Original Article
The effects of miRNA-1180 on suppression of pancreatic cancer

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Abstract: The aim of this study was to evaluate the effects of miR-1180 on pancreatic cancer. We sampled adjacent cancer and carcinoma tissues from 30 pancreatic cancer patients and measured miR-1180 expression by qRT-PCR and NF-κB protein expression by immunohistochemistry. To explore the effects of this miRNA in cell culture, we used pancreatic cancer (PANC-1) cells that received only vehicle (negative control, NC), miR-1180 mimic, or miR-1180-inhibitor. Cells were treated with cisplatin to induce apoptosis. Proliferation, apoptosis, cell cycle progression, cell invasion, and cell migration were assessed in the three cell groups. The expression levels of relevant proteins (TNIP2, NF-κB, MMP-2, MMP-9, Bax, Bcl-2, p21, and cyclin D1) in each cell group were determined by western blotting. Compared with healthy tissue adjacent to carcinoma tissues, miR-1180 expression in cancer tissues was significantly enhanced (P<0.05). NF-κB protein had a similar expression pattern to miR-1180; miR-1180 expression was positively correlated with NF-κB expression. The invasion and wound healing abilities of miR-1180-inhibited cells were significantly reduced compared with the NC or miR-1180-expressing cells (P<0.05). The cell proliferation rate of miR-1180-inihibited cells was also significantly lower than that of NC or miR-1180-expressing cells (P<0.05), while the cell apoptosis and G1 phase rates of miR-1180-inihibited cells were significantly higher than the NC or miR-1180-expressing cells (P<0.05). In conclusion, suppressing miR-1180 expression may exert anti-cancer effects on pancreatic cancer cells via regulation of TNIP 2/NF-κB signaling and the downstream MMP-2/-9, Bax, Bcl-2, p21, and cyclin D1 factors.

Keywords: miR-1180, PANC-1, NF-κB, MMP-2/-9, Bax, Bcl-2, P21, Cyclin D1

Introduction

Since early diagnosis of pancreatic cancer is rare, the rate of radical surgeries is low, the prognosis is poor, and the mortality is high [1, 2]. A better understanding of the mechanisms underlying pancreatic cancer will ultimately improve clinical diagnosis and advance treatment options. Human microRNAs (miRNAs) are non-coding RNAs of approximately 22 nucleotides that interact with target genes to regulate gene expression. miRNAs play important roles in proliferation, apoptosis, and metastasis of tumor cells [3, 4].

NF-κB is transcription factor expressed in many cell types in vivo. NF-κB can bind to nucleotide sequences in many gene promoters and activate gene transcription and promoter activities

of these genes. Furthermore, NF-κB is involved in the pathogenesis of various diseases, not only by affecting cell proliferation, apoptosis, growth, differentiation, cell cycle progression, and immune responses, but also by affecting tumor invasion and metastasis [5-7]. Studies of NF-κB in pancreatic cancer, however, have been limited. In this study, we investigated the role of miR-1180 in the inhibition of pancreatic cancer cell activity via the NF-κB signaling pathway.

Materials and methods

Tissue specimens

Fresh pancreatic cancer tissues and normal pancreas tissues were collected from 30 pancreatic cancer patients, who were diagnosed by histopathology. All samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Using SYBR Green I (Invitrogen) dye according to manufacturer's instructions, qRT-PCR was performed and analyzed using a 7500 Fast Real-Time Sequence detection system and software (Applied Biosystems). The qRT-PCR reactions for miRNAs were performed as follows: 95°C for 3 min, followed by 40 cycles (each 30 s) at 95°C, 58°C, and 72°C. miR-1180 expression levels were normalized to the expression levels of U6 small nuclear RNA (snRNA). The qRT-PCR conditions for genes were set at 95°C for 10 min, followed by 40 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 1 min.

Immunohistochemistry (IHC)

All specimens were fixed with 10% formaldehyde solution and embedded in paraffin. Samples were then sectioned to a thickness of 4 µm. IHC was performed on the samples to detect the expression of NF-κB in pancreatic cancer and adjacent normal tissues using the streptavidin-peroxidase method.

Cell culture and transfection

Human pancreatic cancer (PANC-1) cells were purchased from the American Type Culture Collection (ATCC, USA). Cells were divided into three groups for treatment: NC (negative control), miR-1180 mimic (miR-1180), and miR-1180 inhibitor (miR-1180-in). The miR-1180 mimic, miR-1180 inhibitor, and negative control were purchased from Kingsy Biotechnology Co., Ltd. Cells were grown in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) as recommended. We used Lipofectamine 2000 reagent (Invitrogen) to transfect oligonucleotides according to the manufacturer's protocol. Cells were treated with cisplatin (0.4 µg/ml dissolved in PBS, Sigma) for 24 h as an apoptosis inducer.

Cell proliferation assay

Cell proliferation was assessed using an MTS (3-[4, 5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) assay. Cells (3000) were plated in wells of a 96-well plate and MTS was added. Cells were incubated at 37°C for 4 h. Optical density was measured at 490 nm using a microplate reader (BioTek Instruments Inc.) to compare the treatment groups. Each group was repeated three times.

Flow cytometric analysis

Cells were counted in logarithmic growth phase and seeded in 6 plates. Cells were collected and washed twice with ice-cold PBS. A 70% ethanol solution was then added to fix the cells with an overnight incubation at 4°C. Cells were washed with 500 µL PBS and 10 µL RNase (10 g/L) at 37°C for 30 min. Cells were stained with 10 µL propidium iodide (PI, 1 g/L). Cell apoptosis and cell cycle progression were analyzed by flow cytometry.

