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miR-543 is up-regulated in gefitinib-resistant non-small cell lung cancer and promotes cell proliferation and invasion via phosphatase and tensin homolog

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ABSTRACT

MicroRNAs (miRNAs) play important roles in the pathogenesis of many types of cancers by negatively regulating gene expression at posttranscriptional level. Here, we identified that miR-543 is up-regulated in gefitinib-resistant non-small cell lung cancer (NSCLC) patients comparing gefitinib-sensitive ones. It promotes NSCLC cell proliferation by negatively regulates its target gene PTEN. In NSCLC cell lines, CCK-8 proliferation assay indicated that the cell proliferation is promoted by miR-543 mimics. Transwell assay showed that miR-543 mimics promotes the invasion and migration of NSCLC cells. Luciferase assays confirmed that miR-543 directly binds to the 3'untranslated region of PTEN, and western blotting showed that miR-543 suppresses the expression of PTEN at the protein level. This study indicates that miR-543 promotes proliferation and invasion of NSCLC cell lines by PTEN. The miR-543 may represent a potential therapeutic target for gefitinib-resistant NSCLC intervention.

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1. Introduction

Gefitinib, the first selective inhibitor of epidermal growth factor receptor's (EGFR) tyrosine kinase domain, is used to treat non-small cell lung cancer (NSCLC), which is the one of the most common cause of cancer-related death worldwide [1,2]. However, resistance to gefitinib limits its therapeutic effects on NSCLC patients. The discovery of MicroRNAs (miRNAs) opened a new generation for understanding the carcinogenesis of NSCLC [3]. miRNAs are small non-coding RNAs which are important regulators in cancer [4,5]. Some miRNAs, e.g. miR- 34a [6] and MiR- 451 [7] are found to play roles in the mechanisms underlying drug resistance to NSCLC by regulating their target genes. Underlying the miRNA-related molecular mechanisms underlying gefitinib-resistant NSCLC will be helpful to improve NSCLC therapies.

Therefore, we analyzed the expression pattern of miRNA and genes in gefitinib-resistant and -sensitive NSCLC, and found miR-543 and its predicted target gene PTEN was deregulated in gefitinib-resistant NSCLC patients comparing to sensitive ones. This leads to the hypothesis that miR-543 may play roles in NSCLC by PTEN. Further experiments in NSCLC cell lines confirmed that miR-543 directly bound to the 3'untranslated region of PTEN, and suppressed the expression of PTEN at the protein levels. Moreover, modulation of miR-543 expression regulated proliferation and invasion of NSCLC cell lines.

2. Materials and methods

2.1. miRNA and mRNA profile data

miRNA and mRNA profiles data of NSCLC samples and normal control samples were collected from GEO database (www.ncbi.nlm.nih.gov/gds, GSE51828, GSE34228). After quality control, 4 Gefitinib-resistant NSCLC samples and 4 Gefitinib-sensitive NSCLC samples were used in further miRNA analysis, while 104 Gefitinib-resistant NSCLC samples and 104 Gefitinib-sensitive NSCLC samples were used in further mRNA analysis.

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2.2. Identification of differentially expressed miRNA and mRNA

The identification of differentially expressed miRNA and mRNA in drug-resistant patients with NSCLC tissues were performed with Limma package on R platform using download miRNA and mRNA profiles data as mentioned above. The cutline of significantly differentially expressed miRNA and mRNA is P. value <0.01 (T test) and |lgFC|>1(fold change).

2.3. miRNA target genes prediction

Human miRNA target genes prediction uses miRNA sequences that downloaded from the Rfam website (http://www.sanger.ac.uk/Software/Rfam) and satisfy the established criteria [8]. The target genes of miRNAs were predicted using miRanda [9] and TargetScan [10] methods. The predicted target genes supported by both methods were selected for further analysis.

2.4. Ethics statement

The study was approved by the ethical committee of Weihai Woman and Children's Hospital. Written informed consents were obtained from all the patients. The entire investigation conforms to the principles outlined in the Declaration of Helsinki.

