The importance of microRNAs in pediatric Crohn disease

PhD Theses

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Introduction

Crohn disease (CD) is a chronic immune-mediated disorder, which is frequently characterized by the appearance of lesions in the upper gastrointestinal (GI) tract, primarily in pediatric patients. In adults, upper GI lesions in the cardiac area with bamboo joint-like appearance are regarded to be significant risk factors for the progression of the disease from the inflammatory to stenotic or penetrating form of CD.

In the pathogenesis of CD, epigenetic factors including microRNAs (miRs) have come into focus as potent modulators of the progression of the disease. An increasing number of studies investigates the role of these short single-stranded RNAs in inflammatory bowel disease (IBD). However, the expression profile of miRs in the upper GI region of CD patients is completely unknown.

Our working group has previously investigated the expression of miR-146a, -155 and -122 in the inflamed colonic region of pediatric IBD patients. In that study we observed elevated expression levels of all three analyzed miRs. These miRs affect a number of key biological functions involved in the pathomechanism of CD, including inflammatory response, intracellular signaling cascades and response to the presence of bacteria.

The aim of the present study was to investigate the mucosal expression of miR-146a, -155 and -122 in the upper GI tract of children with CD. Thereafter I compared the obtained results with our previous findings.

The other aim of the study was to examine the effect of anti-inflammatory recombinant human transforming growth factor (rhTGF)-β on these miR's expression on CCL-241 small intestinal epithelial and primary duodenal fibroblast cells.

Although upper GI tract endoscopy is an important tool to diagnose CD in pediatric patients, recently there have been no specific markers to definitely distinguish it from other GI diseases with erosions, ulcerations (Helicobacter pylori infection, peptic ulcer

caused by infection or medication, or eosinophil gastritis), and disorders with granulomas (sarcoidosis, Mycobacterium tuberculosis). Therefore, we suppose that our results may contribute to the identification of potential novel biomarkers or therapeutic targets of CD.

Objectives

1: To investigate the mucosal expression of miR-146a, -155 and -122 in the upper GI tract of children with CD.
2: To compare these results with our previous data from the colonic musosal biopsies of pediatric CD.
3: To examine the effect of the anti-inflammatory rhTGF-β on the expression of the investigated miRs on small intestinal epithelial and primary duodenal fibroblast cells.

Methods

Patients

CD was diagnosed according to the Porto criteria. The disease activity score was assessed regarding the Pediatric Crohn Disease Activity Index (PCDAI). Control children were referred to the outpatient clinic due to recurrent abdominal pain and GI symptoms to exclude organic diseases. Esophago-gastro-duodenoscopy was part of their diagnostic procedure showing normal macroscopic appearance and histology. Duodenal biopsy samples were taken from different patients, macroscopically inflamed (CD inflamed: n=10) and non-inflamed (CD intact: n=10) regions of the duodenal mucosa from children with CD and controls (C: n=10). Biopsies were immediately fixed in buffered formaldehyde and embedded into paraffin (PF). Written informed consent was obtained from the parents prior to the procedure, and the study was approved by the Semmelweis University Regional and Institutional Research Ethics Committee (TUKEB No.: 10408/2012).

RhTGF-β treatment of the small intestinal epithelial and duodenal fibroblast cells

Normal small intestinal epithelial cells (CCL-241) were grown in HybriCare medium supplemented with 10% fetal bovine serum (FBS), 30 ng/mL epidermal growth factor, and 1% Penicillin and Streptomycin mixture. Epithelial cells were grown under standard cell culture conditions (37°C, humidified, 5% CO_2/95% air environment).
Primary duodenal fibroblast cells were freshly isolated from the duodenal mucosa of healthy children. The biopsies were washed in phosphate-buffered saline (PBS) and homogenized in 1 mg/mL collagenase content PBS. Cells were grown in Dulbecco's Modified Eagle Medium supplemented with 1% FBS and 1% Penicillin and

Streptomycin mixture. Cells were grown under standard cell culture conditions (37°C, humidified, 5% CO_2/95% air environment) until a confluent monolayer was obtained. During culturing, the unattached cells were removed after every 24 h culture period.
Epithelial and primary fibroblast cells were seeded into 6-well plates at a density of $5x10^5$ cells/well and treated for 24 hours with rhTGF-β at a concentration of 1 nM or vehicle only for control cells.

RNA isolation

Total RNA was isolated from formalin-fixed, paraffin-embedded biopsies using RNeasy Minikit after removing paraffin from the samples according to the instructions of the manufacturer. All contaminants were efficiently washed away, DNase was used to remove DNA from the samples using on-column DNase treatment. Concentrated RNA was filtrated using RNeasy MinElute spin columns. RNA was eluted in 30 µL water.
Total RNA of the intestinal epithelial and fibroblast cells was isolated by the Quick-RNA MiniPrep isolation kit according to the instructions of the manufacturer. RNA Lysis Buffer purified the RNA using Zymo-Spin™ Columns. All contaminants were washed away (RNA Prep and Wash Buffer) and DNase was used to remove the DNA from the samples. RNA was eluted in 30 µL water and used further immediately.

