ORIGINAL ARTICLE

Role of Altered Expression of miR-146a, miR-155, and miR-122 in Pediatric Patients with Inflammatory Bowel Disease

Nóra J. Béres, MD, Dolóresz Szabó, MD,* Dorottya Kocsis, MD,† Dániel Szűcs, MD,‡ Zoltán Kiss, MSc,* Katalin E. Müller, MD, PhD,* Gábor Lendvai, PhD,§,‖ András Kiss, MD, PhD,§ András Arató, MD, DSc,* Erna Sziksz, PhD,*,¶ Ádám Vannay, MD, PhD,*,¶ Attila J. Szabó, MD, DSc,*,¶ and Gábor Veres, MD, DSc**

Background: Evidence suggests the central role of tumor necrosis factor (TNF)-α in the pathomechanism of inflammatory bowel disease (IBD); however, its effect on epigenetic factors, including small non-coding microRNAs (miRs), is less known. Our present aim was the comparative investigation of the expression of TNF-α and immune response–related miRs in children with Crohn's disease (CD) and ulcerative colitis (UC).

Methods: Fresh-frozen (FF) and formalin-fixed, paraffin-embedded (FFPE) biopsies were used to analyze the expression of miR-146a, -155, -122, and TNF-α by real-time reverse transcription polymerase chain reaction in macroscopically inflamed (CD: 12 FFPE and 24 FF; UC: 10 FF) and intact (CD: 12 FFPE; 14 FF) colonic biopsies of children with IBD and controls (16 FFPE; 23 FF). The expression of miR-146a, -155, and -122 was also determined in TNF-α–treated HT-29 colonic epithelial cells.

Results: Increased expression of TNF-α was observed in the colonic mucosa of children with CD and UC in comparison with controls. Expression of miR-146a and -155 was higher in the inflamed mucosa of children with CD and UC than in the intact mucosa. Expression of miR-122 elevated in the macroscopically intact colonic regions of CD compared with controls and patients with UC. In HT-29 cells, TNF-α treatment increased the expression of miR-146a and -155, but not that of miR-122.

Conclusions: Our results showed altered expression of miR-146a, -155, and -122 in the colonic mucosa of children with IBD and in TNF-α–treated colonic epithelial cells. Our data suggest the TNF-α–related involvement of these miRs in the pathogenesis of IBD.

(Inflamm Bowel Dis 2016;22:327–335)

Key Words: TNF-α, microRNAs, miR-146a, miR-155, miR-122, pediatric, inflammatory bowel disease, Crohn's disease, ulcerative colitis

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder with increasing prevalence; the 2 major subtypes comprise Crohn's disease (CD) and ulcerative colitis (UC).[1,2] Approximately 15% to 30% of all cases begins in childhood leading to a life-long disease, frequently accompanied with serious complications.[3–5] Recent cohorts on pediatric and adult patients suffering from IBD demonstrate numerous differences between pediatric and adult patients suffering from IBD. The pediatric IBD is a more severe phenotype; it shows extensive intestinal involvement, rapid progression, and has several side issues, including growth failure, poor bone density, delayed puberty. Investigating the pathomechanism of early-onset IBD is of outmost importance to understand the initiating events of the disease.[2,6,7]

Increased level of tumor necrosis factor (TNF)-α has a prominent role in the pathomechanism of IBD.[8–11] TNF-α influences numerous IBD-related pathways, for example, nuclear factor kappa B (NF-κB)–mediated signaling, which is a critical pathway to induce the expression of IBD-related genes (e.g., genes encoding interleukin (IL)-1 β, IL-6, and TNF-α). As a key proinflammatory cytokine during chronic inflammation, TNF-α together with other inflammatory mediators contributes to the recruitment and activation of immune cells. Furthermore, TNF-α modifies the epithelial barrier through the induction of chemokines and inflammatory mediators connected to the dysregulation of epithelial surface, leading to increased colonic permeability.[12–14] This complex biological role explains the efficacy of anti-TNF-α therapy, representing a significant therapeutic tool in the management of the hard-to-treat patients, showing a good clinical outcome and leading to mucosal healing.[15]

Recent evidence suggests that epigenetic factors are also potent modulators of IBD. Moreover, it has been suggested that

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.ibdjournal.org).

Received for publication September 29, 2015; Accepted November 4, 2015.

From the *1st Department of Pediatrics, Semmelweis University, Budapest, Hungary; †2nd Department of Internal Medicine, Semmelweis University, Budapest, Hungary; ‡Department of Pediatrics and Pediatric Health Care Center, University of Szeged, Szeged, Hungary; §2nd Department of Pathology, Semmelweis University, Budapest, Hungary; ‖MTA-SE, Tumor Progression Research Group, Budapest, Hungary; and ¶MTA-SE, Pediatrics and Nephrology Research Group, Budapest, Hungary.

Supported by grants OTKA-K105530, -K108688, -K116928, -PD105361, and LP008/2015.

The authors have no conflicts of interest to disclose.

Reprints: Gábor Veres, MD, DSc, 1st Department of Pediatrics, Semmelweis University, Bókay János Street, 53-54, H-1083 Budapest, Hungary (e-mail: veres.gabor@med.semmelweis-univ.hu).

DOI 10.1097/MIB.0000000000000687

Published online 11 January 2016.

TNF-α has essential effects on the expression of epigenetic factors as well, including the expression of microRNAs (miRs), which mediate the interactions between environment, host immune system, and genome.[16,17]

MiRs are 19 to 24 nucleotide-long single-stranded RNAs involved in the regulation of gene expression at transcriptional and posttranscriptional level.[18–21] Altered expression of miRs has been linked to different cell functions including signal transduction, differentiation, proliferation, or apoptosis.[18] Moreover, miRs play a determinative regulatory role in innate and adaptive immune processes.[22,23] Altered expression of miRs was described in adult patients suffering from CD and UC; however, there are only limited number of studies examining miRs in children with IBD.[24–26] MiR-146a, -155, and -122 influence numerous biological functions including inflammatory response, intracellular signaling cascades, regulation of cytokine production, and response to bacteria which all play important role in immune-mediated disease (Fig. 1).[18,20,22,28,29] However, their precise role and regulators of their expression in pediatric IBD are completely unknown.

Therefore, the main purpose of this study was to investigate the expression of miR-146a, -155, and -122 in the inflamed and noninflamed colonic mucosa of children with IBD. Considering the importance of TNF-α in IBD, we tested its effect on the expression of miR-146a, -155, and -122 in HT-29 colonic epithelial cells in vitro, as well.

