Original Article

MiR-296 promotes colorectal cancer cells growth through regulating NF-κB

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Abstract: Tumor reduced people’s quality of life. Colon cancer is hard to cure. MicroRNA abnormal expression induces oncogenesis. We intended to explore miR-296 role in colorectal cancer development. MiR-296 expression in colorectal cancer was tested in clinical samples. MiR-296 overexpression and low expression colorectal cancer cell lines were established to detect NF-κB activation. Western blot was applied to determine P65 and pP65 proteins expression. MTT and Colony formation assay were used to evaluate colorectal cancer cell proliferation. Q-PCR showed that miR-296 overexpressed in colorectal cancer patients, and it also confirmed that miR-296 overexpression and low expression colorectal cancer cell lines were successfully constructed. Western blot revealed that miR-296 overexpression may lead to pP65 level significantly increased, while P65 showed the opposite results. Proliferation assay including MTT and colony formation demonstrated that miR-296 overexpression promoted colorectal cancer cell proliferation, whereas miR-296 low expression weakened cancer cell proliferative ability. MiR-296 promotes colorectal cancer cells growth through regulating NF-κB signaling pathway.

Keywords: miR-296, NF-κB, colorectal cancer

Introduction

Colorectal cancer is considered to have high mortality among common malignant tumors, especially in East Asia and South Africa. Furthermore, its incidence and mortality is also significantly higher in developing countries compared with developed countries. Colorectal cancer onceme is a multi-factor, multi-step, and complex process that related to genes abnormal expression [1, 2]. However, its potential mechanism has not been fully classified. Many colorectal cancer patients quickly death is due to cancer cell rapid growth. Surgical resection is the potential treatment option with the best prognosis for long term colorectal cancer patients. Since about 80% of colorectal cancer patients combined with intestinal inflammation, only 10-15% patients can be surgically resected [3, 4]. The left patients cannot receive surgical resection because of tumor size and potential of colorectal disease. More importantly, numerous patients may appear recurrence after surgery. Research showed that high AFP is a risk factor of colorectal cancer recurrence [5, 6]. It is urgently needed to find the potential pathogenesis of colorectal cancer.

MiRNAs are reported to be a type of small non-coding RNA that can regulate the function of RNA transcription and promote or suppress tumor gene expression. Up to now, more than 1000 human miRNAs have been identified and reported in the RNA database. Some of them have been used as molecular biomarkers for diagnosis, prognosis, and treatment [7, 8]. MiR-296 is located in the chromosome 20q13.32. It was reported to be an oncogene associated with angiogenesis and regulating cell polarity. NF-κB is a signaling pathway related to inflammation. Some scholars considered that tumor was derived from chronic inflammation. Inflammation occurrence caused a variety of genes abnormal expression, and further activated the related signaling pathways [9, 10]. Many cancers are closely related with NF-κB signaling pathway. NF-κB mainly leads to P65 enter the
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nucleus through ectogenic factors, resulting in nucleus phosphorylation to activate the signaling pathway, promote inflammation, and impact tumor occurrence and development.

Materials and methods

Clinical information

51 cases of colorectal cancer tissue samples and 50 cases of normal colon tissue specimens adjacent to carcinoma in the oncology department of Qilu Hospital, Shandong University were collected between July 2014 and December 2014. All the enrolled subjects had signed informed consent and the study was approved by the ethical committee in Qilu Hospital, Shandong University. Clinical staging was based on TNM staging standard published by UICC in 2009. Patient’s medical records and the pathological data were complete.

Q-PCR

Reverse transcription kit was from TaKaRa. The specimen was treated with Trizol and centrifuged at 12,000 r/min and 4°C for 10 min. Then the supernatant was moved to a new EP tube and let stand for 5 min. Next, the fluid was added with 200 μl chloroform and centrifuged at 12,000 r/min and 4°C for 15 min. After moving the supernatant to a new tube and added with equal amount of isopropanol, the tube was centrifuged at 12,000 r/min and 4°C for 10 min. At last, after removing the supernatant, the tube was added with 75% ethanol (solved in DEPC water) and centrifuged at 7,500 r/min and 4°C for 5 min. Total RNA was solved in 20 μl DEPC water. Real time PCR was applied to detect miR-340 expression in gastric cancer. The primers sequences were as follows: miR-296, F-GCGAGCTACATTGTCTGCTGGGTT, R-GTCGAGGTTCGAGGTATTCCG; U6, F-CGGCGGTA-GCTTATACAGCTGATG, R-CCAGTCGAGGGTCCGAGTATT. PCR reaction contained 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The melting curve was analyzed.

MiR-296 stable transfected cancer cell line

HCT116 cells (purchased from ATCC) in logarithm phase were seeded in six-well plate at 3×10^5. 5 μl lipo2000 was solved in 100 μl FBS free medium and stand for 5 min, while 12 μl miR-296 overexpressed or low expressed plasmid (purchased from RiboBio) was solved in 100 μl FBS free medium for 5 min. Then the two fluids were mixed together for 20 min and added to cells in 1800 μl FBS free medium. RNA was extracted after 48 h for detection. MiR-296 overexpressed and low expressed plasmids were synthetized by RiboBio.

