

Original Article

MicroRNA-374 targets JAM-2 regulates the growth and metastasis of human pancreatic cancer cells

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Abstract: Pancreatic cancer is one of the devastating human cancers responsible for tremendous human mortality. Current study was undertaken to explore the therapeutic potential of microRNA-374 in human pancreatic cancer. The results showed that microRNA-374 exhibited lower expression in pancreatic cancer cells and tissues. Overexpression of microRNA-374 in cancer cells resulted in significant decrease in the cell viability. The inhibition of the cell viability was mainly due to the induction of apoptosis as evident from the DAPI and AO/EB staining. Annexin V/PI staining also showed that the overexpression of microRNA-374 enhanced the percentage of apoptotic cells. The western blot analysis showed that microRNA-374 overexpression increases Bax and decreased the Bcl-2 expression. The cleaved PARP and Cleaved caspase-3 expression was also considerably increased upon miR-374 overexpression. The TargetScan analysis together with the dual luciferase assay showed that microRNA-374 targets JAM-2 by binding to mRNA at 3'-UTR. The qRT-PCR analysis showed that JAM-2 was significantly upregulated in the pancreatic cancer cell lines and tissues. Moreover, Overexpression of miR-374 suppressed the expression of JAM-2. Additionally, suppression of JAM-2 also inhibited the viability of the pancreatic cancer cells. *In vivo* studies in xenografted mice models showed that microRNA-374 expression is effective in suppressing the growth and volume of pancreatic tumors. In conclusion, microRNA-374 is downregulated in pancreatic cancers and may exhibit therapeutic implications in the pancreatic cancer treatment.

Keywords: Micro-RNA, pancreatic cancer, cell viability, apoptosis, luciferase assay

Introduction

Pancreatic cancer is currently ranked as the 4th most deadly cancers in developed countries [1]. The five year survival rate of just 9% makes pancreatic cancer as one of the most devastating human malignancies [2]. This is true for both male and female sexes. The most alarming fact is that the pancreatic cancer is generally detected at the advanced stage [3]. The incidence of pancreatic cancer deaths has increased during last four to five decades which can be attributed to its poor diagnosis as it remains very silent, particularly in the elder people. The recent advancements in understanding the molecular patterns associated with development and subsequent progression of human cancers have enlightened some prime aspects of cancer biology. This will assist

not only in the prognosis but also in development of better molecular strategies for the management of cancer [4-7]. Of the molecular markers currently being investigated for predicting the onset of cancerous growth, microRNAs are among the most focused ones as they have been found to abnormally expressed in cancer tissues [8]. The micro-RNAs are a class of small non-coding RNAs of multi-cellular organisms which are approximately 22 nucleotides in length and have regulatory role [9]. Micro-RNAs perform their regulatory functions either by repressing the mRNAs at translational level or by targeting them for their cleavage [10]. Studies have revealed that the expression levels of micro-RNAs are subjected to significant variations in many human cancers [11-13]. Micro-RNAs regulate the apoptotic and necrotic cell death pathways of cancer cells [14].

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MicroRNA-374 has been shown to have a protective role against myocardial ischemia-reperfusion injury in mice [15]. In yet another study, it has been shown to regulate the proliferation and apoptosis of human squamous cell carcinoma (SCC) cells [16]. It also affected the migration and invasion of SCC cells [16]. Given this background, current study was designated to investigate the role of microRNA-374 in regulating human pancreatic cancer growth and development. The microRNA-374 was observed to be aberrantly downregulated in pancreatic cancer tissues and cell lines. The overexpression of microRNA-374 in pancreatic cancer cells resulted into loss of viability and also inhibited the migration and invasion of cancer cells. Further, microRNA-374 was found to be regulating the Junctional Adhesion Molecule (JAM) 2 translation by interacting with 3'-UTR of its mRNA. Taken together, the study revealed microRNA-374 to be an important molecular marker against human pancreatic cancer with a potential to act as a therapeutic tool for its management.

