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

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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.

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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′-CCGCTCGAGATCCCAGTAATG GAATGAAG-3′; (Reverse): 5′-ATAAGAATGCGGCCGCCATCACTTAT TATTGCCTATGT-3′. As a negative control, the mutated binding site of the 3′-UTR sequence (using the reverse complement of the

binding site) was amplified using the primers: (Forward) 5′-CC GCTCGAGTGAAGGTAATATTGTGAAGCTATTGTC-3′; (Reverse): 5′-ATAAGAATGCGGCCGCCA TCACTTATTATTGCCTATGT-3′. Products were reclaimed from agarose gel electrophoresis, and cloned into the luciferase reporter PsiCHECK vector (Promega). All constructs were verified by sequencing. Each group cell was seeded in 5×10^5 cells per 6-well plates and transfected plasmid when cells reached 70% confluence, luciferase reporters using lipofectamine™ 2000 (Invitrogen) following the instructions. Cells were harvested 48 h after transfection, being analyzed for luciferase activity using the Dual-Luciferase Reporter Assay system (Promega).

2.12. Statistical analysis

Statistical analysis was performed using SPSS 18.0. Data's were presented as the mean ± standard deviation. Statistical analyses were done by analysis of variance (ANOVA) or Student's test. *P* value <0.05 was considered statistically significant.

3. Results

3.1. miR-21 increases cell proliferation and inhibits cell apoptosis

In order to investigate the function of miR-21 in HT-29 cells, we tested miR-21 expression in HT-29 cell line using TaqMan real-time PCR after infection 72 h. The result showed that a decrease or increase after infected with miR-21 ASO and pre-miR-21 lentiviral vector (Fig. 1A). We observed a significantly increased in proliferation after infection of pre-miR-21 lentiviral vector, In contrast, miR-21 ASO lentiviral vector significantly decreased cell proliferation (Fig. 1B and C). These results indicate that HT-29 cell proliferation ability can be significantly enhanced by the increase of miR-21 expression.

We further investigated the effect of miR-21 on cell apoptosis. The results showed that increase of miR-21 expression could decrease apoptosis dramatically. In contrast, knockdown of miR-21 could increase the apoptosis of HT-29 cells (Fig. 2B). These results suggests that miR-21 could function as a strong antiapoptotic factor in human HT-29 colon cancer cells.

3.2. Effect of miR-21 on cell cycle in HT-29 cell lines

We further investigated the effect of miR-21 on the cell cycle using flow cytometry. miR-21-overexpression had a significantly lower percentage of cells in the G1/G0 phase and increased the percentage of cells in the S phase. By contrast, downregulation of miR-21 had the opposite effect (Fig. 2A). Collectively, these data suggests that miR-21 could enhance the proliferation of HT-29 cells by promoting the G1/S cell cycle transition.

3.3. Effect of miR-21 on invasion of HT-29 cells

To explore whether miR-21 affects the invasion of HT-29 cells, matrigel invasion assays was performed. Increasing the expression of miR-21 enhanced the invasion of HT-29 cells, while downregulation of miR-21 significantly inhibited the invasion of HT-29 cell

