

miR-20b, miR-98, miR-125b-1*, and let-7e* as new potential diagnostic biomarkers in ulcerative colitis

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Abstract

AIM: To use microarray-based miRNA profiling of colonic mucosal biopsies from patients with ulcerative colitis (UC), Crohn's disease (CD), and controls in order to identify new potential miRNA biomarkers in inflammatory bowel disease.

METHODS: Colonic mucosal pinch biopsies from the

descending part were obtained endoscopically from patients with active UC or CD, quiescent UC or CD, as well as healthy controls. Total RNA was isolated and miRNA expression assessed using the miRNA microarray Geniom Biochip miRNA *Homo sapiens* (Febit GmbH, Heidelberg, Germany). Data analysis was carried out by principal component analysis and projection to latent structure-discriminant analysis using the SIM-CA-P+12 software package (Umetrics, Umea, Sweden). The microarray data were subsequently validated by quantitative real-time polymerase chain reaction (qPCR) performed on colonic tissue samples from active UC patients ($n = 20$), patients with quiescent UC ($n = 19$), and healthy controls ($n = 20$). The qPCR results were analyzed with Mann-Whitney U test. *In silico* prediction analysis were performed to identify potential miRNA target genes and the predicted miRNA targets were then compared with all UC associated susceptibility genes reported in the literature.

RESULTS: The colonic mucosal miRNA transcriptome differs significantly between UC and controls, UC and CD, as well as between UC patients with mucosal inflammation and those without. However, no clear differences in the transcriptome of patients with CD and controls were found. The miRNAs with the strongest differential power were identified (miR-20b, miR-99a, miR-203, miR-26b, and miR-98) and found to be up-regulated more than a 10-fold in active UC as compared to quiescent UC, CD, and controls. Two miRNAs, miR-125b-1* and let-7e*, were up-regulated more than 5-fold in quiescent UC compared to active UC, CD, and controls. Four of the seven miRNAs (miR-20b, miR-98, miR-125b-1*, and let-7e*) were validated by qPCR and found to be specifically upregulated in patients with UC. Using *in silico* analysis we found several predicted pro-inflammatory target genes involved in various pathways, such as mitogen-activated protein kinase and cytokine signaling, which are both key signaling pathways in UC.

CONCLUSION: The present study provides the first evidence that miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with UC. The level of these miRNAs may serve as new potential biomarkers for this chronic disease.

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Key words: Biomarker; Crohn's disease; Diagnostics; Inflammatory bowel disease; Microarray; MicroRNA; Ulcerative colitis

Core tip: This study contributes to the current knowledge on the putative role of microRNAs in inflammatory bowel disease pathogenesis, and it provides the first evidence that miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with ulcerative colitis. The level of these miRNAs may serve as new potential biomarkers for this chronic disease.

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INTRODUCTION

MicroRNAs (miRNAs) are short (about 22 nucleotides in length), endogenous, non-coding single-stranded RNAs that act in concert to regulate expression of their target mRNAs^[1-3]. The biogenesis of these small regulatory miRNA is a multistep process occurring in the cell nucleus and cytoplasm. Briefly, miRNAs are transcribed as long primary miRNA transcripts in the nucleus, and are then cleaved into precursor miRNA hairpin (pre-miRNA) by the Drosha-DGCR8 microprocessor complex^[4-6]. Next, the pre-miRNAs are exported to the cytoplasm and further cleaved to mature miRNAs by Dicer^[7-9]. It is generally believed that the mature miRNA then incorporates into the RNA-induced silencing complex, and guides this complex to the 3'-untranslated region (3'-UTR) of specific target mRNA transcripts to suppress translation or induce their degradation^[1,2,10-13]. However, miRNA-binding sites in coding regions as well as in the 5'-UTRs have also been reported^[14-19].

It is estimated that nearly one-third of the genes in the human genome might be regulated by the more than 2000 mature miRNAs so far identified^[20]. As master regulators of post-transcription in cells, these regulatory miRNAs are involved in key functions in many physiological networks^[21-23], and differentially expression miRNAs have been implicated in the pathogenesis of diverse gastrointestinal disorders, such as cancer and inflammatory diseases^[24,25]. In facts, loss of intestinal miRNAs in mouse models has been shown to impair differentiation

of intestinal cells and epithelial barrier function, resulting in acute inflammation^[23].

Recent studies have demonstrated miRNAs to be involved in inflammatory bowel disease (IBD) susceptibility, as polymorphisms in miRNA-binding sites affect the gene expression and thus seem to play a pivotal part in the pathogenesis of this chronic disorder^[17,26]. Moreover, it has been found that miRNAs are differentially expressed in ulcerative colitis (UC) and Crohn's disease (CD)^[27], the two main forms of IBD. However, the pathogenesis of IBD still remains enigmatic^[28], but the identification of differentially expressed miRNAs and subsequent understanding of their molecular mechanisms appear to provide new ways to reveal the pathophysiology, discover new diagnostic biomarkers, and develop new therapeutics^[27].

The present study aims to analyze the miRNA expression in colonic mucosal biopsies from IBD patients and healthy individuals in order to identify new potential miRNA biomarkers in IBD using miRNA microarray profiling.

