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MiR-519d reduces the 5-fluorouracil resistance in colorectal cancer cells by down-regulating the expression of CCND1

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Abstract. – OBJECTIVE: To investigate the effects of miR-519d on the 5-fluorouracil resistance in colorectal cancer cells and to explore the mechanism.

MATERIALS AND METHODS: Colorectal cancer cells HCT116 and SW480 were transfected with miR519d-mimic or siCCND1 by transient transfection. The sensitivity of cells to 5-Fu was assayed by MTT assay. Dual luciferase assay was used to examine the effect of CCND1 on the sensitivity of cells to 5-Fu mediated by miR-519d.

RESULTS: MiR-519d was overexpressed in colorectal cancer cells after transient transfection with miR-519d mimics. Overexpression of miR-519d increased the sensitivity of colorectal cancer cells to 5-Fu. MiR-519d negatively regulated the expression of CCND1 via directly bound to CCND1 3`UTR. si-CCND1 could downregulate the CCND1 expression in colorectal cancer HCT116 and SW480 cells. si-CCND1 increased the sensitivity of colorectal cancer cells to 5-Fu.

CONCLUSIONS: miR-519d inhibits the expression of CCND1 and then plays a role in alleviating 5-Fu resistance in colorectal cancer cells.

Key Words:
CCND1, miR-519d, Colorectal cancer, 5-fluorouracil, Chemoresistance.

Introduction

Colorectal cancer is one of the most common tumors of the digestive tract and one of the most highly malignant tumors[1]. About 5%-15% of patients with colorectal cancer are under 50 years of age[2-4]. Surgery and chemotherapy are the main treatments for colorectal cancer. 5-fluorouracil (5-Fu) is one of the commonly used chemotherapeutic drugs for colorectal cancer. The emergence of drug resistance seriously affects the therapeutic effect.

Lots of studies have reported that microRNA is implicated in 5-Fluorouracil (5-Fu) resistance in colorectal cancer cells. In colorectal cancer, miR-587 antagonizes 5-Fu-induced cell apoptosis and confers drug resistance *via* controlling PPP2R1B expression[5]. Another work[6] reported that miR-23a antisense promotes 5-fluorouracil chemosensitivity by APAF-1/caspase-9 apoptotic pathway. Li et al[7] revealed that miR-218 is a prognostic indicator in colorectal cancer and promotes 5-fluorouracil-induced apoptosis through regulating BIRC5 gene. Kurokawa et al[8] explored the functions of miR-19b in 5-fluorouracil resistance in colon cancer cells.

MiR-519d functions as a tumor suppressor in breast cancer through inhibiting STAT3 expression[9]. In ovarian cancer, miR-519d suppresses cell proliferation and promotes cisplatin-mediated cytotoxicity *via* controlling XIAP expression[10]. At present, there is no report on the mechanism of the resistance of miR-519d to reverse colorectal cancer cells to 5-Fu. Through the miR-519d regulation of CCND1 expression and its colorectal cancer cells to 5-Fu sensitivity, this topic aimed to explore the CCND1 involved in miR-519d mediated reversal of colorectal cancer cells to 5-Fu resistance mechanism.

Materials and Methods

Cell Line and the Main Reagents

Human colorectal cancer cell lines HCT116 and SW480 were purchased from Proso Bio Co., Ltd. (Shanghai, China). Transient siRNA, miR-519d mimic and inhibitor were purchased from Guangzhou Rui Bo Biotechnology Co., Ltd (Guan-

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gzhou, China). Luciferase activity detection using the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI, USA). For the site mutation experiments the Tailor™ Site-Directed Mutagenesis System (Carlsbad, CA, USA) were used.

qRT-PCR Detection

After small-interfering (si)-CCND1 and micro ribonucleic acid (miR)-519d were used to transiently transfect colorectal cancer cells, 2.5×10^5 cells were taken and paved onto a 6-well plate, ensuring that single cell could adhere to the wall and the density was 70%-80%. Cells transiently transfected with siRNA were divided into experimental group (si-CCND1) and negative control (si-NC) group. The transfection was performed according to instructions of the kit. RNA was extracted from cells after 48 h of the action of transiently-transfected interference fragment. Real-time fluorescence quantitative polymerase chain reaction (PCR) was performed after reverse transcription. Cells transiently transfected with miR-519d mimic were divided into overexpression group (miR-519d mimic) and negative control group (mimics NC), and the transfection was performed according to instructions of the kit. After 48 h, RNA was extracted, and Real-time fluorescence quantitative PCR was performed after reverse transcription to confirm the overexpression efficiency of miR-519d.

