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Serum miRNA 146b-5p a pharmacodynamic biomarker in adult inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) impacts the gastrointestinal tract, resulting in multiple hospitalizations, complications, and diminished quality of life. IBD has two subtypes: Crohn's Disease (CD) and Ulcerative Colitis (UC). Evidence suggested that immune response dysregulation and genetic susceptibility are the main disease pathogenesis. IBD diagnosis is established by clinical, laboratory, radiological, endoscopic and histological criteria. MiRNA-146 suppresses proinflammatory cytokines and activates T regulatory lymphocytes (Tregs). Serum miRNA-146b-5p targets genes and cytokines responsible for inhibiting autophagy and maintaining cell homeostasis. This study aimed to evaluate the role of miRNA -146b-5p in diagnosis of IBD and response to treatment. The study consisted of sixty 60 participants separated into 3 groups. Blood samples were withdrawn from 20 acute IBD cases before treatment (Group I), 20 Chronic IBD patients on treatment (Group II) and 20 apparently healthy controls (Group III) for assay of miRNA-146b-5p using the Real-Time polymerase chain reaction (PCR), fecal calprotectin and C reactive protein (CRP). The study revealed statistically significant variation between the 3 studied groups according to stool fecal calprotectin, CRP and microRNA-146b-5p *(p*<0.001*)*. There was a statistically significant difference in microRNA-146b-5p expression among Ulcerative Colitis cases and the control Group and among Crohn's Disease cases and the control Group III (*p*<0.001). There was no difference in microRNA-146b-5p among the CD and UC patients (*p*>0.05). Multi-Regression analysis showed that smoking was a significant variable for CD but not for UC. In conclusion, MiRNA-146b-5p was proved with a superior performance as a biomarker for early diagnosis of IBD and for follow up response to treatment.

Keywords: Inflammatory bowel disease, Crohn's Disease, Ulcerative Colitis.

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Introduction

Inflammatory bowel disease (IBD) is a chronic, idiopathic inflammatory condition that predominantly impacts the gastrointestinal tract. Cases with IBD have recurrent hospitalizations, numerous surgeries, and diminished quality of life because of illness complications.[1] Crohn's Disease and Ulcerative Colitis are two principal types of IBD.[2] IBD is marked by intense inflammation of the small

intestine and/or colon, resulting in chronic diarrhea, rectal bleeding, recurring abdominal discomfort or pain, weight loss, melena, and/or perianal fistula or abscess.[3]

Crohn's Disease (CD) has areas of transmural inflammation, whereas Ulcerative Colitis (UC) is predominantly confined to the innermost layers and infrequently impacts additional layers of the intestinal wall. CD is related to numerous pathophysiological consequences, and its symptoms differ according to the affected region.[4] UC is more common and primarily impacts the colon, particularly the rectum, typically exhibiting a slower progression, with patients being less prone to problems associated with the condition.[5] The diagnosis of IBD is determined by clinical, laboratory, radiographic, endoscopic, and histological criteria as outlined in the European Crohn's and Colitis Organisation [ECCO] and the European Society of Gastrointestinal and Abdominal Radiology [ESGAR] (ECCO-ESGAR) Diagnostics Guideline.[6]

MiR-146 is a multifunctional microRNA that promotes the death of dendritic cells (DCs), inhibits the generation of proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α), reduces Th1 and Th17 cells, and activates regulatory T lymphocytes (Tregs).[7] Deficiency of MiR-146 causes an overproduction of interlukine-6 (IL-6) and TNF-α, resulting in myeloproliferative and inflammatory disorders.[8] Elevated levels of TNF-α have significantly contributed to the pathogenic mechanisms of IBD through many pathways.[9] For example, signaling mediated by nuclear factor kappa B (NF-kB). The induction of IBD-related gene expression, such as those encoding IL-1β, IL-6, and TNF-α, is a crucial mechanism in chronic inflammation.[10]

The biological function of TNF-α elucidates the effectiveness of anti-TNF-α therapy, serving as a crucial therapeutic instrument in the treatment of moderate to severe cases, demonstrating positive clinical results and promoting mucosal healing.[11] Therefore, the present study aimed to evaluate the use of miRNA-146b-5p in prediction and early diagnosis of IBD and to identify the response to treatment in adults with IBD.

Subjects and Methods

This investigation involved 60 cases, selected from the Department of Internal Medicine at Ain Shams Hospitals.