MATERIALS AND METHODS

Patients and tissue samples

Two cohorts, including both IBD individuals and controls, were analyzed in this study. In order to identify potentially deregulated miRNAs, a miRNA microarray analysis was performed on samples from cohort 1 (microarray cohort) consisting of 4 patients with UC (2 with active and 2 with quiescent disease; mean age 34 years, range 34-37 years, 2 females), 4 patients with CD (2 active and 2 quiescent; mean age 40 years, range 25-73 years, 3 females), and 2 controls (mean age 39 years, range 37-41 years, 2 females). A subsequent quantitative real-time polymerase chain reaction (qPCR) validation study was performed on samples from cohort 2 (validation cohort) including 20 patients with active UC, 19 patients with quiescent UC, and 20 healthy controls (Table 1). In both cohorts, the included subjects underwent a routine colonoscopy at the Department of Gastroenterology, Medical Section, Herlev Hospital, Denmark due to their clinical condition. They were included into the study as UC or CD patients with active disease, quiescent disease, or as controls (*i.e.*, an endoscopy was performed due to gastrointestinal symptoms but all clinical and paraclinical investigations subsequently turned out to be normal). All individuals with IBD had their diagnosis established on well-defined criteria^[29] and disease activity of all UC patients were before the colonoscopy graded in accordance with the Mayo score^[30]: a score ≤ 1 as quiescent UC and > 1 as active UC, and CD patients were graded in accordance with the Harvey-Bradshaw score^[31]: a score ≤ 4 as quiescent CD and > 4 as active CD. Exclusion criteria were age above 80 or below 18 years, clinical evidence of infection, recent (within 14 d) use of antibiotics or probiotics, pregnancy, and severe mental illness.

All mucosal pinch biopsies, each of approximately

Table 1 Patients' characteristics (validation cohort)

Characteristics	Control (n = 20)	Inactive UC (n = 19)	Active UC (n = 20)
Gender (male/female)	10/10	6/13	9/11
Age (yr), mean (range)	48 (24-83)	48 (21-69)	38 (16-79)
Age at diagnosis (< 25/> 25 yr)	-	4/15	7/13
Years with disease (< 10/> 10 yr)	-	8/11	15/5
Mayo score, mean (range)	-	0 (0-1)	6 (2-12)
Extension of disease (P/PS/PC/LC/PH)	-	-	3/3/7/5/2
Smoking/non-smoking	5/15	6/13	3/17
EIM (present/never present)	-	3/16	0/20
Daily medications, n (%)			
Systemic mesalazine (1.6-3.2 mg)	-	14 (74)	17 (85)
Topical mesalazine (1000 mg)	-	3 (16)	6 (30)
Systemic glucocorticoids (75 mg)	-	0 (0)	2 (10)
Topical glucocorticoids (100 mg)	-	0 (0)	2 (10)
Azathioprine (100-200 mg)	-	1 (5)	2 (10)
6-mercaptopurine (50-100 mg)	-	0 (0)	1 (5)
Infliximab (5 mg/kg)	-	0 (0)	1 (5)
None	20 (100)	5 (26)	2 (10)

EIM: Extraintestinal manifestations; LC: Left-sided colitis; P: Proctitis; PC: Pancolitis; PH: Proctitis hemorrhagica; PS: Proctosigmoiditis; UC: Ulcerative colitis.

15 mg, were obtained from endoscopically non-inflamed or inflamed areas of the descending colon using routine endoscopic forceps. The descending colon was preferred to avoid any intersegmental variation. Non-inflamed samples originated from CD patients with endoscopically quiescent Crohn's colitis in the descending colon, where no other segments of the colon or ileum were endoscopically inflamed. Inflamed samples originated from CD patients with endoscopically active Crohn's colitis in the descending colon. Endoscopic activity was defined as areas with mucosal oedema, hyperemia, and friability. The endoscopic diagnosis of active or inactive disease was confirmed by histopathology conducted on parallel biopsies taken within an inch of the 1st biopsy. The biopsies were immediately placed in RNA-Later solution (Ambion, Austin, TX, United States), and following 24 h in RNA-Later at 4 °C the biopsies were stored at -80 °C until RNA extraction.

The study was approved by the Scientific Ethics Committee of the Capital Region of Denmark. All patients gave their informed written consent to participate in the study.

Extraction of total RNA and miRNA microarray profiling

The *mirVana*TM miRNA isolation kit (Applied Biosystems, Carlsbad, CA, United States) was applied to isolate total RNA according to the manufacturer's protocol, and subsequently analyzed with a Geniom Real Time Analyzer (GRTA) (Febit GmbH, Heidelberg, Germany) using the Geniom Biochip miRNA *Homo sapiens*. The total RNA quality was assayed on an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States). The quantity of total RNA was measured using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). The 260/280 nm absorbance

values were consistently above 1.9.