Materials and methods

Collection of clinical specimens and culture of cell lines

The specimens of cancer and normal adjacent pancreatic tissues were taken from cancer patients after informed consent under ethical guidelines. The collection and usage of specimens for research purpose was approved by the Ethics Committee of Renmin Hospital of Wuhan University, Wuhan, Hubei, China. Specimens were frozen and stored in liquid N₂. Normal (H6c7) and cancer lines (Capan-2, Panc 10.05, CFPAC-1, and HPAF-II) of pancreas were procured from Sun Yat-sen University Cancer Center (Guangzhou, China). RPMI-1640 (Invitrogen) culture medium supplemented with FBS (10%) was used for culturing the cell lines. The cell lines were maintained in a humidified incubator with 9% CO₂ at 37°C.

RT-PCR based expression analysis

Total RNA was isolated using TRIzol (Invitrogen) reagent. This was followed by DNase I treatment and cDNA synthesis using High-Capacity cDNA Archive™ Kit following manufacturer's protocol. With human GAPDH gene as internal control of gene expression, RT-PCR based

expression analysis was performed using SYBR Green method. The amplification conditions were 10 min initial denaturation at 95°C followed by 40 cycles of 30 s denaturation at 95°C, 30 s of primer annealing at 58°C and 20 s of extension at 72°C. The primers used were miR-F: 5'-AAAACAACCGCAAGGCAGC-3' and miR-R: 5'-CCTCGGCTGTTCCCTGCT-3', JAM-F: 5'-GGGAAGATGGCGAGGAGGAG-3' and JAM-R: 5'-CCAAGGCCACAAACGGAAATC-3', GAPDH-F: 5'-AGGTCGGTGTGAACGGATTG-3' and GAPDH-R: 5'-GGGGTCGTTGATGGCAACA-3' for amplification of microRNA-374, JAM-2 and GAPDH genes, respectively.

Transfection of cancer cells

RiboBio (Guangzhou, China) was used for the synthesis of miR-NC and miR-374 mimics. The pCDNA3.1 was used for over expression of JAM-2 and RNAi based method was used for JAM-2 gene silencing. Transfection of Capan-2 cancer cells was performed using Lipofectamine 2000 (Invitrogen) reagent following manufacturer's protocol. As the cell reached 80% confluence, the cells were PBS washed followed by treatment with 0.25% trypsin to obtain a homogenous cell suspension.

Cell viability assays

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (Invitrogen) based assessment of cell viability was performed to analyze the proliferation of cancer cells. The 96-well plates were used to plate the cells at the density of 1×10^6 cells/well. MTT (0.5%) was added to each well followed by 37°C incubation for 4 h. DMSO (150 µl) was then added to each well for solubilizing the MTT-formazan. The absorbance at 570 nm was recorded to determine the viability of cancer cells.

Colony formation assay

The viability of cancer cells was further determined with colony formation assay. Briefly the cells were cultured in 6-well plates till cell growth of 1×10^3 per 100 µl of medium was attained. Plates were incubated at 37°C with 5% CO₂ for 48 h. Using centrifugation, the cells were harvested and washed with PBS. Cells were then suspended in 1 mL of 0.1% crystal violet solution followed by 37°C incubation for 30 min. Crystal violet stain was removed from

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the medium by harvesting the cells and washing them with PBS. Finally, the cell colonies were analyzed, counted and photographed.

Analysis of cell apoptosis

The cancer cells (0.6×10^6) transfected either with miR-NC or miR-374 mimics were cultured for 24 h at 37°C. The cells were fixed with 70% ethanol for 20 mins followed by PBS washing. The cells were then stained with AO/EB or DAPI. This was followed by visualization of stained cells under fluorescent microscope for nuclear morphology. To determine the percentage of apoptotic cells, the transfected cells were double stained with Annexin V-FITC/PI. Then the cells were incubated for 15 min in dark and finally examined using flow cytometer.

