European Review for Medical and Pharmacological Sciences 2022; 26: 6310-6315

Circulating miRNA-16 in inflammatory bowel disease and some clinical correlations – a cohort study in Bulgarian patients

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Abstract. – OBJECTIVE: The pathogenesis of the inflammatory bowel disease (IBD) includes chronic inflammation and altered immune reactions. There are several publications, reporting that micro ribonucleic acids (miRNAs) may serve as a diagnostic biomarker with a potential to assess inflammation severity and treatment response[1,2] in IBD patients. The objective of the study is to assess and correlate the serum expression of circulating miR-16 in IBD patients with some clinical parameters, such as extent, activity and severity of the disease.

PATIENTS AND METHODS: 70 IBD patients [35 with ulcerative colitis (UC) and 35 with Crohn's disease (CD)] were included in the study. Serum miR-16 expression in both IBD diseases was assessed using reverse transcription quantitative real time PCR (RT-qPCR). Circulating miR-16 levels were also correlated with disease extent, activity and severity indices [Crohn's Disease Activity Index (CDAI), Montreal classification, Partial Mayo score]. Serum expression of miR-16 in the 70 patients was also compared to miR-16 serum levels in 30 healthy control subjects.

RESULTS: The patients' group showed mean serum miR-16 expression of 3.07 for CD, 1.97 for UC and 1.61 for the control group of healthy subjects with a significant difference in the expression between groups. There is a significant correlation between increased serum expression of miR-16 and disease activity, extent and severity.

CONCLUSIONS: The increased miR-16 serum expression correlates with disease activity, intestinal localization of CD, stenotic and penetrating phenotype. MiR-16 could serve as a potential biomarker to assess inflammation.

Key Words:
MiR-16, Ulcerative colitis, Crohn's disease, Inflammatory bowel diseases.

Introduction

It is widely known that inflammatory bowel diseases (IBD) are complex diseases with unclear pathogenesis. Crohn's disease (CD) and ulcerative colitis (UC) are the most frequent diagnoses within the IBD family. They predominantly involve the gastro-intestinal tract (GIT), clinically presenting with chronic and recurrent symptoms: abdominal pain, diarrhea, bloody and purulent inclusions in the stools, weight loss and significantly worsened quality of life.

The IBD background includes chronic inflammation and altered immune reactions. The inflammatory process may be circumscribed to the mucosa or the submucosa (UC) or could involve all layers of the affected gastrointestinal tract (GIT) segment, as well as the mesenteric fatty tissue and the lymph nodes (CD). CD and UC have different phenotypic presentation: localized inflammation, stenosis, fistulas and abscess formation, involvement of other organs and systems and potential development of colitis-induced carcinoma.

There are several publications, reporting that micro ribonucleic acids (miRNAs) may distinguish patients with active CD and UC from healthy controls. Additionally, miRNAs may serve as a diagnostic biomarker with a potential to assess inflammation severity and treatment response[1,2]. Recent research[3-7] shows that miR-16 is important in IBD. MiR-16 is a small non-coding RNA molecule, localized in 13q14 chromosome. Bioinformatics analyses show that miR-16 targets between 1088 and 8778 aims[8]. It is known that miR-16 may significantly inhibit the cell proliferation; it may stimulate the cell apoptosis and

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suppress the progression of the cell cycle[9]. MiR-16 may transform the M2 to M1 macrophage proliferation and may also activate the CD4 + T-cells by a decrease in the programmed death ligand 1 (PD-L1) expression[10]. Additionally, miR16-1 serves as a regulator of the immune-mediated tissue recovery and the absence of miR-16-1 in CD4 + T-cells increases the Interleukin (IL) IL-22 levels[11]. Research shows that miR-16 participates in some potentially regulatory pathways [nuclear factor of kappa-light-chain-enhancer of activated B cells (NF-kB), lipopolysaccharides (LPS) LPS-induced inflammation, 5-Hydroxytryptamine 4 (5-HT4) receptor signal pathway] in autoimmune diseases and plays a significant role in the etiology of the immune-mediated diseases[12-14].

The objective of this study is to assess the expression of serum miR-16 in IBD patients and to correlate its expression with clinical parameters as disease extent, activity and severity.

