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Original article

Cisplatin upregulates MSH2 expression by reducing miR-21 to inhibit A549 cell growth

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ABSTRACT

miR-21 can act as an oncogene. MSH2 has been reported that it involved in the DNA mismatch repair (MMR) system and overexpression of MSH2 can induce cell apoptosis. We predicted that MSH2-3′-untranslated region (3′-UTR) was targeted by miR-21 using microRNA analysis softwares. To further explore the roles of miR-21 and MSH2 in A549 cells, we constructed pcDNA-GFP-msh-UTR vector (including MSH2-3′-UTR) to transfect A549 cells with miR-21, GFP positive cells were estimated under a fluorescence microscopy and by flow cytometry. We found miR-21 could obviously downregulate the expression of MSH2, which was further proved by western blotting. Moreover, we treated A549 cells with cisplatin and found that cisplatin could inhibit A549 cell growth in vitro and in vivo. We also found that cisplatin could downregulate miR-21 expression, while increase MSH2 expression in A549 cells. Our results demonstrated that cisplatin could upregulate the expression of MSH2 through downregulating miR-21 to inhibit A549 cell proliferation, which provides new gene targets for drug design or cancer therary.

1. Introduction

Lung cancer is the leading cause of cancer-related death in the world, with metastasis being the main reason for the mortality [1]. In China, lung cancer is the most common cancer and the incidence of lung cancer continues to increase [2]. According to statistics in 2012, lung cancer is of the first importance reason for cancer deaths in the United States [3]. 85 % of all lung cancers are non-small-cell lung cancer [4], of which adenocarcinoma is one kind of common histology [5]. The diagnosis of non-small-cell lung cancer is often at an advanced stage and the prognosis is poor, although it can be detected in its early stages [4]. Over the past 30 years, the overall 5-year survival from lung cancer remains dismal at around 16 %, showing little improvement [3,6].

The defections of DNA repair genes *MSH2*, *RECQL4* and *RAD51L1* are typically linked to various cancers [7]. Defection of DNA repair capacity, the susceptibility and risk of lung cancer was increased [8]. MSH2, binding directly to mismatched nucleotides to provide a target for DNA repair, is part of the mismatch repair (MMR) system involved in DNA damage recognition and repair [9,10]. MMR is responsible for the correction of DNA replication errors and therefore essential for maintaining genomic stability or preventing tumor formation [11,12]. Human mutS homolog 2 (hMSH2) and human mutL homolog 1 (hMLH1) are the core MMR proteins, which can form heterodimers with protein homologs hMSH3 or hMSH6 and hMLH3 or hPMS2, respectively [13]. The primarily study about mismatch repair (c-Abl and p73) dependent DNA damage-induced apoptotic response was reported in 1999 [14]. To explore whether MMR machinery was related with apoptotic process, microinjection studies had been used to overexpress MSH2. As a result, overexpression of hMSH2 and hMLH1 induced apoptosis within 24 h [15]. It was reported that more than 95 % of patients with Lynch syndrome were deficiency of MMR [11].

microRNAs (miRNAs) are non-coding RNAs of 20–22–nucleotides (nt) that bind to the 3′-UTRs of cognate mRNAs, negatively regulating the target mRNAs [16,17]. miRNAs has been found that they can modulate growth and death of cells [18]. Dependent upon the nature of their target gene(s), miRNAs may function as tumor suppressors or as oncogenes by downregulating target mRNAs. miR-16, miR-17 and miR-34a-c had been verified to act as tumor

Abbreviations: miRNAs, microRNAs; MMR, mismatch repair; 3′-UTR, 3′-untranslated region; hMSH2, Human mutS homolog 2; hMLH1, human mutL homolog 1; nt, nucleotides; FACS, Flow cytometry; RT-PCR, Reverse transcription-polymerase chain reaction; TPM1, Tropomyosin 1.

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suppressors by downregulating the expression of BCL-2 [19]. miR-150 which can downregulate the expression of p53 was confirmed as an oncogene [19,20]. miR-21 has been reported to function as an oncogene and modulate tumorigenesis through regulation of target genes to promote of cell growth, invasion, metastasis, and evasion of apoptosis [21,22].

Cisplatin was an effective anticancer drug for lung cancer therapy [23]. In our previous study, we found that the expression of miR-98 was decreased after treatment with cisplatin, while TP53 expression was enhanced, which indicated that cisplatin involved in the miR-98 regulating TP53 pathway to inhibit A549 cell growth [24]. In this study, to further explore whether cisplatin could affect the expression of miR-21 and MSH2, A549 cells were treated with cisplatin, then MSH2 expression was detected by western blotting. We found that cisplatin might upregulate the expression of MSH2 through downregulating miR-21 to inhibit A549 cell proliferation.

