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Regulation effect of miR-7 on intervening colorectal cancer rats with HP infection through Akt/GSK-3β/β-catenin pathway

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ABSTRACT

This experiment aimed to analysis of the intervention effects of modulating miR-7 on rats with colorectal cancer complicated with HP infection and the effects on (serine/threonine kinase) Akt / (glycogen synthase kinase 3 β) GSK-3 β/ (β- β - Catenin) β- Influence of the catenin pathway. For this purpose, forty special pathogen-free (SPF) - grade rats of both sexes were randomly divided into 10 control, 10 colorectal cancer and HP infection model groups, 10 up-regulated miR-7, and 10 down-regulated miR-7 groups. Observational analysis of rat colon tissues was performed using the HE staining method. Detection of inflammatory factors [TNF- α‘ IL-8, IL-6], detection of miR-7 expression, detection of Akt, GSK-3 using Western blot β‘β- Catenin protein expression. Results showed that forty special pathogen-free (SPF) - grade rats of both sexes were randomly divided into 10 control, 10 colorectal cancer and HP infection model groups, 10 up-regulated miR-7, and 10 down-regulated miR-7 groups. Observational analysis of rat colon tissues was performed using the HE staining method. Detection of inflammatory factors [TNF- α‘ IL-8, IL-6], detection of miR-7 expression, detection of Akt, GSK-3 using Western blot β‘β- Catenin protein expression. It was concluded that modulation of miR-7 in rats with colorectal cancer and HP infection enables regulation of the Akt / GSK-3 β/β- Catenin pathway to improve serum inflammation condition and alleviate HP infection in rats, which played a better role in intervention.

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Introduction

Colorectal cancer, one of the most common malignant tumors in clinics, has recently presented a rising trend with a high mortality rate (1). Some researchers regarded Helicobacter pylori (HP) as a major attributor to gastric adenocarcinoma and discovered the importance of intestinal flora in the occurrence and development of early rectal cancer (2). Mature miRNA-7 plays an important role in the occurrence and development of tumors by effectively regulating the growth and invasion of tumor cells. Clinical surveys show that miR-7 plays a crucial role in the occurrence and development of colorectal cancer(3). Although the increased incidence of colorectal cancer combined with HP infection has led to a growing number of clinical studies, the mechanism of causing HP infection in colorectal cancer is still unclear. Therefore, it is necessary to actively find out therapeutic pathways and treat the disease(4-5). Hence in this paper, the Akt/GSK-3β/β-catenin pathway was used to regulate miR-7 in rats with colorectal cancer and HP infection, with the aim of analyzing its clinical therapeutic effects.

Materials and Methods

Forty Special Pathogen Free (SPF) grade rats (mixed gender) were selected and randomly divided into 10 control groups. The control group was put at 17-25°C with 50% humidity and fresh and dry chow for ready-to-feed feeding. The remaining 30 SD rats were established as a colorectal cancer and HP infection model, divided into 10 as colorectal cancer and HP infection model group, 10 as the up-regulated miR-7 group and 10 as the down-regulated miR-7 group.

Primary reagents: rat anti-mouse TNF-α, IL-8, IL-6 antibodies (Hyclone); miR-7 mimics, miR-7 inhibitors (Biomics Biotechnologies); mouse anti- rat Akt and GSK-3β antibodies (Research Biological Technology); rat anti-mouse β-catenin antibody (J&I Biological).

Modeling

Ten of the 40 rats were enrolled as the control group, while the remaining 30 rats were modeled according to the literature review (6). The liquid culture was performed on HP Sydney strain to enrich the bacteria, and after reviving the frozen bacteria, they were inoculated in 15% horse serum and antibacterial Brucella broth and shaken at 150r/min for 72h after culturing in the N285%, CO210% and O25% environment. Then, they were observed by staining, and inoculated after uremic toxin, catalase and oxidase became positive and the bacterial concentration was adjusted to 1×109CFU/mL. During the inoculation, the rats were

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gavaged with 5% sodium bicarbonate, followed by an injection of 1.5mL HP bacterial solution (1×109CFU/mL) after 15min. The rats gavaged were fasted for 12 hours and infected five times, one per week. All the selected rats were successfully modeled.

