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Original Article

MiR-17-5p enhances pancreatic cancer proliferation by altering cell cycle profiles via disruption of RBL2/E2F4-repressing complexes

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ABSTRACT

The members of the miR-17-92 cluster are upregulated in various cancers and function as a cluster of oncogenic miRNA. Our study characterized a new function of miR-17-5p, a member of the miR-17-92 cluster, in regulating cell proliferation in pancreatic cancer. Our results indicated that miR-17-5p was up-regulated in pancreatic adenocarcinoma and directly targeted the retinoblastoma-like protein 2 (RBL2), a tumor suppressor belonging to the Rb family. High levels of miR-17-5p and low levels of RBL2 were associated with poor prognosis. RBL2 could interact with the transcription factor E2F4 and bound to the promoter regions of the E2F target genes. Disruption of the RBL2/E2F4 complex by miR-17-5p overexpression shifted the activity of E2F from gene repressing to gene activating, which induced cell cycle entry and proliferation. These results suggested that miR-17-5p promoted proliferation in pancreatic ductal adenocarcinoma cells (PDAC) and altered cell cycle profiles *in vivo* and *in vitro*, by disrupting the RBL2/E2F4-associated gene repressing complexes via direct targeting of RBL2. The new regulatory network, involving miR-17-5p and RBL2, emerges as a new target of PDAC treatment.

Introduction

Pancreatic adenocarcinoma (PDAC) is one of the most aggressive malignancies in humans. The high mortality rate and poor prognosis result from the difficulties in early clinical diagnosis and lack of therapeutic options [1]. Unlike the steady increase in survival, observed with most cancers, the 5-year survival rate for pancreatic cancer is approximately 8%. Advances in therapies have been slow [2], highlighting the need to investigate molecular mechanisms that may contribute to the highly malignant phenotype of this cancer.

MicroRNAs (miRNAs) are short, non-coding RNAs, consisting of 14–24 nucleotides, which regulate gene expression at the post-transcriptional level. The miR-17-92 cluster includes seven miRNAs and is located in the intron of *C13orf25* on chromosome 13q31, a genomic locus that is amplified in many cancers [3–5]. The miR-17-92 cluster can act as an oncogene *in vivo* and is, thus, known as oncomiR-1 [6]. miR-17-5p is a member of the miR-17-92 cluster [7] and is overexpressed in various solid tumors including PDAC [8–13]. Many studies, including this one, have shown that

Abbreviations: CRF, Chromatin remodeling factor; DP, Dimerization partner; HDACs, Histone deacetylases; MuvB, Multivulva class B; PDAC, Pancreatic ductal adenocarcinoma; RBL2, Retinoblastoma-like protein2.

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upregulated levels of miR-17-5p are associated with poor prognosis in pancreatic cancer [9,14].

Evasion of growth suppressors is a hallmark of cancer [15]. Regulators that control the correct entry and progression through the cell cycle are often altered in cancer cells [16]. Alterations in the components of the cell cycle, which directly or indirectly control the progression of G1 and transition into G1/S, are particularly vital for tumorigenesis [16]. miR-17-5p possesses different levels of expression during the cell cycle, and may target numerous genes that encode cell-cycle regulators [17]; therefore, miR-17-5p is considered a key regulator of the G1/S phase in cell cycle transition [17]. However, the specific mechanisms, mediating the interactions between miR-17-5p and cell cycle regulators in carcinogenesis, remain unclear.

RBL2, a well-known tumor suppressor gene in the Rb family, is inactivated in numerous cancers [18–20]. The majority of tumors may acquire shifts in the G1/S transition via alterations in the pRb pathway [21,22]. Functional analysis indicates that RBL2 can form heterodimers with the transcriptional repressor E2F4, thereby transcriptionally repressing the E2F4 target genes [23]. This study shows that RBL2 is a downstream target gene of miR-17-5p in PDAC. Activation of miR-17-5p can repress the expression of RBL2, deregulate the control of G1 progression by disruption of RBL2/E2F4-associated transcription repressors, and result in the rapid entry into the S phase, leading to high proliferation *in vitro* and *in vivo*. Therefore, targeting miR-17-5p, and its downstream signaling, may be a potential therapeutic approach for PDAC.

