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Original Article

Expression of miRNAs in the colon, rectum, plasma, and feces in rats with dextran sulfate sodium

Terutaka Kodama[1], Yuko Togashi[1], Naomi Matsutani[1], Seiichiro Kurashige[1], Toyohiko Aoki[2] and Yasuteru Otagiri[1]

[1]Toxicology and Pharmacokinetics Research Dept., Research InstituteEA Pharma Co., Inc., 1-1 Suzuki-cho, Kawasaki, Kanagawa 210-8681, Japan
[2]DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya, Aichi, 491-0113, Japan

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ABSTRACT — We investigated the expression of miRNAs as a potential biomarker in the colon, rectum, plasma, and feces of the rat dextran sulfate sodium (DSS)-colitis model. A 5% DSS solution with drinking water was administered to male SD rats for 1 week, followed by a 1-week off-dose period. In-life parameters were examined daily, and colon length and pathological changes were assessed post-mortem. A selected panel of miRNAs (miR-16-3p, miR-21-5p, miR-31a-5p, miR-34a-5p, miR-146b-5p, miR-155-5p, miR-181b-5p, miR-221-5p, and miR-223-3p) was also measured in the colon, rectum, plasma, and feces using digital polymerase chain reaction (PCR). A high disease activity index (DAI) and reduction in colon length were observed in DSS-treated rats. Erosion and inflammatory cell infiltration were evident in the colon and rectum after DSS treatment. These parameters tended to recover, and regenerative hyperplasia was observed in the rectum after the recovery period. After the end of the administration period, all nine selected miRNA levels showed lower expression in colonic and rectal tissues. miRNA levels, except miR-21-5p and miR-155-5p, were lower in both plasma and feces at 5% DSS group. In contrast, at the end of the recovery period, miRNA levels tended to increase in all samples. Particularly, a higher expression of miR-31a-5p, miR-181b-5p, and miR-223-3p was observed in feces. We suggest that the miRNAs from colon, rectum, plasma, and feces are potential quantitative and sensitive biomarkers in the rat DSS-colitis model. In particular, miR-31a-5p, miR-181b-5p, and miR-223-3p from feces could be used as non-invasive biomarkers to evaluate the reversibility in DSS-treated rats.

Key words: miRNA, Digital PCR, Dextran sulfate sodium (DSS), Rats

INTRODUCTION

In recent years, up- or down-regulation of several miRNAs during inflammatory bowel disease (IBD) have been reported both clinically and non-clinically. It has been reported that miR-16 is closely related to the occurrence of several autoimmune diseases, including IBD (Yan *et al.*, 2019). Patients with ulcerative colitis (UC) displayed significantly higher levels of miR-223 and miR-1246 than healthy subjects (Verdier *et al.*, 2020). Sera samples from IBD patients showed higher levels of miR-16, miR-21, and miR-223 (Schönauen *et al.*, 2018). Silencing of miR-155 was found to attenuate dextran sulfate sodium (DSS)-induced colitis by regulating Th17/Treg cell balance, and Jarid2/Wnt/β-catenin participates in this process (Zhu *et al.*, 2020). Resveratrol-mediated attenuation of colitis may be regulated by miR-31 through the induction of Tregs, and miR-31 may serve as a therapeutic target for human colitis (Alrafas *et al.*, 2020). The miRNA expression profiles dynamically changed either by overexpression

Correspondence: Terutaka Kodama (E-mail: terutaka_kodama@eapharma.co.jp)

(miR-181b, miR-31, miR-146b, miR-221, and miR-155) or by suppression (miR-34a) through the NF-κB-STAT3 crosstalk in the azoxymethane (AOM)/DSS-induced colon tumorigenesis in mice (El-Daly *et al.*, 2019).

As the expression of selected miRNAs in IBD patients varies widely between individuals, there have been no reports of concomitant measurement of miRNAs in the colon, rectum, plasma, and feces in rats during the DSS treatment. In this study, we examined the relationships between the variability of miRNAs among these samples and investigated the expression of miRNAs in these samples as a potential biomarker in the rat DSS-colitis model.