Migration and Invasion assay

The transwell assay was employed to determine the effects of microRNA-374 overexpression on migration of transfected Capan-2 cancer cells. Concisely, 250 μ l of transfected cells were placed at the density of 4×10^5 /ml in upper chamber. The lower chamber contained only the culture medium. This was followed by 24 h incubation at 37°C. The cells that migrated and entered the lower chamber were fixed using methanol, stained with crystal violet for 30 min and then finally observed under a microscope. For cell invasion assay, the procedure was same but Matrigel was used instead.

Identification of microRNA-374 target and luciferase reporter assay

TargetScan (<http://www.targetscan.org>) online software tool was used to identify the target of microRNA-374. Further the microRNA-374 mimics or microRNA-NC were co-transfected with pGL3-JAM2-3'-UTR-WT or pGL3-JAM2-3'-UTR-MUT plasmid constructs into Capan-2 pancreatic cancer cells. After 2 days of transfection, Dual-Luciferase® Reporter Assay System (Promega Corporation) was used to perform the dual luciferase reporter assay using *Renilla* luciferase for normalization.

Western blot analysis

Western blotting technique was used to examine the concentration of proteins of interest. In brief, the Capan-2 cells were harvested by centrifugation and washed with ice-cold PBS fol-

lowed by lysis with RIPA buffer. The protein content of each cell lysate was estimated by Bradford assay. Exactly 40 μ g of protein from each lysate was loaded and run on SDS-PAGE gel which was then blotted to PVDF membrane. The membranes were treated with TBS and exposed to primary antibodies at 4°C. This was followed by treatment with secondary antibodies and then the protein bands of interest were visualized by using enhanced chemiluminescence reagent.

In vivo experimental study

The xenografted male C57 BL/6 mice were kept in the animal holding capacity of the institute following the guidelines of National Institutes of Health standards for the care and use of animals for experimental purpose. Mice were kept in well aerated rooms at $26 \pm 2^\circ\text{C}$ with free water and pellet feed access. Two random groups of mice were made. The miR-374 transfected Capan-2 cells were injected into the left flanks of the mice belonging to one group as sub-cutaneous injections. The mice of other group were injected with miR-NC. Mice were continuously monitored and then sacrificed after 7 weeks. The pancreatic tumors were excised and processed for further experimental analysis.

Statistical analysis

All the experiments were carried out using at least three experimental replicates. SPSS 21.0 (IBM Corp., Armonk, NY) software was used for statistical analysis and data were represented as mean \pm standard deviation. *t*-test was performed for comparing two values and a *p*-value < 0.05 was considered as indicative of significant difference between two values.

Results

MicroRNA-374 is down regulated in pancreatic cancer

RT-PCR expression analysis of microRNA-374 indicated that microRNA-374 has significantly lesser expression in cancer tissues (**Figure 1A**). When expression studies were extended to the cell lines, all the cancer cell lines exhibited lesser expression levels of microRNA-374 (**Figure 1B**).

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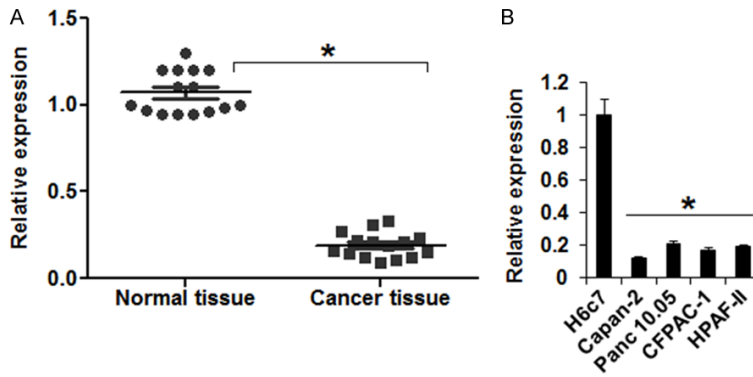


Figure 1. miR-374 inhibited the growth of human pancreatic cancer. A. Expression of miR-374 in normal and pancreatic cancer tissues. B. Expression of miR-374 in normal H6c7 and pancreatic cancer cell lines. The experiments were performed in triplicate and expressed as mean \pm SD (* $P < 0.05$).

