Research Article

Profiling and bioinformatics analyses reveal differential circular RNA expression in NK/T-cell lymphoma-associated hemophagocytic syndrome

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Abstract

Circular RNAs (circRNAs) may be potential biomarkers or therapeutic targets of hemophagocytic syndrome (HPS) due to their high stability, covalently closed structure and implicated roles in gene regulation. The aim of this study was to determine and characterize the circRNAs from NK/T-cell lymphoma-associated hemophagocytic syndrome (NK/T-LAHS). CircRNA in NK/T-LAHS and healthy control patient serum were assessed using next-generation sequencing. 143 differentially expressed circRNAs of which 114 were up-regulated and 29 were down-regulated in NK/T-LAHS patients were identified. Next, GO (Gene Ontology) function and KEGG (Kyoto encyclopedia of genes and genomes) pathway analyses to explore the roles of these circRNAs were utilized, and a miRNA target gene prediction software to predict the interaction of circRNAs and miRNAs was used. Moreover, 5 circRNAs were then selected as NK/T-LAHS candidate circRNAs which were related to tumors and contained NK/T-LAHS-related miRNA binding sites. Using real-time PCR, the significant upregulation of these 5 circRNAs in NK/T-LAHS patient serum were verified. Together these results show that circRNAs may serve as valuable diagnostic biomarkers of early NK/T-LAHS, with potential therapeutic targets in disease progression.

Keywords: NK/T-LAHS, Circular RNA, Second-generation sequencing, Bioinformatics analysis, Biomarker

Introduction

Hemophagocytic lymphohistiocytosis (HLH), also called hemophagocytic syndrome (HPS), is a potentially life-threatening disease characterized by impaired natural killer (NK) and cytotoxic T cell function, cytokine storm and overwhelming inflammation[1, 2]. Recent studies have demonstrated that hyperbilirubinemia, viral infection, NK/T lymphoma, ferritin, serum β2-microglobulin, and serum fibrinogen predict the survival outcomes of sHLH patients [3-6]. However, the prognostic accuracy of these
clinical parameters has not been fully verified in patients with sHLH. Novel prognostic biomarkers mirroring different pathophysiologica|mechanisms need to be identified for individualized risk assessment.

Circular RNA (circRNA) is a novel molecule that is formed by a covalently closed loop with different sizes and sources and represents a class of abundant, stable and widely existing RNA molecules in animals [7, 8]. It plays an important role in biological processes. For example, circRNAs act as miRNA sponges [9], RNA-binding proteins, and mRNA "magnets" to guide protein translation [10] and serve as biomarkers for the diagnosis and prognosis of some diseases [11, 12]. However, the role of circRNAs in NK/T-cell lymphoma-associated hemophagocytic syndrome has not been defined.

Recently, the application of high-throughput RNA sequencing and bioinformatics approaches has revealed a large number of circRNAs in human cells. Emerging evidence indicates that many circRNAs have cell type-specific expression and are linked to physiological development and various diseases [13]. Previous studies have reported that circRNAs are involved in the regulation of the pathogenesis of a number of diseases, such as cancer, cardiovascular diseases, and autoimmune diseases [14-17]. One function of circRNAs is to act as a sponge for miRNAs, and circRNAs may serve as endogenous competing RNAs and affect gene expression by binding to and preventing miRNAs from regulating their downstream target genes[18]. Many circRNAs are differentially expressed between cancers and normal tissues, suggesting that these circRNAs might have potential functions and clinical relevance in cancer [19-21]. These findings suggest that circRNAs play a significant role in the pathogenesis of human diseases and may serve as potential biomarkers for disease diagnosis. However, little is known about the expression profiles and functions of circRNAs in NK/T-LAHS.

In the present study, we identified a group of circRNAs with differentia expression using next-generation sequencing technology and explored the roles of these circRNAs by a miRNA target gene prediction software. With real-time PCR, 5
selected NK/T-LAHS candidate circRNAs were verified for the significant upregulation.

