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Targeting miR-21 enhances the sensitivity of human colon cancer HT-29 cells to chemoradiotherapy in vitro

Jun Deng [1], Wan Lei [1], Jian-Chun Fu [1], Ling Zhang, Jun-He Li, Jian-Ping Xiong *

Department of Oncology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, PR China

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ABSTRACT

5-Fluorouracil (5-FU) is a classic chemotherapeutic drug that has been widely used for colorectal cancer treatment, but colorectal cancer cells are often resistant to primary or acquired 5-FU therapy. Several studies have shown that miR-21 is significantly elevated in colorectal cancer. This suggests that this miRNA might play a role in this resistance. In this study, we investigated this possibility and the possible mechanism underlying this role. We showed that forced expression of miR-21 significantly inhibited apoptosis, enhanced cell proliferation, invasion, and colony formation ability, promoted G1/S cell cycle transition and increased the resistance of tumor cells to 5-FU and X radiation in HT-29 colon cancer cells. Furthermore, knockdown of miR-21 reversed these effects on HT-29 cells and increased the sensitivity of HT-29/5-FU to 5-FU chemotherapy. Finally, we showed that miR-21 targeted the human mutS homolog2 (hMSH2), and indirectly regulated the expression of thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD). These results demonstrate that miR-21 may play an important role in the 5-FU resistance of colon cancer cells.

1. Introduction

Colorectal cancer (CRC) is one of the most common types of cancers in China. Despite advances in surgery, chemotherapy and molecular targeted therapy, the mortality rate has only decreased slightly over the past years [1]. Among classic chemotherapeutic drugs, 5-FU is one of the widely used chemotherapies for the adjuvant and metastatic therapy of CRC. However, primary or acquired resistance to fluoropyrimidine-based chemotherapy is a major clinical obstacle to the successful treatment of CRC. Tremendous efforts have been taken to understand molecular and cellular mechanisms for the action of 5-FU over the past decades, but it remains unclear how these colon cancer cells become resistant to 5-FU. Thus, a better understanding of molecular events underlying this drug resistance is important and necessary for achieving effective therapy against this type of colon cancers.

Recently, studies found that the sensitivity to 5-FU was closely related to drug-metabolizing enzymes, including thymidylate synthase (TS), TP and DPD. TS is a target enzyme of 5-FU, essential for converting intracellular dUMP into dTMP. The high expression of TS mRNA often prompts resistance to 5-FU in colon cancer cells, and a disease-free survival and overall survival rates of those patients are often very low [2]. TP accelerates the activation of 5-fluorodeoxyuridine. Studies showed that the increased expression of TP enhances the sensitivity of colon cancer cells to the prodrug of 5-FU, while decreased expression of TP enzyme provokes cancer cells resistance [3]. The DPD enzyme catalyzes the catabolism of 5-FU, and the high expression of DPD can decompose 5-FU prior to form active antitumor metabolites and presents one mechanism of 5-FU resistance. The mismatch repair (MMR) system is involved in the damage recognition and repair of DNA, and MMR mainly consists of hMSH2 core protein [4]. Clinical trials showed the defect of MMR proteins could be associated with reduced or absent benefit from 5-FU adjuvant chemotherapy [4,5].

In addition to these studies on metabolic enzymes for nucleic acid bio-synthesis involving 5-FU resistance, recent studies have also shown the involvement of microRNAs in this resistance. MicroRNAs are a class of highly conserved noncoding small RNAs in cells, which regulate gene expression by binding to the 3′-UTR of their target mRNAs post-transcriptionally [6]. They have been shown to have both diagnostic and prognostic significances and to serve as a novel target for the development of cancer therapy [7–9]. Recently, miRNAs were found to target 5-FU drug metabolism enzymes and to influence the drug sensitivity. For instance, miR-433 negatively regulated the expression of TS responsible for 5-FU sensitivity [10], and expression of the human DPD protein was repressed by some miRNAs [11]. miR-21 is upregulated in most of human malignancies and involved in each stage of cancer

Abbreviations: CRC, colorectal cancer; qRT-PCR, quantitative real time polymerase chain reaction; TS, thymidylate synthase; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase; MMR, mismatch repair system; UTR, untranslated regions; NC, nonsense control.

* Corresponding author.
E-mail address: jpxiong@medmail.com.cn (J.-P. Xiong).

[1] These authors contributed equally to this work.

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development and progression, including transformation, neoplasia, invasion, metastasis and drug resistance [12–15]. Moreover, miR-21 over-expression is associated with poor prognosis of 5-FU chemotherapy, including lymph node and liver metastasis [16–18]. However, it remains elusive whether and how miR-21 may cause 5-FU resistance in colon cancer cell line. HT-29, which contains high levels of miR-21, we investigated if miR-21 may play a role in the development of 5-FU resistance. As a result, inhibition of this miRNA expression led to hypersensitivity of HT-29 cells to 5-FU, whereas further overexpression of miR-21 promoted cell proliferation. Also, we found that hMSH2 is a direct target of miR-21. These results suggest that miR-21 might be one of the key cellular components important for the development of colorectal cancer 5-FU resistance. Our study also implies that targeting miR-21 may overcome the resistance of colon cancer cells to 5-FU and thus serve as a potential target for the development of cancer therapy specifically against 5-FU resistant cancer cells.