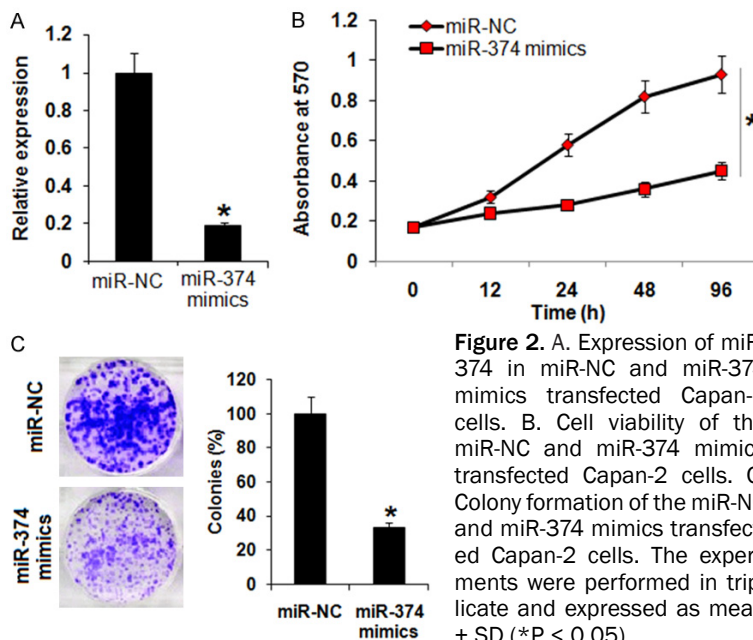


Figure 2. A. Expression of miR-374 in miR-NC and miR-374 mimics transfected Capan-2 cells. B. Cell viability of the miR-NC and miR-374 mimics transfected Capan-2 cells. C. Colony formation of the miR-NC and miR-374 mimics transfected Capan-2 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* $P < 0.05$).

Over expression of microRNA-374 reduced viability of pancreatic cancer cells

To assess the role of microRNA-374 in cancer growth, Capan-2 cells were transfected with miR-NC and miR-374 mimics. The over expression of microRNA-374 using miR-374 mimics was confirmed by RT-PCR (Figure 2A). Assessment of cell viability of miR-NC and miR-374 mimics transfected Capan-2 cells at different time intervals after the cell transfection showed that cell viability was significantly lowered by over expression of microRNA-374 (Figure 2B). This loss of cell viability was also evident as remarkably low colony formation capability of

microRNA-374 over expressing cells (Figure 2C). Analysis of transfected cancer cells for apoptosis using AO/EB and DAPI staining methods showed clear nuclear deformation in cancer cells under fluorescent microscope (Figure 3A, 3B). The induction of apoptosis in cancer cells under microRNA-374 over expression was also confirmed by flow cytometry. The percentage of apoptotic cells was significantly higher in microRNA-374 over expressing cells when compared with NC cells (Figure 3C). Western blot analysis showed that miR-374 overexpression caused upregulation of Bax and downregulation of Bcl-2. Additionally the expression of cleaved PARP and cleaved caspase-3 was significantly increased (Figure 3D).

microRNA-374 inhibited the migration and invasion of cancer cells

Overexpression of microRNA-374 in Capan-2 cells reduced the potential of cancer cells to migrate and invade their surroundings. This was proved by transwell assays. The percentage of migrating cells decreased remarkably under microRNA-374 over expression (Figure 4A).

Similar results were obtained for cell invasion (Figure 4B).