2. Material and methods

2.1. Construction of pcDNA-GFP-msh-UTR vector

Firstly, MSH2-3′-UTR (NM_000251, AK299667) was amplified by PCR from human genomic DNA. The forward primer was: 5′-AGTAATGGAATGAAGGTA-3′; Reverse was 5′-ATAAAATTCAGCA-CATCA -3′. PCR conditions was 30 cycles of denaturation at 94 °C for 45 s, annealing at 47 °C for 45 s and elongation at 72 °C for 60 s, performed in a PCR machine (eppendorf, Germany). The MSH2-3′-UTR production (241 bp) was cloned into T-vector (Takara, Japan) to construct T-MSH2 vector. Then the MSH2-3′-UTR was cut from T-MSH2 and cloned into pcDNA-GFP vector (our previous study [24]) by K*pn* I/H*ind* III to form pcDNA-GFP-msh-UTR vector.

2.2. Cell culture and miRNA transfection

A549 cells (obtained from Shanghai Institute of Cell Biology, China) were maintained in F12 medium (Gibco, USA) supplemented with 10 % calf serum (Hyclone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5 % CO_2.

For transfection, 1×10^6 cells were treated with 0.5 μg miRNA and 0.5 μg pcDNA-GFP-msh-UTR in 2.5 μL of lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instruction. All transfections were carried out in triplicate.

2.3. GFP assays

72 h after transfection, the expression of GFP in A549 cells was observed under one fluorescence microscope. The cells were trypsinized and gently washed with serum-containing medium. The cells was collected and centrifugated at 400 g for 5 min. At last, GFP positive cells were analyzed by flow cytometry (FACS).

2.4. Cisplatin suppressing A549 cell growth

Cells (1×10^4) in each of the 96-well flatbottom microtiter plates were treated with various of concentrations (0 μg/mL, 3 μg/mL, 6 μg/mL, and 9 μg/mL) of cisplatin (synthesized from QiLu Pharmaceutical Co., Ltd, China) for 72 h. The cells in each well were exposed to 10 μL MTT (5 mg/mL) at 4 h before the end of incubation, the supernatant was removed and 100 μL dimethyl sulfoxide (DMSO, signa) was added to determine the OD value at 570 nm using an enzyme-linked immunosorbent assay reader (ELX800, USA). The growth inhibition rate was dectected according to our previous study [20], the growth inhibition rate = $(OD_{control}-OD_{sample})/OD_{control} \times 100$ (%).

Flow cytometry (FACS) was used to further detect cells apoptosis induced by cisplatin. The specific method was as follows: Cells (8×10^4) in each of 12-well flatbottom microtiter plates were exposed to various of concentrations (0 μg/mL, 3 μg/mL, 6 μg/mL, and 9 μg/mL) of cisplatin for 48 h. Then the cells were dyed with Annexin V-FITC/PI, according to the manufacturer's instruction (KeyGEN Biotech., China). Annexin V-FITC/PI positive cells were analyzed by FACS.

2.5. A549 lung adenocarcinoma cell xenografts

After trypsinized, 5×10^6 cells in 100 μL PBS were subcutaneously injected into the lower back of 6-8-week old BALB/C-nu mice (nude mice, HFK Bio-Technology, China). When tumor volume ($V = \text{length} \times \text{width}^2/2$, length > width) is ~150 mm^3, 3 mg/kg of cisplatin was injected into the body of the nude mice every four days for four times. During treated with cisplatin, tumor volume was measured by a caliper everyday. Four days after the last treatment, nude mice were sacrificed and tumors were collected for further analysis. The control mice were treated with physiological saline in the same amount. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Binzhou Medical University.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

miRNAs were isolated by mirVana miRNA Kit (Ambion) and added ploy (A) using poly (A) polymerase (Ambion). The cDNA was synthesized by RT primer 5′-AACATGTACAGTCCATG-GATGd(T)30N(A,G,C or T)-3′. The forward primer used to amplify miR-21 was 5'- AGCTTATCAGACTGATGTTGACTG-3', and the reverse was 5'- ACATGTACAGTCCATGGATG-3'. Then, the Quantitect SYBR-Green kit (Qiagen) was used to assess the expression of miR-21 in one RG3000 system (Corbett Research) as follows: denaturing at 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 60 °C annealing for 20 s and extension at 72 °C for 20 s. At the each extension step of 72 °C, fluorescence was detected at 585 nm.

2.7. Western blotting

After A549 cells or mice were treated with cisplatin, the samples were lysed with lysis buffer (Cell lysis buffer for Western of Beyotime, China). 30 μg protein of each sample was loaded respectively to each lane of polyacrylamide gel, then the protein was transferred to PVDF membranes. After transferring, the membranes were blocked with 5 % non-fat milk in TBST (50 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, 0.1 % Tween-20) for 2 h at room temperature. The membranes were incubated with MSH2 antibody (1:400, Boster) in TBST at 4 °C overnight. The membranes were washed with TBST for three times. The HRP-labeled goat anti-mouse IgG (1:6000, Beijing Zhong Shan-Golden Bridge Technology Co., Ltd., China) was used to incubate the membrane for 1 h at room temperature. At last, the membrane was put in ECL reagent (Boster Immunoleader, China) and detected. Actin was used as the control.

2.8. Statistics

SAS software was used to analyze the significance of all results. The Student's t-test was used for inter-group comparison. A *P* value less than 0.05 was considered significant.