

## High dose glargine alters the expression profiles of microRNAs in pancreatic cancer cells

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### Abstract

**AIM:** To investigate the effect of high dose glargine on the expression profiles of microRNAs in human pancreatic cancer cells.

**METHODS:** Real-time polymerase chain reaction array (RT-PCR) was applied to investigate miRNAs differentially expressed in Sw1990 cells treated with or without 100 IU/L glargine. Stem-loop RT-PCR was used to confirm the results of the array assay in Sw1990 and Panc-1 cells. The effects of miR-95 on cell growth, apoptosis, invasion and migration abilities were respectively examined by CCK8 assay, apoptosis assay, Matrigel invasion and migration assay in Sw1990 and Panc-1 cells. Nude mice xenograft models with Sw1990 cells were built to investigate pancreatic cancer growth *in vivo* after transfection by the lentivirus pGLV3-GFP- miR-95.

**RESULTS:** Ten miRNAs were significantly up-regulated and 2 miRNAs down-regulated in glargine treated Sw1990 cells when compared with non-treated cells (2.48-fold changes on average,  $P < 0.01$ ). miR-95, miR-134 and

miR-34c-3p are the top three miRNAs regulated by glargine (3.65-fold, 2.67-fold and 2.60-fold changes respectively,  $P < 0.01$ ) in Sw1990 cells. Stem-loop RT-PCR confirmed that high dose glargine up-regulated the expression of miR-95 and miR-134 in both Sw1990 and Panc-1 cells. The most obvious change is the apparent increase of miR-95. Forced expression of miR-95 significantly increased cell proliferation (Sw1990:  $2.510 \pm 0.129$  vs  $2.305 \pm 0.187$ ,  $P < 0.05$ ; Panc-1:  $2.439 \pm 0.211$  vs  $2.264 \pm 0.117$ ,  $P < 0.05$ ), invasion (Sw1990:  $67.90 \pm 12.33$  vs  $47.30 \pm 5.89$ ,  $P < 0.01$ ; Panc-1:  $37.80 \pm 8.93$  vs  $30.20 \pm 5.14$ ,  $P < 0.01$ ), migration (Sw1990:  $101 \pm 6.00$  vs  $51.20 \pm 8.34$ ,  $P < 0.01$ ; Panc-1:  $91.80 \pm 9.22$  vs  $81.50 \pm 7.47$ ,  $P < 0.01$ ) and inhibited cell apoptosis (Sw1990:  $22.05\% \pm 1.92\%$  vs  $40.32\% \pm 1.93\%$ ,  $P < 0.05$ ; Panc-1:  $20.17\% \pm 0.85\%$  vs  $45.60\% \pm 1.43\%$ ,  $P < 0.05$ ) when compared with paired negative controls, whereas knockdown of miR-95 obtained the opposite effect. Nude mice xenograft models confirmed that miR-95 promoted the growth of pancreatic cancer *in vivo* when compared with negative control (tumor volume:  $373.82 \pm 23.67$  mL vs  $219.69 \pm 17.82$  mL,  $P < 0.05$ ).

**CONCLUSION:** These observations suggested that modulation of miRNA expression may be an important mechanism underlying the biological effects of glargine.

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**Key words:** Glargine; MicroRNAs; Pancreatic cancer; Lentivirus; Cancer growth

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## INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in Western countries and has the poorest survival rate (< 5%) among the common malignancies<sup>[1,2]</sup>. Recently, antidiabetic therapies have been shown to affect the risk of pancreatic cancer. Some observational studies in humans have linked glargine with a putative increased cancer risk, including pancreatic cancer<sup>[3,4]</sup>.

Glargine (A21Gly, B31Arg, B32Arg human insulin) is a widely used insulin analog in which a 24-h action profile is achieved by altering the amino acid sequence of the alpha ( $\alpha$ ) and beta ( $\beta$ ) chains of the C terminus<sup>[5]</sup>. It has been shown that glargine increases resistance to apoptosis in several tumor cell lines<sup>[6]</sup>. Given the increased affinity to the insulin-like growth factor- I receptor (IGF-IR) *in vitro*<sup>[7]</sup>, glargine may increase the bioavailability of IGF- I by altering the levels of IGF-binding proteins<sup>[8,9]</sup>. IGF- I is a more potent growth factor than insulin, promoting proliferation and inhibiting apoptosis, and plays an important role in facilitating malignant cell survival and metastasis<sup>[10,11]</sup>. This may be the theoretical basis for the potential carcinogenicity of glargine. However, data regarding the effect of glargine on pancreatic cancer are inconsistent. Administration of glargine didn't alter proliferation of Colo-357 pancreatic carcinoma cells and survival of patients with pancreatic carcinoma<sup>[12]</sup>. Thus, the role of glargine in pancreatic carcinogenesis deserves further investigation.

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs, 19-25 nucleotides in length, which are now recognized as crucial post-transcriptional regulators of gene expression<sup>[13-15]</sup>. It has been demonstrated that miRNAs play important roles in biological processes that affect tumor progression including migration, invasion, epithelial to mesenchymal transition (EMT) and metastasis<sup>[16-18]</sup>. miRNAs are promising as early biomarkers, prognostic indicators and therapeutic targets for anticancer treatments<sup>[19-21]</sup>. Aberrant miRNA expression has also been frequently reported in pancreatic cancer<sup>[22]</sup>. However, very few compounds, not to mention glargine, which affect cell growth and/or development, have been shown to affect miRNA expression.

In this present study, we elucidated the miRNAs signature in response to glargine treatment in human pancreatic cancer cells. Our results indicated that glargine alters specific miRNA expression in human pancreatic cells, especially miR-95. The effect of miR-95 on apoptosis, proliferation, migration and invasion ability of pancreatic cancer cells were further investigated. Moreover, nude mice xenograft models were built to investigate pancreatic cancer growth *in vivo* after transfection by the lentivirus pGLV3-GFP-miR-95. It therefore appeared that miR-95-related changes were important effects of glargine.

## MATERIALS AND METHODS

### Cell lines and cultures

Pancreatic ductal cancer cell lines Sw1990 and Panc-1 were

conserved in our own laboratory and were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% fetal bovine serum (FBS; GIBCO) in a humidified incubator at 37 °C with an atmosphere of 5% CO<sub>2</sub>.