Reverse transcription and real-time polymerase-chain-reaction (RT-PCR)

The total RNA was reversely transcribed by the TaqMan MicroRNA Reverse Transcription kit. TaqMan MicroRNA Assay was used to quantify individual microRNA levels with real-time RT-PCR on a LightCycler 480 instrument. Reactions were performed in triplicates. Relative expression of miRs was determined by the 2ΔCq method using U6 as an internal control.

Statistical analysis

Statistical analysis was performed by Graphpad statistical software package. Normality was tested by the Shapiro-Wilk test. Analysis was based on the Mann-Whitney U-test, Kruskal-Wallis-test, Analysis of variance (ANOVA) and Dunn's Post-Hoc test, and $p \leq 0.05$ was considered as statistically significant. Data were presented as mean ± standard error of the mean (SEM).

Results

<u>Expression of miR-146a in the duodenal mucosa of pediatric patients with CD</u>

The expression of miR-146a was significantly higher in the inflamed duodenal mucosa of children with CD compared to the intact mucosa (CD inflamed: 3.21±0.50 vs CD intact: 0.62±0.26, $p \leq 0.01$) and controls (CD inflamed: 3.21±0.50 vs Control: 1.00±0.33, $p \leq 0.05$). There was no significant difference between the uninflamed group and the control one (CD intact vs Control: p=n.s.).
In comparison with our previous study, the expression of miR-146a 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 vs CD intact: 1.79±0.45, $p \leq 0.05$) and controls (CD inflamed: 6.66±1.52 versus Control: 1±0.23, $p \leq 0.001$).

<u>Expression of miR-155 in the duodenal mucosa of pediatric patients with CD</u>

MiR-155 showed significantly elevated expression in the inflamed region of the duodenal mucosa of CD patients compared to the control group (CD inflamed: 4.87±1.02 vs Control: 1.00±0.40, $p \leq 0.001$). There was no significant difference between the uninflamed group and the control one (CD intact: 2.50±0.38 vs Control: 1.00±0.40, p=n.s.).
In comparison with our previous study, the expression of miR-155 showed significant elevation in inflamed CD mucosa in comparison with the intact CD mucosa and controls (CD inflamed: 8.52±1.90 versus Control: 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.

Expression of miR-122 in the duodenal mucosa of pediatric patients with CD

There was no significant difference in the expression of miR-122 between the CD and control groups (CD inflamed: 0.86±0.25, CD intact: 0.96±0.14, Control: 1.00±0.28, p=n.s.).
In comparison with our previous study, the expression of miR-122 was statistically higher in macroscopically intact colonic mucosal biopsies of children with CD than in controls (CD intact: 3.12±0.71 vs Control: 1±0.17, p≤0.05).

Expression of miR-146a, -155 and -122 in rhTGF-β treated small intestinal epithelial cells

RhTGF-β had no effect on the expression of miR-146a (p=n.s.); however, it decreased significantly the expression of miR-155 in CCL-241 small intestinal epithelial cells (TGF-β: 0.7±0.083 vs Control: 1±0.09, p≤0.05). No miR-122 was detected in the small intestinal epithelial cells.

Expression of miR-146a, -155 and -122 in rhTGF-β treated duodenal fibroblasts

RhTGF-β significantly decreased the expression of miR-146a (rhTGF-β: 0.67±0.04 vs Control: 1±0.15, p≤0.01) and miR-155 (rhTGF-β: 0.72±0.09 vs Control: 1±0.06, p≤0.05) in duodenal fibroblasts compared to vehicle treated control cells. There was no difference in the expression of miR-122 when rhTGF-β was administered compared to the control group (p=n.s).

Discussion

Based on our study the following conclusions can be derived:

1. The expression level of miR-146a and -155 is elevated in the macroscopically inflamed duodenal mucosa of newly diagnosed, treatment-naive pediatric patients with CD compared to the control group.

2. These results are in accordance with our previous observations demonstrating the increased expression of miR-146a and -155 in the inflamed colonic region of pediatric IBD patients.

3. In contrary to our earlier observations related to the colon, we found unchanged expression of miR-122 in the duodenal mucosa of children with CD.

4. Increased expression of miR-146a and -155 in the inflamed intestinal mucosa suggests their involvement in the pathomechanism of CD as inflammation-specific markers.

5. The expression of miR-146a and -155 is independent of, and miR-122 is dependent of the localization of CD.

6. Anti-inflammatory rhTGF-β is a negative regulator of miR-146a and -155.

7. Our recent data have provided a baseline to explore the possible role of these miRs as diagnostic markers or their potential as therapeutic targets.

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