MATERIALS AND METHODS

Patients

The diagnosis of CD and UC was based on clinical symptoms, endoscopic findings, and histopathology, according to the Porto criteria,[1] and disease activity score was calculated by Pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Activity Index. The presenting symptoms of CD were weight loss, hematochezia, abdominal pain, diarrhea, bloody diarrhea, anemia, and perianal fistula. Control children were referred to the outpatient clinic because of rectal bleeding, chronic abdominal pain, or weight loss. Colonoscopy was part of their diagnostic procedure, and the mucosa showed normal macroscopic appearance with normal histology in the biopsy specimens. Colonic biopsy samples were taken from children with CD, UC, and from controls. Biopsies were immediately fixed in formaldehyde and embedded in paraffin (FFPE) or were snap-frozen (fresh-frozen [FF]) and stored at −80°C. FFPE sections were as follows: intestinal biopsies of macroscopically inflamed and

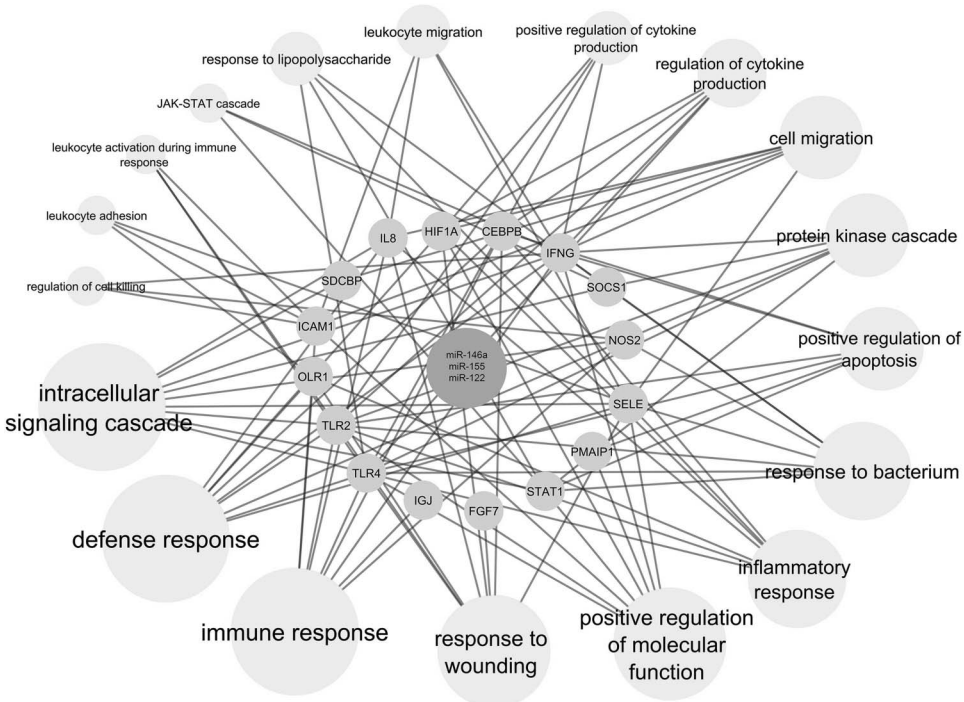

FIGURE 1. Regulatory network of microRNA-146a, -155, and -122 according to IBD-related genes. Target genes MiR-146a, -155, and -122, based on MiRTarBase overlapping with microarray expression data from pediatric patients with CD.[27] The enrichment analysis resulted in 18 gene ontology term categories. The degree of the category nodes are represented by the size of the circles. CEBPB, CCAAT/enhancer-binding protein beta; FGF7, fibroblast growth factor 7; HIF1A, hypoxia-inducible factor 1-alpha; ICAM1, intercellular adhesion molecule 1; IFNG, interferon gamma; IGJ, immunoglobulin J chain; NOS2, nitric oxide synthase 2; OLR1, oxidized low-density lipoprotein (lectin-like) receptor 1; PMAIP1, phorbol-12-myristate-13-acetate-induced protein 1; SDCBP, syndecan binding protein; SELE, selectin E; SOCS1, suppressor of cytokine signaling 1; STAT1, signal transducer and activator of transcription 1; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4.

noninflamed (intact) regions of the colonic mucosa from children with CD (n = 12, mean age: 14.73 ± 0.58, PCDAI: 33.89 ± 5.27) and from controls (C) (n = 16, mean age: 10.56 ± 1.36). Characteristics and laboratory parameters of patients in the FFPE group are listed in Table 1. FF biopsies were as follows: biopsies of macroscopically inflamed (n = 24, mean age: 12.92 ± 1.13, PCDAI: 26.20 ± 2.98) and intact (n = 14, mean age: 12.86 ± 1.16; PCDAI: 26.35 ± 4.22) regions of patients suffering from CD, macroscopically inflamed regions of patients with UC (n = 10, mean age: 11.8 ± 1.75, Pediatric Ulcerative Colitis Activity Index: 36.00 ± 4.00) and biopsies from controls (n = 23, mean age: 8.57 ± 1.09). The noninflamed samples were taken from the same patients with CD as the inflamed ones. Characteristics of patients with CD, UC and controls included in the FF group are shown in Table 2. Most of the newly diagnosed children had a short disease duration and received no previous medications except for 6 children (5-acetylsalicylic-acid (n = 5), methylprednisolone (n = 2), azathioprine (n = 6) at the time of the endoscopy, and one of these 6 children who received also anti-TNF-α antibody 1 year before the endoscopy).

TNF-α Treatment of Colonic Epithelial Cells

Human colonic epithelial (HT-29) cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin mixture (Life Technologies of Thermo Fisher Scientific Inc., Carlsbad, CA) under standard cell-culture conditions (37°C, humidified, 5% CO_2/95% air environment). HT-29 cells were seeded in 6-well plates at a density of 5×10^5 cells per well and treated for 24 hours with recombinant human TNF-α (R&D Systems, Minneapolis, MN) at a concentration of 10 ng/mL or vehicle (phosphate-buffered saline) only for control cells.

RNA Isolation

Total RNA was isolated from FFPE biopsies using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany) after the removal of paraffin applying RNeasy MinElute spin columns (Qiagen). RNA isolation from FF biopsies was performed using TRIzol reagent (Ambion, Austin, TX) combined with Quick-RNA Mini-Prep isolation kit (Zymo Research, Irvine, CA), which was also applied to gain RNA from the cells.

TABLE 1. Clinical Characteristics and Laboratory Parameters of Patients in the FFPE group

FFPE Group	Pediatric CD	Control
No. patients	12	16
Gender	6 M/6 F	11 M/5 F
Age (yr)	14.73 ± 0.58[a]	10.56 ± 1.36
BMI (kg/m^2)	17.76 ± 1.47	19.87 ± 1.78
PCDAI	33.89 ± 5.27	—
Iron (μmol/L)	10.15 ± 2.33[a]	17.23 ± 1.415
Albumin (g/L)	40.32 ± 1.53[b]	46.42 ± 0.7732
Platelet count (Giga/L)	504.7 ± 33.88[c]	340.6 ± 23.78
CRP (mg/L)	33.54 ± 9.02[a]	1.15 ± 0.63

[a]$P \leq 0.05$; [b]$P \leq 0.01$; [c]$P \leq 0.001$ versus control.
BMI, body mass index; CRP, C-reactive protein; F, female; M, male.

