ORIGINAL PAPER

DOI: http://dx.doi.org/10.15403/jgld-4656

Tissue and Circulating MicroRNA-31, MicroRNA-200b, and MicroRNA-200c Reflects Disease Activity in Crohn's Disease Patients: Results from the BIOMIR Study

Cristina Tocia[1,2], Andrei Dumitru[1,2], Bogdan Mateescu[3], Lucian Negreanu[3], Monica State[3], Georgeta Camelia Cozaru[4,5], Anca Florentina Mitroi[4,5], Costel Brinzan[4,5], Razvan Popescu[1], Nicoleta Leopa[1], Miorita Melina Iordache[1], Mihaela Manea[4], Elena Matei[4], Eugen Dumitru[1,2,4], Luana Alexandrescu[1,2]

1) Ovidius University of Constanta, Faculty of Medicine, Constanta;
2) Gastroenterology Clinic, County Clinical Emergency Hospital of Constanta, Constanta;
3) Carol Davila University of Medicine and Pharmacy, Bucharest;
4) Center for Research and Development of the Morphological and Genetic Studies of Malignant Pathology (CEDMOG), Ovidius University of Constanta, Constanta;
5) Clinical Service of Pathology, County Clinical Emergency Hospital of Constanta, Constanta, Romania

Address for correspondence:
Andrei Dumitru
Ovidius University of Constanta, Faculty of Medicine
dr.andreidumitru@gmail.com

Received: 12.10.2022
Accepted: 25.02.2023

ABSTRACT

Background & Aims: MicroRNAs (miR) have altered expression in multiple autoimmune disorders including inflammatory bowel disease. The aim of the study was to assess the tissue and circulating miR-31, miR-200b, and miR-200c expression levels as potential biomarkers for intestinal disease activity in patients with Crohn's disease (CD).

Methods: The study included 45 patients with histopathological confirmed CD and active disease (defined as fecal calprotectin >50 μg/g and Simple Endoscopic Score (SES) of CD >3), and 21 subjects as controls for the validation cohort. Demographic and clinical data, biomarkers (fecal calprotectin), endoscopy data, the expression levels of miR-31, miR-200b, and miR-200c in tissue and serum were assessed (by RT-PCR). Receiver operating characteristic analysis was performed to assess the miR-31, miR-200b, and miR-200c expression levels as potential biomarkers for active CD.

Results: Mean fecal calprotectin was 1540±890 μg/g. Mean SES-CD was 8.9±4.2. Tissue and circulating miR-31 were significantly correlated with fecal calprotectin (r=0.81, r=0.83, p<0.01) and with SES-CD (r=0.82, r=0.79, p<0.01). The expression level of miR-31 was significantly upregulated in CD tissue cases compared to the control tissue samples (6.24±1.57 vs. 3.70±1.44; p <0.01). Similarly, serum miR-31 expression levels in CD patients were significantly upregulated compared to the control serum samples (0.78±0.42 vs. -2.07±1.00; p<0.01). The expression levels of tissue miR-200b and miR-200c were significantly upregulated in CD tissue cases compared to the control tissue samples (-5.25±0.93 vs. -4.69±0.80, p=0.03 for miR-200b, and -0.86±0.96 vs. 0.39±0.66, p<0.01 for miR-200c). Similarly, serum miR-200b and miR-200c expression levels in CD patients were significantly upregulated compared to the control serum samples (p < 0.05). Receiver operating characteristic analysis revealed that the expression levels of the selected miRNAs could help to discriminate active CD patients from healthy controls with very good specificity and sensitivity.

Conclusions: Tissue and circulating miR-31, miR-200b, and miR-200c reflect disease activity in CD patients and can be used as biomarkers for active disease.

Key words: microRNA – disease activity – Crohn's disease – fecal calprotectin – biomarkers.

Abbreviations: AUC: area under the curve; CD: Crohn's disease; EMT: epithelial-mesenchymal transition; fCal: fecal calprotectin; IBD: inflammatory bowel disease; miR: microRNA; ROC: receiver operating characteristic; SES: Simple Endoscopic Score.

INTRODUCTION

Accurate assessment of disease activity in inflammatory bowel disease (IBD) is of paramount importance in decisions regarding treatment strategies. To apply a treat-to-target strategy, a tight assessment of activity is mandatory. Endoscopy is the gold standard for diagnosis and follow-up of IBD patients and provides the most accurate information, but is invasive, time-consuming, costly, and uncomfortable for patients [1]. Clinical activity is easy to assess but does not always correlate with endoscopic activity [2]. Although fecal calprotectin (fCal) has proved its beneficial role and has substantially improved the clinical care of IBD patients, there is still an urgent need for ideal non-invasive biomarkers that could replace the invasive endoscopy [3]. An objective monitoring biomarker that can assess disease activity could

be extremely useful in the follow-up of patients with IBD [4]. Furthermore, such non-invasive biomarkers could be used also as biomarkers for drug response [5].

MicroRNAs (miRs) are small, non-coding short (ribonucleic acid) RNAs that have a role in the regulation of genes and protein expression [6]. MicroRNAs have altered expression in multiple autoimmune disorders, including IBD, as well in inflammation, chronic degenerative disorders, and cancer [7]. Increasing evidence show that these miRs are involved in the pathogenesis of IBD. Among them, miR-31 has a critical role in inflammation, reduces the inflammatory response and promotes the regeneration of colon epithelium in mice. In vitro studies demonstrated that miR-31 has a direct action on IL-25 and regulates Th1/Th17 cell-mediated mucosal inflammation in colitis and suppresses the immune response; miR-31 also promotes epithelial regeneration in the inflamed epithelium by WNT and Hippo signalling pathways [8]. Advanced research suggests that intestinal epithelial cells and epithelial-mesenchymal transition (EMT) play an important role in the pathogenesis of IBD [9, 10]. Few studies have investigated the role of miRs in chronic inflammation, development of fibrosis and stenosis with promising results. The TGF-beta signalling pathway is the mechanism by which miRs are involved in chronic inflammation and fibrosis in IBD [11]. The miR-200 family has been shown to induce EMT in experimental models and in various human diseases [12,13]. Chen et al. [10] demonstrated that miR-200b promoted proliferation of intestinal epithelial cells by inhibiting TGF-beta1-induced EMT.

