RNA Interference Mediates Livin Gene Silencing to Enhance Chemosensitivity of Pancreatic Adenocarcinoma in Vitro

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Research Article

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Abstract

Purpose RNA interference (RNAi) was used to silence the expression of Livin gene in human pancreatic adenocarcinoma cell line (PANC1), and to investigate the chemosensitivity of PANC1on gemcitabine after the Livin gene knockdown.

Methods Using Lipofectamine^{TM}2000 as a carrier, a small interfering RNA (siRNA) targeting Livin gene was transfected into PANC1 cells. The mRNA and protein expression of Livin, cleaved PARP (cPARP) and caspase-3 (cCaspase-3) in PANC1 cells were measured by RT-PCR and Western Blot. The protein bands density was analyzed by Image J software, and expressed by the density ratio with the corresponding internal control protein (β-actin). The density ratios of the cPARP and cCaspase-3 protein bands were analyzed by one-way analysis of variance and Student's t-test for comparison of multiple or two groups. A $p < 0.05$ was considered statistically significant.

Results Livin mRNA expression of PANC1 cells significantly decreased after transfection with Livin-siRNA # 1. The cPARP and cCaspase-3 induced by 50 nM gemcitabine were stronger than those induced by 25 nM after 48 hours and 72 hours in PANC1 cells.But the cPARP and cCaspase-3 induced by 100 nM gemcitabine did not significantly change compared with 50 nM. After transfection of Livin-siRNA # 1, cPARP and cCaspase-3 induced by both 25 nM and 50 nM gemcitabine were enhanced. This indicated that the apoptosis of PANC1 cells induced by gemcitabine was enhanced after Livin knockdown.

Conclusions RNAi-targeted Livin promotes apoptosis of PANCl cells in vitro through PARP and caspase-3 pathways, and enhances its chemosensitivity to gemcitabine.

Introduction

Pancreatic duct adenocarcinoma (PDAC) is increasing in the global population. It is estimated that about 7–9 % of cancer deaths in the world are attributed to PDAC, which is the seventh leading cause of cancer death. By 2030 year, PDAC will become the second leading cause of cancer-related death [1]. In the past 30 years, great progress has been made in the diagnosis and treatment of many diseases, but little progress has been made in improving the survival rate of PDAC and reducing the mortality of PDAC. Early surgical resection is the first choice for the treatment of PDAC, but most patients are in the advanced stage at the initial diagnosis. Only 10–15 % of lesions are resectable and 2 / 3 of them are difficult to tolerate pancreatoduodenectomy [2]. Chemotherapy has become the main palliative treatment for most atients with unresectable PDAC. Gemcitabine has been approved by FDA as a first-line treatment drug for patients with advanced PDAC, but its effectiveness is still controversial. Some studies have proved that gemcitabine can only improve the average survival time of patients with advanced PDAC by 1.5–3 months [2]. Moreover, long-term exposure to gemcitabine lead to drug resistance. And the resistance to gemcitabine is also the main reason for the failure of PDAC chemotherapy. Because the resistance to conventional chemotherapy depends on the ability of apoptosis, it is important to understand the abnormal apoptosis of PDAC. Therefore, the molecular targeted therapy for cell apoptosis and

gemcitabine resistance may provide new ideas to improve the survival rate of patients with advanced PDAC.

The imbalance between apoptosis and cell cycle is closely related to the occurrence and development of tumor. The basic level of apoptosis is strictly controlled by the family of endogenous inhibitor of apoptosis proteins (IAPs). IAPs are a kind of structurally related cytokines, which can inhibit apoptosis. IAPs have at least one characteristic protein domain, which is called baculoviral IAPs repeat, and play a role in preventing apoptosis through a variety of mechanisms, including caspase inhibition or involvement in survival signaling pathways [3]. Livin, a new member of IAPs family, is located in human chromosome 20q13.3, with a total length of 4.6 kb. It contains a characteristic baculoviral IAPs repeat domain of the IAPs family and a carboxyl-terminated ring zinc finger-like domain. And it has two splice isomers, including longer-chain Livin and shorter-chain Livin β. The Livin a encodes 298 amino acids that differ from the Livin β by 18 amino acid residues. This difference lies between the baculoviral IAPs repeat and ring zinc finger-like domains [3]. Livin gene is lowly expressed in most normal tissues, and highly expressed in most malignant tumors, such as ovarian cancer, head and neck squamous cell carcinoma, malignant melanoma [4–8]. The high expression of Livin in most tumors is associated with more aggressive biological behavior, chemotherapy resistance and poor prognosis. Therefore, we believe that Livin will be a potential and attractive molecular target.