Reverse Transcription and Quantitative Polymerase Chain Reaction

For miR analysis, total RNA was reverse-transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) and quantitatively measured in a real-time polymerase chain reaction (PCR) using TaqMan Universal PCR Master Mix No AmpErase UNG (Life Technologies), according to the instructions of the manufacturer. Primers were provided as the following TaqMan MicroRNA Assays (Life Technologies): miR-122 (ID: 002245), miR-146a (ID: 00468), and miR-155 (ID: 002623). For TNF-α analysis, the total RNA from FF biopsies was reverse-transcribed using the Maxima First strand cDNA Synthesis Kit (Thermo Fischer Scientific, Waltham, MA) and quantitatively measured by real-time PCR using LC480 SYBR Green I Master Mix (Roche Diagnostics, Basel, Switzerland), applying TNF-α specific primers (forward: 5′GGC TCC AGG CGG TGC TTG TTCC 3′, reverse: 5′TGG CCC GGC GGT TCA GC provided by IDT, Coralville, IA). The PCRs were performed on a LightCycler 480 instrument (Roche Diagnostics). Relative expression level was calculated by the $2^{\Delta Cq}$ formula, using U6 (ID: 001973) for miR and RPLP0 (forward: 5′GGG GGA ATG TGG GCT TTG TGTT3′, reverse: 5′GGT GCC CCT GGA GAT TTT AGT GGT3′provided by IDT, Coralville, IA) for mRNA as the references.

Bioinformatics Analysis

DESeq normalized RNAseq data were obtained from the public ArrayExpress database for E-GEOD-57945.[30] Gene expression data of biopsy specimens from pediatric patients with CD and controls were analyzed. Data from the microarray were compared with *t* test, and a fold change threshold of 1.5 was set on the genes showing significant overexpression compared with the control samples. Experimentally validated (Western blot, reporter assay, etc.) target genes were selected from the MiRTarBase database (http://mirtarbase.mbc.nctu.edu.tw).[31] The derived data sets were then compared, and the overlapping genes were taken for further analysis. We performed Gene Ontology (GO) analysis on the common genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov).[32,33] We established a 0.05 threshold of the *P* values of the enriched categories adjusted with the Benjamini–Hochberg correction method. The resulting interrelationships were visualized with the Cytoscape 3.2.1. software (www.cytoscape.org).[34]

Statistical Analysis

Statistical analysis was performed using the GraphPad statistical software package (GraphPad Software, La Jolla, CA). Data were analyzed performing Mann–Whitney *U* test, Kruskal–Wallis test, analysis of variance and post hoc test. The threshold

TABLE 2. Clinical Characteristics and Laboratory Parameters of Patients in the FF group

FF Group	Pediatric CD (Intact)	Pediatric CD (Inflamed)	Pediatric Ulcerative Colitis	Control
No. patients	14	24	10	23
Gender	8 M/6 F	9 M/15 F	6 M/4 F	16 M/7 F
Age (yr)	12.86 ± 1.6	12.92 ± 1.13	11.8 ± 1.75	8.57 ± 1.09
BMI (kg/m^2)	17.53 ± 0.82	16.28 ± 0.78	18.38 ± 1.04	16.88 ± 0.86
PCDAI/PUCAI	26.35 ± 4.22[a]	26.20 ± 2.98[a]	36.00 ± 4.00[a]	—
Iron (μmol/L)	6.57 ± 1.29[a]	5.29 ± 0.92[a]	5.70 ± 1.89[a]	16.52 ± 1.45
Albumin (g/L)	39.86 ± 1.69[b]	38.35 ± 1.28[a]	41.60 ± 2.48	46.39 ± 0.56
Platelet count (Giga/L)	513.8 ± 52.46[c]	538.3 ± 37.79[b]	486.1 ± 38.15	368.3 ± 17.74
CRP (mg/L)	27.95 ± 9.18	40.89 ± 15.32[c]	10.41 ± 1.64	0.5167 ± 0.17

[a]$P \leq 0.001$; [b]$P \leq 0.01$; [a]$P \leq 0.05$ versus control.
BMI, body mass index; CRP, C-reactive protein; F, female; M, male; PUCAI, Pediatric Ulcerative Colitis Activity Index.

for statistical significance was set at $P \leq 0.05$. Data are presented as mean ± standard error of the mean and normalized to the control group.

Ethical Consideration

Written informed consent was obtained from the parents before the procedure, and the study was approved by the Semmelweis University Regional and Institutional Committee for Research Ethics (TUKEB No.: 10408/2012).

RESULTS

Expression of miR-146a in the Colonic Mucosa of Children with CD, UC and Controls

The expression of miR-146a in FFPE biopsies was significantly higher in the macroscopically inflamed colonic mucosa of children with CD than in the intact colonic mucosa regions (CD inflamed: 6.66 ± 1.52 versus CD intact: 1.79 ± 0.45, $P \leq 0.05$) and controls (CD inflamed: 6.66 ± 1.52 versus C: 1 ± 0.23, $P \leq 0.001$) (Fig. 2A). A similar tendency was found in the FF biopsies as the expression of miR-146a was elevated in the inflamed intestine of patients suffering from CD and UC when compared with controls (CD inflamed: 2.87 ± 0.70 versus C: 1 ± 0.14, $P \leq 0.05$; UC: 4.68 ± 1.05 versus C: 1 ± 0.14, $P \leq 0.001$). No significant difference was observed between the intact mucosa of CD and controls, and between the inflamed region of CD and UC (Fig. 2D).

Expression of miR-155 in the Colonic Mucosa of Children with CD, UC and Controls

Expression of miR-155 in FFPE biopsies showed a statistically significant elevation in inflamed CD mucosa in comparison with the intact CD mucosa and controls (CD inflamed: 8.52 ± 1.90 versus C: 1 ± 0.21, $P \leq 0.001$; CD inflamed: 8.52 ± 1.90 versus CD intact: 1.73 ± 0.38, $P \leq 0.01$), whereas no significant difference could be observed between the intact and control groups (Fig. 2B). A similar tendency was found in the FF biopsies as the expression of miR-155 was elevated in the macroscopically inflamed colonic CD mucosa as compared with controls (CD inflamed: 4.07 ± 1.2 versus C: 1 ± 0.1, $P \leq 0.05$), whereas no significant difference was present between the intact CD regions and controls. Additionally, the expression of miR-155 was elevated in UC compared with the control group (UC: 2.49 ± 0.36 versus C: 1 ± 0.1, $P \leq 0.05$), but no significant difference was found between UC and CD (Fig. 2E).

Expression of miR-122 in the Colonic Mucosa of Children with CD, UC and Controls

Expression of miR-122 in FFPE biopsies was statistically higher in macroscopically intact colonic mucosal biopsies of children with CD than in controls (CD intact: 3.12 ± 0.71 versus C: 1 ± 0.17, $P \leq 0.05$) (Fig. 2C). A similar tendency was observed in the FF biopsies as elevated expression of miR-122 was found in the intact CD mucosa as compared with controls (CD intact: 4.71 ± 1.45 versus C: 1 ± 0.2, $P \leq 0.01$) and inflamed UC mucosa (CD intact: 4.71 ± 1.45 versus UC: 0.85 ± 0.43, $P \leq 0.01$). In contrast, no significant difference was found between the control and inflamed CD and UC mucosa (Fig. 2F).

