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# Screening of differentially expressed microRNA in ulcerative colitis related colorectal cancer

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## ABSTRACT

**Objective:** To investigate the differential expression of microRNA (miRNA) in colon between ulcerative colitis (UC) and ulcerative colitis related colorectal cancer (UCRCC). **Methods:** An UC mouse model was built by dextran sodium sulfate, and an UCRCC mouse model by dextran sodium sulfate and 1,2-diformylhydrazine. RNAs were extracted from the colon, purified and hybridized with fluorescence-labeled miRNA oligonucleotide gene chip. Real-time fluorescence quantitative PCR was used to verify the expression variation of miRNA. SAM was employed for the data analysis. **Results:** The up-regulated miRNAs in colon cancer included has-miR-194, has-miR-215, has-miR-93, has-miR-192, has-miR-92a, has-miR-29b, and has-miR-20a (median false discovery rate < 5%), while the down-regulated miRNAs were has-miR-1231, has-miR-195, has-miR-143, and has-miR-145 (median false discovery rate < 5%). **Conclusions:** Significant differential expression of miRNA was found between the UC mouse and UCRCC mouse, which may be related to the onset, erosion and transfer of colorectal cancer.

## 1. Introduction

Ulcerative colitis (UC) is a non-specific chronic inflammatory disease in rectum and colon. According to the statistics, the number of UC inpatients increased by 3 times in recent 10 years. It tends to occur in proximal section and even spreads to whole colon, which always leads to diarrhea, bellyache and mucous bloody stool. The disease situation is always repeated. One of severe complications of UC is the UC related colorectal cancer (UCRCC), which has a strong impact on life quality and longevity of

patients. Presently, it has been accepted that the UC and carcinogenesis are related to abnormal immunomodulation of intestinal mucous membrane, constant enteral infection, and barrier injury of intestinal mucous membrane, genetics and environmental factors. MicroRNA is involved in the post-transcriptional regulation of genes and affects the growth and metabolism of cells. To investigate the roles of microRNA in UC and UCRCC, we detected its expression in mice with UC and UCRCC.

## 2. Materials and methods

### 2.1. Mice

Eighty Balb/c mice with the age of 6–8 weeks and weight of 22–30 g were purchased from Guangdong Medical

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Laboratory Animal Center. They were bred in SPF grade rat/mouse breeding room and received 1 week of adaptive breeding under the constant humidity and temperature before the experiment.

## 2.2. Establishment of animal models and sample preparation

The mice were randomly divided into control group ( $n=20$ ), UC group ( $n=30$ ) and colorectal cancer group ( $n=30$ ). The inflammatory agent dextran sodium sulfate (DSS; Sigma-Aldrich, USA) was adopted to induce the mouse model of chronic UC. After 1 week of freely feeding 35 g/L DSS, the mice received the intraperitoneal injection of physiological saline and 2 weeks of normal water drinking. This cycle cost 3 weeks and three cycles were used to establish the UC model. The UCRCC model was established in the same way, except that the mice received the intraperitoneal injection of 1,2-diformylhydrazine (DMH; Sigma-Aldrich) at a dose of 20 mg/kg after 1 week intake of DSS. The mice in the control group had free access to water and were intraperitoneally injected with physiological saline. After 9 week induction, the murine status, stools and survival were observed and the disease activity index (DAI) was recorded according to their weight, stools and occult blood. The scoring standard is as follows: 1 point for the weight loss of 1%–5%, 2 points for 5%–10%, 3 points for 10%–15%, and 4 points for >15%; 1–2 points for the pale stool and 3–4 points for the loose stool; 1–2 points for the occult blood and 3–4 points for macroscopic bloody stool. After the mice were killed by cervical dislocation and laparotomized, the segment from ileocecum to colon of anus was dissociated and longitudinally dissected. The patulous intestinal cavity was then cleaned with physiological saline and cut into two parts. One was immediately transferred into liquid nitrogen and then stored in a refrigerator at  $-80^{\circ}\text{C}$  for mRNA detection, and the other one was fixed by formaldehyde for histopathological observation.

## 2.3. Total RNA extraction and chip hybridization

The samples were milled into powder, and ion-exchange column adsorptive process was adopted to extract total RNA using mirVana™ RNA isolation kit (Ambion). Then the concentration and purity of the total RNA were detected using ultraviolet spectrophotometer and formaldehyde-agarose gel electrophoresis, and the extract with  $A_{260}/A_{280}$  of 1.9–2.1 was qualified. One oligonucleotide was marked at the end of poly-A tail for fluorescence labeled connection. Through a microcirculation pump, chip hybridization was

performed using miRNA oligonucleotide gene chip kit. Then GeneChipR hybridization wash and stain kit (Affymetrix) was used for digital washing and treatment of staining signals, and data were obtained using the professional software miRNA QC tool.

