MiR-216b inhibits pancreatic cancer cell progression and promotes apoptosis by down-regulating *KRAS*

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Abstract

Introduction: Pancreatic cancer is a highly lethal malignancy with high invasion metastasis, which is difficult to diagnose and treat. MicroRNA-216b (miR-216b) plays an important role in many types of tumors. In this study, we explore how miR-216b affected human pancreatic cancer cell development by targeting *KRAS*.
Material and methods: Expression level of miR-216b and KRAS in tissue samples and cells were detected by RT-PCR and western blot. Immunohistochemical assay analysed the expressions of *KRAS* protein in tumor and adjacent tissues. The target relationship between miR-216b and *KRAS* was validated by dual-luciferase reporter assay. Pancreatic cancer cell proliferation, migration, invasion and apoptosis abilities of cells transfected with miR-216b mimics and KRAS-siRNA, Panc-1 were detected by MTT assay, transwell assay and flow cytometry assay respectively. Prognosis of patients with different expression levels of miR-216b and *KRAS* were analyzed by Kaplan-Meier survival analysis and Cox proportional hazards regression model.
Results: The expression of miR-216b in pancreatic cancer tissue and cell line was down-regulated ($p < 0.01$), while KRAS expression was up-regulated ($p < 0.01$) compared with adjacent normal tissues. Both the expressions of miR-216b and KRAS have a strong influence on prognosis of the pancreatic cancer patients ($p = 0.024$ and $p = 0.017$). The dual-luciferase reporter assay verified that miR-216b directly targeted KRAS in pancreatic cancer cells. Overexpression of miR-216b reduced the expression of mRNA and protein of KRAS ($p = 0.013$ and $p = 0.003$), but silencing KRAS had no effect on miR-216b expression ($p = 0.706$). By silencing KRAS or up-regulation of miR-216b could suppress cell proliferation, migration and invasion of pancreatic cancer cells and promote apoptosis.
Conclusions: MiR-216b might inhibit pancreatic cancer cell progression and stimulate apoptosis by silencing *KRAS*.

Key words: pancreatic cancer, miR-216b, *KRAS*.

Introduction

Pancreatic cancer is a devastating cancer with a high morbidity and poor prognosis. It is reported that only 20% of patients will be alive 5 years after pancreatic resection and the survival rate of pancreatic cancer patients is less than 30% [1, 2]. Radiotherapy and chemotherapy are widely used for the treatment of pancreatic cancer. However, as pancreatic cancer is highly resistant to chemotherapy and radiotherapy, the

efficacy of current treatment for pancreatic cancer still cannot reach expectations [3]. Therefore, it is urgent for the treatment of pancreatic cancer to develop an effective therapeutic strategy.

MicroRNAs (miRNAs), as endogenous small and non-coding RNAs, play a crucial role in regulating gene expression within the target messenger RNA (mRNA) 3′-untranslated region (UTR) [4]. There is considerable evidence suggesting that miRNAs present aberrant expressions in multiple cancer tissue and cells [5]. Yuan *et al*. demonstrated that miR-216b inhibited the multi-drug resistance of hepatocellular cancer cells through down-regulating autophagy [6]. Zheng *et al*. revealed that miR-216b may serve as a potential therapeutic agent for hepatocellular carcinoma by targeting an oncogene [7]. Zhang *et al*. inferred that miR-216b suppressed pancreatic cancer by inducing apoptosis [8]. Nonetheless, the mechanism of miR-216b in pancreatic cancer cells needs to be further explored.

In normal tissue signaling, the normal *KRAS* protein has an essential function, while a mutation of the *KRAS* gene may lead to many unpredictable cancers. One study showed that *KRAS* mutation is a critical predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer [9]. Another study revealed that PanINs and PDAC can develop from pancreatic acinar cells when mutant *KRAS* is restricted using an acinar-specific promoter [10].

Table I. Clinical and pathological characteristics of 27 patients with pancreatic cancers

Variables	Numbers
Age [years]:	
≤ 60	9
> 60	18
Gender:	
Male	17
Female	10
Maximum tumor diameter [cm]:	
< 5.0	19
≥ 5.0	8
Clinical stage:	
I and II	14
III	13
Tumor localization:	
Pancreatic head	21
Periampullary	6

Moreover, a recent study revealed that the establishment of a highly metastatic *KRAS* mutant in lung cancer is fatal [11]. All the above studies suggested that *KRAS* plays a significant role in cancer development. However, the relationship between *KRAS* and pancreatic cancer remains incompletely elucidated.

In the current study, we investigated how miR-216b took effect in human pancreatic cancer by interacting with *KRAS*. Our study provided clues for the role of miR-216b-*KRAS* interaction in regulating the cancer cell biology of pancreatic cancer, which distinguished it from previous studies, and indicated the therapeutic potential for miR-216b in pancreatic cancer treatment.

Material and methods

Clinical samples

Twenty-seven pairs of pancreatic cancer tumor tissues and adjacent normal tissues were obtained from pancreatic cancer patients undergoing surgery at the Third Affiliated Hospital of Soochow University from May 2013 to May 2015. Clinical and pathologic characteristics of pancreatic cancer patients are shown in Table I. The adjacent tissues were taken at a distance of 1.5 cm from the tumor. All the tissues were snap-frozen with liquid nitrogen at –80°C. No treatment such as radiotherapy and chemotherapy had been conducted on any patients before surgery. The study was sanctioned by the Ethics Committee of the Third Affiliated Hospital of Soochow University. All patients were informed of the study and gave informed consent.