### miRNAs real time polymerase chain reaction array

Sw1990 cells ( $3 \times 10^5$  per well) were plated on 6-well plates in DMEM with 10% FBS. After 24 h of incubation at 37 °C, the cells were treated with or without 100 IU/L glargine. Glargine was replenished every 24 h. The cultures were incubated for 2 d, then the total RNA was isolated from cell samples using Trizol reagent (Invitrogen) following the manufacturer's protocol. Then, cDNA synthesis was performed using Universal cDNA synthesis kit (Exiqon). The expression levels of 372 human mature miRNAs were examined using the miRCURY LNA™ Universal real time microRNA polymerase chain reaction system, Ready-to-use human panel I (Exiqon, kangchen, China).

Briefly, total RNA containing miRNA was polyadenylated, and cDNA was synthesized using a poly (T) primer with a 3'degenerate anchor and a 5'universal tag. Then, cDNA served as a template for microRNA quantitative real-time polymerase chain reaction (qPCR) using miRCURY LNA Universal RT miRNA PCR kit (Exiqon). The miRNA Ready-to-use human panel I is a 384-well PCR plate containing dried down LNA™ primer sets for one real-time PCR reaction per well. Three small RNA (U6snRNA, SNORD38B, SNORD49A) and three miRNA (miR-103, miR-191 and miR-423-5p) reference genes are included on the panel. The amplification profile was denatured at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 60 s. At the end of the PCR cycles, melting curve analyses were performed. All reactions were conducted three times. Expression levels of mature miRNAs were evaluated using comparative CT method ( $2^{-\Delta CT}$ ).

### Stem-loop real-time reverse transcription-PCR

The miRNAs (miR-95, miR-134 and miR-34c-3p) were quantitated by stem-loop real time reverse transcription (RT)-PCR to confirm the reliability of the miRNA array assay. In brief, Sw1990 and Panc-1 cells ( $3 \times 10^5$  per well) were seeded on 6-well plates in DMEM with 10% FBS. After 24 h of incubation, the cells were treated with different concentrations of glargine (0-150 IU/L) for 48 h or treated with 100 IU/L glargine for different periods (24-72 h). Glargine was replenished every 24 h. Then the total RNA was isolated. 0.2-0.5  $\mu$ g of total RNA was reverse transcribed to cDNA using a target-specific stem-loop primer indicated in Table 1. cDNA in water was added to 5  $\mu$ L of the  $2 \times$  SYBR green master mix (Applied Biosystems Inc, Foster City, United States), 400 nmol/L of gene-specific primer and water used to make the solution up to 10  $\mu$ L. The reactions were amplified at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. U6 small nuclear RNA (U6) served as the endogenous control. The relative amount of each miRNA

**Table 1** Primers used for reverse transcription or polymerase chain reaction of microRNAs

Gene name	Primer sequences (5'-3')	
<i>miRNA-95</i>	Stem-loop primer	CTCAACTGGTGTCTGGAGTCG-GCAATTCAGTTGAGTGCTCAAT
	Sense	CGGGTATTTATTGAGCA
	Antisense	AACTGGTGTCGTGGAG
<i>miRNA-134</i>	Stem-loop primer	CTCAACTGGTGTCTGGAGTCG-GCAATTCAGTTGAGCCCTCTG
	Sense	TGTGACTGGTTGACCAGAG
	Antisense	AACTGGTGTCGTGGAG
<i>miRNA-34c-3p</i>	Stem-loop primer	CTCAACTGGTGTCTGGAGTCGG-CAATTCAGTTGAGCCTGGCCGTG
	Sense	AATCACTAACCACACGG
	Antisense	AACTGGTGTCGTGGAG
U6	Stem-loop primer	CTCAACTGGTGTCTGGAGTCG-GCAATTCAGTTGAGAAAAATAT
	Sense	CAAGGATGACACGCAAAAT
	Antisense	TGGTGTCTGGAGTCG

to U6 was described using the formula  $2^{-\Delta Ct}$  where  $\Delta Ct = (Ct \text{ miRNA} - Ct U6)$ . Each sample was run in triplicate.

#### Vector constructs and lentivirus production

The miR-95 sequence was constructed as follows: (forward) hsa-miR-95-*Bam*H I: GATCCGTTCAACGGGTATT-TATTGAGCATTCAAGAGATGCTCAATATACCC-GTTGAACCTTTTTTG; (reverse) hsa-miR-95-*Eco*R I: AATTCAAAAAAGTTCAACGGGTATATTGAG-CATCTCTTGAATGCTCAATAAATACCCGTT-GAACG. The sequence was amplified and cloned into the pGLV3-GFP vector (GenePharma) to generate pGLV3-GFP- miR-95. The negative control was pGLV3-shRNA-NC. Virus packaging was performed in HEK 293T cells after the co-transfection of 20 mg pGLV3-GFP-miR-95 vector with 15 mg of the packaging plasmid pHelper 1.0 Vector and 10 mg of the envelope plasmid pHelper 2.0 Vector using Lipofectamine 2000 (Invitrogen). Viruses were harvested 48 h after transfection, and viral titers were determined.

#### Oligonucleotide construction

After glargine treatment, the expression of miR-95 was increased most obviously in Sw1990 cells and Panc-1 cells, so we further investigated the functional roles of miR-95 in pancreatic cancer cells. miR-95 mimics, miR-95 inhibitor and negative control siRNA oligonucleotides were chemosynthesized (Shanghai GenePharma Co. Ltd). The oligonucleotides used in these studies were hsa-miR-95 mimics: 5'-UUCAACGGGUAU UUAUUGAG-CA-3' and 5'-CUCAAUAAAUACCCGUUGAAUU-3'. Mimics negative control: 5'-UUCUCCGAACGUGU-CACGUTT-3' and 5'-ACGUGACACGUUCGGAGAA TT-3', hsa-miR-95 inhibitor: 5'-UGCUCUAAAUA-ACCCGUUGAA-3'. MicroRNA inhibitor negative control: 5'-CAGUACUUUUGUGUAGUACA A-3'.

#### Cell transfection

Cells were cultured to 80% to 90% confluence after be-

ing seeded into 6-well plates and were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For transient transfection, Sw1990 or Panc-1 cells in each well of a 6-well plate were transfected with 12.5  $\mu$ L miRNA inhibitor or 7.5  $\mu$ L miRNA mimic oligonucleotides. Transfection efficiency was evaluated by FAM in control vector or real-time PCR. For stably transfected cells, cells were transfected with lentivirus at 80%-90% confluency. Sw1990 cells ( $1 \times 10^5$ ) were infected with recombinant lentivirus in the presence of 5  $\mu$ g/mL polybrene (GenePharma).