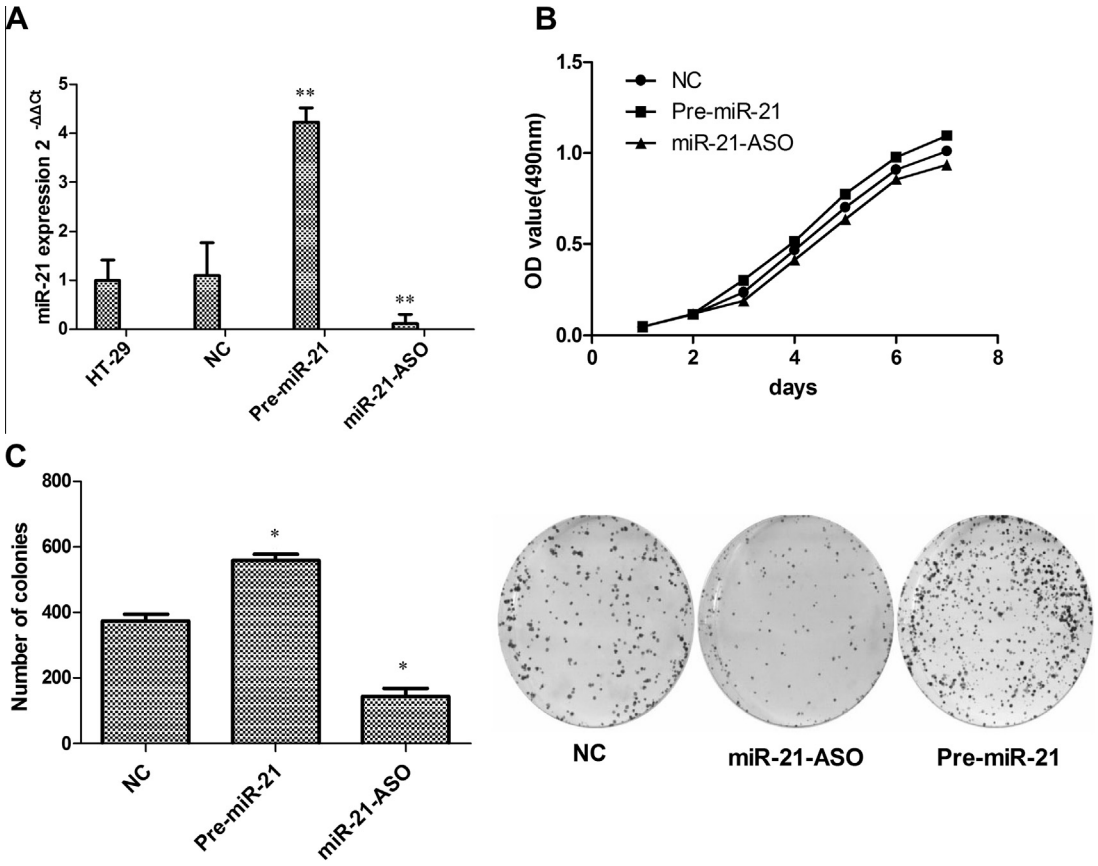

Fig. 1. miR-21 increases cell proliferation and colony formation in HT-29 cancer cells. (A) Analysis of TaqMan qRT-PCR shows that a decrease or increase after infected with miR-21 ASO and pre-miR-21 lentiviral vector, the nonsense sequence lentiviral vector (NC) as negative control (*$P < 0.05$, independent *t* test). (B) miR-21 increases the cell proliferation of HT-29 cells. Cells were infected with miR-21 ASO and pre-miR-21 lentiviral vector, and were plated into 96 well plates, each day harvested a 96 well plates, a total of observation for 7 days. All the experiments were done in triplicate (*$P < 0.05$, independent *t* test). (C) Colonies formed by miR-21 ASO and pre-miR-21 lentiviral vector infected cancer cells were shown 2 weeks after plating (*$P < 0.05$, independent *t* test).

