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Original Article

The expressions of miR-200a and miR-21 in colorectal cancer tissues and their effects on the biological functions of HCT116 cells

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Abstract: Objective: To explore the expressions of miR-200a and miR-21 in colorectal cancer tissues and their effects on the biological functions of human colorectal cancer HCT116 cells. Methods: 51 patients with colorectal cancer who underwent surgical resection were enrolled in this study. 51 specimens of colorectal cancer tissues and 51 specimens of tumor-adjacent tissues were collected from the patients. The miR-200a and miR-21 expression levels in the cancer tissues and in the corresponding tumor-adjacent tissues were quantified using a quantitative real time polymerase chain reaction (qRT-PCR). The expression vectors of miR-200a and miR-21 were established, and after they were transfected with HCT116 cells, their proliferation, invasion, and apoptosis abilities were determined using a cell counting kit-8 (CCK8) assay, a Transwell in vitro invasion assay, and an apoptosis experiment using flow cytometry, respectively. Results: The expression levels of miR-200a and miR-21 in the colorectal cancer tissues were significantly higher than the levels in the adjacent tissues ($P<0.001$). From 24 h to 72 h, the miR-200a inhibitor group and the miR-21 inhibitor group showed a gradual decline in cell proliferation activity ($P<0.001$). At 48 h and 72 h, the miR-200a inhibitor group and the miR-21 inhibitor group showed significantly lower cell proliferation activity than the NC and blank control groups (all $P<0.001$). The miR-200a inhibitor group and the miR-21 inhibitor group showed a significantly lower number of invasive cells than the blank and NC groups (all $P<0.001$). The miR-200a inhibitor group and the miR-21 inhibitor group showed no significant differences in their cell apoptosis rates ($P>0.05$), but their cell apoptosis rates were significantly higher than the rates in the NC and blank groups (all $P>0.05$). Conclusion: miR-200a and miR-21 are highly expressed in colorectal cancer tissues. Inhibiting the expressions of miR-200a and miR-21 can suppress the proliferation and invasion of human colorectal cancer HCT116 cells and promote their apoptosis.

Keywords: miR-200a, miR-21, human colorectal cancer HCT116 cells, apoptosis

Introduction

Colorectal cancer, namely colon cancer, is a common malignant gastrointestinal cancer in the digestive system. Its early symptoms are not obvious. The incidence of colon cancer is affected by factors such as bad dietary habits, and it is increasing annually [1-3]. At present, colorectal cancer is mainly treated with surgical excision, radiotherapy, comprehensive radiotherapy, radiotherapy alone, chemotherapy, and immunotherapy [4-7]. Relevant studies have found that the 5-year survival rate of patients with advanced colorectal cancer and colorectal cancer metastasis was only 15%, and the survival time of most patients with early colorectal cancer can be prolonged through radical colorectal cancer operations, but a large number of patients still suffer a recurrence of colorectal cancer or a poor prognosis after surgery [8]. An analysis of the occurrence and development mechanisms of human colorectal cancer is an important part of colorectal cancer research [9].

A related in-depth study of miRNAs in the pathogenesis of cancer itself found that microRNAs (miRNAs) are endogenous non-coding single-stranded RNAs with cancer promotion and inhibition functions [10, 11]. The current study

Table 1. The primer sequences of miR-200a and miR-21 and their internal references

Group	Upstream primer	Downstream primer
miR-200a	5'-CCTACGCACAATTAACAAG CC-3'	5'-GCCGTCTA ACACTGTCTGGTA-3'
GAPDH	5'-TATGTCGTGGAGTCTACTGGT-3'	5'-GAGTTGTCATATTTCTCGTGG-3'
miR-21	5'-GCGGCGGTAGCTTATCAGACTG-3'	5'-ATCCAGTGCAGGGTCCGAGG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

