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Original Contribution

SERUM EXPRESSION OF MICRORNA-142 IN A COHORT OF BULGARIAN PATIENTS WITH INFLAMMATORY BOWEL DISEASES

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ABSTRACT

The study aims to assess the expression of serum miRNA-142_3p and miRNA-142_5p in IBD patients and its correlation with disease extent, activity and severity. It was performed on 35 patients with ulcerative colitis (UC), 35 patients with Crohn's disease (CD) and 30 healthy controls. Serum miRNA-142 expression was assessed using reverse transcriptase quantitative real time PCR (RT-qPCR), and then correlated with that of a group of 30 healthy subjects. It was also correlated with disease extent and disease activity and severity indices (CDAI, Montreal classification, Partial Mayo score). The patients' group showed mean serum miR-142_3p expression of 2.69±1.96 for CD, 1.66±0.90 for UC and 1.25±0.91 for the control group and serum miR-142_5p expression of 2.42±2.08 for CD, 1.61±1.12 for UC and 1.21±0.78 for the control group with a significant difference between groups. Conclusion: The expression of miR-142_3p and miR-142_5p was significantly higher in patients with CD compared to patients with UC and the control group.

Key words: miR-142, ulcerative colitis, Crohn's disease, inflammatory bowel diseases

INTRODUCTION

Chronic inflammatory bowel diseases (IBD) include ulcerative colitis (UC), Crohn's disease (CD), nondeterministic colitis and microscopic colitis. They are characterized by heterogeneity - genetic, immunological and clinical. Switching from immune tolerance to specific immunoreactivity leads to improper activation of innate and acquired immunity - this is the key point that predisposes to the onset of IBD. Some environmental factors may act as a trigger with initial activation and subsequent disease relapse. A better understanding of the mechanisms involved in inflammation and the immune response provides opportunities to respond therapeutically, control, and possibly modifies the course of IBD.

Numerous studies have revealed that micro-ribonucleic acids (miRNAs) play a significant role in every stage of inflammation. miRNAs are an integral part of the differentiation, regulation and cellular signalling of the immune system. Improper adaptation within these processes can lead to acute or chronic ongoing inflammation, which is characterized by inflammatory disorders in IBD. (1, 2)

A number of authors reveal that miRNAs play a key role in the construction and proper functioning of acquired and innate immunity. (3-5)

They are small 21-24 nucleotide double-stranded RNA molecules that, with the help of a complex of proteins, can cause the cleavage of certain messenger RNA (mRNA) molecules. These mRNAs are their targets and their cleavage causes RNA- silencing of the respective genes.

This paper discusses the expression of miRNA-142_3p and miR-142_5p, as their role in inflammatory processes has recently been established. miR-142_5p plays also a significant role in many pathological processes: progression of Hashimoto's thyroiditis, development of colorectal cancer and renal cell carcinoma (6-8).

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A team of authors discovered that miR-142_5p is overexpressed in patients with UC, releasing inflammatory molecules and suppressing the function of Suppressor of Cytokine Singnaling-1 (SOCS1), and it is necessary to elucidate the role of these miRNAs in colon inflammation (9). Other authors have observed that miR-142_5p enhances Th1 cell differentiation (10, 11), while miR-142_3p contributes to neutrophil maturation and differentiation (12).

On the other hand, miRNA-142_3p regulates the synthesis of proinflammatory Nuclear Factor Kappa B Subunit 1 (NF-κB1), Tumor Necrosis Factor Alpha (TNF-α) and Interleukin 6 (IL-6) in macrophages by targeting the Interleukin-1 Receptor –Associated Kinase 1 (IRAK1) gene and decreasing IRAK1 protein expression (13, 14). In 2014, Zhai et al. published data, which shows that miRNA-142_3p controls autophagy through Autophagy Related16 Like 1 (ATG16L1), with increased miRNA expression leading to decreased autophagic activity in patients with CD. (15) In 2012, Paraskevi et al. discovered that decreased miRNA-142_3p expression was associated with inflammation, which is regulated by the Nucleotide-binding oligomerization Domain-Containing 2 (NOD2) gene, which is associated with autophagy processes (16).

Aim

The aim of the study was to evaluate the serum expression of miR-142_3p and miR-142_5p in patients with IBD and the correlation of the expression of these miRNAs with the extent of disease, activity and severity of CD and UC.