Expression of TNF-α in the Colonic Mucosa of Children with CD, UC and Controls

The mRNA expression of TNF-α was significantly elevated in the inflamed colonic mucosa of children with CD and UC as compared with controls (CD: 2.67 ± 0.43 versus C: 1 ± 0.24, $P \leq 0.05$; UC: 3.30 ± 0.88 versus C: 1 ± 0.24, $P \leq 0.05$), whereas no significant TNF-α expressional differences were observed between UC and CD (Fig. 3A).

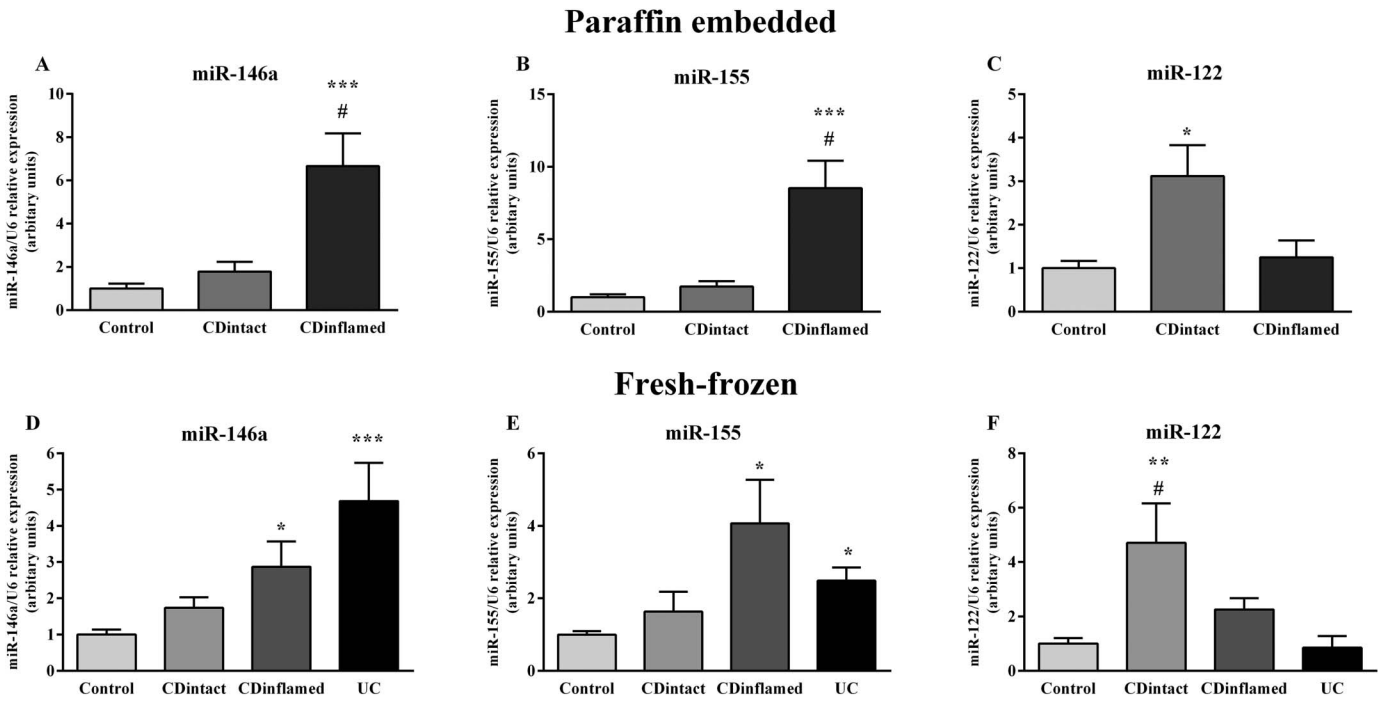

FIGURE 2. Expression of miR-146a (A and D), -155 (B and E), and -122 (C and F) in FFPE and in FF biopsies from the intact and inflamed region of children with CD, UC, and controls. Expression of miR-146a and -155 was significantly higher in the inflamed colonic region of children with CD and UC than in controls; however, the expression of miR-122 was significantly higher in macroscopically intact biopsies of patients with CD than in controls. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control, #$P < 0.01$ versus CD intact.

Expression of miR-146a, -155, and -122 After TNF-α Treatment in Colonic Epithelial (HT-29) Cells

TNF-α treatment significantly enhanced the expression of miR-146a (TNF: 55.3 ± 6.22 versus C: 1 ± 0.24, $P \leq 0.01$) and miR-155 (TNF: 2.7 ± 0.11 versus C: 1 ± 0.09, $P \leq 0.01$) in HT-29 human colonic epithelial cells in comparison with vehicle-treated control cells (Fig. 3B, C), whereas the expression of miR-122 was not statistically different between TNF-α–treated and control groups (Fig. 3D).

Regulatory Network of microRNA-146a, -155, and -122 According to IBD-Related Genes

We annotated the target genes of miR-146a, -155, and -122 overlapping with microarray expression data with GO terms (biological process domain). The enrichment analysis resulted in 18 strongly overlapping categories after the depletion of the redundant terms (Fig. 1, see Table, Supplemental Digital Content 1, http://links.lww.com/IBD/B179, which data shown the GO term categories and the target genes of miR-146a, -155, and -122).

The most abundant terms are the following: intracellular signaling cascade, defense response, immune response, response to wounding, positive regulation of molecular function, inflammatory response, and response to bacterium. The terms can be further grouped into 4 major categories: inflammatory response, regulation of cell killing, cell migration, and leukocyte cell adhesion.

DISCUSSION

IBD is a multifactorial disease, characterized by a chronic inflammation of the gastrointestinal tract. MiRs can offer a potential missing link between the genetic susceptibility, environmental and immunological factors involved in the pathogenesis of IBD.[16,35] Moreover, miRs could serve as new potential biomarkers for the diagnostics and monitoring of disease activity and may also be therapeutic targets in the future.[21,26]

The first study investigating miRs in colonic biopsies of adult patients suffering from CD and UC was published in 2008.[36] Since then, continuous efforts have been made to examine the expression of different miRs in serum and colonic samples of adult patients with CD and UC to find specific regulators of disease activity and outcome.[18,36,37] However, less is known about the mucosal condition and role of miRs in pediatric IBD.