MTT Assay

Cells in the logarithmic growth phase in si-CCND1 group, si-NC group, miR-519d mimics and mimics NC (negative control) group were taken and inoculated into a 96-well plate (1×10^4/well), and 5 repeated wells were set in each group. At the same time, the blank control group with only medium added was set up. Cells were cultured in an incubator with 5% CO_2 at 37°C for 9-12 h, and after cell adherence, 5-fluorouracil (5-Fu) with the mass concentration of 0, 5, 10, 20, 40, and 80 μmol/L was added. After 48 h, the optical density value was measured at 490 nm using a microplate reader.

psiCHECK/CCND1 3'UTR Conduction

According to the prediction of bioinformatics software, PrimerPremier5.0 system was used to construct primers. The 3'-untranslated region (UTR) of CCND1 was amplified *via* PCR, and both upstream and downstream binding sites of miR-519d should be contained in the 3'-UTR. The 5' end of forward primer was added with XhoI restriction site, while the 5' end of reverse primer was added with NotI restriction site. At the same time, a few protective bases were added. The following operations were performed one by one: PCR product recovery, enzyme digestion reaction, purification of enzyme digestion products, ligation reaction, transformation of ligation products, identification of recombinant vector PCR, and extraction of the recombinant vector.

psiCHECK-2/CCND13'UTR Site Mutant

Operations were performed according to instructions of Gene Tailor™ Site-Directed Mutagenesis System. The mutation reaction consists of 3 steps: methylation reaction, mutation reaction, and conversion reaction. Finally, the target mutant plasmid psiCHECK-2/CCND1 mt 3'-UTR was obtained.

Dual Luciferase Assay

Operations were performed according to instructions of Dual-Luciferase Reporter Assay System. Cells lysated by Passive Lysis Buffer were transferred into the 96-well plate dedicated for luciferase assay. First, firefly luciferase was detected. Luciferase detection reagent II was added into the sample, and the resulting optical signal lasted for at least 1 min. After the firefly fluorescence intensity was quantified, Stop & Glo reagent was added into the same sample to terminate the firefly luciferase reaction. At the same time, Renilla luciferase reaction was initiated. The ratio of firefly luciferase activity to Renilla luciferase activity was used as the relative luciferase activity, followed by comparisons and analyses among different groups.

Statistical Analysis

Statistical product and service solutions (SPSS) 20.0 statistical software (Armonk, NY, USA) was used. Independent-samples *t*-test was used for the intra group comparison, and paired *t*-test was used for the comparison between two groups. Unpaired *t*-test was used for the comparison of OD value of the same drug concentration among different groups, and one-way analysis of variance (ANOVA) was used for the comparisons of OD values of different drug concentrations in the same group. Dunnett's statistical method was used for the comparison among different drug concentrations. $p<0.005$ was considered statistically significant.

Results

miR-519d Was Overexpressed in Colorectal Cancer Cells After Transient Transfection With miR-519d Mimics

The qRT-PCR assay was used to detect the expression of miR-519d between the experiment group and the control group, so as to identify the transfection efficiency. Significantly, the results of qRT-PCR showed that the expression of miR-519d in the experiment group (cells transfected with miR-519d mimics) was higher than that in the control group (Figure 1A and 1B).