MATERIALS AND METHODS

Animal study

Nineteen male Sprague-Dawley rats (Crl:CD [SD]), 5 weeks old, were purchased from Charles River Japan, Inc. (Shiga, Japan). The rats were individually housed in a room maintained under controlled temperature (22 ± 3°C) and relative humidity (55 ± 15%), and a 12 hr light/dark cycle (7:00–19:00) in a barrier system. A standard commercial diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water were available *ad libitum*. After acclimatization for 6 days, the rats were randomly allocated to four groups (n = 4 each in control and DSS groups as the dosing groups and n = 4 each in control and DSS groups as the recovery groups) based on their body weight. Animals that were not assigned to any study group were excluded from the study.

DSS was obtained from MP Biomedicals (Santa Ana, CA, USA) and dissolved in tap water to obtain appropriate concentrations. Control rats received only tap water. The dosing solution was administered in drinking water at daily doses of 0 (control) or 5 w/v% DSS for 7 days. After the 7-days dosing, the administration of DSS was withdrawn for the next 7 days. Dosage levels were selected based on the results of the preliminary studies (data not shown). During the dosing and recovery periods, rats were observed once daily for general clinical signs and stool. All animals were weighed, and food consumption and water intake were measured daily from the starting dose to the day of necropsy. The average DSS intake was 7713.18 mg/kg/day in the 5 w/v% group. Based on these data, the disease activity index (DAI; [sum of scores of body weight gain, fecal condition, and bloody stool]/3) was calculated for each individual (Table 1).

Rats were sacrificed under anesthesia with isoflurane and necropsied at the end of the administration period or the recovery period. The thoracic and intraperitoneal organs were then examined macroscopically. The GI tract (stomach to rectum) was then removed. Colon length (the boundary between the cecum and colon to the anus) was measured. The large intestine was fixed with 10% buffered formalin. All the tissues obtained were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Histopathological examination of the large intestine was conducted using light microscopy. Blood was collected from the abdominal aorta, and feces and tissues (distal colon to rectum) were obtained on each day of necropsy for measuring miRNA. All experiments were conducted in conformity with the "Guide for animal experiments done at the DIMS Institute of Medical Science, Inc., Aichi, Japan.

Table 1. Score for DAI.

Score	Body weight gain (%)	Fecal condition	Bloody stool
0	No decrease	Normal	Not detected
1	−5 to −1		
2	−10 to −5	Loose stool	
3	−20 to −10		
4	< −20	Diarrhea	Present

Measurement of miRNAs

Blood samples were obtained from the abdominal aorta of rats under isoflurane anesthesia on the day of necropsy. Blood was collected into tubes containing EDTA-2K, centrifuged at approximately 3,000 g for 15 min at 4°C and filtered. Plasma was collected and preserved below −70°C. Feces from the colon to the rectum were collected at autopsy and preserved below −70°C. Tissues were collected from the rectum and distal colon, 5 mm square × 3 each, and left overnight at 2–10°C, immersed in RNA*later* (Thermo Fisher Scientific K.K., Tokyo, Japan), and then preserved below −70°C.

Digital PCR was conducted for plasma, feces, and tissue samples. Briefly, total RNA was extracted with QIAzol using miRNeasy Serum/Plasma kit (QIAGEN K.K., Tokyo, Japan) for plasma, miRNeasy Micro kit (QIAGEN K.K.) for tissues, and RNeasy PowerMicrobiome Kit (QIAGEN K.K.) for feces. cDNA for miRCURY LNA miRNA PCR assay was synthesized using the miRCURY LNA RT Kit (QIAGEN K.K., Catalog #339340). The synthesized cDNA was stored at −20°C. The cDNA was diluted 10-fold to form a DNA template for PCR. After preparing the PCR reaction solution with 2 × EvaGreen ddPCR SuperMix (Catalog #186-4033, Bio-Rad, Tokyo, Japan), a droplet was prepared using the QX200™ Droplet Digital™ PCR system (Catalog #1864001, Bio-Rad). PCR was conducted on a ProFlex™ PCR Sys-