This emerging role has led to investigations into miR expression profiles in IBD to understand the pathogenesis of these diseases and led to clinical advances in this area [6]. Several studies showed altered miRs expression profiles in IBD [14-16] and discovered different signatures of miRs associated with susceptibility to IBD, with the risk of clinical and histological exacerbation, or associated with disease remission [17].

The majority of the studies examined miRs in intestinal biopsies from patients with IBD [18]. Besides tissue profiling, miRs were also discovered in serum in a cell-free state suggesting their potential use as non-invasive biomarkers [18]. Extra-cellular miRs are very stable and protected from degradation (packed in vesicles of microparticles, nano proteins, or RNA binding proteins) and are found in most biologic fluids like peripheral blood, serum, urine, plasma, milk, or saliva [18, 19].

Although endoscopy with biopsies remains the gold standard of care and especially in the diagnosis of IBD, the discovery of an accurate non-invasive test is useful in disease monitoring. To improve clinical care, circulating miRs have been investigated in IBD with important and useful results [19].

Also, for accurate results in this field of research, it is important to investigate if the dysregulation of the tissue miRs is similar to the dysregulation of circulating miRs [20].

The study aimed to assess the tissue and circulating miR-31, miR-200b, and miR-200c expression levels as potential biomarkers for intestinal disease activity in patients with Crohn's disease (CD).

METHODS

Study Patients

Out of 86 patients with CD admitted to County Clinical Emergency Hospital of Constanta and Clinical Hospital Colentina of Bucharest between January 01, 2019, and December 31, 2020, 45 patients with histopathological confirmed CD and endoscopic active disease (defined as Simple Endoscopic Score (SES) for CD ≥3) were included in the study. Patients with CD and concomitant infectious (such as *Clostridioides difficile*), inactive endoscopic disease (SES-CD < 3), patients who did not receive a colonoscopy on admission, or who did not express their consent, were excluded (41 patients). The study included also 21 subjects for validation cohort (age and sex-matched healthy volunteer's serum samples served as control samples).

Study Design

Demographic and clinical data, endoscopy data (SES-CD score), biomarkers (fCal), the expression levels of the selected miRs (miR-31, miR-200b, and miR-200c) in tissue and serum were assessed in all the subjects included in the study.

The activity of CD was assessed by endoscopy and fCal.

Endoscopy was performed using a Pentax EPK-i7010. Endoscopic disease severity was assessed by SES-CD; decoding was as following: 0–2 = remission, 3–6 = mild endoscopic activity, 7–15 = moderate endoscopic activity, >15 = severe endoscopic activity [21]. Biopsies for the evaluation of tissue miRs were performed as follows: three fragments from the inflamed areas, and three fragments from the endoscopic normal areas (endoscopic normal areas which served as tissue controls). For fCal determination, patients were trained to collect 3 different samples of at least 2 cm from different locations of the feces and samples were stored in a sterile container; all samples were collected in the morning and were immediately brought to the laboratory for processing; the stool sample was analysed using PETIA (Particle Enhanced Turbidimetric ImmunoAssay). Fecal calprotectine <50 μg/g means inactive disease and >50 μg/g means active disease [22]. Blood samples from the patients with CD and controls were collected for the evaluation of circulating miRs (1.4 ml of serum collected in BD Vacutainer® SSTTM II Advance Tubes).

Extraction and Quantification of MiRNAs from Tissues and Serum

The extraction of total RNA from tissues was carried out using a miRNeasy mini kit (Qiagen) according to manufacturer protocol [23]. Regarding the isolation of RNA from serum exosomes (EVs), we employed miRCURY® Exosome Serum/Plasma kit and miRNeasy Serum/Plasma kit following the manufacturer's instructions. Initially, enrichment of exosomes from serum was done by mixing the 1.4 ml of cleared serum (collected in BD Vacutainer® SSTTM II Advance Tubes) in a microcentrifuge tube with 560 μl Precipitation Buffer A. After overnight incubation at 2–8°C, the precipitate was centrifuged at 1500xg for 30 min at room temperature (RT). The resulting supernatant was discarded, and the formed pellet was resuspended in 240 ul resuspension buffer. Thereafter, 1 ml of QIAzol Lysis Reagent was added to the resuspended RNA

pellet and vortexed before standing 5 min at RT. After adding a 300 µl chloroform, the homogenate was shaken vigorously, left to stand for 5 min at RT, and then centrifuged at 12,000xg for 15 min at 4°C. Following centrifugation, the upper aqueous phase containing RNA was transferred and precipitated in an Eppendorf tube by adding 1.5 volumes of 100% ethanol. Next, 650 µl of the mixture was transferred into a RNeasy MinElute spin column placed in an appropriate collection tube and centrifuged at 12,000 rpm for 1 min at RT. This time the flow-through was discarded and 700 µl wash buffer RWT was pipetted and centrifuged at 12.000 rpm for 1 min. The column was then washed sequentially by centrifugation with RPE buffer and 80% ethanol at the same speed and time as the previous step. To dry the membrane, the column was centrifuged with an open lid at maximum speed for 5 min. Finally, 15 µl of RNase-free water was placed onto the column and incubated for 1 min at RT before centrifugation at maxim speed to elute the RNA.

The purity of isolated RNA was assessed by measuring absorbances at 260 and 280 nm using a NanoDrop One™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), where a 260/280 ratio range between 1.9 - 2.1 was considered acceptable. Moreover, the concentration of the samples was measured using the Qubit®3.0 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity number (RIN) was determined using 2200 TapeStation Bioanalyzer (Agilent Technologies GmbH, Waldbronn, Germany).

Reverse Transcription of miRNA to Complementary cDNA and Real-time PCR

Transcription of miR molecules in complementary DNA (cDNA) was achieved using Multiscribe RT enzyme coupled with miRNA-specific stem-looped RT primer (Table 1), reagents of TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, San Diego, CA). A standard amount of 10 ng RNA was loaded in a final volume of 15 µl of the reaction mixture and incubated in a thermocycler at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min to denature the sample.

Quantification level of miR in serum exosomal and tissues was evaluated by quantitative real-time PCR (qRT-PCR) using TaqMan®MicroRNA Assays Inventoried (Applied Biosystems, San Diego, CA). PCR reaction mixture included 10 µl of TaqMan® 2 × Universal PCR Mastermix (no AmpErase UNG), 1.33µl of the product from the reverse transcription reaction, 7.67 µl of RNase-free dH2O, and 1µl of TaqMan® Small RNA assay (20X). All reactions were carried out in duplicate for each sample using the ABI 7500 Fast qPCR instrument in 40 cycles under the following conditions: denaturation at 95 °C for 15 seconds and annealing coupled with extension at 60 °C for 60 seconds. Fluorescence was detected at the end of each cycle and a negative control without a template was used with all the qRT-PCR runs.