MicroRNA-374 targets cell junction protein, JAM-2

The bioinformatics analysis revealed JAM-2 to be the potential intracellular target of microRNA-374. Further it was observed that microRNA-374 targets JAM-2 by binding to its 3'-UTR in a sequence specific manner (Figure 5A). The interaction between the miR-374 and JAM-2 was confirmed by luciferase activity assay. The luciferase activity was higher when the vector construct having wild type 3'-UTR of JAM-2 was

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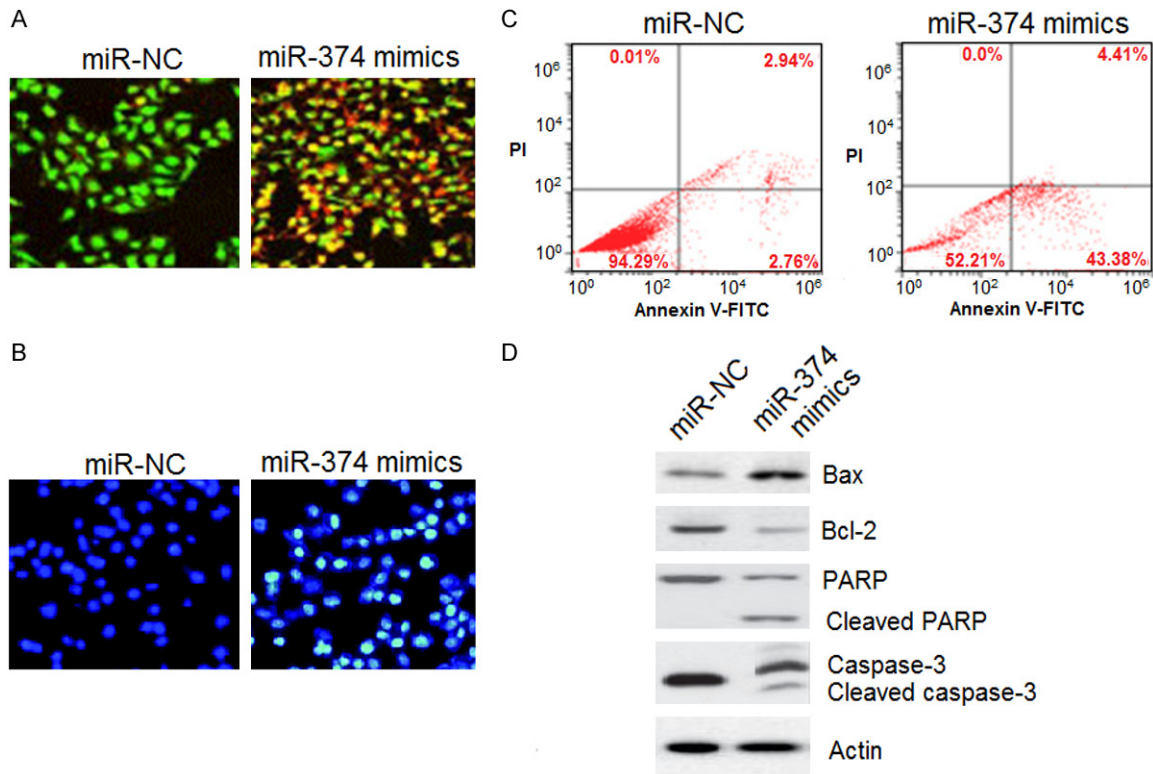


Figure 3. A. AO/EB staining showing the induction of apoptosis in miR-NC and miR-374 mimics transfected Capan-2 cells. B. DAPI staining of the miR-NC and miR-374 mimics transfected Capan-2 cells. C. Annexin V/PI staining of the miR-NC and miR-374 mimics transfected Capan-2 cells. D. Expression of apoptosis related proteins in miR-NC and miR-374 mimics transfected Capan-2 cells. The experiments were performed in triplicate and expressed as mean \pm SD (*P < 0.05).