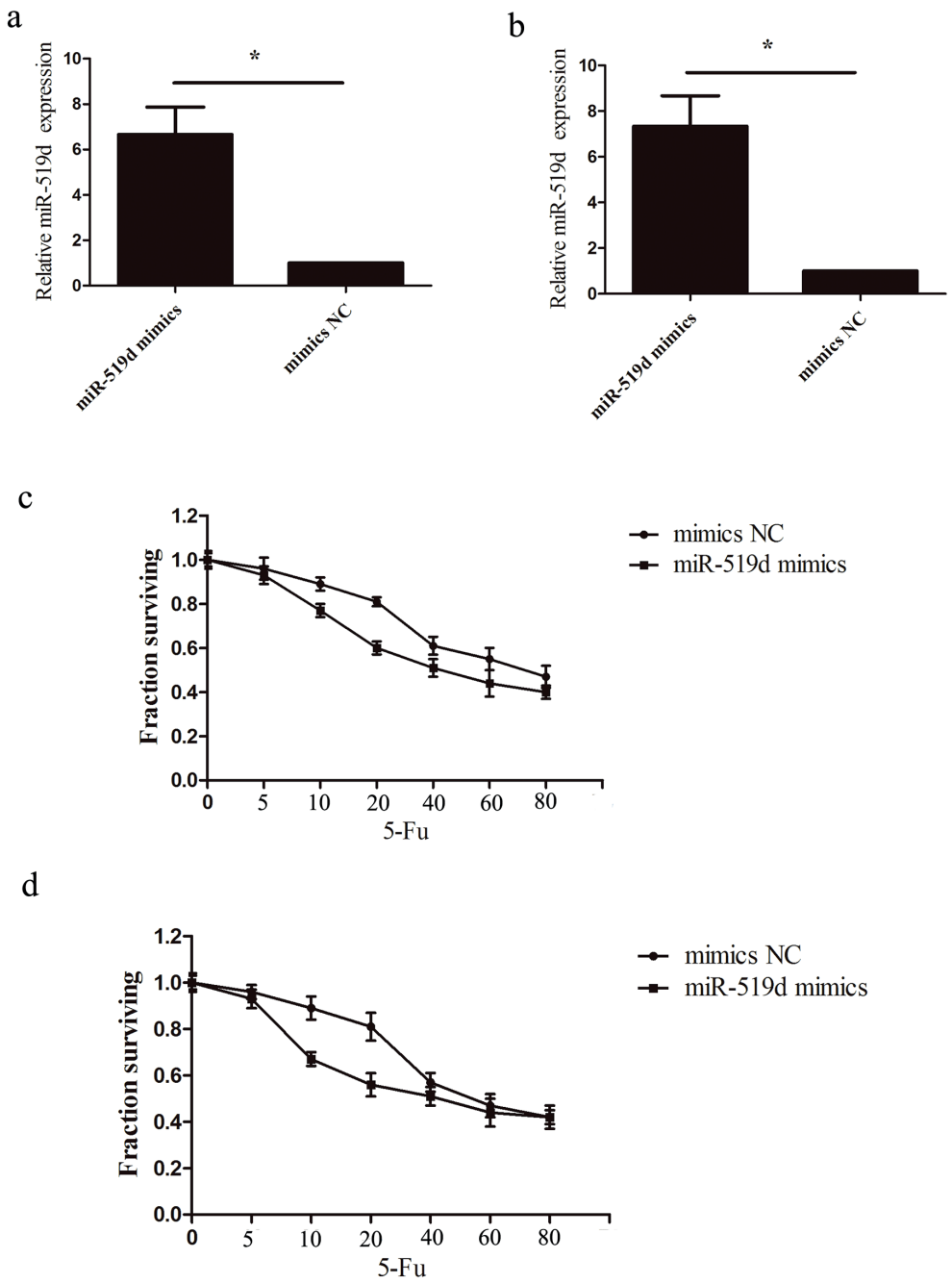

Figure 1. The relative miR-519d expression was detected *via* qRT-PCR. ***A,*** miR-519d was overexpressed in HCT116 after transient transfection with miR-519d mimics. ***B,*** miR-519d was overexpressed in SW480 after transient transfection with miR-519d mimics. *$p<0.05$; ***C,*** (HCT116) and ***D,*** (SW480), MTT analysis showed that overexpression of miR-519d increased the sensitivity of colorectal cancer cells to 5-Fu.

