The Sera miRNA Pattern in Patients Inflammatory Bowel Disease

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Abstract- Inflammatory bowel disease comprising Crohn's disease and ulcerative colitis presents with periods of flares and remission. Many reports have identified different dysregulated miRNAs in patients with IBD. Finding new biomarkers in IBD patients can help to launch a novel non-invasive approach for diagnosis and prognosis for patients with UC and CD. This study aimed to evaluate the plasma expression pattern of the miRNAs panel in IBD patients compared to healthy individuals. 73 plasma samples were included; 58 patients with IBD (33 individuals in flare and 25 in remission phase) and 15 healthy controls were enrolled in the study. The miRNA expression was measured by qRT-PCR using miScript SYBR Green PCR Kit (QIAGEN). Our results showed the expression level of miR-16-5P was significantly increased in the active phase compared to the inactive phase (P=0.0138) and in CD patients compared to UC patients (P=0.0216). There was a significant difference in the expression of miR-29a in Crohn's patients compared to healthy subjects (P=0.04). Measuring the expression of mir-106a; a significant increase was observed compared to healthy individuals (P=0.03) and patients with CD (P=0.0143) in proportion of UC patients' group. The miR-126 expression significantly increased in patients with active disease compared to patients in the inactive phase (P=0.0413) and healthy controls (P=0.0492). This study showed evidence for differential expression levels of plasma panel of miR-16, miR-29a, miR-106a, and miR-126 in IBD patients compare to healthy individuals. We illustrate that miRNAs in plasma correlate with disease activity and can be used as a practical and non-invasive biomarker for early diagnosis and monitoring of the treatment protocol.

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Introduction

Ulcerative Colitis (UC) and Crohn's Disease (CD) (1) are two main features of inflammatory bowel disease (IBD) that commonly causes gastrointestinal tract complication in developed countries (2). Recently studies showed an increase in the prevalence of digestive diseases like inflammatory bowel diseases (3,4). IBD frequently repeats cycles of remission - a decrease or disappearance of the symptoms of IBD and a flare-up period the disease is active with symptoms (5). IBD as a multifactorial disease depends on many factors like environmental and epigenetics, genetics, microbiota, and immunologic condition that are involved in the pathogenesis of the disease (6,7). Epigenetic mechanisms play an important role in the

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development of IBD, as well, environmental factors such as breastfeeding, food, smoking, drugs, etc., have interaction by genome through epigenetic mechanisms mediation (8,2). Also, microRNA can be affected by various epigenetic mechanisms such as methylation of DNA and histone modifications that, play important roles in the pathogenesis of IBD (9,10). MicroRNAs (miRNA) are small (22-24 nucleotides), endogenous, single-stranded noncoding RNAs; that play an important role in regulating gene expression at post-transcriptional levels (11). The microRNAs have many physiological functions such as the immune system, cell cycle, apoptosis, and proliferation regulation (12). Previous studies have confirmed that levels of miRNAs in serum, plasma, and tissue can be used as a non-invasive biomarker for cancer and various diseases, such as IBD (13,14). Finding new biomarkers in IBD individual plasma; can help to introduce new non-invasive biomarkers for prognosis and diagnosis in UC and CD patients (15). Recently studies reported that miR-16 and miR-29 showed a meaningful association between UC and CD (16-18). Also, miR-106 significantly increased in CD compared to healthy individuals and confirmed that miR-126 effect NFkB expression and is related to the pathogenesis of IBD (18,19).

Studies on microRNA panels can develop more suitable markers for the prognosis and diagnosis of diseases. This study aimed to evaluate the plasma expression of miRNAs panel in IBD patients compared to healthy individuals to gain further knowledge about the diagnostic and prognostic value of selected miRNAs.

Materials and Methods

Study subjects

A total number of 73 plasma samples included; 33 patients with IBD in the active phase (flare-up), 25 patients with IBD in the inactive phase (remission), and 15 healthy control samples who were referred to the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (Tehran, Iran) between 2014 and 2016, enrolled in this study. The patients were provided written consent before the sampling procedure. This study protocol was conforming to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the approval by the Ethics Committee of the Gastroenterology and Liver Diseases Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran under the ethical code of IR. SBMU.RIGLD.1394.824. Blood samples were taken from volunteers in 5 ml tubes containing EDTA. The samples were stored in - 80 and RNA extraction was performed.