The relative gene expression values for the selected miRNAs were normalized against RNU44 in tissue and miR-16 in serum and calculated in concordance with the equation $2^{-\Delta\Delta CT}$, which represents the fold change (FC) between samples [24]. If the FC is positive it means the miRNAs gene is upregulated; if the FC is negative, it means it is downregulated.

Ethics

The study was conducted according to good laboratory practice and in accordance with the Declaration of Helsinki and national and institutional standards. Informed consent was obtained from all patients, and the study was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (approval no 16/15.11.2018).

Statistical Analysis

Statistical analysis and graphics were performed using MedCalc® software version 14.8.1. The D'Agostino-Pearson test verified the normality of the data distribution. Parametric tests were used when data were normal distributed.

Data were expressed as a mean and standard deviation, or median and range for quantitative variables or frequency and percentage for qualitative ones.

Independent-samples T-tests were applied to determine the statistical difference of miR species between groups, where a p-value less than 0.05 was considered statistically significant. Receiver operating characteristic (ROC) was employed to evaluate the diagnostic performance of the selected miRNAs as biomarkers useful in discriminate CD cases from control cases. The diagnostic accuracy was asses in terms of area under the curve (AUC), sensitivity (Sn), specificity (Sp), true positive/negative fraction (TP, TN), and false negative/positive fraction (FN, FP), where: sensitivity was calculated as follows = TP/(TP+FN); specificity = TN/(TN + FP).

Pearson rank correlation test was used for correlations between ordinal variables. A p value < 0.05 was considered statistically significant. A value of rho < 0.3 indicated a weak correlation, 0.3-0.7 moderate correlation, and rho > 0.7 indicated a strong correlation [25].

RESULTS

General characteristics are illustrated in Table II. The mean age of the CD patients was 45.5±10.4 years. Out of 45 patients, 29 (64.4%) were female and 16 (35.6%) were male. Regarding location, 10 patients (22.2%) had ileal disease (L1), 5 (11.2%) had colonic disease (L2) and 30 (66.6%) had

Table I. The mature microRNA sequences

microRNA	Lot ID	Mature microRNA sequence
hsa-miR-31	P170311-009 H10	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-200b	P161205-006 D03	CAUCUUACUGGGCAGCAUUGGA
hsa-miR-200c	P161207-003 G05	CGUCUUACCCAGCAGUGUUUGG
hsa-miR-16	P180730-024 E07	UAGCAGCACGUAAAUAUUGGCG
RNU44	P181019-000 G11	UAUUGCACUUGUCCCGGCCUG

ileocolonic disease (L3). Regarding phenotype, 39 patients (86.6%) had inflammatory phenotype (B1) and 6 (13.4%) had stenotic phenotype (B2), and none had penetrant phenotype (B3). Mean fCal was 1540 ±890 µg/g. Mean SES-CD was 8.9± 4.2.

Table II. Characteristics of the patients in the testing cohort

Characteristics	CD	Controls	p
Total (n)	45	21	
Age (mean +/- SD)	45.5±10.4	42±9.8	>0.05
Gender			
Male	16 (35.6%)	9 (42.8%)	>0.05
Location, n (%)			
L1	10 (22.2%)		
L2	5 (11.2%)		
L3	30 (66.6%)		
Behaviour, n (%)			
B1	39 (86.6%)		
B2	6 (13.4%)		
B3	0		
fCal (µg/g)	1540±890		
SES-CD (mean +/- SD)	8.9±4.2		

SD: standard deviation; fCal: fecal calprotectin; SES-CD: Short Endoscopic Score for Crohn's disease.

All selected miRs were significantly correlated with fCal and SES-CD (Table III). MiR-31 and miR-200c showed a strong correlation with SES-CD and fCal, whereas miR-200b showed a moderate correlation.

Table III. Correlations of microRNAs with fecal calprotectin and endoscopic score

MicroRNAs	fCal	SES-CD
Tissue miR-31	r=0.81, p<0.001	r=0.82, p<0.001
Circulating miR-31	r=0.83, p<0.001	r=0.79, p<0.001
Tissue miR-200b	r=0.52, p=0.03	r=0.59, p=0.04
Circulating miR-200b	r=0.55, p=0.04	r=0.51, p =0.03
Tissue miR-200c	r=0.79, p<0.001	r=0.75, p<0.001
Circulating miR-200c	r=0.70, p=0.02	r=0.74, p<0.001

For abbreviations see Table II.

The Expression Level of MiR-31 in Inflammatory Tissues and Serum Samples

The expression of miR-31 in the tissue and plasma of the 45 patients was upregulated. As we presented in Fig. 1A, the mean expression level of miR-31 was found significantly upregulated in CD tissue samples compared to the control tissue samples (6.24±1.57 vs. 3.70±1.44; p<0.001).

Similarly, mean serum miR-31 expression level in CD patients was significantly upregulated than in control volunteers (0.78±0.42 vs. -2.07±1.00; p<0.001, Fig. 1B). The mean FC level of miR-31 in CD tissues and serum samples was upregulated by up to 5.20-fold and 6.23-fold, respectively.

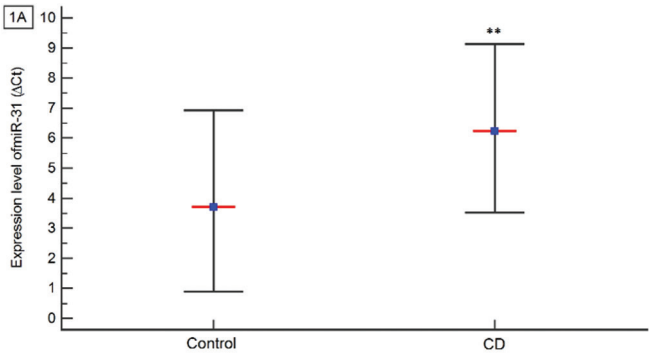

Fig. 1A. The expression level of micrR-31 in tissue samples collected from patients with Crohn's disease and controls.o

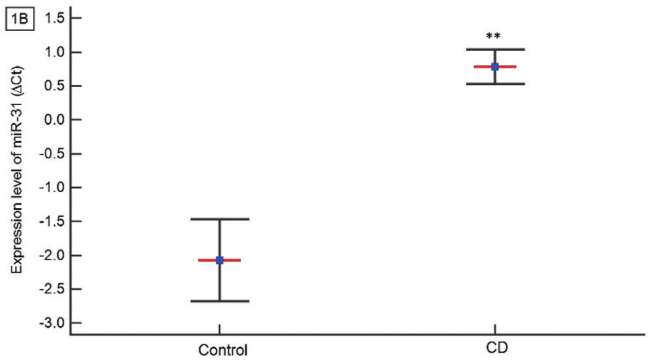

Fig. 1B. The expression level of microRNA-31 in serum samples collected from patients with CD and controls.