revealed that miRNAs play a role in cancer cell inhibition and promotion in different solid tumors. For example, the expression of miR-200a in hepatocellular carcinoma tissues is significantly lower than it is in tumor-adjacent tissues, and its up-regulation suppresses the cancer cell proliferation of hepatocellular carcinoma [12, 13]. Some researchers have confirmed that the expression of miR-21 in prostate cancer, breast cancer, and other cancer tissues is significantly higher than it is in their tumor-adjacent tissues [14]. However, the correlation of the biological characteristics of human colorectal cancer HCT116 cells with miR-200a and miR-21 and the specific effects of the changes in miR-200a and miR-21 expressions on the biological characteristics of human colorectal cancer HCT116 cells are not clear, so we carried out this experimental study of the characteristics of miR-200a and miR-21 expressions in colorectal cancer and their effects on the biological characteristics of human colorectal cancer HCT116 cells, so as to provide a new theoretical basis for the diagnosis and treatment of colorectal cancer in the field of molecular biology.

Materials and methods

Data collection

A total of 51 patients (35 males and 16 females) with a mean age of (50.18±17.34) years admitted to our hospital for surgical resections were enrolled in the study, and 51 specimens of their cancer tissues and corresponding tumor-adjacent tissues were taken during surgery. Inclusion criteria: patients with normal liver and kidney functions, no other malignant tumors, and the tissue sections were diagnosed with colorectal cancer tissues through pathology [15]. All the specimens were placed in liquid nitrogen immediately after the excision. Patients who had undergone chemotherapy, immunotherapy, or radiation therapy before the surgery were excluded. The study was approved by the ethics committee of our hospital, and the patients and their families were informed before the study and signed informed consents.

Main reagents, instruments, and test methods

Main reagents and instruments

Human colorectal cancer HCT116 cells (Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences), Trizol reagent (Invitrogen, USA), a qRT-PCR kit and minScript reverse transcription kit (TaKaRa, Dalian), HBS-1096A enzyme micro-plate reader (Nanjing Detie Experimental Equipment Co., Ltd.), a real-time quantitative PCR system (BioRad, USA), Dulbecco's modified eagle medium (DMEM) (Gibco, USA), fetal bovine serum (FBS), trypsin (Hyclone, USA), a CCK8 kit (Bei Jing Think-Far Technology Co., Ltd.), a Transwell chamber (BD Company, USA), a CyFlow Cube 8 flow cytometer (Partec, Germany), primer sequences of miR-200a, miR-21, internal references including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6, and an miRNA negative control (synthetically designed by Shanghai GenePharma Co., Ltd.). More details are shown in **Table 1**.

Determining the expressions of miR-200a and miR-21

The expressions of miR-200a and miR-21 in colorectal cancer tissues and tumor-adjacent tissues were determined using qRT-PCR. The total RNA was extracted from the tissues according to the Trizol reagent's operating instructions, dissolved in 20 μL of diethylpyrocarbonate (DEPC) water, and then reverse transcribed using a reverse transcription kit. The reaction system was as follows: 1 μl of Moloney murine leukemia virus (M-MLV), 1 μl of Olig (dT), 0.5 μl of RNasin inhibitor, 1 μl of nucleoside triphosphates (NTPs), and RNA-free water (added to make up to 15 μl in total). This was incubated at 38°C for 60 min. cDNA (1 μl) was taken and synthesized at 85°C for 5 s. The synthesized cDNA was used as a template for the qRT-PCR amplification; the PCR reaction system was pre-

pared as follows: 2.5 μl of 10 × PCR buffer, 1 μl of dNTPs, 1 μl of upstream and downstream primers, respectively, 0.25 μl of Taq DNA Polymerase, and ddH_2O (added to made up to 25 μl in total). The reaction conditions were as follows: Pre-denaturation at 95°C for 15 min, denaturation at 95°C for 15 s and annealing at 60°C for 30 min, for a total of 35 cycles, and then extension at 72°C for 15 min. Three replicate wells were set for each sample for three repeated experiments, and GAPDH was taken as an internal reference for the miR-200a, and U6 for the miR-21. After the reaction, the amplification and dissociation curves of real-time PCR were confirmed, and the relative quantity of the target gene was calculated based on the resulting parameters. The relative quantification of the target gene was calculated using 2-ΔCt.