Transwell cell invasion assay

Diluted extracellular matrix (ECM) glue (100 µL) was added to the Transwell chamber and incubated at 37°C for 60 min to ensure complete polymerization. Then, 600 µL RPMI-1640 medium containing 10% fetal bovine serum was added to the lower chamber and incubated for 60 min. Logarithmic PANC-1 cells were digested with 0.25% pancreatin and plated in the upper chambers at a density of 5×10^5 cells/well in 100 µL of cell suspension. After 48 h, the Transwell chamber was removed and a cotton swab was used to wipe the polycarbonate membrane ECM glue. The upper and lower chambers were rinsed with D-Hanks' surface liquid rinse, cooled, and dried. Cells were stained with 0.1% crystal violet for 10 min. After removal of the excess crystal violet dye, plates were observed under a light microscope. Five fields (100× magnification) of each membrane were randomly selected for analysis. The number of cells on the surface of the polycarbonate membrane was counted. The invasion ability of the cells was expressed as the invasion cell number. The experiment was repeated three times.

Wound healing analysis

After transfection for 36 h, suspended cells were seeded in 6-well plates at a concentration of 5×10^5/well. The next day, a 20-µL gun head was used to plate vertical lines on the bottom of wells. Cells were then washed with PBS and incubated in serum-free medium. Photographs were obtained at 48 h to compare the migration ability of the three groups of cells.
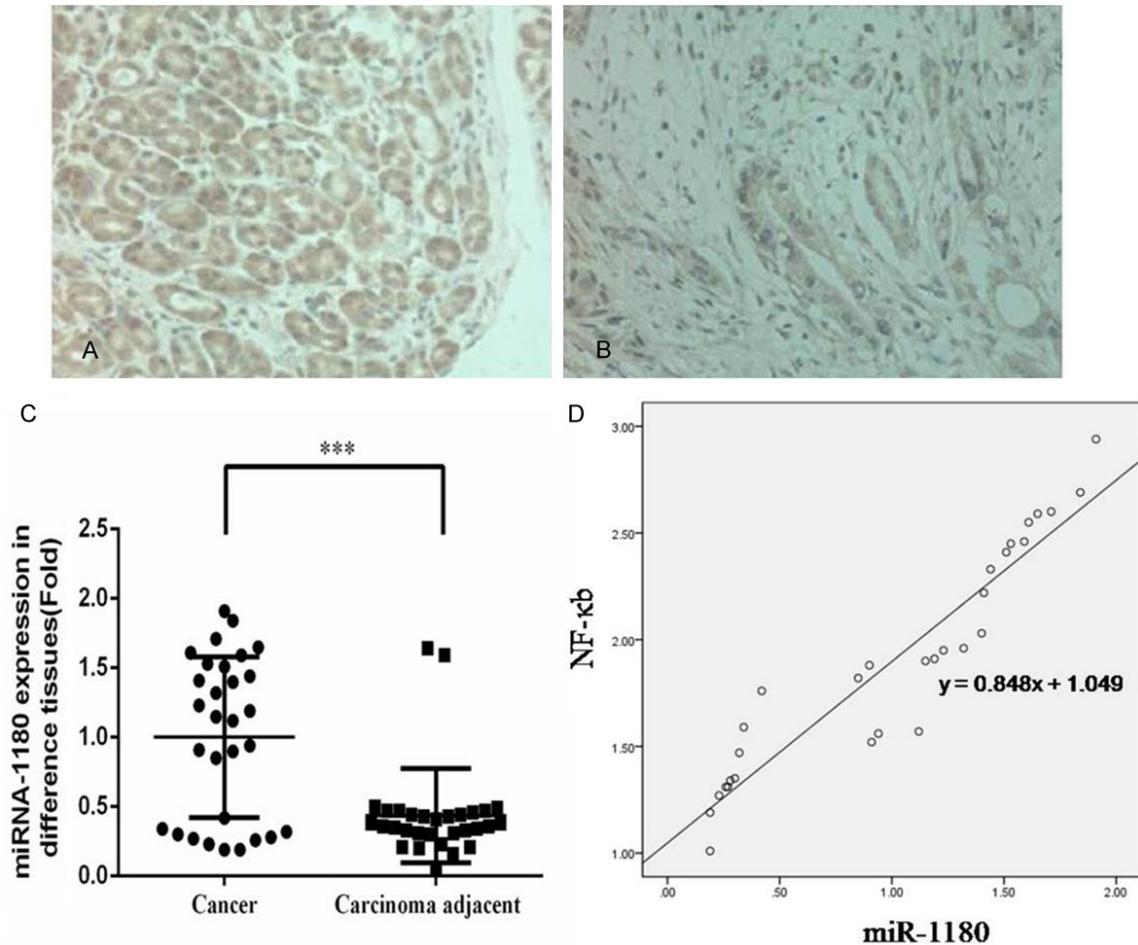
Figure 1. Clinical relevance analysis. A. The NF-κB protein expression in pancreatic cancer tissue by IHC (×200). B. The NF-κB protein expression in carcinoma adjacent tissue by IHC (×200). C. The miR-1180 gene expression in cancer and carcinoma adjacent tissues (Fold). ***: *P<0.05*, compared with NC group. D. The correlation between miR-1180 gene expression and NF-κB protein expression.

