Gene xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Gene

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

Research paper

Over expression of miR-200c suppresses invasion and restores methotrexate sensitivity in lung cancer A549 cells

Wulin Shan [1], Xiaolei Zhang [1], Ming Li *, Fang Deng, Jing Zhang

Department of Clinical Laboratory, Anhui Provincial Cancer Hospital, Hefei, China

ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form 16 June 2016
Accepted 15 July 2016
Available online xxxx

Keywords:
Lung cancer
microRNA-200c
Methotrexate
P53
EZH2

ABSTRACT

MicroRNAs have become recognized as key players in the development of malignancy. MiR-200c can function as a tumor suppressor gene. However, the effect of miR-200c on methotrexate resistance remains unclear to date. This study aims to evaluate the function of miR-200c in lung cancer A549 cells. The data presented in our study demonstrated that the expression of miR-200c was down-regulated in methotrexate-resistant A549 cells. Over expression of miR-200c could significantly inhibit cell proliferation, induce G0/G1 cell cycle arrest and induce cell apoptosis. RT-PCR and Western blot assays showed that the expression of P53 and P21 were significantly increased with miR-200c overexpression. These results indicated that over expression of miR-200c might enhance the sensitivity of A549 cells to methotrexate through the P53/P21 pathway. Furthermore, miR-200c overexpression significantly inhibited cell migration and invasion with increasing the expression of E-cad and decreasing the expression of EZH2. In consequence, we provide a mechanism of acquired resistance to methotrexate that is caused by the loss of miR-200c in lung cancer cells. Along with this, our study demonstrates the complex network of microRNA mediated chemoresistance.

1. Introduction

Methotrexate (MTX) is a widely used and highly successful anti-cancer agent, particularly for some solid tumors, human leukemia and severe psoriasis (Olsen, 1991). MTX ceases intracellular folate metabolism and then blocks the synthesis of purines and thymine which leads to impairment of tumor growth and cell death (Lorico et al., 1988). However, the acquired chemoresistance is a major obstacle in the treatment of cancer (Maheswaran et al., 2008; Chen et al., 2012). Hence, an advancement of the treatment by avoiding drug resistance and a better prediction of chemotherapy efficacy would improve the clinical outcome for cancer patients.

MicroRNAs (miRNAs) are endogenous, small and non-coding RNAs that regulate gene expression by preferentially binding to specific sequences in the 3′-untranslated region (3′-UTR) of their target mRNAs (Bartel, 2004). Accumulating evidence indicated that miRNAs were crucial for the initiation, promotion, and progression of human cancers (Ahn et al., 2012; Gailhouste et al., 2013; Salvi et al., 2013). Moreover, several studies have also shown that miRNAs were able to modulate the sensitivity of cancer cells to chemotherapeutic drugs and inhibit tumors cells migration and invasion (Kutanzi et al., 2011; Leskelä et al., 2011; Bao et al., 2012; Shi et al., 2013). A recent study has found that restoration of miRNA-200c (miR-200c) could reverse the EGFR-resistant phenotype in bladder cancer cells (Adam et al., 2009). In addition, miR-200c has been shown to increase the sensitivity to cisplatin and inhibit cell migration and invasion in lung cancer cells (Ceppi et al., 2010). However, the role of miR-200c in modulating MTX resistance in lung cancer cells remains largely unexplored.

In the present study, we aim to provide insights into the association of miR-200c expression with MTX resistance and cell invasion in human lung cancer A549 cells. The results presented in our study show that up-regulation of miR-200c can reduce the resistance of A549 cells to MTX with the possible mechanism of inducing apoptosis through the P53/P21 pathway. In addition, over expression of miR-200c can reduce MTX-resistant A549 cell migration and invasion through inhibition of EZH2 and up-regulation of E-cad. Hence, our data prove that miR-200c functions as a tumor suppressor in human lung cancer progression. This study may serve as a basis to explain the function of miR-200c in MTX chemoresistance and discover novel targeted combinations agents against cancer.

Abbreviations: MTX, methotrexate; miRNAs, microRNAs; HDAC, histone deacetylase; A549, MTX-sensitive cell line A549; A549/MTX, MTX-resistant cell line A549; RT-PCR, real time-PCR; MTT, 3-(4,5-dlmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FCM, flow cytometry.

* Corresponding author.
E-mail address: 18919685630@sina.cn (M. Li).

[1] Wulin Shan and Xiaolei Zhang contributed equally to this work.

http://dx.doi.org/10.1016/j.gene.2016.07.038
0378-1119/

2. Materials and methods

2.1. Cell culture

Human lung cancer MTX-sensitive cell line A549 (A549) was purchased from the Shanghai Institutes for Biological Sciences (Chinese Academy of Science). In our previous study, 15 μM MTX-resistant cell line A549 (A549/MTX) was successfully constructed. Cells were routinely cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were split at a 1:5 dilution every 2 to 3 days.

2.2. miRNA transfection

A549/MTX cells were seeded in six-well plate plates at a density of 5×10^5 cells/ml one day before transfection. Then, A549/MTX cells were transfected with 200 pmol miR-200c mimics (A549/MTX-M) or negative control (A549/MTX-N), using Lipofectamine 2000 (Invitrogen, USA) and OPTI-MEM I reduced serum medium (Invitrogen, USA) according to the manufacturer's protocol. Cells were harvested for further analysis at 48 h after transfection. The miR-200c mimics and negative control were supplied by GenePharma Tech (Shanghai, China).