Cell culture and transfection

Human colorectal cancer HCT116 cells were placed in a medium containing 10% phosphate buffered saline and Dulbecco's Modified Eagle Medium (PBS DMEM), then transfected in a CO_2 incubator at 37°C to promote cell growth and fusion up to 50%, mixed with pancreatin for digestion, and then cultured in the medium continually after the digestion to complete the passage. Cells at the logarithmic phase were grouped and then transfected. The cells not transfected were divided into a blank group, a negative RNA control group (NC group), an miR-200a inhibitor group, and an miR-21 inhibitor group. Lipofectamine 2000 and DNA were diluted and mixed according to the Lipofectamine 2000 kit's instructions (Shanghai Ke Min Biological Science and Technology; item number: 11668-019), and NC, the miR-21 inhibitor and the miR-200a inhibitor were transfected into human colorectal cancer HCT116 cells using the liposome Lipofectamine 2000, then incubated at room temperature for 5 min. Finally, the mixture and the cells were mixed well, transfected under CO_2 at 37°C, and the expression of the miR-200a in the human colorectal cancer HCT116 cells transfected with miR-200a, miR-200a-NC, miR-21, and miR-21-NC was determined using qRT-PCR at 48 h after the transfection.

Cell proliferation determination using the CCK-8 assay

The colorectal cancer HCT116 cells of each group were inoculated in a 96-well plate at 100 μl of cells per well at 48 h after the transfection and diluted to 4×10^3 cells/ml after the pancreatic digestion. Then the culture plate was placed in a cell incubator for 24 h, and the original culture medium was taken out and discarded, and then NC, the miR-200a inhibitor and the miR-21 inhibitor were added. The cells that were transfected for 48 h were collected, diluted to 2×10^4 cells/ml, inoculated into a 96-well plate at 100 μl of cells per each well and cultured at 37°C under 5% CO_2. Then we added 10 μl of CCK8 solution to each well at 24 h, 48 h, and 72 h after the cells attached to the wall and sprawled out, respectively, and then we cultured them continually in an incubator with the reagent. After 1 h, the optical density was determined at 450 nm using an enzyme mark instrument to detect cell proliferation, and the experiment was repeated three times.

Detection of the in vitro invasion ability of the cells using a Transwell chamber

First, Trypsin digestive cells were centrifuged for 5 min, and the culture medium was discarded. The cells were washed twice with phosphate buffer saline (PBS), and then resuspended in a serum-free medium containing bull serum albumin (BSA) to adjust their density to 5×10^4/ml. 1 mL of a medium containing fetal calf serum (FBS) was added to the lower chamber of a 6-well plate. 2 ml of cell suspension was added to the Transwell chamber. The removed chamber was washed with PBS after removing the supernatant with a cotton swab. The cells in the lower chamber were fixed with a 70% ethanol solution for 30 min, taken out, washed with PBS again, and then stained with 0.1% crystal violet. The invasion of the cells in 5 randomly selected fields was detected with a microscope after the staining, and their average value was calculated. The experiment was repeated three times.

Cell apoptosis detection of each group by flow cytometry

The cells treated with miR-200a, miR-21, and NC for 48 h after digestion with trypsin were collected, fixed with 75% ethanol at 20°C for 24 h, and then centrifuged at a constant temperature of 4°C and 3000 rpm for 5 min, and then the ethanol was discarded. Then the cells were washed with PBS, centrifuged at a constant temperature of 4°C and at 3000 rpm for 5 min, and the supernatant was discarded. Then 500

Table 2. General patient data

Group	[n (%)]
Age (Y)	
≤50	20 (39.22)
>50	31 (60.78)
Gender	
Male	35 (68.63)
Female	16 (31.37)
Tumor size (cm)	
>1-≤3	13 (25.49)
>3-≤5	26 (50.98)
>5	12 (23.53)
Invasion degree	
Tumor invading to the submucosa	8 (15.69)
Tumor invading to the muscular layer	19 (37.25)
Tumor penetrating the muscle layer to the serous layer	14 (27.45)
Tumor penetrating beyond the serous layer	10 (19.61)
Differentiation	
High	11 (21.59)
Medium	27 (52.94)
Low	13 (25.49)
Lymph node metastasis	
Yes	25 (49.02)
No	26 (50.98)
TNM stage	
I	10 (19.61)
II	16 (31.37)
III	12 (23.53)
IV	13 (25.49)