Western blotting

The cells were centrifuged and total protein was extracted. Proteins were separated on a 15% acrylamide gel by electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% skim milk for 1 h and then incubated with rabbit anti-human TNIP2, matrix metalloproteinase (MMP)-2, NF-κB, MMP-9, Bax, Bcl-2, cyclin D1, or p21 polyclonal antibodies (Abcam, 1:500) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Abcam, 1:2000) at room temperature for 2 h. After washing, electrochemiluminescence (ECL) substrate was added, and images were assessed on x-ray film for data analysis and

gray value detection. GAPDH was used as the internal reference.

Statistical analyses

All data were analyzed using SPSS 19.0 statistics software (IBM). Cell counts are expressed as the mean ± standard deviation (SD). Student's t-tests were used to compare two groups. P<0.05 indicated a statistically significant difference.

Results

NF-κB and miR-1180 expression in pancreatic cancer tissue

IHC assays indicated that expression of NF-κB protein was significantly upregulated in pancre-
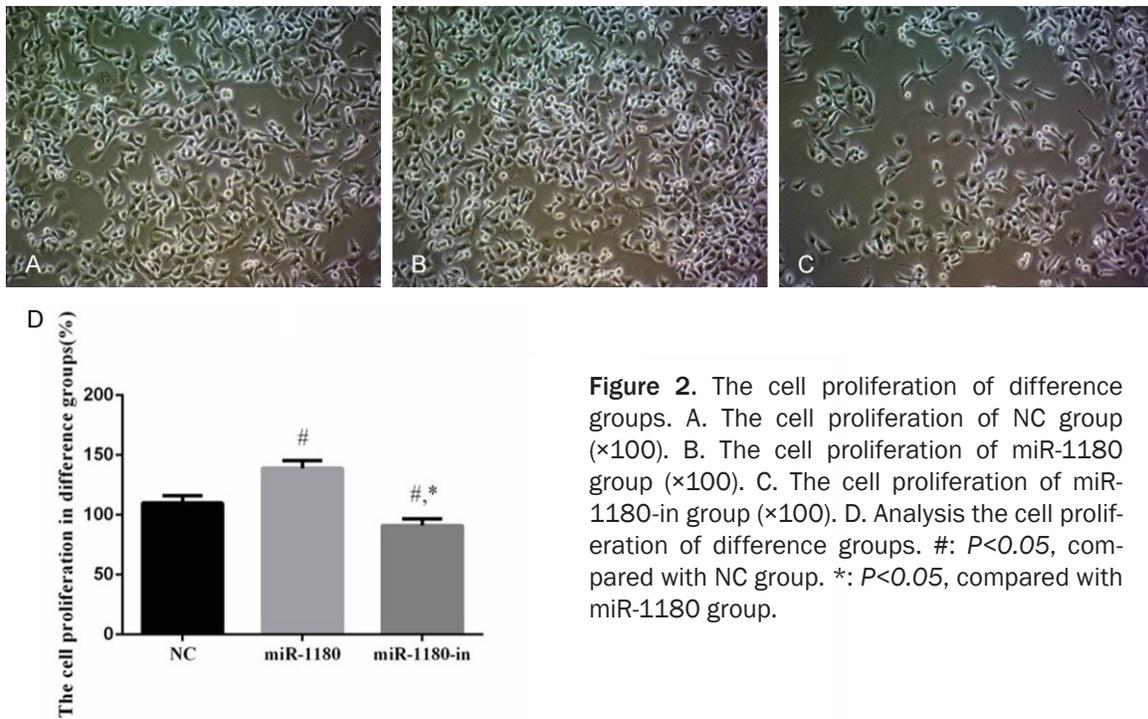
Figure 2. The cell proliferation of difference groups. A. The cell proliferation of NC group (×100). B. The cell proliferation of miR-1180 group (×100). C. The cell proliferation of miR-1180-in group (×100). D. Analysis the cell proliferation of difference groups. #: *P<0.05*, compared with NC group. *: *P<0.05*, compared with miR-1180 group.
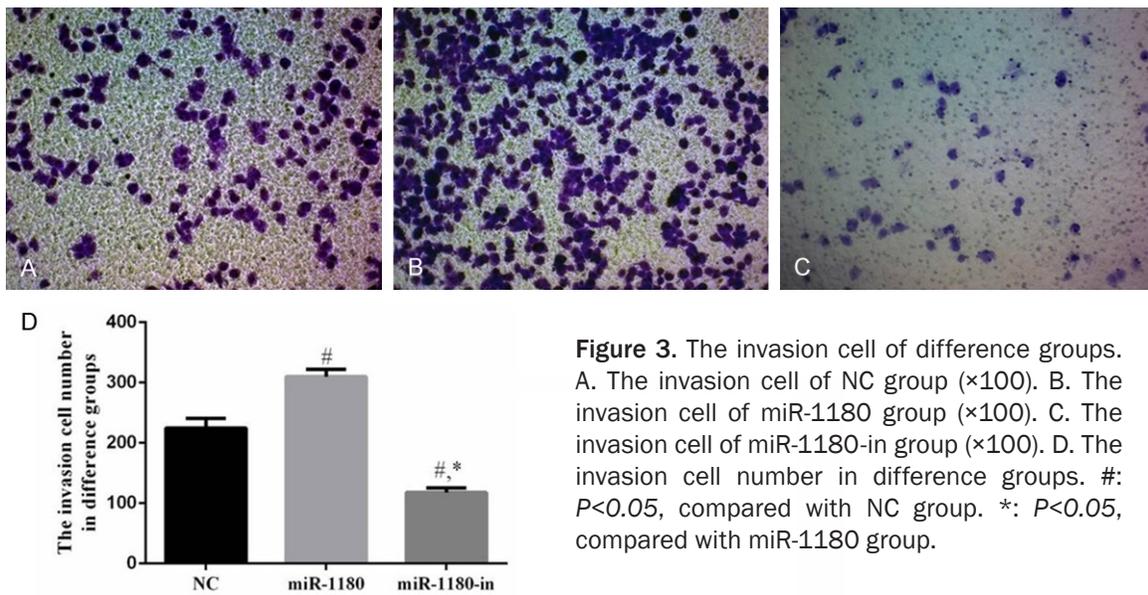
Figure 3. The invasion cell of difference groups. A. The invasion cell of NC group (×100). B. The invasion cell of miR-1180 group (×100). C. The invasion cell of miR-1180-in group (×100). D. The invasion cell number in difference groups. #: $P<0.05$, compared with NC group. *: $P<0.05$, compared with miR-1180 group.