2. Materials and methods

2.1. Cell lines

Human colon cancer cell line HT-29 (ATCC) and HT-29/5-FU were cultured in McCOYS'5A medium with 10% fetal bovine serum (FBS) and 1% antibiotics. All cell lines were incubated at 37 °C in 5% CO_2.

2.2. Cell infection

We obtained fragments of miR-21 ASO and pre-miR-21 sequence through some important references, and created the pre-miR-21 and miR-21-ASO lentiviral vector, the nonsense sequence lentiviral vector (NC) as a negative control, lentiviral vector-U6 snRNA as an endogenous control. All of the lentiviral vectors were synthesized by GenePharma, Shanghai. HT-29 cells were transfected with each group. We used puromycin to screen stable cell lines.

2.3. TaqMan quantitative real-time PCR analysis of mature miR-21 expression

miRNA was isolated from the cell lines with miRNA isolation Kit (Omega), reverse transcribed using TaqMan® miRNA reverse transcription kit, and subjected to real-time PCR using TaqMan® miRNA Assay kit. Real-time PCR was performed using Step One Plus Real-Time PCR System (Applied Biosystems, USA) by standardized protocol. To normalize the expression levels of miR-21, U6 was used as a reference. The relative amount of miR-21 to internal control U6 was calculated by using $2^{-\Delta\Delta CT}$.

2.4. Cell proliferation assay

To determine the biological effect of miR-21 on cell proliferation, cell counting kit-8 was used following manufacturer's protocol. 1×10^2 cells per well were plated into 96 well plates and each day harvested a 96 well plates, a total of observation for 7 days. Then, 10 μl of CCK-8 solution was added to each well, and cells were incubated for 2 h at 37 °C. Absorbance at 450 nm was read on a microplate reader. All experiments were performed for 3 times.

2.5. Colony formation assay

1×10^3 cells were plated into 6-cm plates. Two weeks later, cells were fixed with methanol and stained with 0.1% crystal violet. The number of colonies, defined as ⩾50 cells/colony, was counted. The experiments were performed in triplicate.

2.6. Cell cycle and apoptosis assays by flow cytometry

For cell cycle analysis, pro-pidium iodide (PI) was attributable to cell cycle. The distribution of cells was analyzed by cell flow cytometry. For apoptosis analysis, an Annexin-V-PE/7-AAD apoptosis detection kit was used according the manufacturer' instructions. Apoptosis was analyzed by cell flow cytometry. The cells undergoing apoptosis were Annexin V-PE positive and 7-AAD negative.

2.7. Matrigel invasion assay

Cell invasion was analyzed using a transwell chamber of diameter 6.5 mm with an 8 μm membrane. Cells were added to the upper chamber respectively in each group, whose bottom was coated with 1 mg/ml matrigel for invasion assays. 1×10^6 cells seeded on matrigel coated transwell chamber in a small room.

2.8. Chemoradiotherapy sensitivity assays

Each group of cells was harvested by trypsinization. To adjust the cell suspension to 5×10^4 cells/ml, 100 μl per well were plated into 96 well plates in quadruplicate. The total of 5-FU concentration were set up to 9 different groups, each group was 6.25, 12.5, 25, 50, 100, 200, 400, 600, 800 (μl/ml). 10 μl of the CCK-8 solution was added to each well. After 2 h incubation, the absorbance at 450 nm was measured after 48 h. For radiotherapy sensitivity, each group was treated with dose of irradiation (2 Gy, continuous 5 days, suspended 2 days) and the inhibition ratio of irradiation was detected by MTT assays.

2.9. TaqMan quantitative real-time PCR for mRNA expression

Total RNA from cells was isolated using mRNA isolation Kit (Omega) according to the instructions supplied by the manufacturer. Reverse transcription (invitrogen) was performed with (1 ng–5 μg) of total RNA. Real-time quantitative RT-PCR was performed using TaqMan human miR assay kit. The relative amount of TS, TP, DPD, hMSH2 to internal control (GAPDH) mRNA was calculated by using $2^{-\Delta\Delta CT}$. Each experiment was performed in triplicate.

2.10. Western blotting

Each group of cells was seeded into 6-well plates and the cells were allowed to grow until 100% confluency, then lysed in lysis buffer on ice. Proteins were separated by 12% SDS–PAGE and blotted to nitrocellulose membranes. Membranes were blocked with 10% non-fat milk powder at room temperature for 2 h and incubated overnight with primary antibodies: TS, TP, DPD, hMSH2, GAPDH (Cell signaling, USA). After three 5 min washes in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 4 h at room temperature, then washed again in TBS-T and the membranes were developed with an ECL plus western blotting detection system.

2.11. Luciferase reporter assay

The 3′-UTR of hMSH2 containing miR-21 binding site was amplified using the primers: (Forward) 5′-CCGCTCGAGATCCCAGTAATGGAATGAAG-3′; (Reverse): 5′-ATAAGAATGCGGCCGCCATCACTT ATTATTGCCTATGT-3′. As a negative control, the mutated binding site of the 3′-UTR sequence (using the reverse complement

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