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Original article

MiR-1 targets PIK3CA and inhibits tumorigenic properties of A549 cells

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Abbreviations:
MiR-1, MicroRNA-1
NSCLC, Non-small cell lung cancer
PIK3CA, Phosphoinositide-3-kinase catalytic subunit alpha
3′UTR, 3′ untranslated region
miRNAs, MicroRNAs
CCK-8, Cell Counting Kit-8

ABSTRACT

MicroRNAs are small endogenous RNAs that play important roles in the pathogenesis of human diseases, including malignancy. MicroRNA-1 (miR-1) is downregulated in non-small cell lung cancer (NSCLC); however, the underlying mechanisms by which it suppresses tumorigenesis in NSCLC are largely unknown. We investigated whether phosphoinositide-3-kinase catalytic subunit alpha (PIK3CA) was a novel target of miR-1 in the NSCLC cell line A549, and the mechanism of miR-1 inhibition of the tumorigenic properties of A549 cells is discussed. The influence of miR-1 on A549 cells was studied by transfection with miR-1 mimics or inhibitor. MiR-1 overexpression led to downregulation of PIK3CA protein, but not mRNA by western blot and quantitative real-time PCR, respectively. The dual-luciferase reporter assay confirmed that miR-1 targeted PIK3CA directly. PIK3CA downregulation by miR-1 mimics led to a significant reduction of phosphorylated Akt and survivin protein, the downstream targets of the PI3 K/Akt pathway. Cell proliferation was studied using a cell counting kit. Migration and invasion were evaluated by Transwell and Matrigel assays, respectively. Cell cycle and apoptosis were detected by flow cytometry. The results were that miR-1 upregulation inhibited A549 cell proliferation, migration, and invasion. These findings indicate that miR-1 may play an important role in the pathogenesis of NSCLC by regulating PIK3CA through the PI3 K/Akt pathway. Increasing miR-1 expression may provide a novel approach for NSCLC treatment.

1. Introduction

With nearly two million new cases diagnosed worldwide each year, lung cancer remains one of the most life-threatening tumors [1]. Non-small cell lung cancer (NSCLC), which represents about 75–85% of lung cancers, is widely treated with surgical resection. Unfortunately, the 5-year survival rate of NSCLC patients post-surgery is around 50%, even if they are diagnosed in the early stage [2,3]. Thus, rationally designed and targeted agents that mediate NSCLC progression and that can be used for molecular targeted therapies are urgently required and are of great interest.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate diverse cellular processes by binding to the 3′ untranslated region (3′UTR) of target mRNAs, resulting in the degradation of the mRNAs or inhibition of their translation to functional proteins [4]. It is currently estimated that miRNAs potentially regulate almost one-third of the coding genes in the human genome [5], indicating that miRNAs play substantial roles in physiological and pathological processes. Emerging evidence shows that miRNAs have a variety of functions in regulation and in controlling cancer initiation and progression [6,7]. Depending on their specific target genes, miRNAs can function as oncogenes or tumor suppressors [8–12].

MicroRNA-1 (miR-1) has been identified as a tumor suppressor in various human cancers, such as lung cancer, head and neck squamous cell carcinoma, colorectal cancer, prostate cancer, bladder cancer, thyroid carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma [13–21]. MiR-1 is expressed in the lung and is downregulated in human primary lung cancer tissues and cell lines; exogenous miR-1 significantly reduced the expression of oncogenic targets in NSCLC A549 and H1299 cells, reversing their

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tumorigenic properties [13]. Thus, miR-1 has potential therapeutic application against NSCLC. However, its role in NSCLC and the molecular mechanisms by which it exerts its functions remain largely unknown; identification of novel miR-1 targets would provide new insights into the molecular mechanism underlying miR-1 inhibition of tumorigenic properties and subsequently enable the design of improved therapies.

Phosphoinositide-3-kinase catalytic subunit alpha (PIK3CA), the p110α subunit of PI3 K, functions as an oncogene and plays important roles in many cancers, including NSCLC [22–24]. It was amplified in 70% of squamous cell carcinomas, 38% of large cell carcinomas, and 19% of adenocarcinomas [24]. In this study, we identified PIK3CA as a novel target of miR-1 in the NSCLC cell line A549. MiR-1 overexpression in A549 cells suppressed cell tumorigenic properties through PIK3CA repression and constitutive suppression of the PI3 K/Akt signaling pathway. Our data suggest that miR-1 may be a new therapeutic target for NSCLC.

2. Materials and methods

2.1. Cell culture

The human pulmonary adenocarcinoma cell line A549 was obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). Cells were incubated in Dulbecco's modified Eagle's medium (HyClone, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Auckland, New Zealand), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were used for all assays.

2.2. Transfection

Cells were transiently transfected with miR-1 mimics (50 nM), miR-1 inhibitor (100 nM), and their corresponding negative controls (GenePharma, Shanghai, China) using Lipofectamine™ 2000 (Invitrogen, CA, USA) in Opti-MEM (Invitrogen) according to the manufacturer's recommendations. The negative control (NC) scrambled oligonucleotide does not encode for any known miRNA. All miRNA sequences are listed in Table 1. Transfection efficiency was confirmed by TaqMan real-time PCR detection of miR-1 expression.