Selection of miRNAs

The chosen miRNAs were selected based on a literature review of previous studies. We identified four miRNAs (miR-126, miR-16, miR-29a, miR-106a) to evaluate them as a panel of microRNAs to find non-invasive biomarkers as IBD signatures (20).

Total RNA isolation and cDNA synthesis

Total RNA that contains microRNA was isolated using miRNeasy Mini Kit (Qiagen, Germany, Cat. No.217004) according to the manufacturer's protocols. The quantity and quality of RNA extracted were investigated using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA). Then, cDNA synthesis was performed using the mi Script II RT kit (QIAGEN, Germany, Cat. No. 218161) according to the manufacturer's protocol.

Quantitative real-time PCR

qRT-PCR was carried out on a real-time PCR cycler (Rotor-Gene Q MDx, Qiagen). cDNA fragments were used as templates to amplify the miRNAs (16, 29a,106a, and 126) genes by using miScript SYBR Green PCR Kit (QIAGEN, Germany, Cat. No. 218073), according to the manufacturer's instructions. The experimental protocol was performed as follows: 1) thermal cycling conditions consisted of an initial activation step for 30 seconds at 95° C, 2) 40 cycles at 95° C for 5 seconds and 60° C for 35 seconds, and 3) melting curve analysis. A duplicate, no template controls (NTC), was included in every run for each primer pair to test the DNA contamination in buffers as well as solutions and to assess the primer–dimers. The U6B gene was used as a normalizer. MiScript SYBR Green PCR Kit contains the license Universal reverse primer (miScript Universal Primer). The primers are listed in Table 1. The $2^{-\Delta\Delta CT}$ method was used to determine the expression fold changes (IBD *vs.* normal) (21).

Statistical analysis

Statistical analysis was performed using the SPSS (version 19.0) software (SPSS Inc., Chicago, Ill, USA) and by the application of the Chi-squared test, the independent two-tailed t-test, and the one-way ANOVA test. A *P* less than 0.05 was considered statistically significant. Data was graphed by the GraphPad Prism

(©2010 GraphPad Software; version 5.04). The receiver operating characteristics (22) curve was constructed to describe diagnosis specificity and sensitivity. ROC curve was performed using SPSS v.20 (SPSS Inc., Chicago, IL, USA).

Table 1. Primers were used for Quantitative Real-time PCR in This Study.

Name	Direction	Primer (5′-3′)	Mature sequence
miR-16-5P	Forward	Hs_miR-16_1 mi Script Primer Assay (cat. No. MS00006517, Qiagen)	uagcagcacguaaauauuggcg
miR-29a	Forward	Hs_miR-29a_1 mi Script Primer Assay (cat. No. MS00003262, Qiagen)	uagcaccaucugaaaucgguua
miR-106a	Forward	Hs_miR-106-5p mi Script Primer Assay (cat. No. Mi0000113, Qiagen)	aaaagugcuuacagugcagguag
miR-126	Forward	Hs_miR-126_1 mi Script Primer Assay (cat. No. Mi00003430, Qiagen)	ucguaccgugaguaauaaugcg
U6B	Forward	Hs_RNU6-2_11 mi Script Primer Assay (cat. No. Mi00033740, Qiagen)	-
Universal qPCR primer	Reverse	miScript Universal Primer (Qiagen)	-

Results

Recruitment of patients

In this study, 58 patients and 15 healthy individuals were enrolled to measure the expression level of miR-16-5P, miR-29a, miR-106a, and miR-126. The patients who were in the inactive phase had a history of consuming anti-inflammatory drugs, including 5-aminosalicylic acid (5ASA), prednisone (Pred), azathioprine (18), and infliximab (IFX). No treatment has been used for the active phase patients recruited in the current study. The demographic characteristics and BMI of the individuals were summarized in Table 2. Among the individuals enrolled in this study; there was no background of familial history of IBD, cigarette smoking, and alcohol consumption.