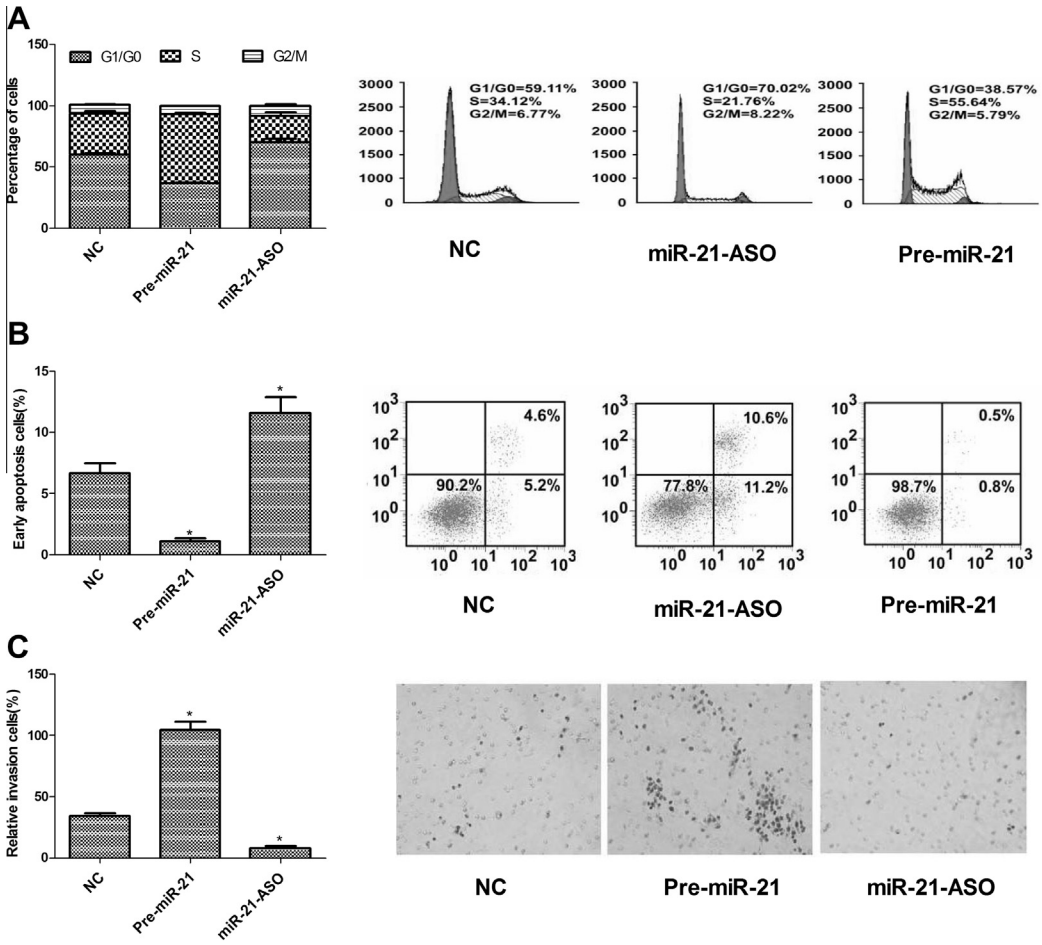

Fig. 2. Impact of miR-21 on cell cycle, apoptosis and invasion. (A) Impact of miR-21 on cell cycle of HT-29 cancer cells. The distribution of cells throughout the cell cycle were analyzed by cell flow cytometry (*$P < 0.05$, independent t test). (B) Impact of miR-21 on cell apoptosis. Apoptosis was analyzed by cell flow cytometry. The cells undergoing apoptosis were Annexin-V-PE-positive and 7-AAD-negative (*$P < 0.05$, independent t test). (C) Impact of miR-21 on cell invasion. Cells in each group were added to the upper chamber respectively, which 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 (*$P < 0.05$, independent t test).

(Fig. 2C). Therefore, these results suggests that miR-21 could promote the invasion ability of HT-29 cells.

3.4. Effect of miR-21 on the sensitivity of HT-29 cells to 5-FU chemotherapy

Next, we will investigate whether miR-21 could affect the sensitivity of HT-29 cells to 5-FU chemotherapy. Compared with the IC50 value of 5-FU in the control group (HT-29 cells), upregulation of miR-21 increased the IC50 value of 5-FU, while down-regulation of miR-21 significantly reduced the IC_{50} value of 5-FU after introducing these agents into HT-29 cells (Fig. 3A). This data indicates that miR-21 is one key player in 5-FU resistance of this colon cancer cell.

3.5. Effect of miR-21 on the sensitivity of HT-29 cells to radiotherapy

We further investigated the effect of miR-21 on the radiosensitivity of HT-29 cells. Based on the above results we speculated that upregulation of miR-21 could inhibit the sensitivity of HT-29 cells to irradiation, while downregulation of miR-21 could significantly increase the sensitivity of HT-29. Indeed, this was the case as shown in (Fig. 3B). Therefore, the expression of miR-21 could affect radiosensitivity of HT-29 cells.

3.6. The hMSH2 3′-UTR is a target for miR-21

We then searched for the potential target of miR-21 by TargetScan and Miranda. These two programs predicted hMSH2 as one of the prime targets of miR-21. In HT-29 cells, hMSH2 could be regulated negatively by miR-21 at both transcriptional and protein levels (Fig. 4 A and B). Next, we performed a luciferase reporter assay and observed a significant decrease or increase in luciferase activity in the presence of pre-miR-21 or miR-ASO. In addition, to validate whether hMSH2 is a direct target of miR-21, we mutated the miR-21 binding site that was located in the 3′-UTR of hMSH2, and this mutant reporter was no longer responsive to this miRNA (Fig. 3C). These results indicate that hMSH2 is a direct target of miR-21.