#### Cell proliferation assay

A total of  $10^4$  Sw1990 or Panc-1 cells per well were plated in 96-well plates before transfection and cultured for 24 h in normal conditions. They were then transfected with hsa-miR-95 mimics or hsa-miR-95 inhibitor along with paired negative controls. The cells were incubated at 37  $^{\circ}$ C for 48 h. Cell proliferation was assessed using Cell Counting Kit 8 (Dojindo, Tokyo, Japan) according to manufacturer's protocol.

#### Apoptosis assay

At 72 h after transfection, apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (Biovision, United States). Results were calculated by the percentage of apoptotic cells in all cells counted.

#### Matrigel invasion assay

At 48 h after transfection, the invasive ability of the cells was assayed using Transwells (8 mm pore size, Corning Costar Corp). The Transwells were put into the 24-well plates. First, 0.1 mL Matrigel (50 mg/mL, BD Biosciences) was added onto the plate surface and incubated for 2 h, and then the supernatant was removed. Freshly trypsinized and washed Panc-1 or Sw1990 cells were suspended in DMEM containing 1% FBS. Then 0.1 mL of the cell suspension ( $1 \times 10^5$  cells) was added to the upper chamber of each insert that was coated with Matrigel. Next, 0.6 mL of DMEM containing 10% FBS was added into the lower compartment, and the cells were allowed to invade for 24 h at 37  $^{\circ}$ C in a 5% CO<sub>2</sub> humidified incubator. After incubation, the cells were fixed with 95% absolute alcohol and followed by crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of  $\times 200$ . Each experiment was performed in triplicate.

#### Cell migration

At 48 h after transfection, the ability of Panc-1 or Sw1990 cells to migrate was detected using Transwells (8 mm pore size, Corning Costar Corp). The Transwells were put into the 24-well plates. Freshly trypsinized and washed cells were suspended in DMEM containing 1% FBS.  $5 \times 10^4$  cells/well were placed in the top chamber of each insert (BD Biosciences, NJ), with the non-coated membrane. 0.6 mL of DMEM containing 10% FBS was added into the lower chambers. After incubating for 24 h at 37  $^{\circ}$ C in



a 5% CO<sub>2</sub> humidified incubator, the cells were fixed with 95% absolute alcohol and stained with crystal violet stain. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 10 fields with magnification of  $\times 200$ . Each experiment was performed in triplicate.

### Mice xenografts

The stable cell line Sw1990 was harvested from tissue culture flasks after transfection with the pGLV3-GFP-miR-95 and control pGLV3-GFP vector using trypsin and washed three times with PBS. About  $1 \times 10^7$  cells were implanted into the right flanks of female nu/nu mice (five in each group). Tumor volume (V) was measured with an external caliper every 4 d and it was calculated as  $V = 0.52 (\text{length} \times \text{width}^2)$ . After four weeks, all the animals were sacrificed and tumors were removed.

### Statistical analysis

Data were expressed as the mean  $\pm$  SD unless otherwise noted. The differences between groups were analyzed using a two-tailed Student's *t*-test when only two groups were present and the null hypothesis was rejected at the 0.05 level.

## RESULTS

### Glargine treatment alters miRNAs expression profiles

To study the responses of miRNAs to glargine, miRNA real time PCR array analysis of miRNA expression was conducted with total RNAs extracted from Sw1990 pancreatic cells treated with or without 100 IU/L glargine. Differential expression between glargine-treated and non-treated cells was defined using a cut off value of 2-fold change. We observed that 10 miRNAs were significantly up-regulated and 2 miRNAs were significantly down-regulated (2.48-fold on average,  $P < 0.01$ ) in glargine treated Sw1990 cells when compared with non-treated cells. miR-95, miR-134 and miR-34c-3p are the top three miRNAs regulated by glargine (3.65-fold, 2.67-fold and 2.60-fold changes respectively,  $P < 0.01$ ) in Sw1990 cells (Figure 1A).

### Confirmatory studies with differentially expressed miRNAs by stem-loop real-time PCR

After treatment with increasing concentrations of glargine (50, 100, 150 IU/L) for 48 h, miR-95 was up-regulated by 1.18 fold ( $P > 0.05$ ), 3.41 fold ( $P < 0.01$ ) and 2.92 fold ( $P < 0.01$ ) on average respectively in Sw1990 cells and 1.45 fold ( $P < 0.01$ ), 3.41 fold ( $P < 0.01$ ) and 2.92 fold ( $P < 0.01$ ) on average respectively in Panc-1 cells, when compared with non-treated cells (0 IU/L). No obvious dose dependent responses were observed; miR-134 was up-regulated in a dose dependent manner (Sw1990: 1.69, 2.10 and 2.93 fold on average respectively,  $P < 0.01$ ; Panc-1: 1.56, 1.99 and 2.88 fold on average respectively,  $P < 0.01$ ) in both Sw1990 and Panc-1 cells; miR-34c-3p showed no significant changes (Sw1990: 1.03, 1.05 and 1.06 fold on average respectively,  $P > 0.05$ ; Panc-1: 1.25, 1.25 and 1.19

fold on average respectively,  $P > 0.05$ ) in both Sw1990 and Panc-1 cells (Figure 1B-1,2).

After treatment with 100 IU/L glargine for different periods (24, 48 and 72 h), miR-95 was up-regulated by 2.50 fold ( $P < 0.01$ ), 3.10 fold ( $P < 0.01$ ) and 2.99 fold ( $P < 0.01$ ) on average, respectively, in Sw1990 cells and 2.31 fold ( $P < 0.01$ ), 2.46 fold ( $P < 0.01$ ) and 2.16 fold ( $P < 0.01$ ) on average, respectively, in Panc-1 cells, when compared with the cells at 0 h; miR-134 was up-regulated by 2.22 fold ( $P < 0.01$ ), 2.37 fold ( $P < 0.01$ ), 2.17 fold ( $P < 0.01$ ) on average, respectively, in Sw1990 cells and 2.10 fold ( $P < 0.01$ ), 2.31 fold ( $P < 0.01$ ) and 2.37 fold ( $P < 0.01$ ) on average, respectively, in Panc-1 cells; miR-34c-3p showed no significant changes (Sw1990: 1.16, 1.14 and 1.13 fold on average respectively,  $P > 0.05$ ; Panc-1: 1.17, 1.24 and 1.13 fold on average, respectively,  $P > 0.05$ ) in both Sw1990 and Panc-1 cells (Figure 1B-3,4).