Table 3. The expressions of miR-200a and miR-21 in colorectal cancer tissues and adjacent tissues

Group	Colorectal cancer tissues	Colorectal cancer tissues	t	P
miR-200a	4.18±0.22	1.02±0.03	101.6	<0.001
miR-21	3.21±0.14	1.28±0.06	90.490	<0.001

ul of DNA staining solution was added to the cells and the solution was mixed well. The prepared solution was transferred to a flow tube, incubated on ice for 30 min in the dark and measured with a CyFlow Cube 8 flow cytometer.

Statistical analysis

SPSS 17.0 software was adopted for the statistical analysis. The enumeration data were expressed as the number of cases/percentage [n (%)], and the comparisons between two groups were subjected to χ^2 tests. The measurement data were expressed as the means standard deviations ($\bar{x} \pm$ sd), and the comparisons within groups were subject to a *t* test or a F test. P<0.05 indicated a significant difference.

Results

General patient patients

The patients' ages, genders, tumor sizes, degrees of invasion, degrees of differentiation, lymph node metastasis, and TNM staging are shown in **Table 2**. They exhibited no significant differences.

The expressions of miR-200a and miR-21 in the colorectal cancer tissues and the tumor-adjacent tissues

The expressions of miR-200a in the colorectal cancer tissues and the tumor-adjacent tissues were (4.18±0.22) and (1.02±0.03), respectively, and the expressions of miR-21 in the colorectal cancer tissues and the tumor-adjacent tissues were (3.21±0.14) and (1.28±0.06), respectively. The comparisons between the two groups indicated that the colorectal cancer tissues showed significantly higher expressions of miR-200a and significantly lower expressions of miR-21 than the tumor-adjacent tissues did (both P<0.001) (**Table 3**).

The relative expressions of miR-200a and miR-21 in each group's cells after the transfection

The expressions of miR-200a in the miR-200a inhibitor group, the NC group, and the blank group were (1.01±0.02), (4.16±0.18) and (4.09±0.21), respectively, so the miR-200a inhibitor group showed a significantly lower expression of miR-200a than the NC group and the blank group (both P<0.001). The expressions of miR-21 in the miR-21 inhibitor group, the NC group, and the blank group were (1.24±0.05), (3.17±0.13) and (3.15±0.12), so

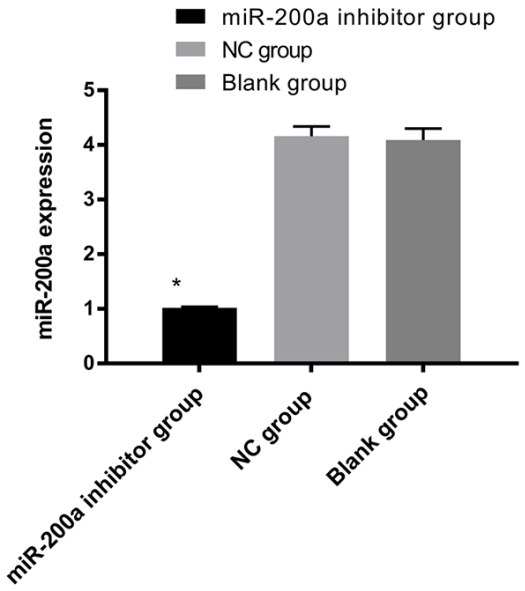

Figure 1. The relative expressions of miR-200a in each group's cells after the transfection. * indicated that the expression of miR-200a in the miR-200a inhibitor group was significantly lower than it was in the NC and blank groups (both P<0.001) and the NC and blank groups had no significant differences in terms of expression (P>0.05).