2.5. Patients and NSCLC samples

Patients undergoing NSCLC at Weihai Woman and Children's Hospital were included. Lung samples from 106 patients with Gefitinib-sensitive NSCLC and 95 patients with Gefitinib-resistant NSCLC were collected between November 2013 and January 2016 at the Weihai Woman and Children's Hospital (Table 1).

2.6. Cell culture and transfection

The human NSCLC cell lines, A427 and A549 was obtained from American Type Culture Collection (ATCC, USA). The A427 and A549 cell line was cultured in RPMI 1640 media (Life Technologies, Shanghai, China) and supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Shanghai, China). Cells were maintained in a humidified atmosphere with 5% CO2 at 37 °C. A427 and A549 cell lines were seeded in 24-well plates at 3×10^5 cells/wells and incubated overnight. Transfection of the miR-543 miRNA mimic (5′-AAACAUUCGCGGUGCACUUCUU-3′), the anti-miR-543 (5′-AAGAAGUGCACCGCGAAUGUUU-3′), inactive control cel-mir-67 (5′-UCACAACCUCCUAGAAAGAGUAGA-3′) (Life Technologies, Shanghai, China), or pMIR-Report vectors was taken using Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China) with 300 nmol of miRNA or 1 μg/ml DNA plasmid, respectively. Total proteins of A427 and A549 cells were isolated at 48 h after transfection.

2.7. Cell proliferation

Cell proliferations were measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). A427 and A549 NSCLC cells were plated in 24-well plates at 3×10^5 cells/well. Then cells were incubated in 10% CCK-8 which was diluted in normal culture medium at 37 °C for color conversion. Proliferation rates were determined at 24, 48 and 72 h after transfection.

2.8. Cell migration and invasion

Cell invasion and migration were measured using a transwell chamber (Corning, Shanghai, China) with and without Matrigel (Invitrogen, Shanghai, China). For the determination of A427 and A549 NSCLC cells invasion, transwell chambers were placed into 24-well plates, and coated with 30 μl Matrigel, then incubated at 37 °C for 40 min. In transwell assays with and without Matrigel, A427 and A549 cells were trypsinized and then seeded in chambers at the density of 8×10^4 cells/well at 48 h after transfection. These cells were cultured in RPMI 1640 medium with 2% serum. Meanwhile 600 μl of 10% FBS-1640 was added to the lower chamber. After 24 h, migrated A427 and A549 cells were fixed in 100% methanol for 30 min. These non-migrated A427 and A549 cells were removed by cotton swabs. After that cells on the bottom surface of the membrane were stained with the 0.1% crystal violet for 20 min. Images of A427 and A549 cells were taken under a phase-contrast microscope.

2.9. Luciferase assay

A427 and A549 cells were seeded in 24-well plates at 3×10^5 cells/well and incubated for 24 h before transfection. In the reporter gene assay, the A427 and A549 cells were co-transfected with 0.6 μg of pGL3-PTEN-3′UTR or pGL3-PTEN-3′UTR Mut plasmid, 0.06 ng of the phRL-SV40 control vector (Promega, Shanghai, China), and 100 nM miR-543 or control RNA using Lipofectamine 2000 (Invitrogen, Shanghai, China). The renilla and firefly luciferase activities were determined with a dual luciferase assay (Promega, Shanghai, China) 24 h after transfection.

2.10. RNA extraction and RT-PCR

miRNA and mRNA expression levels were determined by using the qRT-PCR kit (Life Technologies, Beijing, China). Real-time PCR

Table 1
NSCLC patients' characteristics.

Characteristics	All patients (n = 201)	Gefitinib-sensitive (n = 106)	Gefitinib-resistant (n = 95)
Gender, n (%)			
Male	140 (69.5)	69 (65.1)	71 (74.7)
Female	61 (30.5)	37 (34.9)	24 (25.3)
Age, n (%)			
<70	114 (56.7)	58 (54.7)	56 (58.9)
≥70	87 (43.3)	48 (45.3)	39 (41.1)
Stage, n (%)			
III/IV	190 (94.5)	103 (97.2)	87 (91.6)
Recurrence	11 (5.5)	3 (3.8)	8 (8.4)
Smoking history, n (%)			
Yes	151 (75.1)	80 (75.5)	71 (74.7)
No	51 (24.9)	26 (24.5)	24 (25.3)

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