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The Role of MicroRNA-21 and MicroRNA-145 as Biomarkers in Ulcerative Colitis

Fatimah Ahmed Sabry[1,2*] , Suhad saad Mahmood [1]

[1]*Biotechnology Department, College of science, University of Baghdad, Baghdad, Iraq*

[2]Department of Biology, College of Science, Al-Nahrain University

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Abstract

MicroRNAs (miRNAs) are vital bioactive factors that are important in almost every kind of cellular events. It plays a crucial role in regulating gene expression and is implicated in various diseases. This study aims to identify and characterize the differential expression of miRNAs in ulcerative colitis tissues compared to normal tissues and investigate their potential as biomarkers for early diagnosis. Real-time PCR was used to profile miRNA expression in 20 ulcerative colitis samples and 20 matched normal tissues. Bioinformatics tools were used to analyze expression patterns and identify significantly deregulated miRNAs. The analysis revealed miRNAs with significantly altered expression levels in ulcerative colitis tissues. MiR-21 were found to be upregulated, while miR-145 was downregulated. These changes were validated by quantitative PCR. Specific miRNAs, particularly miR-21 and miR-145, hold promise as potential biomarkers for the diagnosis and prognosis of ulcerative colitis. Further research is needed to explore its roles in cancer and therapeutic potential.

Keywords: MicroRNAs, Ulcerative colitis, Real-time PCR, MiR-21, miR-145.

دور الحمض النووي الريبوزي الميكروي-21 والحمض النووي الريبوزي الميكروي-145 كمؤشرات حيوية في التهاب القولون التقرحي

فاطمة احمد صبري*[1, 2] , سهاد سعد محمود[1]

[1]قسم التقنيات الاحيائية , كلية العلوم , جامعة بغداد , بغداد ,العراق

[2]قسم علوم الحياة ، كلية العلوم ، جامعة النهرين

الخلاصة

الرنا الميكروي (miRNAs) هي عوامل حيوية نشطة بيولوجيًا لها أهمية في كل أنواع الأحداث الخلوية تقريبًا. وتلعب دورًا حاسمًا في تنظيم التعبير الجيني ولها دور في أمراض مختلفة.هدفت هذه الدراسة إلى تحديد وتوصيف التعبير التفاضلي للحمض النووي الريبوزي المييرنا في أنسجة التهاب القولون التقرحي مقارنة بالأنسجة الطبيعية والتحقيق في إمكاناتها كمؤشرات حيوية للتشخيص المبكر.تم استخدام تفاعل البوليميراز

*Email: * Fatima.Ahmed2406p@sc.uobaghdad.edu.iq

المتسلسل في الوقت الحقيقي لتحديد ملامح تعبير الحمض النووي الريبي المرسال في 20 عينة من التهاب القولون التقرحي و20 نسيجًا طبيعيًا. استُخدمت أدوات المعلوماتية الحيوية لتحليل أنماط التعبير وتحديد الحمض النووي الريبي المرسال غير المنظم بشكل كبير.كشف التحليل عن وجود مستويات تعبير متغيرة بشكل ملحوظ في أنسجة التهاب القولون التقرحي. ووُجد أن MiR−21 قد تم تنظيمه بشكل متصاعد ، في حين كان تنظيم miR−145 منخفضا . تم التحقق من صحة هذه التغييرات عن طريق Real−time PCR.وتبين ان نتائج الحمض النووي الريبوزي المرسال الذي تم تحديده، وخاصةً miR−21 و miR−145 واعدة ، كمؤشرات حيوية محتملة لتشخيص التهاب القولون التقرحي وتشخيصه. هناك حاجة إلى مزيد من الأبحاث لاستكشاف أدوارها في علم أمراض السرطان وإمكاناتها العلاجية.

1. Introduction

Ulcerative colitis (UC) is one of the two major inflammatory bowel diseases (IBD), distinguished by chronic generalized inflammation, mainly presenting as chronic inflammation and ulceration of the rectum and colon. Ulcerative colitis typically results in inflammation that is restricted to the mucosa and submucosa, with a continuous distribution from the rectum to the proximal colon [1].