In IBD, excessive amount of TNF-α is produced in the inflamed mucosa mainly by activated T cells.[8–11,38] TNF-α has a central role in the pathomechanism of IBD that is well demonstrated by the efficacy of the anti-TNF-α antibodies, which has a strong therapeutic effect even in the management of the conventional therapy–refractory patients.[15] In this study, we demonstrated the increased expression of TNF-α in the colonic mucosa of pediatric patients suffering from CD and UC. Moreover, we demonstrated the increased expression of miR-146a, -155, and -122 in the colonic mucosa of children with CD, both in FFPE and FF samples, suggesting that both types of samples can be

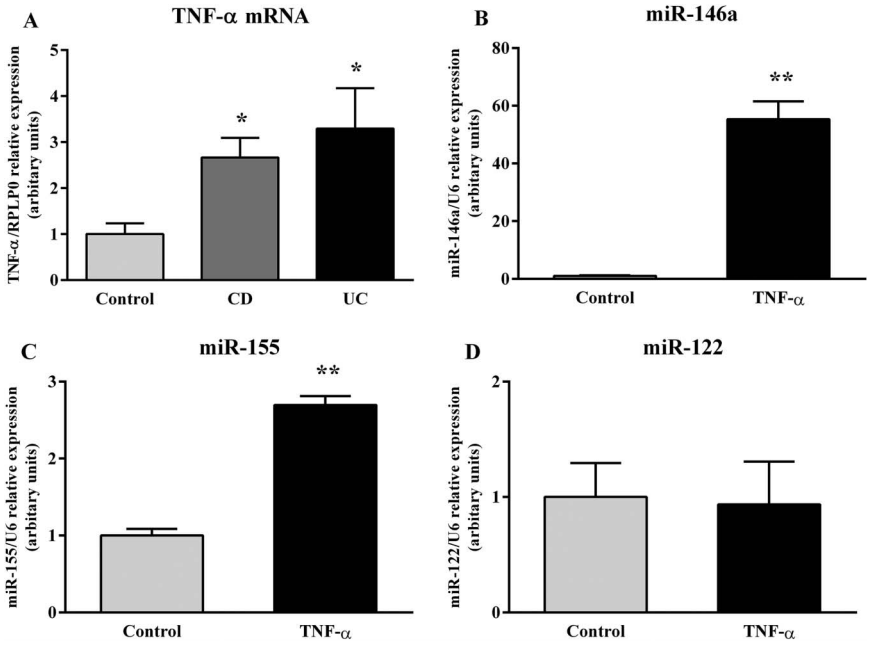

FIGURE 3. Expression of TNF-α in the inflamed colonic mucosa of children with CD and UC and controls (A). Expression of miR-146a (B), -155 (C) and -122 (D) in TNF-α-treated HT-29 colonic epithelial cells. Expression of TNF-α was elevated in the colonic mucosa of children suffering from CD and UC compared with controls. Expression of miR-146a and -155 in human colonic epithelial cells was significantly elevated after TNF-α treatment compared with controls. TNF-α had no effect on the expression of miR-122 in HT-29 cells. *$P < 0.05$; **$P < 0.01$ versus control.

reliably used to investigate the expression of miRs in the colonic mucosa.

Previously, it has been shown that these miRs may regulate the TNF-α signaling pathway (Fig. 4); however, less is known about the effect of TNF-α on the expression of these miRs. In accordance with our findings on human biopsies, we demonstrated that TNF-α treatment increases the expression of miR-146a and -155, but not miR-122 in HT-29 colonic epithelial cells. According to our findings, Min et al[39] proved an elevated expression of miR-155 in TNF-α–treated colonic epithelial cell line. This provides further support about the importance of TNF-α in IBD and indicates that TNF-α contributes to the induction of miR-146a and -155, which, through their effects on the immune-regulatory target genes, have further roles in the activation of TNF-α–mediated signaling. A study examining miRs in the serum during the induction of anti-TNF-α therapy in adult patients with CD could not find differences in the expression of these 2 miRs, and no data are available on their local effects during biological therapy.[40]

Our present observations of elevated miR-146a and -155 expression in the macroscopically inflamed colonic mucosa of children with active CD and UC, but not in the intact colonic regions, is partially in accordance with Zahm et al, who were the first one investigated rectal mucosa of pediatric patients with IBD. They reported increased level of miR-146a, but not miR-155, in the inflamed rectal biopsies of treated pediatric patients with UC in comparison with controls. Moreover, they did not find elevation of these miRs in the rectal biopsies of patients with treated CD with 2 to 3 years of disease duration.[24] Fasseu et al[41] have described elevated expression of miR-146a and -155 in the inflamed and also in the intact colonic mucosa of adult patients with CD as compared with healthy controls. Moreover, increased expression of miR-155 has been demonstrated in the inflamed colonic mucosa of adult patients with CD[42] and UC[42,43] compared with the intact mucosa. Although increased expression of miR-146a and -155 has been found in the inflamed intestinal region of both children and adults, the data regarding the intact mucosal region of patients are not entirely concordant.[35,41–45] This can be probably due to the heterogeneity of the investigated populations and the localization of the biopsies.

As mentioned above, the targets of miR-146a and -155 are involved in immunoregulatory pathways, including the regulation of IL-1 receptor–associated kinase (IRAK) 1 and 2, mitogen-activated protein kinases (MAPKs), and TNF receptor–associated factor 6 (TRAF6).[18,46] Recently, miR-146a and -155 have also been connected to Toll-like receptor (TLR) pattern recognition receptor family. TLRs seem to be important in the pathogenesis of IBD as their expression is elevated in the colonic mucosa of children with IBD, reported in our previous studies.[47,48] Microbes promote TLR-dependent mucosal immune response through the NF-κB and STAT signaling pathways and induce the production of inflammatory cytokines including IL-1β, TNF-α, IL-6, and chemokines, such as chemokine C-C motif ligand 2 (CCL2) and chemokine C-X-C motif ligand 8 (CXCL8).[23,49] Both miR-146a and -155 are upregulated in response to lipopolysaccharides (LPS); however, miR-155 enhances, whereas miR-146a inhibits the inflammatory processes.[50,51] Absence of miR-146a results in

