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Original Article

Detection of miRNA differential expression of necrotizing enterocolitis in newborns by high-throughput sequencing

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Abstract: Objective: To detect miRNA differential expression of necrotizing enterocolitis (NEC) in infants using peripheral blood samples. Method: From October 2014 to November 2015, 25 infants diagnosed as Bell stage II and above at Neonatal Intensive Care Unit (NICU) of Guangzhou Women and Children's Medical Center were included and whole blood samples were collected. In the meantime, whole blood samples from 25 newborns without NEC were collected as the controls. Five peripheral blood samples were collected randomly from the two groups, respectively, for high-throughput sequencing using Illumina technology. Differentially expressed miRNAs were identified and the target genes and the corresponding biological roles were predicted. The remaining samples were used for verification of the high-throughput sequencing result by RT-qPCR. Result: As compared with the control group, 482 miRNAs were differentially expressed significantly (P<0.05). Using the criteria of $P<0.01$, $q<0.001$ and $|\text{Log}_2$ fold change $| \geq 1$, 126 known miRNAs were found to be differentially expressed, including 58 upregulated miRNAs and 68 downregulated miRNAs. The miRNAs showing stable expression and high fold change were verified by RT-qPCR. The verification results agreed with the sequencing results for upregulated miRNAs, hsa-miR-223-5p, -183-3p and -222-5p, and the downregulated miRNAs hsa-miR-23b-5p, -150-5p, -146a-3p and -1298-5p. Bioinformatics analysis indicated that the target genes of differentially expressed miRNAs were regulatory of the Toll-like receptor signaling pathway, MAPK pathway and JAK-STAT pathway. Conclusion: Between newborns with and without NEC, some miRNAs in the peripheral blood leukocytes were differentially expressed and they might be involved in NEC by regulating different target genes.

Keywords: miRNA, necrotizing enterocolitis, high-throughput sequencing, newborns

Introduction

Necrotizing enterocolitis (NEC) is a common and critically severe enteric disease in infants caused by various pathogenic factors, especially for premature infants and very low birth weight infants. miRNA is a highly conservative non-encoding RNAs composed of 20-25 nucleotides. Over 1/3 of human genes are regulated by miRNAs on the posttranscriptional level, which induces the degradation or inhibits the translation of target mRNAs. This is important for the regulation of different cell processes [1]. As to the pathogenesis of NEC, the role of miRNAs in intestinal mucosal barrier, intestinal innate immunity and microbe-host interaction attracts most attention [2]. So far it has been known that miRNAs play important roles in intestinal mucosal inflammation, immunologic tolerance, innate immunity, tumor formation and the proliferation, differentiation and apoptosis of intestinal epithelial cells [3-5]. Besides, miRNAs are involved in various intestinal physiopathological processes through different regulatory pathways associated with intestinal immunity or inflammatory response. To determine the role of miRNA in NEC, we identified differentially expressed miRNAs using high-throughput sequencing technology and verified the results by RT-qPCR. Moreover, the target genes were predicted and gene enrichment analysis was conducted. The findings shed new light into the understanding of pathogenesis of NEC.

Materials and methods

Subjects

Data of newborns treated at NICU and hospitalized at Department of Neonatology in Guang-

zhou Women and Children's Medical Center from October 3rd 2014 to November 15th 2015 were collected. Informed consent was signed by the guardians of all subjects and the experimental protocol was approved by ethics committee.

NEC group

Newborns diagnosed as Bell stage II and above were included [6]. Newborns were excluded if combined with history of severe infection and asphyxia or congenital malformation of brain, heart, gastrointestinal tract, kidney, liver and respiratory tract. Thus 25 newborns with NEC (13 males and 12 females) were included, with an average gestational age of 35.0±3.9 weeks and average birth weight of 2 370±746 g.

Control newborns were included following the criteria below

Non-NEC newborns; high risk newborns matched to NEC cases in gestational age, birth weight and gender; without gastrointestinal inflammation or malformation 2 months after birth. Twenty-five control newborns (13 males and 12 females) were included, with an average gestational age of 35.1±3.9 weeks and average birth weight 2 417±694 g.

The two groups showed no significant difference in gestational age, birth weight and the gender ratio ($P>0.05$).