Cell culture and transfection

BxPC-3, Panc-1, and CFPAC-1 (cell lines of pancreatic cancer) and HPDE6-C7 (a normal pancreatic cell line) were obtained from Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). Cultured in High Glucose Dulbecco's modified Eagle medium (DMEM) (Gibco, Paisley, UK), all cells were supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere at 37°C. Cell

Table II. Sequences for KRAS siRNA

KRAS siRNA	Sequence (5'-3')
Sense1	5′-CCAACAAUAGAGGAUUCCUACAGGA-3′
Antisense1	5′-UCCUGUAGGAAUCCUCUAUUGUUGG-3′
Sense2	5′-CAAGACAGAGAGUGGAGGAUGCUUU-3′
Antisense2	5′-AAAGCAUCCUCCACUCUCUGUCUUG-3′
Sense3	5′-CAUUGGUGAGGGAGAUCCGACAAUA-3′
Antisense3	5′-UAUUGUCGGAUCUCCCUCACCAAUG-3′

Table III. Primer sequences in qRT-PCR

Variables	Forward primer	Reverse primer
miR-216b	5'-GCCGCGCTAAAGTGCTTA-3'	5'-CACCAGGGTCCGAGGT-3'
U6	5'-TGCGGGTGCTCGCTTCGGC-3'	5'-CCAGTGCAGGGTCCGAGGT-3'
KRAS	5'-TCTCCTTCTCAGGATTCCTACAG-3'	5'-ACAAAGAAAGCCCTCCCCAGT-3'
GADPH	5'-ACAACTTTGGTATCGTGGAAGG-3'	5'-GCCATCACGCCACAGTTTC-3'

transfection was not performed until cell growth reached about 80% confluence. MiR-216b mimics and *KRAS*-siRNA were synthesized by Shanghai Bioengineering Engineering Inc. (Shanghai, China) and transfected into Panc-1 cells by Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's manual. The siRNA sequences used for KRAS siRNA experiments are shown in Table II. Four groups of cells were constructed: control group (non-transfection), NC group (transfection with negative control mimics or siRNA), miR-216b group (transfection with miR-216b mimics), and *KRAS*-siRNA group (transfection with *KRAS* siRNA).

qRT-PCR

Trizol reagent (Invitrogen) was first used to lyse cells and extract total RNA. Next, ReverTra Ace qPCR RT Kit (Toyobo, Japan) was used to perform reverse transcription of RNA according to the instructions. Real-time quantitative PCR was then carried out on the MiniOpticon real-time PCR system (Bio-Rad, Hercules, CA, USA), using SYBR-Green RealMasterMix (Bio-Rad). U6 acted as an internal reference for miR-216b, while *KRAS* mRNA expression was normalized relative to GAPDH by the $2^{-\Delta\Delta Ct}$ method. Primers were designed and provided by Sangon Biotech (Shanghai, China); their sequences are displayed in Table III.

Western blot

Panc-1 cells in the logarithmic growth phase were first collected and digested with 0.25% trypsin. Next, radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, Shanghai, China) was applied to extract total protein. A bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was employed for the quantification of protein concentrations and 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad) was utilized for protein separation. After that, polyvinylidene fluoride (PVDF) membranes (Invitrogen) were employed for protein transference following the producer's guidelines and sealed in 5% skim milk at 37°C for 2 h. The membranes were placed into a plastic bag in the next step and incubated overnight with primary antibodies against *KRAS* (ab180772, 1 : 500, Abcam, Cambridge, MA, USA) and GAPDH antibody (ab9485, 1 : 2000, Abcam) at 4°C. After 3 times 5-min washing with phosphate buffered saline (PBS), at room temperature, 1.5-h incubation of membranes with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG H&L (1 : 2000) was conducted, followed by again washing 3 times for 5 min with PBS. An electrochemiluminescent (ECL) detection system (Thermo Scientific, MA, USA) was employed for signal detection. The density of protein bands was quantified or analyzed using Quantity One v4.6.2 software (Bio-Rad).

Immunohistochemistry (IHC)

Immunohistochemical staining was carried out on 4 µm-thick slides. Briefly, the tissues were embedded in paraffin, then the slides were deparaffinized and rehydrated through graded alcohols and washed in phosphate buffered saline (PBS) 2 times for 10 min. Next the sections were incubated on the slides overnight with rabbit polyclonal primary antibody of KRAS (ab55391, Abcam) at a 5 µg/ml concentration. Then the sections were incubated with 45 µl of secondary antibody horseradish peroxidase-conjugated goat polyclonal anti-mouse IgG H&L (HRP) (1 : 500, ab6789, Abcam) at 37°C for 30 min. Slides were stained with 3,3′-diaminobenzidine (DAB) working solution for 3 min, then washed in water for 10 min. Slides were counterstained with hematoxylin. After rewashing the slides in water for 10 min, we finally dehydrated and cleared them. The slides were then ready for microscopic observation.

MTT assay

After 24-h transfection, cells were inoculated into 96-well plates at a density of 1×10^4 cells/well and stored in DMEM with FBS in it. At 37°C, cells were incubated for 4 h with 50 µl of MTT (0.5 mg/ml, Sigma-Aldrich) added into each well and had their growth condition observed at 0, 24, 48, and 72 h. To solubilize the crystals, 150 µl of dimethylsulfoxide (DMSO) was added into each well after the supernatant was subsequently removed. The optical density (OD) was measured at 570 nm by a microplate reader (Bio-Rad).