atic cancer tissue (**Figure 1A**) compared with the tissue adjacent to the carcinoma (**Figure 1B**). In addition, qRT-PCR assays demonstrated that miR-1180 expression was significantly increased in pancreatic cancer tissues (n=30) compared with the tissue adjacent to the carcinoma (P<0.05, **Figure 1C**). Next, we analyzed the relationship between NF-κB and miR-1180 in these samples and found that expression of NF-κB was positively correlated with miR-1180 expression (r=0.848, **Figure 1D**).

miR-1180 expression affects cell proliferation

Among the PANC-1 cells treated with NC, miR-1180 mimic, and miR-1180-in, differences in cell proliferation were noted. Cell proliferation of the miR-1180 cells was significantly increased compared to the NC cells (P<0.05). However, the cell proliferation rate of miR-1180-in cells was significantly decreased compared to that of the NC cells (P<0.05) and the miR-1180 cells (P<0.05, **Figure 2**).
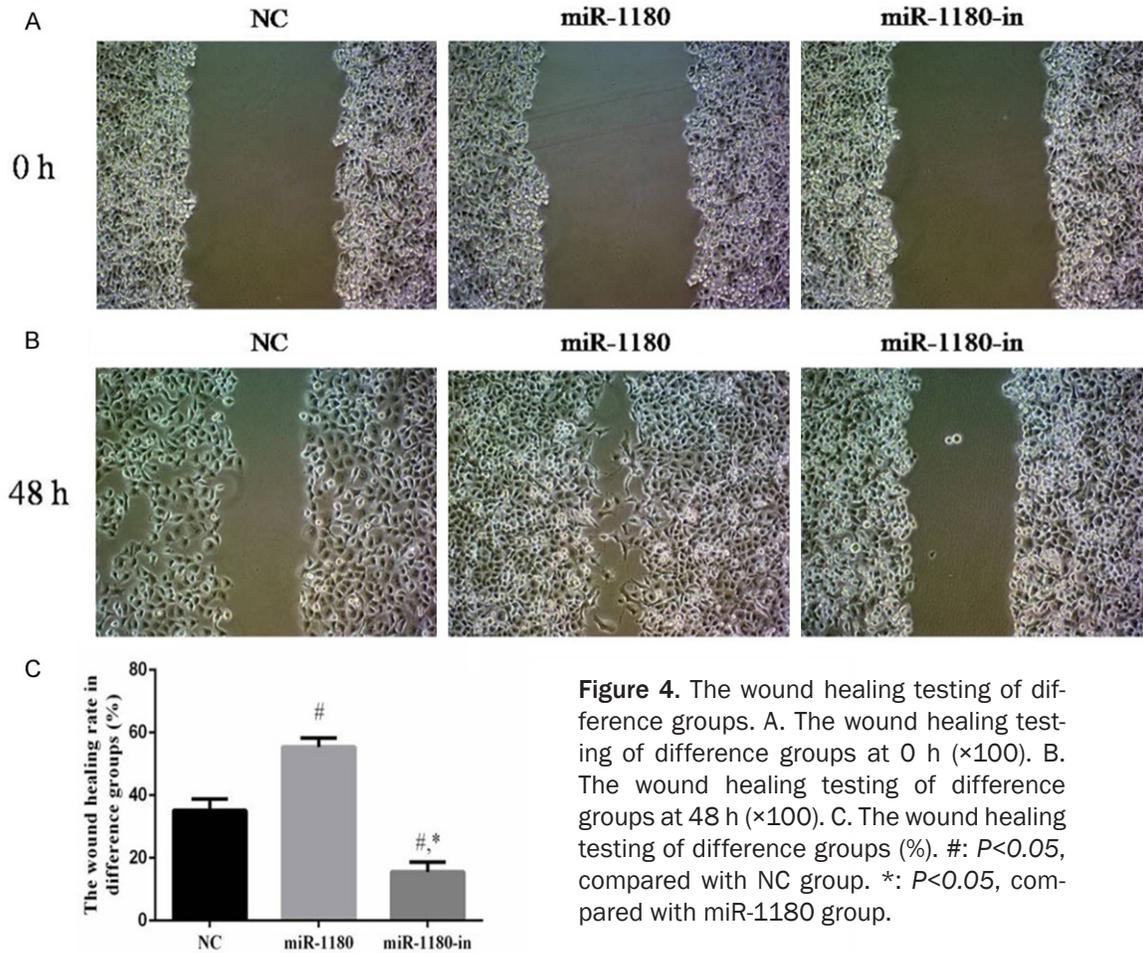
Figure 4. The wound healing testing of difference groups. A. The wound healing testing of difference groups at 0 h (×100). B. The wound healing testing of difference groups at 48 h (×100). C. The wound healing testing of difference groups (%). #: *P<0.05*, compared with NC group. *: *P<0.05*, compared with miR-1180 group.