Method

Sample collection and treatment: From NEC cases after diagnosis and from control cases before discharge, 1.5 ml of peripheral arterial blood was collected, respectively. Blood samples were added with EDTA anti-coagulant and placed at room temperature for 2 h. Into the whole blood samples 5 times volume of 1× red blood cell lysis buffer (Beyotime, Shanghai) was added and the peripheral blood leukocytes were isolated. Then 1 ml of Trizol was added (Invitrogen, USA), mixed well and transferred to a EP tube at -80°C.

Detection of miRNA differential expression using high-throughput sequencing technique. Five samples were randomly selected from the control group and NEC group, respectively. Second-generation high-throughput sequencing was conducted using Illumina.

Total RNA extraction and quality evaluation

Total RNA extraction was performed using Trizol reagent and DNA contamination was removed by DNase I. All procedures were implemented according to the instruction manual. The extracted RNA was evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). RNA was considered qualified by the criteria of RNA integrity number (RIN) ≥7.0 and 28S/18S ≥0.7.

Building of small RNA library and sequencing

Illumina Truseq Small RNA Preparation Kit was used to build small RNA library (Illumina, USA). Adapter was connected to the 3'- and 5'-terminal of RNA and cDNA was synthesized by reverse transcription (RT) using primers complementary to the 3'-terminal of RNA. After qPCR, fragments of 140-160 bp were recovered using 6% polyacrylamide gel. The quality of the small RNA library generated was evaluated using an Agilent Bioanalyzer 2100. RNAs with the concentration above 20 nmol/L, length of 140-160 bp and free from dimer contamination were used for high-throughput sequencing on the Illumina HiSeq 2500 platform. The raw reads were treated to obtain the clean reads for aligning against the full human genome using SOAP Toolkit 2.0 Software. The unaligned reads were removed and the matched ones were aligned against the genomic repeated sequences, GenBank and Rfam10.0 database, respectively. Sequences annotated as other small molecules were removed and alignment was done for the remaining sequences against miRbase21.0 database using BLAST Program. The known miRNAs were classified and annotated and their expression levels were determined (RNA library building and sequencing were accomplished by BGI-Shenzhen).

Detection of miRNA differential expression

miRNA expression profiles of the two groups were determined using DEGseq [7]. Differentially expressed miRNAs were those with $P<0.05$ in the comparison of the two groups. According to the criteria of $P<0.01$, $q<0.001$ and standardized $|\text{Log}_2 \text{(fold change)}| \geq 1$ (i.e., fold change ≥2 times and ≤0.5 times), known miRNAs showing significantly differential expression were identified. The fold change estimate was the ratio of standardized miRNA expression of NEC group to control group.

Table 1. Small RNA libraries for sequencing

Sample	Raw reads	Clean reads	Reads aligned to the genome (%)	Reads aligned to known miRNA (%)
C1	32561880	31885975	22855244 (71.68%)	21152812 (66.34%)
C2	33655601	32438886	24834360 (76.56%)	22467343 (69.26%)
C3	35992672	35197248	23133131 (65.72%)	21602890 (61.38%)
C4	33304780	32777818	23172263 (70.69%)	20607805 (62.87%)
C5	39470860	38875256	28562213 (73.47%)	25024516 (64.37%)
N1	37655684	37008040	26327465 (71.14%)	23731430 (64.13%)
N2	35348695	34778527	28052179 (80.66%)	25147963 (72.31%)
N3	30862334	30161809	22239685 (73.73%)	20132863 (66.75%)
N4	35076566	33466904	25403126 (75.91%)	21321244 (63.71%)
N5	30487718	29745090	21252121 (71.45%)	17759070 (59.7%)
Total for NEC group	174985793	171175183	122557211 (71.6%)	110855366 (64.76%)
Total for control group	169430997	165160370	123274576 (74.64%)	108092570 (65.45%)

Note: N denotes control group and C control group; % represents the percentage of aligned reads to clean reads.