Transwell assay

Cells were first placed on a transwell plate. After being diluted with 100 µl of serum-free medium, Matrigel (200 mg/ml, BD Biosciences, CA, USA) was added to the upper chamber of the bottom membranes. Cells were lysed by trypsin and collected by centrifugation 48 h after transfection. Afterwards, the cells were washed with PBS twice, re-suspended in the serum-free medium with bovine serum albumin (BSA) in it and then inoculated into the upper chamber. At 37°C atmosphere, 250 µl DMEM with 10% FBS in it was added into the lower chamber for 24-h incubation. Non-invading cells were gently removed using a cotton swab. Migratory or invasive cells were fixed with 95% alcohol, stained with hematoxylin for 15 min and counted under an Olympus inverted microscope (200×) (Olympus, Tokyo, Japan).

Flow cytometry assay

After 48-h transfection, cells were digested by 0.25% trypsin and harvested by centrifugation at 800 rpm after 5 min. Next, at 4°C, the cells were washed with 0.01 mol/l ice-cold PBS twice, 5 min each time, and resuspended in binding buffer for 15 min. Annexin-V FITC (5 µl) and 5 µl of propidium iodide (PI; BD Bioscience) were added to the cells. After incubation for 20–30 min in the dark, the apoptotic cells were detected by flow cytometry (EPICS XL; Beckman-Coulter, Fullerton, CA).

Dual-luciferase reporter assay

The sequences of *KRAS* 3′UTR wild-type (wt) and *KRAS* 3′UTR mutated type (mut) were synthesized by Sangon Biotech (Shanghai, China). After amplification, the *KRAS* 3′UTR wt and *KRAS* 3′UTR mut were subcloned into the pmirGLO vector (Promega, Madison, USA). When 80% confluence was reached, using Lipofectamine 2000 reagent (Invitrogen), Panc-1 cells were co-transfected with miR-216b mimic or negative control mimic and pmirGLO-*KRAS* 3′UTR-wt or pmirGLO-*KRAS* 3′UTR-mut. Luciferase activity was detected at 48 h after transfection using a dual-luciferase reporter gene assay kit (Promega, USA).

Statistical analysis

All data were processed and analyzed by SPSS 21.0 statistical software (SPSS, Chicago, IL, USA). Data were presented as the mean ± standard deviation (SD). All *in vitro* experiments were carried out in triplicate. Comparison of data from two groups was performed using Student's *t*-test when the data had a normal distribution; otherwise the Mann-Whitney *U*-test was used. Comparison between three groups or more was performed by one-way ANOVA. The association between miR-216b expression and *KRAS* expression levels was analyzed by Spearman's method. Categorical variables of prognosis were contrasted by the χ^2 test. Kaplan-Meier survival analysis and Cox proportional hazards models were applied to determine the link between the expression levels of miR-216b and *KRAS* and prognosis of patients. A *p*-value less than 0.05 was defined as statistically significant.

Results

Abnormal expression of miR-216b and KRAS in pancreatic cancer tissues and cells

The results of qRT-PCR showed that compared with that of normal pancreatic tissues and cells, miR-216b mRNA expression in pancreatic cancer tissues and cells was obviously lower ($p < 0.01$, Figures 1 A, B). Conversely, the expression level of *KRAS* mRNA and protein were considerably higher in pancreatic tumor tissues (all, $p < 0.01$, Figures 1 C–E). Also, the expression of *KRAS* protein was higher in pancreatic tumor tissues than normal pancreatic tissues according to IHC assay (Figure 1 F). Moreover, pancreatic cancer cells presented remarkably higher expression of *KRAS* than normal pancreatic cells ($p < 0.01$, Figures 2 A, B). As the Panc-1 cell line presented a more significant difference than the other two cancer cell lines, it was selected for further experiments.

The TCGA database was analyzed to compare the clinicopathological characteristics with the expression of miR-216b and *KRAS* in pancreatic cancer patients. Patients were divided into two groups according to the mean expression of miR-216b or *KRAS*. The χ^2 method was used and the results are shown in Tables IV and V. The low miR-216b expression group shows higher lymphatic metastasis and TNM staging compared to the high miR-216b group ($p < 0.05$). Meanwhile, patients with a high KRAS level suffered from a high probability of lymphatic metastasis, a larger tumor size and an advanced TNM staging ($p < 0.05$). In addition, the overall survival (OS) analysis of patients with low-expressed miR-216b was significantly poorer than those with high-expressed miR-216b (Figure 2 C, $p < 0.05$), while the prognosis of patients with low-expressed *KRAS* was observably better than those with high-expressed *KRAS* (Figure 2 D, $p < 0.05$). To further study the factors and their contribution to the prognosis of pancreatic cancers, we performed both univariable and multivariable analysis of overall survival. The results confirmed KRAS as an independent indicator for the prognosis of patients with pancreatic cancers (Table VI).