It has been reported that the Livin gene is highly expressed in PDAC, while it is low or not expressed in paracancerous The high expression of Livin gene is closely related to the biological behavior of PDAC, such as differentiation and lymph node metastasis, and poor prognosis [9]. Antisense oligonucleotide or small interfering RNA (siRNA) mediated knockdown of Livin has been shown to reduce the proliferation potential of tumor cells, such as malignant melanoma, and induce the sensitivity to pro-apoptotic analogues and chemoradiotherapy [5–6]. Therefore, we believe that Livin may be a potential adjuvant drug for PDAC chemotherapy. In this study, we firstly used siRNA-mediated knockdown of Livin in human pancreatic adenocarcinoma cell line (PANC1) to verify whether Livin gene therapy combined with gemcitabine chemotherapy had a synergistic sensitization effect on apoptosis.

Methods

Cell culture

PANC1 cells were purchased from Cancer Institute, Chinese Academy of Medical Sciences (Shanghai, China). Cells were cultured in DMEM medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U / ml penicillin, and 100 μg / ml streptomycin in a 37 ° C constant temperature incubator with 5 % CO_2.

siRNA design

The siRNA sequences were designed on the website of Dharmacon's siRNA Design Center. Two Livin-siRNA sequences with the highest scores were selected for experiments. They were siLivin 1 # (sense

5’GAGAGGUCCAGUCUGAAAGUU3’, antisense 5’CUUUCAGACUGGACCUCUCUU3’) and siLivin 2 # (sense 5’GAGAGAGGUCCAGUCUGAAUU3’, antisense 5’UUCAGACUGGACCUCUCUCUU3’). The siRNA negative control (sictrl sense 5’UUCUCCGAACGUGUCACGUUU3’, antisense 5’ACGUGACACGUUCGGAGAAUU3’) was a random sequence and did not target any mammalian genes. The siRNA sequences were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China).

RNA extraction and RT-PCR

In accordance with the manufacturer's instructions, total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). In the presence of 1 μl random primers, 2 μg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The PCR primer sequences are as follows: Livin sense 5’ATGGAGTCCTGTGGCATCCAC3’, antisense 5’CGGAGTACTTGCGCTCAGGAGG3’, β-actin (internal control) sense 5’ATGGAGTCCTGTGGCATCCAC3’, antisense, 5’CGGAGTACTTGCGCTCAGGAGG3’. The PCR amplification conditions were as follows: 1 cycle at 94 ° C for 5 minutes; 94 ° C for 30 seconds, 55 / 59 ° C for 30 seconds, and 72 ° C for 30 seconds (Livin PCR cycle number 36, β-actin PCR cycle number 21); and finally, 72 ° C for 5 minutes.

Protein cleavage and Western blot (WB)

After washing twice with precooled PBS, the cells were fully lysed in RIPA buffer (50 mM Tris.HCl, pH 7.6, 150 mM NaCl, 1% Deoxycholic acid, 0.1% SDS, 1% NP-40, 2 mM EDTA, 2 mM EGTA). After centrifuge at 12,000 g for 15 minutes, the supernatant was aspirated, and the protein concentration was determined by Bradford. WB used standard experimental methods. According to the molecular weight of the target protein, a suitable concentration of separation gel was selected for SDS-polyacrylamide gel electrophoresis, and the protein was transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The polyvinylidene difluoride membrane was sealed with TBS-T (10 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) solution containing 5% skimmed milk powder or 3% BSA at room temperature for one to two hours, and then incubated with the main antibody for one hour. The antibodies were as follows: poly ADP ribose polymerase (PARP), cleaved caspase-3 antibodies (Cell Signaling Technology, Danvers, MA, USA), and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the secondary antibodies (1: 4000 ~ 1: 10000) were added in TBS-T containing 5% skimmed milk powder, and incubated in a rotating shaking table for one hour at room temperature. The protein bands density was analyzed by Image J software, and expressed as the density ratio of the corresponding β-actin protein.

