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miR-139-5p sensitizes colorectal cancer cells to 5-fluorouracil by targeting NOTCH-1

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ABSTRACT

Multidrug resistance (MDR), a phenomenon that often occurs with drug treatment and is characterized by relapse or attenuation of drug efficacy, is almost unavoidable in colorectal cancer (CRC) patients receiving 5-fluorouracil (5-FU)-based chemotherapy. MicroRNAs (miRNAs) are small noncoding RNAs that post-transcriptionally regulate gene expression. Our previous study has identified miR-139-5p as a potential tumor suppressor in CRC, but its role in chemoresistance of CRC has not been elucidated. In this study, we demonstrated that miR-139-5p was down-regulated either in CRC tumors receiving chemotherapy or in 5-FU-resistant CRC cell lines (HCT-8/5-FU and HCT-116/5-FU). Ectopic expression of miR-139-5p sensitized CRC cells to 5-FU by increasing 5-FU-induced apoptosis. In addition, miR-139-5p inhibited the expression of the miR-139-5p target gene NOTCH-1 and its downstream molecules MRP-1 and BCL-2, two key MDR-associated genes. Furthermore, silencing NOTCH-1 expression promoted the chemotherapeutic effects of 5-FU, and up-regulation of NOTCH-1 abrogated miR-139-5p-mediated sensitization to 5-FU in LoVo and HCT-116 cells. Taken together, our data indicate a new role of miR-139-5p/NOTCH-1 pathway in the drug resistance of CRC cells to 5-FU, which may be a promising therapeutic target for the anti-MDR treatment of CRC.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer-related death worldwide [1]. Systemic chemotherapy is one of the standard treatments for patients who are not candidates for effective surgery. Although many novel drugs have been developed for patients with advanced CRC, 5-fluorouracil (5-FU) is still widely used as the first-line drug for systemic chemotherapy of many human cancers, including CRC. As a base analogue, 5-FU targets thymidylate synthase to exert anti-cancer effects through blocking the synthesis of DNA and disrupting RNA processing [2]. However, the clinical responses to 5-FU vary greatly, and chemoresistance is considered to be a major reason for CRC therapy failure. Therefore, identification of molecular markers that modulate CRC chemoresistance is of great importance.

miRNAs are a group of small noncoding RNAs that negatively regulate target genes expression through direct binding to the 3′-untranslated region (3′-UTR) of their corresponding mRNA [3]. Aberrant expressions of miRNAs have been widely reported in the progression of nearly all tumor types, affecting cell proliferation, apoptosis, invasion and metastasis etc. [3–5]. Now, accumulating evidences showed that miRNAs play important roles in multidrug resistance (MDR) of various cancers. Several miRNAs, such as miR-204-5p, miR-17-5p, miR-22, miR-587, miR-145 and miR-129, have been reported to be associated with MDR of CRC through different mechanisms involved in apoptosis, autophagy, ATP-binding cassette transporter and DNA damage repair [6–11]. However, the detailed mechanism is complicated and still poorly understood. Our and other groups have reported that miR-139-5p is frequently down-regulated in human cancers and show significant anti-tumor activity in multiple cancers, including CRC [12–15].

In this study, we investigated the relevance of miR-139-5p to MDR in CRC cells, and revealed that miR-139-5p induced apoptosis and sensitized CRC cells to 5-FU, a most commonly used chemotherapeutic agents. In addition, we revealed that, as the target gene of miR-139-5p, NOTCH-1 plays key functional roles in miR-139-5p- induced apoptosis and drug re-sensitization. The

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results may contribute to the understanding of CRC resistant to conventional chemotherapy.

2. Materials and methods

2.1. Cell lines and tissues

Human CRC cell lines, including HCT-116, LoVo and HCT-8 were purchased from American Type Culture Collection (ATCC). Drug-resistant CRC sub cell lines (HCT-116/5-FU and HCT-8/5-FU) were developed by repeated exposure to stepwise increasing concentrations of 5-FU over a period of ~6 months as described [16]. These cells were cultured in high-glucose (4.5 g/l) DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin. All cell lines were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2.

Human CRC tissues were collected at Affiliated Hospital of Jiangnan University. All of the patients' materials were obtained with informed consent, and this project was approved by the Clinical Research Ethics Committees of Affiliated Hospital of Jiangnan University. Patients' information was showed in Supplementary Table 1.

2.2. Total RNA extraction and real-time qRT-PCR

Total RNA was extracted from cells or tissues using RNAiso reagent (TaKaRa, Japan). NanoDrop-2000 (Thermo, USA) was used to determine the concentrations of RNA. Complementary DNA (cDNA) was generated using the HiFiScript cDNA kit (CWBIO, China). UltraSYBR Mixture (CWBIO) was used to detect the relative mRNA expression by qRT-PCR assay, with β-actin as an internal control. TaqMan miRNA probes (Applied Biosystems, USA) were used to detect the levels of the mature miRNAs through tem-loop qRT-PCR assays, with U6B as an internal control. Primer sequences were showed in Supplementary Table. 2.