The array contained ten replicates of each human miRNA and miRNA star (*) sequences as annotated in the Sanger miRBase v.11.0^[32]. Sample labelling with biotin was carried out using the *mirVana*TM miRNA labeling kit (Ambion). Following hybridization for 16 h at 42 °C the biochip was washed as indicated by the supplier and signal enhancement was processed with the GRTA. For each array, signal intensities were calculated using the Geniom Wizard Software (Febit GmbH).

qPCR

Total RNA (10 ng) was reverse transcribed into cDNA using miRNA-specific primers (Applied Biosystems) and the TaqMan[®] miRNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. MiRNA expression levels of seven selected miRNAs identified by microarray were measured using commercially available pre-designed miRNA-specific TaqMan[®] miRNA assays (Applied Biosystems) according to the manufacturer's recommendations. All PCR reactions were performed using a Mx3000P thermocycler (Stratagene, La Jolla, CA, United States), and cycles were as follows: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. The expression levels of each miRNA were normalized to endogenous RNU6B expression - a widely used internal control - and analyzed using the 2^{-ΔΔCT} method.

miRNA target gene predictions

Target genes of miRNAs were predicted using the miR-Walk database (<http://www.ma.uniheidelberg.de/apps/zmf/mirwalk/>) that allows simultaneous searches of several databases^[33]. Four additional programs were selected: TargetScan, miRanda, miRDB, and RNA22 as target prediction programs. The search was performed on the 3'-UTR regions of target mRNAs with a *P* value of 0.05 defining the probability distribution of random matches set in the software with a minimum miRNA seed length of 7. When at least three programs co-identified a specific transcript, then the target(s) were selected for our list of potential targets. In addition, due to the limited ability of all algorithms to predict targets of miRNA complementary strands (*), the miRNA* targets were identified using miRWalk and miRanda and only those targets predicted by both programs were examined more closely.

Statistical analysis

miRNA data analysis: The raw microarray-data were background corrected and the ten replicate intensity values of each miRNA were summarized by their median value.

In order to reduce data complexity the unsupervised multivariate data analysis tool principal component analysis (PCA) was applied to determine if any intrinsic clustering existed within the dataset. If intrinsic clustering was found, the supervised multivariate data analysis tool projection to latent structure-discriminant analysis (PLS-DA) was employed. PLS-DA, like PCA, involves reduction of

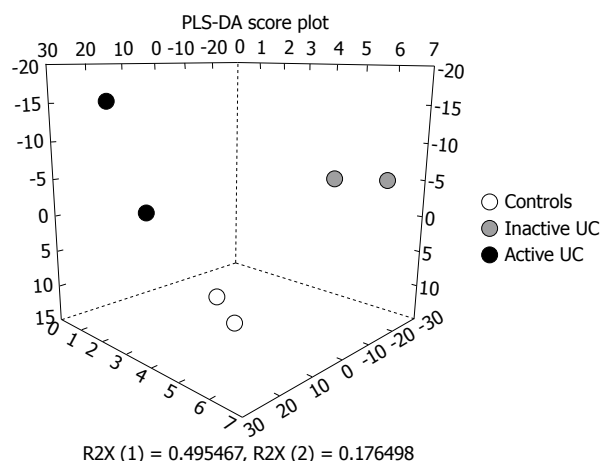


Figure 1 Projection to latent structure-discriminant analysis score-plot of the miRNA microarray expression profiles from mucosal colonic biopsies of controls, active ulcerative colitis, and inactive ulcerative colitis reveals a clear separation of these three groups. All patient with active ulcerative colitis (UC) are positioned in the left part of the space, and all patients with inactive UC are placed in the right space, whereas all control subjects are found in the middle. PLS-DA: Projection to latent structure-discriminant analysis.

data complexity and is commonly used where quantitative or qualitative relationships are sought between a matrix, X , in this case miRNA expression profiles, and another matrix, Y , in this case the class belonging of the samples. Such PLS-DA models offer the opportunity to create lists of miRNAs with the highest regression coefficients for each class, thus making it possible to identify the miRNA expression profiles responsible for the differentiation between the classes and subsequently the unique miRNAs with the strongest differential power. The multivariate data analysis was performed using SIMCA-P+ 12.0 (Umetrics, Umea, Sweden).

qPCR data analysis: Groups were compared using the Mann-Whitney U test, and P values less than 0.05 were considered significant.

RESULTS

Identification of differentially expressed miRNAs by miRNA microarray profiling

We have previously demonstrated that gene expression profiles using microarray studies can differentiate between active UC, inactive UC, and control samples^[34,35]. Thus, in an initial attempt to identify new miRNAs that are differentially expressed in patients with IBD, we performed miRNA microarray profiling of colonic tissue samples from cohort 1. The PCA score-plot indicated a 3-way separation of the samples; controls, active CD, and inactive CD in one cluster, and active UC and inactive UC in two separate clusters (data not shown). The PCA model was described by 2 components explaining a total of 61% (R2X) of the variation in the dataset. The clustering of controls, active CD, and inactive CD in the PCA model indicates a similar miRNA expression profile

in all three groups making further comparisons questionable. Thus, the subsequent PLS-DA model only contained the following three groups: active UC, quiescent UC, and controls. As seen in Figure 1, the PLS-DA score-plot resulted in a clear separation of these three groups. This PLS-DA model contained 2 components explaining a total of 67% (R2X) of the variation in the dataset and with a cross-validation parameter Q2Y (cum) of 0.89, indicating the predictability of the model. In order to substantiate the initial interpretation of the PCA score-plot, *i.e.*, clustering of active CD, inactive CD, and controls, an identical statistical procedure (creation of a PLS-DA model and comparison of regression coefficient lists) was performed with respect to these three groups. As expected, this resulted in an extensive number of miRNA duplicates in the regression coefficient lists, and the few miRNAs that were not duplicates had fold changes below 2 (results not shown).