The Expression Levels of MiR-200b and MiR-200c in Inflammatory Tissues and Serum Samples

The expression levels of miR-200b and miR-200c in the tissue and serum of the 45 patients were upregulated. As we presented in Fig. 2A, the expression level of tissue miR-200b and miR-200c was found significantly upregulated in CD tissue cases compared to control tissue samples: -5.25±0.93 in controls vs. -4.69±0.80 in CD, p=0.03 (for miR-200b), and -0.86±0.96 in controls vs. 0.39±0.66 in CD, p<0.001 (for miR-200c). The mean FC level expressions of miR-200b and miR-200c in CD tissue samples were upregulated by up to 1.65-fold and 2.18-fold, respectively.

Similarly, serum miR-200b and miR-200c expression levels in CD patients were upregulated compared to the

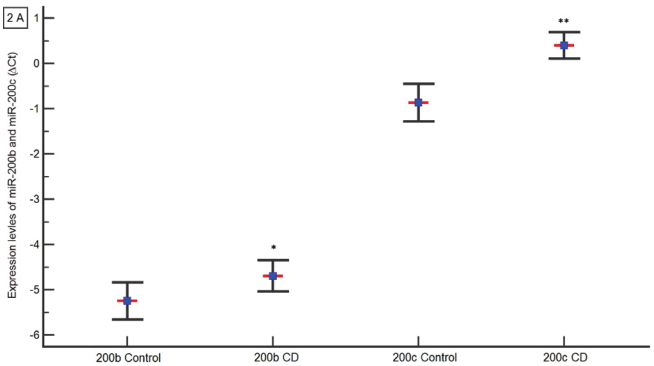

Fig. 2A. The expression level of microRNA-200b and microRNA-200c in tissue samples collected from patients with Crohn's disease and controls.

control volunteers (Fig. 2B). The mean FC level of miR-200b and miR-200c in CD serum samples was upregulated by up to 1.31-fold and 3.16-fold, respectively; however, the statistical analysis revealed significant differences only in the case of miR-200c ($p<0.001$).

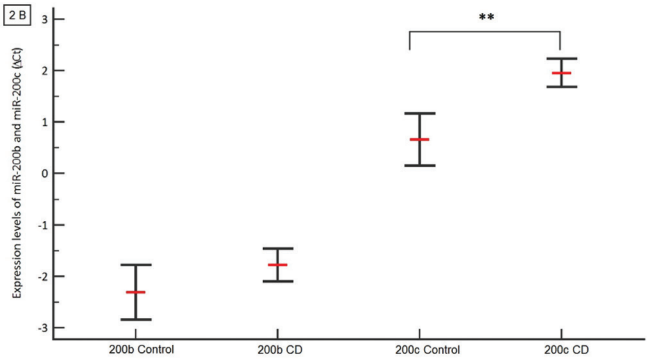

Fig. 2B. The expression level of microRNA-200b and microRNA-200c in serum samples collected from patients with Crohn's disease and controls.

Receiver Operating Characteristic Curve Analysis of the Selected MicroRNAs

Receiver operating characteristic curves were analysed to evaluate the miR-31, miR-200b and miR-200c expression levels as potential biomarkers for active CD. Analysis revealed that the expression levels of tissue miR-31, miR-200b and miR-200c could help to discriminate active CD patients from healthy controls with very good specificity and sensitivity (Table IV and Fig. 3-5).

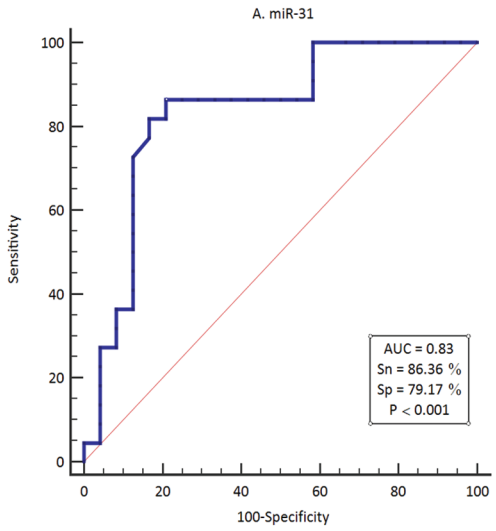

Fig. 3. ROC curve for microRNA-3.

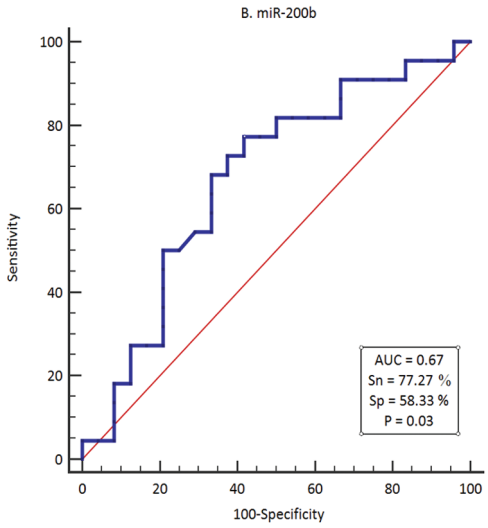

Fig. 4. ROC curve for microRNA-200b.

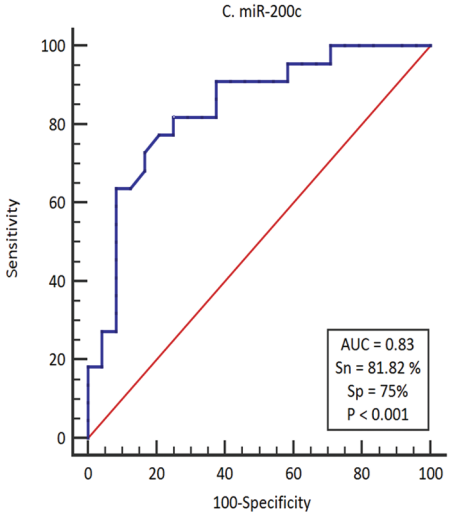

Fig. 5. ROC curve for microRNA-200c.