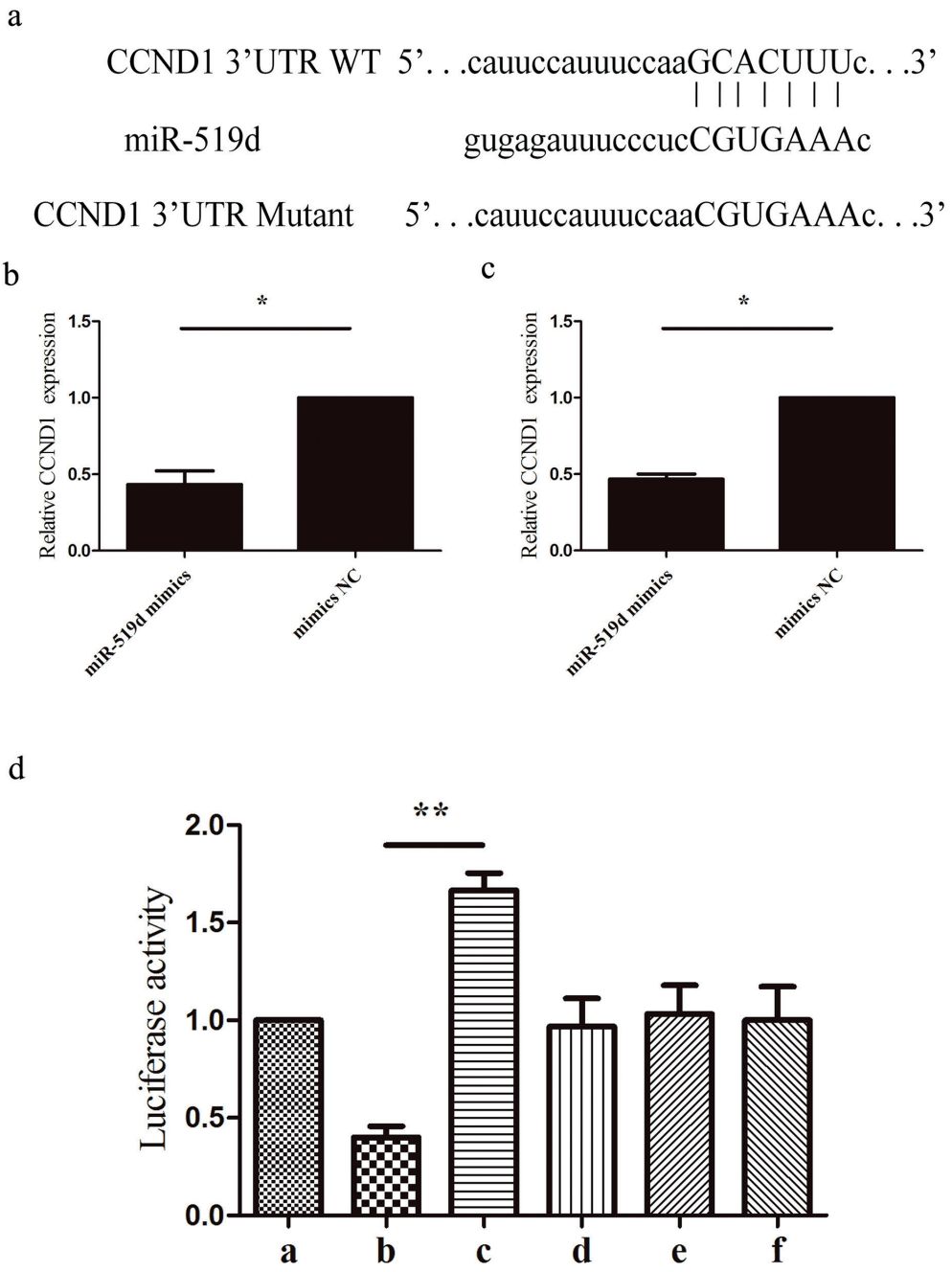

Figure 2. miR-519d negatively regulated the expression of CCND1 through directly bound to CCND1 3`UTR. ***A,*** The potential binding site was predicted through the bioinformatics for miRNA targets analysis (microRNA.org). ***B,*** (HCT116) and ***C,*** (SW480). The relative CCND1 expression was detected by qRT-PCR. *p<0.05; ***D,*** The luciferase activity was examined (*a*=PsiCHECK2+mimics, *b*=3`UTR wild type+mimics, *c*=3`UTR wild type+inhibitor, *d*=PsiCHECK2+inhibitor, *e*=PsiCHECK2+3`UTR wild-type +mimics, f: PsiCHECK2+3`UTR wild-type +inhibitor, ***p*<0.01).

Overexpression of miR-519d Increased the Sensitivity of Colorectal Cancer Cells to 5-Fu

The sensitivity of colorectal cancer cells to 5-Fu was also examined by using MTT detection method. The results of MTT assay (Figure 1C and 1D) showed that the IC50 values of colorectal cancer HCT116 and SW480 cells were decreased after miR-519d overexpression, and the sensitivity of both cell lines to 5-Fu was increased (p<0. 05).

miR-519d Negatively Regulated the Expression of CCND1

Furthermore, through the bioinformatics for miRNA targets analysis (microRNA.org), we focused that CCND1 was a potential target of miR-519d (Figure 2A). The relationship between miR-519d and CCND1 was investigated *via* qRT-PCR assay. The data showed that overexpression of miR-519d inhibited the expression of CCND1 (Figure 2B and 2C). All the above results suggested that miR-519d negatively regulated the expression of CCND1 in colorectal cancer cells.