In order to identify the miRNA expression profiles responsible for the differentiation between UC, CD, and controls, a list of 50 miRNA with the highest regression coefficients was generated for each group. When comparing the lists, no miRNA duplicates were found. Several of the differentially expressed miRNAs (such as miR-23a, miR-155, miR-16, miR-150, miR-346, and miR-126) reported to be associated with UC in previous studies^[36-38] were also identified within our analyses (Tables 2 and 3). However, in addition to these previously reported miRNAs, we additionally identified five potential miRNAs, miR-20b, miR-99a, miR-203, miR-26b, and miR-98 to be differentially up-regulated more than a 10-fold in active UC compared to inactive UC, active CD, inactive CD, and controls (Table 2). Two miRNAs, miR-125b-1* and let-7e*, were differentially up-regulated more than a 5-fold in inactive UC compared to active UC, active CD, inactive CD, and controls (Table 3). These miRNAs have not previously been reported to be involved in IBD^[27]. Thus, in the subsequent study, we focused on these seven new candidate miRNAs.

Verification of differences by qPCR

The expression levels of the seven candidate miRNAs (miR-20b, -99a, -203, -26b, -98, -125b-1*, and let-7e*) were tested in an independent validation cohort on samples from patients with active UC ($n = 20$), inactive UC ($n = 19$), and controls ($n = 20$) using individual miRNA-specific primers. In accordance with the microarray data, qPCR results showed significantly increased miR-20b ($P < 0.05$) expression in active UC *vs* controls. Moreover, miR-20b expression was significantly higher ($P < 0.05$) in inactive UC than in controls (Figure 2). Additionally, the qPCR analysis confirmed a significant ($P < 0.05$) higher expression of let-7e* in inactive UC *vs* controls (Figure 2).

In contrast to microarray results, miR-98 was significantly over-expressed in inactive UC ($P < 0.05$), when compared to both active UC and controls (Figure 2). Similarly, miR-125b-1* was not significantly up-regulated in inactive UC, as predicted by the microarray profiling,

Table 2 Top fifty differentially expressed miRNAs (active ulcerative colitis *vs* inactive ulcerative colitis, active Crohn's disease, inactive Crohn's disease, and controls) and their respective fold-changes from miRNA microarray expression profiling

MicroRNAs	Active UC/ active CD	Active UC/ controls	Active UC/ inactive CD	Active UC/ inactive UC
hsa-miR-15a	64	7	19	24
hsa-miR-199b-3p	55	6	17	61
hsa-miR-20b	53	12	20	35
hsa-miR-20a	51	9	14	47
hsa-miR-106b	43	4	7	40
hsa-miR-27b	36	6	6	15
hsa-miR-99a	33	33	17	31
hsa-miR-222	32	7	12	26
hsa-miR-151-5p	19	4	18	20
hsa-miR-203	19	11	17	24
hsa-miR-30a	14	9	14	7
hsa-miR-25	14	6	9	11
hsa-miR-26b	12	15	10	13
hsa-miR-646	12	19	13	8
hsa-miR-100	10	4	3	10
hsa-miR-125b	10	15	8	4
hsa-miR-98	10	10	10	12
hsa-miR-411*	8	3	8	5
hsa-miR-28-5p	8	3	3	6
hsa-miR-768-3p	7	5	4	17
hsa-miR-195	7	10	35	22
hsa-miR-99b	7	4	4	3
hsa-miR-23a	7	8	18	49
hsa-miR-18a	7	11	7	8
hsa-miR-17	7	5	6	44
hsa-miR-155	6	7	11	19
hsa-miR-23b	6	7	10	18
hsa-miR-1201	6	6	6	8
hsa-miR-130a	6	6	11	26
hsa-miR-199a-3p	6	5	15	37
hsa-miR-93	5	3	4	8
hsa-miR-199a-5p	5	3	3	7
hsa-miR-16	5	8	17	175
hsa-miR-146a	5	3	3	6
hsa-miR-103	5	3	6	30
hsa-miR-126	5	16	39	65
hsa-miR-107	5	3	7	18
hsa-miR-106a	5	5	6	21
hsa-miR-1248	4	5	8	93
hsa-miR-27a	4	6	6	25
hsa-miR-222*	4	4	4	6
hsa-miR-24	3	3	4	17
hsa-miR-182	3	3	3	5
hsa-miR-193a-3p	3	7	3	7
hsa-let-7e	3	2	4	2
hsa-miR-548a-3p	2	2	2	6
hsa-miR-99a*	1	1	1	3
hsa-miR-758	1	1	1	2
hsa-miR-568	1	5	6	7

Differentially up-regulated (≥ 10 -fold) miRNAs are indicated in bold in the lists. *miRNA complementary strands. CD: Crohn's disease; UC: Ulcerative colitis.

but was significantly different ($P < 0.01$) between active UC and controls (Figure 2). The expression levels of the other predicted miRNAs (miR-99a, -203, and -26b) did, however, not reach a statistical significance (Figure 3).