miR-1180 expression affects the invasion ability of PANC-1 cells

The invasion ability of PANC-1 cells treated with the miR-1180 mimic was significantly higher than that of the NC cells (P<0.05). However, the invasion ability of PANC-1 cells treated with miR-1180-in was decreased compared to the NC and miR-1180 cells (P<0.05, **Figure 3**).

miR-1180 expression impacts the migration ability of PANC-1 cells

Compared with the NC cells, the wound healing rates of PANC-1 cells treated with the miR-1180 mimic or miR-1180-in were significantly different (P<0.05). The wound healing ability of miR-1180-in cells was significantly lower than that of the miR-1180 cells (P<0.05, **Figure 4**).

miR-1180 expression affects cell apoptosis

Compared with the NC cells and the cells treated with the miR-1180 mimic, apoptosis of the miR-1180-in cells was significantly increased (P<0.05). The apoptosis rate of miR-1180 cells was significantly reduced due to the miR-1180 overexpression (P<0.05, **Figure 5**).

miR-1180 expression affects cell cycle progression

Compared with the NC cells, the number of cells in the G1 phase of the miR-1180 group was significantly lower (P<0.05). However, the number of miR-1180-in cells in the G1 phase was significantly higher than that of the NC or miR-1180 cells (P<0.05, **Figure 6**).

Comparison of relative protein expression in the different cell groups

The protein expression of NF-κB, MMP-2, MMP-9, Bcl-2, and cyclin D1 was significantly downregulated and the expression of TNIP2, Bax, and p21 proteins were significantly upregulated in cells treated with miR-1180-in compared with expression levels in the NC cells or the
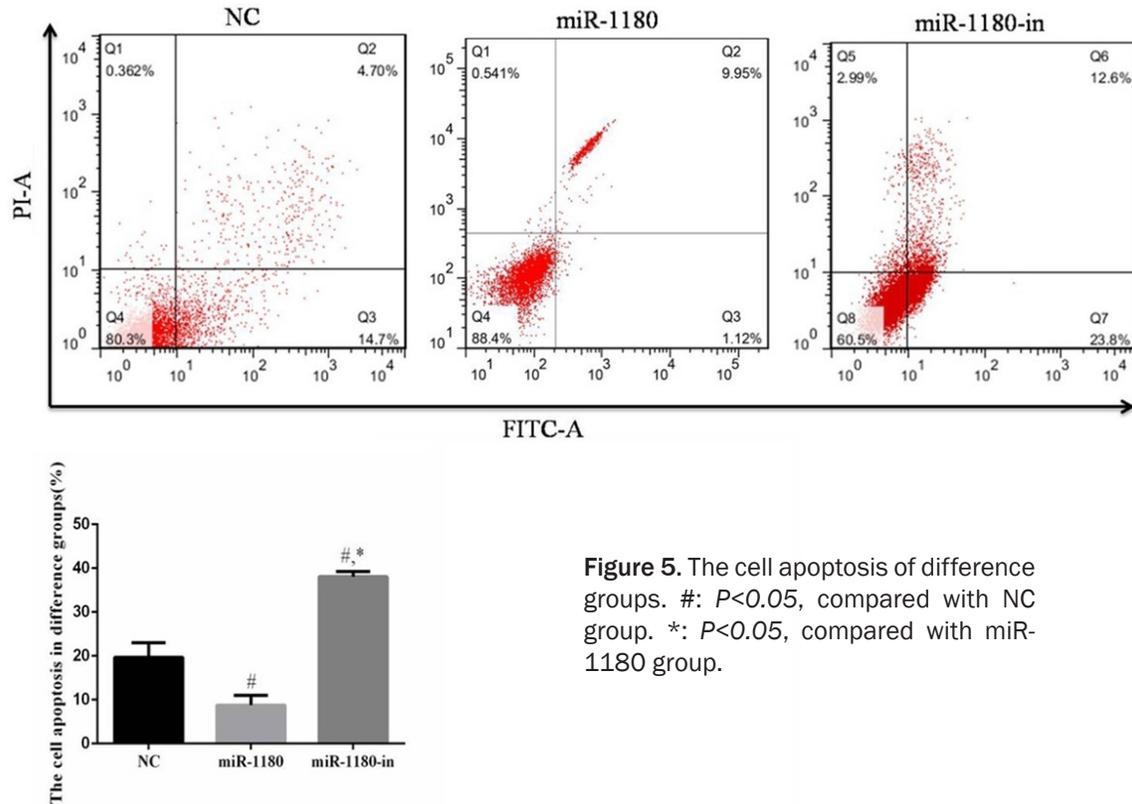
Figure 5. The cell apoptosis of difference groups. #: *P<0.05*, compared with NC group. *: *P<0.05*, compared with miR-1180 group.

cells treated with the miR-1180 mimic (P<0.05, **Figure 7**). Furthermore, these relative protein expression trends were different in the miR-1180 group compared with those of miR-1180 group. The relative expression of these proteins in the miR-1180 cells was significantly different compared with that in the NC cells (P<0.05, **Figure 7**).

Discussion

Previous studies showed that miR-1180 expression is significantly upregulated in cancer tissues and cell lines [8-10]; however, the expression of this miRNA has not been described in pancreatic cancer. Here, we demonstrated that miR-1180 and NF-κB expression were significantly increased in pancreatic cancer tissues and uncovered a positive correlation between their expression (r=0.848).