Materials and methods

Patients and tissue collection

26 pairs of PDAC samples were collected from patients who underwent surgical resection at the Department of General Surgery of Ruijin Hospital between 2012 and 2013. Follow-up duration lasted to September 2016. Surgical specimens were confirmed by pathological examination. Tumor staging was determined by the latest edition of the TNM system of the American Joint Committee. The project was approved by the Ethics Committee of Ruijin Hospital affiliated to Shanghai Jiao Tong University and all subjects signed an informed consent.

RNA isolation and qPCR analysis

miRNA was isolated using the miRNAExtractor (Sangon Biotech, China). The quality and concentration of RNA were evaluated by a spectrophotometer (Bio-Rad, USA). cDNA was synthesized using 100 ng miRNA with miRNA First Strand cDNA Synthesis Kit (Sangon Biotech, China). The miRNAs Quantitation PCR Kit (Sangon Biotech, China) was used for the qPCR analysis of miRNA expression. The relative expression levels of miR-17-5p were quantified in triplicate and calculated using the $2^{-\Delta CT}$ method. The U6 small nuclear RNA was used as endogenous control for the normalization of miR-17-5p expression.

Total RNA was isolated using TRIzol (Invitrogen, USA). cDNA was synthesized with 1 μg RNA using the ReverTra Ace qPCR RT Kit (TOYOBO, Japan). SYBR Green (TOYOBO, Japan) was used for the qPCR analysis of mRNA expression. Quantitation, of the relative expression levels of target genes, was performed in triplicate and calculated using the $2^{-\Delta CT}$ method. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as endogenous control for the normalization of target gene expression.

All the assays, mentioned above, were performed according to the manufacturers' protocols. The primers for targeted genes were as follows: the miR-17-5p forward primer was purchased from TIANGEN Biotech (CD201-0016, China); the U6 forward primer and Universal PCR reverse primer were included in the miRNA First Strand cDNA Synthesis Kit (Sangon Biotech, China); RBL2: 5′-GGAGGAAATTGGGACTCTCTCA-3’ (Forward) and 5′- AGACGACTCAAGCTATGCGTA-3’ (Reverse), GAPDH: 5′- ACAACTTTGGTATCGTGGAAGG-3’ (Forward) and 5′- GCCATCACGCCACAGTTTC-3’ (Reverse), CCND1: 5′- GTGCCACAGATGTGAAGTTCATT-3’ (Forward) and 5′- CTCTGGAGAGGAAGCGTGTG-3’ (Reverse), JUN: 5′-GTGCCGAAAAAGGAAGCTGG-3’ (Forward) and 5′- CTGCGTTAGCATGAGTTGGC-3’ (Reverse) and MYC: 5′- TAGTGGAAAACCAGCAGCCTC-3’ (Forward) and 5′-CTCCTCCTCGTCGCAGTAGA-3’ (Reverse).

Cell culture and reagents

AsPC-1, BxPC-3, HPDE6-C7, MIA PaCa-2, PANC-1 and CFPAC-1 were purchased from ATCC and authenticated using DNA analysis performed by the Analysis Core of the Shanghai Jiao Tong University. AsPC-1, MIA PaCa-2 and PANC-1 were cultured in DMEM. BxPC-3 and HPDE6-C7 were cultured in RPMI 1640. CFPAC-1 was cultured in IMDM. All the media were supplemented with 10% fetal bovine serum (FBS; Gibco, USA), and the cell lines were maintained at 37 °C under 5% CO_2 in a humidified chamber.

Plasmid construction

The lentiviral vector, pHBLV-CMVIE-ZsGreen-Puro, was digested between EcoRI and BamHI by a restriction endonuclease (Fermentas, USA); full-length cDNA of miR-17-5p was cloned and confirmed by DNA sequencing. cDNA was then ligated by T4 DNA ligases (Fermentas, USA).