2.3. Plasmid, si-RNA and transduction

The human pri-miR-139-5p sequence was amplified and cloned into the lentivirus expression vector pWPXL to generate pWPXL-miR-139-5p as we previously described [12]. miR-139-5p mimic and corresponding negative control (NC) were synthesized at Ribobio (Guangzhou, China). NOTCH-1-specific interfering RNA (si-NOTCH-1) was purchased from GenePharma (Shanghai, China) (si-NOTCH-1: 5′-GCUGUCGCCUUGUUAAAUATTUAUUUAACAAGGCGACAGCTT-3′). Oligonucleotide transfection was performed using Lipofectamine 2000 reagents (Invitrogen, USA) according to the manufacturer's instructions.

2.4. Lentivirus production

The pWPXL-GFP and pWPXL-miR-139-5p plasmids were cotransfected into HEK-293T cells along with the packaging plasmid psPAX2 and the envelope plasmid pMD2G using Lipofectamine 2000 (Invitrogen). Virus particles were harvested 48 h after cotransfection. The particles were then individually used to infect HCT-116 and LoVo cells [12].

2.5. Chemotherapy sensitivity assay

The cytotoxic effect of 5-FU was evaluated at 48 h with the CCK-8 (Cell Counting Kit-8, Dojindo, Japan) assay. Cells were seeded in 96-well plates (5000 cells per well) and placed in an incubator at 37 °C for 12 h. The medium was then replaced with fresh medium containing different concentrations of 5-FU. After 48 h, 10 μl CCK-8 was added to each well. Incubation for another 2 h, the absorbance was determined by a microplate reader at 450 nm. The half-maximal inhibitory concentration (IC_{50}) was then calculated by the SPSS 16.0 package (IBM, USA).

2.6. Apoptosis assay

HCT-116 and LoVo cells were seeded in a 6-well plate with 40% density. After about 12 h, cells were transfected with miR-139-5p mimic or negative control (NC), incubated for another 24 h, cells were treated with 0.6 μg/ml of 5-FU for 48 h. Then, the cells were harvested and subjected to apoptosis analysis using an annexin V-FITC and propidium iodide labeling kit (CWBIO) by flow cytometry.

2.7. Western blotting

Cellular proteins were extracted and separated in SDS-PAGE gels, probed with anti-NOTCH-1 (Abcam, USA), anti-MRP-1 (Santa Cruz, USA) and anti-BCL-2 (Santa Cruz). Normalization was performed by blotting the same samples with an antibody against GAPDH (CWBIO), all antibodies were used at a dilution of 1:1000, and the expression levels of proteins were quantified by Quantity One software (Bio-Rad, USA).

2.8. Statistical analyses

The results are presented as the mean values ± SEM. The data were subjected to Student's *t*-test and the Mann-Whitney *U* test. A *P* value of less than 0.05 was considered statistically significant. SPSS 16.0 package (IBM, USA) and GraphPad prism 5.0 (GraphPad Software, USA) were used for statistical analyses and scientific graphing, respectively.

3. Results

3.1. miR-139-5p is down-regulated either in CRC tumors receiving chemotherapy or in 5-FU-resistant CRC cell lines

Intrinsic or acquired drug resistance is the key factors for the failure of chemotherapy. The previous studies showed that down-regulation of miR-139-5p in CRC tissues indicated poor survival of CRC patients [12]. However, whether miR-139-5p is associated with drug resistance is not clear. To investigate the relevance of miR-139-5p to drug resistance, we collected twenty tissues without chemotherapy, and ten tissues receiving 5-FU-based neoadjuvant chemotherapy (NACT) from CRC patients. The results showed that miR-139-5p was significant down-regulated in patients treated with NACT than those did not received ($P = 0.039$) (Fig. 1A).

To further check the potential relationship between the down-regulation of miR-139-5p and 5-FU resistance in CRC, we established two 5-FU-resistant CRC cell lines (HCT-116/5-FU and HCT-8/5-FU). Compared with their sensitive parental cells, the IC_{50} for 5-FU was increased approximately 15- or 110-fold in HCT-116/5-FU or HCT-8/5-FU cells, respectively (Fig. 1B). Interestingly, miR-139-5p expression was also down-regulated in these 5-FU-resistant CRC cells, and reduced to 32.3 ± 1.3% in HCT-116/5-FU cell or 50.9 ± 2.8% in HCT-8/5-FU cell, respectively (Fig. 1C). These results suggest the potential role of miR-139-5p in the chemoresistance of CRC.

3.2. miR-139-5p sensitizes CRC cells to 5-FU and promotes the 5-FU-induced apoptosis

To investigate whether miR-139-5p affect the sensitivity of CRC cells to 5-FU, we established HCT-116 and LoVo cells stably expressing miR-139-5p (Supplementary Fig. 1A). The CCK-8 assay was then