Inhibition of miR-1180 increased cisplatin-induced PANC apoptosis

Previously, miR-1180 was found to directly target TNIP 2 [8]. In the present study, we found that TNIP2 protein expression was significantly

downregulated in cells treated with miR-1180-in. TNIP2 functions as a NF-κB signaling inhibitor. We found that the NF-κB pathway was stimulated by miR-1180 overexpression. Thus, this pathway is important in suppression of cell activation (cell proliferation, invasion, and migration) via regulation of NF-κB and its downstream signaling pathway in PANC-1 cells.

MMP-2 and MMP-9 are two important factors in NF-κB downstream signaling [11-13]. The invasion of tumors is regulated by these MMPs, which are zinc ion-dependent endonucleases that regulate the biological behavior of cells. The MMP family is comprised of 20 different enzymes that are classified as different subtypes according to their substrate specificities and sequence characteristics [14]. MMP-2 and MMP-9 can degrade the main component of type IV collagen in the basement membrane and ECM [15]. MMP-2 and MMP-9 are closely related to tumor invasion and metastasis [16, 17]. Indeed, Farina et al. [18] reported that the expression of MMP-9 is regulated by NF-κB at the transcriptional level. Bond et al. [19] found that activation of NF-κB is essential for the upregulation of MMP-9 expression. Philip et al.
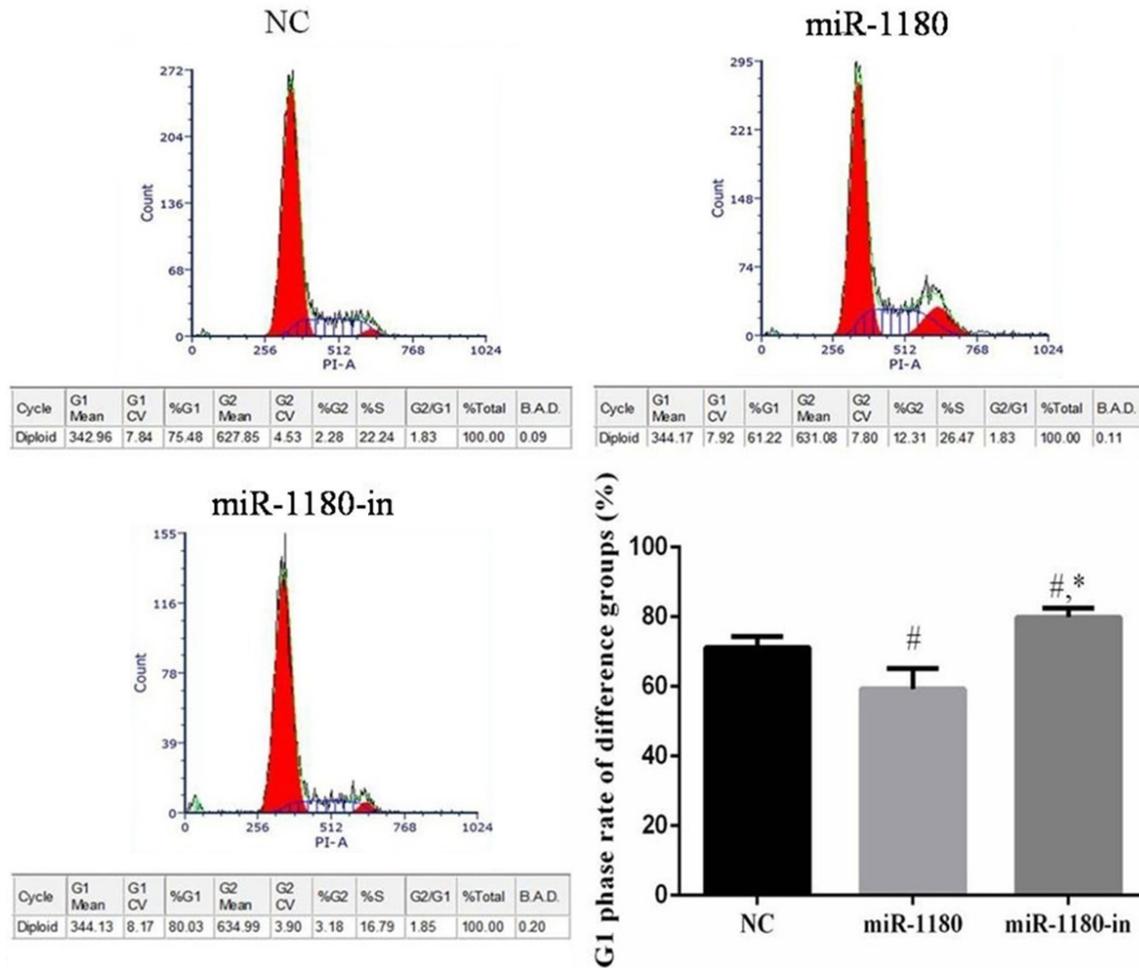
Figure 6. The cell cycle of difference groups. #: *P<0.05*, compared with G1 phase of NC group. *: *P<0.05*, compared with G1 phase of miR-1180 group.

[20] reported an increase in MMP-2 activity in the mouse melanoma cell line B16F10 and a concomitant activation of NF-κB. We found that inhibition of miR-1180 can effectively reduce the invasion and migration of PANC-1 cells. The mechanism underlying this effect may be related to inhibition of the activities of NF-κB, MMP-2, and MMP-9.