Chemosensitivity assay

Gemcitabine was purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). After treating PANC1 cells with different concentrations of gemcitabine (25 nM, 50 nM, 100 nM) for 48 hours and 72 hours, the activity of cleaved PARP (cPARP) and cleaved caspase-3 (cCaspase-3) was detected by WB. And the appropriate concentration of gemcitabine was determined for the next experiment. Then, after treating PANC1 cells with Livin-siRNA for 24 hours and gemcitabine at different concentrations for 48 hours, the activity of cPARP and cCaspase-3 were detected by WB.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software package (SPSS, Chicago, IL, USA). All measurements were repeated three times and the data were expressed as mean ± standard deviation. The density ratios of the cPARP and cCaspase-3 protein bands were analyzed by one-way analysis of variance and Student's t-test for comparison of multiple or two groups. A $p < 0.05$ was considered statistically significant.

Results

Detection of siRNA knockdown efficiency

After transfection of 200 nM Livin-siRNA in PANC1 cells for 64 hours, the knockdown efficiency was measured by RT-PCR with β-actin as the internal control. The expression of Livin mRNA in each group was detected by RT-PCR. The results showed that two pairs of siRNA sequences had decreased expression of Livin mRNA after transfection into PANC1 cells. Among them, the expression of Livin mRNA in siLivin # 1 group was more significantly reduced. Therefore, all subsequent experiments used siLivin # 1 to silence Livin gene.

Gemcitabine induces apoptosis

PANC1 cells were treated with different concentrations of gemcitabine (25 nM, 50 nM, 100 nM) for 48 hours and 72 hours to detect cPARP and cCaspase-3 in Fig. 2. The protein bands density of each group was measured by Image J software. The averages of density radios were shown in Table 1. The results showed that the cPARP and cCaspase-3 induced by 50 nM gemcitabine were stronger than those induced by 25 nM after 48 hours and 72 hours in PANC1 cells. But the cPARP and cCaspase-3 induced by 100 nM gemcitabine did not significantly change compared with 50 nM.

Table 1
Detection of gemcitabine-induced apoptosis

Gemcitabine		**48hrs**			**72hrs**		**F**	**p**
(nM)	25	50	100	25	50	100		
cPARP	0.158 ± 0.0033	0.205 ± 0.0142	0.211 ± 0.0176	0.111 ± 0.0060	0.298 ± 0.0143	0.246 ± 0.0158	14.479; 170.646	0.005; < 0.001
cCaspase-3	0.168 ± 0.0052	0.234 ± 0.0100	0.156 ± 0.0185	0.260 ± 0.0283	0.431 ± 0.0036	0.413 ± 0.0079	34.085; 90.338	0.001; < 0.001
nM: nmol/l; hrs:hours								

Livin knockdown increased apoptosis of PANC1 cells induced by gemcitabine

After Livin knockdown in PANC1 cells for 24 hours, cells were treated with gemcitabine for 48 hours. The cPARP and cCaspase-3 were detected by WB in Fig. 3. And the protein bands density of each group measured by Image J software was shown in Table 2. The results showed that after Livin knockdown, cPARP and cCaspase-3 were enhanced at both 25 nM and 50 nM gemcitabine. This indicated that gemcitabine induced apoptosis of PANC1 cells was enhanced after Livin knockdown.

Table 2
Increased apoptosis of gemcitabine-induced PANC1 cells after Livin knockdown

Gemcitabine		**sictrl**			**siLivin**		**F**	**p**
(nM)	0	25	50	0	25	50		
cPARP	0.103 ± 0.0062	0.597 ± 0.0585	0.899 ± 0.0590	0.402 ± 0.0047	0.978 ± 0.0387	0.980 ± 0.0229	209.570; 488.806	< 0.001; < 0.001
cCaspase-3	0	0.165 ± 0.0068	0.531 ± 0.0387	0	0.418 ± 0.0389	0.679 ± 0.0200		< 0.001; < .0001
nM: nmol/l;sictrl: siRNA negative control; siLivin: Livin-siRNA								

