Identification of Differentially Expressed lncRNAs and mRNAs in Children with Acquired Aplastic Anemia by RNA Sequencing

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1. Introduction

Acquired aplastic anemia (acquired AA) is a life-threatening disorder in children characterized by pancytopenia and bone marrow failure. Successful use of immunosuppressive agents and hematopoietic stem cell transplantation (HSCT) in the treatment of acquired AA lead the way to understanding the pathology of acquired AA [1]. It is now widely acknowledged that at the cell level, the deficiencies of hematopoietic stem and progenitor cells (HSPCs), immune cell dysfunction, and abnormal bone marrow microenvironment are the main factors in the pathology of acquired AA [2, 3]. Besides, with the rapid developments in basic immunology and molecular biology techniques, a large number of studies have been carried out to explore the definitive mechanism at the molecular level in acquired AA. The messenger RNA (mRNA) expression profiles for CD34+ stem/progenitor cells [4, 5], T cells [6, 7], and mesenchymal stem/stromal (MSC) cells [8–10] in acquired AA have been described, and some mRNAs were identified to be involved in the pathology of acquired AA. Furthermore, microRNA (miRNA) expression profiles in acquired AA were also explored [11–13], and some miRNAs were identified to take part in the pathology of acquired AA. However, the long noncoding RNA (lncRNA) expression profiles and their role in children with acquired AA have not been described yet.

In the human genome, about 5%-10% sequences are transcribed, among which 10–20% are protein-coding RNAs and 80%-90% are non-protein-coding RNAs. lncRNAs are a kind of noncoding RNAs longer than 200 bp, and they can serve as signals, decoys, guides, and scaffolds in a large number of bioregulatory processes. Their biological role can be interpreted indirectly through the mRNAs which are regulated by cis-regulation, trans-regulation, or competing...
endogenous (Ce) regulation: the cis-regulation means that lncRNAs affect the expression of their neighboring genes located at the same chromosome, the trans-regulation means that lncRNAs can also act on their target genes through a long-range manner such as conjunction with other transcription factors (TFs), and Ce regulation means that lncRNAs can act as sponges to compete for the miRNA, hence reducing the miRNA’s ability to interfere with the expression of target genes [14, 15].

It has been reported that lncRNAs are regulators of many immune processes and they participate in many immune-mediated disorders such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE) [16, 17]. What is more, lncRNAs were also reported to regulate the hematopoietic stem cell development and play important roles in hematological disease [18]. As acquired AA is an immune-mediated hematological disease, it can be predicted that lncRNAs also play important roles in the pathology of acquired AA.

In this study, differentially expressed lncRNAs (DElncRNAs) and mRNAs (DEmRNAs) between acquired AA children and healthy controls were identified by RNA sequencing. The cis-, trans-, and Ce regulation networks were constructed to predict the DEmRNAs that might be regulated by DElncRNAs. Moreover, literature screen and quantitative real-time PCR (qPCR) validation were performed to identify immune- or hematopoietic-related DElncRNA-DEmRNA pairs, which may lay the foundation for future study of potential effects of lncRNAs in children with acquired AA.

2. Materials and Methods

2.1. Patients and Samples. Peripheral blood (PB) samples of 5 acquired AA children and 5 healthy controls were obtained at the Department of Pediatrics, Shanghai Tongji Hospital. After exclusion of any other marrow failure syndromes, the diagnosis of acquired AA was established by peripheral blood counts and bone marrow biopsy according to Camitta’s criteria in the guideline [19]. Informed consent was obtained according to protocols approved by the Institutional Review Board of Shanghai Tongji Hospital affiliated to Tongji University. Student’s t-test and Fisher’s exact test were used to compare the basic characters of AA children and healthy controls.

2.2. RNA Extraction and Sequencing. Mononucleated cells of PB were separated by Solarbio R1010, and RNA was isolated according to the manufacturer’s instructions. RNA sample sequencing of 5 acquired AA children and 5 healthy controls was performed separately based on the IlluminaHiSeq 2000/2500 platform (Illumina, Inc., San Diego, CA, USA) with a 150 bp read length. The FASTQ sequence data were acquired from the RNA sequencing data. Reads with low quality were removed to obtain the clean reads.

2.3. Identification of DElncRNAs and DEmRNAs. Sequencing reads were aligned to the human genome (hg38) reference sequence, HTSeq was used, and the expression of mRNAs and lncRNAs was normalized. Reads Per Kilobase per Million (RPKM) of lncRNAs and mRNAs were calculated. DESeq2 was used for the differential expression analysis, and DEmRNAs and DElncRNAs were obtained with $|\log_{2}FC| > 1$ and adj. $P$ value < 0.05. By using the R package “pheatmap,” hierarchical clustering analysis of DEmRNAs and DElncRNAs was conducted.

2.4. Functional Annotation of DEmRNAs. To understand the biological functions and potential pathways of DEmRNAs, Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed and visualized by DAVID [20] and the R packages “clusterProfiler,” “enrichplot,” and “GOplot,” and adj. $P$ value < 0.05 was considered to be significant.