Materials and Methods

Study subjects

Serum samples (200 µl per person) from 4 NK/T-LAHS patients who had not received cancer therapy and 4 healthy controls were obtained during 2018-2019 at the Department of Geriatrics Hematology, the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital. The diagnosis of HLH was established based on the 2004 HLH Diagnostic Criteria [22]. At least five of the following criteria had to be satisfied: (1) persistent fever (temperature >38.5°C for more than one week), (2) splenomegaly, (3) cytopenia referring to at least two of the three lineages: hemoglobin <90 g/l, platelets <100×10⁹/l, and neutrophils <1.0×10⁹/l, (4) hypofibrinogenemia (fibrinogen <1.5 g/L) and/or hypertriglyceridemia (≥3.0 mmol/L), (5) hyperferritinemia (serum ferritin ≥500 µg/l), (6) high levels of soluble IL-2R (≥2400 U/ml), (7) low or absent natural killer (NK) cell activity, and (8) hemophagocytosis in the bone marrow, spleen, or lymph nodes. The diagnosis of NK/T cell lymphoma met pathological criteria through biopsy samples according to the 2016 revision of the World Health Organization classification of lymphoid neoplasms [23]. The sera from the 8 blood samples were separated immediately and stored at -80°C until RNA extraction. Second-generation sequencing technology and real-time PCR were then used for circRNA screening and identification. Our study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (Reference Number: 2019-SR-446), and informed consent was obtained from all participants to review their medical records and publish clinical data.

RNA isolation, RNA-Seq library preparation, and sequencing

Total RNA was extracted from the 8 serum samples with TRIzol (Life Technologies, Carlsbad, CA, USA). The NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the concentration of each RNA sample.
All RNA samples involved in this research met standards of quality control based on a qualifying ratio of OD 260 to OD 280 (1.8-2.1). RNA integrity and gDNA contamination were measured by modified agarose gel electrophoresis. The quality of the test library was determined with an Agilent 2100 Bioanalyzer.

RNA high-throughput sequencing was performed by Cloud-Seq Biotech (Shanghai, China). Briefly, total RNA was used to remove the rRNAs with the NEBNext, rRNA Depletion Kit (New England Biolabs, Inc., Massachusetts, USA) following the manufacturer's instructions. RNA libraries were constructed by using the NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England Biolabs, Inc., Massachusetts, USA) according to the manufacturer's instructions. Libraries were quality controlled and quantified using the BioAnalyzer 2100 system (Agilent Technologies, Inc., USA). Library sequencing was performed on an Illumina HiSeq instrument with 150 bp paired end reads. The 10 pM library was transformed into single-stranded DNA molecules, which was captured on an Illumina flowcell (Illumina, USA), amplified into clusters in situ, and sequenced in 150 cycles with PE mode on an Illumina HiSeq (Illumina HiSeq 4000, USA) sequencer.

**CircRNA profiling analysis**

Paired-end reads were obtained from the Illumina HiSeq 4000 sequencer data and were quality controlled by Q30. Then, 3’ adaptor trimming and removal of low-quality reads were performed by cutadapt software (v1.9.3) [24]. The high-quality trimmed reads were used to analyze circRNAs. The high-quality reads were aligned to the reference genome/transcriptome with STAR software (v2.5.1b) [25], circRNAs were detected and identified with DCC software (v0.4.4) [26], and the identified circRNA were annotated with the circBase database and Circ2Traits [27, 28]. EdgeR software (v3.16.5) was used to normalize the data and perform differentially expressed circRNA analysis [29]. The circRNAs that had a fold change value≥2.0 and a p values ≤0.05 were designated as being significantly differentially expressed. GO and KEGG analyses were performed for the differentially expressed circRNA-associated genes. The interaction network between circRNAs and their downstream miRNAs was constructed by Cytoscape software (v2.8.0) based on data
of circRNAs with specific miRNA binding sites and their predicted miRNA partners.

**Quantitative real-time polymerase chain reaction analysis**

To confirm that the data generated through sequencing efforts were reliable, qPCR validation of specific circRNAs was performed. Total RNA obtained from serum with TRIzol (Invitrogen, Carlsbad, CA USA) was used for synthesizing cDNA with the ReverTraAc real-time qPCR kit (Toyobo, Osaka, Japan). First-strand cDNA was used for PCR, which was performed in triplicate with SYBR Green Super Mix (Bio-Rad, Hercules, CA, USA). The specific primers sequences for the 5 upregulated circRNAs used for RT-qPCR are summarized in Table 1.

All reactions were conducted according to the following procedures, 40 PCR cycles (95 °C, 10 seconds; 60 °C, 60 seconds (measure fluorescence)). The fusion curve of PCR products was established as follows: 95 °C, 10 seconds; 60 °C, 60 seconds; and 95 °C, 15 seconds after the amplification reaction, followed by slowly heating from 60 °C to 99 °C (the ramp up rate of the instrument was 0.05 °C/second). Each sample was assayed three times.

**Bioinformatics analysis and target prediction**

The TargetScan and miRanda databases were used to conduct the analysis and predict interactions between circRNAs and miRNAs in which they may act as sponges. Both the Gene Ontology (GO) (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) databases were also used to assess the function of genes associated with detected differentially expressed circRNAs. The interactions between circRNAs and miRNAs predicted by customized Arraystar software based on TargetScan and miRanda together with the top 5 correlated miRNAs are displayed in the form of a network graph produced by Cytoscape software [30-32].