Study subjects were categorized into three groups: Group 1: consisted of 20 cases with untreated acute IBD, while Group 2 comprised 20 chronic IBD cases who received therapy. Group 3 contained 20 apparently healthy volunteers, matched by age and sex, as controls. All individuals with concurrent autoimmune illnesses, such as systemic lupus erythematosus, rheumatoid arthritis, asthma, and ankylosing spondylitis, as well as those with a history of malignancy, continuing infection, or colorectal cancer, were excluded from this trial. Participants in the investigation were categorized regarding the ECCO-ESGAR Diagnostics Guideline.[25]

All participants in the present investigation underwent a full medical history assessment, clinical examination, and laboratory investigations. These included a complete blood count (CBC), was performed using a hematology analyzer (XN-1000 six-part differential hematology analyzer, provided by Sysmex, Egypt), erythrocyte sedimentation rate (ESR) was assayed by the Westergreen method. Serum electrolytes (sodium, potassium, calcium, magnesium), C-reactive protein, aspartate transaminase (AST), alanine transaminase (ALT), serum albumin and total and direct bilirubin were performed on a chemistry analyzer (AU 680 chemistry analyzer, Beckman Coulter, USA) with dedicated reagents supplied by Beckman Coulter. Stool analysis and culture were assayed manually, and fecal calprotectin was assayed using particle enhanced turbidimetric immunoassay on a fully automated analyzer (Cobas C60004, Roche Diagnostics, USA) using the manufacture reagents. MicroRNA 146b-5p was assayed by Quantitative Real Time PCR using commercial reagents (supplied by Qiagen, USA). Radiological investigations which include: computed tomography (CT) or magnetic resonance imaging (MRI) and Colonoscopy (for IBD cases only).

Sample collection

A venous blood sample (10 ml) was collected under complete aseptic precautions from each study subject for the assay of routine laboratory investigations and the MicroRNA 146b-5p assay. Of these, a blood sample (3 ml) was collected into EDTA vacutainer tube for PCR analysis. Plasma was separated and stored frozen at – 80 °C until miRNA extraction. The remaining blood sample (7 ml) was distributed for immediate assay between Westergren tube for assay of ESR, k3 EDTA vacutainer tube for CBC and gel separating tube that was left for 20 minutes to clot then centrifuged for serum separation at 4000 RPM for 10 minutes. The serum was used for immediate assay of serum electrolytes (K^+, Na^+, Mg^{+2}, Ca^{+2}), AST, ALT, serum albumin, total and direct bilirubin and C reactive protein (CRP). Stool samples were collected for immediate stool analysis, fecal calprotectin and stool culture.

Assay of MicroRNA 146b-5p by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

MicroRNA 146b-5p was assessed in four steps: 1. isolation of genomic RNA from peripheral blood leukocytes using EDTA whole blood specimens; 2. Reverse transcription of RNA into complementary DNA (cDNA); 3. Amplification of the extracted DNA and finally, performance of miRNeasy using Mini commercial Kits (Cat no: 217004, Qiagen, Germany) and miRCURY LNA miRNA PCR Starter commercial kit (Cat no:339340, Qiagen, Germany), according to the manufacturer's instructions.

Result interpretation

Interpretation of the results was done by viewing the amplification blots and setting threshold values. The cycle threshold (Ct) was determined for (miRNA-146b-5p), and housekeeping gene (miRNA-103a-3p) in all samples which is the intersection between an amplification curve and a threshold line. Results are stated in relative quantification and adjusted to the endogenous reference gene (housekeeping gene, miRNA-103a-3p). The relative expression level of miRNA-146b-5p in each sample was determined using the comparative cycle threshold delta-delta Ct (2-[ΔΔCT]) technique.

Statistical Analysis

The Statistical Package for Social Science (SPSS, Version 27.0, IBM Corp., USA, 2020) was used for the statistical analysis. Data are presented as median and percentiles for quantitative non-parametric measures, along with both number and percentage for categorical data. The receiver operating characteristic (ROC) curve was used to determine the diagnostic performance of microRNA 146b-5p.

Results

The results of this study are presented in Tables 1-5. Our study population was divided into 20 acute IBD patients before treatment (Group Ⅰ), 20 chronic IBD patients after treatment (Group Ⅱ) and 20 age- and sex-matched control subjects (Group Ⅲ).