2.5. cis-Regulated Inc-mRNA Network. To further explore the potential effects of DElncRNAs in children with acquired AA, the DElncRNA-DEmRNA coexpression networks were constructed. DElncRNA-DEmRNA pairs with an absolute value of the Pearson correlation $> 0.9$ and $P < 0.01$ were defined as coexpressed DElncRNA-DEmRNA pairs. The cis-regulated DEmRNAs were defined as follows: (1) DEmRNA loci were within a 100 kb window downstream of the given DElncRNA and (2) DElncRNAs and DEmRNAs were coexpressed DElncRNA-DEmRNA pairs. The cis-regulated Inc-mRNA network was visualized by Cytoscape.

2.6. trans-Regulated Inc-TF-mRNA Network. For a trans-regulated network, we focused on the manner that lncRNAs play their functions via TFs. The DElncRNAs’ coexpressed DEmRNAs were overlapping with TF target DEmRNAs in DAVID, using hypergeometric distribution to calculate the significance of this overlap, and adj. $P$ value < 0.05 was considered to be significant. If the DElncRNAs’ coexpressed DEmRNAs were overlapping with the target mRNAs of a given TF significantly, it meant that this TF might work with these DElncRNAs and these DEmRNAs could be the trans-regulated target of these DElncRNAs. The trans-regulated Inc-TF-mRNA network was constructed and visualized by Cytoscape.

2.7. Ce-Regulated Inc-micromRNA Network. Some lncRNAs might act as competing endogenous RNAs and influence the posttranscriptional regulation by regulating miRNA. miRNA-binding sites on DEmRNAs and DElncRNAs were predicted by software, and a Ce-regulated Inc-micromRNA network was constructed and visualized by Cytoscape.

2.8. Quantitative Real-Time PCR Validation. Blood samples of 5 acquired AA children and 5 healthy controls were used for qPCR validation, respectively. M-MLV reverse transcriptase was used for cDNA synthesizing. Subsequently, qPCR using SYBR Green assays was conducted in a total reaction volume of 10 μl, including 0.5 μl (10 μM) PCR forward primer and 0.5 μl (10 μM) PCR reverse primer, 2 μl CDNA, 5 μl 2× Master Mix, and 2 μl double-distilled water. The qPCR reaction conditions were denaturation at 95°C for
10 min, followed by 40 cycles of 95°C (10 s) and 60°C (60 s). GAPDH was used as a reference. The relative expression level of each RNA was calculated using the $2^{-\Delta\Delta C_{T}}$ method. Student’s $t$-test was applied to compare the expression levels of two groups, and $P<0.05$ was considered to be significant. The primers are shown in Supplement Table 1.

3. Results

3.1. Clinical Characteristics of 5 Acquired AA Patients and 5 Healthy Controls. The clinical characteristics of 5 acquired AA patients and 5 healthy controls are listed in Table 1. No significant differences were found in age and gender between the two groups.

| Group          | ID | Age (year)* | Gender | Diagnosis | HB (g/l) | WBC ($10^9$/l) | Neutrophils ($10^9$/l) | Platelet ($10^9$/l) |
|----------------|----|-------------|--------|-----------|----------|----------------|-----------------------|---------------------|
| Patients       | 1  | 6           | M      | NSAA      | 110      | 3              | 0.9                   | 25                  |
|                | 2  | 10          | M      | NSAA      | 70       | 3.5            | 0.9                   | 40                  |
|                | 3  | 8           | F      | SAA       | 56       | 3              | 0.4                   | 5                   |
|                | 4  | 2.1         | F      | SAA       | 75       | 2.3            | 0.4                   | 11                  |
|                | 5  | 3.8         | F      | SAA       | 71       | 3.35           | 0.1                   | 2                   |
| Healthy controls| 1  | 8           | M      | —         | 137      | 7.8            | 5.3                   | 211                 |
|                | 2  | 4           | F      | —         | 142      | 6.4            | 3.4                   | 202                 |
|                | 3  | 1.7         | M      | —         | 121      | 7.2            | 3.6                   | 183                 |
|                | 4  | 6           | M      | —         | 142      | 8              | 5.5                   | 316                 |
|                | 5  | 2.7         | F      | —         | 124      | 6              | 2.1                   | 204                 |

*No statistical differences were found between patients and healthy controls (age and gender).
coagulation-related DEmRNAs may work as negative feedbacks and compensate the thrombocytopenia in some degree.

Unlike miRNAs, solely basing on lncRNAs’ sequences to predict their function is difficult. Based on a previous study by Guttman et al. [22], we constructed a coexpression network of DElncRNAs and DEmRNAs. According to this network, the cis-regulation, trans-regulation, and Ce regulation networks were constructed to comprehend the biological functions of DElncRNAs. After literature screen and qPCR validation, 6 immune- or hematopoietic-related DElncRNA-DEmRNA pairs in the networks were identified as key lncRNAs and mRNAs in the pathology of acquired AA.