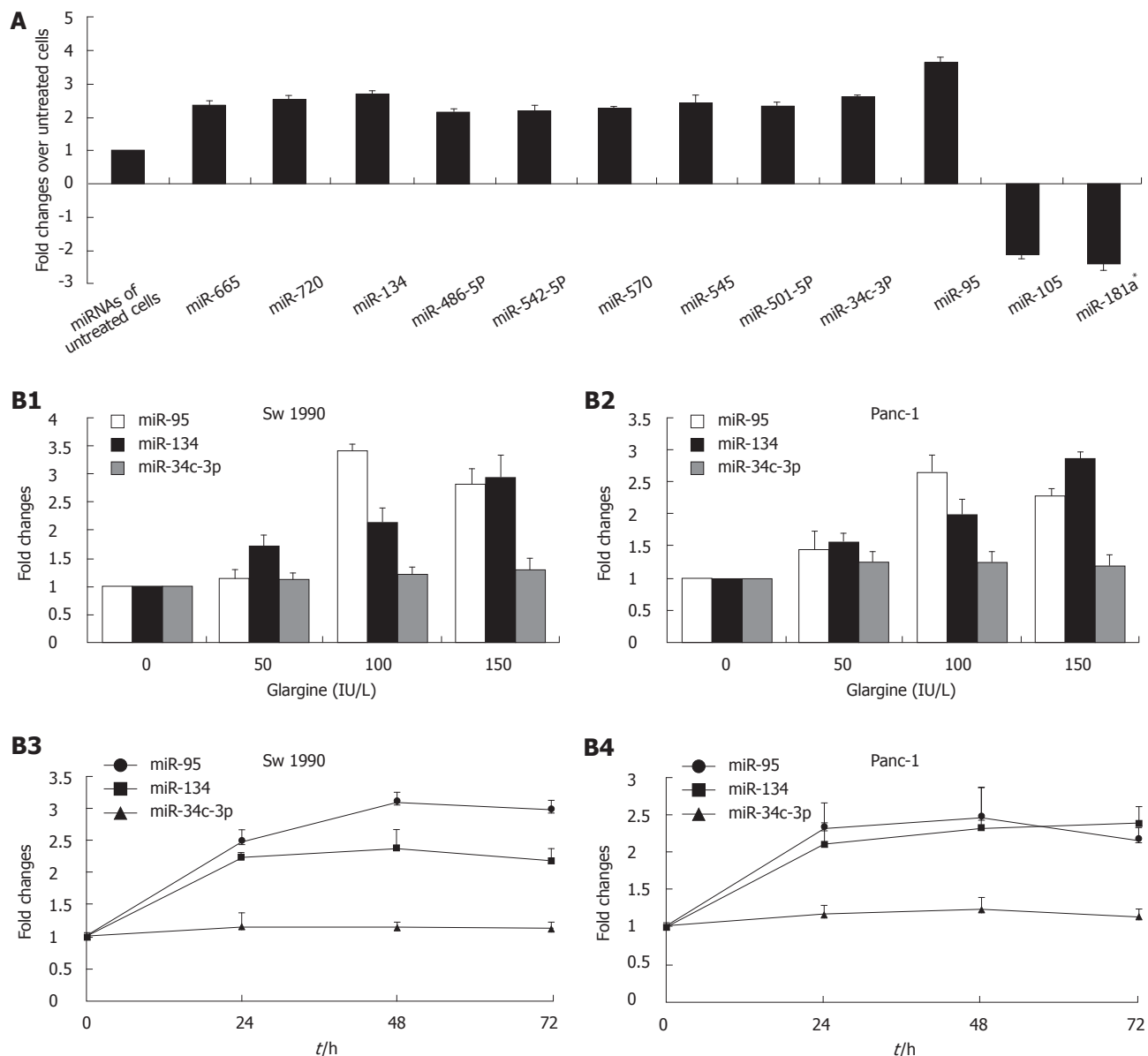
### miR-95 increases cell proliferation and inhibits cell apoptosis

We investigated the potential oncogenic role of miR-95 in Sw1990 and Panc-1 cells. First, we tested miR-95 expression using stem-loop real-time PCR. It increased or decreased after transfected with miR-95 mimics or anti-miR-95 inhibitor. We observed a significant increase in proliferation (Sw1990:  $2.51 \pm 0.13$  vs  $2.31 \pm 0.19$ ,  $P < 0.05$ ; Panc-1:  $2.44 \pm 0.21$  vs  $2.26 \pm 0.12$ ,  $P < 0.05$ ) after transfection of miR-95 mimics (Figure 2A-1). In contrast, anti-miR-95 inhibitor significantly decreased cell proliferation (Sw1990:  $2.11 \pm 0.07$  vs  $2.23 \pm 0.13$ ,  $P < 0.05$ ; Panc-1:  $2.09 \pm 0.09$  vs  $2.31 \pm 0.13$ ,  $P < 0.05$ ) (Figure 2A-2). These data indicate that cell proliferation can be significantly promoted by increase of miR-95 expression.

We further investigated the effect of miR-95 on apoptosis and found that apoptosis decreased dramatically (Sw1990:  $22.05\% \pm 1.92\%$  vs  $40.32\% \pm 1.93\%$ ,  $P < 0.05$ ; Panc-1:  $20.17\% \pm 0.85\%$  vs  $45.60\% \pm 1.43\%$ ,  $P < 0.05$ ) in Sw1990 and Panc-1 cells 72 h after transfection with miR-95 mimics (Figure 2B). It suggested that miR-95 may function as a strong apoptotic suppressor in human pancreatic cancer cells.