DISCUSSION

In the present study, we evaluated the feasibility of several tissues and circulating miRs as potential biomarkers for active CD. Our study showed that miR-31, miR-200b, and miR-200c correlates with active CD and these biomarkers could be novel biomarkers for disease monitoring. In the last years, there was a changing paradigm for the management of IBD, aiming to avoid disease progression, to prevent complications, bowel damage, and disability [26]. The possibility to apply the concept of "treat to target" strategies requires accurate and regular

Table IV. Receiver operating characteristic curve analysis of microRNAs-31, microRNAs-200b, microRNAs-200c

microRNA	AUROC (CI, p value)	Sensitivity	Specificity	TP	TN	Cut-off
miR-31	0.83 (0.69 – 0.92, p < 0.01)	86.36%	79.17%	4.15	0.17	0.65
miR-200b	0.67 (0.51 – 0.80, p = 0.03)	77.27%	58.33%	1.85	0.39	0.35
miR-200c	0.83 (0.70 – 0.93, p < 0.01)	81.82%	75%	3.27	0.24	0.56

miR: microRNA; AUROC: area under the curve; CI: confidence interval; TP: true positive fraction ; TN: true negative fraction.

assessment of disease activity [26]. Data showed that early escalation of the treatment based on regular disease monitoring by clinical data and biomarkers (such as fCal) leads to improved outcomes in IBD [26, 27]. Unfortunately, in IBD patients, treatment based only on symptoms failed to stop disease progression. Many studies showed the discordances between clinical symptoms and objective measures of disease activity, especially in CD [2]. A posthoc analysis of the patients with CD from the SONIC trial proved that in half of the patients, clinical activity did not correlate with endoscopic activity [28].

In our study, we tried to objectively assess disease activity by fCal and endoscopy, excluding clinical activity based on symptoms. The CALM trial was the first study to prove that tight disease control using objective biomarkers of inflammation and early treatment escalation in patients with CD leads to better outcomes compared to strategies driven by clinical activity [29]. Fecal calprotectin proved its important role in the monitoring of IBD patients, being a highly sensitive biomarker of endoscopic disease activity and is an adjunctive measure to monitor activity in both CD and ulcerative colitis (UC) [30]. Despite these facts, the optimal cut-off values best predictive of active disease are still being investigated [31-33].

Substantial efforts have been made to identify novel non-invasive biomarkers that could reflect intestinal activity. Despite these efforts, endoscopy with histology remains the „gold standard" and without any doubt, endoscopy is the most accurate instrument for disease activity assessment, but it is invasive, costly, inconvenient for patients, and may be also associated with serious adverse events [2]. Deep remission is the highest target in patients with IBD and this could be achieved only by stringent patient follow-up and regular monitoring. Objective biomarkers of disease activity as treatment targets are an important step towards this direction of achieving deep remission in patients with IBD [26].

Our study showed that miR-31, miR-200b, and miR-200c can differentiate active CD tissues from healthy tissues and the study of their expression levels may be used as a biomarker for the diagnosis of active CD patients. Similarly, in our study, circulating miR-31, miR-200b, and miR-200c follow the course of endoscopic disease activity and were upregulated in the serum of active CD patients in contrast with healthy controls and may be used as non-invasive biomarkers for disease activity in CD.

MicroRNAs, small non-coding molecules, post-transcriptional gene-regulating RNAs, have been investigated in multiple diseases for the past 10 years. It is already proved that miRs are unique stable molecules and protected from degradation, that may become dysregulated early in the course of a disease and that dysregulation can induce alterations in gene expression and promote inflammation or neoplasia [34-37].

MicroRNAs have altered expression in multiple autoimmune disorders, as well in inflammation, chronic degenerative disorders, and cancer [6]. Current evidence shows that miRs have an important functional role in IBD [38]. The miRs investigated in our study were selected based on previous studies. Intensive research in IBD showed that different miRs are involved in inflammation, cell-to-cell communication, cell integrity, immune response, barrier integrity and gut mucosal permeability, apoptosis of epithelial cells, tissue reparation, or malignancy [39, 40]. Even if it is in its infancy, research in this area showed that miRs can be used as a diagnostic and predictive tool for IBD and its complications and predictors of drug response [39]. Wu et al. [14] were the first to discover a differential pattern of miR expression in patients with active UC and controls or UC in remission. Since then, significant scientific progress has been made in the research of miRNAs in IBD. There is increasing data that IBD patients have dysregulated miRs compared to healthy controls.

Several miRs were investigated in tissue samples from patients with CD. For example, miR-23b, miR-106a, and miR-191 were upregulated, and miR-19b and miR-629 were downregulated in CD compared to healthy controls [41]. Another study showed that miR-9, miR-126, miR-130a, miR-181c, and miR-375 were dysregulated in inflamed biopsies from CD compared to healthy controls [42].

Although altered mucosal miR expressions in IBD are an excellent tool for disease activity in IBD, non-invasive assessment of miR in blood samples may become a more interesting and useful tool. As we also demonstrated in our study, circulating miR reflects mucosal changes. One of the advantages of miRs as biomarkers is the measurement which is performed by RT-PCR analysis which is an established standardized and sensitive method for gene expression [43] in contrast with fCal which test assays lack standardization leading to different results [44-46].

Wang et al. [47] investigated miR-223 level expression in 50 serum samples from patients with CD and UC and demonstrated the correlation of miR-223 with endoscopic scores of disease activity, similar to our results. Cordes et al. [43] demonstrated that circulating miR-320a distinguishes active disease from remission and correlates with endoscopic activity scores in both CD and UC. One previous study investigated several circulating miRs in 14 patients with active CD, 5 patients with CD in remission, 13 patients with active UC, 10 patients with UC in remission, and 13 controls and found that most of the investigated miRs (miR-199, miR-362, miR-532, miR-plus-E1271, miRplus-F1065) were differentially expressed only in active CD and miR-340 and miR-149 were dysregulated in both patients with active or inactive CD [48]. Paraskevi et al. [16] studied a profile of multiple miRNAs and found that miR-151, miR-199, and miR-16 were upregulated in IBD compared to controls. Several other studies that investigated mucosal miRs in IBD showed dysregulated miRNAs in IBD versus healthy controls [49-50].