3.7. miR-21 indirectly target TP and DPD

At last, we determined the effect of miR-21 on the expression of TS, TP and DPD in HT-29 cells. The results showed that miR-21-ASO increases the expression of TS mRNA (1.5-fold), TP mRNA (7.87-fold) and DPD mRNA (4.9-fold). Moreover, pre-miR-21 decreased the expression of DPD mRNA (0.5-fold) and had no effect on TP mRNA and TS mRNA. Those results suggest that miR-21 presents a negative regulator of TP and DPD. It may be partly involved in regulation of the expression of these proteins at transcriptional

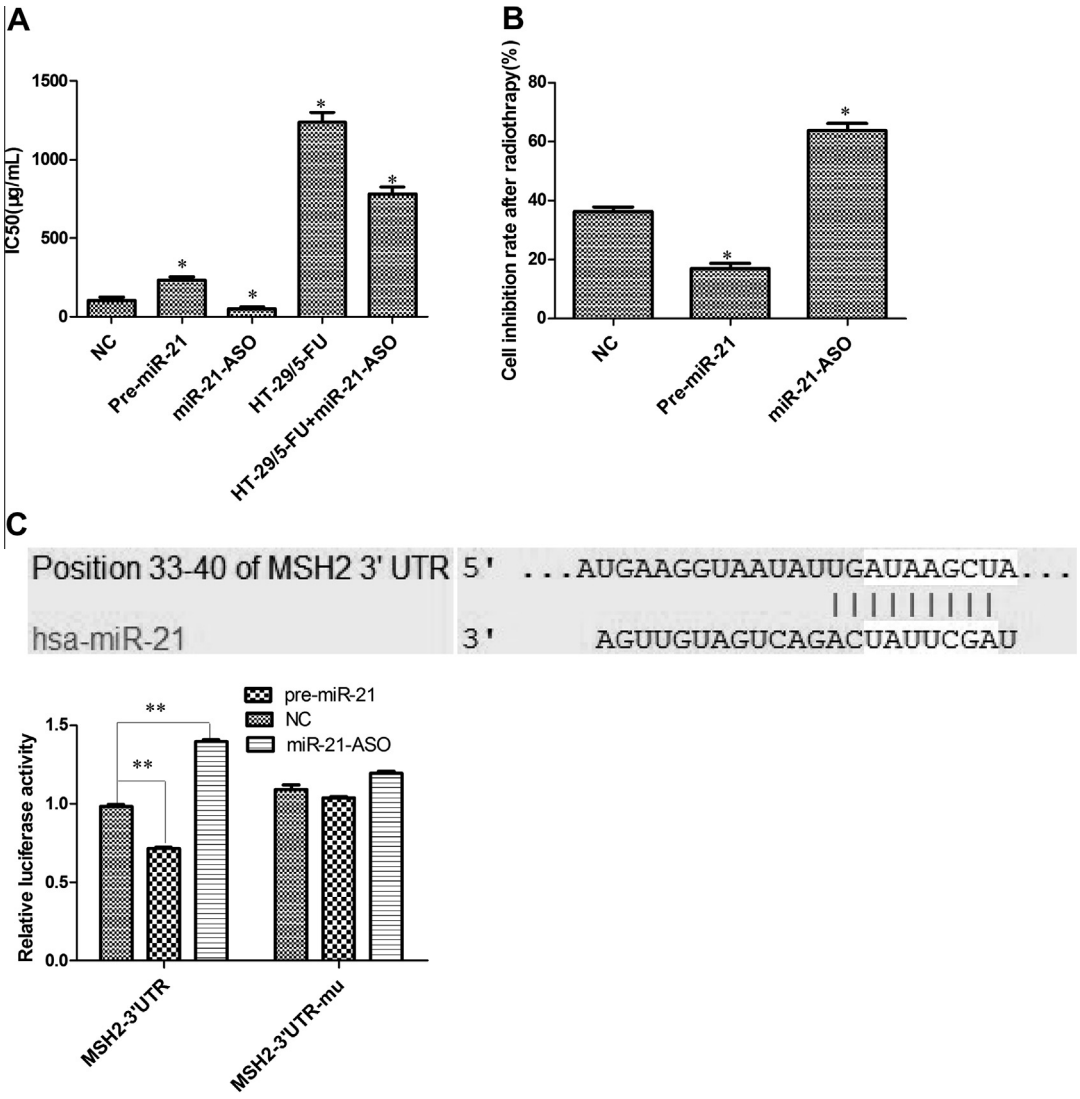

Fig. 3. (A) Effect of miR-21 on the sensitivity of HT-29 cells to 5-FU chemotherapy. The total 5-FU concentration was set up to 9 different groups, each group was 6.25, 12.5, 25, 50, 100, 200, 400, 600, 800 (μl/ml). We used the SPSS 18.0 to calculate the IC50 of each group (*$P < 0.05$, independent t test). (B) Effect of miR-21 on the sensitivity of HT-29 cell lines to radiotherapy. Each group of cells was treated with dose of irradiation and the inhibition ratio of irradiation was detected by MTT assays (*$P < 0.05$, independent t test). (C) The hMSH2 3′-UTR is a target for miR-21 in HT-29 colon cancer cells. Putative miR-21-binding sequence in the 3′-UTR of hMSH2 mRNA. Mutation was generated on the hMSH2 3′-UTR sequence in the complementary site for the seed region of miR-21. Analysis of luciferase activity (**$P < 0.01$, independent t test).