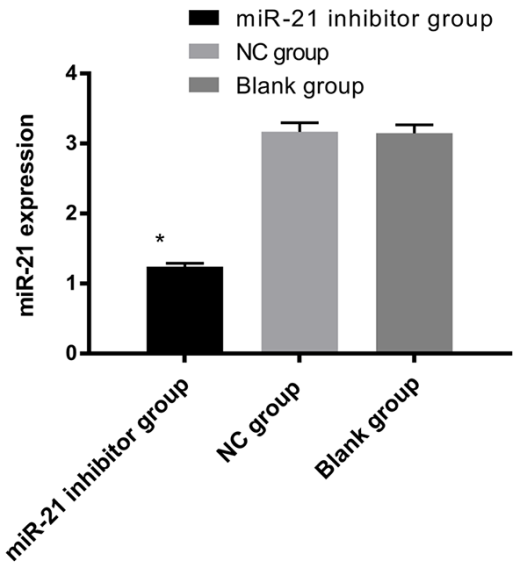

Figure 2. Relative expressions of miR-21 in the each group's cells after the transfection. * indicated that the expression of miR-21 in the miR-21 inhibitor group was significantly lower than it was in the NC and blank groups (both P<0.001) and the NC group and blank group had no significant difference in terms of expression (P>0.05).

the miR-21 inhibitor group showed a significantly lower expression of miR-21 than the NC group and the blank group (both P<0.001), and the NC group and the blank group showed no significant difference in terms of the expression level (P>0.05) (**Figures 1** and **2**).

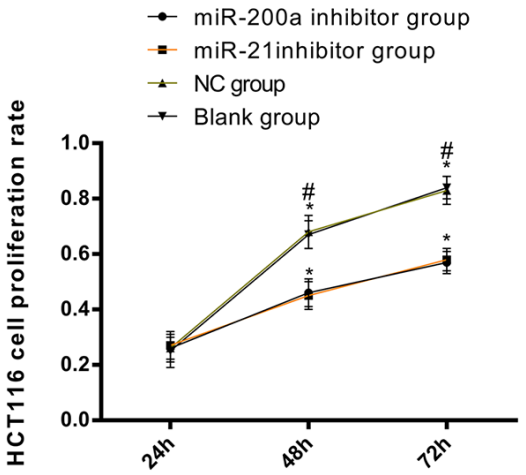

Figure 3. Comparisons between groups in the cell proliferation ability of human colorectal cancer HCT116 cells. The comparisons within the groups indicated that the miR-200a inhibitor group and the miR-21 inhibitor group showed a gradual decline in cell proliferation activity from 24 h to 72 h. * indicated that the comparison within groups in terms of cell proliferation activity at the different time points showed no significant differences (P<0.001); # indicated that at 48 h and 72 h, the miR-200a inhibitor group and the miR-21 inhibitor group showed significantly lower cell proliferation activity than the NC and blank control groups (all P<0.001), and the NC and blank groups had no significant difference in terms of cell proliferation activity (P>0.05).

Comparisons between the groups in terms of the cell proliferation ability of the human colorectal cancer HCT116 cells

The comparisons within the groups indicated that from 24 h to 72 h, the miR-200a inhibitor group and the miR-21 inhibitor group showed a gradual decline in cell proliferation activity, and the comparisons within the groups at the different time points showed no significant differences (P<0.001). At 24 h, the miR-200a inhibitor group, the NC group, and the blank control group showed no significant differences in cell proliferation activity (all P>0.05). At 48 h and 72 h, the miR-200a inhibitor group and the miR-21 inhibitor group showed significantly lower cell proliferation activity than the NC and blank control groups did (all P<0.001), and the NC and the blank groups had no significant differences (P>0.05) (**Figure 3**).