Discussion

PDAC is a very malignant tumor. Although medical science has made great progress in recent years, especially in tumor chemotherapy, the overall survival rate of patients with PDAC has not improved significantly, with the 5-year survival rate less than 5–7 % [1]. In the past few years, the use of the deoxycytidine analog gemcitabine has been shown to have some clinical benefit and slightly extend the average survival time. Therefore, gemcitabine has become a first-line chemotherapy drug for PDAC. However, studies have shown that long-term exposure to gemcitabine can lead to acquired resistance [2]. Among the causes of chemotherapy resistance, apoptotic defects are considered to be the main reason, because many chemotherapeutic drugs work by inducing apoptosis [2]. Therefore, the resistance of tumor cells to cytotoxic drugs may be attributed to resistance to apoptosis. It is of great significance to explore the molecular determinants of apoptosis resistance in PDAC cells for the development of new and more effective treatment strategies. In this study, from the new perspective of Livin as a target gene for apoptosis resistance, RNA interference (RNAi) was used to effectively silence Livin gene expression, and combined with gemcitabine to treat PDAC. This study firstly proved that the combined treatment of Livin knockdown and gemcitabine had synergistic sensitization effect on PDAC in vitro.

Livin is a new member of the IAPs family. It is highly expressed in transformed cells and a variety of malignancies, but it is not expressed in most normal differentiated tissues except placenta, normal testes, and spinal cord [3]. According to reports, Livin's anti-apoptotic mechanism is mainly inhibiting the activity of the mitochondrial apoptotic signaling pathway molecules caspase 3, 7 and 9. They can activate transforming growth factor β-activated kinase1 and its binding protein to mediate mitogen-activated protein kinases pathways, and play the role of apoptosis mediated by tumor necrosis factor-alpha and interleukin 1β converting enzyme [7]. In addition, Livin can inhibit E3 ubiquitin ligase-like activity. It promotes the degradation of Smac / DIABLO, which is a key endogenous regulator of all IAPs [7]. It has been proved that Livin can improve the viability of cancer cells, and make it easy for cancer cells to avoid immune surveillance and cytotoxin effects. Its expression level is closely related to the biological behavior of tumor, sensitivity of radiotherapy and chemotherapy and prognosis, and can inhibit the apoptosis induced by chemotherapy drugs [8].

In view of Livin's overexpression in multidrug-resistant tumor cells, tumor treatment with Livin as target has attracted more and more attention. Studies have shown that down-regulating Livin gene expression by RNAi or antisense oligonucleotides can inhibit the growth of cancer cells and allow tumor cells to redifferentiate towards proapoptotic anticancer drugs [6–8]. Wang H et al. conducted in vitro and in vivo studies using human malignant melanoma cell lines (LiBr), which showed that down-regulation of Livin expression by siRNA could significantly inhibit cell proliferation and induce apoptosis [6]. RNAi is a strategy for suppressing gene expression after highly specific transcriptionMore specifically, siRNA, as a means of reducing the expression of specific proteins in vitro and in vivo, makes almost all protein targets be inhibited by these sequences. The use of siRNA for silencing of the Livin gene is well established. This study designed two pairs of siRNAs based on Livin sequences, which showed differences in silencing efficiency. We chose the one with better efficiency for subsequent experiments. It was successfully transfected into PANC1 cells. the level of Livin mRNA and protein transcription in the stable transfection group was significantly reduced. The results suggest that Livin-siRNA can specifically down-regulate the expression of Livin in PANC1 cells, and the siRNA silencing efficiency of different parts of the same target mRNA may be different.

The concentration of gemcitabine used in the treatment of PANC1 cells in this experiment showed that the apoptosis induced by gemcitabine at 50 nM was more significant than that at 25 nM. But the apoptosis induced by 100 nM gemcitabine was not changed from 50 nM. This indicates that there may be an upper limit for the concentration of gemcitabine and chemotherapy resistance. After Livin knockdown in PANC1 cells, the apoptosis induced by gemcitabine was enhanced at both 25 nM and 50 nM, which indicates that Livin-siRNA targeted silencing of Livin gene expression combined with gemcitabine has a synergistic effect on PDAC. Liu Y et al. found that siRNA-Livin could reduce the multidrug resistance-related protein gene expression in glioblastoma cell lines (U251 cells and U251 stem cells) to reduce the resistance of glioblastoma to chemotherapy [10]. Wang Z et al. found that silencing Livin could induce apoptosis and autophagic death of renal cancer cells, and improve the sensitivity of renal cancer cells to cisplatin [11]. Oh BY et al. found that siRNA-mediated down-regulation of Livin gene expression could significantly inhibit the growth of colon cancer cell lines (HCT116) and enhance the