TABLE 2. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF THE STUDY POPULATION

Variable	Patients (n=58)	Controls (n=15)
Age (mean years±SD)	36.85 ± 12.03	26.30 ± 12.09
BMI (mean±SD)	23.55±6.85	26.30±3.19
Gender, n (%)		
Female	38 (66%)	3 (20%)
Male	20 (34%)	12 (80%)
Disease Phase, n (%)		
Flare-up (Active)	33 (57%)	-
Remission (Inactive)	25 (43%)	-
Disease Type n (%)		
Ulcerative Colitis (UC)	40 (69%)	-
CROHN'S DISEASE (1)	18 (31%)	-

Comparison of the miRNAs gene expression pattern in patients and healthy individuals

miR-106a

The miR-106 expression was significantly upregulated in patients with active diseases in comparison with healthy individuals (P=0.03) (figure 1A). The differences in the expression levels of miR-106 in the samples were statistically increased significantly between CD patients with UC and healthy controls (P>0.05, P=0.0143, respectively). However, there was no significant difference between UC patients and control individuals (P=0.073) (Figure 1A, B).

miR-126

The expression of miR-126 was measured in the plasma samples of patients and healthy individuals. According to the results, the mean expression values of the mir-126 have been significantly increased in the patients in the active phase compared to the inactive and healthy individuals (P=0.413, P=0.049, respectively) (Figure 1C). In addition, the expression of miR-126 in patients with CD was significantly increased compared to the healthy individuals (P=0.0241) (Figure 1D).

miR-16-5p

The Expression level of miR-16-5p in the active phase significantly increased compared to the patients in the inactive phase (*P*=0.0138) (Figure 1E). Also, a significant increase in miR-16-5p level of healthy individuals was observed compared to patients who were in their inactive phase (*P*=0.0078) (Figure 1E). In addition, the expression of miR-16 in CD patients was significantly increased compared to UC patients (*P*=0.0216) (Figure 1F).

miR-29a

Comparison of expression of miR-29a in patients with active and inactive phases and healthy individuals did not show any significant difference (Figure 1G). Also, no significant alteration in the expression of miR-29a in UC patients was observed compared to healthy individuals. However, there was a significant difference in the expression of miR-29a in CD patients compared to healthy individuals (*P*=0.037) (Figure 1H).