### miR-95 regulates pancreatic cancer cell invasion and migration in vitro

In the cell invasion and migration assay, we observed that depletion of miR-95 significantly impaired the ability of Sw1990 cells to migrate and invade through the matrigel-coated membranes or the non-matrigel-coated membranes towards serum-containing medium (invasion:  $49.40 \pm 6.59$  vs  $65.80 \pm 5.09$ ; migration:  $52.30 \pm 10.87$  vs  $88.90 \pm 10.46$ ,  $P < 0.01$ ), when compared with a paired negative control (Figure 3B); Increased expression of miR-95 significantly promoted the ability of Sw1990 cells to migrate and invade through matrigel-coated membranes or non-matrigel-coated membranes towards serum-containing medium (invasion:  $67.90 \pm 12.33$  vs  $47.30 \pm 5.89$ ; migration:  $101.00 \pm 6.00$  vs  $51.20 \pm 8.34$ ,  $P < 0.01$ ), when compared with the paired negative control (Figure 3A). Similar results were found in Panc-1 cells (depletion of miR-95,



**Figure 1** Effect of insulin glargine on miRNAs expression in Sw1990 cell line (A), insulin glargine up-regulated the expression of miR-95 and miR-134, miR-34c-3p showed no obvious changes in Sw1990 and Panc-1 cells (B). Ten miRNAs were significantly up-regulated and two miRNAs were down-regulated after 100 IU/L insulin glargine treatment for 48 h compared with non-treated cells. The cut off line for miRNA expression change was 2-fold. Data were presented as mean  $\pm$  SD. Sw1990 and Panc-1 cells were incubated with increasing concentrations of insulin glargine (50-150 IU/L) for 48 h or treated with 100 IU/L insulin glargine for different periods (24-72 h). Then the expression of the three miRNAs were detected by stem-loop real-time reverse transcription-polymerase chain reaction array. miR-95 was significantly up-regulated, but no dose or time dependent changes were observed; miR-134 was up-regulated in a dose-dependent manner. miR-181a: The miRNA of lower abundance.

invasion:  $57.90 \pm 10.55$  vs  $73.80 \pm 11.95$ , migration:  $66.40 \pm 10.1$  vs  $99.50 \pm 8.85$ ,  $P < 0.01$ ; forced expression of miR-95, invasion:  $37.80 \pm 8.93$  vs  $30.20 \pm 5.14$ ; migration:  $91.80 \pm 9.22$  vs  $81.50 \pm 7.47$ ,  $P < 0.01$ ) (Figure 4A and B). These results indicated that miR-95 may be important in the progression of pancreatic cancer through increasing cell invasion and migration.

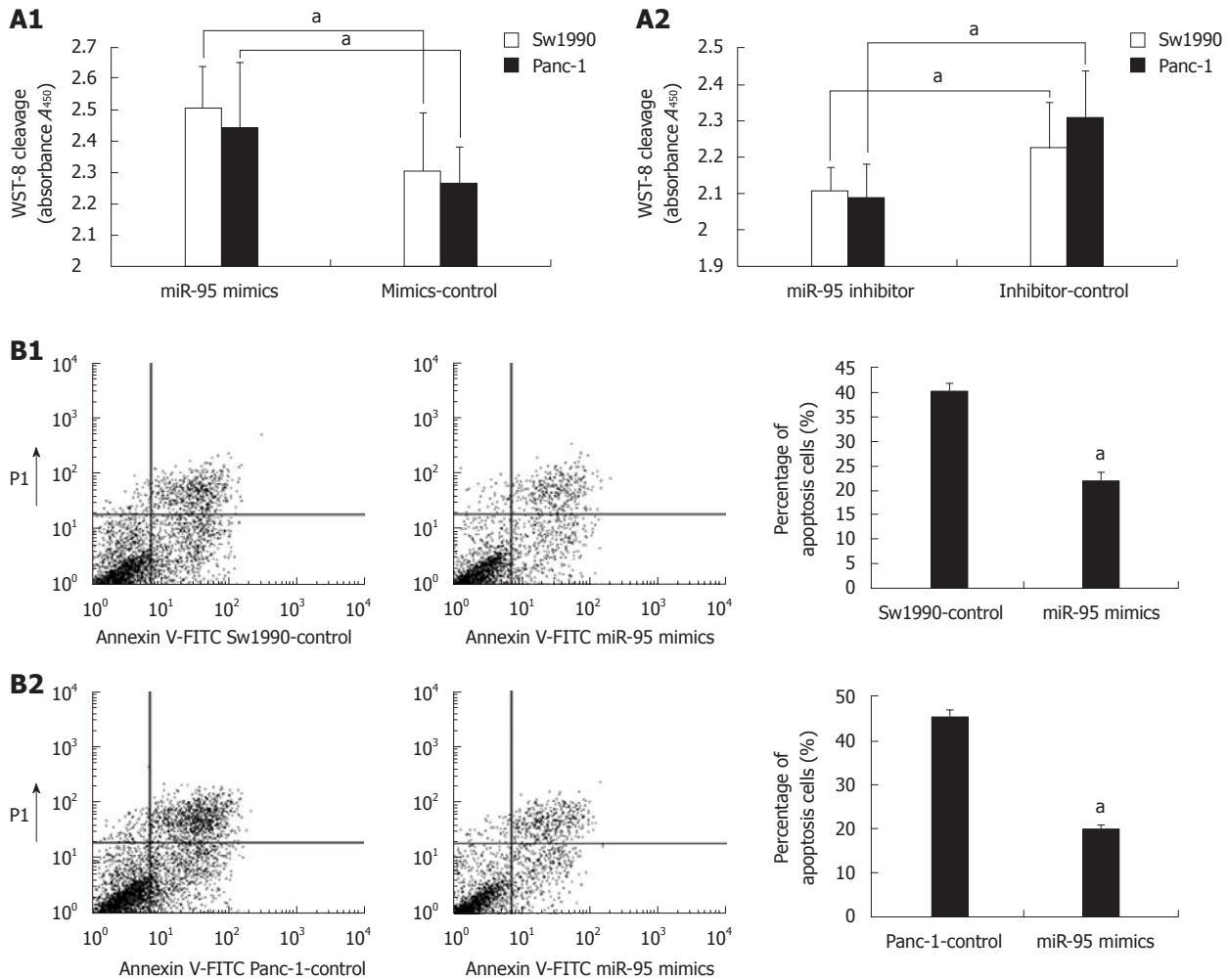
**miR-95 promotes the growth of Sw1990 xenografts**

Sw1990 cells transfected with pGLV3-GFP-miR-95 or negative control pGLV3-GFP were injected into the right flank of nude mice. Four weeks later, the tumor volumes of xenografts were  $373.82 \pm 23.67$  mm<sup>3</sup> in the miR-95

transfected group and  $219.69 \pm 17.82$  mm<sup>3</sup> in the negative control group. The weight of xenografts was  $0.40 \pm 0.08$  g in the miR-95 transfected group and  $0.23 \pm 0.05$  g in the negative control group. miR-95 significantly increased the growth of the Sw1990 xenografts (Figure 5,  $P < 0.05$ ).