The progression of ulcerative colitis is characterized by alternating active and inactive phases [2]. Individuals in the active phase usually experience abdominal pain, diarrhea, hemorrhage, and weight loss [3]. UC is thought to originate from a complex interplay of environmental variables, genetic predisposition, modified gut flora, and immunological dysfunction. UC frequently relapses, potentially resulting in intestinal perforation, toxic megacolon, colorectal cancer (CRC), and further problems; UC is not treatable. Currently, treatment aims towards clinical reduction in symptoms, mucosal restoration, and histological healing [4, 5].

At present, the diagnosis of UC is achieved using a combination of endoscopic examination, clinical symptoms, histological analysis, laboratory tests, and imaging studies [6]. However, UC is a condition wherein 15-30% of IBD patients are unable to attain a definitive diagnosis of either UC or Crohn's disease (CD) [7]. Due to the rising incidence and prevalence of UC, it has emerged as a significant worldwide health burden. There is an urgent clinical need for improved diagnosis, monitoring, and treatment of UC [8].

Various types of miRNAs with diverse expression levels have a strong association with inflammatory and autoimmune disorders, including psoriasis [9], rheumatoid arthritis [10], multiple sclerosis [11], and IBD [12]. Mature miRNAs are single-stranded noncoding RNAs that are approximately 22 nucleotides in length. Pre-miRNA hairpins, approximately 80 nucleotides in length, form them. These hairpins are frequently processed from original miRNA transcripts, which may contain hundreds of nucleotides due to the presence of numerous pre-miRNAs. Both intronic and intergenic nucleotides can produce pri-miRNA, which can show signs of evolutionary conservation. MicroRNAs (miRNAs) are very important for controlling gene expression after transcription. The mature form of miRNA binds to its target, which is usually made up of six nucleotides from positions 2 to 7, and the rest of the sequence often interacts with different nucleotide bulges [13].

The expression of miRNAs is crucial in cell cycle arrest, apoptosis, and the proliferation of tumor cells. Multiple studies indicate that variably generated miRNAs affect mRNA at distinct regulatory levels: transcriptional, posttranscriptional, modification of chromatin, and genome-specific imprint. miRNAs can affect biological processes through endogenous RNA competition, modulating the RNA transcription process, sequestration of the protein, and also

regulation of translation [14]. Such regulations may lead to reduced stability and repression of translation, impacting many basic biological processes, such as proliferation, migration, cell signaling, autophagy, and cell suicide. MiRNAs manage approximately 60% of mRNA through complementary pairing at the 3′ untranslated regions (UTRs) [15].miRNAs function as local regulators within cells and are also present at distant sites from their source, directly or indirectly influencing nearly all forms of biological regulation in living organisms [16]. Moreover, several miRNAs exhibit stability in biological fluids, including serum, plasma, urine, saliva, and other tissues [17–19]. This study aims to investigate the expression exacerbation of miR-21 and miR-145 in UC tissues using quantitative real-time PCR (RT-PCR). By comparing patients miRNAs levels with control tissues, seeking to elucidate the role of these miRNAs in UC pathogenesis and their potential as biomarkers for disease progression and response to treatment.

2. Materials and Methods

2.1 samples collection

This study included 40 patients with active UC who consulted the Gastroenterology and Hepatology specialized hospital, a tertiary centre in the Directorate of Medical City in Baghdad, Iraq, between February 2023 and August 2023. The patients were divided into two groups: 20 patients with ulcerative colitis and 20 healthy individuals. Each biopsy was immediately preserved in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to ensure RNA integrity and stability during transport and storage. The 40 biopsies include 16 males and 24 females with a median age of 39.5 years. The Medical Ethics Committee of the University of Baghdad, College of Science, approved the investigation under the reference number CSEC/1224/0119. All participants gave informed consent and agreed to donate endoscopic samples for this case-control study.

2.2 RNA Extraction

2.2.1 Total RNA isolation

Total RNA, including miRNA, was extracted using a modified TRIzol method, firstly by homogenization of 0.25 g of biopsy sample in 600 µL of TRIzol reagent using a tissue homogenizer. This is followed by phase separation, which involves adding 250 µL of chloroform to the mixture, vortexing for 3 min, freezing for 2-3 min, centrifugation at 10,000 rpm for 10 min at 4°C, and collection of the upper aqueous phase containing RNA. Lastly, precipitation by mixing the phase with 500 µL of pre-chilled isopropanol, and then incubating at -20°C for at least 10 min to precipitate the RNA. The samples were then centrifuged at 10,000 rpm for 10 min at 4°C. The RNA pellet was washed with 70% ethanol, centrifuged at 10,000 rpm for 5 min, air-dried for 3 min, and resuspended in RNase-free water.

