Abstract. microRNA-96 (miR-96) is known to be downregulated in pancreatic cancer. The overexpression of miR-96 in MIA PaCa-2 pancreatic cancer cells has been shown to inhibit cell proliferation, migration and invasion; however, the mechanisms involved have not yet been fully elucidated. Novel (nua) kinase family 1 (NUAK1) functions as an onco-gene in non-small cell lung cancer (NSCLC), melanoma, glioma, breast cancer, hepatocellular carcinoma and pancreatic cancer. In this study, firstly, we demonstrate that NUAK1 expression is specifically upregulated in pancreatic cancer and that it promotes the proliferation, migration and invasion of MIA PaCa-2 pancreatic cancer cells. Secondly, we performed an analysis of potential microRNA (miRNA) target sites using three commonly used prediction algorithms: miRanda, TargetScan and PicTar. All three algorithms predicted that miR-96 targets the 3' untranslated region (3' UTR) of NUAK1. Further experiments confirmed this prediction, namely that miR-96 suppresses the expression of NUAK1 by targeting its 3' UTR. Finally, we demonstrate that the introduction of NUAK1 cDNA lacking predicted sites of the 3' UTR abrogates miR-96 cellular function.

Introduction

MicroRNAs (miRNAs or miRs) are short non-coding RNAs that regulate the target mRNA by binding mostly to the 3' untranslated region (3' UTR), inducing either translational repression or the degradation of the target (1-3). The aberrant expression of miRNAs has been reported in multiple human cancer types and miRNAs are known to play an oncogenic or tumor suppressor role. They are also known to play key roles in cell survival, proliferation, apoptosis, migration, invasion, as well as in other processes that are associated with human cancers (4,5). More than 50% of the known miRNAs have been shown to participate in human tumorigenesis and/or metastasis by directly targeting oncogenes or tumor suppressor genes (6,7). miR-96 is markedly downregulated in pancreatic cancer compared to normal tissue and it suppresses KRAS and functions as a tumor suppressor gene (8). However, the mechanisms of action of miR-96 as a tumor suppressor in pancreatic cancer have not yet been fully elucidated.

Novel (nua) kinase family 1 (NUAK1), also known as KIAA0537/ARK5, and is identified as the fifth member of the adenosine monophosphate (AMP)-activated protein kinase (AMPK)-related kinase (ARK) family (9). Akt phosphorylates NUAK1 at Ser600, a C-terminal site outside the catalytic domain, which leads to the activation of this 74-kDa kinase. During glucose deprivation or response to adenosine monophosphate, NUAK1 supports the survival of cells in an Akt dependent manner (9). NUAK1 suppresses cell death induced by nutrient starvation and the activation of death receptors through the inhibition of caspase-8, as well as through the negative regulation of pro-caspase-6 (10,11). NUAK1 is strongly associated with tumor invasion and metastasis, and is a factor associated with tumor survival and progression (12-14). Recently, it has been reported that a high NUAK1 expression correlates with a poor prognosis and plays an important role in human non-small cell lung cancer (NSCLC) cell migration and invasion (15). The inhibition of miR-211 has been shown to increase NUAK1 expression and decreases melanoma cell adhesion, whereas the upregulation of miR-211 restores cell adhesion through the suppression of NUAK1 expression (16). NUAK1 has been shown to promote glioma cell invasion, and its elevated expression correlates with a poor clinical outcome (17). NUAK1 has also been shown to be associated with a more invasive phenotype and metastatic potential in human breast cancer dependent on Akt (18). In addition, the overexpression of NUAK1 is associated with a poor prognosis in hepatocellular carcinoma (19). NUAK1 has been shown to stimulate the invasion, metastasis, tumorigenesis and to suppress the necrosis of PANC-1 pancreatic cancer cells (14).
In this study, firstly, we demonstrate that NUAK1 expression is specifically upregulated in pancreatic cancer and that it promotes the proliferation, migration and invasion of MIA PaCa-2 pancreatic cancer cells. Secondly, we performed an analysis of potential miRNA target sites using three commonly used prediction algorithms: miRanda, TargetScan and PicTar. All three algorithms predicted that miR-96 targets the 3' UTR of NUAK1. Further experiments confirmed this prediction, namely that miR-96 suppresses the expression of NUAK1 by targeting its 3' UTR. Finally, we demonstrate that the introduction of NUAK1 cDNA lacking predicted sites of the 3' UTR abrogates miR-96 cellular function.