Grouping and intervention

The rats successfully modeled were randomly grouped, among which 10 were divided into colorectal cancer and HP infection model group, 10 into the miR-7 up-regulated group, and 10 into the miR-7 down-regulated group. The colorectal cancer rats with HP infection were injected with 10 mL/kg normal saline, the miR-7 up-regulated rats intraperitoneally with 10 mg/kg ago miR-7, and the down-regulated rats with 10 mg/kg antago miR-7.

Indicator analysis

Materials selection

At the end of the experiment, 5mL of blood was drawn from the abdominal aorta of the rats, centrifuged to separate the serum and plasma, and stored at -80℃ for use as a substitute. The rats were then executed under general anaesthesia to separate their GI tracts and then remove colon tissue and stomach tissue. The mean diameter of the tumor was measured using a vernier caliper. With the ≥1mm diameter as the standard, the tissues were divided into two parts, one was rinsed with saline and then fixed in 4% paraformaldehyde solution, and the other was stored at -40°C for future use.

HE staining

The fixed tissue was taken out and sliced serially at 5mm intervals. The slices were dried, dewaxed and rehydrated with alcohol for 3 min. After staining with hematoxylin for 15 min, the slices were cleaned with water three times and differentiated with acid alcohol for 30s. Then, they were thoroughly cleaned with 1% eosin for staining and dewaxed after dehydrating with alcohol. The microscope was used for observation after sealing.

Inflammation analysis

The extracted serum was removed and serum TNF-α, IL-8 and IL-6 levels were evaluated using the ELISA method in strict accordance with the manual for the kit. After the reaction ended, the absorbance value of the serum at 450 nm was determined by a microplate reader (Elx800 from Bio-Tek, USA) for subsequent measurement.

miR-7 expression examination

The expression of miR-7 was determined by PCR. The total RNA of colon tissue in mice was extracted by TRIzol method, and its integrity was detected by 1% agarose gel electrophoresis. The concentration and purity of total RNA were determined, and RNA was reversely transcribed into cDNA. PCR reaction system: 25mmol/L MgCl22μL, 4μL 5×PCR buffer solution, 0.5μL respectively for upstream and downstream primers, 2μL polymerase, 2μL cDNA, 9μL RNase-freeH2O; Reaction process: the reaction continued at 94℃ for 2min; 94℃ for 40s; 50~65 ℃ for 40s; 72℃ for 1min; repeated the cycle for 30 times at 72℃ for another 5min before terminating the reaction. Primer sequence: forward: 5'-GTGTGCGGAAATGCTTCTGCTA', reverse: 5'-GCGAGCACAGAATTAATACGAC-3', internal reference GADPH forward: 5'-ACCACAGTCCATGCCATCAC-3', reverse: 5'-TCCACCACCCTGTTGCTGTA-3. After the PCR reaction products were processed by 2% agarose gel electrophoresis, the integral of optical density was determined by grayscale scanning of the gel imaging analysis system. The expression of the target gene was indicated as the ratio of the gray value of the electrophoresis band of the target gene product to the gray value of the internal reference GAPDH band.

HP clearance rate

The stomach tissue was removed and washed out using stroke-physiological saline solution for HP culture. After 4d, the growth of HP was observed and colonies matching the morphology of HP were isolated and extended. DNAase, oxidase and urease were identified after 3d. Gram staining of smears was performed to determine HP positivity with specimens that were positive for DNAase, oxidase and urease and whose smears matched the HP morphology.

Akt, GSK-3β, and β-catenin detection

The Western blot was utilized to detect the Akt, GSK-3β and β-catenin. Cell lysates were prepared to measure the protein density with the BCA protein assay reagent. An equal amount of protein (10-30 μg) was electrophoresed on 8% or 12% SDS-polyacrylamide gels and transferred to PVDF membranes. The transferred proteins were then sealed in 5% non-fat dry milk of the phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBST) for 2h at room temperature. Later, membranes, together with the primary antibodies containing 3% non-fat dry milk, were incubated in PBS for the night at 4°C. The washed membranes were incubated with HRP-conjugated secondary antibodies that were diluted at 1:3000 for 2h, before being cleaned again with PBST. Protein expression was displayed with an ECL chemiluminescence kit.