levels. Furthermore, we found that miR-21 had no significant effect on the expression of TS, but might influence the expression level of TP to some extent. In addition, increasing the expression of miR-21 could inhibit the expression of DPD protein, but inhibition of miR-21 expression had no effect on DPD at protein level (Fig. 4A and B). To our knowledge, it is the first time to verify that miR-21 can indirectly target TP and DPD.

4. Discussion

MiR-21 is an oncomiR in human cancer, several studies have shown that it is significantly upregulated in many human malignancies, including lung cancer [19], hepatocellular cancer [18], glioma [20], colorectal cancer [15] and so on. A number of studies also indicate that miR-21 plays a very important role in tumor cell proliferation, apoptosis and invasion. In consistence with these studies, here we showed that miR-21 can promote cell proliferation and inhibit apoptosis in colon cancer HT-29 cells. In this study, we also found that the tumor suppressor hMSH2 was negatively regulated by miR-21. Moreover, it was the first time that we show that miR-21 targets TP and DPD indirectly. Thus, miR-21 may be involved in 5-FU resistance of colon cancer by regulating the expression of hMSH2, TP and DPD.

5-Fu primary and secondary drug resistances have been the bottleneck in improving the efficacy of chemotherapy. miR-21 has been considered to function as an oncogenic molecule and may play a role in this drug resistance. Several reports suggest that miR-21 is one of the key miRNAs which plays a broad role in sensitivity to chemotherapeutic agents. A study [21] showed that co-treatment of breast cells with topotecan and anti-miR-21 can sensitize the tumor cells to anticancer agents. This was partly due to the downregulation of Bcl-2 expression by this miRNA. Recently, one report showed that miR-21 overexpression contributes to trastuzumab resistance in $HER2^+$ breast cancers. Since PTEN is a direct target of miR-21, loss of PTEN function in $HER2^+$ cancer cells led to trastuzumab resistance by enhancing downstream PI3K/AKT phosphorylation [22]. Similarly, another report also found that miR-21 modulates gemcitabine-induced apoptosis by PTEN-dependent activation of PI3K signaling [23], and activation of PI3K could upregulate MDR-1 expression. Silencing miR-21