2.6. **Statistical Analysis.** All data are means ± standard deviation (SD) based on triplicate experiments. Student’s t-tests were used to compare circRNA levels between groups, with $P < 0.05$ as the threshold of significance.

**Results**
Profiling of circRNAs in NK/T-LAHS patient serum

We utilized an Illumina HiSeq instrument to profile circRNA expression in 4 NK/T-LAHS serum samples and 4 healthy control serum samples (Table 2). In the 4 NK/T-LAHS samples, the total reads were 85,833,954, 88,495,030,79,053,298, and 86,398,392, yielding mapped read counts of 61,953,110, 65,427,958, 58,369,924 and 63,262,850, respectively. For the healthy controls, the read counts were 83,389,774, 83,205,550, 86,771,462 and 82,877,972, yielding mapped read counts of 47,656,180, 44,744,062, 50,073,200 and 46,639,532, respectively (Here to remove).

In total, we were able to identify 11,481 different putative circRNAs in these serum samples, of which 2698 were consistent with circRNAs in circBase (circBase; http://www.circbase.org) and 42 were documented in the literature. The remaining 8,741 are novel circRNAs first identified in this document (Figure 1(a)). Using the RefSeq database, we annotated the identified circRNAs and found that 2,990 contained protein-coding exons, while 4,288, 2,110, 1,126 and 967 circRNAs aligned with introns, antisense, intergenic and sense overlapping regions of known transcripts, respectively (Figure 1(b)). The length of the majority of identified exonic circRNAs (2,990 total) was under 1500 nucleotides (nt), with the majority being 200-1000 nt (Figure 1(c)). The chromosome distributions of circRNAs are shown in Figure 1(d), with the majority being concentrated on chromosomes 1 and 2.

Differentially expressed circRNAs in NK/T-LAHS serum

The differential circRNAs between the NK/T-LAHS and normal control groups were determined through fold change and p values (fold change ≥2.0; p values ≤0.05) statistical criteria. Finally, of the identified circRNAs, 143 were specifically and significantly differentially expressed in NK/T-LAHS samples. In contrast to those in the normal control group, a total of 114 circRNAs were markedly upregulated, and 29 were significantly downregulated in the NK/T-LAHS group, as shown by a cluster heatmap (Figure 2(a)). The most significantly upregulated and downregulated circRNAs were chr22:20981567-20981750+ (fold change 219.967531 up; p values < 0.05) and hsa_circ_0123217 (fold change 369.5452384 down; p values < 0.05).
respectively.

Differentially expressed circRNAs between the NK/T-LAHS group and normal controls are displayed by scatter plots (Figure 2(b)) and volcano plots (Figure 2(c)). Vertical green lines indicate 2-fold (log₂ scaled) up- or downregulation, and the horizontal green line indicates p values of 0.05 (-log₁₀ scaled). Red squares represent circRNAs with statistically significant differences in expression.

**Functional analysis of target genes**

The Gene Ontology project provides a controlled vocabulary to describe gene and gene product attributes in any organism (http://www.geneontology.org). The ontology covers three domains: biological process (BP), cellular component (CC) and molecular function (MF). The top 10 GO terms were identified. “Positive regulation of pseudopodium assembly,” “regulation of pseudopodium assembly,” and “pseudopodium assembly” were the top three BPs (Figure 3(a)); “beta-catenin destruction complex,” “complex of collagen trimers,” and “clathrin coat of trans-Golgi network vesicle” were the top three CCs (Figure 3(b)); and “receptor antagonist activity,” “GTP-Rho binding,” and “hydrolase activity, acting on ether bonds” were the top three MFs (Figure 3(c)).

When we performed a KEGG pathway analysis, a total of 21 pathways were enriched in these circRNAs. The top 10 pathways were identified (Figure 4(a)). Insulin signaling pathway(Figure 4(b)), endometrial cancer(Figure 4(c)), Hippo signaling (Figure 4(d)) pathways regulated by the upregulated circRNAs in NK/T-LAHS were the top three signaling pathways. These top three signaling pathways were connected with expression of GSK-3β, which played a crucial role in pathogenesis of NK/T-LAHS. These pathway analyses indicated that upregulated circRNAs in the serum of NK/T-LAHS patients may have substantial implications for this disease.