Verification of the differentially expressed miRNAs

The remaining 20 samples from each group were used for RT-qPCR as verification. According to high-throughput sequencing, 7 differentially expressed miRNAs were chosen for stem-loop RT-qPCR. Total RNA (500 ng) was extracted and cDNA was synthesized by using the reverse transcription kit (RR037A, TaKaRa, Japan). Then qPCR was performed using the kit (RR820B, TaKaRa, Japan) on the ABI7500 instrument (ABI, USA) by taking 2 μl of the synthesized cDNA as the template. RNU6 SnRNA was used as internal control gene. Primers for stem-loop RT-qPCR and PCR were designed and synthesized by RiboBio Co. PCR conditions were as follows: predenaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, 40 cycles; 95°C 15 s, 60°C 1 min. PCR dissociation curve was plotted. Three replicates were set up for each sample and the average was taken. Results were analyzed using SDS 1.4 software and miRNA relative expression was calculated by using $2^{-\Delta\Delta Ct}$ method.

Bioinformatics analysis

Target genes were predicted for 126 differentially expressed miRNAs using RNAhybrid, miRanda, targetscan and PITA. GO annotations and analysis of KEGG metabolic pathway and signal transduction pathways were done for all target genes using DAVID bioinformatics software. The threshold for significant difference was set as $P \leq 0.05$ after adjustment.

Statistical analysis

SPSS 17.0 software was used for statistical analyses. Measurements obeying the normal distribution were reported as $\bar{x} \pm s$. The independent two-sample t-test was adopted for intergroup comparison. Counts were expressed as frequencies or percentages. miRNA differential expression was analyzed in the two groups using DEGseq R language, with $P<0.05$ indicating significant difference. *q* was defined as the probability of a differentially expressed miRNA being a false negative; it was a re-statistic amount of *P*. Differentially expressed miRNAs were divided into different groups by clustering analysis using the MeV4.9.0 software.

Results

Results of high-throughput sequencing

Small RNA libraries: Ten small RNA libraries were built by sequencing (**Table 1**), with 174 985 793 and 169 430 997 raw reads in NEC group and control group, respectively; there were 171 175 183 and 165 160 370 clean reads, respectively, having 18-22 nucleotides generally. These small RNA sequences were qualified for alignment against the sequences from the databases (**Figure 1**). Alignment results indicated that NEC group and control group had 110 855 366 and 108 092 570 miRNA sequences, accounting for 64.76% and 65.45% among total clean reads, respectively.

Differentially expressed miRNA: Ten miRNA libraries in **Table 1** were analyzed separately for

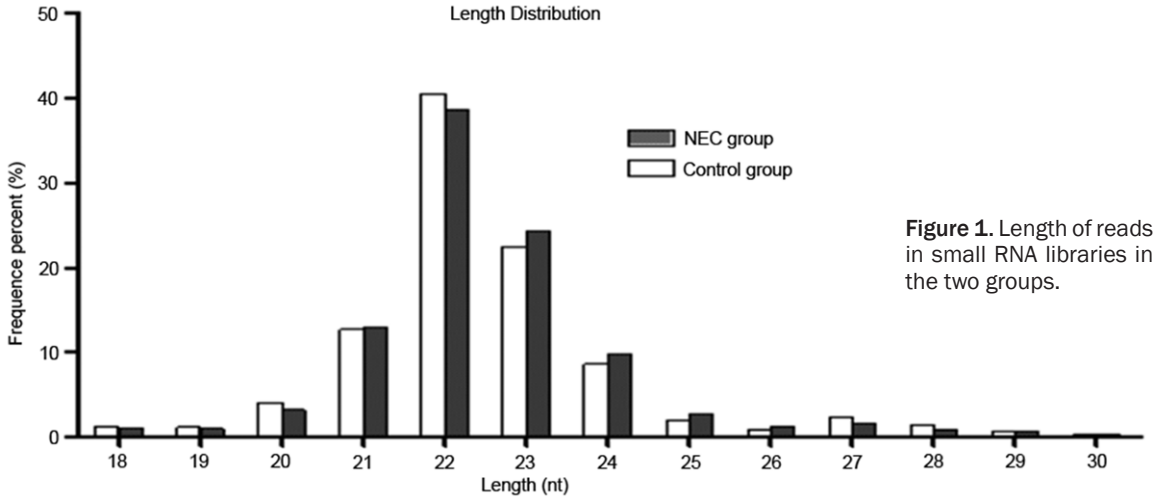

Figure 1. Length of reads in small RNA libraries in the two groups.

