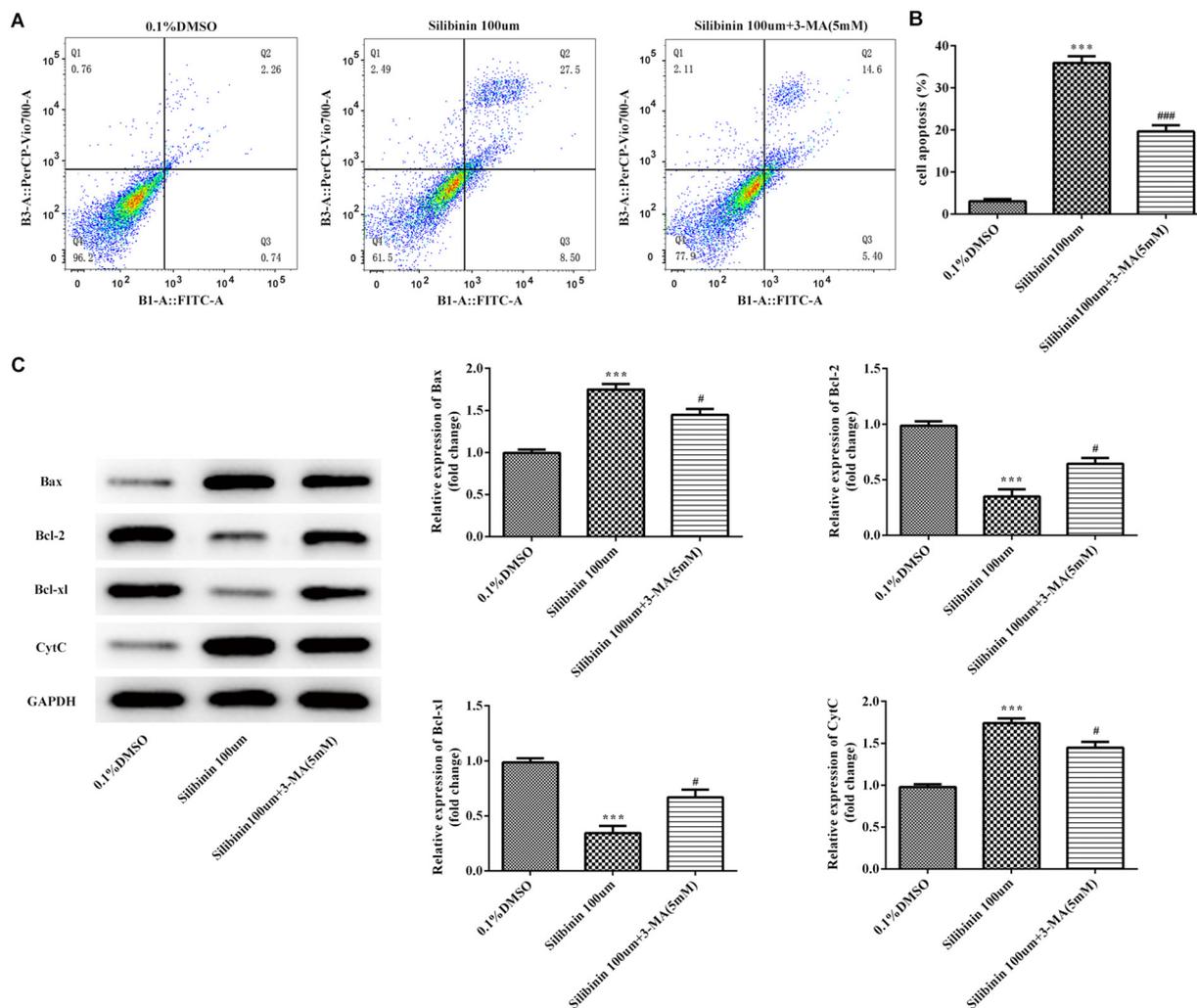


**Fig. 6.** Silibinin induced autophagy flux in pancreatic carcinoma. SW1990 cells were treated with 0.1% DMSO control or 100  $\mu$ M Silibinin or for 48 h, or pretreated JNK/SAPK specific inhibitor (SP600125) for 1 h before 100  $\mu$ M Silibinin treatment. (A) The expression of LC3II was determined by cell immunofluorescence. (B) The expression levels of autophagic proteins were detected by western blotting. Data shown are mean  $\pm$  SD of triplicate samples for each treatment. \*\*\*P < 0.001 compared with the control; #P < 0.05 compared with Silibinin 100  $\mu$ M.