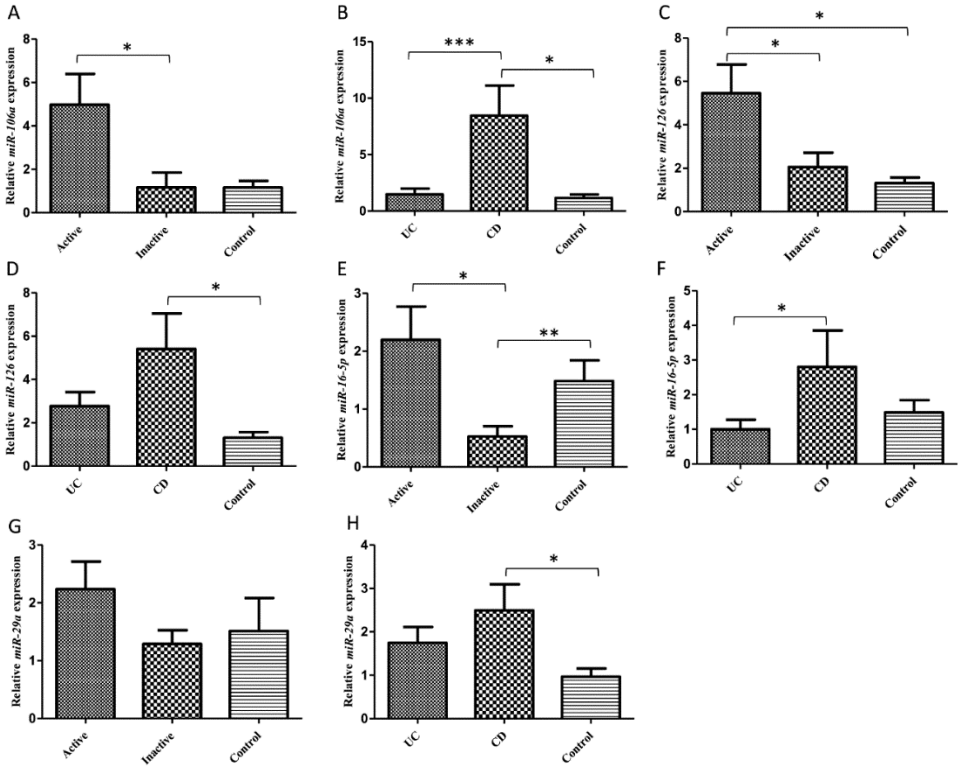

Figure 1. Relative expression of each gene between the serum sample of inflammatory bowel disease patients compared to healthy subjects. (A, B) Relative expression of miR-106a between the different phases and subtypes of IBD and healthy individuals. (C, D) Relative expression of miR-126 between the different phases and subtypes of IBD and healthy individuals. (E, F) Relative expression of miR-16-5p between the different phases and subtypes of IBD and healthy individuals. (G, H) Relative expression of miR-29a between the different phases and subtypes of IBD and healthy individuals. CD, Crohn's disease; UC, ulcerative colitis; (*P<0.05) (**P<0.01) (***P<0.001)

Evaluation of miR-16, miR-29a, miR-106a and miR-126 as biomarkers

To investigate if miR-16, miR-29a, miR-106a, and miR-126 can be considered as the potential biomarkers for IBD, the receiver operating characteristics (23) curves and the area under the ROC curves (AUC) were quantified in IBD patients and healthy individuals. The plasma levels of miR-16, miR-106, and miR-126 had diagnosis values in active and inactive phases. The ROC curves analysis results showed an AUC of 0.743 (95 percent CI: 0.614-0.871) for miR-16, 0.721 (95 percent CI: 0.589-0.854) for miR-106 and 0.698 (95 percent CI: 0.563-0.833) for miR-126. Combination ROC curve analyses of the three miRNAs in plasma as predictive IBD-related biomarkers in active and inactive phases showed an AUC of 0.812 (95 percent CI: 0.693-0.931) (Figure 2 A, B). Comparison of miR-126 in active and miR-16 in inactive phases with healthy individuals showed strong separation AUC of 0.714 (95 percent CI: 0.569-0.859) for miR-126, 0.780 (95 percent CI: 0.625-

0.935) for miR-16 (Figure 2C and D). Comparison between individuals with CD and healthy individuals in miR-126, miR-29, and miR-106 showed substantial AUC of 0.65 (95 percent CI: 0.444-0.871) for miR-126, 0.70 (95 percent CI: 0.501-0.899) for miR-29 and 0.75 (95 percent CI: 0.574-0.931) for miR-106. Combination ROC curve analyses of the three miRNAs (miR-126, miR-29, miR-106) in plasma as predictive biomarkers in CD and healthy individuals showed an AUC of 0.822 (95 percent CI: 0.650-0.955) (Figure 2E and F). Comparison of miR-106 and miR-16 in CD and UC patients showed AUC of 0.790 (95 percent CI: 0.657-0.924) for miR-106, 0.715 (95 percent CI: 0.563-0.867) for miR-16. Also, Multi ROC curve analyses of the two miRNAs (miR-16, miR-106) in plasma as predictive biomarkers in CD and UC individuals showed AUC of 0.825 (95 percent CI: 0.703-0.947) for miR-106 and, 0.715 (95 percent CI: 0.563-0.867) for miR-16. Combination ROC curve analyses of the two miRNAs (miR-16, miR-106) in plasma as predictive biomarkers in CD and UC subjects showed an AUC of 0.855 (95 percent CI: 0.741-0.970) (Figure 2G and H.). The diagnosis value of miRNAs in plasma as predictive Biomarkers in IBD the cut-off points, sensitivity, specificity, PPV (Positive predictive value), NPV (Negative predictive value), and diagnosis efficiency of the miRNAs where shown in Table 3.