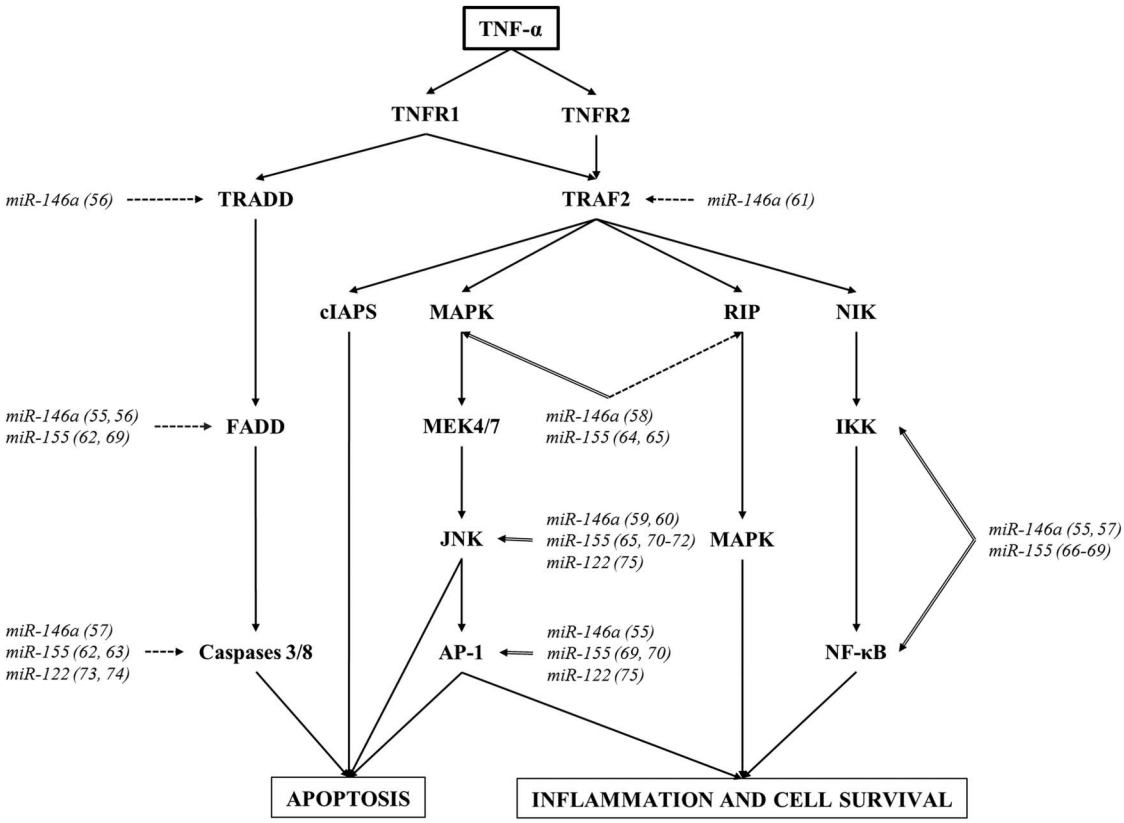

FIGURE 4. Schematic representation of the target interactions between TNF-α—microRNA-146a, -155, and -122. TNF-α is considered as a major inflammatory mediator which play important role in the pathogenesis of IBD. MiR-146a, -155, and -122 act as possible regulators of the TNF-α signal transduction, with capacity to induce apoptosis, inflammation, and cytokine production. The data of the figure based on the database of MiRTarBase and manually enriched with the data from the recent publications. Dotted-line arrow represents the experimentally validated (Luciferase assay, MiR-mimic, MiR-inhibitor studies) direct targets, and the dubble-line arrow presents both the direct and indirect targets (signaling pathways, with or without direct target gene). AP1, activator protein 1; cIAPS, cellular inhibitors of apoptosis; FADD, Fas-associated death domain; IKK, NF-κB inhibitor kinase; JNK, c-Jun N-terminal kinases; MAP3K1, mitogen-activated protein kinase kinase kinase 1; MEKK 4/7, mitogen-activated protein kinase kinase kinase 4/7; NIK, NF-κB–inducing kinase; RIP, receptor-interacting protein; TNFR1, 2, tumor necrosis factor alpha receptor 1, 2; TRADD, tumor necrosis factor receptor type 1–associated DEATH domain protein; TRAF2, TNF receptor-associated factor 2.

hyperresponsiveness to bacterial LPS in mice and is a classical sign of autoimmune diseases.[50,52] Indeed, miR-146a is upregulated during T-cell receptor–mediated and TLR-mediated NF-κB signaling, and suppresses NF-κB pathway and inhibits IRAK-1 and TRAF-6, being targets of the TLR-signaling cascade.[51,53,54] Moreover, miR-155 stimulates the expression of proinflammatory cytokines including TNF-α, IL-6, and IL-1 and has a negative effect on IL-8 signaling,[18,46] in which pathways are important for intestinal immune tolerance and also for establishing the protection against harmful bacteria.[20,36,51,53,54] Furthermore, miR-155 is a negative regulator of the TLR-signaling pathway by repressing the expression of suppressor of cytokine signaling 1 and SHIP-1.[46] MiR-155 knockout mice are immunodeficient with the failure of T-cell activation and antigen presentation.[50] In agreement with this, miR-155–deficient B cells are unable to differentiate into antibody-producing plasma cells, which leads to an insufficient T-cell function, suggesting a immunoregulatory function of miR-155.[46]

Contrary to the expressional changes of miR-146a and -155, we did not find any difference in the expression of miR-122 in the inflamed mucosal region; however, increased expression of miR-122 was found in the macroscopically intact colonic region of children with CD. Only a few studies have investigated miR-122 in relation to IBD; however, a recent publication identified it as a possible marker of disease progression.[44] Additionally, overexpression of miR-122 has been shown to inhibit LPS-induced apoptosis in HT-29 intestinal epithelial cells by suppressing NOD2, leading to consequential inhibition of the NF-κB pathway.[28] Moreover, the upregulation of miR-122 has been found to increase the level of anti-inflammatory cytokines (IL-4 and IL-10) and decrease the expression of proinflammatory cytokines (TNF-α and IFN-γ), indicating that miR-122 may mitigate the intestinal epithelial cell injury.[20,28] Thus, we hypothesize that miR-122 has a protective effect as it may interfere with the progression of CD, which could explain the increased expression of miR-122 in the intact colonic region of patients with CD.

Because the risk for a malignant transformation of chronic inflammation is increasing in IBD during the disease course,[44] it is important to follow pediatric patients suffering from IBD and to find markers indicating malignant transformation. An miR study investigating patients with IBD with different colorectal carcinoma states (nonneoplastic, dysplastic, and cancer) simultaneously present in the same resected colon samples has found increased expression of miR-122 in dysplastic state as compared with nonneoplastic state, whereas demonstrated reduced levels of miR-122 in cancer in comparison with nonneoplastic and dysplastic states. In colon cancer cell lines (HT-29 and HCT-116), transfection with miR-122 was associated with the downregulation of p53 tumor suppressor and cyclin G-cell–cycle regulator.[44]

The exact role of miR-146a, -155, and -122 in the pathomechanism of IBD is still unclear. To better understand the function of these miRs, we analyzed microrarray data of pediatric patients with CD and the possible target genes of miR-146a, -155, and -122 based on MiRTarBase. The categories of the GO analysis are strongly connected to immune-regulatory processes and network mapping of the genes showed a strong internal cohesion in the data set. These findings underline the relevance of the miRs in the context of acute and chronic inflammation (Fig. 1). In addition, these miRs are key modulators of the innate and adaptive immune responses as they affect the differentiation of T and B cells, the activation of macrophages and neutrophil granulocytes and induce immune responses against bacterial and viral infection.[18,50,52] In previous studies, it has been shown that miR-146a and -155 are involved in several physiological processes including phagocytosis, autophagy, endocytosis, and cytokine secretion.[50]

Our study presenting altered expression of miR-146a, -155, and -122 in the colonic mucosa of children with CD and UC suggests that these miRs are involved in the pathogenesis of pediatric IBD. Furthermore, the fact that TNF-α upregulated the expression of miR-146a and -155 indicates that these miRs contribute to the mediation of the diverse biological effects of TNF-α, the key regulatory element of IBD.

ACKNOWLEDGMENTS

The authors are grateful to Maria Bernáth for her skillful technical assistance and Zsófia Sztupinszki for the bioinformatics support.