**circRNA-targeted miRNA-gene network generation**

Of the 143 differentially expressed circRNAs, we selected 5 circRNAs for further investigation. They are chr5:112090570-112137080+ (hsa_circ_0073586), chr1:77620134-77635080- (hsa_circ_0114150), chr12:62715245-62749256+
(hsa_circ_0027353), chr20:34304662-34320057- (hsa_circ_0115056), chr6:56989532-57025950+ (hsa_circ_0131996). These 5 circRNAs had increased differential expression in NK/T-LAHS compared with normal samples. Moreover, these 5 circRNAs all contained target sites for miRNAs associated with NK/T-LAHS. We therefore inferred that these 5 circRNAs might be closely related to NK/T-LAHS. As circRNAs can have many miRNA target sites, only the top 5 sites for each circRNA are compiled in Table 3. The graph shows that circRNAs interacted with miRNAs in numerous way (Figure 5).

**Verification of selected circRNAs**

The serum samples from 10 NK/T-LAHS patients and 10 healthy volunteers were used for verification by real-time qPCR. We focused our attention on upregulated circRNAs with certain circBase IDs and selected five of these circRNAs, with copy numbers that were significantly higher and were evenly distributed in each sample of the NK/T-LAHS group, for validation of the NGS data. As expected, circRNAs were upregulated in the serum of NK/T-LAHS patients, further supporting the notion that these circRNAs have potential as viable biomarkers of this disease (Figure 6).

**Discussion**

Hematological malignancies, for example, non-Hodgkin’s lymphoma, are some of the most common risk factors for HLH. Most of them have poor outcomes, with the outcome of patients with LAHS being the poorest. Moreover, in LAHS patients, the NK/T cell lymphoma hemophagocytic lymphohistiocytosis (NK/T-LAHS) subtype is one of the most common pathologies, accounting for up to 35% of the LAHS patient population. Early diagnosis of the underlying conditions, especially NK/T-LAHS, may lead to better outcomes [33, 34]. At present, there are no biomarkers of NK/T-LAHS sensitive enough for diagnostic purposes. Therefore, it is necessary to identify new biomarkers and explore their functions. In this study, a new useful marker for the early diagnosis of LAHS was identified in the search for biomarkers associated with disease progression capable of sensitively and effectively diagnosing early NK/T-LAHS, which should be confirmed in further investigations.
CircRNAs are novel endogenous noncoding RNAs with a special circular structure that have been found to play critical roles in development and diseases. Recent studies have revealed that circRNAs are involved in gene regulation during the transcriptional and posttranscriptional stages [35]. CircRNAs can interact with RNA-binding proteins to regulate the expression of targeted genes [9]. These circRNAs can perform unique functions; they can be detected at high levels in exosomes, and as such, they can be a good target for cancer biomarker diagnostics [36].

Many studies have proven that circRNAs are potential biomarkers and participate in the pathogenesis of various diseases [14, 37-39]. Some research results have shown that circRNAs are moderately effective assistant diagnostic biomarkers for lymphoma. However, to date, few studies have assessed these molecules as biomarkers for NK/T-LAHS. Mei M et al. found that circCDYL was highly expressed in the plasma of MCL (mantle cell lymphoma) patients and that circCDYL knockdown inhibited MCL cell proliferation. These findings indicate that circCDYL might serve as a potential diagnostic biomarker in clinical practice [40]. Hu Y et al. analyzed the involvement of circRNAs in diffuse large B-cell lymphoma (DLBCL) and found that circ-APC (hsa_circ_0127621) was downregulated in DLBCL tissues, cell lines and plasma, suggesting that circ-APC is a novel proliferation inhibitor and that restoring circ-APC expression may be a promising therapeutic approach for DLBCL patients [41]. Deng L et al. showed that circ-LAMP1 was overexpressed in T-LBL (T-cell lymphoblastic lymphoma) tissues and cell lines and modulated cell growth and apoptosis by regulating the miR-615-5p/DDR2 pathway in T-LBL. These findings demonstrated that circ-LAMP1 might be an oncogene in T-LBL, which might be useful in developing promising therapies for T-LBL [42]. Dahl M et al. provided a map of circRNA expression in B-cell malignancies and presented an enzyme-free digital counting methodology, which has the potential to become a new gold standard for circRNA quantification [43]. However, the functional mechanism of circRNAs in NK/T-LAHS is poorly understood.