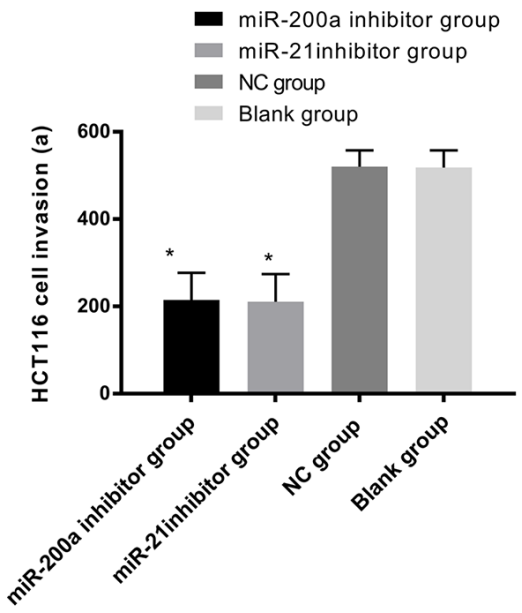

Figure 4. Comparison between the groups in the invasion of human colorectal cancer HCT116 cells. * indicated that the miR-200a inhibitor group and miR-21 inhibitor group showed a significantly lower number of invasive cells than the blank and NC groups (all P<0.001).

Comparison between the groups in the invasion of human colorectal cancer HCT116 cells

The numbers of invasive cells in the miR-200a inhibitor group, the miR-21 inhibitor group, the NC group and the blank control group were (214.86±62.42), (210.92±63.51), (520.20±37.74), and (518.39±39.30), respectively, so the miR-200a inhibitor group and miR-21 inhibitor group showed a significantly lower number of invasive cells than the blank and NC groups did (P<0.001), but there was no significant difference between the miR-200a inhibitor and miR-21 inhibitor groups, or between the blank and NC groups (both P>0.05) (**Figure 4**).

Comparison between the groups in the apoptosis ability of human colorectal cancer HCT116 cells

The cell apoptosis rates of the miR-200a inhibitor group and the miR-21 inhibitor group were (18.23±3.01)% and (19.74±3.46)%, respectively, so the miR-200a inhibitor group and the miR-21 inhibitor group showed no significant differences in terms of the cell apoptosis rate (P>0.05), but they showed a significantly higher cell apoptosis rate than the NC ((3.13±0.21)%) and blank groups ((3.49±0.45)%) (both P< 0.05). The NC and blank groups had no significant difference in their apoptosis rates (P> 0.05) (**Table 4**; **Figure 5**).

Discussion

Changes in biological functions such as tumor cell growth and apoptosis are closely related to the occurrence and development of tumors [16]. In recent years, with an increase in the incidence and mortality of colorectal cancer, health factors leading to colorectal cancer have been extensively studied in clinical research [17-19].

In this study, the expressions of miR-200a and miR-21 in colorectal cancer tissues and tumor-adjacent tissues were determined, and it was found that their expressions in the colorectal cancer tissues were significantly higher than those in the tumor-adjacent tissues. Both miR-200a and miR-21 act as important regulatory factors of cancer progression. Related studies found that miR-200a was down-regulated in hepatocellular carcinoma, and an overexpression of miR-200a can inhibit the proliferation of hepatocellular carcinoma [20]. Another study pointed out that miR-200a was up-regulated in the serum of patients with colorectal cancer, and miR-21 was highly expressed in the serum and cancer tissues of patients with colorectal cancer [21]. Therefore, we thought that both miR-200a and miR-21 were up-regulated in colorectal cancer tissues, and appropriately regulating the expression of miRNA may affect the biological ability of colorectal cancer cells. Then the relative expressions of miR-200a and miR-21 in the cells of each group after the transfection were observed, and the proliferation of the human colorectal cancer HCT116 cells in each group after the transfection was compared after making the expression of miR-200a in the miR-200a inhibitor and the expression of miR-21 in the miR-21 inhibitor group were significantly lower than those of the NC and blank groups. Our data revealed that from 24 h to 72 h, the miR-200a inhibitor group and miR-21 inhibitor group showed a gradual decline in cell proliferation activity; at 24 h, the miR-200a inhibitor group, the NC group, and the blank control group had no significant differences in cell proliferation activity; at 48 h and 72 h, the miR-200a inhibitor group and the

Table 4. Comparisons between the groups in terms of their cell apoptosis rates (%)

Group	miR-200a inhibitor group	miR-21 inhibitor group	NC group	Blank group	F	P
Cell apoptosis rate (%)	18.23±3.01	19.74±3.46	3.13±0.21	3.49±0.45	1803.000	<0.001