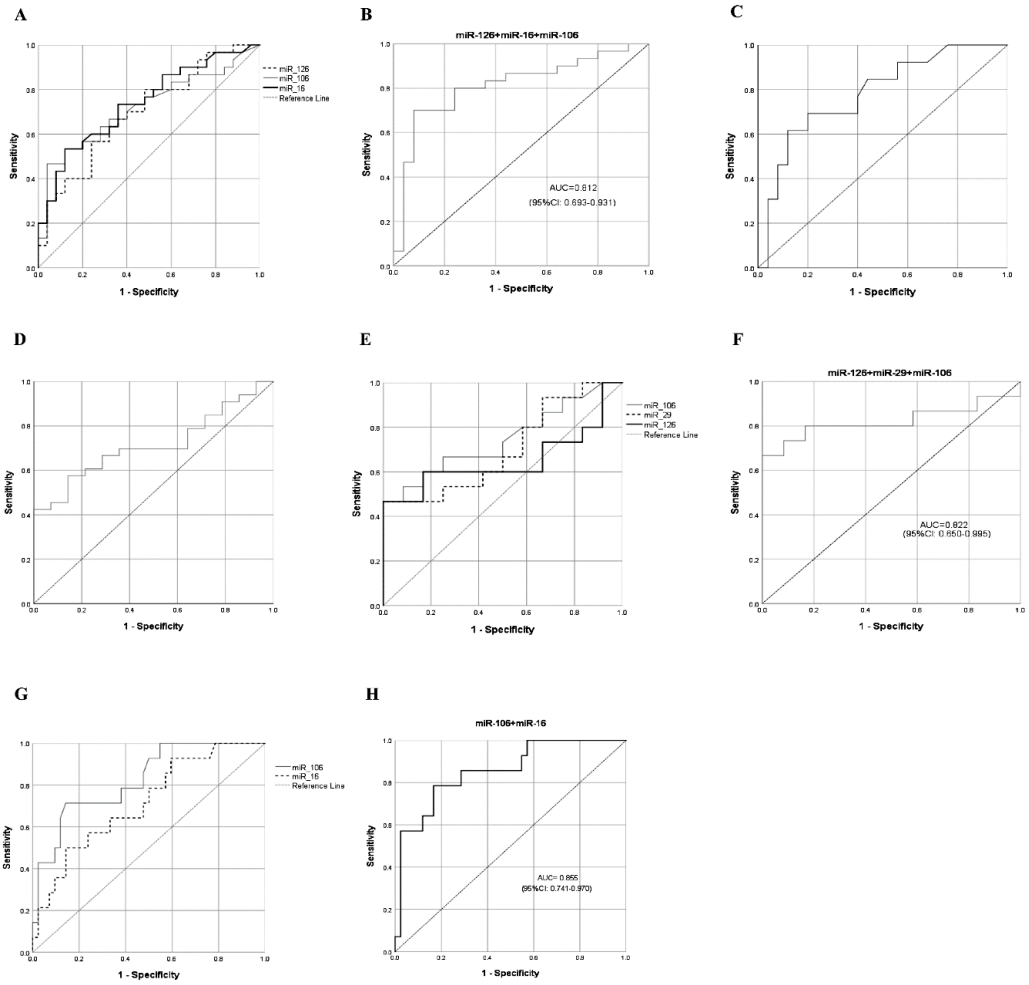

Figure 2. ROC curve analysis with multiple markers compared with a combination of ROC curve analyses of the panel miRNAs. (A, B) ROC curves for the single and combination ROC curve miRNAs (miR-126, miR-16, miR-106) for the diagnosis between active and inactive groups. (C) ROC curves for the single miR-16 diagnosis between inactive and healthy control groups. (D) ROC curves for the single miR-126 diagnosis between active and healthy control groups. (E, F) ROC curves for the single and combination ROC curve miRNAs (miR-126, miR-29, miR-106) for the diagnosis between CD and healthy groups. (G, H) ROC curves for the single and combination miRNAs (miR-106, miR-169) for the diagnosis between CD and UC groups. miRNA, microRNA; ROC, receiver operating characteristic