Descriptive and comparative statistics of all studied parameters among the three study groups using Kruskal-Walis test are mntioned in Table 1. Comparative statistics of various studied parameters among Group 1 and Group 2 utilizing Wilcoxon Rank-Sum test, showed significant variation in microRNA 146b-5p between the two groups *(p<0.001)* and significant variance in stool fecal calprotectin between the two groups *(p< 0.05)*. However, there was statistically insignificant differences for other laboratory parameters. Descriptive and comparative Statistics using Wilcoxon Rank-Sum test for microRNA 146b-5p showed insignificant distinction between CD and UC patients *(p >0.05).*

Table 1. Comparative and descriptive Statistics, Utilizing Kruskal-Walis Test, for all studied parameters in Group 1, Group 2, and Group III.

Parameter	Group I (n=20)	Group II (n=20)	Group III (n=20)	*p- value*
	Median (IQR) (25^{th}-75^{th})			
Hemoglobin (g/dL)	11.45(9.55-12.7)	12.35(11.2-13.07)	13.4 (12.8-14)	<0.001
Platelets (10^3/μl)	324 (9.55-12.7)	320.5 (267.5-347)	245 (198-285)	<0.001
Stool.Fecal Calprotectin (μg/g)	500(225.7-1237.2)	84 (50-1159.925)	22 (17-31)	<0.001
CRP (mg/L)	8.2(3.75-25.275)	5 (2.625-8.65)	1.75 (0.8-2.5)	<0.001
Na (mmol/L)	135 (133-141)	136.5 (133.2-142)	141.5 (136-143)	0.019
K (mmol/L)	3.85 (3.4-4.175)	4.2 (3.72-4.57)	4.15 (3.7-4.7)	0.040
AST (IU/L)	20 (17-22)	15.5 (14-18)	14 (12-16)	<0.001
Albumin (g/dL)	3.85 (3.42-4.27)	3.75 (3.42-4.17)	4.55 (4.1-5)	<0.001
ESR (mm/hr)	50 (25-83.75)	15.5 (7.25-23.75)	10.5 (8-13)	<0.001
MicroRNA146b_5p expression (ΔC_t)	12.99(6.7-19.6)	2.28 (1.65-2.97)	0.085(0.07-0.18)	<0.001

IQR, interquartile range (25th -75^{th}); ΔCt, change in threshold cycle; Na, sodium; K, potassium; AST, aspartate aminotransferase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n, sample size. $p \leq 0.05$ is significant.

Table 2 illustrated a statistical comparison, using Wilcoxon Rank-Sum test, revealed a statistically significant difference in the studied parameters between the control group, UC and CD patients.

Table 2. Statistical Comparison, Using Wilcoxon Rank-Sum Test, for All Studied Parameters between Group III Vs CD Patients and Group III Vs UC Group.

Parameter	Group III (n=20) Vs CD patients (n=17)	Group III (n=20) Vs UC patients (n=23)
	p- value [z]	
Hemoglobin (g/dL)	0.038	<0.001
Platelets (10^3/μl)	0.0003	<0.001
Stool.Fecal Calprotectin (μg/g)	<0.001	<0.001
CRP (mg/L)	<0.001	0.0002
Na (mmol/L)	0.034	0.012
AST (IU/L)	0.046	<0.001
Albumin (g/L)	0.0004	<0.001
ESR (mm/hr)	0.047	<0.001
MicroRNA146b_5p expression (ΔC_t)	<0.001	<0.001

ΔCt, change in threshold cycle; Na, sodium; K, potassium; AST, aspartate aminotransferase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n, sample size. $p \leq 0.05$ is significant.

As regards the relation of all possible variables affecting development of CD, multi-Regression analysis was used and revealed that smoking was a significant variable for CD, however, there was no significant difference for UC as mentioned in Table (3) and Table (4).

Table 3. Correlation Study, Using Multi-Regression Analysis, Between Possible Predictors for Crohn's Disease (CD).

Parameter	Reg. Coef.	^{t}*p- value*	$^{F\text{-}Ratio}$*p*- value
Age	-0.007	NS	
Gender	0.189	NS	
Smoking	0.544	0.04	0.034

Reg. Coef., regression coefficient; t, t-statistic; probability value; F-Ratio, F-statistic. $p > 0.05$ is not significant (NS).

Table 4. Correlation Study Using Multi-Regression Analysis Between Possible Predictors for Ulcerative Colitis (UC).

Parameter	Reg.Coef.	^{t}*p- value*	$^{F\text{-}Ratio}$*p- value*
Age	0.026	NS	
Gender	-0.201	NS	
Smoking	0.507	NS	NS

Reg.Coef., regression coefficient; t, t-statistic; F-Ratio, F-statistic for overall model fit. $p > 0.05$ is not significant (NS).