miR-519d Directly Bound to CCND1 3`UTR

To further explore the regulatory mechanism, we used dual luciferase reporter assay. The results (Figure 2D) showed that co-transfection of CCND1 3'UTR wild-type plasmid and miR-519d mimic significantly reduced the luciferase activity. Co-transfection of CCND1 3'UTR wild-type plasmid with miR-519d inhibitor significantly increased luciferase activity. However, the luciferase activity did not change after co-transfection with CCND1 3'UTR mutant plasmid and miR-519d mimic or inhibitor respectively. This finding demonstrated that miR-519d directly bound to CCND1 3'UTR.

si-CCND1 Could Downregulate CCND1 Expression in Colorectal Cancer HCT116 and SW480 Cells

Moreover, to confirm the interference efficiency of si-CCND1, we performed qRT-PCR methods. The result of qRT-PCR (Figure 3A and 3B) showed that the interference efficiency of si-CCND1 on the siRNA-1 was higher than the siRNA-2, which indicated that si-CCND1 could down-regulate CCND1 expression in colorectal cancer HCT116 and SW480 cells. Thus, we chose siRNA-1 to perform the follow experiments.

si-CCND1 Increased the Sensitivity of Colorectal Cancer Cells to 5-Fu

The sensitivity of colorectal cancer cells to 5-Fu was examined by using MTT detection method. The results of MTT assay showed that the IC50 value of siCCND1 group was decreased in colorectal cancer HCT116 and SW480 cells (Figure 4A and 4B). This result demonstrated that the sensitivity of 5-Fu to HCT116 and SW480 cells after transient transfection with siCCND1 was increased.

Discussion

With a wide range of biological functions, miRNAs are involved in a series of important processes in human life. They can directly target the gene by recognizing and complementing the 3'UTR end of the target gene, and then down-regulate the expression of the target gene so as to regulate the biological functions.

It can be seen that miR-519d acts as an anti-oncogene to suppress tumor progression. This topic mainly discusses the correlation between the expression level of miR-519d and the drug resi-

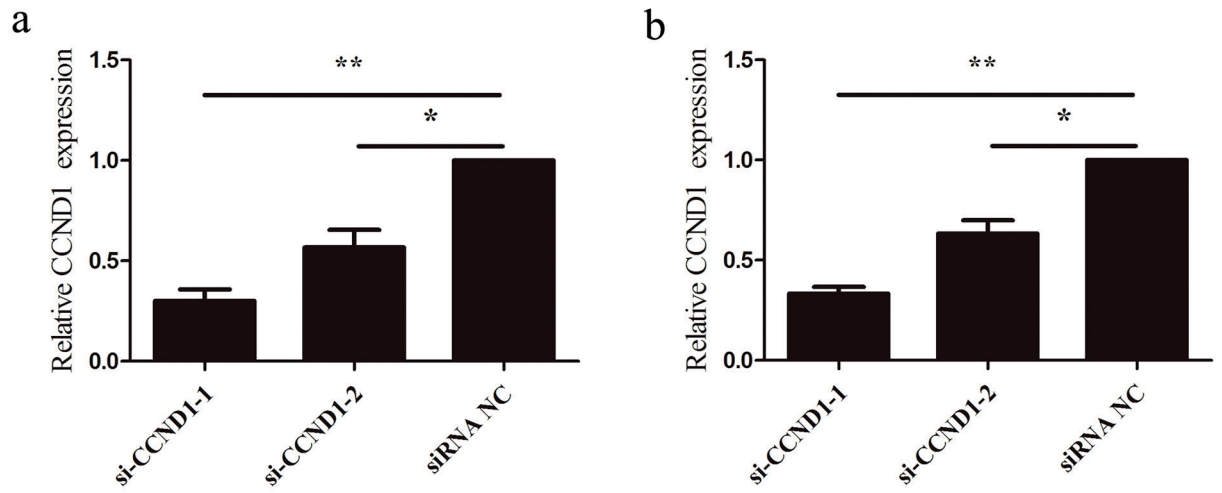

Figure 3. CCND1 was down-regulation *via* transfecting si-CCND1-1. ***A,*** (HCT116) and ***B,*** (SW480). The relative CCND1 expression was detected by qRT-PCR method. *$p<0.05$; **$p<0.01$

