Alimentary Tract

Altered mucosal expression of microRNAs in pediatric patients with inflammatory bowel disease

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A B S T R A C T

Introduction: MicroRNAs (miRs) came recently into focus as promising novel research targets offering new insights into the pathogenesis of inflammatory bowel diseases (IBD).

Aims: The aim of our study was to identify a pediatric IBD (pIBD) characteristic miR profile serving as potential Crohn’s disease (CD) and ulcerative colitis (UC) specific diagnostic pattern and to further analyze the related target genes.

Methods: Small RNA sequencing was performed on inflamed and intact colonic biopsies of CD, and control patients. Selected miRs were further investigated by RT-PCR, complemented with an UC group, in order to address the differential diagnostic potential of miRs in the two IBD subtypes. To analyze network connection of differentially expressed miRs and their target genes MiRTarBase database and previous transcriptome sequencing data from pediatric patient groups were used.

Results: Sequencing analysis identified 170 miRs with altered expression. RT-PCR analysis revealed altered expression of miR-31, -125a, -142-3p, and -146a discriminating between the inflamed mucosa of CD and UC. Enrichment analysis identified main IBD-related functional groups.

Conclusions: We demonstrated a characteristic colonic miR pattern in pIBD that could facilitate deeper understanding of the pathomechanism of IBD and may serve as a diagnostic tool.

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1. Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are the most prevalent forms of inflammatory bowel diseases (IBD) [1–3] characterized by inappropriate immune response of the gastrointestinal tract in genetically susceptible hosts [4]. Approximately 15–25% of all cases begin in childhood resulting in a life-long disease, frequently accompanied by serious complications [2,5–9]. Despite many years of research the exact pathomechanism and etiology of pediatric IBD (pIBD) is still unknown, however, many factors including genetic and epigenetic predispositions, environmental exposures, and microbiota are proved to significantly contribute to the disease progression [10–15].

Recently, special epigenetic mechanisms came into view with increasing interest toward the involvement of small non-coding RNAs called microRNAs (miRs) that are able to alter protein translation [16]. The field of miR research is rapidly expanding in adult IBD, however, little is known about their role in pIBD [17–21]. Nevertheless, functional analysis of IBD-related miR-genes with determined functions could highlight several previously unknown IBD-related biological processes [12].

Therefore, in the present study, our primary aim was to identify a novel IBD-specific miR pattern in pediatric patients. In addition, our secondary goal was to analyze the network connection of differentially expressed miRs and target genes in pIBD [22,23].

The authors have no competing interests.

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Colonic samples from all patients enrolled into the present study were obtained at the 1st Department of Pediatrics, Semmelweis University, Hungary. Clinical characteristics and laboratory parameters of the patients are shown in Table 1. Diagnosis of CD and UC was based on clinical symptoms, endoscopic findings and histopathology according to the Porto criteria [24]. Control samples were obtained from patients undergoing endoscopy for suspicion of polyp, Meckel-diverticulum or IBD. The specimens selected into the control group were identified as histologically normal showing an intact macroscopic- and microscopic appearance. Collected biopsies were snap-frozen and stored at −80 °C until further analysis. Written informed consent was obtained from the parents prior to the procedure, and the study was approved by the Semmelweis University Regional and Institutional Committee for Research Ethics (TUKEB No.: 10408/2012).

### 2.2. RNA isolation

Total RNA of colonic mucosa samples was extracted for next-generation sequencing using a TRIzol reagent (Ambion, Austin, TX, USA). For the real-time reverse transcription polymerase chain reaction (RT-PCR) measurements Quick-RNA MiniPrep isolation kit (Zymo Research, Irvine, CA, USA) were used to extract RNA from the colonic specimens according to the manufacturer’s protocol.