2.3. Plasmid construction and dual-luciferase reporter assay

To construct a luciferase reporter vector, the PIK3CA 3′UTR fragment containing putative miR-1 binding sites was PCR-amplified from A549 complementary DNA. The 3′UTR was digested with XbaI and inserted into a pGL3-promoter vector (Invitrogen), which is downstream of the luciferase reporter gene, and termed pGL3-PIK3CA 3′UTR-wt (wild type). Site-directed mutagenesis of the miR-1 target site in pGL3-PIK3CA 3′UTR was performed using a Stratagene QuikChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions to construct pGL3-PIK3CA 3′UTR-mut (mutant). Constructs were validated by DNA sequencing. A549 cells were cultured in 24-well plates (5×10^4 cells/well); one day later, each well was cotransfected with 100 ng pGL3-PIK3CA 3′UTR-wt or pGL3-PIK3CA 3′UTR-mut, and 5 ng pRL-SV40 (Promega, WI, USA) and miR-1 mimics or inhibitor using Lipofectamine™ 2000. A Renilla luciferase pRL-SV40 vector was used as a transfection control. Cells were harvested 48 h after transfection and dual-luciferase activity was measured with a Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions and normalized to Renilla signals. Each experiment was performed in triplicate.

Table 1
Sequences of primers for qRT-PCR and miR-1 related sequence.

Name	Sequences (5′ to 3′)
Primers used for mRNA detection	
PIK3CA (Forward)	CCACGACCATCATCAGGTGAA
PIK3CA (Reverse)	CCTCACGGAGGCATTCTAAAGT
β-actin (Forward)	CCAACCGCGAGAAGATGA
β-actin (Reverse)	CCAGAGGCGTACAGGGATAG
miRNA sequences	
miR-1 Mimics (Forward)	UGGAAUGUAAAGAAGUAUGUAU
miR-1 Mimics (Reverse)	ACAUACUUCUUUACAUUCCAUU
Mimics NC (Forward)	UUCUCCGAACGUGUCACGUTT
Mimics NC (Reverse)	ACGUGACACGUUCGGAGAATT
miR-1 Inhibitor	AUACAUACUUCUUUACAUUCCA
Inhibitor NC	CAGUACUUUUGUGUAGUACAA

2.4. Conventional RT-PCR of mRNA expression

We used qRT-PCR to examine the level of *PIK3CA* mRNA in A549 cells. Total RNA was extracted from cells using TRIzol (Invitrogen) as instructed by the manufacturer 48 h after transfection. Total RNA (500 ng) was reverse-transcribed using a PrimeScript RT Reagent Kit (Takara, Dalian, China) at 37 °C for 15 min and 85 °C for 30 s. Results were observed using an ABI PRISM 7900HT unit (Applied Biosystems, CA, USA) using a SYBR Premix Ex Taq™ kit (Takara) according to the manufacturer's instructions. Cycle threshold (Ct) fluorescence values were determined using SDS 2.4 software (Applied Biosystems). PIK3CA expression was normalized to that of β-actin using the $2^{-\Delta Ct}$ method. All primers (Invitrogen) are listed in Table 1.

2.5. Real-time PCR of mature miR-1 expression

For reverse transcription, 1 μg total RNA was converted to cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer protocol. The resulting cDNA was diluted 1:40 and used for PCR with 1 μL miR-1 or U6 TaqMan primers in triplicate wells using TaqMan Universal Master Mix II with no UNG (Applied Biosystems) in the ABI PRISM 7900HT. Ct values were calculated using SDS 2.4 software. MiR-1 expression was normalized to that of U6 with the $2^{-\Delta Ct}$ method. The TaqMan probes for miR-1 (Assay ID: 002222) and U6 (Assay ID: 001973) were purchased from Applied Biosystems.

2.6. Western blot

Forty-eight hours after transfection, cells were lysed in lysis buffer (50 mM Tris [pH 7.4], 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM Na_3VO_4, 1 μg/mL leupeptin) containing freshly added protease inhibitor. Equivalent amounts of protein lysates and loading buffer were loaded on 7.5%/12.5% polyacrylamide gels and separated by SDS-PAGE, and electrophoretically transferred onto PVDF membranes. Membranes were blocked for 1 h with 5% non-fat milk in TBST buffer (20 mM Tris [pH 8.0], 150 nM NaCl, 0.05% Tween 20) at room temperature and incubated overnight at 4 °C with the following primary antibodies: anti-PIK3CA (Abgent, CA, USA), anti-Akt (Cell Signaling Technology, MA, USA), rabbit monoclonal anti-phosphorylated (p)-Akt (Thr308) (244F9; Cell Signaling), biotinylated anti-human survivin (R&D Systems, MN, USA), and anti-GAPDH (Beyotime, Nantong, China). Membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (Beyotime) for 1 h at room temperature, and proteins were visualized using an ECL Chemiluminescence Kit (Millipore, MA, USA).