MATERIAL AND METHODS

35 patients with CD, 35 patients with UC and 30 healthy controls were studied. Serum expression was assessed by reverse transcriptase - real time quantitative polymerase chain reaction PCR (RT-qPCR).

Levels of miRNAs were assessed in blood serum. 5 ml of blood was obtained via peripheral venous puncture with closed system BD Vacutainer™ SST™ II Advance (Becton Dickinson, USA). After withdrawal, the blood sample was held 30 minutes at room temperature for clothing. Subsequently, it was centrifuged at 500×g for 15 minutes at room temperature and the serum was separated and divided into aliquots of 500 µl that were immediately stored at −80 °C until the moment of the analysis.

miRNAs were isolated from 200 µl serum using a pre-existing commercial miRNeasy Serum/Plasma Kit, as per protocol of the manufacturer. 3,5 µl (1,6×108 copies/ µl) control miRNA C. elegans miR-39: miRNeasy Serum/Plasma Spike-In Control, was added to each sample for normalization control; the samples were afterwards eluted in 14 µl RNA-ase free water.

Each of the samples was subsequently submitted to reverse transcription via ready-to-use commercial kit miScript II RT Kit, as per manufacturer's protocol from 2,5 µl eluted miRNA in a final volume of 10 µl with HiFlex buffer and it was incubated at 37 °C for 60 minutes and the enzyme was inactivated at 95 °C for 5 minutes.

Each of the samples was then submitted toe quantitative real time polymerase chain reaction (rt-PCR) via a ready-to-use commercial kit miScript SYBR Green PCR Kit, and prepared primers miScript Primer Assay, as per manufacturer's protocol: 1 µl complementary DNA (cDNA) in 10 µl reactions in 3-times repetitions for 4 target miRNA in 102 well plates. The used miScript Primer Assay primers, are as follows (the reference number is in the brackets): Hs_miR-142-5p_1, Hs_miR-142-3p_2, Hs_RNU6-2_11, Ce_miR-39_1,. The used temperature parameters are as follows: maintenance for 15 minutes at 95 °C for enzyme activation; 40 cycles of 15 seconds at 94 °C; 30 seconds at 70 °C with fluorescent reading; analysis of the melting curve in order to prove the specificity of the amplification: primary denaturation for 15 seconds at 95 °C and cooling to 55 °C for 60 seconds with an increase to 95 °C with velocity of +0,05 °C per second and fluorescent reading. The analysis was done by Quant Studio Dx instrument of Applied Biosystems (USA) company; a threshold cycle (Ct) was assessed for each sample.

The results were statistically processed with SPSS v. 20.0 for Windows, using variation, ANOVA, correlation, comparative analysis, ROC curve analysis and risk assessment analysis.

The clinical trial was initiated after approval and permission №82 / 28.03.2019 of the Ethics commission for scientific research at the Medical University – Varna, Bulgaria. All trial participants have signed an informed consent form.

RESULTS

Table 1 shows the characteristics of patients with CD, UC, and those of the healthy control group. There was no significant difference in the mean age of patients with UC and CD. The gender distribution also did not show a significant difference, although women predominated in the UC patients group. According to the localization, in CD patients predominate those with intestinal localization (L1) - 54.3%, while in UC patients predominate those with pancolitis (E3) - 65.7%. According to the Montreal classification, in patients with CD, those with an inflammatory phenotype (B1) predominate - 48.6%, and in patients with UC, 80.0% of cases have a chronic recurrent course of the disease. Considering the patients' age at the onset of the disease, a significant difference was discovered ($p = 0.044$), as the patients with UC have a significantly longer duration (66.91 months for CD compared to 113.03 months for UC). Over 2/3 (68.6%) of the patients with CD are on biological therapy, while 34.3% of the patients with UC are on 5 aminosalicylic acid drugs (5-ASA) and on biological treatment.