REFERENCES

1. Levine A, Koletzko S, Turner D, et al. The ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr.* 2013;58:795–806.
2. Muller KE, Lakatos PL, Arato A, et al. Incidence, Paris classification, and follow-up in a nationwide incident cohort of pediatric patients with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr.* 2013;57:576–582.
3. Perrin JM, Kuhlthau K, Chughtai A, et al. Measuring quality of life in pediatric patients with inflammatory bowel disease: psychometric and clinical characteristics. *J Pediatr Gastroenterol Nutr.* 2008;46:164–171.
4. Kim SC, Ferry GD. Inflammatory bowel diseases in pediatric and adolescent patients: clinical, therapeutic, and psychosocial considerations. *Gastroenterology*. 2004;126:1550–1560.
5. Baldassano RN, Piccoli DA. Inflammatory bowel disease in pediatric and adolescent patients. *Gastroenterol Clin North Am.* 1999;28:445–458.
6. Rigoli L, Caruso RA. Inflammatory bowel disease in pediatric and adolescent patients: a biomolecular and histopathological review. *World J Gastroenterol.* 2014;20:10262–10278.
7. Grover Z, Muir R, Lewindon P. Exclusive enteral nutrition induces early clinical, mucosal and transmural remission in paediatric Crohn's disease. *J Gastroenterol.* 2014;49:638–645.
8. Murch SH, Braegger CP, Walker-Smith JA, et al. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut*. 1993;34:1705–1709.
9. Mouzaoui S, Djerdjouri B, Makhezer N, et al. Tumor necrosis factor-alpha-induced colitis increases NADPH oxidase 1 expression, oxidative stress, and neutrophil recruitment in the colon: preventive effect of apocynin. *Mediators Inflamm.* 2014;2014:312484.
10. Komatsu M, Kobayashi D, Saito K, et al. Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. *Clin Chem.* 2001;47:1297–1301.
11. Dionne S, Hiscott J, D'Agata I, et al. Quantitative PCR analysis of TNF-alpha and IL-1 beta mRNA levels in pediatric IBD mucosal biopsies. *Dig Dis Sci.* 1997;42:1557–1566.
12. Reimund JM, Hirth C, Koehl C, et al. Antioxidant and immune status in active Crohn's disease. A possible relationship. *Clin Nutr.* 2000;19:43–48.
13. Roach DR, Bean AG, Demangel C, et al. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J Immunol.* 2002;168:4620–4627.
14. Veres G, Baldassano RN, Mamula P. Infliximab therapy in children and adolescents with inflammatory bowel disease. *Drugs*. 2007;67:1703–1723.
15. Ruemmele FM, Veres G, Kolho KL, et al. Consensus guidelines of ECCO/ESPGHAN on the medical management of pediatric Crohn's disease. *J Crohns Colitis.* 2014;8:1179–1207.
16. Ventham NT, Kennedy NA, Nimmo ER, et al. Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics. *Gastroenterology*. 2013;145:293–308.
17. Iborra M, Bernuzzi F, Correale C, et al. Identification of serum and tissue micro-RNA expression profiles in different stages of inflammatory bowel disease. *Clin Exp Immunol.* 2013;173:250–258.
18. Iborra M, Bernuzzi F, Invernizzi P, et al. MicroRNAs in autoimmunity and inflammatory bowel disease: crucial regulators in immune response. *Autoimmun Rev.* 2012;11:305–314.
19. Papaconstantinou I, Stamatis K, Tzathas C, et al. The role of variations within microRNA in inflammatory bowel disease. *Eur J Gastroenterol Hepatol.* 2013;25:399–403.
20. Chen WX, Ren LH, Shi RH. Implication of miRNAs for inflammatory bowel disease treatment: Systematic review. *World J Gastrointest Pathophysiol.* 2014;5:63–70.
21. Whiteoak SR, Felwick R, Sanchez-Elsner T, et al. MicroRNAs in inflammatory bowel diseases: paradoxes and possibilities. *Inflamm Bowel Dis.* 2015;21:1160–1165.
22. Raisch J, Darfeuille-Michaud A, Nguyen HT. Role of microRNAs in the immune system, inflammation and cancer. *World J Gastroenterol.* 2013;19:2985–2996.
23. Rebane A, Akdis CA. MicroRNAs: essential players in the regulation of inflammation. *J Allergy Clin Immunol.* 2013;132:15–26.
24. Zahm AM, Hand NJ, Tsoucas DM, et al. Rectal microRNAs are perturbed in pediatric inflammatory bowel disease of the colon. *J Crohns Colitis.* 2014;8:1108–1117.
25. Zahm AM, Thayu M, Hand NJ, et al. Circulating microRNA is a biomarker of pediatric Crohn disease. *J Pediatr Gastroenterol Nutr.* 2011;53:26–33.
26. Koukos G, Polytarchou C, Kaplan JL, et al. A microRNA signature in pediatric ulcerative colitis: deregulation of the miR-4284/CXCL5 pathway in the intestinal epithelium. *Inflamm Bowel Dis.* 2015;21:996–1005.
27. *Deleted in proof.*
28. Chen Y, Wang C, Liu Y, et al. miR-122 targets NOD2 to decrease intestinal epithelial cell injury in Crohn's disease. *Biochem Biophys Res Commun.* 2013;438:133–139.
29. Bai J, Li Y, Shao T, et al. Integrating analysis reveals microRNA-mediated pathway crosstalk among Crohn's disease, ulcerative colitis and colorectal cancer. *Mol BioSyst.* 2014;10:2317–2328.