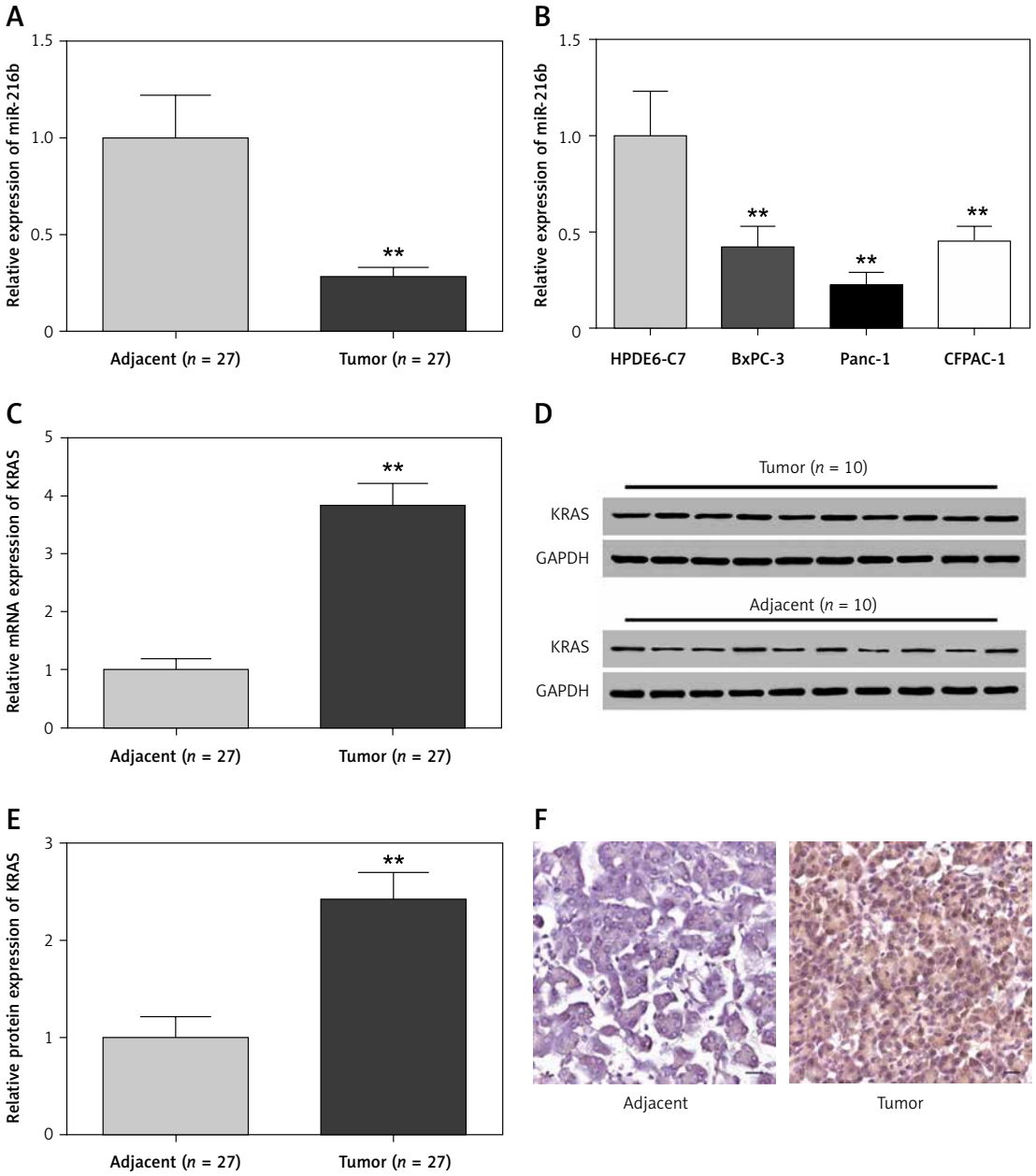

Figure 1. The expression levels of miR-216b were lower and the expression levels of *KRAS* were higher in pancreatic tissues and cell samples. **A**, **B** – qRT-PCR indicated that the expression levels of miR-216b were significantly lower in pancreatic cancer tissues and cells than in adjacent tissues and normal pancreatic cells HPDE6-C7. $^{**}P < 0.01$, compared with adjacent tissues and HPDE6-C7 cells. **C–E** – qRT-PCR and western blot results indicated that the expression levels of *KRAS* mRNA and protein in tumor tissues were significantly higher than those in adjacent tissues. $^{**}P < 0.01$, compared with adjacent tissues. **F** – IHC assay results suggested that the expression levels of *KRAS* protein in tumor tissues were higher than those in adjacent tissues

MiR-216b directly targeted at *KRAS*

We first predicted the potential binding sites of miR-216b in the 3′UTR of *KRAS* through the TargetScan database (Figure 3 A). In the meantime, as Figure 3 B shows, the luciferase activity of *KRAS* 3′UTR-wt in Panc-1 cells transfected with miR-216b mimics was remarkably weaker than cells transfected with negative control mimics ($p < 0.01$). Nonetheless, no significant difference of luciferase activity of *KRAS* 3′UTR-mut was observed between the miR-216b group and NC group ($p > 0.05$). The above results indicated that miR-216b directly targeted at *KRAS* and overexpression of miR-216b significantly repressed the luciferase activity of the wild-type 3′UTR of *KRAS* in Panc-1 cells.

MiR-216b overexpression inhibited proliferation, migration and invasion, and induced apoptosis of pancreatic cancer cells by down-regulating *KRAS*.