Patients and Methods

Seventy IBD patients: 35 UC patients (20 with active disease and 15 in quiescent disease) and 35 CD patients (20 with active disease and 15 in quiescent disease) were included in the study. All patients were classified according to the Montreal classification. Serum Hs_miR-16_2, Hs_RNU6-2_11 and Ce_miR-39_1 expression was assessed with reverse transcription quantitative real time PCR (RT-qPCR), and then, were correlated with the serum Hs_miR-16_2, Hs_RNU6-2_11 and Ce_miR-39_1 level expression in a group of 30 healthy subjects.

We used blood serum as a test for miRNAs. It was obtained *via* peripheral puncture with a closed system BD Vacutainer™ SST™ II Advance 5 ml (Becton Dickinson, Franklin Lakes, NJ, USA). After sampling, the blood was left at room temperature for 30 minutes to coagulate. It was then centrifuged at 1500×g for 15 minutes at room temperature and the serum was separated and distributed into aliquots of 500 µl and was then kept at -80°C until the time of the analysis.

Isolation of miRNA was done from 200 µl serum *via* a commercial ready-to-use set miRNeasy Serum/Plasma Kit (50), catalogue No. 217184 (QIAGEN, Germany) as per the protocol of the manufacturer. 3.5 µl (1,6×108 copies per µl) control miRNA *C. elegans* miR-39 was added to every serum sample for normalization control: miRNeasy Serum/Plasma Spike-In Control, catalogue No. 219610 (QIAGEN, Germany), and then, the samples were eluted in 14 µl RNAse-free water.

Each of the samples was subsequently submitted to reverse transcription *via* ready-to-use commercial kit miScript II RT Kit (50), catalogue No. 218161 (QIAGEN, Germany) as per the 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 to quantitative real time polymerase chain reaction (rt-PCR) *via* a ready-to-use commercial kit miScript SYBR Green PCR Kit (200), catalogue No. 218073 (QIAGEN, Germany) and prepared primers miScript Primer Assay (100), catalogue No. 218300 (QIAGEN, Germany) as per the manufacturer's protocol: 1 µl complementary DNA (cDNA) in 10 µl reactions in 3-times repetitions for 15 target miRNA in 384 well plates. The used miScript Primer Assay primers (100), catalogue No. 218300 (QIAGEN, Germany) are as follows (the reference number is in the brackets): Hs_miR-16_2 (MS00031493), Hs_RNU6-2_11 (MS00033740) and Ce_miR-39_1 (MS00019789). 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 QuantStudio Dx instrument of Applied Biosystems (Waltham, MA, USA) company; a threshold cycle (Ct) was assessed for each sample.

There are no defined referent or validated cut-off levels for the expression of miRNAs in healthy individuals or in IBD patients. In order to differentiate IBD patients from healthy individuals, a cut-off expression level of miRNA-16 was calculated for our cohort of patients, using receiver operating characteristic curve (ROC curve) analysis. This cut-off is specific as it was calculated for the tested population. It may also serve as a marker of the miRNA-16 expression in UC and CD. The expression levels of miR-16 were analyzed and correlated with some IBD characteristics, disease duration, clinical behavior and type of treatment.

Statistical Analysis

The results we processed with Statistical Product and Service Solutions (SPSS Corp., Armon, NY, USA) v. 20.0 for Windows. We used variation, correlative, regression, ROC curve, risk assessment and comparative analyses (x^2, *t*-test). The significance of the obtained results was judged at the 5% level. The level of significance used for all analyzes was $p < 0.05$.

Results

The threshold miRNA-16 cut-off in this trial was calculated at 1.63 (AUC 0.701 (0.585-0.817) with a sensitivity of 62.9% and specificity of 63.3% (Figure 1).

Disease activity, localization and behavior of UC and CD was assessed as per the Montreal classification. MiR-16 expression was measured in patients with both CD and UC and was assessed according to the level of the disease localization in the GIT. The miR-16 expression was distributed according to some IBD characteristics and features of healthy controls.

The current age of IBD patients with active disease was: CD 41.1±12.9, (18-71)/UC 37.9±13.8 (19-73). Distribution according to gender was: CD: n=18 (male)/n=17 (female); UC n=15 (male)/n=20 (female). Localization of CD according to the Montreal classification was: CD: ileal localization /L1/n=19, colonic localization /L2/n=5, ileocolonic localization / L3/ n=11. Localization of UC according to the Montreal classification was: proctitis /E1/ n=1, left-sided colitis /E2/n=11, pancolitis /E3/n=23. The phenotype presentation of CD patients was: inflammatory phenotype /B1/n=17, stenotic phenotype /B2/n=11, penetrating phenotype /B3/n=6, B2+B3/n=1. The IBD patients in this study received different treatment regiments: -5-aminosalicylic acid /5ASA/n=9, corticosteroids (CS)/n=20, immune modulators/n-13 and biologic treatment/n=28. This patients' characteristics was published as a part of a different study of our team as well[15,16].