30. Haberman Y, Tickle TL, Dexheimer PJ, et al. Pediatric Crohn disease patients exhibit specific ileal transcriptome and microbiome signature. *J Clin Invest.* 2014;124:3617–3633.
31. Hsu SD, Tseng YT, Shrestha S, et al. miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. *Nucleic Acids Res.* 2014;42:D78–D85.
32. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009;4:44–57.
33. Gene Ontology Consortium. Gene ontology consortium: going forward. *Nucleic Acids Res.* 2015;43:D1049–D1056.
34. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 2003;13:2498–2504.
35. Lin J, Welker NC, Zhao Z, et al. Novel specific microRNA biomarkers in idiopathic inflammatory bowel disease unrelated to disease activity. *Mod Pathol.* 2014;27:602–608.
36. Wu F, Zikusoka M, Trindade A, et al. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha. *Gastroenterology*. 2008;135:1624–1635 e1624.
37. Wu F, Zhang S, Dassopoulos T, et al. Identification of microRNAs associated with ileal and colonic Crohn's disease. *Inflamm Bowel Dis.* 2010; 16:1729–1738.
38. Pak S, Holland N, Garnett EA, et al. Cytokine profiles in peripheral blood of children and adults with Crohn disease. *J Pediatr Gastroenterol Nutr.* 2012;54:769–775.
39. Min M, Peng L, Yang Y, et al. MicroRNA-155 is involved in the pathogenesis of ulcerative colitis by targeting FOXO3a. *Inflamm Bowel Dis.* 2014;20:652–659.
40. Fujioka S, Nakamichi I, Esaki M, et al. Serum microRNA levels in patients with Crohn's disease during induction therapy by infliximab. *J Gastroenterol Hepatol.* 2014;29:1207–1214.
41. Fasseu M, Treton X, Guichard C, et al. Identification of restricted subsets of mature microRNA abnormally expressed in inactive colonic mucosa of patients with inflammatory bowel disease. *PloS One*. 2010;5:10.
42. Schaefer JS, Attumi T, Opekun AR, et al. MicroRNA signatures differentiate Crohn's disease from ulcerative colitis. *BMC Immunol.* 2015;16:5.
43. Takagi T, Naito Y, Mizushima K, et al. Increased expression of microRNA in the inflamed colonic mucosa of patients with active ulcerative colitis. *J Gastroenterol Hepatol.* 2010;25(suppl 1):S129–S133.
44. Kanaan Z, Rai SN, Eichenberger MR, et al. Differential microRNA expression tracks neoplastic progression in inflammatory bowel disease-associated colorectal cancer. *Hum Mutat.* 2012;33:551–560.
45. Pekow JR, Kwon JH. MicroRNAs in inflammatory bowel disease. *Inflamm Bowel Dis.* 2012;18:187–193.
46. O'Connell RM, Rao DS, Chaudhuri AA, et al. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol.* 2010;10:111–122.
47. Szebeni B, Veres G, Dezsofi A, et al. Increased mucosal expression of Toll-like receptor (TLR)2 and TLR4 in coeliac disease. *J Pediatr Gastroenterol Nutr.* 2007;45:187–193.
48. Szebeni B, Veres G, Dezsofi A, et al. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol.* 2008;151:34–41.
49. Dunne A, O'Neill LA. Adaptor usage and Toll-like receptor signaling specificity. *FEBS Lett.* 2005;579:3330–3335.
50. Montagner S, Orlandi EM, Merante S, et al. The role of miRNAs in mast cells and other innate immune cells. *Immunol Rev.* 2013;253:12–24.
51. Taganov KD, Boldin MP, Chang KJ, et al. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A.* 2006;103: 12481–12486.
52. Boldin MP, Taganov KD, Rao DS, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med.* 2011;208:1189–1201.
53. Ceribelli A, Satoh M, Chan EK. MicroRNAs and autoimmunity. *Curr Opin Immunol.* 2012;24:686–691.
54. Chassin C, Kocur M, Pott J, et al. miR-146a mediates protective innate immune tolerance in the neonate intestine. *Cell Host Microbe.* 2010;8: 358–368.
55. Curtale G, Citarella F, Carissimi C, et al. An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes. *Blood.* 2010;115:265–273.
56. Sandhu R, Rein J, D'Arcy M, et al. Overexpression of miR-146a in basal-like breast cancer cells confers enhanced tumorigenic potential in association with altered p53 status. *Carcinogenesis*. 2014;35:2567–2575.
57. Park H, Huang X, Lu C, et al. MicroRNA-146a and microRNA-146b regulate human dendritic cell apoptosis and cytokine production by targeting TRAF6 and IRAK1 proteins. *J Biol Chem.* 2015;290:2831–2841.
58. Cheng HS, Sivachandran N, Lau A, et al. MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. *EMBO Mol Med.* 2013;5:949–966.
59. Miyata R, Kakuki T, Nomura K, et al. Poly(I: C) induced microRNA-146a regulates epithelial barrier and secretion of proinflammatory cytokines in human nasal epithelial cells. *Eur J Pharmacol.* 2015;761:375–382.
60. Lamer-Svensson HM, Williams AE, Tsitsiou E, et al. Pharmacological studies of the mechanism and function of interleukin-1beta-induced miRNA-146a expression in primary human airway smooth muscle. *Respir Res.* 2010;11:68.
61. Tanic M, Zajac M, Gomez-Lopez G, et al. Integration of BRCA1-mediated miRNA and mRNA profiles reveals microRNA regulation of TRAF2 and NFkappaB pathway. *Breast Cancer Res Treat.* 2012;134:41–51.
62. Wang HQ, Yu XD, Liu ZH, et al. Deregulated miR-155 promotes Fas-mediated apoptosis in human intervertebral disc degeneration by targeting FADD and caspase-3. *J Pathol.* 2011;225:232–242.
63. Palma CA, Al Sheikha D, Lim TK, et al. MicroRNA-155 as an inducer of apoptosis and cell differentiation in Acute Myeloid Leukaemia. *Mol Cancer.* 2014;13:79.
64. Liu J, van Mil A, Vrijsen K, et al. MicroRNA-155 prevents necrotic cell death in human cardiomyocyte progenitor cells via targeting RIP1. *J Cell Mol Med.* 2011;15:1474–1482.
65. Rouquette-Jazdanian AK, Kortum RL, Li W, et al. miR-155 controls lymphoproliferation in lat Mutant mice by restraining t-cell apoptosis via SHIP-1/mTOR and PAK1/FOXO3/BIM pathways. *PloS One*. 2015; 10:e0131823.
66. Subedi A, Park PH. Autocrine and paracrine modulation of microRNA-155 expression by globular adiponectin in RAW 264.7 macrophages: involvement of MAPK/NF-kappaB pathway. *Cytokine*. 2013;64:638–641.
67. Bao JL, Lin L. MiR-155 and miR-148a reduce cardiac injury by inhibiting NF-kappaB pathway during acute viral myocarditis. *Eur Rev Med Pharmacol Sci.* 2014;18:2349–2356.
68. Gerloff D, Grundler R, Wurm AA, et al. NF-kappaB/STAT5/miR-155 network targets PU.1 in FLT3-ITD-driven acute myeloid leukemia. *Leukemia*. 2015;29:535–547.
69. Xiao B, Liu Z, Li BS, et al. Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response. *J Infect Dis.* 2009;200:916–925.
70. Yin Q, Wang X, McBride J, et al. B-cell receptor activation induces BIC/miR-155 expression through a conserved AP-1 element. *J Biol Chem.* 2008;283:2654–2662.
71. Wu T, Xie M, Wang X, et al. miR-155 modulates TNF-alpha-inhibited osteogenic differentiation by targeting SOCS1 expression. *Bone*. 2012;51:498–505.
72. Wang XY, Wu MH, Liu F, et al. Differential miRNA expression and their target genes between NGX6-positive and negative colon cancer cells. *Mol Cell Biochem.* 2010;345:283–290.
73. Huang X, Huang F, Yang D, et al. Expression of microRNA-122 contributes to apoptosis in H9C2 myocytes. *J Cell Mol Med.* 2012;16:2637–2646.
74. Lin CJ, Gong HY, Tseng HC, et al. miR-122 targets an anti-apoptotic gene, Bcl-w, in human hepatocellular carcinoma cell lines. *Biochem Biophys Res Commun.* 2008;375:315–320.
75. Chen YJ, Chang LS. Hydroquinone-induced miR-122 down-regulation elicits ADAM17 up-regulation, leading to increased soluble TNF-alpha production in human leukemia cells with expressed Bcr/Abl. *Biochem Pharmacol.* 2013;86:620–631.