Materials and methods

Human tissue samples. Ten pairs of human pancreatic tissue samples were obtained from patients who underwent surgical resection at the Second Artillery General Hospital of PLA (Beijing, China) or Tianyou Hospital Affiliated to Wuhan University of Science and Technology (Wuhan, China) between 2013 and 2014 and were diagnosed with pancreatic cancer based on a histopathological evaluation. The matched non-tumor adjacent tissue was obtained from a segment of the resected specimens that was the farthest from the tumor. The samples were snap-frozen in liquid nitrogen and stored at -80°C until use. No local or systemic treatment was conducted on these patients prior to surgery.

The use of human tissue samples followed internationally recognised guidelines, as well as local and national regulations. All research carried out on human participants followed international and national regulations. Ethics approval for this study was obtained from the Medical Ethics Committee of Tianyou Hospital Affiliated to Wuhan University of Science and Technology and all participants provided written informed consent prior to enrollment.

Cell culture, plasmids and transfection. The pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C with 5% CO2. NUAK1-expressing plasmids and empty vector (pcDNA3.1; mock) were purchased from R&D Systems (Abingdon, UK). Pre-miR-96 and control-miR (scramble) were purchased from Ambion (Austin, TX, USA). Anti-miR-96 and control-anti-miR (scramble) also were purchased from Ambion (Austin, TX, USA). For the transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluence for 24 h, and then transfected with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Following incubation for 6 h, the medium was removed and replaced with normal culture medium for 48 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effects of treatments on cell proliferation were assessed by MTT assay (Sigma) which was carried out as previously described (20). Briefly, MTT (Sigma) was added to a final concentration of 1 mg/ml, the reaction mixture was incubated for 3 h at 37°C, and the absorbance was measured at 570 nm. The absorbance was directly proportional to the number of surviving cells.

BrdU cell proliferation assay. Cell proliferation was also assessed using a colorimetric BrdU proliferation kit according to the manufacturer's instructions (Cat. no. 1164729001; Roche, Indianapolis, IN, USA). The cells transfected with NUAK1-expressing plasmids or the empty vector (pcDNA3.1; mock) were labeled with BrdU for 3-4 h. The genomic DNA was fixed and denatured, and then incubated with peroxidase-conjugated anti-BrdU antibody for 90 min. A substrate for the conjugated peroxidase was then added and the reaction product was quantified by measuring the absorbance. The results were then normalized to the number of total viable cells.

Western blot analysis. Western blot analysis was performed as previously described (20). Briefly, following incubation with rabbit anti-NUAK1, anti-Ki67, anti-proliferating cell nuclear antigen (PCNA), anti-p27, anti-p21, c-myc, anti-CDK1, anti-CDK2, anti-p53 or anti-β-actin antibodies (all from Abcam, Cambridge, MA, USA) overnight at 4°C, IRDye™-800-conjugated anti-rabbit secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used for 30 min at room temperature. The specific proteins were visualized using the Odyssey™ Infrared Imaging System (Gene Company, Ltd., Hong Kong, China).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the cultured cells, with efficient recovery of small RNA, was isolated using the mirVana miRNA Isolation kit (Cat. no. AM1561, Ambion). Detection of the mature form of miRNAs was performed using the mirVana qRT-PCR miRNA Detection kit and qRT-PCR Primer Sets, according to the manufacturer's instructions (Ambion). The U6 small nuclear RNA was used as an internal control.

Bioinformatics analysis. The analysis of potential miRNA target sites was carried out using three commonly used prediction algorithms: miRanda (http://www.microrna.org/), TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/).