Prediction of miRNA target genes associated with UC

Having identified significant changes in the four miRNA

Table 3 Top fifty differentially expressed miRNAs (inactive ulcerative colitis *vs* active ulcerative colitis, active Crohn's disease, inactive Crohn's disease, and controls) and their respective fold-changes from miRNA microarray expression profiling

MicroRNAs	Active UC/ inactive UC	Inactive UC/ controls	Active CD/ inactive UC	Inactive UC/ inactive CD
hsa-miR-506	13	4	5	5
hsa-miR-125b-1*	12	6	27	5
hsa-let-7e*	6	6	6	6
hsa-miR-512-5p	5	5	5	4
hsa-miR-637	5	3	4	4
hsa-miR-1288	4	4	4	5
hsa-miR-330-3p	4	5	9	4
hsa-miR-623	4	6	6	6
hsa-miR-34b	4	9	9	7
hsa-miR-138-1*	3	6	6	5
hsa-miR-154	3	7	7	7
hsa-miR-760	3	3	17	3
hsa-miR-1296	3	5	11	4
hsa-miR-523	3	3	3	3
hsa-miR-149	3	5	10	5
hsa-miR-509-3-5p	3	3	3	3
hsa-miR-1276	3	3	19	4
hsa-miR-1178	3	4	2	5
hsa-miR-885-5p	3	3	3	3
hsa-miR-1264	2	2	2	2
hsa-miR-521	2	6	6	5
hsa-miR-218-2*	2	3	2	2
hsa-miR-551a	2	8	13	4
hsa-miR-505*	2	2	3	2
hsa-miR-1226	2	3	15	4
hsa-miR-495	2	4	9	3
hsa-miR-220c	2	3	3	4
hsa-miR-550*	2	4	2	3
hsa-miR-371-3p	2	3	3	3
hsa-miR-596	2	4	4	3
hsa-miR-216a	2	6	9	4
hsa-miR-1293	2	3	15	3
hsa-miR-1247	2	3	47	2
hsa-miR-34c-3p	2	4	12	5
hsa-miR-1233	2	3	6	3
hsa-miR-346	2	3	4	3
hsa-miR-211	2	8	9	11
hsa-miR-302c*	2	5	11	6
hsa-miR-520a-3p	2	2	2	2
hsa-miR-485-3p	2	5	14	3
hsa-miR-92a-2*	2	2	4	3
hsa-miR-328	1	2	2	2
hsa-miR-661	1	3	7	4
hsa-miR-453	1	7	8	5
hsa-miR-30c	1	5	7	7
hsa-miR-520h	1	2	2	2
hsa-miR-193b	1	4	10	4
hsa-miR-483-3p	1	2	4	2
hsa-miR-150	1	3	6	2

Differentially up-regulated (≥ 5 -fold) miRNAs are indicated in bold in the lists. *miRNA complementary strands. CD: Crohn's disease; UC: Ulcerative colitis.

expression profiles (miR-20b, miR-98, miR-125b-1*, and let-7e*) between UC and controls, we next examined the biological relevance of these miRNAs by identifying their target genes. *In silico* analysis using miRWalk, which combines the output of multiple prediction algorithms, was used to identify putative targets. Among the large number of predictive targets identified by this approach,

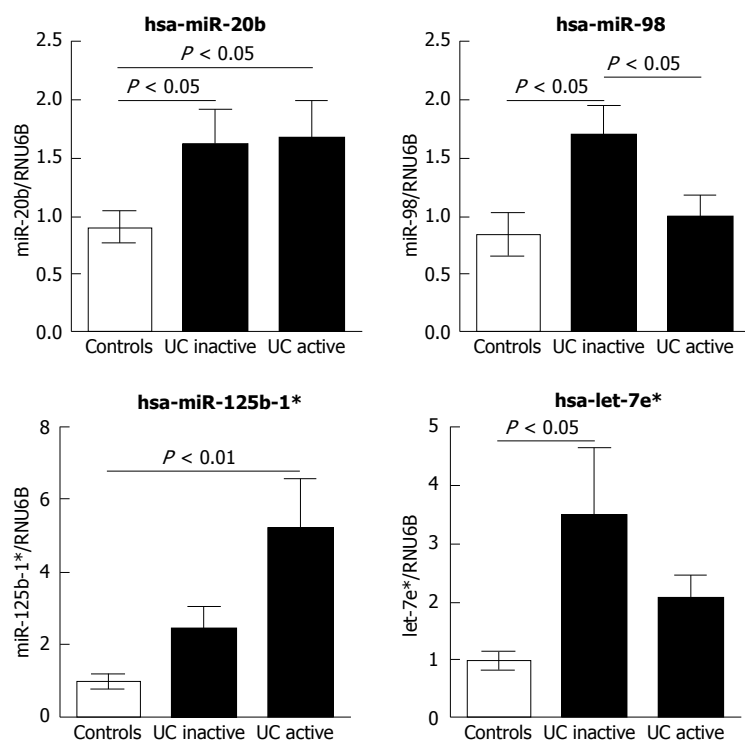


Figure 2 Expression levels of four miRNAs in mucosal colonic biopsies were significantly up-regulated in ulcerative colitis. Expression differences of miRNA in active ulcerative colitis (UC) ($n = 20$), inactive UC ($n = 19$), and controls ($n = 20$). Levels were determined using quantitative real-time polymerase chain reaction. Relative expression differences of each miRNA were normalized to endogenous RNU6B expression and calculated using the $2^{-\Delta\Delta CT}$ method. P value was calculated by Mann-Whitney U test and data are represented as medians with inter-quartile ranges.