Studies of circulating miRs have demonstrated that they are surrogate biomarkers for the diagnosis and monitoring of IBD and various other diseases [51-53].

MicroRNA-21, miR-155, and miR-31 are among the most investigated miRNAs in IBD [3]. miR-31 was found to be specific for IBD in a study by Zhang et al. [54, 55]. Tian et al. [8] showed that miR-31 is upregulated in tissue samples from patients with IBD and has a positive correlation with biomarkers of active disease. This study clearly demonstrated the molecular pathway of miR-31-5p activation in the inflammatory phase of IBD; in vivo mouse model showed that miR-31-5p activated the Wnt targets proteins and inhibited Hippo signalling target proteins [8,56].

In contrast with a previous study [57] which showed that miRNAs from the miR-200 family were downregulated in surgical specimens of stenotic CD, in our study, miR-200b and miR-200c were upregulated. Chen et al. [10, 58] found miR-200b to be downregulated in the inflamed mucosa and upregulated in the serum of the patients with IBD. Previous studies showed that the miR-200 family induces EMT in experimental models and humans [10, 59, 60]. Fibrosis results after myofibroblasts activation and excessive accumulation of extracellular matrix proteins. The relationship between inflammation and fibrosis and how inflammations lead to fibrosis is poorly understood, but most likely EMT is a key contributor [57]. The discrepancies between the results of our study and previous literature could be explained by this hypothesis: the mentioned studies included patients with severe fibrosis and without inflammation (upon histological examination) and miR-200 family were downregulated; in our study, patients had stenosis but also with active disease and miR-200 family were upregulated. It is worth studying miR-200 only in patients with CD and long-standing stenosis.

MicroRNA-200b was found one of the most important regulators of EMT by targeting ZEB transcription factors and also controlling E-cadherine and vimentin. Chen et al. [10,58] reported that ZEB1 was decreased by miR-200b and concluded that miR-200b plays an important role in the EMT pathogenesis. Furthermore, recent research shows that miR-200b-3p could regulate the gut microbiota and contribute to intestinal homeostasis [61].

Analysis of the ROC curves and AUCs revealed that expression levels of tissue miR-31, miR-200b, and miR-200c could help to discriminate active CD patients from healthy controls with very good specificity and sensitivity. The results of our study support the potential role of the selected miRNAs as biomarkers for active CD.

Regarding the design of our study, the most common design described in studies was to compare patients with IBD versus healthy controls. Other studies, in concordance with the design of our study, have investigated mucosal miR expressions in biopsies from areas with active disease and areas without inflammation with normal endoscopic appearance [18].

The limitations of the study could be the small sample size (45 patients), the mixed phenotype (patients with inflammatory and stricturing disease analysed together), and the absence of healthy control volunteers for tissue samples.

Knowledge about miRNAs involvement in fibrosis is limited, which highlights a growing need for discovering the mechanisms behind the pathogenesis of strictures in IBD. Future directions of IBD research may focus on the role of miRNAs in assessing fibrosis. Our results can be improved in the future with more extensive research which may help solidify differential miRNAs in IBD patients with consideration for specific disease phenotype variation amongst inflammatory, stricturing (with inflammation and without inflammation), and fistulizing phenotypes.

CONCLUSIONS

Tissue and circulating miR-31, miR-200b, and miR-200c reflect disease activity in CD and can be used as biomarkers for active CD.

Conflicts of interest: None to declare.

Authors' contributions: C.T., A.D., L.A. conceived the study, wrote and revised the paper. E.D., B.M., M.S., L.N. performed colonoscopies and tissue samples for analysis. R.P., N.L., M.M.I. data acquisition and database. G.C.C., A.F.M., M.M., E.M., C.B. performed DNA extraction, quantification of miRNAs and RT-PCR in the laboratory and interpreted the results. C.B. and E.D. performed statistics. All authors critically revised the paper and approved the final version.

Acknowledgements: Molecular analysis was performed at the Center for Researh and Development of the Morphological and Genetic Studies of Malignant Pathology (CEDMOG), Ovidius University of Constanta, Romania. This research was funded by the Ovidius University of Constanta Romania through the BIOMIR grant number 3 / 14.11.2018 Ovidius University of Constanta

REFERENCES

1. Annese V, Daperno M, Rutter MD, et al. European evidence based consensus for endoscopy in inflammatory bowel disease. *J Crohns Colitis* 2013;7:982–1018. doi:10.1016/j.crohns.2013.09.016
2. Walsh AJ, Bryant RV, Travis SP. Current best practice for disease activity assessment in IBD. Nat Rev Gastroenterol Hepatol 2013;13:567–579. doi:10.1038/nrgastro.2016.128
3. Schönauen K, Le N, von Arnim U, Schulz C, Malfertheiner P, Link A. Circulating and Fecal microRNAs as Biomarkers for Inflammatory Bowel Diseases. Inflamm Bowel Dis 2018;24:1547-1557. doi:10.1093/ibd/izy046
4. Dulai PS, Peyrin-Biroulet L, Danese S, et al. Approaches to integrating biomarkers into clinical trials and care pathways as targets for the treatment of inflammatory bowel diseases. Gastroenterology 2019;157:1032–1043.e1. doi:10.1053/j.gastro.2019.06.018
5. Batra SK, Heier CR, Diaz-Calderon L, et al. Serum miRNAs Are Pharmacodynamic Biomarkers Associated With Therapeutic Response in Pediatric Inflammatory Bowel Disease. *Inflamm Bowel Dis 2020*;26:1597–1606. doi:10.1093/ibd/izaa209
6. Pekow JR, Kwon JH. MicroRNAs in inflammatory bowel disease. Inflamm Bowel Dis 2012;18:187-193. doi:10.1002/ibd.21691
7. Brînzan C, Aşchie M, Cozaru G, Dumitru E, Mitroi A. The diagnostic value of miR-92a,-143, and-145 expression levels in patients with colorectal adenocarcinoma from Romania. Medicine 2020;99:e21895. doi:10.1097/MD.0000000000021895
8. Tian Y, Xu J, Li Y, et al. MicroRNA-31 Reduces Inflammatory Signaling and Promotes Regeneration in Colon Epithelium, and Delivery of Mimics in Microspheres Reduces Colitis in Mice. Gastroenterology 2019;156:2281–2296.e6. doi:10.1053/j.gastro.2019.02.023
9. Flier SN, Tanjore H, Kokkotou EG, Sugimoto H, Zeisberg M, Kalluri R. Identification of epithelial to mesenchymal transition as a novel source of fibroblasts in intestinal fibrosis. J Biol Chem 2010;285:20202–20212. doi:10.1074/jbc.M110.102012
10. Chen Y, Xiao Y, Ge W, et al. MiR-200b inhibits TGF-β1-induced epithelial-mesenchymal transition and promotes growth of intestinal epithelial cells. Cell Death Dis 2013;4:e541. doi:10.1038/cddis.2013.22
11. Xiao X, Mao X, Chen D, Yu B, Hui Y, Wang J. miRNAs can affect intestinal epithelial barrier in inflammatory bowel disease. Front Immunol 2022;13:868229. doi:10.3389/fimmu.2022.868229