Statistics process

The statistical analysis was conducted using IBM SPSS Statistics 20.0 for sample analysis and data processing. The between-group error was measured and compared by ($\bar{x} \pm s$), while the error in the comparative results of the between-group frequency was determined by the t difference value; the measurement data was subject to the % test, and the F value between multiple groups was calculated. The error in the comparative results of the between-group frequency was subjected to the between-group mean error x^2 test. $P<0.05$ represented effective statistical learning and practical significance.

Results

HE staining results

Fig. 1A showed the HE staining results of the control rats, Fig. 1B gave the HE staining results of the colorectal cancer rats with HP infection, Fig. 1C illustrated the HE staining results of the miR-7 down-regulated rats and Fig. 1D revealed the HE staining results of the miR-7 up-regulated rats.

Inflammation of rats in each group

As shown in Fig. 2, for the colorectal cancer rats with HP infection, the miR-7 up-regulated and down-regula-

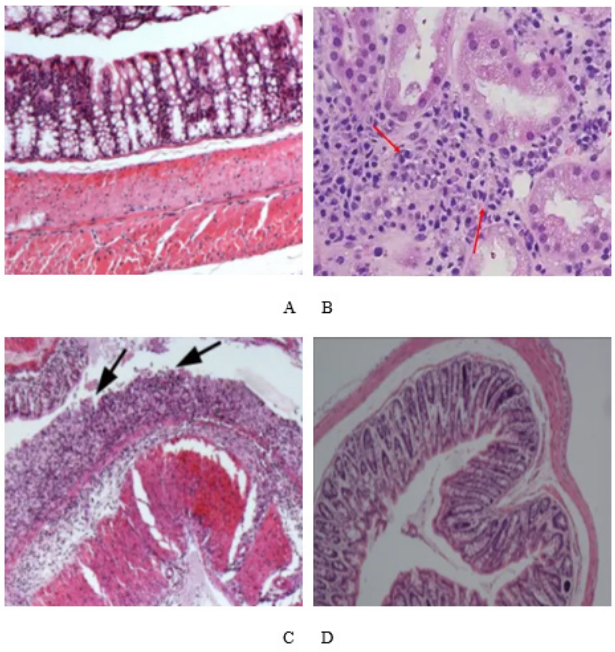

Figure 1. Colorectal cancer HE staining results.

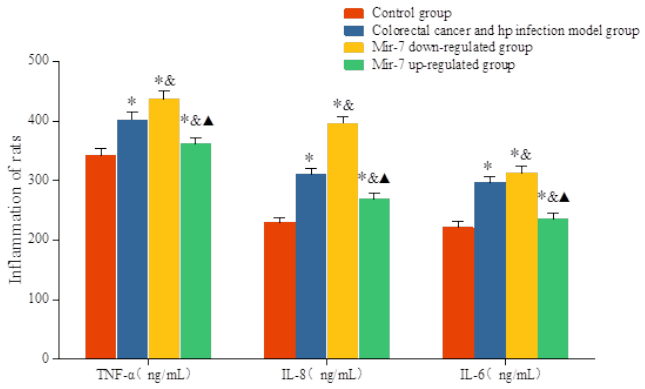

Figure 2. Inflammation of rats in each group ($\bar{x} \pm s$). Note: Compared with the control group, $^{*}P<0.05$; compared with the colorectal cancer and HP infection model group, $^{\&}P<0.05$; compared with the miR-7 up-regulated group, $^{\blacktriangle}P<0.05$.

ted rats had a higher level of TNF-α, IL-8, and IL-6 than the control rats, which showed a statistical difference (P<0.05). The colorectal cancer rats with HP infection presented a lower level of TNF-α, IL-8, and IL-6 than the miR-7 down-regulated rats, but a higher level of TNF-α, IL-8, and IL-6 than the control and miR-7 up-regulated rats. There was a statistical difference (P<0.05). There was also a statistical difference (P<0.05) when the level of TNF-α, IL-8 and IL-6 in the miR-7 down-regulated rats was higher than that in control, miR-7 up-regulated and colorectal cancer rats with HP infection. The miR-7 up-regulated rats had a higher level of TNF-α, IL-8 and IL-6 than the control rats but a lower level of TNF-α, IL-8 and IL-6 than the miR-7 down-regulated rats and colorectal cancer rats with HP infection. Such a difference was statistically significant (P<0.05).