Luciferase reporter assay. The luciferase reporter plasmid of the 3' UTR of NUAK1 (NUAK1-WT-luc) was donated by Dr Jun Zhou, Department of Gastroenterology and Hepatology, University Medical Center, Utrecht, the Netherlands. Site-directed mutagenesis of the miR-96 target-sites in the 3' UTR of NUAK1 was carried out using the QuikChange Site-Directed Mutagenesis kit (Cat. no. 200519; Stratagene, Heidelberg, Germany), with NUAK1-WT-luc as a template. For reporter assays, the MIA PaCa-2 cells were transiently transfected with the wild-type (WT) or mutant reporter plasmid and miR or anti-miR (as indicated in Fig. 3G) using Lipofectamine 2000 (Invitrogen). Reporter assays were performed 36 h post-transfection using the Dual-Luciferase® Reporter Assay System (Promega), normalized for transfection efficiency by co-transfecting with Renilla-luciferase.

Migration and invasion assay. For the Transwell migration assays, 2.5x10^4-5x10^4 cells were plated in the top chamber
with a non-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences, Franklin Lakes, NJ, USA). For the invasion assays, $1.25 \times 10^5$ cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences). In both assays, the cells were plated in medium without serum, and medium supplemented with serum was used as a chemotactant in the lower chamber. The cells were incubated for 24 h and the cells that did not migrate or invade through the pores were removed using a cotton swab. The cells on the lower surface of the membrane were stained with the Diff-Quick Stain Set (Dade Behring, Inc., Deerfield, IL, USA) and counted.

**Statistical analysis.** Data are expressed as the means ± SE, and were derived from three independent experiments. The data were analyzed using the Student's t test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

**Results**

**Aberrant NUAK1 expression in pancreatic cancer.** In order to determine the expression levels of NUAK1 in the pancreatic cancer tissue samples, western blot analysis was conducted in 10 pairs of pancreatic cancer tissue and matched adjacent normal tissue samples. The expression of NUAK1 was consistently higher in the pancreatic cancer tissue samples than in the normal tissue samples (Fig. 1A). Moreover, the analysis of NUAK1 expression in two pancreatic cancer cell lines (MIA PaCa-2 and PANC-1) revealed that NUAK1 was upregulated in the tumor cell lines as well (Fig. 1B). These data support the notion that NUAK1 functions as an oncogene in pancreatic cancer.

The overexpression of NUAK1 in MIA PaCa-2 pancreatic cancer cells promotes cell proliferation, migration and invasion. In an attempt to identify the role of NUAK1 in regulating the proliferation of MIA PaCa-2 cells, the cells were transfected with NUAK1 plasmids. Following stable transfection, NUAK1 expression was detected by western blot analysis and the expression of eight proliferation-associated markers of MIA PaCa-2 cells (Ki67, PCNA, p27, p21, c-myc, CDK1, CDK2 and p53) was also determined by western blot analysis. The results revealed that NUAK1 plasmids evidently increased NUAK1 protein expression, and suppressed $p53$, $p21$ and $p27$ expression and promoted CDK1, CDK2 and Ki67 expression in the MIA PaCa-2 cells (Fig. 2A). Moreover, the proliferation rates of the MIA PaCa-2 cells were examined by MTT assay. The results revealed that overexpression of NUAK1 significantly increased the proliferation rate of the MIA PaCa-2 cells and that this increase in cell proliferation occurred in a dose-dependent manner (Fig. 2B). This was further revealed by BrdU incorporation assay, showing that transfection with NUAK1 resulted in increased DNA synthesis activity per viable cell in the MIA PaCa-2 cells also in a dose-dependent manner (Fig. 2C).

Given that NUAK1 markedly promoted MIA PaCa-2 cell proliferation, we then wished to determine whether NUAK1 has an effect on the migration and invasion of MIA PaCa-2 cells. The cell migration and invasion assay of MIA PaCa-2 cells revealed that the overexpression of NUAK1 not only induced the migration of the MIA PaCa-2 cells, but also promoted the invasion of these cells (Fig. 2D).