Odd's ratio (OR) was used in the study to determine the association between Group I and Group II, and showed that age was a risk factor in these groups (OR: 2.25%; 0, 0.808%), respectively with 95th percentile CI 0.635-7.973 and 0.224-2.912, respectively). And gender as risk factor in the two groups (OR:1.556%, 1.909%, respectively) with 95th percentile CI 0.420-5.763 and 0.520-7.007, respectively. Expression of microRNA146b-5p was shown as a risk factor in Group I and Group II (OR: 3.182%, 0.870%, respectively) with 95th percentile CI (1.244-8.142 and 0.756-1.000, respectively.

A ROC curve was plotted to study the diagnostic performance of microRNA 146b-5p in Group I, Group II, Group III, UC, and CD patients as illustrated in Table (5).

Table 5. Receiver Operating Characteristic (ROC) Curve for Diagnostic Performance of MicroRNA146b-5p Expression Between the Different Studied Groups.

	Best Cutoff (ΔC_t)	% SP	% SN	% NPV	% PPV	% Eff	AUC
Group II Vs Group III	0.4	100.0	90.0	90.9	100.0	95.0	0.270
Group I Vs Group III	0.4	100.0	100.0	100.0	100.0	100.0	0.000
Group II Vs Group I	3.0	85.0	95.0	94.4	86.4	90.0	0.910
CD Vs Group III	0.4	100.0	100.0	100.0	100.0	100.0	0.000
UC Vs Group III	0.4	100.0	91.3	90.9	100.0	95.3	0.274

ΔCt, change in threshold cycle; % SP, percent specificity; % SN, percent sensitivity; % NPV, percent negative predictive value; % PPV, percent positive predictive value; % Eff, percent efficiency; AUC, area under the receiver operating characteristic curve; CD, Crohn's disease; UC, ulcerative colitis.

Discussion

IBD includes a range of inflammatory disorders that induce varying degrees of swelling in the gastrointestinal tract (GIT) due to diverse etiologies. It is marked by chronic swelling or inflammation of the tract, which may damage the tract and lead to recurrent inflammation of the gastrointestinal tract. Primarily, there are two variants of this inflammatory condition: UC and CD,[12] with diagnosis primarily reliant on the ECCO-ESGAR Diagnostic Guideline.[6]

IBD was once seen as a disease of the western world but now it is known to affect people in the Middle East, Asia, and South America.[13] IBD typically is presented clinically in the 2nd or 3rd decade of life, but patients can also present symptoms between 40 and 70 years.[14] However, children under the age of 18 years contribute to about 25% of IBD cases.[15]

The main aim in managing IBD is to reduce inflammation, manage symptoms, prevent complications and increase quality of life. The specific management plan depends on severity of symptoms including aminosalicylates, corticosteroids, immunomodulators and biologics.[16]

Serum miR-146b-5p was shown as a reliable biomarker for pathophysiology of IBD and used for follow up of IBD.[17] It was shown that miRNA 146b-5p reduce Forkhead box class O3 (FOXO3 or FOXO3a) expression to inhibit autophagy.[18]

In the view of the above data, our research aimed to assess the use of miRNA -146b-5p as a marker in diagnosis of IBD and to identify the response to treatment in adults with IBD. Our study included 60 subjects recruited from the Department of Internal Medicine, Ain Shams hospitals. They were classified into 20 acute IBD cases before management with anti TNF-α (group I). In addition to group II with 20 chronic IBD cases following treatment with anti TNF-α, and group III, consisted of 20 age and sex matched normal controls.

The results of present study revealed an increase expression of microRNA 146b-5p in IBD group in comparison to the control group (p <0.001). This finding agreed with that of a study by El Sabbagh et al., 2023, who revealed that miR146b-5p can be used to discriminate between IBD patients and healthy controls among an Egyptian population. The study reported that significant difference in miR146b-5p expression among cases with IBD and healthy controls. MiRNA146b-5p is down regulated in healthy controls and up regulated in IBD due to its main role in inflammation through targeting and down regulating pro-inflammatory cytokines as TNF-α, IL-1β, IL-6, suppressing Th1 and Th17 and promoting Tregs. Thus, miR146b-5p can influence the activity of immune cells like macrophages and T- cells which play important roles in the inflammatory response and development of IBD.[17]