Therefore, it is significant to profile the circRNA expression and find new biomarkers, which may help to provide new directions and strategies for the diagnosis and
treatment of diseases, and is reasonable to hypothesize that there might be certain circRNAs expression styles which were characteristic of NK/T-LAHS. In this study, we compared circRNA expression patterns between NK/T-LAHS and healthy control serum samples using second-generation circRNA sequencing. Through our sequencing efforts we were able to identify a vast large number of special circRNAs, including many that are not current in available circRNA databases. Furthermore, to exploring this full content of circRNAs, we described their lengths and chromosomal distribution models in the serum of NK/T-LAHS patients. Progressively, we identified 143 circRNAs differentially expressed between NK/T-LAHS and control serum samples, with the number majority of these being upregulated in NK/T-LAHS patients, suggesting a possible together with NK/T-LAHS progression. Others have also obtained similar views to ours, such as Huang et al., who found that significant circRNA up-regulation was detected in bladder urothelial carcinoma and renal clear cell carcinoma[44]; and Wang et al., who showed that 178 differentially expressed circRNAs were detected in the serum of patients with ovarian cancer, of which 175 were up-regulated and only 3 were down-regulated [15]. However, there are also opposite results. For example, Zheng et al found that significant circRNA downregulation was detected in colorectal and gastric cancers[45]. Although the specific role of these circRNAs in cancer remains uncertain, we believe that their altered expression is widespread in the context of disease. While the specific role of these circRNAs in NK/T-LAHS remains uncertain, we believe their altered expression to be wide-ranging in the context of disease. We performed a GO analysis that compiles broad sets of information about gene function based on a series of defined terms [46]. The ontology covers three domains: biological process (BP), cellular component (CC) and molecular function (MF) (Here to remove). GO function analysis of the host genes of differentially expressed circular RNAs was performed to annotate and predict the function of these circular RNAs. KEGG pathway analysis is the process of mapping molecular data sets in genomics, transcriptomics, proteomics, and metabolomics to KEGG pathway maps to explain the biological functions of these molecules. By analyzing the pathways of differentially expressed
genes derived from circular RNAs, we can infer the pathways in which they participate and their biological functions. (Here to remove) Through KEGG pathway analysis, we determined that these circRNAs were enriched in pathways including insulin signaling, endometrial cancer, Hippo signaling, basal cell carcinoma, colorectal cancer, PI3K-Akt signaling, prolactin signaling, focal adhesion, ECM-receptor interaction, and regulation of actin cytoskeleton. These pathway analysis methods together emphasize the broad regulatory role that circRNAs can play in the context of some diseases, and circRNAs may affect a variety of cellular functions by changing their regulation. The majority of Akt substrates are involved in the regulation of cell survival and cell cycle progression, underlying the central role of the PI3K/AKT pathway in promoting the progression of human malignancies. Several reports exist on the implication of AKT signaling in tumourigenesis and cancer progression[47, 48]. The relationship between dysregulation of PI3K activity and abnormal proliferation has been well documented. PI3K activity is associated with a range of human tumors, such as breast cancer, lung cancer, melanoma and leukemia. In addition, evidence suggests that AKT is a downstream enzyme of PI3K and is also associated with malignant transformation[49]. However, there is no study on PI3K/AKT signaling pathway in NK/T-LAHS. Our study shows that up-regulated circRNA participates in this important signaling pathway, and we still need to further explore how the signaling pathway works and whether it can become a new therapeutic target.

In addition, substantial evidence has suggested that circRNAs can play many significant roles, such as acting as competitive endogenous RNAs to sponge microRNAs (miRNAs) efficiently, attenuating or compromising the inhibitory effect of miRNAs on target genes, and modulating protein synthesis accordingly [9, 35, 50]. CircRNAs can decrease or eliminate the inhibition of miRNAs on target genes and regulate protein synthesis correspondingly [51]. There has been little research on how circRNAs and miRNAs interact in the context of NK/T-LAHS, so we constructed a circRNA-miRNA network for NK/T-LAHS using our sequencing results. Based on miRNA binding site predictions, we selected 5 significantly upregulated circRNAs (chr5:112090570-112137080+ (hsa_circ_0073586), chr1:77620134-77635080-
(hsa_circ_0114150), chr12:62715245-62749256+ (hsa_circ_0027353), chr20:34304662-34320057- (hsa_circ_0115056) and chr6:56989532-57025950+ (hsa_circ_0131996)) that may be closely related to NK/T-LAHS. We further confirmed that these circRNAs are upregulated in the serum of NK/T-LAHS patients by qPCR, indicating that these 5 circRNAs may be potential biomarkers for NK/T-LAHS. All these circRNAs have multiple binding sites for NK/T-LAHS-related miRNAs. For example, hsa-circ_0131996 has a miR-4268 binding site, miR-6514-3p binding site, miR-146a-3p binding site, miR-6741-5p binding site and miR-1273d binding site. Previous studies have reported that miR-142-3p, miR-451, miR-144, miR-143-3p, miR-106b, and miR-101 are upregulated in HLH and may be associated with the immune/inflammatory response in patients with HLH [52]. Li W et al. found that the mean level of miR-133 was significantly higher in LAHS than in benign disease-associated HLH, and serum miR-133 is a new very useful marker for diagnosing LAHS [53]. Bay A et al. evaluated the plasma microRNA expression levels in secondary hemophagocytic lymphohistiocytosis and showed that miR-205-5p and miR-194-5p were upregulated and miR-30c-5p was downregulated in HLH, which could be useful plasma biomarkers for HLH [54]. We thus predict that hsa_circ_0131996 is a miRNA sponge and that by binding to many different miRNAs at once, it is able to prevent them from in turn binding to their target genes. This allows such circRNAs to indirectly regulate target genes of the miRNAs to which they are able to bind. Using a circRNA-miRNA network, we were able to further understand the role of circRNAs in NK/T-LAHS, demonstrating the potential value of exploring these complex regulatory networks to gain specific insights into disease progression.