### 2.3. cDNA library preparation and next-generation sequencing

Paired biopsies with macroscopically inflamed (CD inflamed, n = 4) and intact (CD intact, n = 4) colonic mucosa of pediatric CD patients and controls (C, n = 4) were selected for next-generation sequencing (Table 1A). CDNA library for small RNA-Seq was generated from 1 μg total RNA using TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s protocol. Fragment size distribution and molarity of libraries were checked on Agilent BioAnalyzer DNA1000 chip (Agilent Technologies, Santa Clara, CA, USA). Concentration of small RNA libraries was set to 10 nM and cluster generation was done using TruSeq SR Cluster kit v3-cBot-HS kit on cBot instrument, then single read 50 bp sequencing run was performed on Illumina HiScan SQ instrument (Illumina, San Diego, CA, USA) carried out by UD-GenoMed Medical Genomic Technologies Ltd. (Debrecen, Hungary). The dataset is accessible in the ArrayExpress (www.ebi.ac.uk/arrayexpress) repository under the accession number E-MTAB-5010.

### 2.4. Real-time RT-PCR

Real-time RT-PCRs were performed on colonic biopsy samples of CD (CD inflamed, n = 15, CD intact, n = 10), UC (n = 10) patients and controls (n = 11) (Table 1B). Intact, non-inflamed samples were collected from the same CD patients as the inflamed ones. Total RNA was reverse-transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Based on our next generation sequencing data we used miRs with a corrected p-value <0.05, and fold change ≥1.5. Moreover, we made efforts to keep the biological relevance of the selection. The final 18 miRs were selected according to their relevance in adult IBD, or in mechanisms strongly related to IBD reported in the current literature [20,25–31]. Selected miRs (miR-18a: ID: 002422, miR-20a: ID: 000524, miR-221: ID: 000524, miR-223: ID: 002295, U6: ID:001973) were miR-qPCR using TaqMan MicroRNA Assays (Life Technologies, Carlsbad, CA, USA). A 25 μl qPCR mixture included: 12.5 μl TaqMan MicroRNA Assay, 12.5 μl TaqMan Universal PCR Master Mix (N.E.B. Inc., Ipswich, MA, USA), 1 μl primer probe mix, 1 μl cDNA sample and 9 μl nuclease-free water (Invitrogen, Carlsbad, CA, USA). Primer and probe sequences are described in Table 1B. Real-time RT-PCR measurements were carried out on additional samples (CD inflamed, n = 15 + 8, UC, n = 10 + 12) on a subset of miRs, showed a statistically significant difference or a tendency comparing the original copy number of CD inflamed and UC patients (miR-31, -125a, -142-3p, -142-5p). Condition of real-time RT-PCR were performed using TaqMan Universal PCR Master Mix No AmpErase UNG (Life Technologies, Carlsbad, CA, USA). For hypoxia-inducible factor 1-alpha (HIF1A), interferon-γ (IFNg), PH domain and leucine rich repeat protein phosphatase 2 (PHLPP2) and the...
ATP-binding cassette sub-family G member 2 (ABCG2) analysis, the total RNA from fresh-frozen biopsies was reverse-transcribed using the Maxima First strand cDNA Synthesis Kit (Thermo Fischer Scientific, Waltham, MA, USA) and quantitatively measured by real-time PCR using LC480 SYBR Green I Master Mix (Roche Diagnostics, Basel, Switzerland). Relative expression level was calculated by the ΔΔCq formula, using U6 for miR and GAPDH (forward: 5′ GCC GAT CAC ATC ATC ATC AA 3′, reverse: 5′ ACC ATC CAA GCC TTT CAA ATAA 3′) provided by IDT, Coralville, IA, USA. IfNg (forward: 5′ TTT GCC TTC TCG CTG TTA CT 3′, reverse: 5′ TTT GCC TCT GCA TTA TTT TTC TGT 3′), PHLP2 (forward: 5′ CAC CTG CGA ATC TTG CAC CT 3′, reverse: 5′ CAG GCA AAG CCT GTG CAA TC 3′) and ABCG2 (forward: 5′ ATG AAC TCT GTG AG 3′, reverse: 5′ ACG GCT GAA ACA CTG CT 3′) specific primers.

The PCRs were performed on a LightCycler 480 instrument (Roche, Basel, Switzerland). Relative expression level was calculated by the 2−ΔΔCq formula, using U6 for miR and GAPDH (forward: 5′ AGC ATG GCC TCC TGC ACC ACC AA 3′, reverse: 5′ GGC GCC ATC AGG CCA CAG TTT 3′) provided by IDT, Coralville, IA, USA) for mRNA as an internal standard of the same samples.