tem (Thermo Fisher Scientific K.K.) according to the manufacturer's instructions, and the fluorescence intensity was read using a QX200 Droplet Reader (Bio-Rad) and the QuantaSoft software (Bio-Rad). The target genes evaluated were: miR-16-3p, miR-21-5p, miR-31a-5p, miR-34a-5p, miR-146b-5p, miR-155-5p, miR-181b-5p, miR-221-5p, and miR-223-3p. The relative fold-change of individual values to the mean value of the control group was calculated for each miRNA.

Statistical analysis

For DAI, colon length, and each miRNA, mean values and standard deviation (SD) were calculated for each measurement day and for each group. In addition, for colon length and each miRNA for each group, the data were first analyzed using F test (two-tailed, significance level: $p < 0.05$) to determine homogeneity of variance. When the variance was homogeneous, the Student's t-test (two-tailed, significance level: $p < 0.05$) was used to compare the averages between the control and 5% DSS groups. When the variance was heterogeneous, the Welch test (two-tailed, significance level: $p < 0.05$) was used. As a minimum number of animals required from the viewpoint of animal welfare was used in this study, a comprehensive evaluation was conducted by adding a biological evaluation to the statistical analysis.

RESULTS

Clinical observations

No animal died during the experiment. In general clinical observations, anal bleeding was observed in 4 rats in the 5% DSS group. In the 5% DSS group, food intake tended to be lower than that in the control group from Day 2 to recovery Day 5 (data not shown). Loose stool was observed in all animals in the 5% DSS group during the administration period, and bloody stool was observed in 4 rats. Loose stool stopped by Day 2 of recovery, except for 1 rat in the 5% DSS group. DAI increased in the 5% DSS group with the duration of DSS dosing, and then decreased with the lapse of the recovery period (Fig. 1).

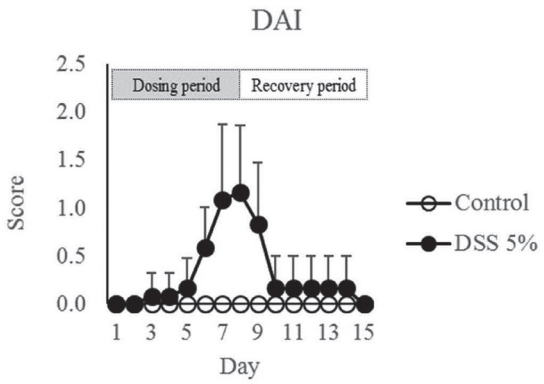

Fig. 1. DAI during the 1-week repeat dosing and recovery periods (Mean ± S.D.). N = 8/group during the dosing period. N = 4/group during the recovery period.

Pathological findings in colon and rectum

On macroscopic examination, no notable changes were observed in DSS-treated rats at the end of the administration and recovery periods. The colon length was significantly shortened in the 5% DSS group at the end of the administration period compared to that in the control group, and no recovery was detected after the recovery period (Table 2). Histopathological examination revealed erosion and inflammatory cell infiltration in the colon of 1 rat at a minimum grade and in the rectum of all 4 rats at a relatively higher grade and larger distribution in the 5% DSS group (Table 3). At the end of the recovery period, erosion or inflammatory cell infiltration in the colon resolved, but were still observed in the rectum with partial recovery. Regenerative hyperplasia was also observed in the rectum.

miRNA levels in colon, rectum, plasma, and feces

Figure 2 shows the results of miRNA levels in the colon, rectum, plasma, and feces of the control group, obtained using digital PCR. For all miRNAs, although there was a difference in absolute values depending on the sampling location, the levels on Day 8 and on Day 8 of recovery (recovery Day 8) were similar, and no time-dependent difference was observed.

Table 2. Colon length at the end of the administration and recovery periods.