miR-7 expression and HP improvement

As shown in Table 1, the control group showed lower expression in miR-7 than both the colorectal cancer and HP infection model group and the miR-7 up-regulated group and higher expression than the miR-7 down-regulated group, which revealed a statistical difference (P<0.05). The expression of miR-7 in colorectal cancer and HP infection model group was higher than that in the control group and the miR-7 up-regulated group with a statistical difference (P<0.05); the expression of miR-7 in the miR-7 down-regulated group was higher than that in the other three groups. There was a statistical difference (P<0.05); the expression of miR-7 in the miR-7 up-regulated group was lower than that in the other three groups with a statistical difference (P<0.05). Colorectal cancer and HP infection model group had the same positive rate of HP as the miR-7 down-regulated group, which was significantly higher than that in the control and miR-7 up-regulated groups, hence a statistical difference (P<0.05).

Akt, GSK-3β and β-catenin expression

As shown in Fig. 3 and 4, the control group showed higher expression in Akt, GSK-3β and β-catenin than the colorectal cancer and HP infection model group. The miR-7 up-regulated group, and the miR-7 down-regulated group revealed a statistical difference(P<0.05). Expression of Akt, GSK-3β and β-catenin in colorectal cancer

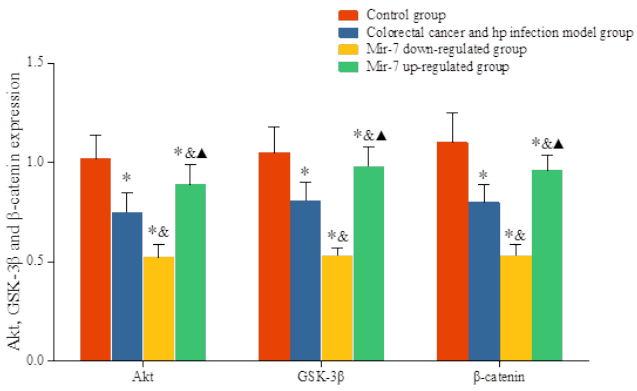

Figure 3. Akt, GSK-3β and β-catenin expression ($\bar{x} \pm s$). Note: Compared with the control group, $^{*}P<0.05$; compared with the colorectal cancer and HP infection model group, $^{\&}P<0.05$; compared with the miR-7 up-regulated group, $^{\blacktriangle}P<0.05$.

Table 1. Analysis of miR-7 expression and HP improvement in two groups [($\bar{x} \pm s$) , (n,%)]

Group	Quantity (n)	miR-7	HP positive rate (n,%)
Control group	10	0.86±0.15	0 (0.00)
Colorectal cancer and hp infection model group	10	2.05±0.32*	10 (100.00)
Mir-7 down-regulated group	10	0.51±0.10$^{*\&\blacktriangle}$	10 (100.00)
Mir-7 up-regulated group	10	3.08±0.52$^{*\&\blacktriangle}$	2 (20.00)$^{\&\blacktriangle}$

Compared with the control group, $^{*}P<0.05$; with colorectal cancer and HP infection model group, $^{\&}P<0.05$; compared with the miR-7 up-regulated group, $^{\blacktriangle}P<0.05$.

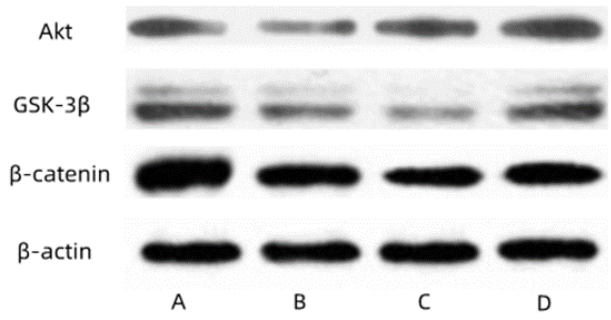

Figure 4. Akt, GSK-3β and β-catenin W B expression map. Note: Compared with the control group, $^{*}P<0.05$; compared with colorectal cancer and HP infection model group, $^{\&}P<0.05$; compared with the miR-7 up-regulated group, $^{\blacktriangle}P<0.05$. Note: A: the control group; B: colorectal cancer and HP infection model group; C: the miR-7 down-regulated group; D: the miR-7 up-regulated group.

