Experimental Cell Research 363 (2018) 310–314

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Primate-specific miRNA-637 inhibited tumorigenesis in human pancreatic ductal adenocarcinoma cells by suppressing Akt1 expression

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ARTICLE INFO

Keywords:
miR-637
Pancreatic ductal adenocarcinoma
Akt1
Tumor-suppressor
Apoptosis

ABSTRACT

As a primate-specific microRNA, miR-637 has been discovered for nearly 10 years. Our previous study demonstrated that miR-637 acted as a suppressor in hepatocellular carcinoma. However, its biomedical significance in pancreatic cancer remains obscure. In the present study, miR-637 was found to be significantly downregulated in pancreatic ductal adenocarcinoma (PDAC) cell lines and most of the PDAC specimens. Furthermore, the enforced overexpression of miR-637 dramatically inhibited cell proliferation and induced apoptosis of PDAC cells. Akt1, as a serine/threonine-protein kinase, has been identified as an oncogene in multiple cancers including pancreatic cancer. Our data confirmed that Akt1 was a novel target for miR-637, and its knockdown also induced cell growth inhibition and apoptosis in PDAC cells. In conclusion, our data indicated that miR-637 acted as a tumor-suppressor in PDAC, and the suppressive effect was mediated, at least partially, by suppressing Akt1 expression.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer deaths, and the most common type is pancreatic ductal adenocarcinoma (PDAC). As one of the most lethal malignancies, it is responsible for hundreds of thousands of deaths each year [1,2]. Recent advances in therapeutic strategies have improved the quality of the patients with early-stage PDAC [3]. However, most patients were detected with advanced stages and the 5-year survival rate remains about 6% [4]. Thus, further investigations are urgently needed to find the potential therapeutic targets for PDAC patients.

MicroRNAs are a family of small non-coding RNAs which act as gene regulators in various biological activities [5]. They modulate the target gene expression by repressing translation or regulating mRNA degradation *via* binding to the 3′UTR of their target genes [6]. It is well known that miRNAs involve in human carcinogenesis and aberrantly expressed miRNAs play essential roles in pancreatic cancer progression [7]. Several miRNAs have been considered as potential biomarkers for pancreatic cancer, such as miR-21, miR-155, miR-196a, and miR-210, which were shown to be upregulated in cancer tissues [8–10], serum [11–14], fecal specimens [15,16] and pancreatic juices [17,18] derived from pancreatic cancer patients. Other miRNAs have been reported to directly regulate cell proliferation and survival, *i.e.* miR-216 [19], miR-372 [20], miR-195 [21], miR-186 [22] were downregulated in pancreatic cancer specimens and served as suppressors in tumorigenesis. As for miR-637, our previous study showed that it inhibited cell proliferation and tumorigenesis of hepatocellular carcinoma [23]. However, the role of miR-637 in pancreatic cancer remains elusive.

In the present study, miR-637 was found to be downregulated in PDAC cells and clinical specimens. Further investigation revealed that miR-637 overexpression suppressed cell proliferation and induced apoptosis of PDAC cells *via* directly suppressing oncogene Akt1

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https://doi.org/10.1016/j.yexcr.2018.01.026
Received 16 October 2017; Received in revised form 18 January 2018; Accepted 19 January 2018

expression. Therefore, miR-637 may be a promising molecular target for PDAC patients.

2. Materials and methods

2.1. Cell culture

A panel of human PDAC cell lines including SW1990, BxPC-3 and Capan-2 and the pancreatic duct epithelial cells (HPDE) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% Penicillin-Streptomycin.

2.2. Clinical samples

Twenty-five paired primary PDAC specimens and matched adjacent non-tumor tissues were collected from tumor resections at the Eighth Affiliated Hospital, Sun Yat-sen University and the people's Hospital of Baoan Shenzhen. All tissues were confirmed by histological staining. Informed consents were obtained from all patients.

2.3. RNA oligoribonucleotides transfections

MiRNAs were transfected at the concentration of 100 nM by using Lipofectamine 2000 (Invitrogen, CA). The miR-637 mimics, siRNA duplexes consisting of random sequence used as negative control (NC), anti-miR-637, anti-NC and siRNA of Akt1 (siAkt1) were all purchased from GenePharma (Shanghai, China).