2.5. Bioinformatics analysis

DESeq normalized RNAseq data were obtained from the public ArrayExpress database for E-GEOD-57945 [22] and GSE10616 [23]. Gene expression data of inflamed colonic biopsy specimens from pediatric CD, UC patients and controls were analyzed, and compared with t-test. Multiple-testing correction was applied using the Benjamini–Hochberg method, and results were filtered after a threshold of 0.05. A fold change threshold of ±1.5 was set on the genes showing significant overexpression compared to the control samples. Experimentally validated (western blot, reporter assay, etc.) target genes were selected from the MiRTarBase database (http://mirtarbase.mbc.nctu.edu.tw) [32]. The derived datasets were then compared, and the overlapping genes were further analyzed. We performed Gene Ontology (GO) analysis on the common genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov) [33,34]. We established a 0.05 threshold of the p-values of the enriched categories adjusted with the Benjamini–Hochberg correction method. The resulted GO terms were filtered after evidence codes referring experimental validation (Inferred from Direct Assay (IDA), Inferred from Mutant Phenotype (IMP), Inferred from Expression Pattern (IEP)), REVIGO (http://revigo.irb.hr/), a web server which is able to summarize long GO term lists by removing functional redundancies utilizing an algorithm based on semantic similarity scores (Resnik) was used to simplify the related GO terms [35]. The resulting interrelationships were visualized with the Cytoscape 3.2.1. software (www.cytoscape.org) [36].

2.6. Statistical analysis

Statistical analysis was performed using the GraphPad statistical software package (GraphPad Software, La Jolla, CA, USA) and MedCalc statistical software (MedCalc Software, Ostend, Belgium). Data were analyzed performing Mann–Whitney U-test, ANOVA and post-hoc test, Benjamini–Hochberg false discovery rate algorithm. The threshold for statistical significance was set at p ≤ 0.05. The receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of miR-142-3p, -146a, -185 and -223 in the biopsies. The cut-off values of the statistical power analysis were the following: type I error: 0.05, type II error: 0.2. To determine the optimum cut-off point Youden’s J index was applied (J = sensitivity + specificity – 1). Data are presented as mean ± standard error of the mean (SEM) and normalized to the control group.

Table 2

<table>
<thead>
<tr>
<th>miR</th>
<th>CD inflamed vs. Control</th>
<th>CD intact vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-18a</td>
<td>3.27</td>
<td>3.68</td>
</tr>
<tr>
<td>miR-20a</td>
<td>–1.94</td>
<td>1.11</td>
</tr>
<tr>
<td>miR-21</td>
<td>1.93</td>
<td>2.14</td>
</tr>
<tr>
<td>mir-31</td>
<td>6.30</td>
<td>3.00</td>
</tr>
<tr>
<td>miR-99a</td>
<td>3.21</td>
<td>–</td>
</tr>
<tr>
<td>miR-99b</td>
<td>2.56</td>
<td>1.91</td>
</tr>
<tr>
<td>miR-100</td>
<td>4.90</td>
<td>2.39</td>
</tr>
<tr>
<td>miR-125a</td>
<td>2.00</td>
<td>2.23</td>
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<td>mir-126</td>
<td>2.64</td>
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<tr>
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<tr>
<td>miR-150</td>
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<td>1.09</td>
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<tr>
<td>miR-185</td>
<td>2.27</td>
<td>1.86</td>
</tr>
<tr>
<td>miR-204</td>
<td>–4.66</td>
<td>–1.60</td>
</tr>
<tr>
<td>miR-221</td>
<td>2.80</td>
<td>2.66</td>
</tr>
<tr>
<td>miR-223</td>
<td>5.31</td>
<td>2.84</td>
</tr>
</tbody>
</table>

CD: Crohn’s disease.

3. Results

3.1. CD-specific miR expression pattern identified by next-generation small RNA sequencing

Using next-generation sequencing of colonic small RNAs, 148 frequently dysregulated miRs were identified in the inflamed mucosa of pediatric CD patients compared to their intact mucosa or to the controls. 99 out of these 148 miRs were dysregulated in the inflamed colonic mucosa of CD patients compared to the intact ones, and altered expression of 114 miRs were detected in the inflamed region of the patients compared to the controls. Moreover, 22 miRs were differentially expressed in the intact mucosa of CD patients compared to controls (Table S1).

