Effect of MicroRNA-149 on Proliferation and Apoptosis of SW480 Cells and Preliminary Molecular Mechanism Study

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Abstract

Objective: Colon cancer is a common malignant tumor in today's society. The incidence of colon cancer in China ranks third in the world. Colon cancer is mainly treated with surgery and chemotherapy now. For patients with early colon cancer, the survival rate of post operation is higher. However, for patients with colon cancer who have already had tumor metastasis, a higher mortality rate will occur if they were treated by operation. In recent years, the targeting of microRNAs (miRNAs) in tumorigenesis has received great attention. miR-149 has also been found to be involved in various diseases such as breast cancer and lung cancer. This study has designed the miR-149 mimics of miR-149 and explored the effects of miR-149 on various biological behaviors in colon cancer SW480 cells, which provided a new idea for the early detection and treatment of colon cancer. Methods: The miR-149 mimics and NC sequences were designed and synthesized, and the human colon cancer SW480 cells were transfected in this experiment. After 24 hours, the expression levels of mRNA in SW480 cells were detected by qPCR. After 24, 48 and 72 hours of transfection, the effect of miR-149 on the proliferation of SW480 cells was detected by CCK8 method. The effects of miR-149 on apoptosis of SW480 cells were detected by flow cytometry after 24h and 48h transfection. The effect of miR-149 on cell migration and invasion was explored by Transwell experiments. Results: (1) The miR-149 mimics sequence which were designed and synthesized can promote the expression of mRNA in miR-149. (2) The experiment results of CCK8, Transwell and flow apoptosis showed that miR-149 can inhibit the proliferation of SW480 cells, reduce the migration and invasion of SW480 cells, and promote the apoptosis of SW480 cells. Conclusion: (1) The miR-149 mimics sequence which were designed and synthesized can promote the expression of mRNA in miR-149. (2) miR-149 can inhibit the proliferation, migration and invasion of SW480 cells and promote the apoptosis of SW480 cells.

Keywords

MiR-149; Transfection; Colon cancer; Apoptosis.

1. Introduction

1.1 Colon cancer

1.1.1 Malignancy

Malignant tumor, usually also known as cancer, is a major disease that can pose a serious threat or even life-threatening to human health. Affected by many factors such as people's daily poor dietary structure, living habits and deterioration of the environment in which they live, the incidence of malignant tumors is increasing year by year. Malignant tumors have become one of the most important causes of death in humans and are the main factor to reduce human life span. The formation of cancer is a multi-step process, in the process of development tumors will slowly have six biological capabilities, and eventually become cancer. These features include: maintenance of proliferative signals, evasion of growth inhibitory factors, resistance to cell death, immortalization of replication, induction of angiogenesis, and activation of invasion and metastasis. Cancer cells have the ability to acquire and stably maintain proliferation signals through different pathways, and they themselves can produce growth factor ligands that bind to ligands through the expression of cognate receptors, thereby stimulating their own production of proliferation. In addition, cancer cells have the potential to emit stimulating signals that stimulate normal cells within the supporting tumor-associated stroma

that provide various growth factors to cancer cells[1] .Cancer cells have a significant feature, which is the ability to continuously grow and proliferate, thereby forming tumor masses[2]. The role of proto-oncogenes and tumor suppressor genes in this process is critical.

Proto-oncogenes are highly evolutionarily conserved genes closely related to cell proliferation and essential for maintaining the normal life activities of the body. If proto-oncogenes are abnormal in structure or regulatory regions, when the amount or activity of gene products is increased, cells will proliferate and accumulate in large numbers, which will lead to the formation of tumors. In the occurrence and development of malignant tumors also located in the colon, the activation of proto-oncogenes is a very important reason, in which it plays a crucial role. Nowadays proto-oncogenes known to be associated with colon cancer include C-scr, Ras family, and C-myc.

Tumor suppressor genes, also known as anti-oncogenes, can inhibit cell proliferation in the activated state and are present in normal cells, but sometimes this gene is inhibited or even absent, which will weaken or disappear the tumor suppressor effect. Under normal circumstances, tumor suppressor genes play an important role in the regulation of cell development, growth and differentiation. Nowadays, tumor suppressor genes that are definitely known to be related to colon cancer are APC, DCC, and MMR.

In recent years, most of the research on colon cancer has focused on the related pathogenesis and molecular mechanism of proto-oncogenes and tumor suppressor genes. Changes in its gene expression can be used as an effective colon cancer marker, which is important for early detection of colon cancer and identification of whether it is a clinical diagnosis such as malignant tumors.

1.1.2 Overview of Colon Cancer

Due to the high incidence, rapid progression, and easy resistance to chemotherapy of colon cancer, it has brought about a serious social and medical burden and attracted great public attention[3]. Colon cancer, the most common and dangerous form of cancer, is a heterogeneous disease that arises through the cumulative effects of genetic alterations and environmental factors. Genomic alterations deregulate conserved signaling pathways involved in cell metabolism, proliferation, differentiation, survival, and apoptosis. Colon cancer, as the name implies, is a cancer that occurs in the colon of the digestive tract and is more frequent at the junction of the rectum and sigmoid colon, with the highest incidence in the age group of 40 to 50 years, and the incidence is higher in men than in women, and the incidence of colon cancer ranks in the top three among gastrointestinal tumors[4]. With the gradual improvement of living standards of Chinese residents, dietary habits have also changed, the incidence of colorectal cancer is also increasing, and the number of deaths is increasing. Its incidence is increasing at a rate of 4.71% per year, much higher than the world average of 2%. There are more than 170,000 new cases per year, which has leapt to the third place of high incidence of malignant tumors. Especially in large cities, its increase is faster and greater. Statistics released by Shanghai Center for Disease Control and Prevention point out that the incidence of colorectal cancer in the city from 2003 to 2007 has ranked second in malignant tumors and fourth in mortality[5]. Therefore, it can be speculated that the incidence and mortality of colorectal cancer in China will continue to rise in the future, and it will become one of the very common malignant tumors with a rapid increase in incidence in China.