NC: 5′UUCUCCGAACGUGUCACGUUU3′;
anti-NC: 5′GUGGAUAUUGUUGCCAUCA3′;
miR-637: 5′ACUGGGGGCUUUCGGGCUCUGCGU3′;
anti-miR-637: 5′ACGCAGAGCCCGAAAGCCCCCAGU3′;
siAkt1: 5′CCAUGAACGAGUUUGAGUATT3′

2.4. Lentiviral miR-637 overexpression plasmid construction and lentivirus production

The lentiviral miR-637 overexpression plasmid was constructed according to previous publication [23]. Briefly, a 295 bp fragment of pre-miR-637 encompassing the stem-loop was amplified and then cloned into lentiviral vector (termed as Lv-miR-637). The production and purification of the lentivirus were performed as previously described [23]. The lentiviral vector expressing a scramble RNA was used as control.

2.5. MTT and apoptosis analyses

The Capan-2 and BxPC-3 cells were seeded into a 96-well plate at density of 5×10^3 cells per well and cultured for 72 h after RNA oligoribonucleotides transfection. The cell growth was determined by MTT assays at wavelength 550 nm by a Wallace victor-1420 multilabel counter (Perkin-Elmer). The apoptotic cells were examined by using a FITC-labeled AnnexinV/propidium iodide (PI) Apoptosis Detection Kit (Invitrogen, CA).

2.6. Colony formation assay

Capan-2 cells were infected with Lv-miR-637 or Lv-Ctrl and were replated in 6-well plates at density of 5×10^2 per well. After two weeks, the cells were fixed with methanol/acetic acid (3:1, v: v), and stained with 0.5% crystal violet. The number of colonies was counted under a microscope [23].

2.7. RNA extraction, reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by Trizol reagent (Invitrogen, CA), and it was reversely transcribed by using NCode™ miRNA First-Strand cDNA Synthesis kit (Invitrogen, CA). The qRT-PCR samples were performed by using SYBR Green PCR master mix (Roche) on an ABI 7500 Real Time PCR System. U6 was used as an endogenous control, and fold changes were calculated according to $2^{-\Delta\Delta Ct}$ analyses.

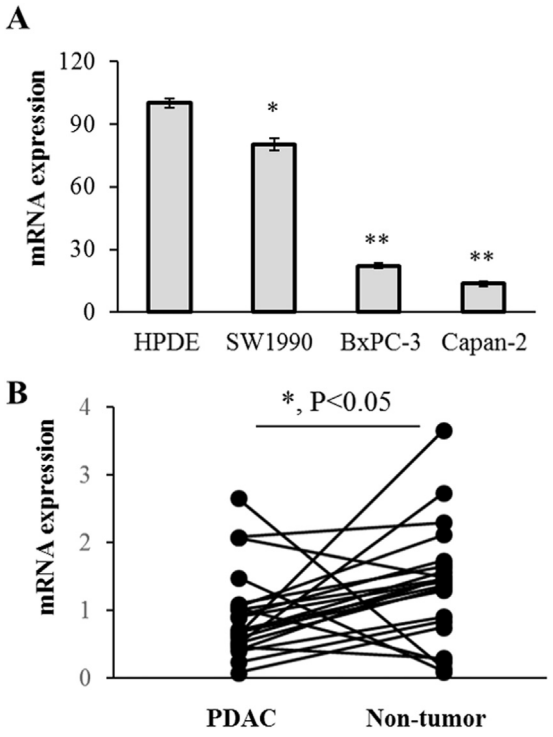

Fig. 1. MiR-637 was downregulated in PDAC cells and clinical specimens. A, miR-637 was downregulated in three PDAC cells. The miR-637 abundance was normalized to U6 RNA. *, $P < 0.05$, *vs* HPDE; **, $P < 0.01$, *vs* HPDE. B, the expression of miR-637 in human pancreatic cancer specimens was suppressed in non-tumor pancreatic tissues (n = 25). *, $P < 0.05$.

2.8. Bioinformatics analyses

Online programs including Targetscan (http://www.targetscan.org), miRanda (http://www.microrna.org) and Findtar (http://bio.sz.tsinghua.edu.cn), were used to predict the targets of miR-637.

2.9. Luciferase assays

A 386 bp fragment of Akt1 3′UTR (nt 280-665, WT) was amplified by PCR and the predicted target site of the fragment was mutated by site-directed mutagenesis (Mu). These Wt and Mu fragment were cloned into the pMIR reporter vector (Promega, WI). For luciferase assays, The Wt or Mu vector and miRNAs were co-transfected into capan-2 cells and each experiment was repeated in triplication. Luciferase activity was measured at 28–30 h after co-transfection by the luciferase reporter assay system (Promega, WI). The total protein concentration was determined at 595 nm by using a Bradford assay (Bio-Rad, USA) on a spectrophotometer (Tecan, Austria). And the luciferase activity was normalized by the total protein.

2.10. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed by using the independent *t*-test. A *p*-value of less than 0.05 was considered statistically significant.