***Table 1**. Characteristics of patients with IBD according to the Montreal classification*

Clinical Characteristics		**CD (n=35)**	**UC (n=35)**	**Control group (healthy people) (n=30)**
Age, years (mean±SD, range)	Current age	41.51±13.55 (18-75)	41.54±15.21 (18-73)	26.2±6.2 (18-42)
	Onset of the complaints	33.97±13.44 (11-75)	31.28±12.05 (15-47)	-
	Diagnosis	36.11±13.02 (14-75)	32.74±12.77 (15-62)	-
Sex	Male	18/51.4 %	15/ 42.9%	15/50,0%
	Female	17/48.6%	20/ 57.1 %	15/50,0%
Localization/ Extension CD/UC	L1/E1	19/54.3 %	1/ 2.9 %	-
	L2/E2	5/4.3%	11/ 31.4 %	-
	L3/E3	11/31.4 %	23/ 65.7 %	-
Behaviour of disease	B1	17/48.6 %	-	-
	B2	11/31.4 %	-	-
	B3	6/17.1 %	-	-
	B2-B3	1/ 2.9 %	-	-
	Chronically recurrent	-	28/ 80.0 %	-
	Chronically persistent	-	7/ 20.0 %	-
Onset of IBD, months	(mean±SD, range)	66.91±59.67 (3-204)	113.03±119.10 (1-492)	-
CRP	(mean±SD, range)	23.80±38.33 (0.09-160.0)	14.55±25.12 (0.13-91.90)	-
	> 5 mg/L	19/54.3%	16/48.5%	-
FCP	(mean±SD, range)	251.36±306.06 (2.0-910.0)	680.03±559.35 (12.5-1800.0)	-
	> 50μg/g	13/76.5%	12/75.0%	-
CDAI	Remission	18/52.9%	-	-
	Mild activity	5/14.7%	-	-
	Moderate activity	11/32.4%	-	-
S (severity)	Remission	-	8/22.9%	-
	Mild activity	-	13/37.1%	-
	Moderate activity	-	4/11.4%	-
	Severe activity	-	10/28.6%	-
Partial (Endoscopic) Mayo score	Remission	-	8/22.9%	-
	Mild activity	-	10/28.6%	-
	Moderate activity	-	8/22.9%	-
	Severe activity	-	9/25.7%	-
Treatment	5-ASA	2/ 5.7 %	12/ 34.3 %	-
	Corticosteroids	7/ 20.0 %	8/ 22.9 %	-
	Immunomodulators	2/ 5.7 %	3/ 8.6 %	-
	Biological treatment	24/ 68.6 %	12/ 34.3 %	-

In the study of miR-142_3p expression, a significant difference was observed between the studied groups (p <0.001), as in patients with CD the serum expression of miRNA was significantly higher compared to patients with UC and the control group. The results were similar for the expression of miR-142_5p, where again the expression of miRNA was significantly higher in patients with CD (p = 0.004) **(Figure 1)**. No significant difference was noted in the expression of the two miRNAs in the study groups.