Test article	Control		DSS	
Dose (w/v%)	0		5	
Day	Day 8	Rec. Day 8	Day 8	Rec. Day 8
Number of animals	4	4	4	4
Colon length (cm)	19.6 ± 0.6	19.1 ± 0.8	16.8 ± 0.5**	16.4 ± 1.7*

Data are shown as mean ± SD. *: $p < 0.05$, **: $p < 0.01$

Table 3. Histopathological findings in large intestine at the end of the administration and recovery periods.

Test article		Control		DSS	
Dose (w/v%)		0		5	
Day		Day 8	Rec. Day 8	Day 8	Rec. Day 8
Number of animals		4	4	4	4
No remarkable changes		4	4	0	0
Colon, mid					
Erosion, focal	Minimal	0	0	1	0
Colon, distal					
Erosion, focal	Minimal	0	0	1	0
Infiltrate inflammatory cell, mucosal, focal	Minimal	0	0	1	0
Rectum					
Erosion, focal	Minimal	0	0	1	0
	Slight	0	0	0	2
Erosion, diffuse	Slight	0	0	2a	1a
	Moderate	0	0	1a	1a
Regenerative hyperplasia		0	0	0	4
Infiltrate inflammatory cell, mucosal, diffuse	Minimal	0	0	0	1
	Slight	0	0	1	0
Infiltrate inflammatory cell, submucosal, diffuse	Slight	0	0	2	3
	Moderate	0	0	1	0

a: Granulation was accompanied.

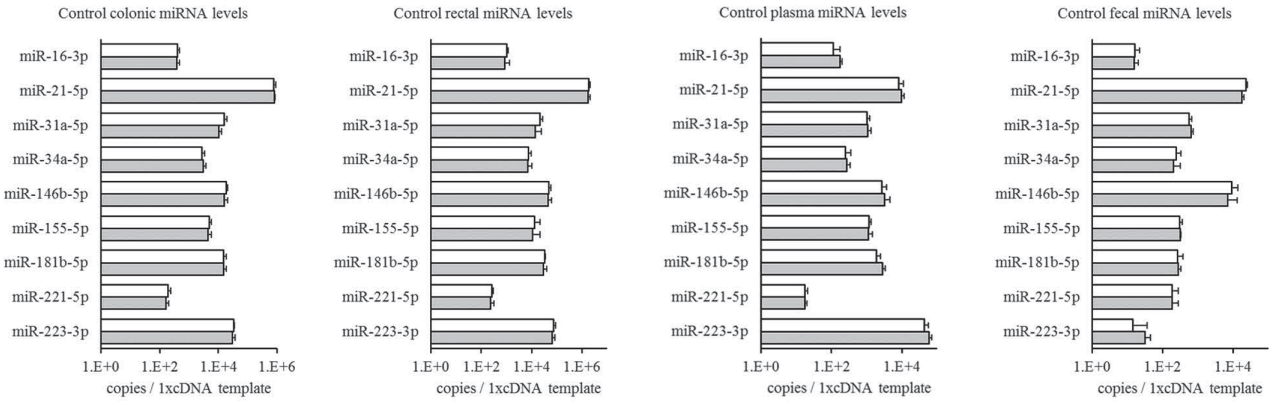

Fig. 2. miRNA levels in the colon, rectum, plasma, and feces in the control group (Mean ± S.D.). The white bar represents miRNA levels at the end of the dosing period (Day 8). The gray bar represents miRNA levels at the end of the recovery period (recovery Day 8). N = 4/group.

Figure 3 shows the miRNA levels in the colon, rectum, plasma, and feces. On Day 8, colonic miRNA levels were significantly lower for all miRNAs compared to the levels in each control group (Fig. 3A). Rectal miRNA levels were significantly lower than those in each control group for all miRNAs, except for miR-155-5p. miR-155-5p also showed similarly low values as those of other miRNAs (Fig. 3B). Plasma miRNA levels were significantly lower than those in each control group for all miRNAs except for miR-31a-5p and miR-181b-5p. The miR-181b-5p value was also similar to those of other miRNAs (Fig. 3C). Fecal miRNA levels were significantly lower for miR-16-3p, miR-31a-5p, miR-34a-5p, miR-181b-5p, and miR-221-5p as compared to the levels in each control group. In addition, although the difference was not significant, miR-146b-5p and miR-223-3p showed lower values, similar to those of other miRNAs (Fig. 3D). On recovery Day 8, colonic miRNA levels (Fig. 3E) and plasma miR-