MTT assay

Single cell suspension in medium with 10% FBS was seeded in 96-well plate at 6,000 cells/200 μl. After HCT116 adherence, 20 μl MTT solution at 5 mg/ml was added to the well for 4 h. After removing the supernatant, 150 μl DMSO was added and the plate was vibrated for 10 min. At last, the plate was read at 490 nm to draw the cell growth curve.

Colony formation assay

100 cells were seeded in the petri dish after counting. The medium was removed after macroscopic colony formation. The cells were fixed with 5 ml 4% paraformaldehyde for 15 min. Then the fixing solution was removed and changed to crystal violet staining solution for 10~30 min. At last, the dish was dried in air after washing.

Western blot

Tissue protein was extracted using protein kit purchased from Beyotime and quantified by BCA. Reagent A was mixed with reagent B at 50:1 to form BCA solution. 2 μl cellular supernatant was added to 18 μl PBS and 200 μl AB mixture. The protein was separated by electrophoresis and transferred to PVDF membrane. After blocked by 5% skim milk for 1 h, the mem-
brane was washed by TBST and incubated in primary antibody overnight. On the second day, the membrane was incubated in secondary antibody at room temperature after rewarming and washing. PVDF membrane was developed by ECL luminous fluid.

Results

MiR-296 expression

Q-PCR was applied to test miR-296 expression in 51 cases of colorectal cancer tissues and 50 cases of normal colon tissues adjacent to carcinoma. The results showed that miR-296 significantly overexpressed in colorectal cancer (P < 0.001) (Figure 1). Our results suggested that miR-296 may play an important role in colorectal cancer occurrence and development.

MiR-296 activated NF-κB signaling pathway

At first, we successfully constructed miR-296 overexpressed and low expressed cancer cell lines (Figure 2A and 2B). Western blot showed that P65 protein declined, while pP65 level elevated and nuclear import enhanced in miR-296 overexpressed HCT116 cells. They were opposite in miR-296 low expressed HCT116 (Figure 2C), suggesting that NF-κB signaling pathway was activated.

MiR-296 overexpression promoted cell proliferation

We further tested miR-296 impact on cell proliferation. Proliferation assay including MTT and colony formation demonstrated that miR-296 overexpression promoted colorectal cancer cell proliferation (Figure 3A and 3B), whereas miR-296 low expression weakened cancer cell proliferative ability (Figure 3C and 3D).

Discussion

Colorectal cancer is a tumor with high malignancy and complicated pathogenesis. Gene abnormal expression led to tumor. MicroRNA received more and more study in colorectal cancer. A variety of miRNAs were discovered in early stage colorectal cancer screening, and some had been recognized as tumor suppressor genes or oncogenes. Colorectal cancer development was a typical multiple factors and multi-step process involving abnormal cell proliferation, apoptosis, invasion, and metastasis. MiR-296 connected to the 3' UTRs of the base sequence that mainly regulated posttranscriptional level [11, 12]. Previous studies considered miRNAs as tumor suppressor genes or oncogenes mainly through regulating cell proliferation, apoptosis, metabolic pathway, and signaling pathways [13]. Many studies reported microRNA involved in colorectal cancer occurrence and development. There were more and more microRNAs had been used as tumor markers applied to clinical research, as a study reported that miR-21 can be treated as the marker of colorectal cancer. It was also considered that AFP can be used for colorectal cancer early screening and diabetes diagnosis. Infinite proliferation made tumor continuous division and proliferation, resulting in protein synthesis metabolism faster than catabolism and even captured normal cells protein metabolism products. It exacer-
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Bated disease state and led to cachexia. Therefore, inducing tumor cells apoptosis became an effective method to restrain tumor unlimited growth. Our results also showed that miR-296 overexpression promoted colorectal cancer cell proliferation. Thus, we aimed to focus on whether miR-296 was related to the speed of apoptosis induction in the future work.

Recent studies demonstrated that miR-296 expression was suppressed in normal tissue compared with malignancy, while miR-296 overexpression may contribute to tumor formation and development [14, 15]. For example, someone found miR-296 overexpressed in osteosarcoma cell line significantly increased cell proliferation, migration, in vitro invasion, and metastasis in xenograft mice model [16]. Studies have reported that high miR-296 expression was related to lymph node metastasis, high pathological grade, clinical stage, and breast cancer patients’ overall survival [17, 18]. It was also revealed that miR-296 expression aggravated colorectal cancer cell growth, and associated with colorectal cancer prognosis [19, 20]. As widely investigated as a typical inflammatory signaling pathway, NF-κB was found activated in colorectal cancer. In colorectal cancer, tumor suppressor gene APC inactivation can activate β-catenin importing to nuclear, further activate NF-κB signaling pathway to exacerbating inflammation. Our results

Figure 3. MiR-296 overexpression promoted cell proliferation. A. Cell proliferative ability enhanced in miR-296 overexpressed HCT116; B. Single cell proliferative ability enhanced; C. Cell proliferative ability weakened in miR-296 low expressed HCT116; D. Single Cell proliferative ability reduced. *P < 0.05, **P < 0.01.
showed that miR-296 overexpressed in colorectal cancer compared with paratumor tissue (P < 0.001). Our study further confirmed miR-296 promoted colorectal cancer cell proliferation mainly through activating the NF-κB signaling pathway.

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Disclosure of conflict of interest

None.

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