12. Zeisberg M, Kalluri R. Cellular mechanisms of tissue fibrosis. Common and organspecific mechanisms associated with tissue fibrosis. Am J Physiol Cell Physiol 2013;304:C216–C225. doi:10.1152/ajpcell.00328.2012
13. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009;139:871–890. doi:10.1016/j.cell.2009.11.007
14. Wu F, Zikusoka M, Trindade A, et al. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha. Gastroenterology 2008;135:1624–1635. e24. doi:10.1053/j.gastro.2008.07.068
15. Archanioti P, Gazouli M, Theodoropoulos G, Vaiopoulou A, Nikiteas N. Micro-RNAs as regulators and possible diagnostic bio-markers in inflammatory bowel disease. J Crohns Colitis 2011;5:520–524. doi:10.1016/j.crohns.2011.05.007
16. Paraskevi A, Theodoropoulos G, Papaconstantinou I, Mantzaris G, Nikiteas N, Gazouli M. Circulating microRNA in inflammatory bowel disease. J Crohns Colitis 2012;6:900–904. doi:10.1016/j.crohns.2012.02.006
17. Berezin A, Poplyonkin E. Diagnostic and therapeutic value of micro-RNAs in inflammatory bowel disease. Biomedl Res Ther 2020;7:3622-3632. doi:10.15419/bmrat.v7i2.589
18. Schaefer JS. MicroRNAs: how many in inflammatory bowel disease?. Curr Opin Gastroenterol 2016;32:258-266. doi:10.1097/MOG.0000000000000284
19. Kalla R, Ventham NT, Kennedy NA, et al. MicroRNAs: new players in IBD. Gut 2015;64:504-513. doi:10.1136/gutjnl-2014-307891
20. James JP, Riis LB, Malham M, Hogdall E, Langholz E, Nielsen BS. MicroRNA biomarkers in IBD – differential diagnosis and prediction of colitis – associated cancer. Int J Mol Sci 2020;21:7893. doi:10.3390/ijms21217893
21. Daperno M, D'Haens G, Van Assche G, et al. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. Gastrointest Endosc 2004;60:505-512. doi:10.1016/s0016-5107(04)01878-4
22. Pathirana WGW, Chubb SP, Gillett MJ, Vasikaran SD. Faecal Calprotectin. Clin Biochem Rev 2018;39:77-90.
23. Brînzan C, Aschie M, Matei E, Mitroi A, Cozaru G. Molecular expression profiles of selected microRNAs in colorectal adenocarcinoma in patients from south-eastern part of Romania. Medicine 2019;98:e18122. doi:10.1097/MD.0000000000018122
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta DeltaC(T)) method. Methods 2001;25:402-408. doi:10.1006/meth.2001.1262
25. Dancey CP, Reidy J. Statistics Without Maths for Psychology: Using Spss for Windows (4th ed.). Prentice Hall International (UK)Ltd., Hertfordshire, UK. 2007.
26. Gonczi L, Bessissow T, Lakatos PL. Disease monitoring strategies in inflammatory bowel diseases: What do we mean by "tight control"? World J Gastroenterol 2019;25:6172-6189. doi:10.3748/wjg.v25.i41.6172
27. Im JP, Ye BD, Kim YS, Kim JS. Changing treatment paradigms for the management of inflammatory bowel disease. Korean J Intern Med 2018;33:28-35. doi:10.3904/kjim.2017.400
28. Peyrin-Biroulet L, Reinisch W, Colombel JF, et al. Clinical disease activity, C-reactive protein normalisation and mucosal healing in Crohn's disease in the SONIC trial. Gut 2014;63:88-95. doi:10.1136/gutjnl-2013-304984
29. Colombel JF, Panaccione R, Bossuyt P, et al. Effect of tight control management on Crohn's disease (CALM): a multicentre, randomised, controlled phase 3 trial. Lancet 2018;390:2779-2789. doi:10.1016/S0140-6736(17)32641-7
30. Mosli MH, Zou G, Garg SK, et al. C-Reactive Protein, Fecal Calprotectin, and Stool Lactoferrin for Detection of Endoscopic Activity in Symptomatic Inflammatory Bowel Disease Patients: A Systematic Review and Meta-Analysis. Am J Gastroenterol 2015;110:802-819. doi:10.1038/ajg.2015.120
31. D'Haens G, Ferrante M, Vermeire S, et al. Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. Inflamm Bowel Dis 2012;18:2218-2224. doi:10.1002/ibd.22917
32. Zhulina Y, Cao Y, Amcoff K, Carlson M, Tysk C, Halfvarson J. The prognostic significance of faecal calprotectin in patients with inactive inflammatory bowel disease. Aliment Pharmacol Ther 2016;44: 495-504. doi:10.1111/apt.13731
33. De Vos M, Louis EJ, Jahnsen J, et al. Consecutive fecal calprotectin measurements to predict relapse in patients with ulcerative colitis receiving infliximab maintenance therapy. Inflamm Bowel Dis 2013;19:2111-2117. doi:10.1097/MIB.0b013e31829b2a37
34. Link A, Goel A. MicroRNA in gastrointestinal cancer: a step closer to reality. Adv Clin Chem 2013;62:221–268. doi:10.1016/b978-0-12-800096-0.00006-8
35. Link A, Balaguer F, Shen Y, et al. Fecal microRNAs as novel biomarkers for colon cancer screening. Cancer Epidemiol Biomarkers Prev 2010;19:1766–1774. doi:10.1158/1055-9965.EPI-10-0027
36. Gracias DT, Stelekati E, Hope JL, et al. The microRNA miR-155 controls CD8(+) T cell responses by regulating interferon signaling. Nat Immunol 2013;14:593–602. doi:10.1038/ni.2576
37. Shi C, Liang Y, Yang J, et al. MicroRNA-21 knockout improve the survival rate in DSS induced fatal colitis through protecting against inflammation and tissue injury. PLoS One 2013;8:e66814. doi:10.1371/journal.pone.0066814
38. Fisher K, Lin J. MicroRNA in inflammatory bowel disease: translational research and clinical implication. World J Gastroenterol 2015;21:12274–12282. doi:10.3748/wjg.v21.i43.12274
39. Berezin AE, Poplyonkin EI. Diagnostic and therapeutic value of micro-RNAs in inflammatory bowel disease. Biomed Res Ther 2020;7:3622-3632. doi:10.15419/bmrat.v7i2.589
40. Popescu RC, Tocia C, Brinzan C, et al. Molecular profiling of the colon cancer in South-Eastern Romania: results from the MERCUR study. Medicine 2021;100:e24062. doi:10.1097/MD.0000000000024062
41. Wu F, Zhang S, Dassopoulos T, et al. Identification of microRNAs associated with ileal and colonic Crohn's disease. Inflamm Bowel Dis 2010;16:1729–1738. doi:10.1002/ibd.21267
42. Fasseu M, Tréton X, Guichard C, et al. Identification of restricted subsets of mature microRNA abnormally expressed in inactive colonic mucosa of patients with inflammatory bowel disease. PLoS One 2010;5:e13160. doi:10.1371/journal.pone.0013160
43. Cordes F, Demmig C, Bokemeyer A, et al. Micro-RNA-320a monitors intestinal disease activity in patients with inflammatory bowel disease. Clin Transl Gastroenterol 2020;11:e00134. doi:10.14309/ctg.0000000000000134
44. Whitehead SJ, French J, Brookes MJ, Ford C, Gama R. Between-assay variability of faecal calprotectin enzyme-linked immunosorbent assay kits. Ann Clin Biochem 2013;50:53–61. doi:10.1258/acb.2012.011272
45. Labaere D, Smismans A, Van Olmen A, et al. Comparison of six different calprotectin assays for the assessment of inflammatory bowel disease. United Eur Gastroenterol J 2014;2:30–37. doi:10.1177/2050640613518201
46. Kristensen V, Malmstrøm GH, Skar V, Røseth A, Moum B. Clinical importance of faecal calprotectin variability in inflammatory bowel