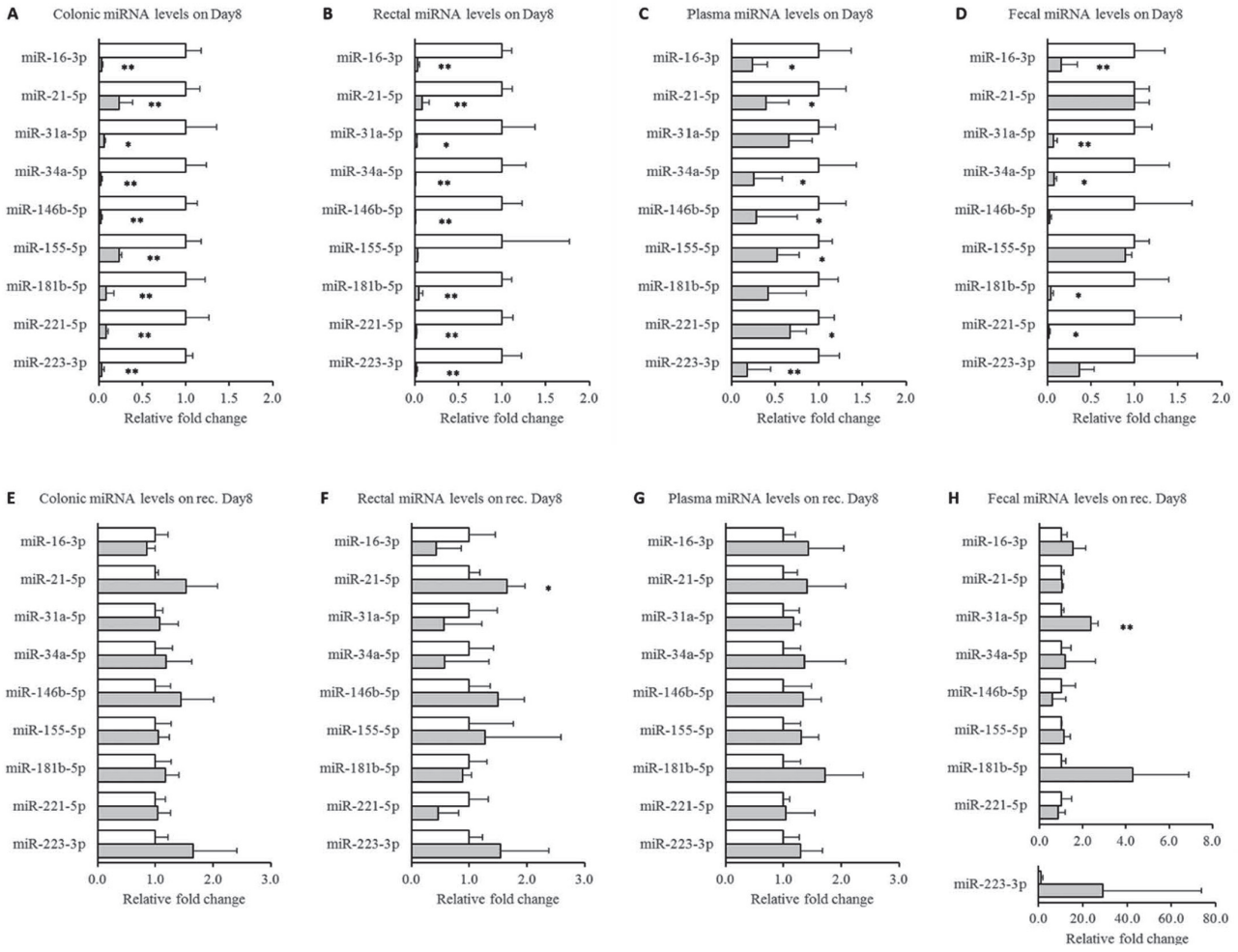

Fig. 3. miRNA levels in the colon, rectum, plasma, and feces (Mean ± S.D.). The white bars represent miRNA levels in the control group. The gray bars represent miRNA levels in the 5% DSS dosing group. N = 4/group. *: $p < 0.05$, **: $p < 0.01$.