Colon cancer can be mainly divided into adenocarcinoma, mucinous adenocarcinoma and undifferentiated carcinoma. Its morphology mainly presents polypoid and ulcerated type. The tumor can grow circumferentially along the intestinal wall, develop longitudinally along the intestinal canal, and even penetrate the intestinal wall and infiltrate deeper. The routes of metastasis include: lymphatic metastasis, blood flow metastasis, local invasion, intraperitoneal implantation and spread and metastasis along the suture or incision surface. In recent years, with the continuous development of immunology, molecular biology and cytogenetics and other tumor-related disciplines, people's understanding of the mechanism of tumor development has been deepening, and their research points are also slowly shifting to the regulatory mechanism of small molecules in tumors. The occurrence

and development of colon cancer is a very complex and comprehensive process regulated by multiple factors.

Colon cancer, one of the most common malignancies today, has a poor prognosis with its treatment. Therefore, early detection, early diagnosis and treatment as soon as possible are important to reduce the mortality of colon cancer and improve the prognosis.

1.1.3 Development of colon cancer

In recent years, with the advancement of research, people's understanding of tumors is getting deeper and deeper, and various treatments are gradually applied in clinical practice[6]. The occurrence of colon cancer is a process regulated by multiple genes and divided into multiple steps. The traditional theory suggests that the process of tumor metastasis is simultaneously regulated by various different factors: when the initial metastatic mechanism is just initiated, epithelial cadherin (E-cadllerin), immunoglobulin (Ig) superfamily, selectins, integrins, etc. are involved in the detachment between tumor cells and the adhesion of tumor cells to the extracellular matrix; at this time, proteolytic enzymes and their inhibitors secreted by tumor cells or stromal cells are involved in the degradation of the basement membrane of extracellular matrix and blood vessels, and also play an important role; under the action of motility factors, growth factors, homing factors, etc., tumor cells move to the target organs; vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin (IL). The increase of angioproliferative factors such as platelet-derived factor (PDGF) promotes the formation of new blood vessels, has the nutrient supply of new blood vessels, a large number of tumor cells proliferate, and finally the tumor metastasizes to a new place and completes the metastatic process. Activation of tumor proteins or inactivation of tumor suppressor genes can affect colon cancer in multiple aspects such as cell proliferation, apoptosis, invasion and metastasis[7].

The occurrence of colon cancer is not regulated by a single gene, which is the result of the combined action of multiple genes, and colon cancer is triggered if there is an imbalance between related proto-oncogenes and tumor suppressor genes, continuous high expression of proto-oncogenes, and inactivation but no expression of tumor suppressor genes.

The growth and proliferation of colon cancer cells are closely related to vascular endothelial growth factor (VEGF) signaling pathway and epidermal growth factor receptor (EGFR) signaling pathway. Therapeutic agents have been developed to target these pathways, their activity is well-established, and they have been incorporated into routine cancer therapy worldwide. In the United States, 60% to 70% of patients with colon cancer will receive these drugs during the course of treatment[8].

Studies have pointed out that the occurrence of colon cancer is initially caused by pathological hyperplasia due to abnormal continuous stimulation of the intestinal mucosa, and the development process of colon cancer generally goes through the four stages of benign polyps - adenomas - colon cancer - distant metastasis of colon cancer. This process involves the abnormal function of a variety of proto-oncogenes and tumor suppressor genes, or the functional changes caused by the mutation or structural change of related genes[9].

1.1.4 Current status of treatment of colon cancer

At present, in clinical practice, surgical resection of tumor tissue is still the most common and relatively good treatment option for tumors during treatment, especially for patients with early detection of cancer, the survival rate within 5 years after surgical treatment is relatively high[10]. However, once the tumor tissue produces metastasis, surgical treatment is rarely continued in clinical practice, resulting in a relatively high mortality rate in patients with advanced disease[11]. Therefore, tumor metastasis is currently the most common cause of high morbidity and mortality in colon cancer. The metastatic ability and degree of tumor tissue were positively correlated with the migration ability of colon cancer cells[12].

In addition, 20% of patients diagnosed with colon cancer are found to have metastatic disease at the time of diagnosis. Thirty percent of patients with early stage colon cancer will eventually develop metastatic disease. The incidence of colorectal cancer can be reduced by means of colonoscopy and surgical resection. However, the widespread use of these methods is limited by their invasiveness,

cost, and discomfort. At present, the biomarkers of CRC include carcinoembryonic antigen (CEA), but they lack sufficient sensitivity and specificity. The prognosis of patients with colon cancer is poor, so it is urgent to further improve the existing treatment strategies and develop new targeted treatment options.

At present, most of the treatment of colon cancer is based on surgery, supplemented by chemotherapy, targeted therapy, immunotherapy and traditional Chinese medicine treatment. Although great progress has been made in a variety of treatment modalities including surgery, radiotherapy, chemotherapy, and adjuvant therapy with traditional Chinese medicine, the 5-year survival rate of colon cancer patients is still limited. So the study of the molecular mechanism of colon cancer is essential. Malignant tumors not only have expression differences at the level of gene expression encoding proteins, but in many studies, it has been found that differential expression of microRNAs is also closely related to a variety of cancers.