cytotoxic effect of anti-cancer drugs such as 5-fluorouracil [12]. Our results are consistent with these research. As for the exact mechanism of how Livin down-regulation can enhance the chemosensitivity of gemcitabine, it is currently believed that Livin down-regulates cell death through the mitochondrial signaling pathway or increases cell death through the death receptor pathway. Our study also showed that Livin knockdown induced apoptosis by activating caspase 3 and PARP. Yoon TM et al. showed that siRNA-mediated Livin gene knockdown resulted in the down-regulation of Livin expression and the increase of apoptosis in three human head and neck squamous cell carcinoma cell lines (SNU1041, PCI1 and PCI50 cells). It was caused by the activation of mitochondrial apoptotic signaling pathways caspase-3 and caspase-7. At the same time, the knockdown of Livin gene increased the sensitivity of here cell lines to cisplatin, 5-fluorouracil and docetaxel [5]. This is basically consistent with our results. These results indicate that Livin may be an important determinant of tumorigenesis. And RNAi targeting Livin is expected to be a new strategy for chemotherapy sensitization on PDAC.

In conclusion, Livin-siRNA can effectively silence the overexpression of Livin gene in pancreatic cancer PANCl cells, and regulate the apoptosis of PANCl cells through PARP and caspase-3 proteolytic pathway. Livin-targeted RNAi can significantly promote the apoptosis of PANC1 cells in vitro and increase its sensitivity to gemcitabine. Therefore, the combined application of RNAi targeting Livin and gemcitabine may be an effective and safe strategy for PDAC treatment.

Declarations

Funding

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Conflicts of interest

All authors have no any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Authors' contributions

In this study, LY designed the research and drafted the paper. WYJ made substantial contributions on analysis and interpretation of the data. YNN conducted research and acquisition data. LJ made great contributions on statistical analysis. HW made critical revision for this manuscript. WZM made great contributions on study supervision, obtained funding and final approval of the version. All authors read and approved the final manuscript.

Ethics approval

Not applicable

Consent to Participate (Ethics)

Not applicable

Consent to Publish (Ethics)

All authors consent to publish

Compliance with Ethical Standards

Not applicable

Informed consent

Not applicable

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References

1. Sielaff CM, Mousa SA (2018) Status and future directions in the management of pancreatic cancer: potential impact of nanotechnology. J Cancer Res Clin Oncol 144(7):1205–1217
2. Miller AL, Garcia PL, Yoon KJ (2020) Developing effective combination therapy for pancreatic cancer: an overview.Pharmacol Res. Mar 2:104740
3. Zareifar S, Ghorbani S, Monabbati A et al (2018 May) Expression of antiapoptotic proteins livin and survivin in pediatric AML patients, as prognostic markers. Pediatr Hematol Oncol 35(4):250–256
4. Zhang Z, Dou X, Yang H et al (2020 Feb) Association of expression of p53, livin, ERCC1, BRCA1 and PARP1 in epithelial ovarian cancer tissue with drug resistance and prognosis. Pathol Res Pract 216(2):152794
5. Yoon TM, Kim SA, Lee DH et al. Livin enhances chemoresistance in head and neck squamous cell carcinoma.Oncol Rep. 2017 Jun;37(6):3667–3673
6. Wang H, Yang Y, Wang W et al (2017 May) Single-chain antibody-delivered Livin siRNA inhibits human malignant melanoma growth in vitro and in vivo. Tumour Biol 39(5):1010428317701645
7. Badr EA, El TE Sayed, Assar I MF, et al. A pilot study of Livin gene and Yes-associated protein 1 expression in hepatocellular carcinoma patients. Heliyon. 2019 Nov 21;5(11):e02798
8. Liu S, Li X, Li Q (2018 May) etal. Silencing Livin improved the sensitivity of colon cancer cells to 5-fluorouracil by regulating crosstalk between apoptosis and autophagy. Oncol Lett 15(5):7707–7715
9. Lopes RB, Gangeswaran R, McNeish IA et al (2007) Expression of the IAP protein family is dysregulated in pancreatic cancer cells and is important for resistance to chemotherapy. Int J Cancer.