**DISCUSSION**

In the present study we demonstrated that glargine altered specific miRNA expression in human pancreatic cells at 50-150 IU/L, which is equivalent to 300-900 nmol and is much higher than the physiological concentration of



**Figure 2** miR-95 promotes cell proliferation (A) and enhancement of miR-95 inhibit apoptosis (B). A total of 10<sup>4</sup> Sw1990 or Panc-1 cells per well were plated in 96-well plates for 24 h and then were transfected with hsa-miR-95 mimics or hsa-miR-95 inhibitor along with paired negative controls. The cells were incubated at 37 °C for 48 h and then cell proliferation was assessed using Cell Counting Kit 8. Data were shown as mean ± SD. <sup>a</sup>P < 0.05 vs pairing negative control.

insulin (0.1-1 nmol). Our miRNA real time PCR array showed that high dose glargine (100 IU/L) up-regulated the expression of 10 miRNAs and down-regulated 2 miRNAs in Sw1990 pancreatic cancer cells. The most obvious change was the apparent increase of miR-95. Stem-loop real-time PCR confirmed the aberrant changes of miR-95 after treatment of high dose glargine in Sw1990 and Panc-1 pancreatic cancer cells. Then miR-95 showed significant anti-apoptotic and growth-promoting effects *in vitro* and *in vivo*. Ectopic expression and siRNA knock-down of miR-95 confirmed its invasion-promoting activity *in vitro*. Therefore, these results highlighted the miR-95-related changes as important effects of glargine.

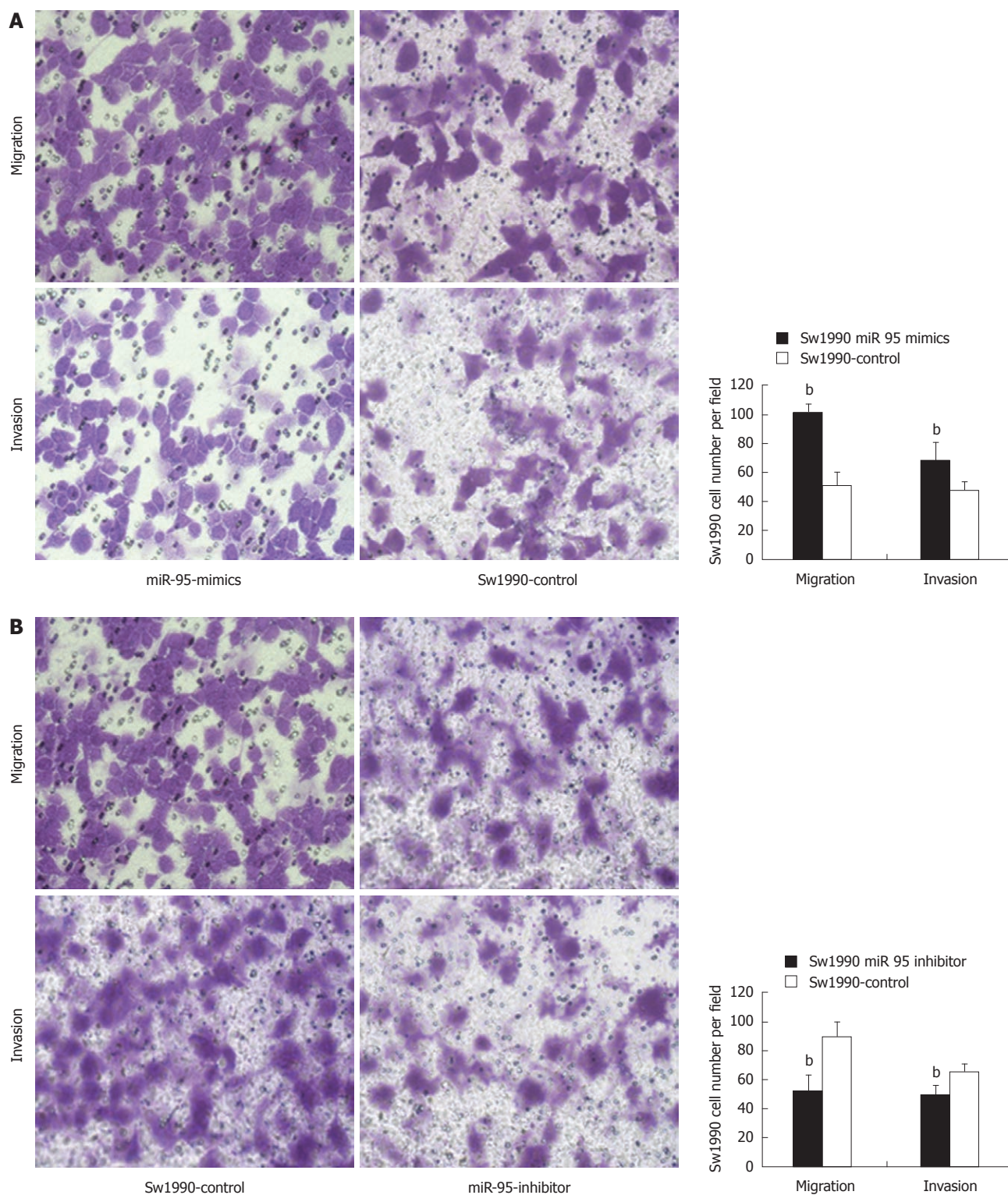
Recent studies linked the use of glargine with increased risk of cancer. Hemkens *et al*<sup>[4]</sup> published a registry study that demonstrated a significantly increased risk of cancer diagnosis associated with high dosages of glargine. However, the Scottish study found a non-significant increased risk for specifically breast cancers<sup>[23]</sup>. The UK study found no link between glargine and cancer<sup>[3]</sup>. In addition, although glargine has been shown to increase resistance to apoptosis in several tumor cell lines, administration of

glargine didn't alter proliferation of Colo-357 pancreatic carcinoma cells *in vitro*<sup>[12]</sup>. Therefore, all epidemiological and laboratory evidence remains inconclusive and new indicators are needed to determine the role of glargine in carcinogenesis.

miRNAs have been recognized as promising diagnostic and prognostic markers for cancer diagnosis or treatment. For example, miR-34a family members were found to be directly regulated by TP53 and act as tumor suppressors<sup>[24]</sup>; miR-217 inhibited pancreatic cancer cell growth through targeting KRAS<sup>[25]</sup>; miR-10b promoted pancreatic cancer invasiveness and correlates with a poor prognosis<sup>[26]</sup>; The miRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) were found to inhibit tumor invasion and metastasis by regulating EMT<sup>[27]</sup>; miR-126 can inhibit cell adhesion, migration and invasion through the suppression of CRK<sup>[28]</sup>. Our study confirmed, for the first time, that miR-95 and miR-134 were primary glargine-responsive miRNAs.