1.2 MiRNA

1.2.1 Overview of miRNAs

Noncoding RNAs (NcRNAs) are composed of long and short RNAs, which do not encode functional proteins, but have regulatory functions. MicroRNAs (Micro RNAs) are regulatory NcRNAs capable of regulating gene expression. MicroRNA (Micro RNA) is a non-protein-coding single-stranded RNA molecule encoded by eukaryotic DNA, which is 18 to 24 nucleotides in length and has gene regulatory functions. MiRNA biogenesis is initiated by transcription by RNA polymerase II and forms a primary transcript (pri-miRNA), which is several kilobases in length. Then, pri-miRNAs undergo a complex process, including drosha (an rnase III enzyme) and dicer (a double-stranded RNA endonuclease), to form a mature miRNA. MiRNAs regulate gene expression and translation through mRNA degradation or inhibition of proteins. MiRNAs are essential for organisms because they regulate various cellular processes such as nervous system patterning, cell proliferation and death during normal development[13].

In recent years, the targeting of miRNAs in tumorigenesis has received great attention. It has been shown that aberrant expression of different miRNAs can regulate tumor growth, carcinogenesis, or response to chemotherapy in different malignancies. So far, dozens of miRNAs have been implicated in a variety of tumor phenotypes[14]. Most miRNAs are located in tumor-associated genomic regions or fragile sites, each has hundreds or thousands of target genes, and almost all coding genomes are under the control of miRNAs. MiRNA expression has been reported in several malignancies, among them leukemia, lung cancer, breast cancer, especially in studies of colorectal cancer. Mature miRNAs are stably associated with RNA-induced silencing complexes and regulate target messenger RNAs, resulting in inhibition of gene expression levels, while others can promote gene expression. Therefore, some miRNAs mainly act as tumor suppressor genes, and others act as proto-oncogenes. MiRNAs are detected in tissues and all body fluids, and miRNAs are considered a potentially useful disease biomarker because of their small size, good stability, and contribution to cellular regulatory functions. Therefore, the discovery of tumor-derived miRNAs has prompted us to improve the understanding of these miRNAs for the screening and monitoring of human malignancies.

1.2.2 Mechanism of miRNA action

Depending on the function of their targets, miRNAs can be oncogenes or tumor suppressors. After DNA transcription, miRNAs do not act by translation into proteins, but directly partially or completely complement the 3 ′ UTR region of target genes by base complementary pairing, and then play a role in post-transcriptional regulation of target gene expression by cleavage of transcription products of target genes, or inhibition of translation of transcription products. Although miRNAs do not have typical open reading frames, they play this irreplaceable important role in development, protein secretion and gene regulation. More than half of human coding genes are able to selectively pair with miRNAs, which can degrade target mRNAs or repress the translation process of mRNAs according to different degrees of sequence complementarity, and then inhibit protein synthesis to achieve the purpose of regulating genes. Since miRNAs act through partial complementary binding

of relatively short sequences, they target multiple mRNAs and thus regulate the entire gene network. In this way, miRNAs organize basic biological cellular processes, such as development, proliferation, and apoptosis. In addition, miRNAs have been implicated in various diseases including cancer, making them targets or diagnostic markers for therapeutic intervention. In recent years, investigators have found in studies of miRNA profiling in different tumor types that the expression of miRNAs in tumors is different from that in normal tissues and is significantly altered. While certain upregulated miRNAs, so-called oncogenes, have transforming potential, there are also examples where miRNAs with intrinsic cancer-suppressing activity are downregulated in cancer cells. Among those miRNAs closely related to metastatic progression, some are key regulators involved in cytoskeletal reorganization. For example, by combined inhibition of integrin α5, the adaptation protein Radisin, and the small GTPase RhoA, its expression level was found to be inversely correlated with disease recurrence in breast cancer patients. When miRNAs regulate the expression of target genes, they can be one-to-many or many-to-one, that is, one miRNA regulates multiple target genes, and multiple miRNAs can also act on one target gene at the same time to synergistically regulate gene expression in a variety of tissues and cells. In the cytoplasm, miRNAs assemble with proteins in the RISC ribonucleoprotein complex, and the miRNA components of the RISC complex bind complementary sequences of mRNA targets depending on the degree of complementarity and result in affected protein synthesis. MiRNAs are tissue- and time-specific in expression. In the process of regulating the expression of functional genes, miRNAs, as an important regulatory molecule, play a crucial role in the process of biological growth, development, and various physiological activities. Genome-wide association analysis shows that many human miRNA genes are found in genomic regions associated with cancer, and it has also been shown that in tumor tissues, the absolute expression level of miRNAs is significantly reduced, miRNAs are the central nodes of biological networks, which can affect multiple target genes, and miRNA expression profiles are more representative in cancer classification compared with mRNA expression profiles, so miRNAs are also used as biomarkers to assist in the early diagnosis of cancer.

MiRNA genes can be encoded by intrinsic transcripts or by introns present between protein-coding genes. They can all be transcribed by RNA polymerase II to produce an initial transcript with a cap-shaped structure and a Poly A tail. The DROSHA/DGCR8 complex processes the initial transcript to obtain a stem-loop shaped molecule of about 60 bp, which is transported out of the nucleus by the transporters expotin and Ran-GTP. In the cytoplasm, the Dicer enzyme cleaves and processes it further, eventually forming a double-stranded dimer of 21 – 23 bp in length, and one of the chains in the duplex will integrate to the RNA silencing complex to function. The seed sequence of MiRNA and the 3 'non-coding region of the target gene combine complementarily through the principle of base complementary pairing, so that the translation of the target gene is inhibited or the mRNA of the target gene is directly degraded, so as to achieve negative regulation of the target gene.