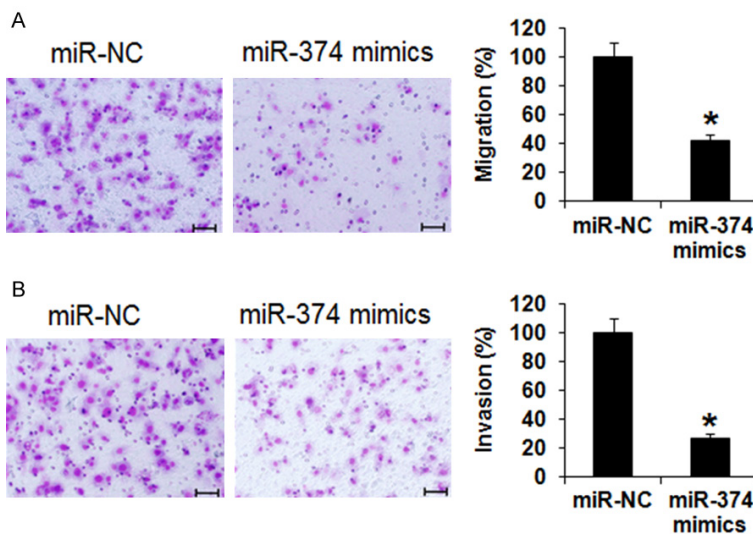


Figure 4. Transwell assay showing (A) migration and (B) invasion of the miR-NC and miR-374 mimics transfected Capan-2 cells. The experiments were performed in triplicate and expressed as mean \pm SD (*P < 0.05).

of microRNA-374 was further supported by expression analysis of JAM-2 in cancer tissues and cell lines wherein its expression was found to be significantly upregulated (Figure 5C, 5D). Additionally, overexpression of miR-374 caused significant decrease in the expression of JAM-2 (Figure 5E).

Silencing of JAM-2 inhibited cancer cell proliferation

RNAi based silencing of JAM-2 in cancer cells reduced the cell viability (Figure 5F). This indicates the role of JAM-2 in growth and development of human pancreatic cancer. When pcDNA-JAM2 was co-transfected into Capan-2 cancer cells, the inhibitory effects which were seen under microRNA-374 almost fully diminished and the transfected

co-transfected with miR-374 mimics but it was significantly lower when mutated 3'-UTR sequence was used (Figure 5B). JAM-2 as a target