Table 2. Top 10 miRNAs showing upregulated or downregulated expression

miR_name	miRNA expression in NEC group	miRNA expression in control group	Standardized log_2 fold change	Expression change
hsa-miR-219a-2-3p	35	0	6.092020725	Upregulated
hsa-miR-124-3p	28	0	5.77009263	Upregulated
hsa-miR-3117-3p	33	1	5.007131827	Upregulated
hsa-miR-182-3p	53	2	4.690658162	Upregulated
hsa-miR-449b-5p	12	0	4.547700209	Upregulated
hsa-miR-4662a-3p	12	0	4.547700209	Upregulated
hsa-miR-6797-5p	11	0	4.422169327	Upregulated
hsa-miR-6762-3p	19	1	4.210665221	Upregulated
hsa-miR-6855-5p	14	1	3.77009263	Upregulated
hsa-miR-6895-3p	14	1	3.7700926	Upregulated
hsa-miR-6874-5p	15	1	-3.944152888	Downregulated
hsa-miR-6087	16	1	-4.037262292	Downregulated
hsa-miR-6851-5p	17	1	-4.124725133	Downregulated
hsa-miR-5581-3p	37	2	-4.246715658	Downregulated
hsa-miR-548ba	24	1	-4.622224793	Downregulated
hsa-miR-3142	13	0	-4.73770201	Downregulated
hsa-miR-5699-3p	13	0	-4.73770201	Downregulated
hsa-miR-5090	14	0	-4.844617214	Downregulated
hsa-miR-3941	17	0	-5.124725133	Downregulated
hsa-miR-3161	59	0	-6.919905341	Downregulated

NEC group and control group using DEGseq, and 428 differentially expressed miRNAs were identified (P<0.05). According to the screening criteria of *P*<0.01, q<0.001 and standardized $|Log_2$ (fold change) $| \geq 1$, 126 known miRNAs were differentially expressed, including 58 upregulated miRNAs and 68 downregulated miRNAs (**Table 2**). For these miRNAs clustering analysis was performed. The two groups differed significantly in color, but within either group, the color of each sample was similar, indicating good correlation and repeatability of the samples.

Verification by RT-qPCR

As compared with the control group, hsa-miR-183-3p, -223-5p and -222-5p were significantly upregulated in NEC group; hsa-miR-23b-5p, -150-5p, -146a-3p and -1298-5p were downregulated significantly. These 7 miRNAs were expressed stably and abundantly in each group with high fold change. For verification, RT-qPCR was performed for the 7 miRNAs and the amplification curves for the miRNAs and internal reference snRNA U6 all had an S-shape. Ct value from each amplification curve was below 35. All dissociation curves of miRNAs were unimodal, indicating no specific amplification. As compared with the control group, hsa-miR-183-3p, -223-5p and -222-5p were significantly upregulated in NEC group; hsa-miR-23b-5p, -150-5p, -146a-3p and -1298-5p were downregulated significantly (P<0.05) (**Table 3**). RT-qPCR results basically agreed

Table 3. Relative expression of miRNA ($\bar{x}\pm s$)

miRNA	miR-223-5p	miR-183-3p	miR-222-5p	miR-23b-5p	miR-150-5p	miR-146a-3p	miR-1298-5p
NEC group	7.254±0.243	5.186±0.592	3.636±0.157	0.743±0.095	0.444±0.326	0.296±0.219	0.247±0.202
Control group	0.955±0.834	1.219±0.850	1.024±0.408	1.227±0.516	1.132±0.587	1.066±0.823	1.394±0.549
t value	4.13	6.432	3.586	2.434	3.321	2.565	5.823
p value	0.001	<0.001	0.003	0.048	0.004	0.034	<0.001

Note: P<0.05 compared with the control group.