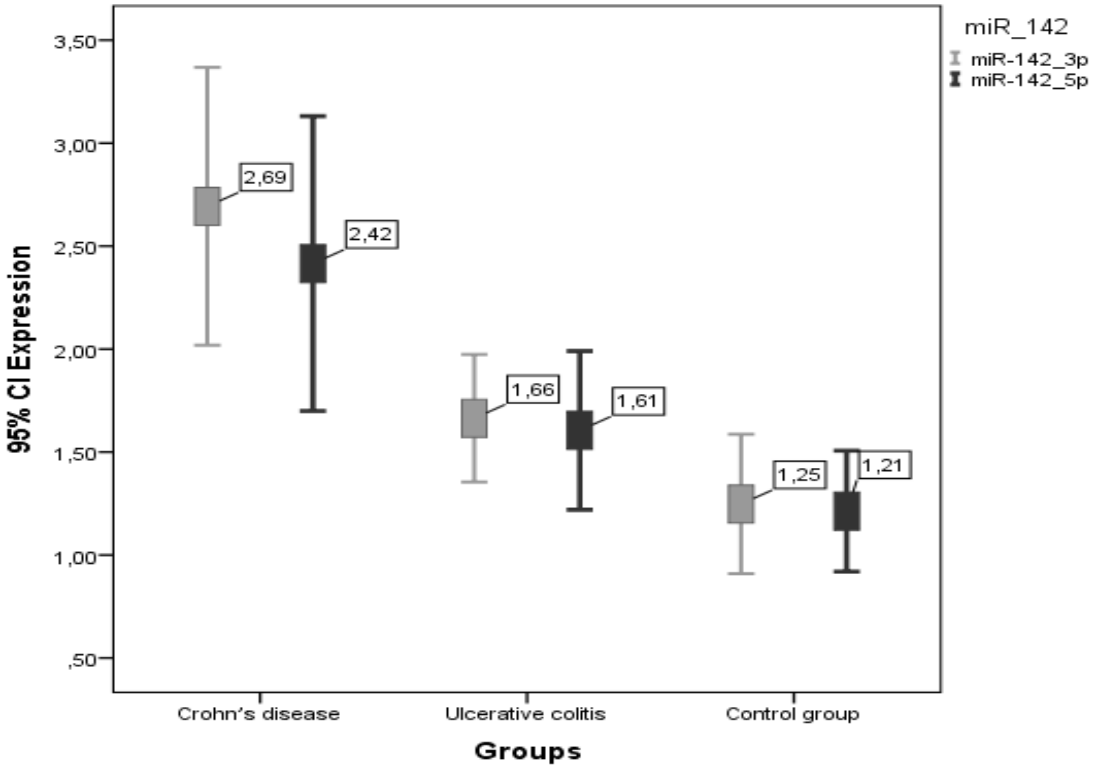

Figure 1. Expression of miR-142_3p and miR-142_5p

There are no validated reference cut-off levels of miRNA expression. The current study established the cut-off values of the expression of miR-142_3p and miR-142_5p to differentiate patients with IBD from the healthy controls. They are specific to a given population and serve as a guide for assessing the expression in CD and UC. In our study, the cut-off value of miR-142_3p was 1.32 (AUC 0.716 (0.609-0.823); p = 0.001) with a sensitivity of 65.7% and a specificity of 66.7% **(Figure 2),** and the cut-off value of miR-142_5p was 1.17 (AUC 0.671 (0.556-0.785); p = 0.007) with a sensitivity of 62.9% and a specificity of 63.3% **(Figure 3**).

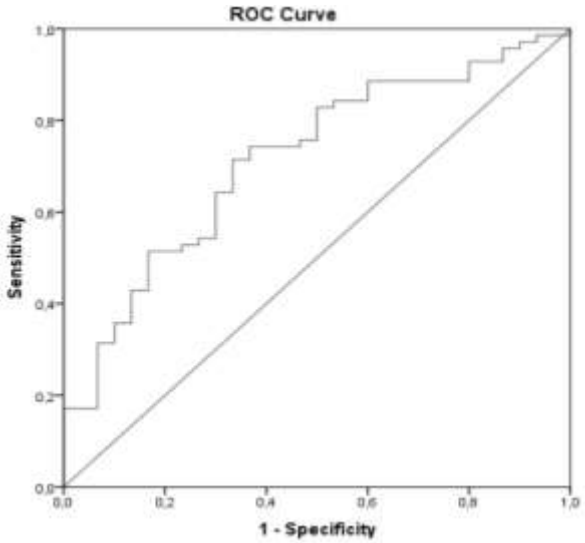

FIG. 2. ROC curve analysis to determine the expression threshold of miR-142_3p (healthy control group)

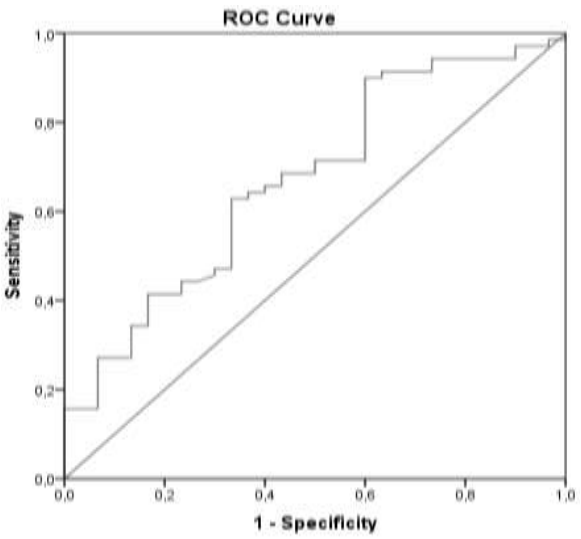

FIG. 3. ROC curve analysis to determine the expression threshold of miR-142_5p (healthy control group)

Table 2 and **Table 3** show the expression of miR-142_3p and miR-142_5p, according to the main characteristics of patients with CD and UC.