3.2. PCR validation of miR expression

18 miRs with dysregulated expression showing a fold change of at least 1.5 in the colonic mucosa of CD patients compared to controls were selected for method validation and further analysis with RT-PCR (Fig. 1, Table 2).

3.2.1. CD inflamed specimens

The expression of miR-18a, -21, -31, -99a, -99b, -100, -125a, -126, -142-5p, -146a, -150, -185, and -223 was significantly elevated in the inflamed colonic mucosa compared to the controls, and that of miR-141 and -204 was markedly decreased in the inflamed colonic mucosa of CD patients compared to the controls and the non-inflamed mucosa of CD patients. The expression of miR-142-3p was increased in the inflamed mucosa compared to the intact group.

3.2.2. CD intact specimens

In the intact mucosa of CD patients the expression of miR-18a, -20a, -21, -31, -99a, -99b, -100, -125a, -126, -142-5p, -146a, -185, -204, -221, and -223 showed statistically significant elevation compared to the controls. The expression of miR-20a, -204 and -221 was elevated exclusively in the intact region of CD patients compared to the controls. The level of miR-142-3p was significantly decreased in the intact colonic samples compared to the controls.

We performed ROC analysis of the differently expressed miRs. ROC analysis of miR-185 resulted in an area under the ROC curve (AUC) value of 0.81 (p < 0.05) between the macroscopically intact mucosa of CD patients and controls. At the optimum cut-off point the sensitivity was 62.5% with a specificity of 100% (J = 0.63)
Fig. 1. Expression of 18 microRNAs selected for further validation in the intact and inflamed colonic mucosa of children with Crohn's disease (CD), ulcerative colitis (UC) and controls (*: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001 vs. Control, #: p < 0.05, ##: p < 0.01, ###: p < 0.001 vs. CD intact, $: p < 0.05, $$: p < 0.01 vs. CD inflamed) (mean ± SEM).
Fig. 2. Receiver operating characteristic (ROC) curve analysis of miR-185 (A), -233 (B), 142-3p (C), -146a (D). The different expression of miR-185 and -223 could efficiently discriminate between the intact mucosa of pediatric Crohn’s disease (CD intact) patients and that of healthy controls (control). Analysis illustrated that the expression of miR-142-3p and -146a could serve as potential biomarker to differentiate between the inflamed mucosa of Crohn’s disease (CD inflamed) and ulcerative colitis (UC) patients. AUC: area under the ROC curve.

(Fig. 2A). The ROC analysis of miR-223 resulted in an AUC value of 1 (p < 0.001) between the CD intact and control specimens. At the optimum cut-off point the sensitivity was 100% with a specificity of 100% (J = 1) (Fig. 2B).

3.2.3. UC specimens

The RT-PCR experiments were complemented with an UC group, in order to analyze the diagnostic potential of the validated miRs in the context of IBD subtypes. The expression of miR-18a, -21, -31, -99a, -99b, -125a, -126, -142-5p, -146a, and -223 was elevated in the UC group compared to the controls. MiR-141 and -204 levels were significantly decreased in the colonic mucosa of UC patients compared to the controls and to the CD intact groups. Expression of miR-31 was elevated in the colonic mucosa of UC patients compared also to the intact and intact regions of the CD patients. Expression of miR-31, -125a and -146a was elevated in the colonic mucosa of UC patients compared to the intact mucosa of CD patients. Expression of miR-142-3p decreased in the UC group compared to the inflamed regions of CD patients.

ROC analysis of miR-142-3p resulted in an AUC value of 0.888 (p < 0.01) between the inflamed mucosa of CD and UC. At the optimum cut-off point the sensitivity was 77.78% with a specificity of 90.31% (J = 0.69) (Fig. 2C). The ROC analysis of miR-146a resulted in an AUC value of 0.838 (p < 0.01) between the inflamed mucosa of CD and UC. At the optimum cut-off point the sensitivity was 80% with a specificity of 76.92% (J = 0.57) (Fig. 2D).