almost fully diminished and the transfected

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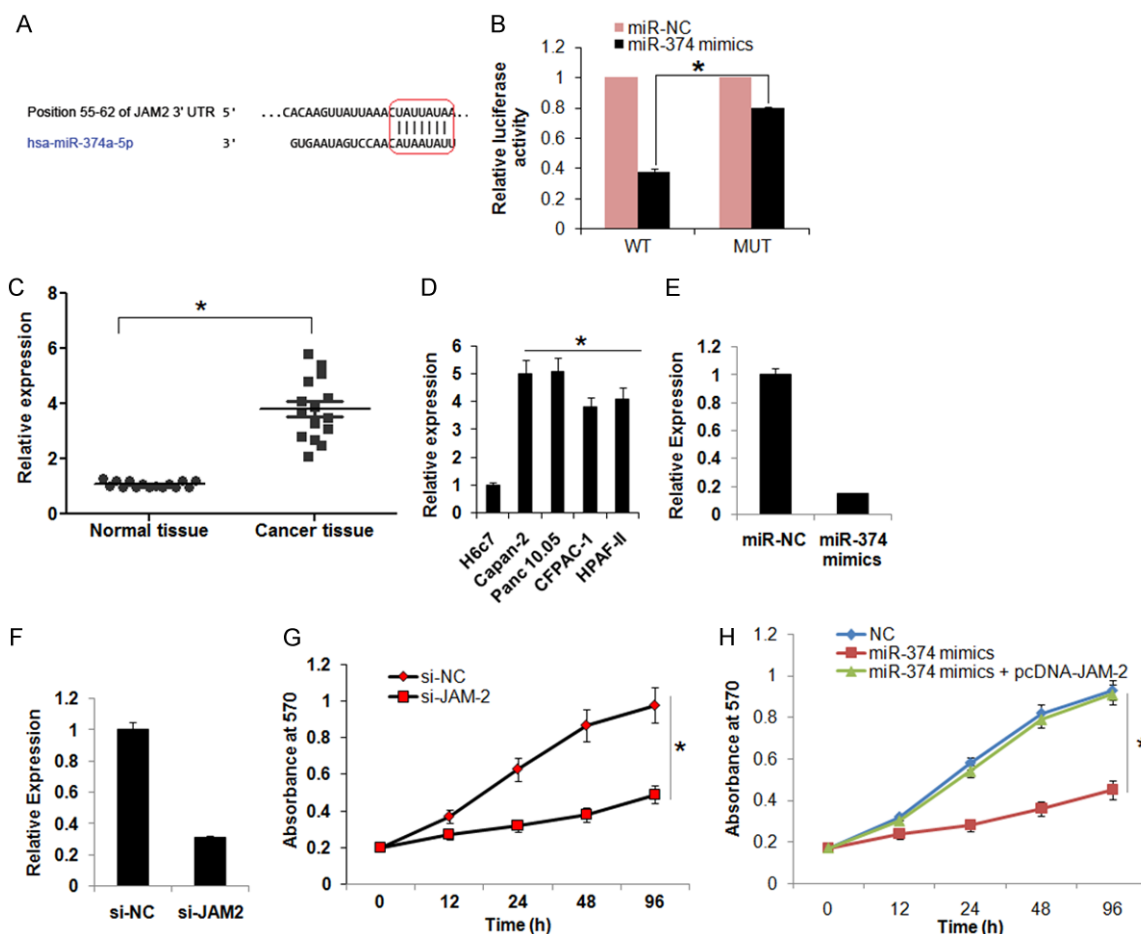


Figure 5. A. TargetScan analysis showing JAM-2 as the target of miR-374. B. The expression of JAM-2 in normal and cancer tissues. C. Dual luciferase assay. D. Expression of JAM-2 in normal H6c7 and pancreatic cancer cells. E. Expression of JAM-2 in miR-NC and miR-374 mimics transfected Capan-2 cells. F. Expression of JAM-2 in si-NC and si-JAM-2 transfected Capan-2 cells. G. Cell viability of si-NC and si-JAM2 pancreatic cancer cell lines. H. Cell viability of miR-NC, miR-374 mimics and miR-374 mimics ± pcDNA-JAM-2 transfected Capan-2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

cancer cells were seen to behave as good as normal cancer cells (Figure 5G and 5H).

MicroRNA-374 inhibited the growth of xenografted tumors in vivo in mice

The *in vivo* study of tumor development in xenografted mice injected with miR-NC or miR-374 mimics showed that the weight and volume of xenografted tumor was remarkably lower in the mice bearing cancer cells transfected with miR-374 mimics than the ones injected with miR-NC transfected cancer cells (Figure 6A-C). The results suggested the regulatory role of microRNA-374 in human pancreatic cancer development.

Discussion

The pancreatic cancer with a five year survival rate of only 9% is ranked among the most death

causing cancers of lung, liver and esophagus [17]. The higher mortality rates for pancreatic cancer are attributed to very poor prognosis and detection is commonly made when the cancer has already reached an advanced developmental stage. Recently, the researchers have focused on understanding the molecular basis of human cancer growth and development with an objective to unravel the specific molecular markers and to develop better therapeutic strategies to combat this disastrous malignancy [18, 19]. Micro-RNAs, a class of very small regulatory RNA molecules, have been reported fundamental to development and progression of human pathologies like cancer [20]. Micro-RNAs are generally down regulated in human cancers [21]. In our current study, microRNA-374 was also seen to be having reduced expression levels in human pan-