Bcl-2, Bax, p21, and cyclin D1 are additional players in the NF-κB signaling pathway [21-23]. Bcl-2 abrogates apoptosis induced by free oxygen radicals, inhibits the transmembrane transport of Ca2+, and inhibits cell apoptosis induced by Ca2+ [24-26]. Cyclin D1 is a key positive regulator of the G1/S phase, as this protein forms a complex with CDK4 to promote cell proliferation. In vitro studies showed that downregulation of cyclin D1 expression effec-

tively inhibits the development of tumors [27, 28]. In addition, p21 is an inhibitor of a wide range of cyclin-dependent kinases (CDKs) [29, 30] and combinations of the corresponding cyclins/CDKs. Thus, CDK inactivation by over-expression of p21 halts the progression of cells in the G1 phase, leading to cell apoptosis. The results of this study confirmed that miR-1180 effectively inhibits the increase of the apoptosis factor Bax and p21 expression and inhibits the expression of Bcl-2 and the cancer-promoting factor cyclin D1, stranding many PANC-1 cells in G1 phase, thus resulting in increased apoptosis.

In conclusion, our findings suggest that suppressing miR-1180 expression may exert anti-cancer effects on pancreatic cancer cells via regulation of the TNIP2/NF-κB signaling path-
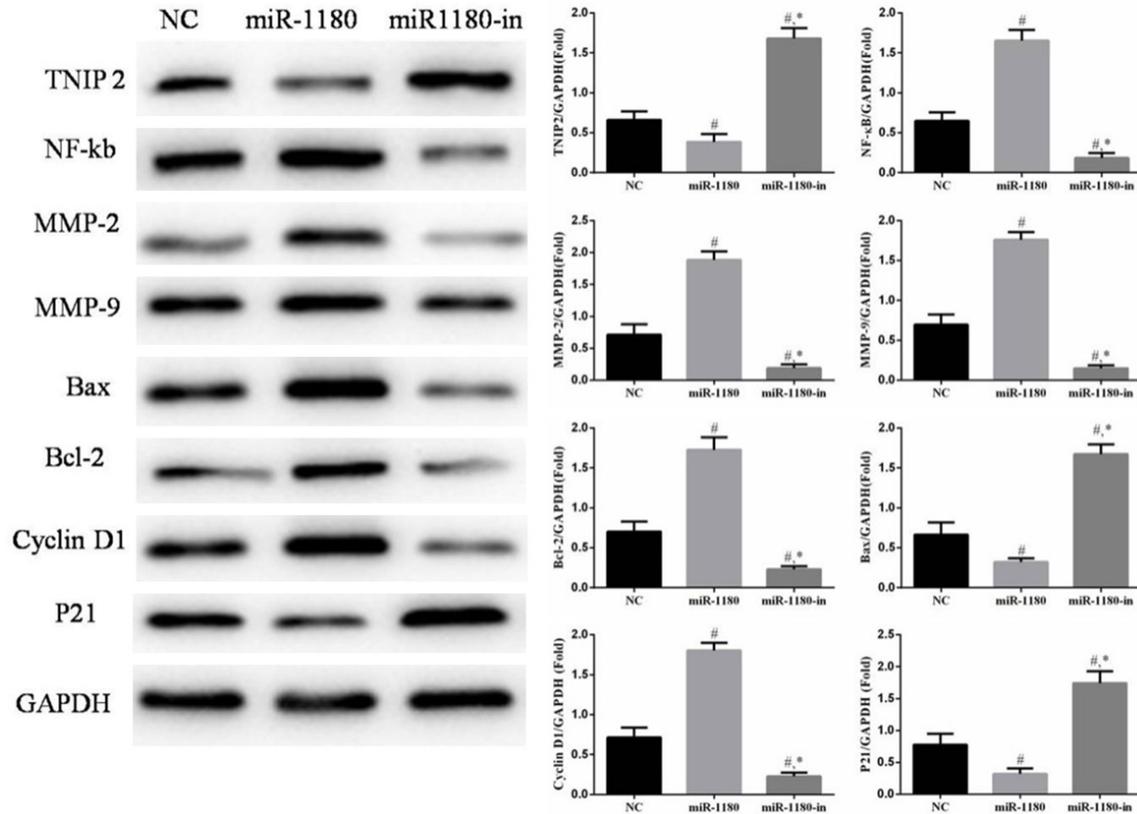
Figure 7. The relative proteins of difference groups. #: *P<0.05*, compared with NC group. *: *P<0.05*, compared with miR-1180 group.

way and its downstream proteins, including MMP-2, MMP-9, Bax, Bcl-2, p21, and cyclin D1.

Disclosure of conflict of interest

None.

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References

[1] Vincent A, Herman J, Schulick R, Hruban RH and Goggins M. Pancreatic cancer. Lancet 2011; 378: 607-620.

[2] Siegel R, Ma J, Zou Z and Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64: 9-29.

[3] Okada H, Kohanbash G and Lotze MT. MicroRNAs in immune regulation-opportunities for cancer immunotherapy. Int J Biochem Cell Biol 2010; 42: 1256-1261.

[4] Llave C, Xie Z, Kaschau KD and Carrington JC. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 2002; 297: 2053-2056.

[5] Nomura A, Majumder K, Giri B and Carrington JC. Inhibition of NF-kappa B pathway leads to deregulation of epithelial-mesenchymal transition and neural invasion in pancreatic cancer. Lab Invest 2016; 96: 1268-1276.

[6] Farhadi E, Zaker F, Safa M and Rezvani MR. miR-101 sensitizes K562 cell line to imatinib through Jak 2 downregulation and inhibition of NF-κB target. Tumor Biol 2016; 37: 14117-14128.

[7] Zhong Z, Cao Y, Yang S and Zhang S. Overexpression of RRM2 in gastric cancer cell promotes their invasiveness via AKT/NF-κB signaling pathway. Pharmazie 2016; 71: 280-284.