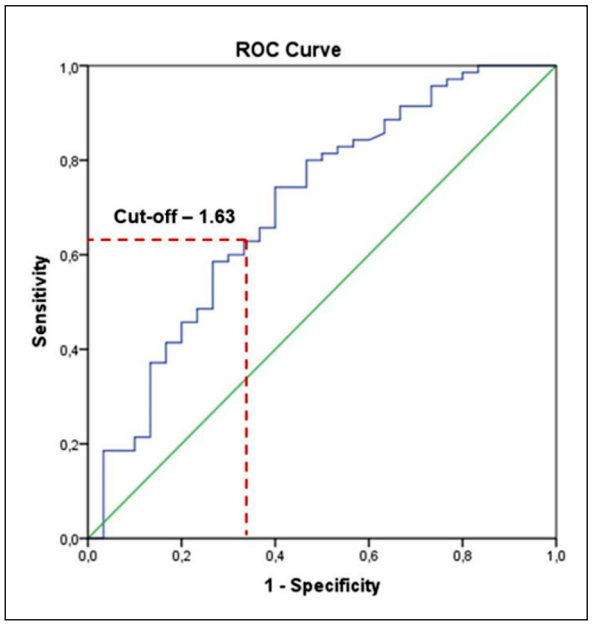

Figure 1. A ROC curve analysis of serum miR-16 expression, calculated to identify UC and CD cases from healthy controls.

MiR-16 expression is increased in CD patients, with disease localized in the small intestine (3.54±2.33) and in pancolitis E3 of UC (2.01±1.54). There is a significant difference in miR-16 expression in inflammatory (B1), stenotic (B2) and penetrating (B3) form of CD (p=0.010) with higher expression levels in the more severe B2 and B3 forms. There is no significant difference in miR-16 expression according to the forms of evolution in patients with UC. Table I shows the distribution of miRNA-16 expression levels according to localization, behavior, clinical activity, severity and treatment in patients with CD and UC.

In CD patients, there is a moderate direct correlation between the expression of miR-16 and the Crohn's Disease Activity Index (CDAI) (r=0.424; p=0.015). MiR-16 expression levels increase progressively in correlation with the activity of CD. Patients with moderate activity of their CD have significantly higher miR-16 expression (4.27±3.00) as compared to patients with mild activity (2.38±1.06) or in remission (2.41±1.23) of the disease (p=0.048). The increase expression levels of miR-16 are a risk factor for CDAI > 150 levels [OR=4.80 (0.506-45.495); p=0.015].

In UC patients, there is no correlation between the miR-16 expression levels and the severity (S) index, measured as per the Montreal classification or the endoscopic activity, measured as per the Partial Mayo score. In patients with severe disease, measured by both S index (1.38±0.89) and Partial Mayo score (1.80±1.12), there is a decrease in miR-16, close to normal controls. miR-16 expression levels do not correlate with any treatment that was given in patients with either CD or UC.

Table I. MiRNA-16 expression levels according to the characteristics of CD and UC patients.

Baseline characteristics		miR-16 expression (mean ± SD)	
		CD (n = 35)	UC (n = 35)
Localization (CD)	L1	3.54 ± 2.33	-
	L2	2.64 ± 0.90	-
	L3	2.45 ± 2.03	-
Localization (UC)	E1	-	2.53
	E2	-	1.82 ± 0.91
	E3	-	2.01 ± 1.54
Behavior	B1	2.43 ± 1.13	-
	B2	3.27 ± 2.49	-
	B3	3.51 ± 2.15	-
	B2-B3	9.20	-
	Chronic recurrent	-	1.97 ± 1.43
	Chronic persistent	-	1.97 ± 0.99
CDAI	Remission	2.41 ± 1.23	-
	Mild	2.38 ± 1.06	-
	Moderate	4.27 ± 3.00	-
S (severity)	Remission	-	1.45 ± 1.11
	Mild	-	2.41 ± 1.65
	Moderate	-	3.01 ± 0.08
	Severe	-	1.38 ± 0.89
Mayo score	Remission	-	2.33 ± 2.05
	Mild	-	2.01 ±1 .02
	Moderate	-	1.74 ± 1.23
	Severe	-	1.80 ± 1.12
Treatment	5-ASA	2.83 ± 0.90	1.44 ± 0.82
	Corticosteroids	3.11 ± 2.25	2.29 ± 0.82
	Immune modulators	2.27 ± 0.82	2.47 ± 1.91
	Biological treatment	3.51 ± 2.26	2.15 ± 1.81