In summary, miRNAs are key regulators of abnormal gene expression in tumor cells and are directly or indirectly involved in multiple important processes of tumor development. MiRNAs are involved in each step of colon cancer formation as well as progression, some playing a promoting role and some playing an inhibitory role. MiRNAs can directly or indirectly regulate physiological processes such as growth and proliferation, angiogenesis, and apoptosis of colon cancer cells, while miRNAs are also associated with migration of colon cancer cells. Therefore, the development of miRNA-based therapeutic agents is likely to be a brand-new therapeutic strategy for colon cancer treatment.

1.3 Test purpose and significance

As one of the most serious malignant tumors in the world, colon cancer is particularly important for researchers to clearly understand its pathogenesis for subsequent studies, but there is still a lack of understanding of its pathogenesis, which brings difficulties to subsequent studies. At present, there are different degrees of abnormal expression of miRNAs in colon cancer, indicating that there is a certain correlation between miRNA expression and the occurrence of colon cancer, so it is particularly important to deeply reveal the role of miRNAs in colon cancer for understanding the pathogenesis of

colon cancer and finding its etiology, and it is important to identify new biomarkers with important prognostic value for the early diagnosis of colon cancer and the establishment of effective miRNA-targeted therapy. It has been shown that miR-149 is lowly expressed in a variety of malignant tumors and acts as a tumor suppressor gene in a variety of tumors, such as non-small cell lung cancer, malignant glioma, and gastric cancer. Therefore, it is necessary to explore the regulatory effect of miR-149 on colon cancer, and this study can provide basic data for subsequent colon cancer research, which can provide important theoretical significance and experimental basis for the treatment of colon cancer.

2. Effect of miR-149 on the proliferation and migration of SW480 cells

2.1 Experimental equipment

2.1.1 Experimental materials

Human colon cancer SW480 cells, were purchased from Shanghai Cell Resource Center, Chinese Academy of Sciences.

MiR-149 mimics (5 '→ 3': F: AGGGAGGGACGGCUGUGC; R: GCACAGCCCCCGUCCCUCCCU);

SimiR-149 (5 '→ 3': AGCTCTGGCTCCGTCTTCACT);

MiR-149 NC (5 '→ 3': F: UCAACCUCCUAGAAAGUAGA; R: UCUACUCUUUCUAGGAGGUUGUGA); the sequence was self-designed and synthesized by TAKARA.

2.1.2 Main reagents

Reagent/Consumable Name	Manufacturer/Source
DMEM medium	GIBCO
Fetal bovine serum	GIBCO
Penicillin-Streptomycin	Biyuntian
Lipofectamine2000	Invitrogen
CCK-8	DOJINDO
DMSO	Sigma
Annexin V-FITC/PI Apoptosis Kit	Bebe

2.1.3 Main instruments

仪器名称	厂商
恒温细胞培养箱	Thermo 公司
倒置生物显微镜	OLYMPUS 公司
低速台式离心机	Eppendorf
无菌超净台	苏州净化设备仪器公司
倒置荧光显微镜	Olympus
酶标仪	BioTek
流式细胞仪	Guava easyCyte

2.1.4 Preparation of conventional solutions

(1) Sterile PBS: Respectively weigh 8.0g of NaCl, 0.2g of KCl, 2.9g of Na2HPO4 • 12H2O and 0.2g of KH2PO4, add deionized water to fully mix them and then dilute them to 1000 mL, use 0.45 μm filter membrane for filtration and then perform autoclaving for future use.

(2) DMEM complete medium: 10 mL of fetal bovine serum, 1 mL of double antibody solution (penicillin + streptomycin), and DMEM medium were added to make 100 mL.

(3) Trypsin solution (containing EDTA): trypsin powder 2.5 g, EDTANa2 0.2 g, add 800 mL 1 × PBS for stirring and dissolution, make constant volume to 1 L, filter and sterilize with 0.22 μm filter, dispense and store at -20℃ for future use.

(4) Cell freezing solution: 9 mL FBS and 1 mL DMSO were taken in a 15 mL centrifuge tube according to the ratio of 9:1, mixed well and stored at 4 ° C until use.

(5) Double antibody: Add 5 mL of normal saline to the Abe bottle of 100 units of streptomycin sulfate; add 8 mL of normal saline to the Abe bottle of 1.6 million units of penicillin, take out 5 mL after thorough mixing, respectively, mix well, dilute to 100 mL with normal saline, dispense and store at -20℃ for later use.

2.2 Experimental Methods

2.2.1 Recovery of SW480 Cells

(1) The cryotube is quickly removed from the liquid nitrogen tank and placed in a 37℃ water bath to quickly melt it, and attention should be paid to avoid contamination in the process of melting.

(2) When the cells dissolve the remaining rice grains in a water bath for as large as possible, they are taken into the cell room, the cryotube tube body is disinfected with 75% alcohol, and the medium liquid is transferred to a 15 ml centrifuge tube with a pipette, 1500 rpm/min, and the centrifugation time is 5 min, at which time the supernatant is removed.

(3), 1 mL of DMEM complete medium was added to uniformly resuspend the cell pellet, at which time the resuspended cells were transferred to a cell culture flask with a pipette and placed in a 37 ° C, 5% CO2 incubator to start culture.

2.2.2 Cell Passage

When the cell density of culture reaches 80% -90% above, it is necessary to make passage. The passage steps are as follows:

(1) After discarding the old culture medium in the culture flask, wash the cells with sterilized PBS for 2 ~ 3 times, add about 1 mL of 0.25% trypsin, and slightly shake well.

(2) Place the culture flask in the incubator for digestion for 3 min and then observe the cells under the microscope. If the intercellular space in the culture flask increases, when the cell digestion tends to be round, tap the flask wall to make the cells detach from the flask wall, and add 1 mL of complete medium to terminate the digestion.