Jun 1;120(11):2344-52

10. Liu Y, Guo Q, Zhang H et al (2015 Oct) Effect of siRNA-Livin on drug resistance to chemotherapy in glioma U251 cells and CD133 + stem cells. Exp Ther Med 10(4):1317–1323
11. Wang Z, Liu S, Ding K et al (2016 Nov) Silencing Livin induces apoptotic and autophagic cell death, increasing chemotherapeutic sensitivity to cisplatin of renal carcinoma cells. Tumour Biol 37(11):15133–15143
12. Oh BY, Kim KH, Chung SS et al (2016 Dec) Silencing the livin gene enhances the cytotoxic effects of anticancer drugs on colon cancer cells. Ann Surg Treat Res 91(6):273–277

Figures

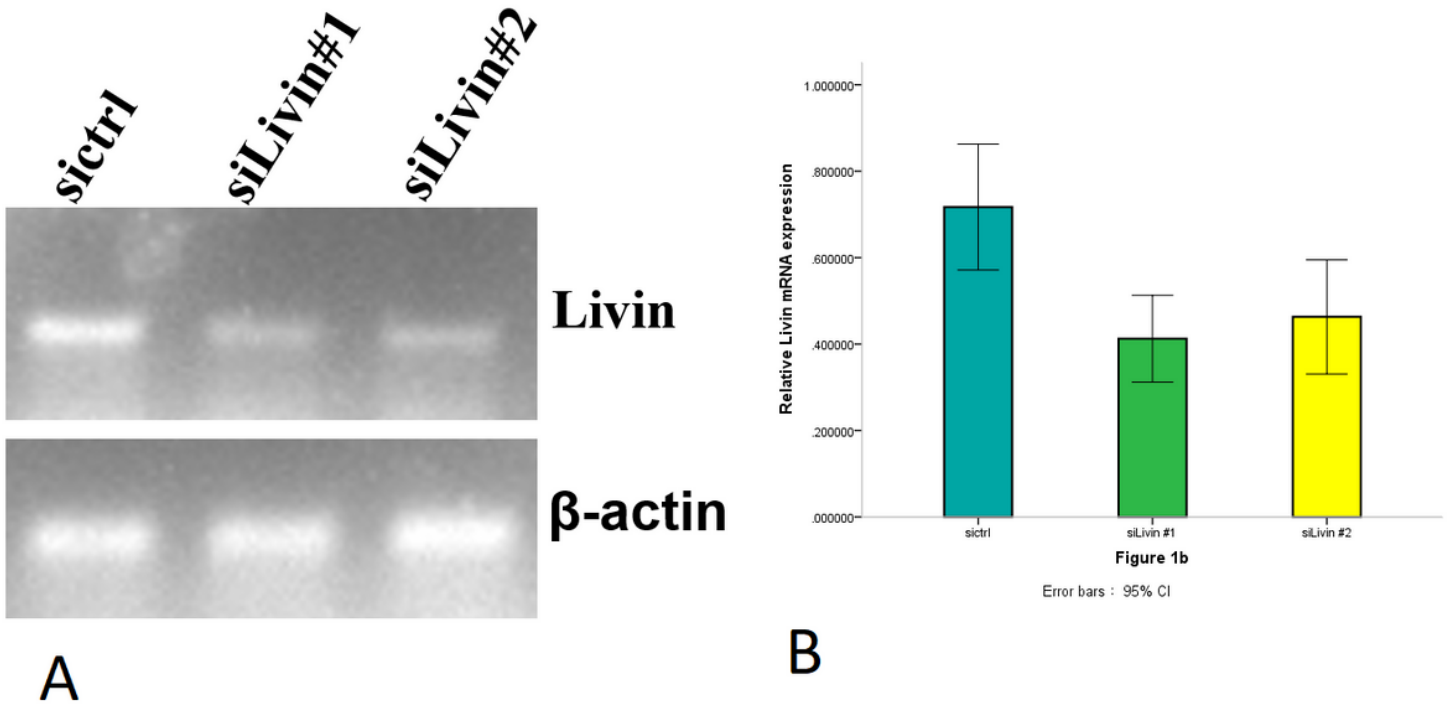

Figure 1

The expression of Livin mRNA in each group was detected by RT-PCR. The decrease of Livin mRNA expression in siLivin # 1 group was more significant.