The adenoviral vector, pHBAd-MCMV-RFP, was digested at EcoRI by a restriction endonuclease (Thermo Fisher Scientific, USA); full length cDNA of RBL2 was cloned and then ligated using the One Step Cloning Kit (#C112, Vazyme Biotech, China).

Empty vectors were used as negative controls.

Virus packing and infection

Lentiviral plasmids were transfected into human embryonic kidney cells 293T. (HEK-293T), using virus-packing plasmids from Lipo2000 (Invitrogen, USA), to produce the lentivirus. The culture supernatants were collected at 48 and 72 h, and added to the pancreatic cancer cell lines to construct stable cell lines using selection by puromycin.

Adenoviral plasmids were transfected into HEK-293A cells using virus-packing plasmids from Lipo2000 (Invitrogen, USA). The culture supernatants were added to the pancreatic cancer cell lines to overexpress RBL2.

Colony formation and cell proliferation assays

For the colony formation assay, AsPC-1 or BxPC-3 cells (at the density of 1000 cells per well) were seeded into 6-well plates. After 2 weeks of culture at 37 °C, under 5% CO_2, in a humidified chamber, cells were washed with PBS, stained with 0.1% crystal violet for 15 min, and washed with PBS twice. Proliferation assay was performed using the Cell Counting Kit 8 (CCK8; Dojindo, Japan). Cells were plated in 96-well plates, at a density of 4×10^3 cells per well, and cultured overnight. The number of viable cells was quantified every 24 h for 5 days by measuring OD450 with a microplate reader (Epoch, BioTek, USA).

Tumor xenograft assay

Total amount of 5×10^6 BxPC-3 cells were resuspended in 200 μl phosphate buffered saline (PBS) and injected subcutaneously into each 4-week-old male nude mice. The mice were monitored weekly for tumor growth and size, and sacrificed 4 weeks post injection. All the tumors were harvested, weighed, and embedded in paraffin for immunohistochemical (IHC) staining.

Immunochemistry analysis

All samples were fixed overnight in 40% formaldehyde (pH 7.4) and then dehydrated in graded concentrations of ethanol. Immunochemical staining and analysis were performed using previously published methods [24]. Anti-RBL2 (#13610, Cell Signaling Technology, USA), anti-Ki67 (#27309, Proteintech, USA), anti-cyclin D1 (#2978, Cell Signaling Technology, USA), anti-*c*-Jun (#9165, Cell Signaling Technology, USA) and anti-*c*-Myc (#10828-1-AP, Proteintech, USA) were used as primary antibodies.

Flow cytometry

Cells were harvested and fixed in 75% ethanol at 4 °C overnight, then washed twice with PBS before staining. DNA was stained using the PI/RNase Staining Buffer (BD Biosciences, USA) according to the manufacturer's protocol. All assays were repeated at least three times. Cells were synchronized using serum starvation for 48 h and replated in 10% FBS medium for 3 h before harvesting.

miRNA and siRNA transfection

The miR-17-5p mimics, miR-17-5p antisense oligonucleotides, RBL2 siRNA, and the negative controls for miRNA mimics, antisense oligonucleotides and siRNA, were synthesized by GenePharma Co, Ltd (China). The RNA sequences were as follows. miR-17-5p mimics: 5′- CAAAGUGCUUACAGUGCAGGUAG-3’; miR-17-5p antisense oligonucleotides: 5′-CUACCUGCACUGUAAGCACUUUG-3’; RBL2 siRNA: 5′- GCCCUGUACUGUGUCUGAATT-3’: negative control for miRNA antisense oligonucleotides: 5′-CAGUACUUUUGUGUAGUACAA-3’; negative control for miRNA mimics and siRNA: 5′-UUCUCCGAACGUGUCACGUTT-3’. One day before transfection, the cells were plated at a density of 2×10^5 cells/well in 6-well plates. Transfection was performed using RNAiMAX (Invitrogen, USA) according to the manufacturer's protocol.

Western blot analysis

The western blot assay was performed using standard protocol. Total protein was extracted with RIPA buffer (Solarbio, China). Nuclear protein lysis and cytoplasmic protein lysis were extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) according to manufacturer's protocol.

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