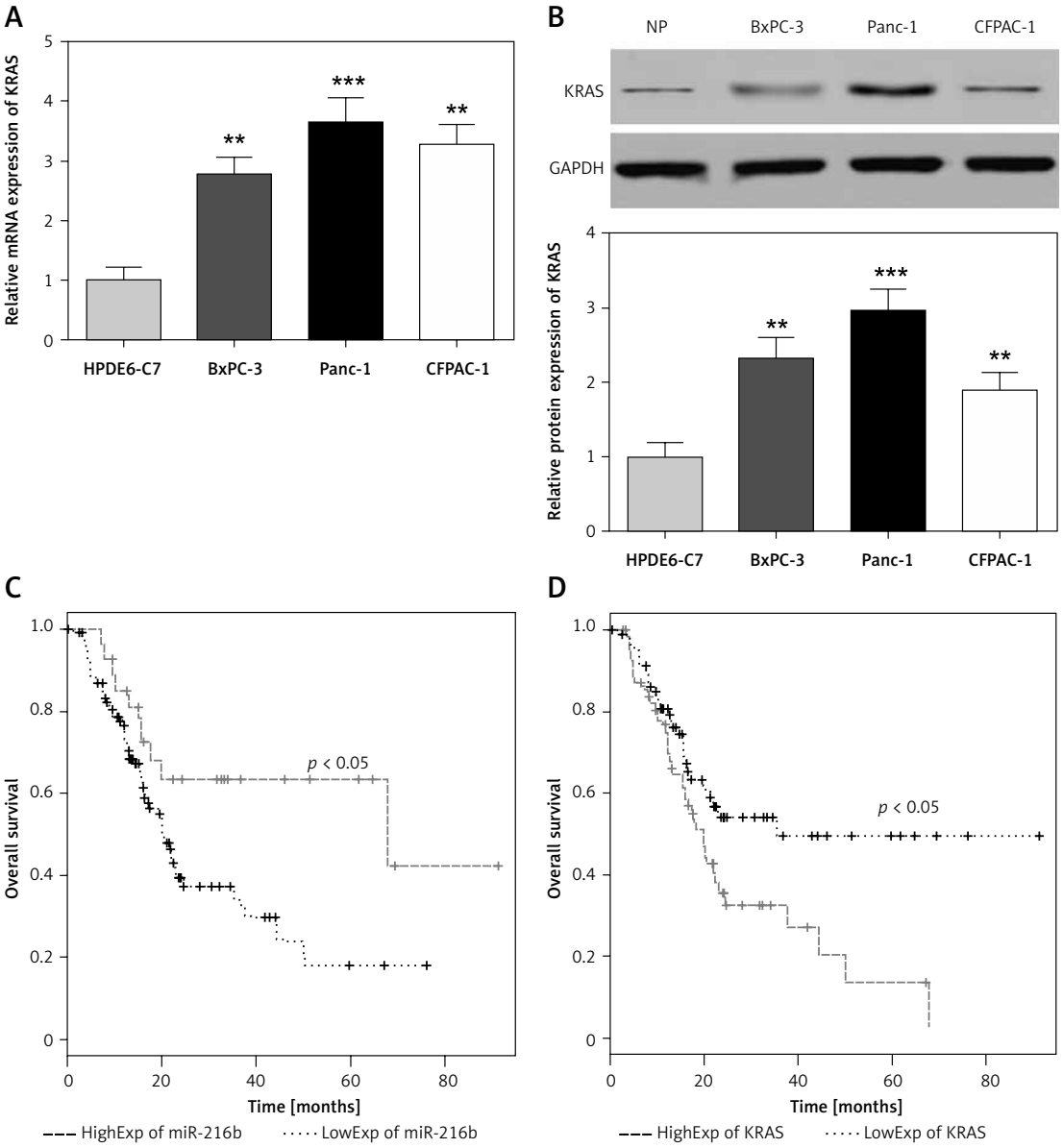

Figure 2. High expression of miR-216b and low expression of *KRAS* was beneficial for the prognosis of pancreatic cancer patients. **A**, **B** – qRT-PCR and western blot results showed that the expression levels of *KRAS* mRNA and protein in pancreatic cancer cell lines (BxPC-3, Panc-1, and CFPAC-1) were remarkably higher than those of normal pancreatic cell line HPDE6-C7. $^{**}P < 0.01$, $^{***}p < 0.001$, compared with HPDE6-C7 cells. **C**, **D** – Kaplan-Meier survival analysis confirmed that the high expression of miR-216b and low expression of *KRAS* were related to the better prognosis of pancreatic cancer patients (log-rank test, all $p < 0.05$)

We determined the transfection efficiency of three KRAS siRNA transfected groups by qRT-PCR (Figures 4 A, C) and found that the third KRAS siRNA transfection group had the lowest KRAS mRNA and protein expression. Hence, we used the third KRAS siRNA transfection group for subsequent experiments. Western blot and qRT-PCR attested that after transfection with miR-216b mimics, miR-216b expression was remarkably up-regulated, while that of *KRAS* dramatically decreased in comparison with the control group (Figures 4 B, D, E). At the same time, as Figure 4 F shows, the cell viabilities in the miR-216b overexpression group and *KRAS*-siRNA group were significantly attenuated ($p < 0.05$), suggesting that miR-216b could prevent the proliferation of pancreatic cancer cells by down-regulating *KRAS*.

In addition, results from transwell assay suggested that the number of migratory and invasive cells in the miR-216b group and *KRAS*-siRNA group was lower than that of the control group ($p < 0.05$, Figures 5 A, B), indicating that miR-216b overexpression and under-expression of *KRAS* could hinder cell migration and invasion abilities. Furthermore, flow cytometry assay results indicated that the apoptosis rates of Panc-1 cells in

Table IV. Relationship between miR-216b expression and clinicopathological characteristics in pancreatic cancer patients

Characteristic	Number	High expression of miR-216b (*n* = 89)	Low expression of miR-216b (*n* = 89)	*P*-value
Age [years]:				
> 60	101	52	47	0.546
≤ 60	77	37	42	
Gender:				
Male	96	49	46	0.764
Female	82	40	43	
Tumor location:				
Pancreatic head	110	59	55	0.640
Periampullary	68	30	34	
Tumor size [cm]:				
< 5	109	55	47	0.289
≥ 5	69	34	42	
Lymphatic metastasis:				
Yes	107	35	57	0.002*
No	71	54	32	
Neural invasion:				
Present	91	40	52	0.099
Absent	87	49	37	
TNM staging:				
Early stage (I–II)	82	49	33	0.012*
Advanced (III–IV)	96	40	56	

**Categorical variables were compared by the χ^2 test. *P < 0.05 was recognized as a significant difference.*

the miR-216b group and *KRAS*-siRNA group were observably higher than the control group (both $p < 0.05$, Figure 5 C). Taken together, miR-216b could inhibit pancreatic cancer cell migration and invasion, and promote cell apoptosis via down-regulation of *KRAS* expression.