2.2.2 Total RNA to cDNA

The RNAs were converted to cDNA using the TaqMan MicroRNA Reverse Transcription Kit. In each reaction, 1 µg of RNA was mixed with a primer set that included primers specific for miRNA-21 and miRNA-145, along with a primer for miRNA-16 as a normalization control. The reaction was incubated at 70°C for 5 min, then at 4°C for 5 min to anneal the primer, followed by 42°C for 30 min to allow reverse transcription, and terminated at 85°C for 5 min to inactivate the enzyme. The synthesized cDNA was then used in quantitative PCR (qPCR) to assess the expression levels of miRNA-21 and miRNA-145, normalizing the results to miRNA-16 to account for any variability in RNA input. This approach enabled accurate quantification of the target miRNAs in the context of ulcerative colitis

2.3 Quantitative RT-PCR (qRT-PCR)

Primers for miRNA-21, miRNA-145, and reference miRNA-16 were synthesized by Macrogen®, Korea) were prepared according to the manufacturer's protocol (Table 1). The relative expression of mRNAs was examined by qRT-PCR using LightCycler FastStart RNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol. Primers for miR-16 were used as internal controls. All of the reactions were run in triplicate. A comparative threshold cycle (ΔCT) method was used to compare each condition with the controls, and values are expressed as ΔΔCT or fold change. As indicated in Table 2, the reaction was carried out for both genes under optimal PCR conditions.

Table 1: The primers used in the amplification of RT-PCR

Primer Name	Sequence (5′-3′)	Tm (C)	References
FORWARD PRIMERS			
miR-21-F	5′-GGTTTTTTTTAGCTTATCAGACTGA-3′	54.6	Design in This study
miR-145-F	5′-GGTTTTTTGTCCAGTTTTCCCAGGA-3′	63.4	
miR-16-F	5′-GGTTTTTTTTTAGCAGCACGTAAAT-3′	55.2	
RT- PRIMERS			
miR-21-RT	5′-GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACTCAACA-3′	79.1	
miR-145-RT	5′-GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACAGGGAT-3′	80.5	
miR-16-RT	5′-GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACCGCCAAT-3′	80.9	
REVERSE PRIMERS			
UNIVERSAL-R	5′-GTGCAGGGTCCGAGGT-3′	64.2	

Table 2: RT-PCR conditions of the desired genes

Steps	Temperature	Time	Number of cycles
Activation	95°C	3 mins.	1
Cycling 1	95°C	20 sec.	40
Cycling 2	60°C	20 sec.	
Cycling 3	72°C	20 sec.	
Extension	60°C to 95°C	30 mins.	1

2.4 Gene Expression analysis

The qRT-PCR data results were determined by directly comparing the Ct values (cycle threshold) between the target (miRNA-21 and miRNA-145) and reference (miRNA-16 as housekeeping gene) genes. The genes were examined using the following equations, which are based on the relative quantification of gene expression levels using the Livak formula described by Schmittgen [20] as demonstrated by the equations below:

1. Calculating the ΔG of the target gene to the reference gene was determined for each sample.

ΔG = Ct target gene – Ct reference gene

2. Calculating the differences between the ΔCt of the unknown and the ΔCt of the calibrator

$\Delta\Delta Ct$ = (Ct target gene – Ct reference gene) sample – (Ct target gene – Ct reference gene) control

3. The sample's normalized target quantity was equalized to $2^{-\Delta\Delta Ct}$, and the expression levels of the samples were compared using this value:
Fold change = $2^{-\Delta\Delta Ct}$
4. Finally, the relative expression changes in mRNA expression levels were calculated using the comparative threshold cycle (CT) value method (= $2^{-\Delta\Delta Ct}$).

2.5 Statistical analyses

The analysis of miR-21 and miR-145 expression levels normalized to the housekeeping gene miR-16 expression was performed using GraphPad Prism (8.0.2). A p-value of < 0.05 was considered statistically significant.

3. Results and Discussion

This study evaluated the expression levels of microRNAs, miRNA-21 and miRNA-145, in 40 endoscopic biopsies; 20 UC biopsies were compared to 20 control biopsies by RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-PCR). Expression levels were normalized to miRNA 16.