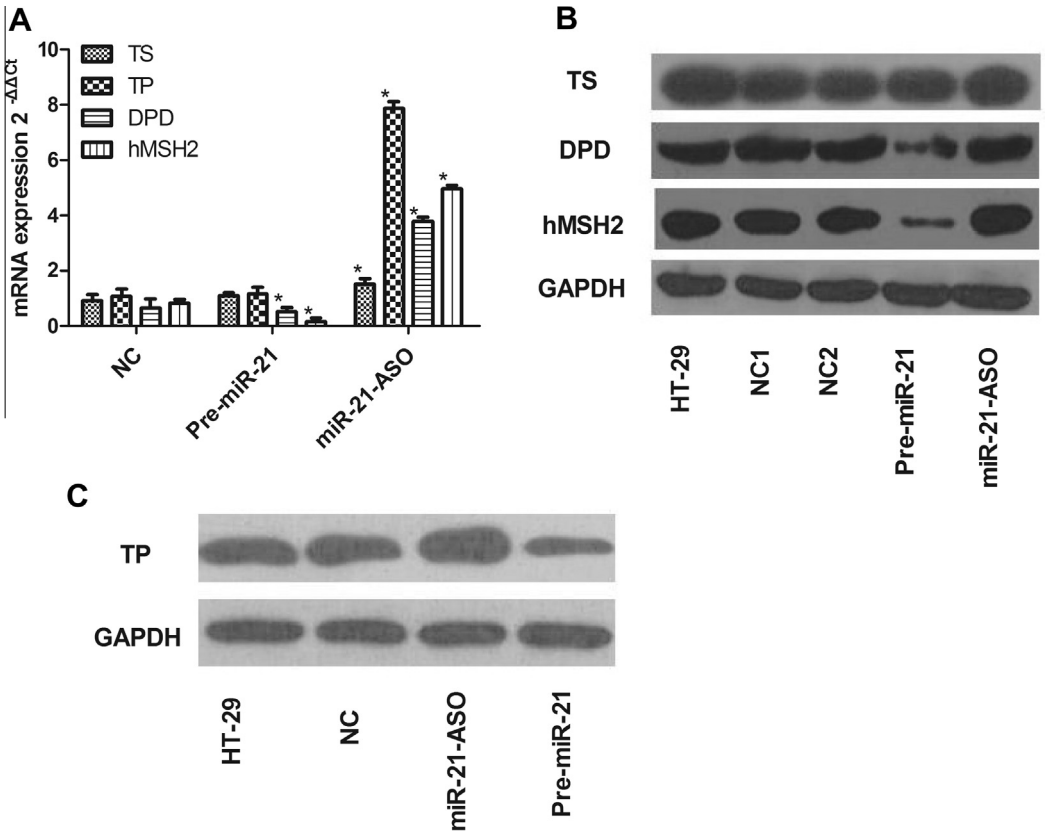

Fig. 4. miR-21 regulates the expression of mRNA and protein levels of TS, TP, DPD, and hMSH2. (A) Analysis of expression levels of endogenous TS, TP, DPD and hMSH2 mRNA in HT-29 colon cancer cells infected with miR-21 ASO, pre-miR-21 lentiviral vector and the nonsense sequence lentiviral vector (NC) by using qRT-PCR (*$P < 0.05$, independent *t* test). (B and C) Analysis of expression levels of endogenous TS, TP, DPD and hMSH2 protein in HT-29 colon cancer cells infected with miR-21 ASO, pre-miR-21 lentiviral vector and two negative control (NC1, NC2) by western blot.

expression could sensitize the cells to docetaxel by directly targeting PDCD4 [24]. miR-21 was a potentially suitable marker for predicting the response of the IFN-α/5-FU combination therapy in HCC. Inhibition of miR-21 could improve HCC cells sensitive to IFN-α/5-FU, whereas such sensitivity was weakened by transfection of siRNAs of target molecules, including PTEN and PDCD4 [18]. Therefore, those studies provide part of molecular mechanisms for how miR-21 contributes to drug resistance. miR-21 may play an important role in multi-drug resistance by regulating cell proliferation, cell cycle, apoptosis, and DNA repair.

The MMR system is necessary for the maintenance of genomic stability. Mismatch binding initiates subsequent events, including cleavage and excision of the error-containing strand followed by new synthesis and ligation [25]. MMR gene mutation increased the risk of a wide variety of cancers, especially in colon cancer [26]. MMR protein deficiency causes resistance to the cytotoxic effects of certain DNA-damaging agents, such as 5-FU and cisplatin [4,5,27]. Valeri et al. [28] determined that miR-21 appeared to directly target the 3′-UTR of both the hMSH2 and hMSH6 mRNAs, and correlated to 5-FU drug resistance in colorectal tumors. We further validated this in HT-29 cancer cells. We also found that miR-21 could indirectly alter the expression of TP and DPD. TP catalyzes the conversion from the prodrug of 5-FU to 5-FU, which is active in cells. We found that downregulation of miR-21 could markedly increase the expression of TP. Hence, we propose that miR-21 may increase 5-FU resistance by regulating the expression of TP. A number of studies showed that DPD activity affects the resistance to 5-FU [29,30]. However, we found that the increased expression of miR-21 could inhibit the expression of DPD, while downregulation of miR-21 could increase the DPD expression. These results verify that miR-21 could regulate DPD expression, leading to the increase of 5-FU resistance. Of note, this different from previous that suggest that lower expression of DPD increases the sensitivity to 5-FU. Our study provides a new piece of evidence supporting the involvement of DPD in miR-21-caused 5-FU resistance, though it still remains unclear how miR-21 regulates TP and DPD.

In conclusion, we have shown that miR-21 plays a significant role in proliferation, apoptosis, invasion and the response to 5-Fu and radiotherapy in HT-29 cancer cells. Moreover, this role might be attributed to targeting of hMSH2 as well as TP and DPD via miR-21 targeted hMSH2 and indirectly targeted TP and DPD to influence 5-FU chemotherapy sensitivity. Our finding suggests miR-21 can be a potentially useful marker for prediction of the clinical response to 5-FU therapy, and miR-21 may be a potential target for colorectal cancer therapy.

Acknowledgments

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