## 2.4. RT-PCR

According to the results of miRNA microarray, some miRNAs with the differential expression was further detected by real-time quantitative PCR. The 5S rRNA was used as the reference gene, and about 80 nucleotide long cDNA was obtained. The upstream primer sequences are presented in Table 1. The downstream primer provided by the kit was used (5′-GCTGTCAACGATACGCTACGTAA-3′). PCR was performed using the following temperature profile: denaturalization at  $95^{\circ}\text{C}$  for 2 min, 40 cycles of  $94^{\circ}\text{C}$  for 20 s, at  $58^{\circ}\text{C}$  for 20 s and  $72^{\circ}\text{C}$  for 30 s. After the reaction, the results were analyzed using the  $2^{-\Delta\Delta\text{CT}}$  equation. All primers were purchased from Shanghai Biotech Co., Ltd.

**Table 1**

Upstream primer sequences for differentially expressed miRNAs.

miRNAs	Upstream primer sequence
has-miR-20a	5′-AAAGTCCTTATAGTCAGGTAGAAAA-3′
has-miR-29b	5′-ACACTCCAGCTGGGTAGCACCATTGAAATC-3′
has-miR-92a	5′-TGTCGCCGGCCTGTAAAAA-3′
has-miR-93	5′-TGCTGTTTCGTGCAGGTAGAA-3′
has-miR-143	5′-GAGATGAAGCACTGTAGCTCAAAA-3′
has-miR-145	5′-TTTTCCAGGAATCCCTAAAA-3′
has-miR-192	5′-ACACTCCAGCTGGGCTGACCTATGAATG-3′
has-miR-194	5′-ACACTCCAGCTGGGTGTAACAGCAACTCCA-3′
has-miR-195	5′-TAGCAGCACAGAAATATGG-3′
has-miR-215	5′-ACACTCCAGCTGGGATGACCTATGAATG-3′
has-miR-1231	5′-ACACTCCAGCTGGGGTGTCTGGCCGGAC-3′

## 2.5. Histopathological observation

After the fixation with formaldehyde, the lesion part of distal colonic tissue was embedded by paraffin and cut into sections with the thickness of  $4\ \mu\text{m}$ . Then the sections were stained by hematoxylin and eosin (HE) solution (Beijing Zhong Shan Golden Bridge Biological Technology) and observed under an optical microscope.

## 2.6. Statistical analyses

The microarray data analysis software for the significant difference was employed to analyze the differential expression of miRNA. The median false discovery rate (FDR) indicates the probability to cause the differential gene expression by accidental factors.  $\text{FDR}<0.05$  means

the statistical significance for the differential expression of miRNA. The results of real-time fluorescence quantitative PCR were used for the statistical analysis on the differences of gene expression.  $P < 0.05$  was considered statistically significant.

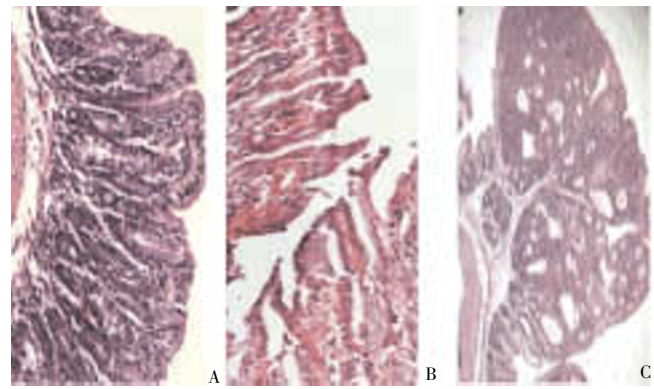
### 3. Results

#### 3.1. Comparison on DAI between the groups

The mice in the control group had normal mental state and diet, no weight change, and no diarrhea or bloody stool. The DAI score of the control group was 0. Diarrhea began to occur after 3–4 days in the mice of the UC group, and all mice in this group suffered diarrhea, bloody stool or adhesion of loose/bloody stool to anus after 1 week. They were also faint, slouchy and disheveled. During the 2nd and 3rd cycles, they were careless and usually stayed still. The DAI score of the UC group was  $18.2 \pm 1.2$ , which was significantly higher than that of the control group ( $P < 0.05$ ). The mice in the colorectal cancer group also had similar manifestations as those in the UC group, but they were weaker during the 2nd and 3rd cycles. The DAI score of the colorectal cancer group was  $33.5 \pm 1.4$ , which was significantly higher than that of the UC group ( $P < 0.05$ ).