Our results thus highlight a marked circRNA dysregulation in NK/T-LAHS, with the majority of detectable circRNAs being substantially overexpressed in the serum of these patients. These novel disease-associated circRNAs have the potential to both offer new insights into various diseases and to serve as novel biomarkers useful for NK/T-LAHS patient diagnosis.
This study had several limitations. The human NK/T-LAHS samples of the present study were only from 4 patients, and the sample size was too small. Furthermore, identification of differentially expressed circRNAs in subjects with NK/T-LAHS based on RNA-seq and verification of the circRNA profile by qPCR need to be conducted in another independent large-size sample cohort. Additionally, because functional validation assays were not included in the present study, the specific mechanisms by which circRNAs may be involved in NK/T-LAHS development were not speculated on. Finally, the role of circRNA in the pathogenesis of the disease has not been explored in this experiment. In the future, the peripheral blood monocytes of patients can be collected to detect the expression of circRNA in the cells, the target genes of circRNA can be predicted by bioinformatics techniques, and the predicted target genes can be verified by double luciferase report for signal pathway analysis.

In summary, we identified 11,481 circular RNAs in human NK/T-LAHS samples. Based on comparisons with current databases, 76% of circRNAs were identified as novel. Further analysis showed that 114 up- and 29 downregulated circRNAs were distinctly associated with NK/T-LAHS disease. The results suggested that the 143 identified circRNAs might serve as valuable diagnostic biomarkers of early NK/T-LAHS.

Competing Interests
The author(s) declare(s) that they have no conflicts of interest associated with the manuscript.

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Authors Contribution
C.F.M., Y.F., and G.L.Y. contributed equally to this study. C.F.M. and Y.F. designed the experiment. C.F.M., G.L.Y., J.Y.H., J.J.W. organized the clinical materials and
performed the data analysis. CFM wrote the paper. HXQ revised the manuscript. All authors contributed to the final approval of the manuscript.

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Ethics approval and consent to participate

All procedures in studies were performed in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The methods were performed in accordance with the approved guidelines, and all experimental protocols were approved by the ethics committee of the Nanjing Medical University and the First Affiliated Hospital of Nanjing Medical University (Reference Number: 2019-SR-446).

Abbreviations

1. Circular RNAs, circRNAs
2. HLH, hemophagocytic lymphohistiocytosis
3. HPS, hemophagocytic syndrome
4. sHLH, secondary hemophagocytic lymphohistiocytosis
5. LAHS, lymphoma-associated hemophagocytic syndrome
6. NK/T-LAHS, NK/T-cell lymphoma-associated hemophagocytic syndrome
7. GO, Gene Ontology
8. BP, biological process
9. CC, cellular component
10. MF, molecular function
11. KEGG, Kyoto encyclopedia of genes and genomes
12. miRNA, microRNA
13. NK, natural killer
14. RNA-seq, RNA-sequencing
15. NGS, next-generation sequencing
16. qRT-PCR, quantitative real-time polymerase chain reaction
17. SD, standard deviation
18. MCL, mantle cell lymphoma
19. DLBCL, diffuse large B-cell lymphoma
20. T-LBL, T-cell lymphoblastic lymphoma

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Table 1  The 5 up-regulated circRNAs specific primer sequences for RT-qPCR. F: Forward; R: Reverse

| CircRNA ID | Sequence (5’ to3’) |
|------------|--------------------|
| chr5:112090570-112137080+ | F: AGCGGCAGAATGAAGGTCAA  
|                      | R: GCCATCCTTGGAAGGTTTGG |
| chr1:77620134-77635080- | F: GGAAGTGTCGAAAAGTGGA  
|                      | R: GGCCATATCATCTGCAAGCATT |
| chr12:62715245-62749256+ | F: GCCTCTCTGCGCTGAAGTAAC  
|                       | R: CTGGTCCTTCCTGATCTGGA |
| chr20:34304662-34320057- | F: GTAAGGAAACGGAAGCGAAGT  
|                       | R: TCGCCTCTCTTCCTTCTACT |
| chr6:56989532-57025950+ | F: ACATGGGT GCCAAAATACTTC  
|                      | R: GACGAGCCAGAGTTAAAGCAAC |
| GAPDH         | F: GCCCTCAAAGGAGTAAGACC  
|              | R: AGGGGAGATTACGTGTTG }
### Table 2  Summary of Reads statistics of NK/T-LAHS and normal control

