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

PhD Theses

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the most prevalent forms of inflammatory bowel diseases (IBD). Approximately 15-30% of all cases begin in childhood resulting in a life-long disease, frequently accompanied by serious complications. 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.

Increased level of tumor necrosis factor (TNF)-α has a prominent role in the pathomechanism of IBD. 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 ß, IL-6, and TNF-α). As a key pro-inflammatory 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. 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.

Recently, special epigenetic mechanisms came into view with increasing interest towards the involvement of small non-coding RNAs called microRNAs (miRs) that are able to alter protein translation. MiRs are 19-24 nucleotide-long single-stranded RNAs involved in the regulation of gene expression at transcriptional and posttranscriptional level. The field of miR research is rapidly expanding in adult IBD, however, little is known about their role in

pIBD. Nevertheless, functional analysis of IBD-related miR-genes with determined functions could highlight several previously unknown IBD-related biological processes.

Objectives

1: to identify a novel IBD-specific miR pattern in pediatric patients
2: to analyze the network connection of differentially expressed miRs and target genes in pIBD
3: to investigate the expression of miR-146a, -155 and -122 in the inflamed and non-inflamed colonic mucosa of children with IBD in fresh-frozen (FF) and formalin-fixed, paraffin-embedded (FFPE) to compare the conservation method
4: to test the effect of TNF-α on the expression of miR-146a, -155 and -122 in HT-29 colonic epithelial cells

Methods

Patients

The diagnosis of CD and UC was based on clinical symptoms, endoscopic findings and histopathology, according to the Porto criteria and disease activity score was calculated by Pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Activity Index (PUCAI). The presenting symptoms of CD were weight-loss, hematochezia, abdominal pain, diarrhea, bloody diarrhea, anaemia, perianal fistula. Control children were referred to the outpatient clinic due to 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 non-inflamed (intact) regions of the colonic mucosa from children with CD, and from controls.
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. Based on our next generation sequencing data we used miRs with a corrected p-value <0.05, and fold change $\geq \left|1.5\right|$. Moreover, we made efforts to keep the biological relevance of the selection. The final 18 miRs were selected for further validation with real-time PCR according to their relevance in adult IBD, or in mechanisms strongly related to IBD reported in the current literature. 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). Intact, non-inflamed samples were collected from the same CD patients as the inflamed ones.

FFPE sections were as follows: intestinal biopsies of macroscopically inflamed and non-inflamed (intact) regions of the colonic mucosa from children with CD (n=12), and from controls (C) (n=16). FF biopsies were as follows: biopsies of macroscopically inflamed (n=24) and intact (n=14) regions of patients suffering from CD, macroscopically inflamed regions of UC patients (n=10) and biopsies from controls (n=23). The non-inflamed samples were taken from the same CD patients as the inflamed ones.

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, USA) under standard cell culture conditions (37°C, humidified, 5% CO2/95% air environment). HT-29 cells were seeded in 6-well plates at a density of 5x105 cells/well and treated for 24 hours with recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) 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, Düsseldorf, Germany). RNA isolation from FF biopsies was performed using TRIzol reagent (Ambion, Austin, TX, USA) combined with Quick-RNA MiniPrep isolation kit (Zymo Research, Irvine, CA, USA), which procedure was also applied to gain RNA from the cells.

cDNA library preparation and next-generation sequencing

CDNA library for small RNA-Seq was generated from 1μg 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 10nM and cluster generation was done using TruSeq SR Cluster kit v3-cBot-HS kit on cBot instrument, then single read 50bp 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).

Reverse transcription and quantitative polymerase-chain-reaction (RT-qPCR)

For miR analysis, total RNA was reverse-transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) and quantitatively measured in a real-time 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). 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, USA) and quantitatively measured by real-time PCR using LC480 SYBR Green I Master Mix (Roche Diagnostics, Basel, Switzerland), applying TNF-α specific primers (IDT, Coralville, IA, USA). The PCRs were performed on a LightCycler 480 instrument (Roche Diagnostics). Relative expression level was calculated by the 2ΔCq formula, using U6 for miR and RPLP0 (IDT, Coralville, IA, USA) for mRNA as the references.

Bioinformatics analysis

DESeq normalized RNAseq data were obtained from the public ArrayExpress database for E-GEOD-57945 and GSE10616. Gene expression data of biopsy specimens from pediatric CD patients 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 to the control samples. Experimentally validated (western blot, reporter assay, etc.) target genes were selected from the MiRTarBase database. The derived datasets were then compared, and the overlapping genes went into further analysis. We performed Gene Ontology (GO) analysis on the common genes using the Database for Annotation, Visualization and Integrated Discovery. 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.

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, Benjamin-Hochberg false discovery rate algorithm. The threshold for statistical significance was set at $p \leq 0.05$.

Results

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 was 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.

PCR validation of miR expression

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.

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.

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 inflamed 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 inflamed mucosa of CD patients. Expression of miR-142-3p decreased in the UC group compared to the inflamed regions of CD patients.

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. The terms could be further grouped into 12 major categories. 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. Enrichment analysis resulted in 192 GO term categories, further reduced into 48 GO terms, which were grouped into 11 major categories. The most abundant terms overlapped with

the full dataset. Based on the dataset of pediatric UC patients (GSE10616), 4 genes, the ATP-binding cassette sub-family G member 2 (ABCG2), PH domain and 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).

Expression of miR-146a, -155 and -122 in the colonic mucosa of children with CD, UC and controls

The expression of miR-146a, and -155 was elevated in the macroscopically inflamed colonic mucosa of children with IBD compared with the controls in the FF and FFPE samples. Expression of miR-122 in FFPE and FF biopsies was higher in macroscopically intact colonic mucosal biopsies of children with CD in comparison to controls.

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, whereas no significant difference related to TNF-α expression was observed between UC and CD.

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 and miR-155 in HT-29 human colonic epithelial cells in comparison to vehicle-treated control cells, whereas the expression of miR-122 was not statistically different between TNF-α treated and control groups.

Duscussion

Based on our study the following conclusions can be derived:

1. The next-generation RNA sequencing of intestinal biopsy samples identified 170 miRs with altered expression in the colonic mucosa of pediatric patients with CD compared to the controls, in which 22 miRs were dysregulated in the non-inflamed mucosa of patients with pediatric CD compared to the controls.

2. 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.

3. 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

4. The highlighted 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. These distinct miR patterns may underline the differences and similarities between pediatric and adult IBD.

5. 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 underlining the potential importance of these regulatory elements in the pathomechanism of pIBD.

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

7. 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.

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