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miR-200c enhances sensitivity of drug-resistant non-small cell lung cancer to gefitinib by suppression of PI3K/Akt signaling pathway and inhibites cell migration via targeting ZEB1

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ABSTRACT

Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) is a major obstacle in the treatment of non-small cell lung cancer (NSCLC) patients. We explored the role of miR-200c in modulating the sensitivity of gefitinib-resistant NSCLC cells and examined the underlying mechanism. The gefitinib-resistant cell line PC-9-ZD and its parental PC-9 cells were used. Growth inhibition was detected by MTT assay. The cell apoptosis was detected by Annexin V/PI assay. Cell migration was assessed by wound-healing assay. RT-PCR was used to detected levels of miR-200c and ZEB1. The PI3k, Bcl-2, Bax, caspase-3 and ZEB1 protein expression were detected using Western blot analysis, and TUNEL, Immunohistochemistry for xenograft model. PC-9-ZD cells had low level of miR-200c expression compared to its parental PC-9 cells. PC-9-ZD cells with miR-200c transfection were more sensitive to gefitinib treatment. Apoptosis induced by gefitinib was observed in PC-9-ZD cells with miR-200c transfection significantly. The levels of phosphorylated-Akt and Bcl-2 expression decreased and levels of Bax and Caspase-3 expression increased in PC-9-ZD cells with miR-200c transfection. Cell migration was inhibited and ZEB1 mRNA level and protein expression were significantly decreased in PC-9-ZD cells with miR-200c transfection. Further in gefitinib resistant xenograft model, miR-200c enhanced sensitivity of gefitinib and induced apoptosis significantly through PI3K/Akt signaling pathway and targeting ZEB1. These results provided insights into the functions of miR-200c and offered an alternate approach in treating gefitinib-resistance NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality in the world [1]. Non-small cell lung cancer (NSCLC) represents the predominant subgroup with a high recurrence and metastasis rate of lung cancer. NSCLC patients with activating epidermal growth factor receptor(EGFR) mutation could benefit from the first-generation reversible EGFR-tyrosine kinase inhibitor(TKI), such as Gefitinib,which has been used extensively in clinical settings [2]. Unfortunately, the patients who initially respond to Gefitinib will gradually develop acquired resistance and result in therapy failure eventually within 6–12 months [3]. The mechanisms underlying resistance to EGFR-TKI include Secondary mutation of EGFR (T790M mutation), overexpression of HGF, MET amplification and additional genetic alterations [3]. However, almost 1/4 of the underlying mechanisms of acquired resistance still remain unclear. Currently, there is lack of effective approach to reverse the drug resistance to EGFR-TKI. It is necessary to explore novel strategies to produce promising clinical outcomes.

MicroRNAs (miRNAs) are endogenous, single-stranded and non-coding RNAs which bind to specific sequences of their target mRNAs at the 3′ untranslated regions and regulate gene expressions. miRNAs have gained greatly attentions worldwide recently due to its effects of transcriptional silence and modulating multiple signalling cascades. One miRNA can target many genes and act as tumor suppressors [4]. It provides a novel function of miRNAs for therapeutic purposes. miRNAs play important role in initiation, promotion, and progression of carcinomas [5–7]. It has

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been reported that miRNAs modulated the sensitivity of cancer cells to chemotherapy and inhibit migration and invasion of tumors cells [8–11]. Moreover, a study have shown that introduction of miRNA may reduce the expression, activity and signalling transduction of EGFR and recover the cytotoxicity of gefitinib in NSCLC [12]. However, the role of miR-200c in modulating the sensitivity of gefitinib-resistant NSCLC cells remains unexplored.

In the present study, we aim to evaluate the effects of miR-200c in overcoming gefitinib-resistance and inhibiting cell migration and invasion in gefitinib-resistant NSCLC cells. Gefitinib-resistant PC-9-ZD cell line was generated by long-term exposure to the drug. PC-9-ZD cells were more resistant to gefitinib than their parental PC-9 cells [13]. The results in the present study showed that restoration of miR-200c enhanced sensitivity of drug-resistant PC-9-ZD to gefitinib by inducing apoptosis through suppression of PI3K/Akt signaling pathway. In addition, over expression of miR-200c inhibited cell migration via targeting ZEB1. These data revealed a potential mechanism by which NSCLC may acquire resistance to EGFR antagonists and proved that miR-200c would be a novel targeted agent in overcoming acquire resistance to EGFR-TKIs.

2. Materials and methods

2.1. Cell culture

The gefitinib-resistant cell line PC-9-ZD and its parental PC-9 cells were obtained from the Laboratory of Biochemistry and Molecular Biology (Tongji University, Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA) at 37 °C with atmosphere of 5% CO_2.