The amplification is reported in Figure 1 as a Ct value. In terms of gene expression, a higher Ct value of the target gene indicates that there are fewer copies of DNA, leading to lower gene expression, while at lower Ct value indicates a high level of gene expression due to the presence of high copies of DNA [21]. The main goal of this study was to quantify gene expression for the miR-21 and miR-145 genes. The miR-16 gene was used as a housekeeping reference gene due to its consistent expression across various conditions in the examined cells. The results were then calculated using the ΔΔCt method (Livak method), based on data recorded from the qRT-PCR software for the selected biopsies [22, 23].

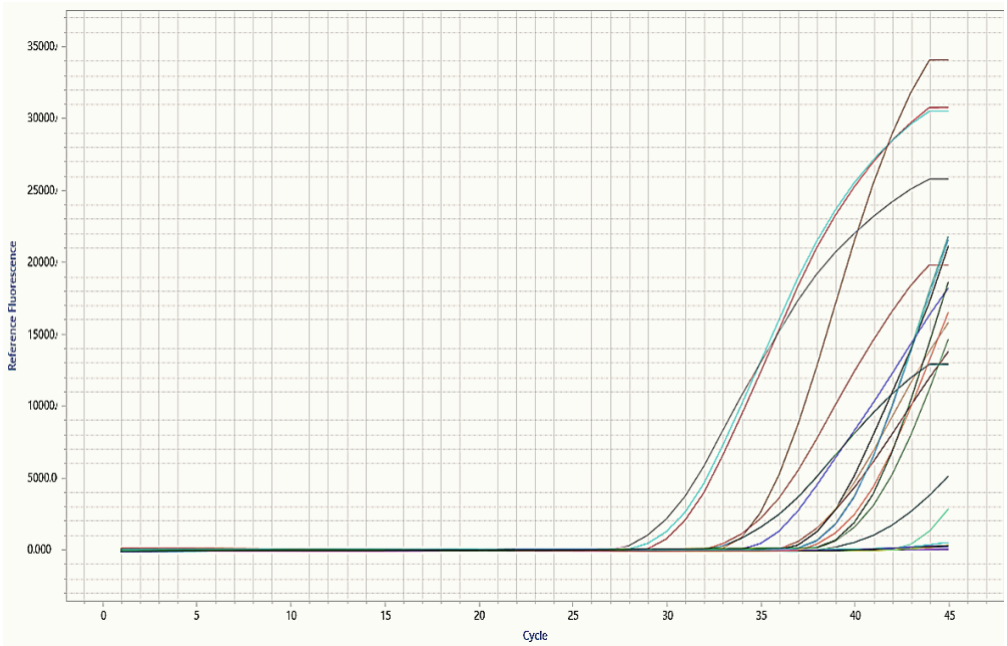

Figure 1: RT-PCR results of related MicroRNAs expression tested by threshold cycle (Ct).

The contrasting roles of these miRNAs in the context of UC in Table 3 show a significant upregulation of miRNA-21 in UC biopsies, with a calculated fold change of 3.86 ($p < 0.01$), compared to a fold change of 1.45 for the control samples. This suggests a potent increase in miRNA 21 expression among patients with UC. Conversely, miRNA-145 exhibited significant downregulation, with a fold change of 1.16 in UC samples versus 0.33 in controls ($p < 0.01$).

Table 3: mir-21 and mir-145 gene expression in UC patients and controls

miRNA	Mean ΔCt For controls	Mean ΔCt For patients	p- value	ΔΔCt	Folding Expression
miRNA-21	9.55	7.97	0.0181	-1.5834	3.8640
miRNA-145	4.56	8.81	<0.0001	4.25	0.1906

A bar chart (Figure 2 a and b) illustrating these findings was generated using GraphPad Prism, displaying significant differences in expression levels between the UC and control groups. The t-test confirmed statistical significance for both miRNAs, reinforcing the notion that dysregulation of these microRNAs is associated with the inflammatory processes inherent in UC.