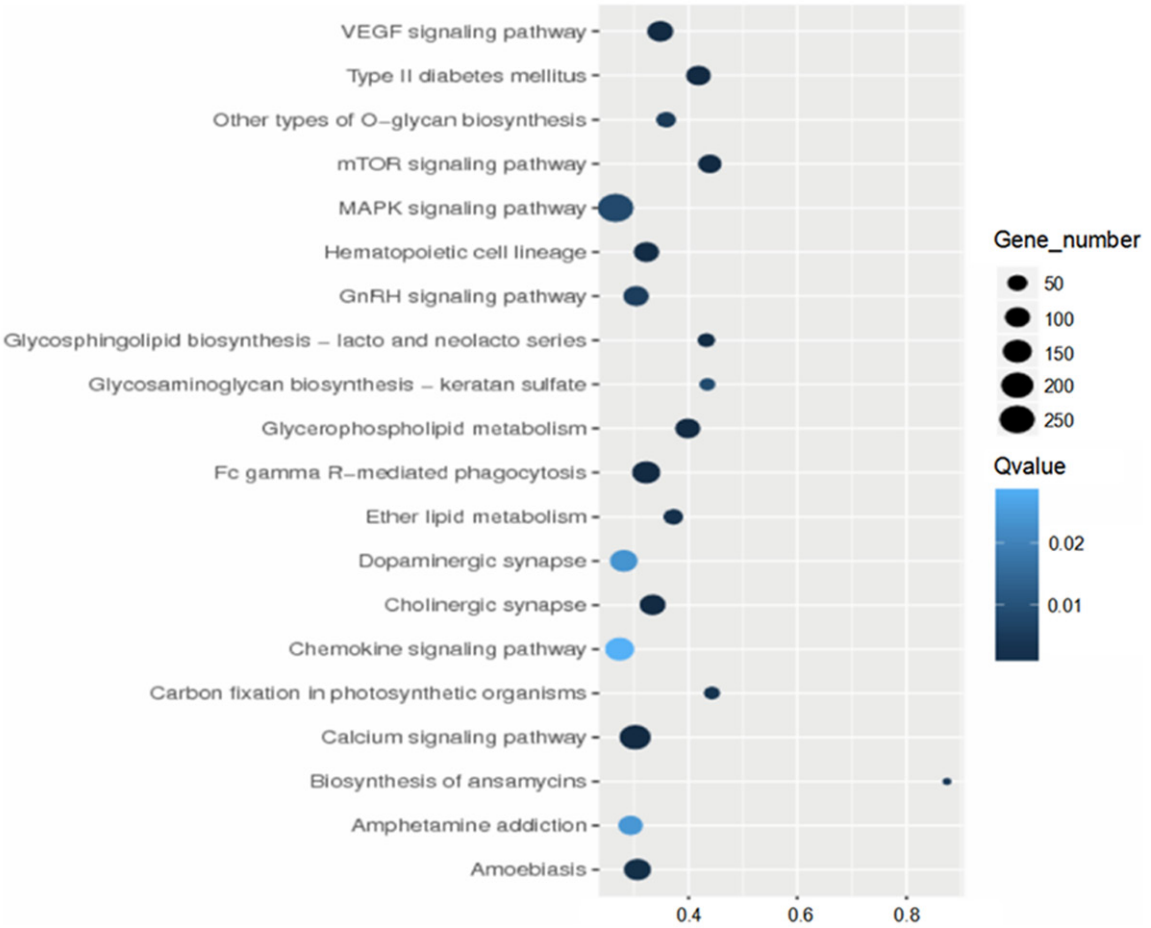

Figure 2. Top 20 pathways showing differential expression. Note: X axis represents the enrichment factor. The larger the dot, the higher the enrichment level and the more candidate genes exist in this pathway. The color representing each dot indicates different range of q value; the darker the color, the greater the difference is. Y axis represents the name of the pathways, which are identified as follows: VEGF, type II diabetes mellitus, other types of O-glycan biosynthesis, mTOR signaling pathway, MAPK signaling pathway, hematopoietic cell lineage, GnRH signaling pathway, glycosphingolipid biosynthesis-lacto and neolacto series, glycosaminoglycan biosynthesis-keratan sulfate, glycerolipid metabolism, Fc gamma R-mediated phagocytosis, ether lipid metabolism, dopaminergic synapse, cholinergic synapse, chemokine signaling pathway, carbon fixation in photosynthetic organisms, calcium signaling pathway, biosynthesis of ansamycins, amphetamine addiction and amoebiasis.

with the sequencing, indicating reliability of the sequencing.