Our study showed increased miR146b-5p expression in acute IBD in comparison to chronic IBD (p<0.001). Also, the ROC curve analysis for the diagnostic performance of miR146b-5p revealed 85% specificity and 95% sensitivity for discrimination between acute IBD from chronic IBD. So, it can detect favorable response to treatment. This finding is in accordance with the results of a research done by Chen et al., 2019, who revealed that serum miR-146b-5p level was significantly higher in clinically active IBD cases compared to IBD patients in the remission stage.[19] On the other hand, our results are different from the results of a study done by El Sabbagh et al., 2023, who found no statistically significant variance among cases with active UC than those in remission and a non-significant variance among cases with active CD compared to those in remission. They explained their findings by the hypothesis that active cases recruited did not comply to either biological nor glucocorticoid therapy.[17]

In the current work, higher expression levels of serum miRNA146b-5p in IBD cases with activity compared to controls and to the remission group. The expression levels tended to decrease with response to anti-TNF therapy compared to patients in activity. In accordance to our results, Batra et al., 2020, stated that serum miRNA-146b correlated with clinical response with anti-TNF treatment and corticosteroids in peripheral blood samples and tissue biopsies of children suggesting a potential noninvasive biomarker for clinical monitoring. The study proposed this effect of anti-TNF as a negative regulator of innate immune signaling, acting on pathway of NF-κB and inhibiting pro inflammatory genes like TNF-α, IL-1β and Toll-like receptors 4 (TLR4). MiRNA-146b-5p and anti-TNF-α in treatment of IBD have synergistic effect in reducing inflammation as anti-TNF-α therapy, directly blocks the activity of TNF-α while miRNA-146b-5p can reduce production of TNF-α and other pro-inflammatory cytokines.[20]

The ROC curve analysis for the diagnostic performance of miR146b-5p revealed 100% specificity and 100% sensitivity for diagnosis of CD and 100% specificity and 91% sensitivity for diagnosis of UC. Meanwhile, miRNA-146b-5p could differentiate between UC and CD only by 58.8% specificity and 56.5% sensitivity. So miRNA-146b-5p has no significant impact in differentiation between UC and CD. Our findings disagreed with those of a research carried out by El Sabbagh et al., 2023, who revealed that miRNA-146b-5p level was significantly higher in

the UC patients than in CD patients with 100% specificity and 48.5% sensitivity for diagnosis of UC, and 100% specificity and 41.1% sensitivity for diagnosis of CD.[17] This can be attributed to the involvement of miRNA-146b in the TLR signaling pathway and the pathogenesis of CD. The expression of miRNA 146b in monocytes is stimulated by flagellin, lipopolysaccharide, peptidoglycan, and exposure to TLR ligands. The induction of miRNA-146b by these stimuli is part of innate immune response helping to regulate inflammation and prevent excessive immune activation.[21]

Moreover, the present study revealed that smoking is considered a significant variable for the development of CD but not for the development of UC. A study done by Berkowitz and her colleagues, 2018, enforced the results of our study.[22] They revealed that smoking is considered an independent risk factor in the development of CD but not a risk factor for UC. The contradiction consequences of smoking remain inadequately understood; yet they appear to be based upon the etiological distinctions among the two illnesses, in addition to site-dependent influences. Components of cigarette smoking have been linked to alterations in gut microbiota and immune factors. Smoking can attribute to development of CD such as disruption of the delicate balance of bacteria in GIT leading to overgrowth of harmful bacteria, alteration of tight junctions with increase intestinal permeability. This permits deleterious compounds to reach the bloodstream, resulting in the disruption of the vascular endothelial growth factor (VEGF) pathway, and thus increasing gut ischemia. This results in the synthesis of pro-inflammatory chemokines and cytokines (CCL20, CCR6, IL-8) in the ileum, which can recruit immune cells such as CD4+ & CD8+ T cells and CD11b+ dendritic cells, contribute to inflammation and tissue damage.[22]

In conclusion, this study concluded that serum microRNA146b-5p is upregulated in IBD patients and might be used as a non-invasive biomarker for identifying IBD and propose the future role of miRNA-146b-5p for early diagnosis, prognosis of disease and monitoring the response of IBD cases to treatment.

Author Contributions

NHM; designed and approved the whole research protocol. DMA; contributed to the protocol design, revised laboratory work, and approved the final paper version to be published. ASA; supervised sample collection according to inclusion criteria, revised clinical data, diagnosis, and patient classification. AMA; monitored data collection process and the laboratory work, interpreted the data, shared in the statistical analysis of the results and critically revised the paper. AMA; collected the samples and patient's clinical data, carried out the laboratory work and analyzed it, carried out statistical analysis and wrote the manuscript. All authors have read and approved the manuscript.

Declaration of Conflicting Interests

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Ethical approval

The protocol of the study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Ain Shams University (FMASU MS28/2023).

Informed consent

A verbal informed consent was obtained from all study individuals before being included in the study.

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