NA levels (Fig. 3G) were not significantly different for all miRNAs compared to levels in each control group. The rectal miRNA level was significantly higher in the miR-21-5p group than in the control group (Fig. 3F). The fecal miRNA level was significantly higher in the miR-31a-5p group than in the control group. In addition, miR-181b-5p and miR-223-3p showed significantly high values that were equal to or higher than those of miR-31a-5p (Fig. 3H).

DISCUSSION

In this study, we examined the relationships between the variability of miRNAs derived from the colon, rectum, plasma, and feces and investigated the expression of miRNAs as a potential biomarker in the rat DSS-colitis model. Treatment with DSS is known to induce colitis in rats and mice and is widely used as a non-clinical IBD model. DSS (5%) was administered to rats with drinking water for 1 week, and general clinical signs, stool condition, body weight, food consumption, water intake, colon length, pathological examination, and changes in miRNAs were evaluated.

One-week administration of 5% DSS in drinking water resulted in high DAI, shortened colon length, and showed histopathological changes characteristic of colitis, such as erosion and inflammatory cell infiltration in the colon and rectum. During the 1-week recovery period, DAI decreased, and histopathological changes showed a tendency to recover and regenerative hyperplasia at the end of a 1-week recovery period.

After the end of the administration period, all nine selected miRNA levels, which changed during colitis,

showed lower expression in colonic and rectal tissues. Expression levels of miRNAs, except miR-21-5p and miR-155-5p, were lower in both plasma and feces in the 5% DSS group. Changes in plasma and fecal miRNAs are largely linked to the colonic and rectal miRNA levels. However, the degree of decrease in plasma miRNA levels was smaller than that of colon and rectal miRNA levels. It has been suggested that miRNAs in plasma may originate from sources other than the colon and rectum. As miR-21-5p and miR-155-5p showed low levels in the colon and rectum but not in feces, it was considered that fecal miR-21-5p and miR-155-5p may have originated from sites other than the colon and rectum. These selected plasma and fecal miRNAs, except for miR-21-5p and miR-155-5p, were considered as useful biomarkers for evaluating GI effects after DSS treatment.

On the other hand, at the end of the recovery period of DSS treatment, miRNA levels in all examined matrices showed substantially high levels. In particular, fecal miR-31a-5p levels increased on recovery Day 8. Although there was no statistically significant difference, the fecal miR-181b-5p and miR-223-3p levels also showed a markedly high tendency on recovery Day 8 (Fig. 3H). The histopathological changes tended to recover, but they did not show complete recovery. Thus, fecal miR-31a-5p, miR-181b-5p, and miR-223-3p could be used to evaluate the reversibility of DSS treatment.

Some miRNAs are known to be elevated in patients with IBD. Olaru *et al.* (2011) revealed that miR-31 expression levels increase with IBD progression. In IBD patients, fecal and serum miR-223 levels were higher than in healthy controls (Schönauen *et al.*, 2018). In this study, it was confirmed that selected miRNAs were decreased when DSS was administered to rats for 1 week and tended to increase at the end of the recovery period. Further assessment may be needed to determine the similarity between post-recovery pathology and IBD patients.

Based on the above-mentioned results, it was concluded that most of the selected miRNAs from the colon, rectum, plasma, and feces may act as potential quantitative and sensitive biomarkers in the rat DSS-colitis model. In particular, miRNAs such as miR-31a-5p, miR-181b-5p, and miR-223-3p, found in feces, could be used as non-invasive biomarkers to evaluate the reversibility in DSS-treated rats.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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