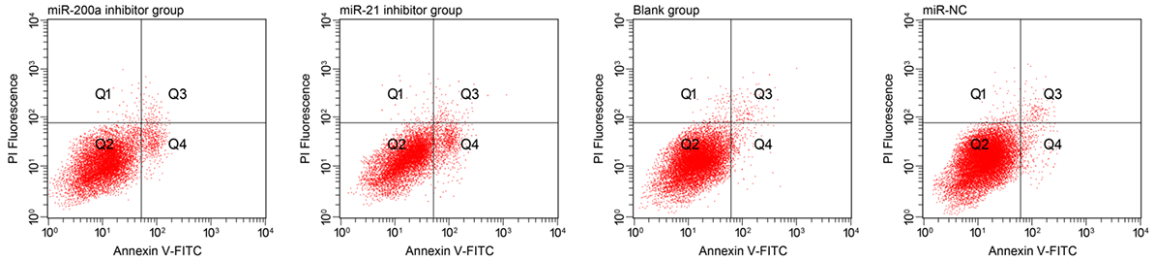

Figure 5. Comparison between the groups in terms of the cell apoptosis rate (%). The miR-200a inhibitor group and miR-21 inhibitor group showed no significant differences in terms of their cell apoptosis rates (P>0.05), but they showed significantly higher cell apoptosis rates than the NC and blank groups (both P<0.05).

miR-21 inhibitor group showed significantly lower cell proliferation activity than the NC group and the blank control group, and the NC group and the blank group had no significant difference in cell proliferation activity. A recent study about the targeted regulation of miRNA expression found that the expression of miR-200a was down-regulated in vulvar squamous cell carcinoma, but the proliferation of vulvar squamous cell carcinoma can be effectively suppressed if the miR-200a is over-expressed and taken as a cancer cell inhibitor [22]. The abnormal up-regulation of miR-21 in colorectal cancer cells is an important reason for the proliferation promotion of colorectal cancer cells [23]. Based on the results of this study, we believe that the inhibition of miR-200a and miR-21 in human colorectal cancer HCT116 cells can effectively inhibit the proliferation of colorectal cancer cells. Finally, the invasion and apoptosis of human colorectal cancer HCT116 cells in each group were observed, and it was found that the miR-200a inhibitor group and the miR-21 inhibitor group showed a significantly lower number of invasive cells than the blank and NC groups, but the miR-200a inhibitor group and the miR-21 inhibitor group showed no significant difference in their cell apoptosis rates, and they showed a significantly higher cell apoptosis rate than the NC and blank groups did. It is very important to control the number of invading cancer cells in alleviating patients' disease, because the invasion and metastasis of cancer cells are often the main causes of death [24, 25]. If the number of cancer cells is controlled and their apoptosis rate is accelerated, the continued deterioration from the cancer has been relieved to a certain extent [26]. Studies on miRNAs and cancer cells have revealed that the number of invasive cells and the amplitude of cancer cell apoptosis can be significantly increased by the overexpression or silencing of miRNAs [27]. Therefore, we believe that regulating miRNAs can affect the number of invasive cells and the apoptosis rate of human colorectal cancer HCT116 cells.

This study still has some shortcomings. For example, this study did not explore the detailed mechanisms of miR-200a and miR-21 on human colorectal cancer HCT116 cells, so it is hoped that more scholars will conduct more in-depth discussions. This experiment suggests that miR-200a and miR-21 are involved in the biological processes of human colorectal cancer HCT116 cells, or they may serve as diagnostic markers and therapeutic targets for colorectal cancer. Therefore, we will detect miRNAs in different types of colorectal cancer cells at a later stage and continue to explain the relationship between miR-200a and miR-21 in other pathological types of colorectal cancer cells.

To sum up, miR-200a and miR-21 are highly expressed in colorectal cancer tissues, and down-regulating the expressions of miR-200a and miR-21 can suppress the proliferation and invasion of human colorectal cancer HCT116 cells and promote their apoptosis.

Disclosure of conflict of interest

None.

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