several putative targets well known to be associated with the inflammatory response were revealed. For example, predicted target genes of miR-20b included interleukin-6 receptor (*IL-6R*), *IL-8*, *IL-10*, *REL* [a member of the nuclear factor (NF)- κ B family], autophagy related 16-like 1 (*ATG16L1*), NOD-LRRs containing pyrin domain 3 (*NLRP3*), *CASP8* and FADD-like apoptosis regulator (*CFLAR*), extracellular signal-regulated kinase 2 (*ERK2*), *p38- α* (MAP kinase), MAP kinase kinase kinase 1 (*MEKK1*), and signal transducer and activator of transcription 3 (*STAT3*). Putative target genes of miR-98 included *IL-8*, *IL-10*, toll-like receptor 4 (*TLR4*), *MEKK1*, *ATG16L1*, *CD95*, claudin-1 (*CLDN1*) and *STAT3*. Finally, miR-125b-1* and let-7e* were predicted to regulate genes such as, hepatocyte nuclear factor-1 α (*HNF1 α*) and *p38- α* , respectively.

The progress in gene discovery in complex disease genetics has increased with the genome-wide association studies (GWAS). Until recently, 99 IBD susceptibility loci were reported: 71 associated with CD, 47 with UC, and 28 with both CD and UC^[39,40]. However, new IBD susceptibility loci were recently added bringing the total number of IBD loci to 163, most of which are associated with both CD and UC^[41]. Thus, in order to identify which of the predicted miRNA targets that are actually reported as UC associated susceptibility genes, we used the web application BioVenn^[42] (<http://www.cmbi.ru.nl/cdd/biovenn/>) to compare the list of the miRNA predicted target genes and UC associated susceptibility genes reported in the literature^[40,41,43-45]. The UC associated predicted miRNA target genes have been illustrated in a Venn diagram (Figure 4). The four circles represent predicted miRNA targets found in the list of reported UC susceptibility genes. Interestingly, 25 of the miR-20b

predicted targets were identified as UC associated susceptibility genes, and 14 miR-98 targets were found to be associated with UC, with 6 common target genes (Figure 4). Additionally, we found four UC associated target genes for miR-125b-1*, however, only one let-7e* target gene were predicted to be associated with UC susceptibility (Figure 4).

DISCUSSION

In recent years, miRNA profiling studies using tissue or blood samples from IBD patients have provided us with new ways to understand this otherwise enigmatic disease, and identifying differentially expressed miRNAs is a first step in the development of miRNA profile-based diagnostic tools.

Thus, in this study, using miRNA microarray profiling followed by qPCR analysis, we identified new miRNAs that were altered in patients with UC *vs* controls. Previous studies have demonstrated different mRNA expression profiles of mucosal colonic biopsies from IBD patients and healthy individuals^[34,35,46,47]. Furthermore, Olsen *et al.*^[34] demonstrated that active and inactive UC could be distinguished from CD patients and controls. However, these studies could not differentiate between inactive CD and controls^[34,48]. Similarly, in the PCA and PLS-DA score-plots of miRNA expression profiles, we observed a clear separation of active and inactive UC patients from CD patients and controls. These results suggest that miRNA and mRNA profiles might follow the same pattern in UC patients and could be combined to differentiate between UC *vs* CD and controls in order to identify potential diagnostic biomarker panels. However, Wu *et al.*^[49] claimed to be able to discriminate active CD from controls using

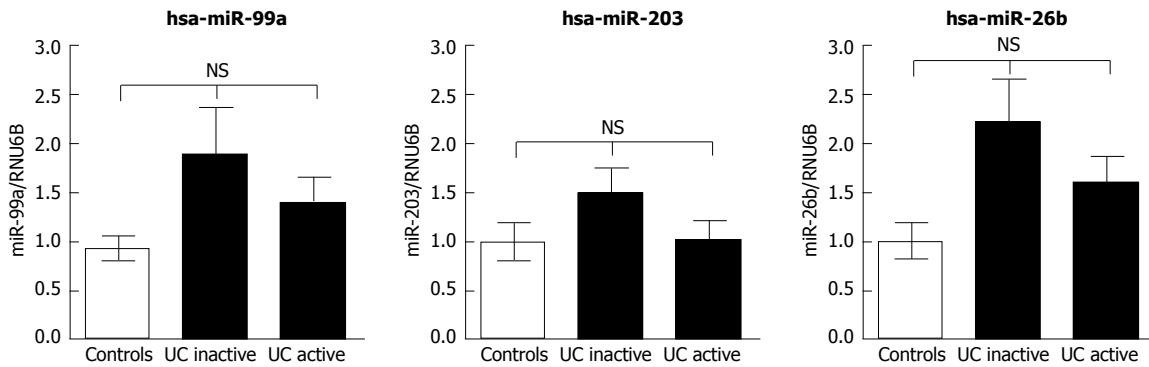


Figure 3 Expression of other miRNAs identified by microarray profiling. Expression differences of miRNA in active ulcerative colitis (UC) ($n = 20$), inactive UC ($n = 19$), and controls ($n = 20$). Levels were determined using quantitative real-time polymerase chain reaction. Relative expression differences of each miRNA were normalized to endogenous RNU6B expression and calculated using the $2^{-\Delta\Delta CT}$ method. NS: Not significant.