#### 3.2. Comparison on histopathological changes between the groups

As revealed by HE staining, the mice in the control group had complete and clear structure of mucous colonic epithelium, orderly arranged epithelia, and complete glandular organs. Obvious histopathological changes were observed in the mice of the UC group, as displayed by remarkably shortened colon, thickened and hardened intestinal wall, comprehensively congested and swollen intestinal mucosa, local erosion and hemorrhage, and distinct ulcerative regions. Moreover, large-area disappearance of mucosa, incomplete structure of most glandular organs, and wide infiltration of inflammatory cells also indicated the typical inflammatory responses in these mice. In the mice of the colorectal cancer group, tuberculum minus appeared in the colonic wall. In addition, thickened cavity, hardened wall, large-area disappearance of mucosa and incomplete structure of most glandular organs were also observed. No distinct changes were found in the small intestine. Colonic adenocarcinoma and senior dysplasia suggested typical carcinogenesis in these mice (Figure 1).



**Figure 1.** Histopathological changes in colon.

A. Control group; B. Ulcerative colitis group; C. Colorectal cancer group.

#### 3.3. Effects of UCRCC on miRNA levels

According to the analysis of the miRNA chip results by SAM software, the miRNA levels in mucous membrane varied in the UC group, compared with those in the control group, but the FDRs were not significantly different between the two groups (Table 2). The miRNA levels in mucous membrane significantly varied in the colorectal cancer group, and the FDRs was significantly different from those in the control and UC groups ( $P < 0.05$ ) (Table 2). The levels of seven miRNAs were up-regulated significantly, including has-miR-194, has-miR-215, has-miR-93, has-miR-192, has-miR-92a, has-miR-29b, and has-miR-20a, while the levels of four miRNAs were down-regulated significantly, including has-miR-1231, has-miR-195, has-miR-143 and has-miR-145 (Table 3). The real-time quantitative PCR also confirmed the same results and demonstrated the reliability of the miRNA chip (Table 3).

**Table 2**

Differential expression of miRNA between the groups as detected by RT-PCR.

miRNAs	Control group	Ulcerative colitis group	Colorectal cancer group
has-miR-194	7.6	7.7	8.6* $\Delta$
has-miR-215	11.5	11.7	13.1* $\Delta$
has-miR-192	14.0	14.3	16.3* $\Delta$
has-miR-93	14.3	14.7	17.5* $\Delta$
has-miR-29b	16.7	17.1	20.7* $\Delta$
has-miR-92a	10.9	11.3	12.5* $\Delta$
has-miR-20a	13.4	13.7	16.5* $\Delta$
has-miR-1231	18.6	18.3	13.0* $\Delta$
has-miR-195	14.5	14.3	10.6* $\Delta$
has-miR-143	16.5	16.2	13.5* $\Delta$
has-miR-145	22.5	22.0	18.4* $\Delta$

\* $P < 0.05$  vs. the control group;  $\Delta P < 0.05$  vs. the ulcerative colitis group.

**Table 3**

Differential expression levels of miRNAs as detected by chip and RT-PCR.

miRNAs	RT-PCR	Chip
Up-regulated miRNAs		
has-miR-194	3.32	3.26
has-miR-215	3.56	4.33
has-miR-192	7.64	6.84
has-miR-93	1.83	1.54
has-miR-29b	1.74	1.89
has-miR-92a	1.58	1.56
has-miR-20a	1.43	1.39
Down-regulated miRNAs		
has-miR-1231	0.69	0.75
has-miR-195	0.56	0.53
has-miR-143	0.52	0.48
has-miR-145	0.57	0.58

The differential time in chip microarray is the ratio of standard value of UCRC group to that of control group. The differential times in RT-PCR were obtained using the  $2^{-\Delta\Delta CT}$  equation.