disease: Intra-individual variability and standardisation of sampling procedure. Scand J Gastroenterol 2016;51:548–555. doi:10.3109/00365521.2015.1117650

47. Wang H, Zhang S, Yu Q, et al. Circulating microRNA223 is a new biomarker for inflammatory bowel disease. Medicine 2016;95:e2703. doi:10.1097/MD.0000000000002703
48. Wu F, Guo NJ, Tian H, et al. Peripheral blood microRNAs distinguish active ulcerative colitis and Crohn's disease. Inflamm Bowel Dis 2011;17:241–250. doi:10.1002/ibd.21450
49. Feng X, Wang H, Ye S, et al. Up-regulation of microRNA-126 may contribute to pathogenesis of ulcerative colitis via regulating NF-kappaB inhibitor IκBα. PLoS One 2012;7:e52782. doi:10.1371/journal.pone.0052782
50. Min M, Peng L, Yang Y, Guo M, Wang W, Sun G. MicroRNA-155 is involved in the pathogenesis of ulcerative colitis by targeting FOXO3a. Inflamm Bowel Dis 2014;20:652–659. doi:10.1097/MIB.0000000000000009
51. Coskun M, Bjerrum JT, Seidelin JB, Nielsen OH. MicroRNAs in inflammatory bowel disease - Pathogenesis, diagnostics and therapeutics. World J Gastroenterol 2012;18:4629–4634. doi:10.3748/wjg.v18.i34.4629
52. Thakral S, Ghoshal K. MiR-122 is a unique molecule with great potential in diagnosis, prognosis of liver disease, and therapy both as miRNA mimic and antimir. Curr Gene Ther 2015;15:142–150. doi:10.2174/1566523214666141224095610
53. Oliveira DNP, Carlsen AL, Heegaard NHH, et al. Diagnostic plasma miRNA-profiles for ovarian cancer in patients with pelvic mass. PLoS One 2019;14:e225249. doi:10.1371/journal.pone.0225249
54. Zhang C, Zhao Z, Osman H, Watson R, Nalbantoglu I, Lin J. Differential expression of miR-31 between inflammatory bowel disease and microscopic colitis. Microrna 2014;3:155-159. doi:10.2174/2211536604666150209115444
55. Mohammadi A, Kelly OB, Smith MI, Kabakchiev B, Silverberg MS. Differential miRNA expression in ileal and colonic tissues reveals an altered immunoregulatory molecular profile in individuals with Crohn's disease versus healthy subjects. J Crohns Colitis 2019;13:1459–1469. doi:10.1093/ecco-jcc/jjz076
56. Gupta P, Yadav RP, Baranwal S. Commentary: MicroRNA-31 Reduces Inflammatory Signaling and Promotes Regeneration in Colon Epithelium, and Delivery of Mimics in Microspheres Reduces Colitis in Mice. Front Immunol 2019;10:2649. doi:10.3389/fimmu.2019.02649
57. Zidar N, Boštjančič E, Jerala M, et al. Down-regulation of microRNAs of the miR-200 family and up-regulation of Snail and Slug in inflammatory bowel disease – hallmark of epithelial-mesenchymal transition. J Cell Mol Med 2016;20:1813-1820. doi:10.1111/jcmm.12869
58. Chen Y, Ge W, Xu L, et al. MiR-200b is involved in intestinal fibrosis of Crohn's disease. Int J Mol Med 2012;29:601–606. doi:10.3892/ijmm.2012.894
59. Mongroo PS, Rustgi AK. The role of the miR-200 family in epithelial-mesenchymal transition. Cancer Biol Ther 2010;10:219–222. doi:10.4161/cbt.10.3.12548
60. Korpal M, Kang Y. The emerging role of miR-200 family of microRNAs in epithelial mesenchymal transition and cancer metastasis. RNA Biol 2008;5:115–119. doi:10.4161/rna.5.3.6558
61. Shen Q, Huang Z, Ma L, et al. Extracellular vesicle miRNAs promote the intestinal microenvironment by interacting with microbes in colitis. Gut Microbes 2022;14:2128604. doi:10.1080/19490976.2022.2128604