| Sample name   | Raw Reads     | Q30  | Clean Reads | Clean Ratio | Mapped Reads | CircRNA Number |
|---------------|---------------|------|-------------|-------------|--------------|---------------|
| NK/T-LAHS 1   | 85,833,954    | 90.79% | 85,454,974  | 99.56%      | 61,953,110   | 1,362         |
| NK/T-LAHS 2   | 88,495,030    | 91.36% | 88,239,244  | 99.71%      | 65,427,958   | 1,828         |
| NK/T-LAHS 3   | 79,053,298    | 90.85% | 78,702,788  | 99.56%      | 58,369,924   | 3,301         |
| NK/T-LAHS 4   | 86,398,392    | 90.63% | 86,143,752  | 99.71%      | 63,262,850   | 818           |
| NC-1          | 83,389,774    | 90.20% | 82,762,386  | 99.25%      | 47,656,180   | 1,187         |
| NC-2          | 83,205,550    | 89.99% | 82,129,898  | 98.71%      | 44,744,062   | 1,542         |
| NC-3          | 86,771,462    | 90.12% | 86,368,674  | 99.54%      | 50,073,200   | 1,390         |
| NC-4          | 82,877,972    | 90.56% | 82,220,232  | 99.21%      | 46,639,532   | 1,646         |

**Abbreviation:** NK/T-LAHS: NK/T-cell lymphoma-associated hemophagocytic syndrome; NC: normal control
Table 3  Predicted miRNAs for the 5 selected differentially expressed circRNAs linked to NK/T-LAHS

| CircRNA ID            | miRNA       | miRNA       | miRNA       | miRNA       | miRNA       |
|-----------------------|-------------|-------------|-------------|-------------|-------------|
| chr5:112090570-112137080+ | hsa-miR-6821-3p | hsa-miR-6728-3p | hsa-miR-6800-3p | hsa-miR-337-5p | hsa-miR-7-5p |
| chr1:77620134-77635080- | hsa-miR-215-3p   | hsa-miR-485-3p   | hsa-miR-5089-5p | hsa-miR-1302 | hsa-miR-593-3p |
| chr12:62715245-62749256+  | hsa-miR-29b-1-5p | hsa-miR-6843-3p | hsa-miR-7-5p   | hsa-miR-1299 | hsa-miR-6876-3p |
| chr20:34304662-34320057-  | hsa-miR-138-1-3p | hsa-miR-425-3p   | hsa-miR-183-5p | hsa-miR-6841-3p | hsa-miR-5009-5p |
| chr6:56989532-57025950+   | hsa-miR-4268   | hsa-miR-6514-3p | hsa-miR-146a-3p | hsa-miR-6741-5p | hsa-miR-1273d |
Figure 1: The overall circRNA sequencing.

(a) The source of circRNAs in NK/T-LAHS

11,481 different putative circRNAs in these serum samples were identified, of which 2698 were consistent with circRNAs in circBase. 42 were documented in the literature and 8,741 are novel circRNAs first identified.

(b) The catalog of circRNAs in NK/T-LAHS

Using the RefSeq database, 2,990 contained protein-coding exons, while 4,288, 2,110, 1,126 and 967 circRNAs aligned with introns, antisense, intergenic and sense overlapping regions of known transcripts, respectively.

(c) The length range of circRNA in NK/T-LAHS

The length of the majority of identified exonic circRNAs (2,990 total) was under 1500 nucleotides (nt), with the majority being 200-1000 nt.

(d) The chromosome distribution of circRNA in NK/T-LAHS

The chromosome distributions of circRNAs with the majority being concentrated on chromosomes 1 and 2.
**Figure 2: Differentially expressed circRNAs in NK/T-LAHS**

(a) Cluster heatmap of intersection expressed circRNAs in NK/T-LAHS patients compared with normal controls (NC)

In the clustered heatmap identified by next-generation sequencing, each column represents serum from a patient or a healthy volunteer, each line represents a circRNA. The red lines are upregulated circRNAs, and the green ones are downregulated circRNAs. Five upregulated circRNAs which were verified by real-time qPCR were marked.

(b) Scatter plot of intersection expressed circRNAs in NK/T-LAHS patients compared with NC

Vertical green lines indicate 2-fold (log$_2$ scaled) up- or downregulation, and the horizontal green line indicates a p value of 0.05 (-log$_{10}$ scaled). Red squares represent circRNAs with statistically significant differences in expression.