MiRs discriminating between the inflamed mucosa of UC and CD were further analyzed in an extended patient group. The expression of miR-31, -125a and -146a was elevated, while the expression of miR-142-3p was decreased in the UC group compared to the CD group. In the case of miR-223, only a tendency has been observed between the inflamed mucosa of UC and CD patients in the original patient group, increasing the sample number resulted a difference between the CD and UC group. These results highlighted another differential diagnostic view of the miR-223 (Fig. 3).

3.3. Regulatory network of miRs with IBD-related genes

We annotated the target genes of miRs according to the data of the present next-generation sequencing, and overlapping with previous transcriptome sequencing expression data with GO terms (biological process domain). Based on the transcriptome sequencing dataset of pediatric CD patients (E-GEOD-57945), 126 genes showed connection with our sequencing data. The enrichment analysis resulted in 248 GO term categories, further reduction revealed 50 major GO terms, based on evidence codes (IDA, IMP, and IEP). The terms could be further grouped into 12 major categories (Table S2). The most abundant terms are the following: regulation of apoptotic process, response to wounding, response to bacterium, immune response, cell proliferation, adhesion, migration and activation, blood vessel development, regulation of gene expression and cell–cell signaling. 64 genes were found to be potentially regulated by our selected and validated miRs (Fig. 5A).

Enrichment analysis resulted in 192 GO term categories, further reduced into 48 GO terms, which were grouped into 11 major categories (Fig. 5B, Table S3). The most abundant terms overlapped with the full dataset. We verified two genes (HIF1A and IFNg) in our samples, both of them showed an elevated expression in CD patients compared to the controls, more about in the dataset of pediatric CD patients (E-GEOD-57945) (Fig. 5). Based on the dataset of pediatric UC patients (GSE10616), 4 genes, the ATP-binding cassette sub-family G member 2 (ABCG2), PH domain and
Fig. 3. Expression of 5 microRNAs selected for further validation in the inflamed mucosa of children with Crohn’s disease (CD inflamed) and Ulcerative Colitis (UC) in an extended patient group ($: p < 0.05, $$: p < 0.01$ vs. CD inflamed) (mean ± SEM).

Fig. 4. Gene ontology (GO) term categories of target genes of dysregulated microRNAs in the colonic mucosa of children with Crohn’s disease (CD) resulting from the sequencing data. Validated target genes were selected from the MiRTarBase database and the derived datasets were then compared with previous transcriptome sequencing expression data. We performed Gene Ontology (GO) analysis on the common genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID). The target gene screening, annotation, and enrichment analysis of microRNAs identified 12 major IBD-related functional groups and processes including inflammation, fibrosis, and angiogenesis.
leucine rich repeat protein phosphatase 2 (PHLPP2), ATP-Binding cassette, sub-Family B 1 (ABCB1) and Ras homolog family member U (RHOU) were related to 5 of our validated miRs (miR-20a, -126, -141, -142 and -223). We verified two of the four genes, which were related to differently expressed miRs in UC, based on the dataset of pediatric UC patients with negative fold change value. The mRNA expression of PHLPP2 and ABCG2 was significantly reduced in the inflamed mucosa of pediatric patients with UC compared to the controls (Fig. S1).

4. Discussion

15–25% of all IBD cases begin in childhood [5–7]. Pediatric onset IBD is often more severe showing rapid progression and serious side issues, including growth failure, poor bone density or delayed puberty. Despite its significance our knowledge is still limited regarding to the pathomechanism of childhood IBD, therefore it is of outmost importance to understand the initiating events of the disease [2,12,13,15]. Although previous studies identified several IBD-related miRs in adults [17,37], only a few data are available about their role in pIBD. Therefore, in the present study, we assessed the miR pattern characteristic for the mucosal deterioration of children with IBD. Moreover, bioinformatics analysis using publicly available transcriptomic datasets of pediatric patients was carried out to identify the significant miR-regulated biological processes involved in the pathomechanism of pIBD.