Table 2. *Expression of micro-RNA 142, according to the main characteristics of patients with CD (n=35)*

Clinical characteristics		**Expression of miR-142_3p (mean ± SD)**	**Expression of miR-142_5p (mean ± SD)**	**P value**
Localization CD	L1	3.06±1.94	3.08±2.54	> 0.05
	L2	1.73±0.88	1.78±0.59	> 0.05
	L3	2.48±2.29	1.54±1.06	0.044
Behaviour	B1	2.03±1.47	2.05±2.12	> 0.05
	B2	2.92±1.74	2.33±1.90	> 0.05
	B3	3.82±3.06	3.67±2.35	> 0.05
	B2-B3	4.66	1.92	N/A
CDAI	Remission	**1.94±1.58**	2.06±2.02	> 0.05
	Mild activity	**2.94±1.32**	3.06±3.02	> 0.05
	Moderate activity	**3.64±2.40**	2.52±1.82	0.031
	P value	**0.047**	> 0.05	-
Treatment	5-ASA	3.02±1.49	0.82±0.08	0.004
	Corticosteroids	3.17±2.77	2.16±1.61	0.05
	Immune modulators	1.37±0.54	1.88±0.37	> 0.05
	Biological treatment	2.63±1.83	2.66±2.33	> 0.05

Table 3. *Expression of micro-RNA 142, according to the main characteristics of patients with UC*

Clinical characteristics		**Expression of miR-142_3p (mean ± SD)**	**Expression of miR-142_5p (mean ± SD)**	**P value**
Extension UC	E1	1.99	**2.38**	N/A
	E2	1.52±0.74	**1.01±0.54**	0.047
	E3	1.72±0.99	**1.85±1.24**	> 0.05
	P value	> 0.05	**0.042**	-
Behaviour	Chronically recurrent	**1.51±0.86**	1.61±1.23	> 0.05
	Chronically persistent	**2.29±0.85**	1.59±0.59	0.021
	P value	**0.033**	> 0.05	-
S (severity)	Remission	1.22±0.72	1.85±1.64	0.031
	Mild activity	1.77±0.94	1.69±1.14	> 0.05
	Moderate activity	1.88±0.36	1.69±0.57	> 0.05
	Severe activity	1.78±1.01	1.25±0.79	0.029
Partial (Endoscopic) Mayo score	Remission	1.69±1.18	2.16±1.65	0.023
	Mild activity	1.54±0.64	1.59±1.16	> 0.05
	Moderate activity	1.69±1.19	1.21±0.64	0.037
	Severe activity	1.74±0.69	1.48±0.76	> 0.05
Treatment	5-ASA	1.54±0.65	1.19±0.54	0.018
	Corticosteroids	2.13±1.02	1.70±0.85	0.036
	Immune modulators	1.67±0.53	2.40±1.95	0.002
	Biological treatment	1.47±1.08	1.75±1.43	0.023

Although there is no significant difference in the expression of miRNAs according to the localization of the disease in patients with CD, it was observed that in patients with intestinal localization (L1) there is an increased expression of miR-142_3p and miR-142_5p

(3.06 and 3.08, respectively). No significant difference in miR-142_3p expression was discovered in patients with UC according to localization, whereas a study of miR-142_5p expression revealed significantly elevated miRNA levels in patients with pancolitis (E3) (1.85) (p = 0.042). In the present study, there was only one patient with proctitis (E1) in whom the expression of the miRNAs tested was not taken into account in the analysis.

According to the disease behaviour, in patients with CD, increased expression was observed in both miRNAs in patients with a penetrating phenotype (B3) (3.82 for miR-142_3p and 3.67 for miR-142_5p, respectively). In patients with UC, miR-142_3p expression was significantly higher in patients with the chronic persistent form of activity (2.29 versus 1.51 for the chronic relapsing form, p = 0.036). The expression of miR-142_5p did not show a significant difference between the two forms of disease behaviour.

In patients with CD, there was a significant difference in the expression of miR-142_3p (p = 0.047), with a proportional correlation with the severity of the disease calculated with CDAI (r = 0.353; p <0.05). Increased miR-142_3p expression carries a 4-fold higher risk of increased activity in patients with CD (OR = 4.13 (0.726-23.429), p <0.05), whereas miR-142_5p expression does not show such a correlation.

There was no significant difference in the expression of the two miRNAs when assessing the severity of the disease (in patients with UC evaluated with two indexes and in patients with CD evaluated with CDAI).

Commonly used biomarkers for measuring IBD activity are C-reactive protein (CRP) and faecal calprotectin (FCP). In patients with CD, miR-142_3p was determined to correlate strongly inversely with FCP (r = -0.663; p = 0.004), while in patients with UC the same miRNA correlated in direct proportion to CRP (r = 0.275; p = 0.021). In patients with UC, elevated miR-142_3p expression is a risk factor for increased serum CRP levels (OR = 3.25 (0.733-14.402), p = 0.012).