Bioinformatics analysis

Target genes were predicted for 126 differentially expressed miRNAs, and only the intersections of the same target genes predicted by the same miRNAs were counted. Thus 6344 potential target genes were predicted. GO enrichment analysis was performed and 78 significant GO terms were identified. The target genes of these differentially expressed miRNAs were mainly found in the cell membranes, protein

channels and receptor complexes. The main biological processes identified by GO enrichment analysis included immune response, chemotaxis-related regulation, cell junction, T-cell activation, regulation of receptor-mediated endocytosis, positive stress-activated protein kinase cascade, formation of epithelial cells, multi-process regulation and positive regulation of histone acetylation. As to molecular function, the gene sets are mainly involved in laminin binding, vascular endothelial growth factor receptor binding and protein kinase binding. Enrichment analysis of KEGG pathway indicated that the target genes of the differentially expressed miRNAs were involved in 61 pathways. They were typically mTOR signaling pathway, Toll-like receptor signaling pathway, VEGF signaling pathway, NF-κB signaling pathway, NOD-like receptor pathway, FcγR-mediated phagocytosis, MAPK signaling pathway, B-cell signaling pathway, T-cell receptor signaling pathway and JAK-STAT signaling pathway ($P<0.05$) (**Figure 2**).

Discussion

NEC, a common enteric disease for newborns, especially premature babies, has high incidence and mortality. Many factors are believed to play a role in NEC, including premature delivery, pathogen colonization and hypoxic and ischemic injury of the intestinal tract. These risk factors are associated with the inflammatory cascade, leading to intestinal inflammation, hemorrhagic necrosis and apoptosis of intestinal epithelial cells and finally resulting in NEC [8]. miRNAs are important components in many physiopathological processes, including development, cell migration, proliferation, necrosis and apoptosis. They are also involved in intestinal inflammation and immune response via several pathways. For example, in inflammatory bowel disease, miR-132 maintains inflammation-dependent homeostasis by regulating the cholinergic signaling pathway [9]. miR-124 targeting *STAT3* regulates intestinal injury in ulcerative colitis [10]. However, the correlation between miRNA correlation and NEC is not fully understood yet. We applied high-throughput sequencing to miRNA expression profile. With the criteria of $P<0.01$, $q<0.001$ and standardized $|\text{Log}_2 \text{ fold change}| \geq 1$, 126 miRNAs were expressed differentially, including 58 upregulated miRNAs and 68 downregulated miRNAs. For verification, 7 miRNAs showing high and stable expression and high fold change in each group were subjected to RT-qPCR. Bioinformatics analysis was conducted for 126 differentially expressed miRNAs.

miR-146a-3p, -23b-5p, -150-5p and -1298-5p were significantly downregulated in NEC group and all of them regulated intestinal inflammation by acting on the target genes. Target gene prediction of miR-146a showed that the target genes of miR-146a-3p were intedeukin-1 receptor-associated kinase-1 (IRAK1) and signal transducer and activator of transcription-1 (STAT1). These target genes mediated intestinal inflammation and immune regulation through Toll-like receptor signaling pathway and NF-κB signaling pathway. Study shows that miR-146a plays a key regulatory role in intestinal mucosal barrier and immune function. Acting as an anti-inflammatory factor, miR-146a is associated with abnormal regulation of all inflammation and immunity-related signaling pathways by inhibiting the genes related to intestinal barrier and inflammation [11]. miR-146a can mitigate inflammation and immune response related to TLR2 and MyD88-NF-κB signaling pathway through the negative feedback regulation of tumor necrosis factor receptor-associated factor 6 (*TRAF6*) and IRAK1, which causes the generation of interleukin-6 (IL-6) and tumor necrosis factor (TNF) in the downstream of the NF-κB signaling pathway [12]. For the newborns, miR-146a mediates IRAK1 downregulation, by which the intestinal epithelial injury caused by bacteria can be prevented [13]. miR-146a is involved in the regulation of intestinal immune cells by targeted inhibition of STAT1 [11]. Based on the results obtained in this study, miR-146a, the anti-inflammatory factor, was significantly downregulated and it finally led to intestinal inflammation and abnormal immune response by targeting TRAF6, IRAK1 and STAT1. However, the specific regulation process needs to be verified experimentally. Other miRNAs were also expressed differentially in NEC, but the details are still unknown. However, some inspirations can be drawn from other intestinal diseases such as inflammatory bowel disease. miR-23b is involved in intestinal inflammation by regulating Marcksl-1 [14]. miR-150 induces inflammatory change of intestinal epithelial cells through the target gene c-myb [15]. Moreover, miR-150-5p plays a part in the generation and regulation of intestinal immune cells and also in inflamma-

tory changes of the bowels [16]. The present study indicated that miR-1298-3p was significantly downregulated in newborns with NEC, but the regulatory mechanism requires further understanding.