Discussion

There was a previous study proving the targeting relationship between miR-216 and *KRAS* in the ELa-*KRAS*^{G12D} mouse model [10]. In our study, we laid more emphasis on the human tissues in an *in vitro* experiment. We came to the conclusion that by silencing *KRAS*, miR-216b could slow down the progression of human pancreatic cancer cells.

It is well known that *KRAS* is a critical gene leading to the deterioration of pancreatic cancer. One previous study has already reported that *KRAS* gene mutation was a major cancer initiating event and may incur pancreatic carcinogenesis [12]. According to previous studies, *KRAS* had high expression when endometriosis occurred [13]. It was also revealed that *KRAS* had high expression in lung adenocarcinoma [14]. Another study revealed that *KRAS* mutations led to poor prognosis in patients with microsatellite-stable stage III colon cancer [15]. Similarly, *KRAS* gene mutations were verified to lead to a poorer prognosis of patients with pancreatic cancer [16]. Intriguingly, in this research, we substantiated that *KRAS* was highly expressed in pancreatic cancer and the prognosis with low *KRAS* expression was better, which are also consistent with some previous study results.

MiR-216 has been found to modulate gene expression and presented down-regulated expression in various cancers. For instance, one typical study revealed that miR-216b expression was down-regulated in pancreatic ductal adenocarcinoma (PDAC) and that miR-216b expression was reduced 2.7-fold in the cases that did not benefit

Table V. Relationship between KRAS expression and clinicopathological characteristics in pancreatic cancer patients

Characteristic	Number	High expression of KRAS (n = 89)	Low expression of KRAS (n = 89)	P-value
Age [years]:				0.0961
> 60	101	56	45	
≤ 60	77	33	44	
Gender:				0.1327
Male	96	53	43	
Female	82	36	46	
Tumor location:				0.6437
Pancreatic head	110	53	57	
Periampullary	68	36	32	
Tumor size [cm]:				0.0089*
< 5	109	63	46	
≥ 5	69	26	43	
Lymphatic metastasis:				< 0.0001*
Yes	107	68	39	
No	71	21	50	
Neural invasion:				0.1335
Present	91	51	40	
Absent	87	38	49	
TNM staging:				0.0238*
Early stage (I–II)	82	33	49	
Advanced (III–IV)	96	56	40	

**Categorical variables were compared by the χ^2 test. P < 0.05 was recognized as a significant difference.*

Table VI. Univariate and multivariate analysis for overall survival of patients with pancreatic cancer

Factors	Univariate analysis		Multivariate analysis	
	Relative risk	P^a	Relative risks	P^b
Age	1.83	0.233		
Gender	0.61	0.674		
Tumor location	2.83	0.081		
Tumor size	6.22	0.002*	4.11	0.284
Lymphatic metastasis	5.31	0.005*	5.17	0.023*
Neural invasion	4.88	0.061		
TNM staging	6.29	0.001*	7.12	0.002*
miR-216b expression	3.97	0.004*	2.74	0.325
KRAS expression	4.69	0.004*	6.17	0.007*

*Analysis was performed using Kaplan-Meier method (a) and Cox proportional hazards regression model (b). *P < 0.05 represents a significant difference.*

from therapy [17]. Another study revealed that there was significantly decreased expression of miR-216b in human gastric adenocarcinoma [18]. What is more, researchers also found out that the high expression of miR-216 was related to higher overall survival [19]. In this study, we have already verified the low expression of miR-216b and the better prognosis with high miR-216b expression in pancreatic cancer.

There is considerable evidence suggesting that miR-216b functions as a key inhibitor in regulating gene expression through base pairing within the target messenger RNA (mRNA) 3′-untranslated region (UTR) [4]. Our study results demonstrated that miR-216b targeted *KRAS* in pancreatic cancer cells. One previous study suggested that miR-216b directly targeted *KRAS* in RInk-1 cells in pancreatic cancer [20]. Another study indicated that miR-216b targeted *KRAS* in nasopharyngeal carcinoma [21]. A similar article also revealed that miR-216a could actually target *KRAS* in PDAC [10]. These lines of evidence all support our conclusion.

In addition, some researchers hypothesized that miR-216a induced apoptosis in pancreatic cancer cells by silencing MALAT1 expression [8], indicating that up-regulation of miR-216a may inhibit the oncogene and suppress the cancer. In the current study, we mainly investigated the role miR-216b played in pancreatic cells through regulating *KRAS* and validated that miR-216b down-regulated the expression of *KRAS*, inhibiting pancreatic cancer cell proliferation, migration and invasion and promoting apoptosis. Moreover, some previous studies have demonstrated that *KRAS* could induce invasion and proliferation of pancreatic cancer cells [22]. The results in a similar study showed that kinase Pim-1 could regulate the oncogene *KRAS*, and hence suppressed human PDAC cell growth [23], while other results indicated that miR-216a could also significantly inhibit cell growth and promote cell apoptosis in pancreatic cancer [24]. Furthermore, some researchers also found out that miR-216a exerted its tumor suppressor function through inhibiting the *KRAS*-related pathway [21]. Our conclusions were strongly supported.

However, some limitations need to be overcome in future studies. For one thing, the signaling pathway is still under investigation. For another, the number of samples in our study was also limited due to the shortage of funds.