Table 3. The diagnosis value between Predictive IBD-related Biomarkers

Variable	CD and UC	CD and Control	Active and Inactive
Pooled sensitivity (%)	50.00%	26.67%	23.34%
Pooled specificity (%)	90.50%	100.00%	92.00%
Pooled PPV	63.64%	100.00%	77.78%
Pooled NPV	84.4%	52.17%	50.00%
Accuracy	80.36%	69.56%	54.54%

Discussion

The incidence of IBD as a chronic gastrointestinal disorder has increased in developing countries in the world (23). Despite numerous studies that have been carefully scrutinized, researchers have not yet been able to find suitable non-invasive biomarkers for detecting IBD. Although colonoscopy is still the common way for diagnosis, it is an invasive method with many complications (24). Previous studies introduced different microRNA signatures obtained from patients (25). Suggested miRNA biomarkers don't have suitable sensitivity and specificity so far, therefore, in this study, a panel of functional miRNAs were evaluated to assess more accurate and non-invasive biomarkers to diagnose or predict prognosis of IBD. Our data showed a significant correlation between *miR-106*, *miR-16*, and *miR-126* levels in different phases of IBD and patients and healthy individuals as well. Compared to colonoscopy and stool calprotectin testing using ELISA, serum miRNA testing has numerous advantages as a detecting technique for IBD. Quantitative RT-PCR used for miRNA quantification is more specific and sensitive than ELISA in calprotectin measurement and has more cost benefits as well. In addition, fecal calprotectin measurement cannot distinguish between UC and CD. Although other serum markers of inflammation such as C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are used in IBD diagnosis, they are not sufficiently sensitive or specific. The previous study by Bo Lv and colleagues showed miR-29a level upregulated in UC patients and UC mice model induced by dextran sodium sulfate (DSS) (26). They identified a miR-29a binding on the 3'UTR site of mcl-1 and Knockout of Mcl-1 caused apoptosis in the colonic epithelial HT29 cells (26). Furthermore, another study showed Serum expression of miR-29a was higher in UC patients compared to healthy subjects (15). However, our results showed no significant association of miR-29a in the plasma samples of UC patients, however, we showed a significant correlation in the plasma samples of CD patients. In 2011, it was shown that an increase of mir-29 by IFN could be one of the regulators of the innate and adaptive immune response (27). Also, in two different studies, mir-29 was introduced as a biomarker for early diagnosis of colorectal cancer (28,29). MiR-16 is known to be crucial in immunity via contribution to the release of inflammatory factors and increased in the T-cell subtypes (30,16). In 2008 it was shown that the levels of miR-16, miR-29a, and miR-126 were significantly increased in active UC tissues (17). Moreover, they showed that miR-16 induces the degradation of mRNAs containing the AU-rich elements on 3′UTRs from the TNF, IL-8, and IL-6 (17). According to a recent report, miR-16 overexpressed in chronic active terminal ileal CD tissues compared to healthy individuals (31). Furthermore, upregulation of miR-16 in PBMC was found in rheumatoid arthritis patients (32). In addition, in 2010, it was shown that miR-106 specifically upregulated in tissues from colonic CD (16). Similar to our result, it was shown that miR-106a was significantly up regulated in the peripheral blood in CD patients compared to the healthy individuals (33). In another study, the same results were repeated. They showed that MiR-106a significantly upregulated in the peripheral blood in an active phase of CD and UC compared to controls (34). Although, our findings showed no significant association of MiR-106a in the serum sample of UC patients compared to control individuals. The expressions of miR-126 in the biopsy samples of IBD patients were significantly higher than in the control group (35). They suggested that the negatively regulated correlation between S1PR2 expression and miR-126 might be explained by this miR-126 overexpression contributed to intestinal mucosal barrier dysfunction (35). In accordance with the previous study, it was demonstrated that miR-126 expression was elevated in UC patients and expressed in endothelial cells (36). Xiao Feng in 2012 showed that the expression of mir-126 in patients with active UC is associated with NFKB inhibitor, which leads to increased NFKB (19). On the other hand, another study on sigmoid colon tissue in active UC patients showed a significant increase of mir-126 compared to healthy subjects (17). In contrast to the mentioned findings, similar to our results, Archanioti Paraskevi (2012)

suggested that miR-126 overexpressed in the peripheral blood in CD patients compared to healthy individuals (33).

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