The next-generation RNA sequencing of intestinal biopsy samples identified 170 miRs with altered expression in the colonic mucosa of therapeutic naive pediatric patients with CD among them 148 miRs were dysregulated in the inflamed mucosa of CD
patients compared to their own non-inflamed mucosa or that of controls. Expression of 22 further miRs were dysregulated in the non-inflamed mucosa of patients with pediatric CD compared to the controls.

Validation of 18 miRs was carried out with RT-PCR measurements, what was extended to a UC patient group in order to analyze the differential diagnostic potential between the two IBD subtypes.

Identification of these disease-specific miR patterns could be of great relevance, since in clinical practice, it is difficult to differentiate between CD and UC especially in case of pancolitis. Extensive colitis is present in 55–75% of pediatric UC patients [11,38–40]. In addition, based on the data of the Hungarian Pediatric IBD Registry Group [HUPIR], approximately 4–6% of CD patients show symptoms of pancolitis with intact terminal ileum—without granulomas. The differentiation of the two pancolitis subtypes by means of specific biomarkers would be important for further therapeutic decisions.

In our present study we found elevated expression of miR-31, -125a, -146a and -223, and decreased miR-142-3p level especially in the inflamed mucosa of pediatric UC patients compared to that of children with CD. The expression of miR-100, -150, -185 was specifically increased in the colonic mucosa of CD patients, but not in UC specimens compared to the controls. Moreover, ROC analysis depicted that the different expression of miR-142-3p and -146a could serve as a potential biomarker to differentiate between the macroscopically inflamed region of CD and UC patients. Based on our results we suppose more predictive potential in combinations of miRs. We suggest that the combination of miR-31, -100, -125a, -142-3p, -146a, -150, -185 and -223 have a potential as biomarkers to identify pediatric CD and discriminate it from UC. The different expression of miR-31, -125a, -146a, -142-3p and -223 could serve as a potential biomarker to differentiate between the inflamed region of CD and UC patients, and the expression of miR-100, -150 and -185 could facilitate the diagnosis of CD as a positive predictive value. Moreover, these miRs may be target of therapeutic miR silencing with antisense oligomers [41].

Diagnosis of IBD is often challenging especially in cases when the colon and upper gastrointestinal tract are intact, or there is no information regarding the terminal ileum (e.g.: technical difficulties, stenosis) [42]. Isolated ileal involvement of pediatric CD appear in 0.4–7% of cases [2,38,43]. Moreover, isolated terminal ileal disease (±limited coecal disease) was seen in 16% of pIBD patients according to the data of EUROKIDS Registry [44]. The expression of miR-18a, -21, -31, -99a, -99b, -100, -125a, -126, -142-5p, -146a, -185, -204, -221, and -223 was elevated in the intact mucosa of pediatric CD patients compared to controls, referring to the differences between the two types of non-inflamed mucosa. Moreover, ROC analysis depicted that miR-185 and -223 could serve as potential biomarkers to differentiate between the macroscopically intact region of CD patients and controls. Nevertheless, small sample size is a limitation of AUC analysis, however according to the statistical power analysis our sample size was sufficient to perform the ROC analysis.

Moreover, we were able to replicate the same results on the extended sample size. In the case of miR-223 only a tendency was seen in the original patient group, but the difference came up significant in the analysis of the extended number of patients. The limitation of our study that we were unable to measure the whole 18 miRs in the extended number of patients. Further analyses needed in those cases where tendency shown.

Moreover, similarly to our recent results in pIBD, Fasseu et al. found elevated expression of miR-21, -31 and -146a in the inflamed and non-inflamed colonic mucosa of adult CD patients compared to controls. Furthermore, increased expression of miR-126 was demonstrated in both adult CD and UC, and elevated expression of miR-185 in the inflamed regions of CD, but not in UC patients. These data further support the hypothesis that miR-185 could serve as a CD specific diagnostic marker discriminating between CD and UC, both in adult and pIBD patients. However, in contrary to our findings, elevated expression of miR-150 was observed in both inflamed and non-inflamed colonic regions of adult patients and expression of miR-223 was elevated only in the quiescent mucosa of adult CD patients compared to the controls [18]. These distinct miR patterns may underline the differences and similarities between pediatric and adult IBD.