2.3. RNA extraction and quantitative real-time RT-PCR analysis

Total RNA from tissues and cells was extracted by Trizol method (Invitrogen, USA) completely following the instructions. Single strand cDNA was synthesized using cDNA Synthesis Kit (Invitrogen, USA) with 1 μg total RNA according to the manufacturer's instructions. Real time-PCR (RT-PCR) was performed on a LightCycler 480 system (Roche, Germany) using SYBR mix (Invitrogen, USA). The PCR condition for miR-200c was: 95 °C 30 s, followed by 40 cycles of 95 °C 10 s, 60 °C 30 s. U6 snRNA was used for control. The PCR condition for mRNAs was: 95 °C 30 s, followed by 40 cycles of 95 °C 15 s, 58 °C 20 s. GAPDH was used as normalized control. For miRNAs, the relative expression was computed by $2^{-\Delta\Delta CT}$ method. Primer sequences have been shown in Table 1.

2.4. Cell proliferation analysis

A549, A549/MTX-M and A549/MTX-N cells treated with 0, 5, 10 and 20 μM MTX were seeded immediately at 100 μl per well in 96-well plates and incubated for 48 h at 37 °C in a humidified 5% (*v*/v) CO_2 atmosphere. Cell proliferation was measured using 3-(4,5-dlmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Each data point was determined three times before analysis.

Table 1
Primers sequences for miR-200c, EZH2, E-cad, P53 and P21 detection.

Primer		Sequences(5′–3′)
miR-200c	Forward	ACACTCCAGCTGGGTTAATACTGCGGGTA
	Reverse	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCCATCAT
U6	Forward	CGCTTCGGCAGCACATATAC
	Reverse	TTCACGAATTTGCGTGTCAT
EZH2	Forward	CATTCGGTAAATCCAAACTGC
	Reverse	CGACATACTTCAGGGCATCA
E-cad	Forward	GGTCTCTCTCACCACCTCCA
	Reverse	CCTCGGACACTTCCACTCTC
P21	Forward	TTAGCAGCGGAACAAGGAGT
	Reverse	CGTTAGTGCCAGGAAAGACA
P53	Forward	GTCAGAAGCACCCAGGACTT
	Reverse	CTCCCAAACATCCCTCACAG
GAPDH	Forward	AGAAGGCTGGGGCTCATTTG
	Reverse	AGGGGCCATCCACAGTCTTC

2.5. Cell cycle analysis

A549, A549/MTX-M and A549/MTX-N cells were seeded at a density of 5×10^5/ml in a twelve-well plate. Cells were then treated with 20 μM MTX for 48 h in medium. Cells were harvested by centrifugation at 1000 rpm for 5 min, washed twice in cold PBS. Cell cycle analysis was performed using the cell cycle kit according to the manufacturer's specification (Bestbio, Guangdong, China) followed by flow cytometry (FCM). All experiments were performed in triplicate.

2.6. Cell apoptosis analysis

A549, A549/MTX-M and A549/MTX-N cells were seeded at a density of 5×10^5/ml in a twelve-well plate. Cells were then treated with 0, 10 and 20 μM MTX for 48 h in medium. The cells were harvested by centrifugation at 1000 rpm for 5 min, washed twice in cold PBS. Cell apoptosis was measured by Annexin V/PI staining. All experiments were performed in triplicate.

2.7. Cell migration and invasion analysis

The wound-healing assay was performed to test cell migration ability. The artificial wounds were produced on the confluent cell monolayer with FBS free, using a 200 μl pipette tip at 24 h post miR-200c transfection. The images were taken at 24 h after wound creation.

Then the transwell assays was performed to evaluate cells' migration and invasion ability. Cells suspended in 200 μl medium without FBS were placed on the upper chamber of each insert with 430 μl of 1 mg/ml matrigel (matrigel wasn't used for cell migration, while it was used for cell invasion) (Millipore, USA). The 600 μl medium with 10% FBS as the nutritional attractant was put in the lower chamber. After 24 h, the cells attached to the lower surface of chamber were fixed 20 min by 20% methanol and stained for 10 min with 10% maygruwald-giemsa. The invasion membranes were then cut down and embedded under cover slips. We counted the cells in 3 different vision fields in condition with 40× magnification which were then used as the average number of cells. All assays were performed in three independent experiments.

2.8. Western blotting

Proteins were separated on 10% SDS-PAGE and then transferred to 0.45 μm PVDF membranes (Amersham, UK). After a blocking incubation with 5% milk-TBST, the membranes were incubated overnight in primary antibodies against EZH2, E-cad, P53 and P21 at appropriate dilutions, followed by 1 h incubations in a secondary antibody that was conjugated to horseradish peroxidase (1:10,000 dilution). After incubations in an enhanced chemiluminescence reagent (Life-technologies, NY, USA), the images were captured on the image reader LAS-4000 system (Olympus, Tokyo, Japan).

2.9. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical differences between each group were determined using the one-way ANOVA and Student's *t*-test. All statistical analyses were performed using SPSS 16.0. A value of $P < 0.05$ was considered statistically significant.

Please cite this article as: Shan, W., et al., Over expression of miR-200c suppresses invasion and restores methotrexate sensitivity in lung cancer A549 cells, Gene (2016), http://dx.doi.org/10.1016/j.gene.2016.07.038