(3), the adherent cells were gently blown with a 1 mL pipette to detach the cells from the flask wall, transferred to a 15 mL centrifuge tube, and centrifuged at 1500 rpm/min for 5 min.

(4) At this time, the supernatant was discarded, and then 1 mL of DMEM complete medium was added, and the cells were gently pipetted with a 1 mL pipette to resuspend them. Transfer to a new bottle at a ratio of 1:3 or 1:4 to continue incubation.

2.2.3 Cell cryopreservation

The cells in logarithmic growth phase with cell density of 70% -80% and good growth status were selected for cryopreservation. The cryopreservation steps were as follows:

(1) Discard the old culture medium in the culture flask, wash the cells with sterilized PBS for 2 ~ 3 times, add about 1 mL of 0.25% trypsin, and slightly shake well.

(2) Place the culture flask in the incubator for digestion for 3 min and observe the cells under the microscope. If the intercellular space in the culture flask increases and tends to be round, tap the flask

wall gently to make the cells detach from the flask wall, and add 1 mL of complete medium to terminate the digestion.

(3), the adherent cells were gently blown with a 1 mL pipette to detach the cells from the flask wall, transferred to a 15 mL centrifuge tube, and centrifuged at 1500 rpm/min for 5 min.

(4) Discard the supernatant and resuspend the cells with an appropriate amount of freezing solution (complete medium containing 10% dimethyl sulfoxide DMSO and 20% fetal bovine serum). Cells were transferred to cryovials. They were sequentially placed in a refrigerator at 4 ° C for 30 min, a freezer at − 20 ° C for 30 min, an ultra-low temperature freezer at − 80 ° C overnight, and finally placed in a liquid nitrogen tank for long-term storage.

2.2.4 Cell Transfection

The experiment was divided into three groups: miR-149 mimic group, mimics negative control group, and blank control group. The specific steps are as follows:

(1) Cell plating: On the day before transfection, the cells were plated into 6-well plates, the cell concentration was adjusted to 3 × 105 cells/well, 2 mL of culture medium without any antibiotics was added to each well, and the plated six-well plates were placed in an incubator at 37 ° C and 5% CO2, and the cell confluence reached 70% ~ 90% on the second day as the optimal state.

(2) During transfection, the medium was discarded and washed twice with basal medium, and 1.5 mL of basal medium was added to each well after washing.

(3), 10 uL Lipofectamine 2000 was added to 250 μL serum-free culture medium and incubated at room temperature for 5 min.

(4), 100 pM miRNA mimic was added to 250 μL serum-free culture medium.

(5), the liquids of steps 3 and 4 were evenly mixed after preparation and incubated at room temperature for 20 minutes to form the MiR149-transfectant complex.

(6) Gently drip the above complex into a six-well plate, gently shake the plate back and forth, and place it in a 37 ° C, 5% CO2 cell incubator to continue the culture.

(7) After 6 hours, the culture medium was replaced with DMEM complete medium containing antibiotics and serum. The culture was continued at 37 ° C in 5% CO2. Transfected cells were collected after 48 hours for the next assay.

2.2.5 Effect of miR-149 on SW480 proliferation detected by CCK8 assay

(1) Discard the old culture medium in the culture flask, wash the cells 2 ~ 3 times with PBS, add about 1 mL of 0.25% trypsin, and shake slightly to mix well.

(2) Place the culture flask in the incubator for digestion for 3 min and observe the cells under the microscope. If the intercellular space in the culture flask increases and tends to be round, tap the flask wall gently to make the cells detach from the flask wall, and add 1 mL of complete medium to terminate the digestion.

(3), the adherent cells were gently blown with a pipette to detach the cells from the bottle wall, transferred to a 15-mL centrifuge tube, and centrifuged at 1500 rpm/min for 5 min.

(4) At this time, the supernatant was discarded, and then 1 mL of complete medium was added, and the cells were gently pipetted with a pipette to resuspend them. Adjust the cell concentration to 3 × 105 cells/mL, and inoculate 100 μL cells per well in a 96-well plate. Place the inoculated 96-well plate into an incubator containing 5% C02 at 37℃ for culture, and set the blank control group. The blank control only contains the complete culture medium. Add the sterilized PBS to the outermost well of the 96-well plate.

(5), a predesigned MiR149 mimics transfection group, a negative control group, and a blank control group were added, and three duplicate wells were set for each group. Continue to cultivate in 37℃, 5% C02 incubator.

(6) Color reaction: 48 hours after drug addition, 100 μL of prepared CCK8 solution was added to each well and incubated in an incubator for 1 hour in the dark before testing on the machine.

(7) Colorimetric reaction: Preheat the microplate reader, then measure and record the absorbance value at OD 450 nm for each well.

(8), the relative cell survival rate of each group was calculated.

2.2.6 Transwell assay of the effect of miR-149 on SW480 migration and invasion

2.2.6.1 Effect of miR-149 on SW480 Migration

(1) Logarithmic phase cells were collected, 5 × 105 cells/mL cells were counted, resuspended with serum-free medium, mixed well and 100 uL of cells were taken, added to the upper chamber of transwell inserts, and 700 uLDMEM high-glucose complete medium was added to the lower chamber.

(2) After 6 hours in a 37 ° C, 5% CO2 incubator, the inserts were removed, the cells in the upper chamber were carefully wiped off with a cotton swab, fixed with 4% paraformaldehyde for 15 min, washed once with PBS, stained with crystal violet for 10 min, and washed again with PBS. Whether the cells passed through the wells was detected, and if any, the other experimental groups were terminated, and photographic statistics were performed.