Integrative genomics approaches are becoming necessary tools for discovering new miR-target gene-biological function-disease relationships. Bioinformatics analysis performed in our present study provided evidence for a complex relationship between differentially expressed miRs and their target genes in pIBD. We applied well-founded pathway analysis, which is able to highlight biologically relevant association signals. Enrichment analysis offers several advantages for unveiling the molecular basis of IBD [45]. In our analysis, 49 miRs and their 99 target genes were identified as key regulators of pediatric CD based on the MiRTarBase dataset and previous transcriptome sequencing data of CD patients. Identification of miR target genes suggested biological processes that are involved in the pathomechanism of pIBD, including inflammation, fibrosis, response to microbe, apoptosis, and angiogenesis.

However, most of the miR-related target genes are connected to the regulation of immune- and inflammatory responses, including cell migration, activation, proliferation, cytokine production with special focus on leukocyte migration and activation. Most of these miR-connected genes are well studied in IBD, such as the genes of different cytokines (TNF, IFNγ, IL-1β, IL-6, IL-8), matrix-metalloproteinases (MMP7), adhesion molecules (ICAM1), transcription factors (HIF-1α, FoxP3, HBEFG, SOX2, STAT1), chemokines (CCL3, CR1, CXCL2), growth factors (CTGF, FGF7) [46–52]. However, there are also several miR-related genes with less known functions in IBD (CEBPA, ESR1, BDNF), which necessitates further investigations. Moreover, our data analysis revealed several genes involved in bacterial responses (TLR2, TLR4, IRAK1) or cell adhesion process (SELE, ICAM1) [53–55]. As members of key pathways several unveiled miR-related genes play an important role in apoptosis (FOS, FOSL, FOSB), epithelial cell turnover, and ulcer formation [56]. In addition, some of the identified miR-related genes are connected to the regulation of angiogenesis (HIF1A, IL1B, IL6, ICAM1, MMP13, SELE, SERPIN1), also a well-studied process in IBD [47,48,52,57]. Moreover, the endothelium regulates the recruitment of inflammatory cells, and the production of several inflammatory mediators (VEGF, IL-17 and TNF-α) [48].

Analyzing the target genes of differentially expressed miRs in the colonic mucosa of UC patients we identified a connection between 5 miRs (miR-20a, -126, -141, -142 and -223) and 4 genes (ABC2G, ABCB1, PHLPP2, RHOU). ABCG2 and ABCB1 are efflux transport proteins playing a significant role in intestinal barrier protection against external stimuli, such as drugs. It is also known that these transporters have a key role in the development of resistance against a broad range of therapeutic agents. According to our results decreased expression of ABCG2, moreover ABCB1 transporters have been found in patients with active IBD [58,59] and in the present study we found increased level of miR-142, -181 and -223 in the colonic mucosa of IBD, that are important regulators of these transporters [60–62]. Other important miR-target gene connection is that of miR-20a and -141 to PHLPP2, which directly targets the gene of a novel member of protein phosphatases, a candidate player in regulation of inflammation-induced epithelial cell apoptosis and tumor growth suppression [63–65]. Interestingly, loss of PHLPP2 protects against dextran sulfate sodium salt (DSS)-induced colitis in mice, however the level of PHLPP2 is decreased in the colonic mucosa of DSS-treated mice and in patients with human IBD, like in our samples too, which corresponds with elevated phosphorylation of Akt [64]. Rho protein family is involved in cell morphology
and migration, however less is known about the regulatory role of the atypical Rho GTPases, the RHOU, in the patomechanism of pIBD [66].

In summary, high-throughput sequencing of colonic biopsy samples revealed a pIBD-characteristic miR profile showing a statistically robust expression pattern, which is predominantly in accordance with the low-scale measurements reported in the relevant literature. In the present study, we identified several miRs, which could contribute to the differentiation between CD and UC. The target gene screening, annotation and enrichment analysis identified several IBD-related functional groups and processes including inflammation, fibrosis, apoptosis and angiogenesis, providing further evidence for the specificity of the miR profile and underlying the potential importance of these regulatory elements in the patomechanism of pIBD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dld.2016.12.022.

References