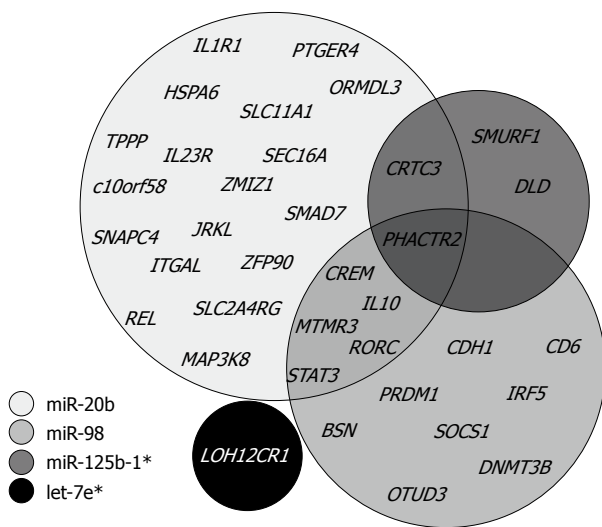


Figure 4 Venn diagram illustrating the miRNA-specific and overlapping ulcerative colitis associated target genes. The four circles represent predicted miRNA targets found in the list of reported ulcerative colitis susceptibility genes.

microarray analysis of intestinal biopsies, whereas other studies have mainly examined the expression of miRNAs by qRT-PCR analysis^[27], which is inherently a much more sensitive technique than microarray^[50]. When comparing our study with the study by Wu *et al.*^[49] there is, however, a striking difference in the miRNA microarray platforms used; Wu *et al.*^[49] used a miRNA microarray platform from NCode (Invitrogen, La Jolla, CA, United States) detecting about 470 unique human miRNAs, while we used a platform from Geniom Biochip miRNA (Febit) detecting almost 850 unique human miRNAs. Thus, the overall miRNA expression panel may possibly not follow the same pattern due to a dissimilar number of detected miRNAs in different arrays. Furthermore, the stringent significance and fold change criteria set by the current study might very well exclude subtle differences present in the expression profiles of patients with CD and controls. However, it cannot be excluded that the limited size of the microarray cohort as well as the medical therapy that each patient received also contributed to the inability to discriminate between groups.

Among the miRNAs with the strongest differential power from the microarray expression data we identified several differentially expressed miRNAs (such as miR-23a, miR-155, miR-16, miR-150, miR-346, and miR-126) previously reported to be associated with UC^[36-38]. However, we also identified seven miRNAs (miR-20b, miR-99a, miR-203, miR-26b, miR-98, miR-125b-1*, and let-7e*) that were not previously reported to be involved in IBD^[27]. Therefore, we focused on these seven new candidate miRNAs. The four significantly up-regulated human miRNAs (miR-20b, miR-98, miR-125b-1*, and let-7e*) identified in UC patients in the present paper contributes to the current knowledge of the roles of miRNAs in IBD. In particular, we show that miR-20b expression is increased in both active and quiescent UC as compared to controls. This could make miR-20b a potential biomarker to differentiate between controls and UC as it is not dependent on disease activity. Increased levels of miR-20b have been reported in human cancers including lung cancer, gastric cancer and leukemias where miR-20b facilitates cellular adaption to normoxia and hypoxia *in vitro* by regulating the transcription factor hypoxia-inducible factor 1- α ^[51]. Therefore, it is possible that miR-20b might be involved in the pathophysiology of colitis-associated colorectal cancer^[52].

We also found miR-125b-1* to be significantly up-regulated in active UC. The miR-125 family has 2 mature isoforms: miR-125a and miR-125b (encoded by miR-125b-1 and miR-125b-2). Deregulated expression of miR-125 family members in various cancers has been reported^[53-55]. Tili *et al.*^[56] has earlier demonstrated that miR-125b is of importance for the innate immune response, as lipopolysaccharide (LPS) stimulation of a murine macrophage cell line caused suppressed miR-125b levels. LPS is the principal component of bacteria in terms of pro-inflammatory properties as it activates the innate immune system through toll-like receptors to produce pro-inflammatory cytokines, including interferon- γ or tumor necrosis factor- α ^[57].