#### 4. Discussion

UC is a non-specific colorectal inflammation caused by unknown reasons. The pathological changes mainly occur in the mucous membrane and submucosa of colon, showing the continuous and diffuse distribution. The risk of UC carcinogenesis tends to be increased year by year. The possibility for patients with UC to suffer the colorectal cancer is 20–30 times higher than that for the general population<sup>[1]</sup>. The miRNA is a group of endogenous non-coding RNA with the length of 19–24 nucleotides. Its primary function is to complementarily pair with specific bases from mRNA of target gene and to negatively regulate the gene expression by degrading the target mRNA and restraining the translation of target mRNA<sup>[2,3]</sup>. Current researches have shown that miRNA is closely related to the growth development of animals and plants, cell differentiation and death, fat metabolism and some serious diseases in human<sup>[4–7]</sup>. As miRNA plays a regulatory role in gene expression, protein translation and occurrence of tumors, miRNA may be a possible oncogene or tumor suppressor gene. The miRNA expression profile in tissues or cells is thus of critical significance for the in-depth researches to investigate the biological roles of miRNA in human diseases.

Depending on building the mouse model of ulcerative colitis and related colorectal cancer, this paper adopted miRNA expression profile microarray to detect the expression profile of miRNA in mucous membrane of mouse ulcerative colitis/normal large intestine and colorectal cancer/ulcerative colitis, and discuss the reliability of results of miRNA profile microarray.

Experimental results showed that the mental state, activity and stool of mice in the control group were all

normal, without situations of diarrhea or bloody stool. Mice in the ulcerative colitis group had the diarrhea, bloody stool or loose/bloody stool adhered to anus 3–5 days after establishing the model. The diarrhea was most serious in the 1st cycle. In the 2nd and 3rd cycles, the clinical manifestations of ulcerative colitis such as diarrhea were weakened, but the mental state became worse and the body gradually became thin. Mice in the colorectal cancer group had similar manifestations to ones in the ulcerative colitis group in the 1st cycle. But in the 2nd and 3rd cycles, manifestations of diarrhea, bloody stool or loose/bloody stool adhered to anus were more serious than ones in the ulcerative colitis group, and the weight loss was also faster. It indicated the smooth model building and it was confirmed by the histopathological analysis. The combined methods of DSS and DMH can build the ideal animal model of ulcerative colitis associated carcinogenesis and provide the important animal model for the further study on the mechanism of ulcerative colitis associated carcinogenesis.

A great number of researches showed that there were abnormal expressions of miRNA in many human tumors<sup>[8–12]</sup>. According to our experimental results, the difference in miRNA was not significant between the ulcerative colitis group and control group; but there was significant change in miRNA in the colorectal cancer, where 7 miRNAs were significantly up-regulated, including has-miR-194, has-miR-215, has-miR-93, has-miR-192, has-miR-92a, has-miR-29b, and has-miR-20a and 4 miRNAs were significantly down-regulated, including has-miR-1231, has-miR-195, has-miR-143 and has-miR-145. Link *et al*<sup>[13]</sup> indicated the high expression of miR-92a in tissues of colorectal cancer, even in the stool of patients with colorectal cancer, and its increase in expression was reversely proportional to the survival period and prognosis, which was in line with results of this research. Wang *et al*<sup>[14]</sup> showed the low expression of miR-143 and miR-145 in colorectal cancer cells. The over-expression of miR-143 can also reduce the colony formation of colorectal cancer cells on soft agar culture medium<sup>[15]</sup>, and miR-143 and miR-145 were recognized as tumor suppressors<sup>[16]</sup>. Xu *et al*<sup>[17]</sup> drew the conclusion that the expression level of miR-195 was reversely proportional to the growth of colorectal cancer cells and its high expression could suppress the growth of cancer cells. Thus it can be regarded that the specific miRNA is closely related to the occurrence and pathologic process of colorectal cancer, which may provide new indicators for the diagnosis, treatment and prognosis of colorectal cancer. According to results of real-time fluorescent quantitative RT-PCR experiment, results of quantitative PCR detection was in line with the microarray results, which indicated that our experimental results were reliable. At present, researches show that miRNA microarray is characterized by high-throughput united detection, high detection efficiency and sensitivity and small sample size, which is significantly better than the traditional RNA detection. It



is a technology that can detect the expression of miRNA in cells or tissues under different physiological conditions and it will contribute to the researches on biological functions of miRNA[18].

In conclusion, there was high expression of has-miR-194, has-miR-215, has-miR-93, has-miR-192, has-miR-92a, has-miR-29b and has-miR-20a, but low expression of has-miR-1231, has-miR-195, has-miR-143 and has-miR-145 in the colorectal cancer group. A significant differential expression of miRNA could be found between intestinal mucosas of colorectal cancer and ulcerative colitis, which indicated that these miRNAs might participate in the onset and migration of colorectal cancer. It's worthy to take the further study on the molecular mechanism of miRNAs in colorectal cancer.

### Conflict of interest statement

We declare that we have no conflict of interest.

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