(c) Volcano plot of intersection expressed circRNAs in NK/T-LAHS patients compared with NC

Differentially expressed circRNAs between NK/T-LAHS and NC are displayed by volcano plots. Vertical green lines indicate 2-fold (log$_2$ scaled) up or down changes, and the horizontal green line indicates a p value of 0.05 (-log$_{10}$ scaled). Red squares represent circRNAs with statistically significant differences in expression.
Figure 3: Gene ontology (GO) annotations of expressed circRNAs in NK/T-LAHS

(a) Biological processes (BP)
(b) Cellular components (CC)
(c) Molecular functions (MF)

Gene ontology (GO) enrichment analysis was made up of (a) biological processes, (b) cellular components, and (c) molecular functions. The top 10 predicted functions of the source gene regulated by 114 upregulated circRNAs in serum from patients with NK/T-cell lymphoma-associated hemophagocytic syndrome (NK/T-LAHS) were explored by GO analysis. Fold enrichment indicated the regulated extent of the predicted functions by upregulated circRNAs in NK/T-LAHS patients compared with normal controls (NC).
Figure 4: Kyoto encyclopedia of genes and genomes (KEGG) pathway annotations of expressed circRNAs in NK/T-LAHS

(a) The top 10 KEGG pathway analysis enriched in circRNAs
(b) Insulin signaling pathway
(c) Endometrial cancer
(d) Hippo signaling pathway

Insulin signaling pathway, endometrial cancer, Hippo signaling pathways regulated by the upregulated circRNAs in NK/T-LAHS. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed 21 signaling pathways related to the 114 upregulated circRNAs in patients with NK/T-LAHS. (b) Insulin signaling pathway, (c) endometrial cancer pathway, and (d) Hippo signaling pathways were connected with expression of GSK-3β, which played a crucial role in pathogenesis of NK/T-LAHS.
### Pathway Analysis

| Pathway                                      | SelectionCounts |
|----------------------------------------------|-----------------|
| Regulation of actin cytoskeleton              | 0.024           |
| Progesterone signaling pathway               | 0.020           |
| P38-Akt signaling pathway                    | 0.016           |
| Insulin signaling pathway                    | 0.013           |
| Hippo signaling pathway                      | 0.007           |
| Focal adhesion                               | 0.002           |
| Endometrial cancer                           | 0.003           |
| ECM-receptor interaction                     |                 |
| Colorectal cancer                            |                 |
| Basal cell carcinomas                        |                 |

#### Diagrams

(a) Pathway Analysis

(b) Signal Pathway

(c) Enzyme Inhibition

(d) Immunological Pathway
Figure 5: The interaction network between differentially expressed circRNAs and miRNAs in NK/T-LAHS.

5 upregulated circRNAs were selected for validation (yellow), are displayed in a network diagram. The graph shows that circRNAs interacted with miRNAs in numerous way. The top 5 miRNAs regulated by each circRNA are shown (green). Circles indicate circRNAs and arrows represent miRNAs.
Figure 6: Five upregulated circRNAs in patients with NK/T-LAHS compared with normal controls.

The serum from 10 NK/T-LAHS patients and 10 healthy volunteers were used for verification by real-time qPCR on the basis of circRNAs sequencing. The relative expression of the five upregulated circRNAs in serum from NK/T-LAHS compared with NC were verified by real-time qPCR. hsa_circ_0073586 (p = 0.001), hsa_circ_0114150 (p = 0.000), hsa_circ_0027353 (p = 0.003), hsa_circ_0115056 (p = 0.000) and hsa_circ_0131996 (p = 0.036) exhibited the same growing trend toward the sequencing results. *P<0.05, ** P<0.01, and *** P<0.001.
**FIGURE LEGENDS**

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| chr12:62715245-62749256+ | F: GCCTCTGTGCGCTAAGTAC  R: CTGGTCTCCTTTTCTTGCA     |
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| chr6:56989532-57025950+ | F: ACATGGGTGCCAAAAATACTTC  R: GACGAGCCAGAGTTAAGCAAC |
| GAPDH               | F: GGCCTCCAAGGAGTAAGACC  R: AGGGGAGATTCAGTGTTGTG   |
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| chr20:34304662-34320057-| hsa-miR-138-1-3p | hsa-miR-425-3p | hsa-miR-183-5p | hsa-miR-6841-3p | hsa-miR-5009-5p |
| chr6:56989532-57025950+ | hsa-miR-4268 | hsa-miR-6514-3p | hsa-miR-146a-3p | hsa-miR-6741-5p | hsa-miR-1273d |