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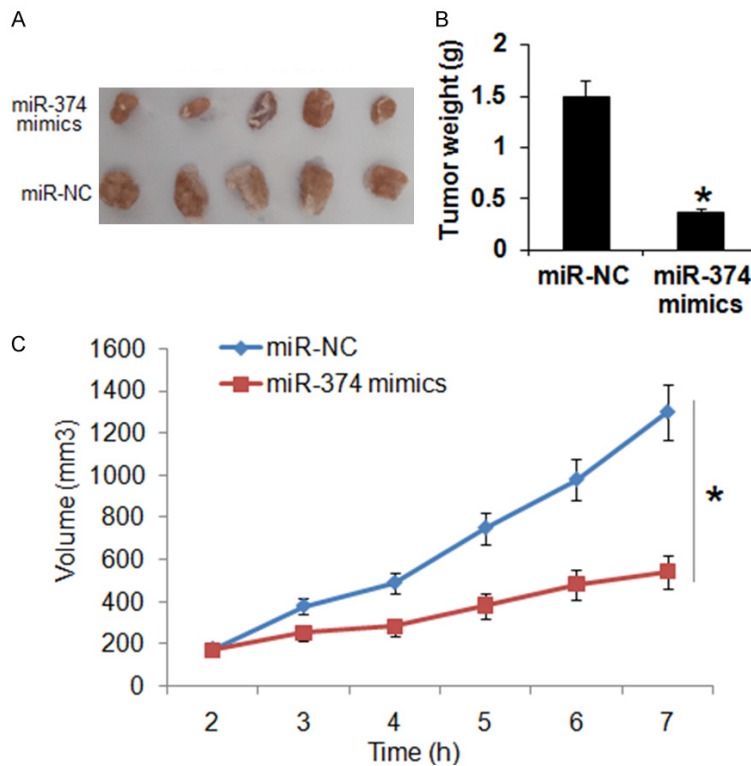


Figure 6. (A) Images showing the miR-NC and miR-374 mimics tumors (B) tumor weight and (C) tumor volume of the miR-NC and miR-374 mimics xenografted tumors. The experiments were performed in triplicate and expressed as mean \pm SD (* $P < 0.05$).

creatic cancer tissues. The over expression of microRNA-374 was seen to considerably reduce the viability of cancer cells. The loss of viability was due to the induction of apoptotic cell death in cancer cells. The induction and promotion of cell apoptosis by microRNA-374 is in coherence with a previous study wherein microRNA-195 was found to promote apoptosis in colorectal cancer cells [22]. Western blot analysis of apoptosis related proteins, Bax and Bcl-2 suggested that the apoptotic signal is mediated through Bax/Bcl-2 signalling. The cancer cell migration and invasion was seen to be affected negatively to a fair level in cancer cells over expressing microRNA-374. Similar results have been obtained for microRNA-142-3p against breast cancer [23]. Junction associated protein, JAM-2 is believed to play a role in development of gastric adenocarcinoma [24]. MicroRNA-374b was reported to inhibit the cervical cancer growth by binding to JAM-2 target [25]. The present study also exhibited the similar results. JAM-2 was having higher expression in pancreatic cancer tissues and cancer cell lines. Luciferase activity assay proved that JAM-2 is

targeted by microRNA-374. Further, the role of JAM-2 in pancreatic cancer was depicted by silencing of JAM-2 in cancer cells which exhibited very low proliferation. The reduction of tumor growth and volume in *in vivo* xenografted mice models by microRNA-374 envisaged the tumor suppressing role of microRNA-374 against human pancreatic cancer and suggests its importance to be used in molecular therapeutic strategy against human pancreatic cancer.

Conclusion

To sum up, the current study is indicative that microRNA-374 may prove as vital molecular marker in diagnosis and management of pancreatic cancer due to very low expression levels of microRNA-374 in cancer tissues and its potential to inhibit the cancer cell proliferation

through targeting of JAM-2, junction-associated protein.

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Disclosure of conflict of interest

None.

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