[8] Tan G, Wu L, Tan J, Zhang B, Tai WC, Xiong S, Chen W, Yang J and Li H. MiR-1180 promotes apoptotic resistance to human hepatocellular carcinoma via activation of NF-κB signaling pathway. Sci Rep 2016; 6: 22328.

[9] Zhou X, Zhu HQ, Ma CQ, Li HG, Liu FF, Chang H and Lu J. MiR-1180 promoted the proliferation of hepatocellular carcinoma cells by repressing TNIP2 expression. Biomed Pharmacother 2016; 79: 315-320.

[10] Ge Q, Wang C, Chen Z, Li F, Hu J and Ye Z. The suppressive effects of miR-1180-5p on the proliferation and tumorigenicity of bladder cancer cells. Histol Histopathol 2017; 32: 77-86.

[11] Poudel B, Lee YM and Kim DK. DDR2 inhibition reduces migration and invasion of murine metastatic melanoma cells by suppressing MMP2/9 expression through ERK/NF-κB pathway. Acta Biochim Biophys Sin 2015; 47: 292-298.

[12] Yang CJ, Liu YP, Dai HY, Shiue YL, Tsai CJ, Huang MS and Yeh YT. Nuclear HDAC6 inhibits invasion by suppressing NF-κB/MMP2 and is inversely correlated with metastasis of non-small cell lung cancer. Oncotarget 2015; 6: 30263-30276.

[13] Miao C, Ma J, Zhang Y, Chu Y, Li J, Kuai R, Wang S and Peng H. Perfluorooctanoic acid enhanceds colorectal cancer DLD-1 cells invasiveness through activating NF-κB mediated matrix metalloproteinase-2/-9 expression. Int J Exp Pathol 2015; 8: 10512-10522.

[14] Sternlicht MD and Werb Z. How matrix metallo proteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001; 17: 463-516.

[15] Curran S and Murray GI. Matrix metalloproteinases in tumor invasion and metastasis. J Pathol 1999; 189: 300-308.

[16] Bjorklund M and Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. Biochim Biophys Acta 2005; 1755: 37-69.

[17] Chang YZ, Yang MW and Wang GJ. A new spectrometry electrospray tip obtained via precise mechanical micromachining. Anal Bioanal Chem 2005; 383: 76-82.

[18] Farina AR, Tacconelli A, Vacca A, Maroder M, Guling A and Mackay AR. Transcriptional up-regulation of matrix metalloproteinase-9 expression during spontancous epithelial to neuroblast phenotype conversion by SK-N-Shneuroblastoma cell, involved in enhanced invasivity, dependes upon GT-box and nuclear factor-kappa B elements. Cell Growth Differ 1999; 10: 363-367.

[19] Bond M, Fabunmi RP, Baker AH and Newby AC. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcrition factor NF-κB. FEBS Lett 1998; 435: 29-34.

[20] Philip S, Bulbule A and Kundu GC. Osteopontin stimulates tumor growth and activation of pro-matrix metalloproteinase-2 through nuclear factor-kappa B-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells. J Biol Chem 2001; 276: 44926-44935.

[21] Zhang S, Qin F, Yang L, Xian J, Zou Q, Jin H, Wang L and Zhang L. co-operation induces malignant conversion via p53 loss, elevated NF-κB and tenascinc associated rigidity, but p21 inhibits ROCK2/NF-κB-mediated progression. Oncogene 2016.

[22] Li S, Zhou Y, Zheng X, Wu X, Liang Y, Wang S and Zhang Y. Sphk1 promotes breast epithelial cell proliferation via NF-κB-p65-mediated cyclin D1 expression. Oncotarget 2016; 7: 80579-80585.

[23] Hockenbery DM, Zutter MK, Hickey W, Nahm M and Korsmeyer SJ. Bcl-2 protein is topo-graphically restricted is tissues characterized by apoptotic cell death. Proc Natil Sci U S A 1991; 88: 6961-6965.

[24] Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP and Wang X. Prevention of apoptosis by bcl-2: release of cytochromec from mitochondria blocked. Science 1997; 275: 1129-1132.

[25] Pezzella F, Tse AGD, Cordell JL, Pulford KA, Gatter KC, Mason DY. Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 1990; 137: 225-232.

[26] Hwang TS, Han HS, Hong YC, Lee HJ and Paik NS. Prognostic value of combined analysis of Cyclin D1 and estrogen receptor status in breast cancer patients. Pathol Int 2003; 53: 74-80.

[27] Koroxenidou L, Chison LC and Porsch Hallstrom I. Long-term 17alpha-ethinyl estradiol treatment decreases cyclin E and cdk2 expression, reduces cdk2 kinase activity and inhibits S phase entry in regenerating rat liver. J Hepatol 2005; 43: 478-484.

[28] Li G, Liu Z, Sturgis EM, Shi Q, Chamberiain RM, Spitz MR and Wei Q. Genetic polymorphisms of p21 are associated with risk of squamous cell carcinoma of the head and neck. Carcinogenesis 2005; 26: 1596-1602.

[29] Huang WS, Kou YH, Kou HC, Hsieh MC, Huang CY, Lee KC, Lee KF, Shen CH, Tung SY and Teng CC. CIL-102-induced cell cycle arrest and apoptosis in colorectal cancer cells via upregulation of p21 and GADD45. PLoS One 2017; 12: e0168989.

[30] Huang WS, Kou YH, Kou HC, Hsieh MC, Huang CY, Lee KC, Lee KF, Shen CH, Tung SY and Teng CC. CIL-102-induced Cell Cycle Arrest and Apoptosis in Colorectal Cancer Cells via Upregulation of p21 and GADD45. PLoS One 2017; 12: e0168989.