Discussion

Wu et al[17] reported that in comparison to healthy individuals, the expression levels of miR-16 are increased in patients with either UC or CD[18]. These results are also confirmed by Paraskevi et al[19] who additionally report that the expression of miR-16 is significantly higher in patients with CD in comparison to patients with UC (6.38±1.46 *vs.* 2.98±0.35, $p<0.05$). Schönauen et al[3] establish significantly increased miR-16 levels in serum and feces in IBD patients in comparison to healthy individuals and the expression levels positively correlate with the activity of the disease. Additionally, miR-16 levels in feces significantly correlate with the C reactive protein (CRP) levels, which identifies miR-16 expression as a potential inflammation biomarker, deserving future research[3]. Our results also confirm that there is a relation between the increased serum miR-16 expression and the disease activity in CD patients. On the contrary, in UC patients with severe activity (as measured by the S index and the Partial Mayo score), the expression of miR-16 paradoxically drops to the levels, measured in healthy controls.

There are some limitations in our cohort and some of them are the small number of patients, the absence of patients with severe CD activity and the different duration of the IBD. Additionally, the serum miR-16 levels reflect a cross-sectional study, and we were not able to assess serial measurements during longitudinal study. Until present, we cannot provide any explanation for the non-increased as compared to healthy control expression in severe UC.

Tian et al[4] establish that there is an increased miR-16 expression in UC patients in comparison to IBD patients and healthy controls. This suggests that the altered expression of miR-16 may be related to IBD and demonstrates that miR-16 inhibits the expression of adenosine A2a receptor protein (A2aAR) at a post-transcriptomic level[4]. These results show that both miR-16 and A2aAR may be potential therapeutic targets for control of the inflammation and a treatment, targeting these molecules may be beneficial for IBD patients[4]. Some reports[20] associate the increased miR-16

expression with the decreased levels TNF-α and IL-12p40 in colonic macrophages. This may, at least partly, demonstrate the role of miR-16 in the suppression of the inflammatory processes within the mucosa.

MiR-16 targets a number of genes, participating in the apoptosis control, the cell cycle regulation and survival as well as in the macrophage differentiation. The NF-kB signal pathway directly participates in the control of the inflammation processes, as well as in the tumor genesis and progression of the colorectal cancer. This justifies future research and serial measurements, reflecting the dynamics of miR-16 expression levels in IBD patients with chronic and difficult to treat inflammatory processes.

Conclusions

MiR-16 is important for the inflammatory processes and the signal pathways that participate in the immune system regulation. The increased miR-16 serum expression correlates with disease activity, intestinal localization of CD, stenotic and penetrating phenotype. miR-16 could also serve as a potential biomarker to assess inflammation. The holistic role of miR-16 is not yet entirely known, which justifies its future exploration in larger prospective cohort studies in IBD patients in order to assess the role of miR-16 in the processes of inflammation.

Conflict of Interest
The Authors declare that they have no conflict of interests.

Ethics Approval
The clinical study was conducted after approval and permission No. 82/ 28.03.2019 of the Ethics Commission for Scientific Research at the Medical University – Varna, Bulgaria.

Informed Consent
The informed consent for patients was taken before the beginning of the study.

Availability of Data and Material
All data generated or analyzed during this study are included in this article.

Funding
None.

Authors' Contribution
Antonia Atanassova: concept and design of the study, drafted the manuscript, statistical analysis. Avgustina Georgieva: analyzed the data and interpreted the results, bibliography (other published reviews/articles, online materials).