There was no significant difference in the expression of the considered miRNAs according to the drug treatment.

DISCUSSION

Table 4 presents a comparative analysis of miR-142_3p and miR-142_5p expression in the results of the present study and those of other authors. Schaefer et al. discovered a significant difference in the increased expression of miR-142_3p in patients with CD compared to healthy controls. The authors also observed a significant difference in the increased expression of miR-142_5p compared to healthy controls in both patients with CD and UC.

Table 4. *Comparative analysis of patients with increased and decreased expression of miR-142_3p and miR-142_5p*

Author	**Disease**	**Expression of miR-142_3p**		**Expression of 1bmiR-142_5p**	
		Decreased expression	**Increased expression**	**Decreased expression**	**Increased expression**
Schaefer et al. (2015) (17)	CD	n=2 p=0.096	n=8 p=0.018	n=2 p=0.133	n=8 p=0.032
	UC	n=1	n=6 p=0.053	-	n=7 p=0.017
Zahm et al. (2014) (18)	UC	-	n=12 p=0.048	-	-
Atanassova A. (2021)	CD	n=11 p=0.005	n=24 p=0.01	n=11 =0.005	n=24 p=0.01
	UC	n=13 p=0.018	n=22 p=0.034	n=15 =0.055	n=20 p=0.016

Zahm et al. discovered a significant difference in the increased expression of miR-142_3p in patients with CD compared to healthy controls (18). In the present study, a significant difference was observed in the miRNAs studied in both groups of patients, with the exception of decreased miR-142_5p expression in patients with UC.

The regulatory role of different miRNAs in many cellular processes, as well as their role in the inflammatory process in patients with IBD should be studied. There are no studies in the literature that evaluate the differential expression of circulating miRNAs and their role in IBD (16, 19-21).

In 2013, Iborra et al. published data on significantly increased expression of miR-142_5p in patients with UC compared to controls (1.71; p = 0.024). (22) The results of the present study also showed increased

expression of miR-142_3p and miR-142_5p in both patients with CD and patients with UC compared to healthy controls.

A study by Zahm et al. (2014) noted increased expression of miR-142_3p in pediatric patients with UC versus healthy controls (18). These results are also confirmed in our study in adult patients with UC.

In 2015, Schaefer et al. observed increased expression of miR-142_3p in patients with UC and increased expression of miR-142_5p in patients with CD (17). Unlike Schaefer et al., in the present study, we discovered elevated levels of the studied miRNAs in patients with CD.

Wohnhaas et al. discovered increased expression of miR-142_5p in patients with CD (23). The authors observed nearly 10 times higher expression of miR-142_5p in patients with CD compared to healthy controls.

In determining the threshold compared to healthy controls, our results were close to those of Zahm et al., who reported miR-142_3p with AUC = 0.723, p = 0.0078 with a sensitivity of 75.86% and a specificity of 66.67% (18). The sensitivity and specificity of miR-142_3p in the present study were 65.7% and 66.7%, respectively.

In their study, Han et al., (2018) discovered that miR-142_5p has overexpression in UC patients. The authors also noted that this microRNA stimulates inflammation of the colon. Its target is SOCS1, where a moderate inverse relationship is observed between the expression of SOCS1 and that of miR-142_5p (r = -0.4835; p = 0.0167). The authors discovered that miR-142-5p can inhibit the expression and release of inflammatory molecules by suppressing the function of SOCS1. miR-142-5p is a key regulator in colon inflammation and functions as a proinflammatory factor. (9) It also acts as an oncogenic microRNA (oncomiR) and stimulates the development of colorectal cancer (6).

CONCLUSION

The expression of miR-142_3p and miR-142_5p was significantly higher in patients with CD compared to patients with UC and the control group. There was no difference in the expression of the two miRNAs in terms of localization in patients with CD, while in patients with UC there was a significant difference in the expression of miR-142_5p, which is increased in mild activity and in extensive colitis (E3). The increased expression of miR-142_3p is a factor for the onset of a chronic persistent course of UC. The high inflammatory activity in CD, measured with CDAI, was associated with increased expression of miR-142_3p. Isolated reports and conflicting results in the literature indicate the need for larger cohort studies of IBD patients.

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