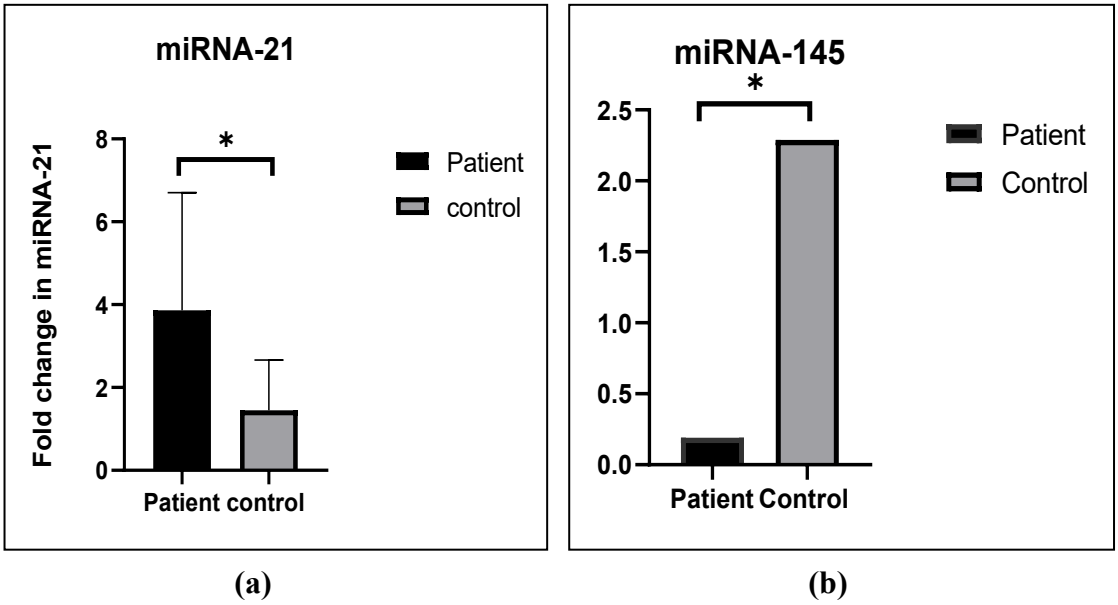

Figure 2: This figure depicts (a) The expression levels of miRNA-21 extracted from a diagnosed ulcerative colitis colon (patient) and normal biopsies (control) by qRT-PCR, P value = 0.0181.(b) The expression levels of miRNA-145 extracted from ulcerative colitis (patient) and normal biopsies (control) by qRT-PCR, P value = 0.0363.

The upregulation of miRNA-21, as shown in Figure 2 a, is consistent with previous studies that explored miRNA-21's significant role in gastrointestinal inflammation and tissue repair, demonstrating miRNA-21's regulation key metabolic pathways in gut microbiota, affecting biosynthesis of indole and L-tryptophan metabolites for controlling inflammation and colonic motility. This novel pathway highlights the interaction of miRNA-21 with the microbiome, linking it to gastrointestinal pathophysiology, including inflammatory bowel diseases and irritable bowel syndrome [24]. In contrast, in Figure 2 b, the significant downregulation of miRNA-145 is consistent with findings that emphasize its critical role in maintaining epithelial integrity and modulating immune responses. Research indicates that miRNA-145 directly influences the regulation of immune cytokines through its interaction with specific targets like Fascin actin bundling protein 1, a factor involved in epithelial cell stability and immune signalling pathways. For instance, during infections or inflammatory conditions,

miRNA-145 is observed to modulate cytokine secretion, helping restore epithelial function and immune homeostasis [25].

These findings suggest that miRNA 21 may play a role in the pathogenesis of ulcerative colitis, potentially serving as a biomarker for disease progression [26]. Downregulation of miRNA 145 also supports the dysregulation of miRNA expression in inflammatory bowel diseases [27], consistent with previous studies highlighting the involvement of these miRNAs in immune regulation and inflammation and cerebral cortical cancer progression [28, 29]. Further research is warranted to clarify the mechanisms underlying miRNAs regulation and their ideal therapeutic targets [30].

4. Conclusion

In conclusion, microRNAs (miRNAs) have opposing roles in inflammation and cellular proliferation pathways, which are crucial in the pathophysiology of UC. MiRNA-21 has been identified for its role in amplifying inflammatory responses and possibly promoting fibrosis or epithelial-mesenchymal transition, thus contributing to the chronic inflammation observed in UC. In contrast, miRNA-145 suppresses tumor progression and maintains the integrity of epithelial cells. Additional investigations are required to elucidate the mechanisms underlying these changes and their specific function in UC pathogenesis

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Disclosure and conflict of interest

The authors declare that they have no conflicts of interest.

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