and HP infection model group was higher than that in the miR-7 down-regulated group but lower than that in the control and up-regulated groups, with statistical difference (P<0.05); the expression of Akt, GSK-3β and β-catenin in the miR-7 down-regulated group was lower than that in the other three groups. There was a statistical difference (P<0.05); the expression of Akt, GSK-3β and β-catenin in the miR-7 up-regulated group was lower than that in the control group but higher than that in colorectal cancer and HP infection model group, and the miR-7 down-regulated group, suggesting a statistical difference (P<0.05).

Discussion

Colorectal cancer is a malignant tumor of the digestive tract that usually occurs in the colon and rectum with high clinical morbidity and fatality rate. As the eating habit of Chinese people has changed in recent years, the incidence of colorectal cancer is on the rise, which seriously affects the physical and mental health and further endangers the life safety of patients(7-8). HP, as one of the common bacteria for chronic infection, is closely related to several stomach diseases and has a certain correlation with the occurrence of colorectal cancer(9). Currently, although there are a variety of clinical methods, including surgery, Western medicine and traditional Chinese medicine, to treat the disease, all of them have certain limitations.

At present, after an in-depth research on the molecular mechanism of colorectal cancer, many clinical scholars believe that the occurrence and development of colorectal cancer are marked by distinct molecular characteristics that mainly include activation of oncogenes, inactivation of tumor suppressor genes, and instability of the genome(10-11). Despite the great achievements in researches, there are still problems such as the poor signaling pathway mechanism, and the failure to reach a consensus on the relationship between genes and colorectal cancer. As a special non-coding RNA, miR has become a new hotspot in RNA research(12). Among three clinical subtypes of miR, namely miR-7-1, miR-7-2 and miR-7-3, which have different locations in the genome, miR-7 shows a decreasing trend in terms of expression in colorectal cancer according to findings of clinical studies. Thus, up-regulation of miR-7 plays a role in effectively intervening in HP infection-induced rats with colorectal cancer(13). Some scholars have pointed out in their studies that the inflammatory factors see an upward trend in colorectal cancer rats with HP infection. Detection of TNF-α, IL-8 and IL-6, as three common clinical indicators for inflammation detection, can contribute to an accurate appraisal of the inflammation(14-15). In this paper, after miR-7 is up-regulated, the level of TNF-α, IL-8, and IL-6 of colorectal cancer rats with HP infection decrease indicating that the up-regulated miR-7 has an intervention effect by effectively improving the inflammation in colorectal cancer rats with HP infection. Akt/GSK-3β/β-catenin pathway is a cancer regulatory pathway newly discovered in recent years. Clinical studies have found the abnormal expression of Akt/GSK-3β, Wnt and other key pathways in the colorectal cancer tissues(16-17). β-catenin, which acts as an effector molecule to activate target genes at the end of the typical Wnt pathway in downstream Wnt protein can be continuously degraded by the degradation complex formed by GSK-3β. Among the factors affecting the content of β-catenin, inactivation of GSK-3β is the key attribute for the aggregation. GSK-3β has a variety of cell functions and can participate in the physio-pathology process of the body by phosphorylating the substrates(18-19). Abnormal activation of Akt, as an upstream regulatory factor of GSK-3β, leads to the deterioration of normal cells through multiple pathways(20). In this paper, we find that up-regulation of miR-7 in colorectal cancer rats with HP infection can contribute to an increase in the expression of Akt, GSK-3β, and β-cateni, indicating the good effect of miR-7 in treating colorectal cancer rats with HP infection through the 3β/β-catenin pathway.

In conclusion, the up-regulated miR-7 has an intervention effect on colorectal cancer rats with HP infection through the Akt/GSK-3β/β-catenin pathway by inhibiting the level of inflammatory factors and improving the HP clearance rate.

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