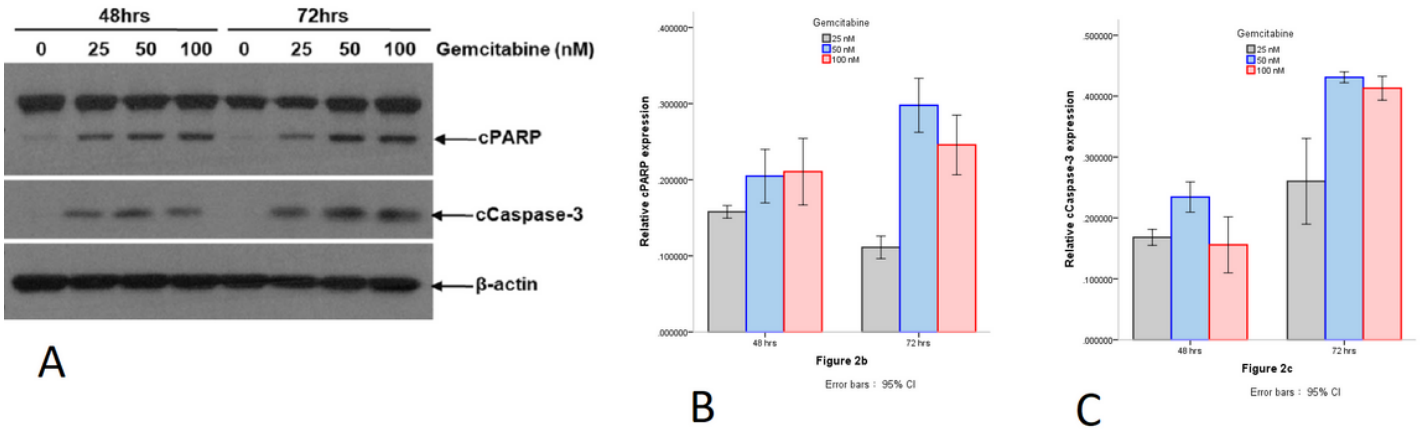

Figure 2

The expression of cPARP and cCaspase-3 protein in each group were detected by WB. 50 nM gemcitabine-induced cPARP and cCaspase-3 were stronger than those induced by 25 nM after 48 hours and 72 hours.

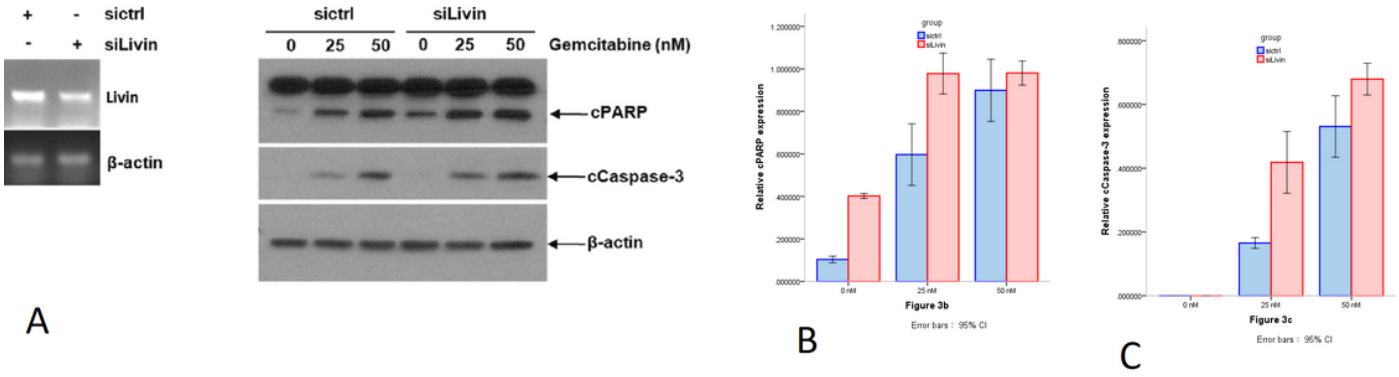

Figure 3

The expression of cPARP and cCaspase-3 protein in each group were detected by WB. After Livin knockdown in PANC1 cells, the apoptosis induced by gemcitabine was enhanced at both 25 nM and 50 nM. Livin knockdown increased the apoptosis of PANC1 cells induced by gemcitabine.