miR-95 has been shown to be involved in carcinogenesis. A highly characterized example is colorectal carcinoma, in which miR-95 can promote cell proliferation by

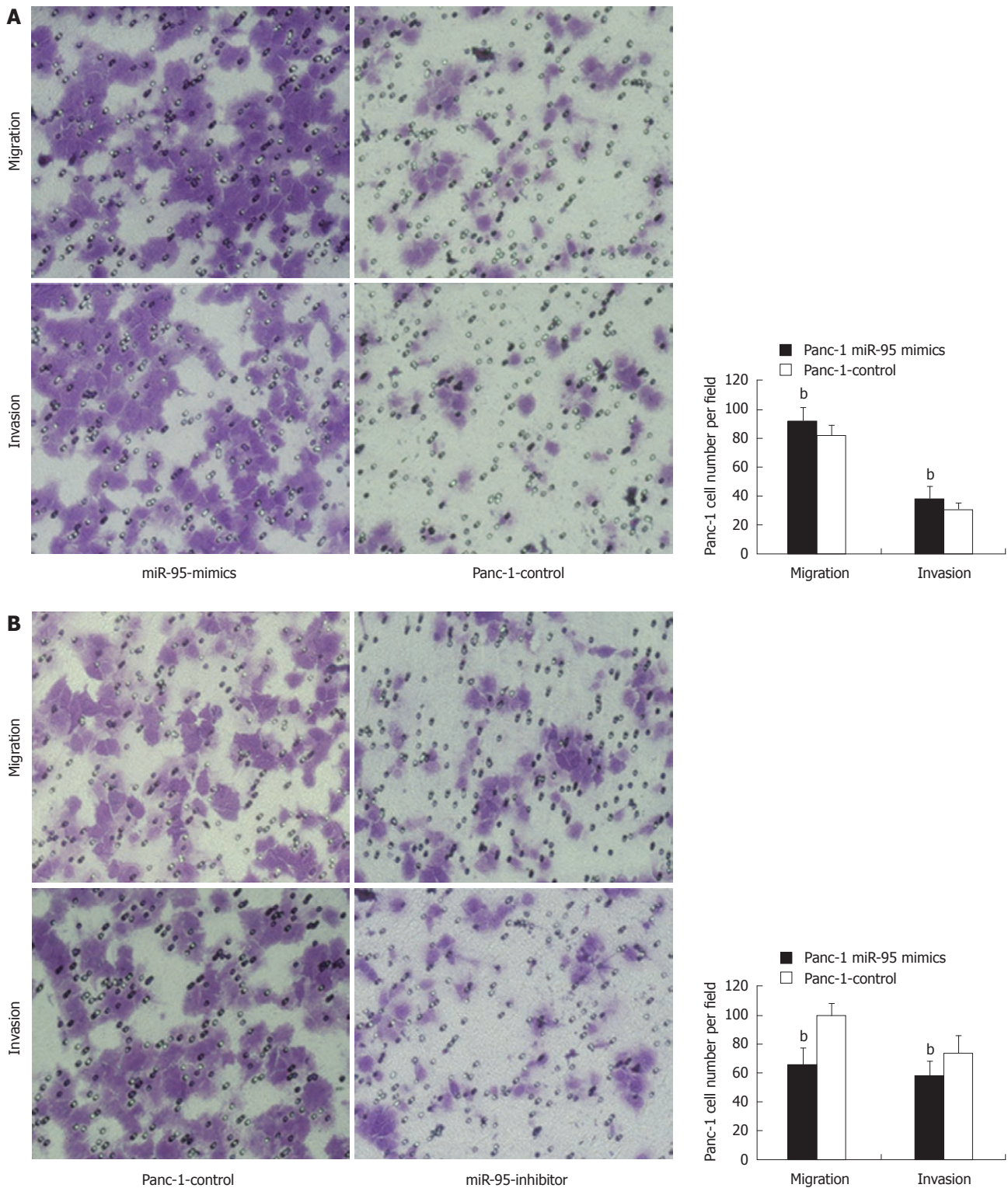




**Figure 3** Effect of miR-95 on tumor cell migration, invasion of Sw1990 cells. A: Invasion and migration assay. Representative fields of invasion (down) or migration (up) cells on the membrane (left, magnification of  $\times 200$ ). Average invasion or migration cell number per field (right). The invasion or migration cell number of Sw1990 transfected with miR-95-mimics drastically increased than that transfected with pairing negative control; B: The invasion or migration cell number of Sw1990 cells transfected with miR-95-inhibitor dramatically decreased than that transfected with pairing negative control. <sup>b</sup> $P < 0.01$  vs Sw1990-control,  $n = 10$ .

regulating sorting Nexin 1<sup>[29]</sup>. In pancreatic cancer, miR-95 is significantly upregulated in most tissues and cell lines<sup>[30]</sup>. In HeLa cells, inhibition of miR-95 caused a decrease in cell growth<sup>[31]</sup>. *miR-134* gene is located at 14q32, and is involved in several physiological and pathological pro-

cesses. For example, miR-134 plays an important role in translation-dependent guidance of nerve growth cones<sup>[32]</sup>; miR-134 is regarded as a potential plasma biomarker for the diagnosis of acute pulmonary embolism<sup>[33]</sup>; plasma miR-134 in bipolar disorder serves as a potential periph-