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References

1) 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.
2) Wu F, Guo NJ, Tian H, Marohn M, Gearhart S, Bayless TM, Brant SR, Kwon JH. Peripheral blood microRNAs distinguish active ulcerative colitis and Crohn's disease. Inflamm Bowel Dis 2011; 17: 241-250.
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.
4) Tian T, Zhou Y, Feng X, Ye S, Wang H, Wu W, Tan W, Yu C, Hu J, Zheng R, Chen Z. MicroRNA-16 is putatively involved in the NF-κB pathway regulation in ulcerative colitis through adenosine A2a receptor (A2aAR) mRNA targeting. Sci Rep 2016; 6: 30824.
5) Chung SH, Park YS, Kim OS, Kim JH, Baik HW, Hong YO, Kim SS, Shin JH, Jun JH, Jo Y, Ahn SB. Melatonin attenuates dextran sodium sulfate induced colitis with sleep deprivation: possible mechanism by microarray analysis. Dig Dis Sci 2014; 59: 1134-1141.
6) Te Velde AA, Pronk I, de Kort F, Stokkers PC. Glutathione peroxidase 2 and aquaporin 8 as new markers for colonic inflammation in experimental colitis and inflammatory bowel diseases: an important role for H2O2?. Eur J Gastroenterol Hepatol 2008; 20: 555-560.
7) Min MI, Peng LH, Gang SU, Guo MZ, Qiu ZW, Yang YS. Aquaporin 8 expression is reduced and regulated by microRNAs in patients with ulcerative colitis. Chin Med J 2013; 126: 1532-1537.
8) Yan L, Liang M, Hou X, Zhang Y, Zhang H, Guo Z, Jinyu J, Feng Z, Mei Z. The role of microRNA-16 in the pathogenesis of autoimmune diseases: A comprehensive review. Biomed Pharmacother 2019; 12: 108583.
9) Zhan XH, Xu QY, Tian R, Yan H, Zhang M, Wu J, Wang W, He J. MicroRNA16 regulates glioma cell proliferation, apoptosis and invasion by targeting Wip1-ATM-p53 feedback loop. Oncotarget 2017; 8: 54788.

10) Jia X, Li X, Shen Y, Miao J, Liu H, Li G, Wang Z. MiR-16 regulates mouse peritoneal macrophage polarization and affects T-cell activation. J Cell Mol Med 2016; 20: 1898-1907.

11) Lu Z, Liu J, Liu X, Huang E, Yang J, Qian J. MiR-15a, 16-1 suppresses AHR-dependent IL-22 secretion in CD4 (+) T cells and contributes to immune-mediated organ injury. Hepatology 2018; 67: 1027-1040.

12) Huang Y, Chen G, Wang Y, He R, Du J, Jiao X, Tai Q. Inhibition of microRNA-16 facilitates the paclitaxel resistance by targeting IKBKB via NFkB signaling pathway in hepatocellular carcinoma. Biochem Biophys Res Commun 2018; 503: 1035-1041.

13) Wang W, Lou C, Gao J, Zhang X, Du Y. LncRNA SNHG16 reverses the effects of miR-15a/16 on LPS-induced inflammatory pathway. Biomed Pharmacother 2018; 106: 1661-1667.

14) Chen Y, Shan T, Qu H, Chen Y, Wang N, Xia J. Inhibition of miR-16 Ameliorates Inflammatory Bowel Disease by Modulating Bcl-2 in Mouse Models. J Surg Res 2020; 253: 185-192.

15) Atanassova A, Georgieva A, Chervenkov T, Konsoulova A. Micro-RNA 155 - On the Crosspoint of Inflammation and Carcinogenesis. International Journal of Current Research and Review 2021; 12: 9-15

16) Atanassova A. Serum expression of microrna-142 in a cohort of bulgarian patients with inflammatory bowel diseases. Trakia Journal of Sciences 2021; 2: 156-163.

17) Wu F, Zikusoka M, Trindade A, Dassopoulos T, Harris ML, Bayless TM, Brant SR, Chakravarti S, Kwon JH. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptid-2 alpha. Gastroenterology 2008; 135: 1624-1635.

18) Wu F, Zhang S, Dassopoulos T, Harris ML, Bayless TM, Meltzer SJ, Brant SR, Kwon JH. Identification of microRNAs associated with ileal and colonic Crohn's disease. Inflamm Bowel Dis 2010; 16: 1729-1738.

19) Paraskevi A, Theodoropoulos G, Papaconstantinou I,Mantzaris G, Nikiteas N, Gazouli M. Circulating MicroRNA inflammatory bowel disease. J Crohns Colitis 2012; 6: 900–904.

20) Huang Z, Ma J, Chen M, Jiang H, Fu Y, Gan J, Dong L, Zhang J, Chen J. Dual TNF-α/IL-12p40 interference as a strategy to protect against colitis based on miR-16 precursors with macrophage targeting vectors. Mol Ther 2015; 23: 1611-1621.