open the permeability transition pore on mitochondrial membrane to promote the Cytc releasing into the cytoplasm [24]. Subsequently, Cytc activate caspase-9/caspase-3 mediated cascade to induce apoptosis [25]. In the present study, results indicated that Silibinin decreased the expression of Bcl-2 and Bcl-xl and increased the expression of Bax and Cytc. In addition, one of the most pivotal function of mitochondria is to produce energy. The generation of ATP, the direct energy source, was significantly decreased with the treatment of Silibinin. Besides, excessive generation of ROS damaged cells, eventually resulted in apoptosis. In the current study, Silibinin increased the expression of Cytc and

furthermore increased the ROS generation. Therefore, Silibinin may promote mitochondrial apoptosis to exert anti-tumor effects.

Apoptosis and autophagy can be resulted by same upstream signals and share molecular interaction between apoptosis and autophagy [26]. Previous investigation indicated that the Bcl-2 family of proteins regulates cell death both in apoptosis and non-apoptotic programmed cell death depended on the autophagy [27]. Bcl-2 binds to Beclin-1 to induce non apoptotic programmed cell death, and Bcl-2 influence the formation of autophagosomes partly by regulation of Beclin-1 [28]. Autophagy induction with Silibinin in pancreatic cancer cells was



**Fig. 7.** Silibinin promoted apoptosis via activating autophagy. (A and B) Cell apoptosis was detected by flowcytometry after treated with silibinin and autophagy inhibitor, 3-MA. (C) Apoptotic proteins were detected by western blotting. \*\*\*  $p < 0.001$  compared with control. #  $p < 0.05$ , ###  $p < 0.001$  compared with Silibinin 100  $\mu\text{M}$ .

determined by Western blot and immunofluorescence. The hallmark of autophagy LC3II in pancreatic cancer cells is accumulated by the treatment of Silibinin. In addition, the expression of autophagosome formation protein, Atg5 and Beclin-1, was significantly increased in Silibinin treatment compared with control. p62/SQSTM1 is another indicator of autophagy by directly linking to LC3II. And decrease in the expression of p62/SQSTM1 with Silibinin treatment was shown in pancreatic cancer cells. In spite of the expression of p62/SQSTM1 during autophagy process is controversial, our results are in accordance with previously investigated [29]. Under extreme conditions, JNK/SAPK-1 induces phosphorylation of Bcl-2, and dissociates the binding of Bcl-2 and Beclin-1, promoting autophagy; in addition, phosphorylation of Bcl-2 is also separated from Bax, thereby facilitating apoptosis [30]. In the present study, Silibinin inhibited the expression of Bcl-2 and promoted the expression of Beclin-1 and Bax to promote the autophagy and apoptosis in pancreatic cancer.

At present, the molecular pathways of pancreatic cancer apoptosis and autophagy by Silibinin, and the expression of apoptotic and autophagic molecules are rarely investigated. Silibinin can induce pro-apoptotic autophagy by activated p53 via ROS-p38 pathway and JNK/SAPK in HT1080 cells [31]. Our previously investigation also indicated that Silibinin activated the JNK/SAPK signaling in pancreatic cancer [13]. In order to clarify Silibinin may mediate apoptosis and autophagy through JNK/SAPK in pancreatic cancer, JNK/SAPK-specific inhibitor,

SP600125, was performed to co-treated with Silibinin in pancreatic cancer cells. The expression of apoptotic proteins of intracellular mitochondrial pathway was analyzed, and the results revealed that the expression levels of Bax, CytC in cells treated with Silibinin and SP600125 were significantly lower than in cells treated with Silibinin, and the expression changes of autophagic proteins of Atg5 and Beclin-1 were the same as the apoptotic proteins. These results indicated that SP600125 reversed the effects of Silibinin on apoptosis and autophagy.

### 5. Conclusion

In summary, silibinin can significantly induce apoptosis and autophagy in pancreatic cancer cells; activating JNK/SAPK pro-apoptotic and pro-autophagic signaling. Moreover, Our study suggest that Silibinin can to a promising anti-pancreatic cancer agent to be further investigated.

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