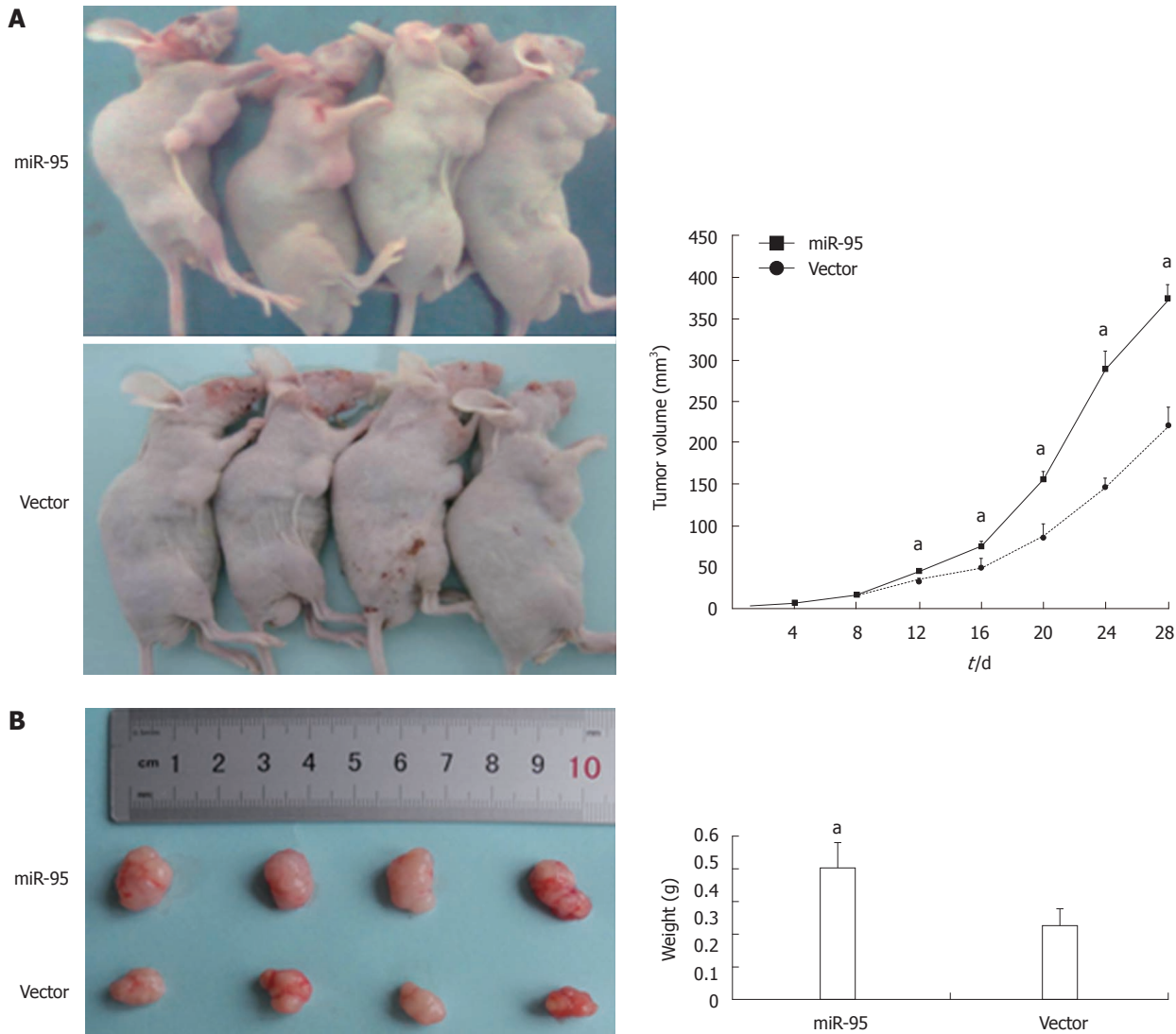


**Figure 4** Effect of miR-95 on tumor cell migration, invasion of Panc-1 cells. A: Invasion and migration assay. Representative fields of invasion (down) or migration (up) cells on the membrane (left, magnification of  $\times 200$ ). Average invasion or migration cell number per field (right). The invasion or migration cell number of Panc-1 transfected with miR-95-mimics drastically increased compared with that when transfected with the pairing negative control. B: The invasion or migration cell number of Panc-1 cells transfected with miR-26a-inhibitor dramatically decreased compared with that when transfected with the pairing negative control. <sup>b</sup> $P < 0.01$  vs Panc-1-control,  $n = 10$ .

eral marker that can respond to acute manic episodes and is associated with effective mood stabilizer treatment<sup>[34]</sup>. Interestingly, recent studies indicated that miR-134 may also be involved in carcinogenesis. p53/p63/p73, the tu-

mor suppressors, were believed to be regulators of the miR-134 processing complex<sup>[35]</sup>. Our study showed that high dose glargine can significantly upregulate the expression of miR-95 (no time or dose dependent manner) and





**Figure 5** miR-95 promotes the growth of Sw1990 xenografts. A: miR-95 strikingly increased the growth of Sw1990 cells xenografted in nude mice; B: At the end of the experiment, all animals were sacrificed and the tumors were removed. The tumors were much heavier in miR-95 group than in the vector group. Data were shown as mean  $\pm$  SD ( $^*P < 0.05$  vs vector).

miR-134 (dose dependent manner) *in vitro*, and for the first time investigated the role of miR-95 in pancreatic cancer.

In conclusion, our study demonstrated that alterations of specific miRNAs and miRNA-related changes were important effects of glargine, suggesting an important and novel new mechanism by which glargine mediates its potent effects on cell growth and apoptosis.

## COMMENTS

### Background

Glargine is widely used in the treatment of type 1 and type 2 diabetes mellitus. Recently, this insulin analogue has been suspected to be associated with an increased risk of cancer, including pancreatic cancer, but available evidence remains inconclusive.

### Research frontiers

Anti-diabetic therapies have been shown to affect the risk of pancreatic cancer. Metformin use was associated with reduced risk, and insulin or insulin analogue use was suspected to be associated with increased risk of pancreatic cancer in diabetic patients. However, data regarding the effect of glargine, one of the long-acting insulin analogues, on pancreatic cancer are inconsistent. Several researchers

believed glargine to be a motigen, not a carcinogen. New biomarkers are needed to determine the role of glargine in the carcinogenesis of pancreatic cancer.

### Innovations and breakthroughs

Recent reports have highlighted that miRNAs play important roles in biological processes that affect tumor progression and are promising biomarkers for cancer diagnosis or treatment. This is the first study to show the effects of high dose glargine on miRNA expression in pancreatic cancer cells. miR-95 was proved to be affected by high dose glargine and to be involved in the carcinogenesis of pancreatic cancer.

### Applications

By understanding the effects of glargine on pancreatic cancer cells, this study may help to clarify the role of glargine in the progress of pancreatic cancer.

### Terminology

Glargine is a widely used insulin analog in which a 24 h action profile is achieved. MicroRNAs (miRNAs) are endogenous, non-coding small RNAs, 19-25 nucleotides in length. It has been demonstrated that miRNAs play important roles in biological processes that affect tumor progression including migration, invasion and metastasis.

### Peer review

The authors examined the effects of high dose glargine on pancreatic cancer cells *in vitro*; miR-95 is up-regulated significantly by glargine. miR-95 can significantly increase pancreatic cancer cell proliferation, invasion and migration and inhibit cell apoptosis. Moreover, miR-95 is proved to inhibit pancreatic

cancer growth *in vivo*. The results are interesting and may represent the role of glargine in pancreatic carcinogenesis.

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