In conclusion, our results revealed a correlation between miR-216b and *KRAS* in human pancreatic cancer cells. All these data suggested that miR-216b was functional in inhibiting oncogenic *KRAS* and could act as a novel therapeutic target for the treatment of pancreatic cancer.

Our study demonstrated that miR-216b suppressed pancreatic cancer cell proliferation, migration and invasion and promoted apoptosis via down-regulation of the oncogene *KRAS*, providing a novel approach for pancreatic cancer treatment.

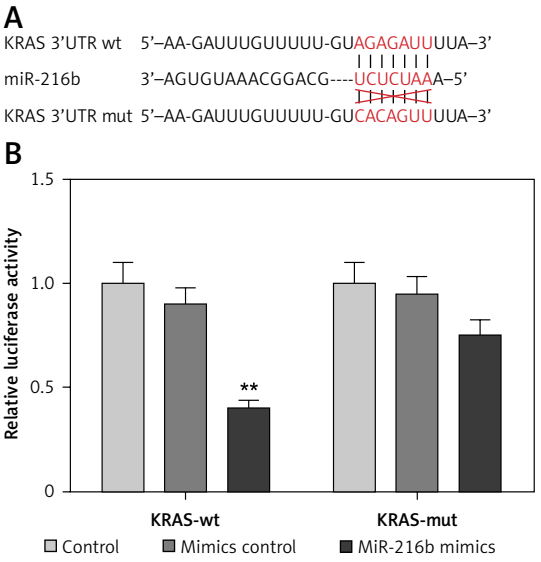

Figure 3. MiR-216b directly targeted *KRAS*. **A** – TargetScan algorithm was used to predict the binding site of miR-216b in *KRAS* 3′UTR. **B** – The luciferase activity of *KRAS* 3′UTR-wt in Panc-1 cells transfected with miR-216b mimics was significantly weaker compared with the cells transfected with negative control mimics. However, there was no significant difference in the luciferase activity of *KRAS* 3′UTR-mut between the miR-216b group and NC group

***P < 0.05, compared with NC group*

Acknowledgments

Xinquan Wu and Weibo Chen contributed equally to this work.

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Conflict of interest

The authors declare no conflict of interest.

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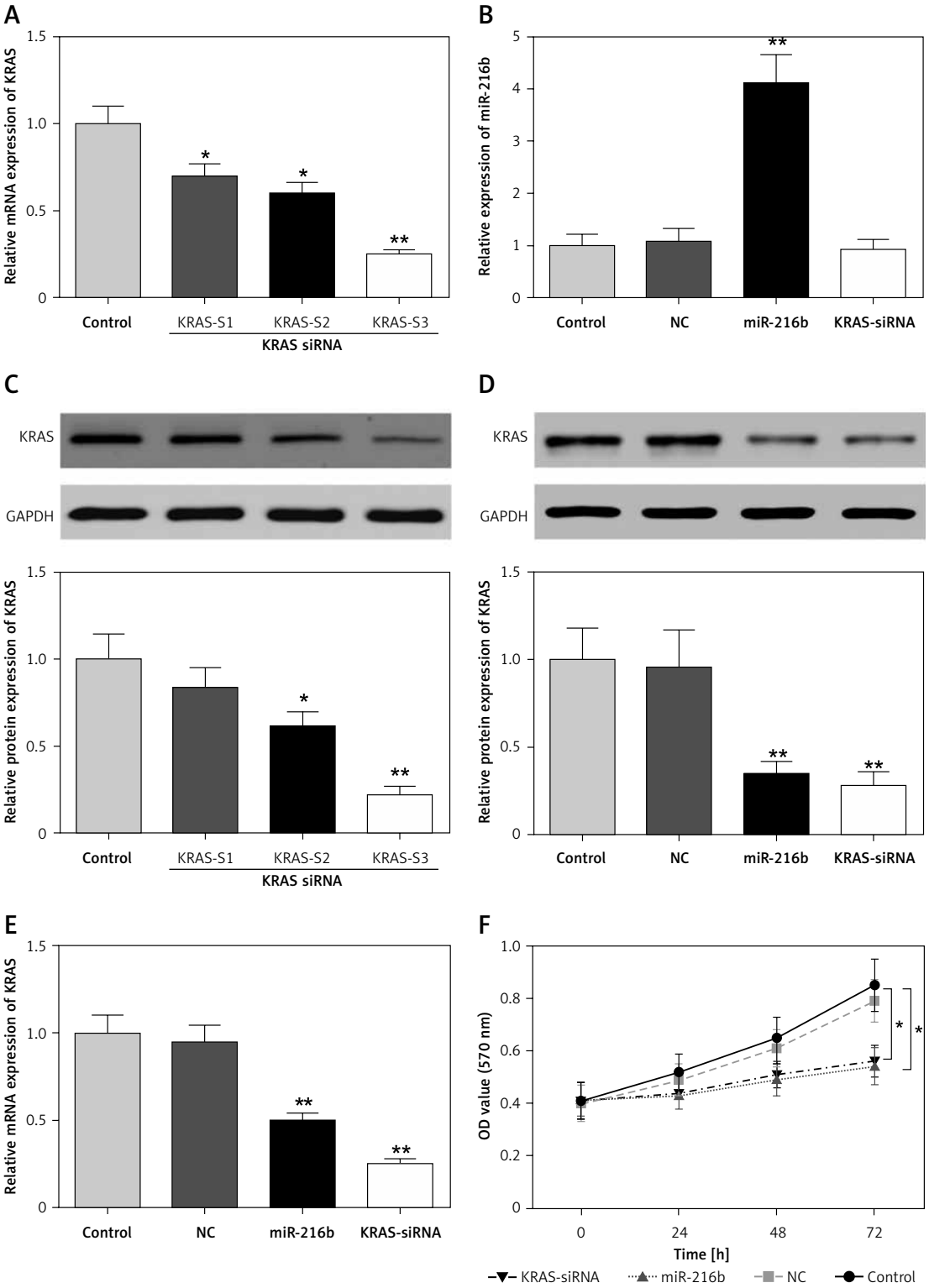