2.2.6.2 Effect of miR-49 on SW480 Invasion

(1) Matrigel was dissolved overnight in a refrigerator at 4 ° C, and Matrigel was diluted in a volume ratio of 1:3 using precooled serum-free medium, mixed well and 40 μL was added to precooled transwell inserts and incubated at 37 ° C for 2 h to solidify Matrigel.

(2), the excess liquid in the insert was aspirated, and 100 μL and 600 μL of serum-free medium were added to the upper and lower chambers, respectively, and equilibrated overnight at 37 ° C.

(3) At this time, the cells in the logarithmic phase were collected, 1 × 106 cells/mL cells were counted, resuspended with serum-free medium, 100 uL cells were taken, added to the upper chamber of the transwell chamber, and 600 μL DMEM high glucose was added to the complete culture in the lower chamber.

(4) After incubation at 37 ° C, 5% CO2 for 48 hours, the inserts were removed, the cells in the upper chamber were carefully wiped off with a cotton swab, fixed with 4% paraformaldehyde for 15 min and washed once with PBS, stained with crystal violet for 10 min, and washed again with PBS to detect whether the cells passed through the wells, and if any, other experimental groups were terminated, and photographs and statistics were taken.

2.2.7 Effect of miR-149 on SW480 apoptosis by flow cytometry

AnnexinV/FITC versus PI double staining can effectively distinguish cells in early apoptotic and other apoptotic stages. The specific method is as follows:

(1) After cell transfection, the cells transfected with miR-149 mimics, negative control and blank control groups were collected and counted, and 3 × 105 cells/well were seeded on 6-well plates with 2 mL complete medium per well.

(2) Place the plated plate in 5% CO2 and culture in an incubator at 37 ° C for 24 h. After 48 h, wash it with PBS once and add an appropriate amount of trypsin cell digestive juice (which may contain EDTA) to digest the cells. When the adherent cells could be blown down by incubation at room temperature until gentle blowing, the trypsin cell digestive juice was sucked off, complete medium was added to terminate the digestion, and the cells were detached from the wall. Excessive digestion by pancreatic enzymes needs to be avoided.

(3) Add the cells collected in step (2), mix well, transfer them to a centrifuge tube, centrifuge at 1000 g for 5 min, discard the supernatant, collect the cells, gently resuspend the cells with PBS and count.

(4), 50,000 ~ 100,000 resuspended cells were centrifuged at 1000 g for 5 min, the supernatant was discarded, and 100 μL of 1X Annexin V buffer was added to gently resuspend the cells.

(5), 5 μL of Annexin V-FITC and 5 μL of propidium iodide staining solution were added and gently mixed.

(6), incubated at room temperature in the dark for 15 min.

(7), followed by flow cytometry, with Annexin V-FITC as green fluorescence and PI as red fluorescence.

2.3 Experimental Results and Analysis

2.3.1 Proliferation of SW480 cells detected by CCK8

In this study, miR-149 mimics sequence was designed and synthesized. The OD value at 450 nm was measured by CCK8 assay at four time points: 0 h, 24 h, 48 h, and 72 h after transfection into SW480 cells, and the proliferation inhibition rate was calculated, 2.1. After statistical analysis of the data by SPSS software, it could be seen that after transfection for 24 h, 48 h, and 72 h, miR-149 transfected SW480 cells produced a significant difference compared with the blank control group, while mimics NC in the negative control group had no significant difference compared with the blank control group. According to the calculation method of cell proliferation inhibition rate: cell proliferation inhibition rate = 1- (OD450 value of experimental group - OD450 value of blank group)/(OD450 value of negative control group - OD450 value of blank group) × 100%, it was calculated that the proliferation inhibition rate of transfected SW480 cells was 12.52% at 24 hours, 13.00% at 48 hours, and 11.93% at 72 hours.

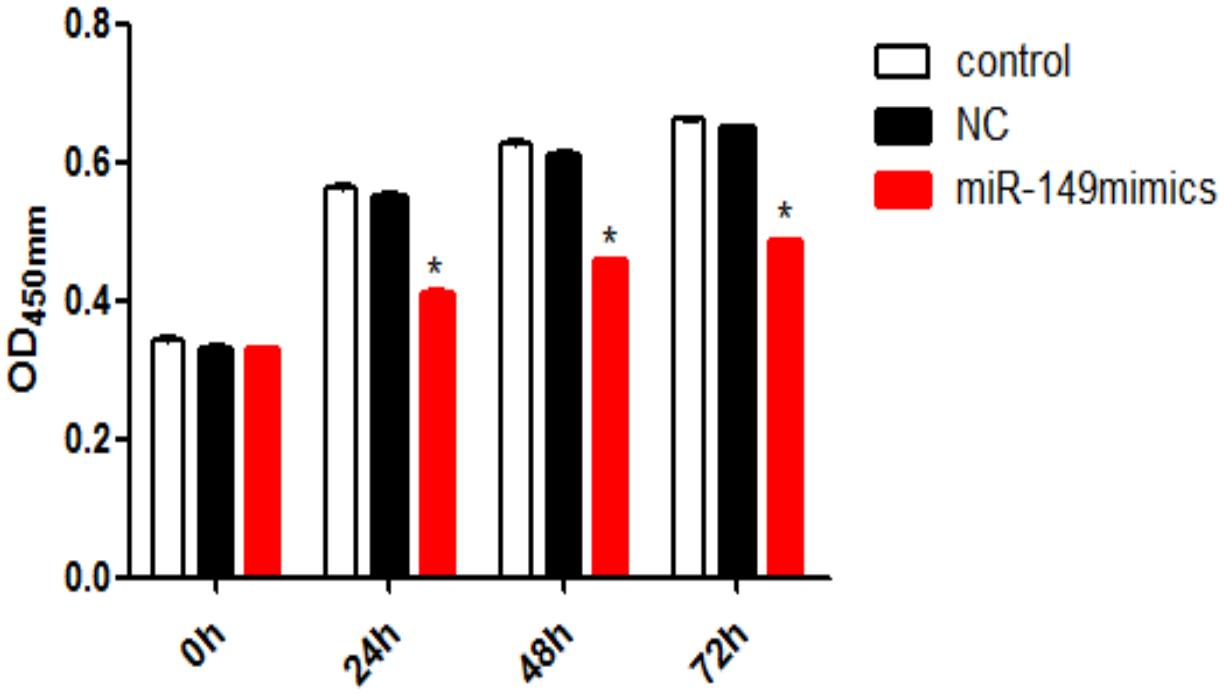
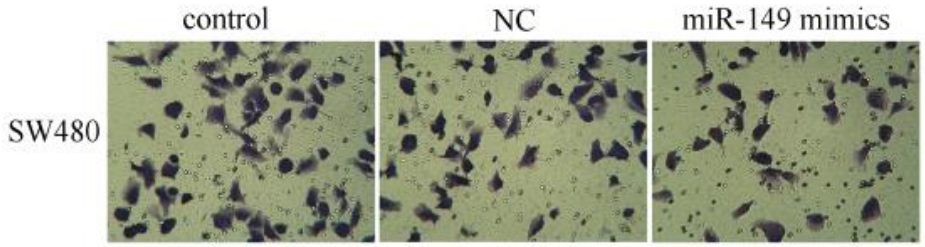