Moreover, miR-223-5p, -34a, -222-3p and -183-3p were upregulated significantly in NEC. Bioinformatics analysis indicated that the target genes of miR-223-5p were claudin-8 (CLDN8), forkhead box O 3a (FOXO3a), nucleotide-binding and oligomerization domain-like receptor 8 (NLRP8) and intercellular cell adhesion molecule-1 (ICAM1). These target genes are part of the intestinal barrier and involved in intestinal inflammation and immune regulation through the PPAR, NF-κB and MAPK signaling pathways. CLDN8 is an important component of the intestinal barrier and miR-223 as the pro-inflammatory factor is upregulated significantly in intestinal inflammation. miR-223 inhibits the expression of target gene CLDN8 and activates IL23/Th17 pathway, causing damage to the intestinal barrier and leading to intestinal inflammation and immune response [17]. In intestinal tissues from rats with inflammatory bowel disease, upregulated miR-223 inhibits the expression of inhibitors of nuclear transcription factor kappa B-α (IκBα), thus activating NF-κB and NF-κB signaling pathway [18]. miR-34a is associated with the apoptosis of intestinal epithelial cells. In intestinal ischemia/reperfusion injury, upregulated miR-34a-5p can cause active oxygen species accumulation and intestinal cell apoptosis by inhibiting the target gene STRT1 [19]. Combining with target gene prediction, the target genes of miR-34a-5p include STRT1 in newborns with NEC. Therefore, miR-34a-5p regulates apoptosis of intestinal epithelial cells and intestinal inflammation by targeting STRT1, but the specific mechanism is unknown. miR-222-3p induces the inflammation and migration of endothelial cells through angiotensin converting enzyme-2 (ACE2), thus causing the inflammation [20]. Besides, miR-222 mediates the Toll-like receptor signaling pathway and regulates intestinal inflammation [21]. According to our results, target genes of miR-183-3p, namely, FOXO4 and PTS1, are regulatory of peroxisome signaling pathway. These target genes, along with the signaling pathway, play an important mediatory role in inflammation, cell differentiation, proliferation and apoptosis. From this we can infer that miR-183-3p may be involved in the regulation of intestinal cell apoptosis and necrosis in newborns with NEC. In brief, the above-mentioned miRNAs can regulate the necrosis and apoptosis of intestinal epithelial cells, intestinal barrier, intestinal immunity and activation of intestinal inflammatory cells through the related target genes. This represents an important mechanism of the pathogenesis of NEC.

Bioinformatics analysis over 126 differentially expressed miRNAs indicates that the enriched GO terms were immune response, chemotaxis-related regulation, cell junction, T-cell activation, regulation of receptor-mediated endocytosis, positive stress-activated protein kinase cascade and formation of epithelial cells. These differentially expressed miRNAs may be involved in the regulation of intestinal barrier, intestinal immunity and inflammation in newborns with NEC. Enrichment analysis of KEGG pathway indicated that the target genes of the differentially expressed miRNAs were involved in mTOR signaling pathway, Toll-like receptor signaling pathway, VEGF signaling pathway, NF-κB signaling pathway, NOD-like receptor pathway, FcγR-mediated phagocytosis, MAPK signaling pathway, B-cell signaling pathway, T-cell receptor signaling pathway and JAK-STAT signaling pathway. All these pathways are related to intestinal immunity, inflammation, cell proliferation and apoptosis. Particularly, abnormal activation of Toll-like receptor signaling pathway is deemed as an important pathogenesis of NEC [22]. According to relevant studies, MAPK signaling pathway mediates the injury and apoptosis of intestinal epithelial cells in newborns with NEC [23]. But the specific miRNAs and their target genes involved in this process are unknown and investigation into the working mechanism of the miRNAs is needed.

This study applied high-throughput sequencing to miRNA differential expression in peripheral blood samples collected from newborns with NEC. Bioinformatics analysis was conducted on the sequencing results in order to understand the role of miRNA in NEC. In spite of some discoveries of the target genes along with their function related to NEC, we are still uncertain as to the relationship between differentially expressed miRNAs and NEC. Is differential expression of miRNAs the cause or effect of NEC? And how are the target genes of the differentially expressed miRNAs regulated?

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Disclosure of conflict of interest

None.

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