Figure 4. MiR-216b suppressed pancreatic cancer cell proliferation through targeting *KRAS*. **A**, **C** – The transfection efficiency of three KRAS siRNA transfected groups was determined. *$P < 0.05$, **$p < 0.01$, compared with control group. **B**, **D**, **E** – The expression of miR-216b in Panc-1 cells was significantly up-regulated, while the expression of *KRAS* was remarkably down-regulated after being transfected with *KRAS*-siRNA detected by qRT-PCR and western blot. **$P < 0.01$ compared with control group. **F** – The proliferation ability of Panc-1 cells after transfection with miR-216b mimics and *KRAS*-siRNA was significantly repressed, as observed by MTT assay. *$P < 0.05$, compared with control group

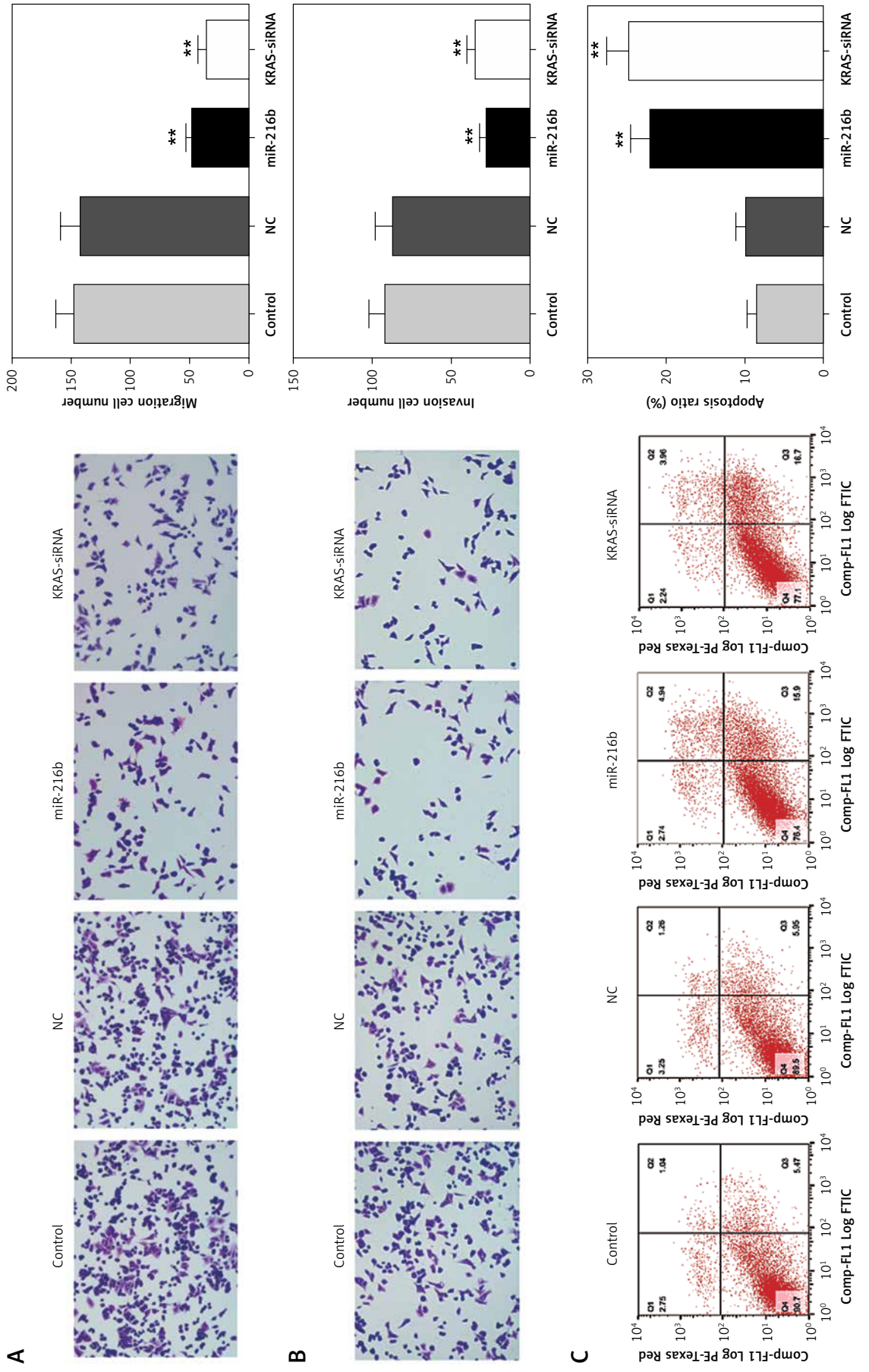

Figure 5. MiR-216b inhibited pancreatic cancer cell migration and invasion and promoted apoptosis by targeting *KRAS*. **A**, **B** – The migration and invasion abilities of Panc-1 cells significantly decreased after transfection with the miR-216b mimics and *KRAS*-siRNA observed by transwell assay. **C** – The apoptosis ability of Panc-1 cells significantly increased after transfection with the miR-216b mimics and *KRAS*-siRNA detected by flow cytometry assay. $^{**}P < 0.01$, compared with control group

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