Figure 2.1 Exploration of miR-149 on the proliferation of SW480 cells detected by CCK8 assay (t test, * indicates P < 0.05, with significant difference; * indicates P < 0.01, with extremely significant difference)

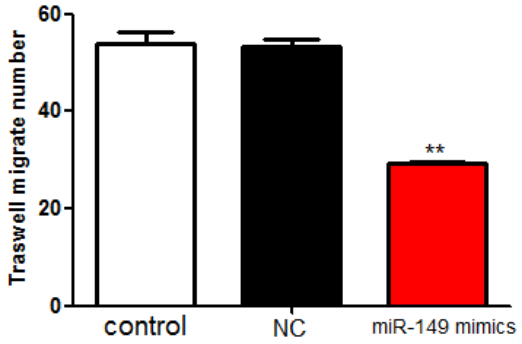
Figure 2.1 CCK8 assay for detection of proliferation of SW480 cells by miR-149

2.3.2 Effect of mi-RNA on SW480 migration and invasion detected by transwell

To further validate the effect of miR-149 on SW480 cell migration and invasion, transwell migration and invasion assays were performed on SW480 cells transfected with miR-149 mimics. The results of transwell migration assay showed that after 48 hours of plating, the cell migration rate was significantly smaller than that of control cells (see Figure Figure2.2). The results of transwell invasion assay showed that the number of SW480 cells entering the lower chamber through the microwell membrane was significantly smaller than that of the control after 48 hours of plating (see Figure 2.3). Both were statistically different.



A

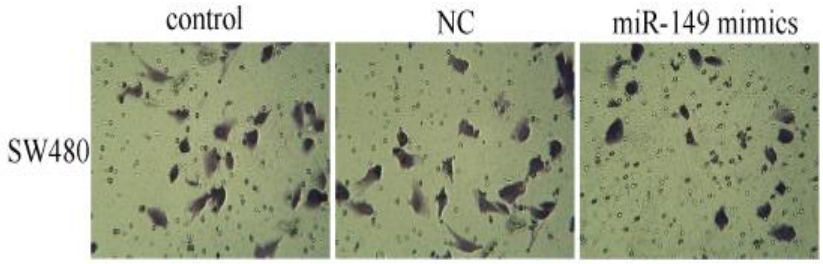


B

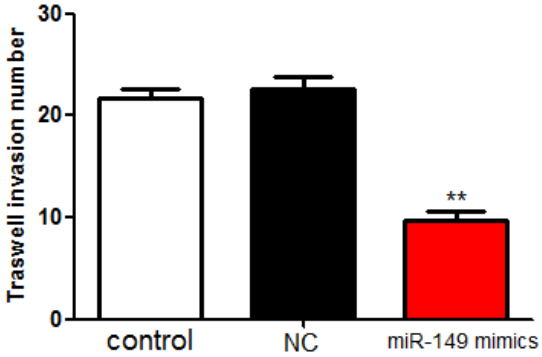
Figure 2.2 Transwell assay of the effect of miR-149 on SW480 cell migration

Note: A. Results of cell migration under microscope; B. Statistical map of cell migration number (t test, * indicates P < 0.05, with significant difference; * indicates P < 0.01, with extremely significant difference)

Figure 2.2 Effect of transwell detection of miR-149 on migration of SW480 cells



A



B

Figure 2.3 Effect of miR-149 on invasion of SW480 cells detected by transwell

Note: A. Results of cell invasion under microscope; B. Statistical map of cell invasion number (t test, * indicates P < 0.05, with significant difference; * indicates P < 0.01, with extremely significant difference)

Figure 2.3 Transwell invasion assay for miR-149 on SW480 cells

2.4 Discussion

(1) In this paper, the proliferation of SW480 cells was detected by CCK8. The results showed that miR-149 colon cancer cells had a significant inhibitory effect on the proliferation of SW480 cells. The proliferation of SW480 cells was significantly slowed down at 0 h, 24 h, 48 h and 72 h after miR-149 mimics transfection, which may indicate that the inhibitory effect on the proliferation of SW480 cells was the greatest between 24 and 48 h after transfection. At the later stage, the time point between 24 and 48 h could be used for other experiments.

