Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Faecal microRNA as a biomarker of the activity and prognosis of inflammatory bowel diseases

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ARTICLE INFO

Article history:
Received 19 June 2018
Accepted 30 June 2018
Available online xxx

Keywords:
Faecal miRNA
IBD
Intestinal microbiota

ABSTRACT

Reasons underlying the individual differences in the clinical manifestations of inflammatory bowel diseases (IBD) and the mechanism by which the host screens the intestinal microbiota remain unclear. The presence of miRNA in faeces might be a potential clue into differences in gut microbiota among these patients. In this study, we analysed the differences in miRNA levels in faecal samples from 117 patients diagnosed with IBD. There was a significant difference in faecal miRNAs between healthy subjects and those with inactive IBD. Further analysis showed that some miRNAs might indicate the severity of IBD activity and prognosis. Sequencing analysis of the 16S RNA V4 region in faecal microbiota in these IBD patients revealed significant differences in the phylogenetic architecture between subjects with active or inactive IBD and between IBD patients and healthy subjects. Finally, in vitro studies showed that these differentially expressed miRNAs have different effects on the proliferative activity of the intestinal microorganisms *Fusobacterium nucleatum* (*Fn*), *Escherichia coli* (*E. coli*) and *segmental filamentous bacteria* (*SFB*). We observed the dynamic uptake of miRNA by these bacteria using flow cytometry. This study reveals a potential link between faecal miRNA, intestinal microbiota, IBD activity and prognosis.

1. Introduction

Inflammatory bowel diseases (IBDs), namely, Crohn's disease (CD) and ulcerative colitis (UC), affect 5 million people worldwide. To date, the causes of IBD remain elusive because they involve complex interactions among genetic environmental factors and immune regulation. UC occurs in the colon mucosa, and CD might occur in the gastrointestinal tract; UC involves persistent severe inflammation, and CD is immunoreactive in the terminal ileum [1,2]. At present, the pathogenesis of IBD is mainly thought to involve the imbalance of the innate immune response triggered by microbial antigens and damage to the intestinal mucosa [3,4]. The clinical characteristics of individuals with IBD, such as disease location, activity intensity, and disease behaviour, vary widely, and the reasons for the individual differences in clinical manifestation remain unknown.

The human intestine harbours microbial strains derived from the mother and those obtained through breastfeeding and other methods of contact [5]. Around the age of 3, the human intestinal flora has gradually developed into stable microbial communities [6]. Many factors affect the formation of the intestinal microflora, including host genetics, diet and disease [7,8]. The number and diversity of gut microbiota vary widely in the intestines of various mammals. However, there is significant individuality in the composition of the intestinal flora, and the intestinal microflora is therefore referred to as the second human genome. When the intestinal microflora of mice is transplanted into recipient mice, it gradually returns to the state before transplantation, indicating that there is a host mechanism regulating the populations of specific microorganisms in the intestine [9]. Since the intestinal flora plays an important role in immunity and disease, the study of how these microbials regulate mechanisms in the intestine is beneficial to our understanding of the individual differences in the clinical manifestations of IBD.

miRNAs are non-coding RNAs of 18–23 nucleotides in length and are widely present in cells involved in various physiological and pathological processes. An increasing number of studies have identified miRNAs in extracellular and humoural fluids [10]. In the

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https://doi.org/10.1016/j.bbrc.2018.06.174
0006-291X/

intestine, miRNAs have been identified in faeces as potential biomarkers of intestinal malignancies [11,12]. In addition, the regulation of the diversity of intestinal microflora by functional miRNAs in faeces has been studied [13]. Herein, our main task was to identify the differences in gut miRNAs in the faeces of IBD patients and to demonstrate their role in regulating and screening the composition of the gut microbiota.

2. Materials and methods

2.1. Subjects

Faecal specimens were collected from 117 IBD patients and 66 healthy subjects (66% males). The basic characteristics of all subjects are shown in Table 1. Written consent was obtained from all subjects, and this study protocol was approved by the Ethics Review Committee of Xi'an Jiaotong University. All authors and investigators who participated in the study read and strictly observed the ethics guidelines of the World Medical Association (Declaration of Helsinki). Samples were taken over the past 12 months, and healthy subjects were excluded if they had other gastrointestinal disorders or had taken antibiotics. The subjects were between 17 and 64 years old. All faecal samples were collected and stored at −80 °C until further processing.