2.2. miRNA transfection

PC-9-ZD cells were seeded at a density of 1×10^5 cells/ml per well. Then, PC-9-ZD cells were transfected with miR-200c mimics (50 nM) or ZEB1 siRNA(100 nM) (GenePharma Co., Ltd., Suzhou, China) by using Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) and OPTI-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Medium was removed and cells were harvested at 48 h after transfection.

2.3. Quantitative RT-PCR

Total RNA from PC-9 and PC-9-ZD cells was extracted by Trizol reagent (Invitrogen, MA, USA) following manufacture's protocol. cDNA was synthesized by cDNA Synthesis Kit (Invitrogen,MA,USA) and RT-PCR was performed on a LightCycler 480 system (Roche, Germany) using SYBR Premix Ex Taq II (Takara Bio Inc, Shiga, Japan). U6 RNA was used as a miRNA internal control. The following primers were used: for miR-200c, 5′-ACACTCCAGCTGGGTTAATACTGCGGGTA-3′ (forward) and 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCCATCAT-3′(reverse); for U6, 5′-CGCTTCGGCAGCACATATAC-3′ (forward) and 5′-TTCACGAATTTGCGTGTCAT-3′ (reverse); for ZEB1, 5′-ACCCTTGAAAGTGATCCAGC-3′(forward) and 5′-CATTCCATTTTCTGTCTTCCGC-3′ (reverse); for GAPDH, 5′-AGAAGGCTGGGGCTCATTTG-3′(forward) and 5′-AGGGGCCATCCACAGTCTTC-3′(reverse). All RNA expression values were computed by 2-ΔΔCT method.

2.4. Cell viability assay

The cell viability was detected by MTT assay according to the manufacturer's instruction (Sigma-Aldrich Co., St. Louis, MO, USA). PC-9-ZD cells with miR-200c transfection were treated by Gefitinib (0.5 μmol/L) for 24 h, 48 h, and 72 h. The control group with no treatment, the miR-200c group with miR-200c transfection and the gefitinib group without miR-200c transfection treated by Gefitinib (0.5 μmol/L) only were established and each group had triplicate treatments. The absorbance at a wavelength of 490 nm was measured by Multiskan (Thermo Fisher Scientific Inc., MA, USA).

2.5. Clonogenic assay

Four experimental groups were established as follows: The control group with no treatment, the miR-200c group with miR-200c transfection, the gefitinib group without miR-200c transfection treated by Gefitinib (0.5 μmol/L) only, the miR-200c + gefitinib group with miR-200c transfection treated by Gefitinib (0.5 μmol/L). PC-9-ZD cells were trypsinized and counted after 48 h treatment. Then the cells were seeded at flask(1000 cells/well) and cultured for fourteen days at 37° C in a 5% CO2 humidified atmosphere. The colonies were then fixed with mixture of methanol and glacial acetic acid and stained with crystal violet (Sigma-Aldrich Co. LLC. St. Louis, MO, USA). Colonies containing more than 50 cells were scored under the Olympus CKX41 light microscope(Olympus Co., Tokyo, Japan).

2.6. Apoptosis analysis

Cell apoptosis rates were determined by using Annexin-V/PI assay. PC-9-ZD cells with miR-200c transfection were treated by Gefitinib (0.5 μmol/L) for 24 h, 48 h, and 72 h. Cells were harvested and examined by Annexin V-FITC Apoptosis Detection kit(BD Biosciences, CA, USA). Cells were stained by Annexin V-FITC and PI, then analyzed by FACSCalibur flow cytometry (BD Biosciences, CA, USA).

2.7. Western blot analysis

The control group with no treatment and the miR-200c group with miR-200c transfection were established. After cell lysing, proteins were separated on 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Logan, UT, USA). After a blocking incubation with 5% milk-TBST, then primary antibodies against PI3K, p-Akt, Bcl-2, Bax, Caspase-3, ZEB1 and GAPDH antibody (1:1,000) were incubated with membranes, followed by incubation of the HRP conjugated secondary antibody (1:10,000)(Cell Signaling Technology, Inc. Danvers, MA,USA). The membranes were visualized using an image reader LAS-4000 system (Olympus Co., Tokyo, Japan).

2.8. Wound healing assay

The ability of cell migration was assessed by using wound-healing assay. The artificial wounds were produced on the confluent cellmonolayer by scraping the cells with a 200 μL pipette tip. The wound areas were measured by ImageJ software.

2.9. Animal treatment and tumor formation assay

BALB/c nude mice (4–6 weeks, weighing 20 ± 2 g) were purchased from the Laboratory Animal Center of Zhejiang University (Hangzhou, China) and housed under a pathogen-free condition. The animal care and experimental protocols were carried out according to the guidelines established by National Institutes of Health(NIH) and approved by the Zhejiang hospital Research Ethics Committee. PC-9-ZD ells that stably expressed miR-200c or miR-NC were injected subcutaneously into the flank.