In the present study, we additionally found two let-7 miRNA family members, miR-98 and let-7e*, significantly altered in UC. MiR-98 was significantly up-regulated in

inactive UC compared to controls and active UC. Moreover, miR-98 has been reported to negatively regulate the anti-inflammatory cytokine, IL-10, production in macrophages - an important factor in the immune response that protects the host from excessive inflammation^[58]. Additionally, a recent study has revealed that miR-98 targets the Fas-receptor mRNA, and decreases Fas-mediated apoptosis^[59]. The increased expression of miR-98 could explain the attenuated Fas-mediated cell death response of lamina propria T-cells in IBD^[60]. This resistance of mucosal T-cells to Fas-mediated apoptosis might explain the sustained mucosal inflammation seen in IBD^[61].

In accordance with the microarray data, we found significantly increased let-7e* levels in inactive UC when compared to healthy individuals. The let-7 family of miRNAs is highly conserved across diverse animal species from worms to humans, and plays important roles in the regulation of cell proliferation and differentiation^[62]. Humans have ten mature let-7-family sequences that are produced from 13 precursor sequences^[63]. Most of the human *let-7* genes map to regions altered or deleted in human tumours^[64], and are reported to be down-regulated in various cancerous conditions^[65,66]. In addition to cancer, an altered expression of let-7 has been reported in inflammation. In allergic airway inflammation, administration of let-7 mimic to mice represses IL-13 production and reduces the inflammation^[67]. Furthermore, other let-7 family members than let-7e* described in this study have previously been linked to IBD. Wu *et al.*^[36] found let-7f differentially expressed in the intestinal tissue of active UC patients when compared to controls, and Zahm *et al.*^[68] found higher concentrations of let-7b miRNA in sera of pediatric CD patients *vs* controls. It has been reported that there is a regulatory interaction between NF- κ B activation and expression of let-7 members^[69-72], which suggests a significant importance for the pathogenesis of IBD. Let-7 miRNAs has also been demonstrated to be repressed in inflammation, which result in increased expression of pro-inflammatory cytokines and enhanced inflammatory responses^[72].

The miRNAs described and analyzed in the present study were initially selected based on the microarray results. The subsequent validation procedure using qPCR resulted in partial discrepancy between the microarray and qPCR results. This was, however, to be expected as correlation coefficients between microarray and qPCR assays have been found to be as low as 0.4^[73], and just substantiates the microarray technology as a hypothesis generating tool and illustrates the importance of verifying microarray expression data.

Another challenge in miRNA research is the identification of genes regulated by miRNAs. One common method to address this challenge is by predicting targets by computer algorithms. Through *in silico* predictions we identified multiple potential miRNA targets in the inflammatory response well-known to be associated with UC. Interestingly, the most target genes were associated with the mitogen-activated protein kinase pathway and cytokine signaling, which are important key signaling path-

ways in IBD^[74]. These findings further suggest that the miRNA molecules found in this study may have a pivotal influence on the clinical course of IBD.

In conclusion, this study contributes to the current knowledge on the putative role of miRNAs in IBD pathogenesis, and it provides the first evidence that miR-20b, miR-98, miR-125b-1*, and let-7e* are deregulated in patients with UC. The level of these miRNAs may serve as new potential biomarkers for this chronic disease. However, this area of research is in its infancy, and studies in the field of IBD need to identify all of the miRNAs that are consistently deregulated in IBD.

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COMMENTS

Background

MicroRNAs (miRNAs) are small, non-coding single-stranded RNA molecules that regulate the expression of target genes and are involved in many cellular and physiological mechanisms. The discovery of miRNAs in inflammatory bowel disease (IBD), particularly their role in cell signaling, offers a novel way of understanding this chronic disease and gives rise to new potential diagnostic tools and therapeutic strategies. In this paper, the authors identified new potential miRNA biomarkers in ulcerative colitis (UC).

Research frontiers

The pathogenesis of IBD remains largely unknown, but involves a complex interaction between genetic, environmental, and immunological factors. However, to date, there are still no ideal methods to assess the severity of inflammation and to differentiate between UC and Crohn's disease (CD). In this context, research on miRNAs is a promising new research, providing novel insights into the pathogenesis of IBD, biomarker identification, and treatment. Thus, there is great promise that miRNAs will aid in the early diagnosis of IBD, and in the development of more targeted, personalized therapies.

Innovations and breakthroughs

Recent reports have highlighted the importance of using miRNAs in IBD pathogenesis, diagnostics and therapeutics. Through microarray-based miRNA profiling of colonic mucosal biopsies from patients with UC, CD, and controls, the authors found that the expression of miR-20b, miR-98, miR-125b-1*, and let-7e* was upregulated in patients with UC. This is the first study to report that these four miRNAs may serve as potential biomarkers for UC.

Applications

The present study suggest that miR-20b, miR-98, miR-125b-1*, and let-7e* levels are deregulated in patients with UC. The level of these miRNAs may serve as new potential biomarkers for this chronic disease.

Peer review

The authors analyzed the miRNA expression in IBD patients and healthy individuals in order to identify new potential miRNA biomarkers in IBD using miRNA microarray profiling of colonic mucosal biopsies. Among the most differentially expressed miRNAs, the authors found that the levels of miR-20b, miR-98, miR-125b-1*, and let-7e* were specifically upregulated in patients with UC. These data are quite important and may help to clarify the complex pathogenesis of UC and further suggest that the level of these miRNAs may serve as new potential biomarkers for this disease.

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