2.2. Faecal miRNA extraction

Briefly, 10 mg of a subject's faecal sample was homogenized in 50 μl of sterile PBS. Total RNA was extracted from faecal samples using a mirVana™ miRNA Isolation Kit (Ambion). The RNA was purified by a 1:1.25 vol mixture of chloroform and anhydrous ethanol and then filtered on a glass fibre filter. The quality of the purified miRNA was detected using a UV spectrophotometer (A260/A280 = 1.8–2.0, A260/A230 ≥ 1.3).

2.3. Microarray screening and qPCR detection of faeces miRNAs

For microarray screening, total miRNAs were labelled using a miRCURY™ Power Labelling Kit (Exiqon, Denmark) and hybridized to a miRCURY LNA microRNA Array (Exiqon, Denmark). After scanning with the Axon GenePix 4000B Microarray Scanner (Molecular Devices, USA), data were extracted using Agilent Feature Extraction v10.7 software. The original data were normalized using GeneSpring GX v11.5.1, and volcano plots of miRNA expression (log [fold change]>2 or < -2, $p < 0.001$) were created using GraphPad Prism 6.0 software. A heat map was generated using MeV4.9 software to show the 60 miRNAs with the most significant differential expression. For miRNA detection by qPCR, mirVana™ miRNA kits were used to extract total miRNA from the samples. miRNAs were reverse transcribed using specific stem-loop primers (GenePharma, CHN) and quantified using RT-PCR with a TaqMan miRNA Assay Kit (Applied Biosystems, USA). U6 was used to standardize miRNA expression.

2.4. Assessment of the severity of IBD activity

The Modified Mayo score index (MSI) [14,15] for the clinical and research evaluation of UC, which includes defecation frequency, blood in the stool, endoscopy, and physician evaluation, was used. Subjects were used as controls; scores of 1–2 indicated clinical remission, 3 to 5 indicated mild activity, 6 to 10 indicated moderate activity, and 11 to 12 indicated high activity. The Crohn's disease activity index (CDAI) [16] was used to assess the severity of CD activity and to evaluate efficacy; this index includes the number of loose stools, abdominal pain and masses, extra-intestinal manifestations and complications, hematocrit, and percent deviation from standard weight; CDAI <150 indicates remission, 150–220 indicates mild activity, 221–450 indicates moderate activity, and >450 indicates severe activity.

2.5. 16S RNA analysis

16S rRNA gene analysis was used to examine the diversity of faecal microbiota in the subjects. Using the NEXT flexTM 16S V4 Amplicon-Seq Kit (Bioo Scientific, USA), primers were used to generate amplicons that span the variable region 4 (V4) of the 16S rRNA gene that were subsequently sequenced on an Illumina MiSeq (Illumina, USA). Data were processed using QIIME software (v1.9.0). More than 250,000 sequences were detected per sample, and unique sequences were classified and grouped into 1823 operational taxonomic units (OTUs) based on 97% nucleotide sequence identity (97% ID OTU). The principal coordinates analysis (PCoA) plot was generated based on the UniFrac distance matrix and was used to test the similarity among individual subject groupings of intestinal bacteria.

2.6. Effect of miRNA on bacterial activity

Equal amounts of *Fn*, *SFB* and *E. coli* in logarithmic growth phase were plated in 96-well plates and treated with artificially synthesized miRNA mimics at 1.25 μM and the corresponding inhibitor sequences. In addition, control groups were treated with vehicle, scramble or mutant miRNA sequences. The absorbance at 600 nm (OD_{600}) was measured using an Infinite F200 Spectrophotometer (Tecan, Switzerland), and measurements were taken every hour for 24 h.

2.7. Flow cytometry

Equal amounts of *Fn.*, *SFB*, and *E. coli* in logarithmic growth phase were plated in 12-well plates and treated with artificially synthesized Cy3-labelled miRNA mimics (1.25 μM; GE Dharmacon, USA). The cell number was determined by flow cytometry (Beckman, USA).

Table 1
Clinical characteristics of the study subjects.

Phenotype	n	Age (mean)	Disease duration (mean, year)	Current IBD medications		
				Prednisone	AZA/6MP	Anti-TNF
Healthy controls	66	49.5	N/A	N/A	N/A	N/A
Inactive UC	25	43.6	11.5	32%	48%	0%
Active UC	41	50.9	6.5	54%	37%	0%
Inactive CD	22	42.3	13.5	18%	18%	32%
Active CD	29	40.7	6.1	34%	34%	0%

Please cite this article in press as: Y. Ji, et al., Faecal microRNA as a biomarker of the activity and prognosis of inflammatory bowel diseases, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.06.174