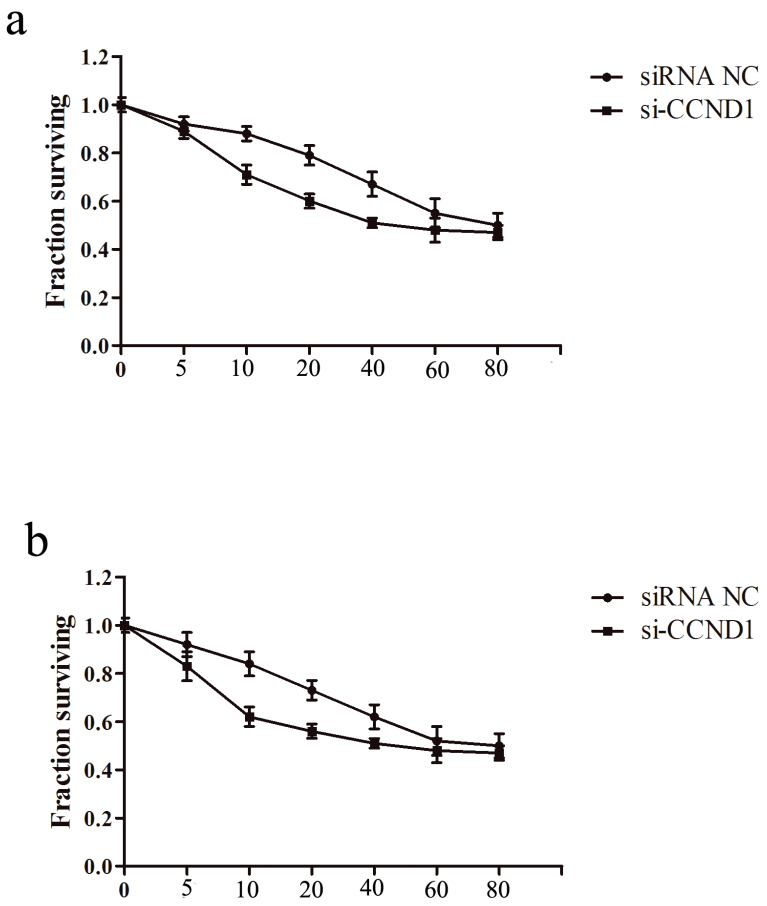

Figure 4. ***A,*** (HCT116) and ***B,*** (SW480), MTT analysis showed that downregulation of CCND1 increased the sensitivity of colorectal cancer cells to 5-Fu with the mass concentration of 0, 5, 10, 20, 40 and 80 μmol/L.

stance of colorectal cancer cells, further clarifies its mechanism of action on the sensitivity of colorectal cancer cells to 5-Fu.

Some studies[11-16] found that CCND1 located in human chromosome 11q13, and is highly expressed in liver cancer, gastric cancer, lung cancer, nasopharyngeal cancer, and ovarian cancer. In addition to the involvement of CCND1 in the regulation of cell cycle and tumor proliferation, recent studies have found that CCND1 overexpression influences the sensitivity of multiple chemotherapeutic drugs, such as cisplatin resistance in testicular cancer[17], breast cancer[18], head and neck squamous cell carcinoma[19], pancreatic cancer[20], and is also associated with myeloma[21].

We found that both CCND1 and miR-519d could affect the sensitivity of colorectal cancer cells to 5-Fu. Furthermore, our work predicted that CCND1 might be miR-519d by computerized biological prediction. To verify the results of bioinformatics analysis, we found that the changes of miR-519d and CCND1 were synchronized by the luciferase reporter assay, that is, the inhibition of the expression of miR-519d can promote the increase of CCND1 expression, miR-519d can act directly on CCND1 by directly binding to the 3'UTR region, and target CCND1 to achieve CCND1 inhibition. As a result, it reduces colorectal cancer drug resistance to 5-Fu and increases its chemosensitivity.

Colorectal cancer is the most common tumor of the digestive system. The 5-Fu-based chemotherapy regimen is one of the most important treatments. However, the drug resistance of colorectal cancer cells to 5-Fu drugs seriously affects the clinical efficacy. How to reverse its drug resistance and increase the sensitivity of colorectal cancer cells to 5-Fu is particularly important for improving clinical efficacy.

Conclusions

We showed that miR-519d may be used as a therapeutic target to further study how to overcome the drug resistance of colorectal cancer cells to 5-Fu drugs and to improve the clinical efficacy of chemotherapy and benefit the masses of tumor patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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