**NUAK1 is a target of miR-96 in MIA PaCa-2 pancreatic cancer cells.** Having demonstrated that NUAK1 expression is
specifically upregulated and that it promotes the proliferation, migration and invasion of MIA PaCa-2 cells, then investigated the mechanisms responsible for promoting NUAK1 expression. miRNAs are a new class of small (~22 nucleotide) non-coding RNAs that negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition (1-3). We hypothesized that NUAK1 expression was upregulated due to a defect in a specific miRNA in pancreatic cancer.

To further confirm this hypothesis, on the one hand, we used three commonly used prediction algorithms: miRanda (http://www.microrna.org/), TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/) to analyze the 3' UTR of NUAK1. All three algorithms predicted that miR-96, miR-145, miR-203, miR-23a and miR-23b target the 3' UTR of NUAK1 (Fig. 3A). miR-96 is downregulated in pancreatic cancer and the overexpression of miR-96 in pancreatic cancer has been shown to suppress cell proliferation, migration and invasion (8). Thus, we hypothesized that NUAK1 expression was upregulated due to a defect in a specific miRNA in pancreatic cancer.

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To determine whether NUAK1 can be downregulated by miR-96, we transfected the MIA PaCa-2 cells with pre-miR-96 and then RT-qPCR was performed to detect the mRNA expression of miR-96 and NUAK1 in the cells. The results revealed that transfection with pre-miR-96 markedly increased miR-96 expression (Fig. 3C), but it did not affect NUAK1 mRNA expression in the MIA PaCa-2 cells (Fig. 3D). Since miRNAs can suppress mRNA translation without degrading the mRNA, we also performed western blot analysis to determine whether miR-96 affects NUAK1 protein expression. The results of western blot analysis demonstrated that NUAK1 protein expression was markedly downregulated following transfection with pre-miR-96 in the MIA PaCa-2 cells (Fig. 3E).

To further demonstrate the direct regulation of NUAK1 by miR-96 through its 3' UTR, we constructed luciferase reporters with the targeting sequences of wild-type (NUAK1-WT-luc) and mutated 3' UTRs of NUAK1 (NUAK1-MUT-luc) (Fig. 3F). To determine whether miR-96 targets the 3' UTR of NUAK1, a luciferase assay was performed. Our results revealed that pre-miR-96 inhibited the effects of the NUAK1-WT-luc plasmids, but not those of the NUAK1-MUT-luc plasmids (Fig. 3G). Our data confirm that miR‑96 negatively regulates the expression of the protein-coding gene, NUAK1, by targeting its 3' UTR; thus, this suggests that the upregulation of NUAK1 is associated with the low expression of miR-96 in pancreatic cancer.

Silencing miR-96 promotes the expression of NUAK1. To determine whether NUAK1 is indeed regulated by miR-96 and that the upregulation of NUAK1 is associated with the low expression of miR-96, we transfected the MIA PaCa-2 cells with anti-miR-96 or control anti-miR and RT-qPCR was performed to determine the expression levels. Our results revealed that anti-miR-96 effectively inhibited miR-96 expression in the cells (Fig. 4A). We then performed RT-qPCR and western blot analysis to determine NUAK1 expression levels in the cells transfected with anti-miR-96. The results revealed that transfection with anti-miR-96 did not inhibit NUAK1 mRNA expression (Fig. 4B), although it inhibited NUAK1 protein expression (Fig. 4C). To further demonstrate the direct regulation of NUAK1 by anti-miR-96, both the wild-type and mutant reporter constructs were introduced.
into the MIA PaCa-2 cells. The luciferase activity induced by NUAK1-WT-luc, but not that induced by NUAK1-MUT-luc was significantly promoted by transfection with anti-miR-96 in the MIA PaCa-2 cells (Fig. 4D).

Introduction of NUAK1 cDNA lacking predicted sites of 3' UTR abrogates miR-96 cellular function. Since miR-96 directly targets NUAK1 through its 3' UTR, we reasoned that the ectopic expression of NUAK1 by transfection with cDNA that did not contain the predicted target of 3' UTR (in this study, the NUAK1-expressing plasmids did not contain the target of miR-96 in its 3' UTR predicted by bioinformatics analysis) would allow NUAK1 to evade regulation by miR-96 and would thus attenuate or decrease miR-96 function. To this end, we transfected NUAK1-expressing plasmids or pcDNA3.1 into control-miR- or pre-miR-96-treated MIA PaCa-2 cells. Western blot analysis revealed that transfection with NUAK1 plasmids eliminated the effects of pre-miR-96 on NUAK1 protein expression (Fig. 5A).