(2) The results of transwell migration assay showed that the cells entering the lower chamber were significantly reduced in the SW480 cell group transfected with miR-149 mimics compared with the control mimicsNC group cells ($P < 0.05$), indicating that miR-149 mimics could inhibit the migration ability of human colon cancer cell SW480. The results of transwell invasion assay showed that the cells entering the lower chamber were significantly reduced in the SW480 cell group transfected with miR-149 mimics compared with the control mimics NC group cells, indicating that miR-149 could inhibit the invasive ability of human colon cancer SW480 cells. Note that the experimental results of CCK8 in detecting the cell proliferation inhibition rate showed that the total number of cells would change significantly after 24 hours, so the transwell invasion and migration assay should be performed within 24 hours after plating, which can ensure the stability of the total number of cells.

(3) In this study, the apoptotic effect of miR-149 on human colon cancer cell line SW480 was detected by flow cytometry and Annexin V-FITC/PI double staining, and the results showed that the apoptotic rate of cells transfected with miR-149 mimics was significantly higher than that of cells in the control mimics NC group. The apoptosis rate of SW480 cells in the experimental group at 24 hours was higher than that in the experimental group at 48 hours, and 24 hours after transfection in the experimental group may be used as the optimal transfection time in subsequent experiments.

According to the cell proliferation and migration assay in this chapter, as well as the results of flow cytometry detection of apoptosis of transfected SW480 cells, the cell proliferation and migration ability of the experimental group were weakened, and the apoptosis rate was significantly increased, indicating that up-regulation of miR-149 can indeed reduce the proliferation and migration ability of SW480 cells and promote the apoptosis of SW480 cells. Westen Blot can be used at a later stage to further validate the molecular mechanism of its apoptosis.

2.5 Summary

In this paper, the proliferation rate of SW480 cells transfected with miR-149 was significantly inhibited by CCK8 assay. The migration and invasion assay of transwell showed that the migration and invasion ability of transfected SW480 cells were also decreased, while the flow cytometry results showed that the apoptosis rate of transfected SW480 cells was significantly increased, which was consistent with the results of CCK8 and transwell, indicating that the designed miR-149 mimics sequence inhibited the proliferation and migration of SW480 cells.

3. Summary and Prospect

3.1 Summary

In this study, CCK8, transwell and flow cytometry were used to investigate the effects of miR-149 on the proliferation and migration of colon cancer cells, and it was found that the efficiency of proliferation and migration of transfected SW480 was significantly reduced.

3.2 Outlook

In this study, we investigated the effects of miR-149 on the proliferation and migration of colon cancer cells, the number of cells studied was small, and it was only verified at the cellular level, so the cell line could be expanded in the next experiment, verified at both the cellular and tissue levels, and the relationship between miR-149 and different cell lines of colon cancer could be verified at the animal level. In addition, miR-149 can regulate multiple target genes, while each target gene is regulated by multiple miRNAs, and subsequent experiments can search for other target genes

involved in colon cancer cells. In summary, exploring different target sites of miR-149 at different cellular, tissue and animal levels will certainly provide a theoretical basis and new ideas for the treatment of colon cancer.

References

[1] Cheng N, Chytil A, Shyr Y, et al. Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion[J]. Mol Cancer Res, 2008, 6(10): 1521-1533.

[2] Hanahan D, Weinberg R A. Hallmarks of cancer: The next generation[J]. Cell, 2011, 144(5): 646-674.

[3] He J, Pei L, Jiang H, et al. Chemoresistance of colorectal cancer to 5-fluorouracil is associated with silencing of the bnip3 gene through aberrant methylation[J]. J Cancer, 2017, 8(7): 1187-1196.

[4] Bordonaro M, Lazarova D. Hypothesis: Induction of biomarkers for detection of colonic neoplasms[J]. J Cancer, 2018, 9(1): 166-173.

[5] Center M M, Jemal A, Smith R A, et al. Worldwide variations in colorectal cancer[J]. CA Cancer J Clin, 2009, 59(6): 366-378.

[6] Shibuya H, Iinuma H, Shimada R, et al. Clinicopathological and prognostic value of microrna-21 and microrna-155 in colorectal cancer[J]. Oncology, 2010, 79(3-4): 313-320.

[7] Arnold M, Sierra M S, Laversanne M, et al. Global patterns and trends in colorectal cancer incidence and mortality[J]. Gut, 2017, 66(4): 683-691.

[8] Peeters M, Price T, Van Laethem J L. Anti-epidermal growth factor receptor monotherapy in the treatment of metastatic colorectal cancer: Where are we today?[J]. Oncologist, 2009, 14(1): 29-39.

[9] Remco N, Carlos L S, Bego?A D, et al. Regulation of the adenomatous polyposis coli gene by the mir-135 family in colorectal cancer[J]. Cancer Research, 2008, 68(14): 5795-5802.

[10] Bloomston M, Frankel W L, Petrocca F, et al. Microrna expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis[J]. Jama, 2007, 297(17): 1901-1908.

[11] Pedersen I M, Otero D, Kao E, et al. Onco-mir-155 targets ship1 to promote tnfalpha-dependent growth of b cell lymphomas[J]. EMBO Mol Med, 2009, 1(5): 288-295.

[12] Valeri N, Gasparini P, Fabbri M, et al. Modulation of mismatch repair and genomic stability by mir-155[J]. Proc Natl Acad Sci U S A, 2010, 107(15): 6982-6987.

[13] Calin G A, Croce C M. Microrna-cancer connection: The beginning of a new tale[J]. Cancer Res, 2006, 66(15): 7390-7394.

[14] Garzon R, Marcucci G, Croce C M. Targeting micrornas in cancer: Rationale, strategies and challenges[J]. Nat Rev Drug Discov, 2010, 9(10): 775-789.