As the overexpression of miR-96 in pancreatic cancer inhibits proliferation, migration and invasion (8), in order to determine whether NUAK1 abrogates the roles of miR-96 in cell proliferation, control-miR- or pre-miR-96-transfected MIA PaCa-2 cells were treated with either NUAK1-expressing plasmids or pcDNA3.1. We then performed MTT and BrdU incorporation assays and found that the pre-miR-96-treated MIA PaCa-2 cells displayed a 30-40% decrease in proliferation compared with the control-miR-treated cells (Fig. 5B) and in DNA synthesis (Fig. 5C). The overexpression of NUAK1 reversed the loss in proliferation observed in the pre-miR-96-treated cells.

We then treated the control- miR- or miR-96-transfected MIA PaCa-2 cells with either NUAK1-expressing plasmids or pcDNA3.1 and performed migration and invasion assays. The results revealed that the overexpression of NUAK1 reversed the loss in migration and invasion observed in the pre-miR-96-treated cells (Fig. 5D). Hence, the suppression of NUAK1 expression may account for the reduced cell migration and invasion following treatment with pre-miR-96.

Discussion

The prognosis of patients with pancreatic cancer remains dismal (21,22). The disease is extremely aggressive and is profoundly resistant to all forms of therapy (23). Given the frequent failure of conventional treatment strategies, many cancer-related molecules have been characterized toward the goal of developing novel anti-cancer therapies, such as molecular-targeted drugs and antibodies or cancer vaccines (24,25). Tumor malignancy, including invasion and metastasis, accelerated by Akt activation has been well documented for breast cancer, ovarian cancer, squamous cell carcinoma, colorectal cancer and pancreatic cancer (26-29). Suzuki et al suggested that NUAK1 overexpression is involved in the tumor progression of colon cancer (14). However, the role of NUAK1, as a tumor-associated factor downstream of Akt signaling (14), in pancreatic cancer has not yet been elucidated. In the present study, we demonstrated that NUAK1 expression was specifically upregulated in pancreatic cancer and that it promoted the proliferation, migration and invasion of MIA PaCa-2 pancreatic cancer cells by targeting NUAK1.
miRNAs are a new class of small (~22 nucleotide) non-coding RNAs that negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition (1-3). Profiling studies have revealed the contribution of aberrant miRNA expression to pancreatic initiation and progression by perturbing the function of target genes (30-32). It has been previously demonstrated that miR-96 is poorly expressed in human pancreatic cancer and that miR-96 deregulates KRAS by targeting its 3' UTR, ultimately functioning as a tumor suppressor gene in pancreatic cancer (8). Although miR-96 is downregulated and suppresses cell proliferation, migration and invasion, and targets KRAS in human pancreatic cancer, the mechanisms of action of miR-96 functioning as a tumor suppressor gene have not yet been fully clarified. Consistent with a previous study (8), we found that miR-96 inhibited the proliferation, migration and invasion of MIA PaCa-2 pancreatic cancer cells by targeting NUAK1; other mechanisms of action of miR-96 in regulating proliferation, migration and invasion are emerging.

The miR-96/NUAK1-mediated regulation of the proliferation, migration and invasion of MIA PaCa-2 pancreatic cancer cells demonstrated in this study has potential basic and clinical implications. On the one hand, miR-96 is a powerful tumor suppressor by exerting anti-proliferative, anti-migratory and anti-invasive effects in human pancreatic cancer and the pharmacological restoration of miR-96 expression may represent a promising therapeutic strategy in pancreatic cancer. On another hand, NUAK1, as an oncogene, may be a therapeutic target in patients with pancreatic cancer. However, further
studies are required to fully elucidate the comprehensive roles of miR-96 and NUAK1 in pancreatic cancer.

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