For the immune-related genes, DHRS9 [23] was reported to be a specific marker of the human regulatory macrophage and HRH4 [24] can downregulate Th1-related chemokines. As acquired AA is an immune-mediated disease, these two DEmRNAs in our networks may be involved in the pathology of acquired AA. Our work showed that these two downregulated DEmRNAs can be regulated by lncRNA AC007556.1 and AC007922.2 in cis- and Ce regulation manners. Hence, we can conclude that lncRNA AC007556.1 and AC007922.2 may be involved in the
Figure 2: Functional annotation of 364 DEmRNAs. (a) Top 10 GO terms of 364 DEmRNAs and the DEmRNAs enriched in each term. The red and blue colors represent the up- and downregulated RNAs, respectively. (b) Top 10 KEGG terms of 364 DEmRNAs.
pathology of acquired AA by regulating DHR9 and HRH4.

For the hematopoietic-related genes, PDGFA [25] and GFI1B [26, 27] were crucial for the hematopoiesis and they may be related to acquired AA. Our work showed that these downregulated DEmRNAs can be regulated by lncRNAs AC147651.1 and AC111000.4 in cis- and trans-regulation manners. Hence, we can conclude that lncRNAs...
AC147651.1 and AC111000.4 may be involved in the pathology of acquired AA by regulating PDGFA and GFI1B.

What is more, IDO1 [28] and SEMA7A [29] were reported to be important in the immunomodulatory effect of mesenchymal stromal cells in acquired AA. In our study, IDO1 and SEMA7A were downregulated and they were shown to be cis- and trans-regulated by lncRNAs AC007991.2 and RHOXF1P1. We can also conclude that lncRNAs AC007991.2 and RHOXF1P1 may be involved in the pathology of acquired AA by regulating IDO1 and SEMA7A.

There are limitations in our study. Firstly, our study is only a small sample size study which needs further validation. Another limitation is that we merely predict the
potential link between lncRNAs and their target mRNAs and the definite connections between them could not be confirmed by the present study. Further study will be continued to validate the cis-, trans-, and Ce regulation networks.

5. Conclusion

In summary, our study describes the expression profiles of lncRNAs and mRNAs in acquired AA children by RNA sequencing. The cis-, trans-, and Ce regulation networks of DElncRNAs and DEmRNAs were identified, and 6 immune- or hematopoietic-related DElncRNA-DEmRNA pairs were identified as key RNAs in the pathology of acquired AA, which lay the foundation for future exploration of potential effects of lncRNAs and their target mRNAs in children with acquired AA.

Data Availability

The clinical data of our patients are shown in Table 1.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

Authors’ Contributions

Shuanglong Lu and Xiaoxiao Song contributed equally to this work.

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Supplementary Materials

Supplement Table 1: the clinical features of the patients in our manuscript. (Supplementary Materials)

References

[1] N. S. Young, "Aplastic anemia," The New England Journal of Medicine, vol. 379, no. 17, pp. 1643–1656, 2018.

[2] L. Wang and H. Liu, "Pathogenesis of aplastic anemia," Hematology, vol. 24, no. 1, pp. 559–566, 2019.

[3] C. Liu, Y. Sun, and Z. Shao, “Current concepts of the pathogenesis of aplastic anemia,” Current Pharmaceutical Design, vol. 25, no. 3, pp. 236–241, 2019.

[4] W. Zeng, G. Chen, S. Kajigaya et al., "Gene expression profiling in CD34 cells to identify differences between aplastic anemia patients and healthy volunteers," Blood, vol. 103, no. 1, pp. 325–332, 2004.

[5] U. Fischer, C. Ruckert, B. Hubner et al., "CD34+ gene expression profiling of individual children with very severe aplastic anemia indicates a pathogenic role of integrin receptors and the proapoptotic death ligand TRAIL," Haematologica, vol. 97, no. 9, pp. 1304–1311, 2012.

[6] W. Zeng, S. Kajigaya, G. Chen, A. M. Risitano, O. Nunez, and N. S. Young, "Transcript profile of CD4+ and CD8+ T cells from the bone marrow of acquired aplastic anemia patients," Experimental Hematology, vol. 32, no. 9, pp. 806–814, 2004.

[7] A. Franzke, R. Geffers, J. K. Hunger et al., "Identification of novel regulators in T-cell differentiation of aplastic anemia patients," BMC Genomics, vol. 7, no. 1, 2006.

[8] J. Li, S. Yang, S. Lu et al., "Differential gene expression profile associated with the abnormality of bone marrow mesenchymal stem cells in aplastic anemia," Plos One, vol. 7, no. 11, article e47764, 2012.

[9] Y. H. Chao, K. H. Wu, S. H. Chiou et al., "Downregulated CXCL12 expression in mesenchymal stem cells associated with severe aplastic anemia in children," Annals of Hematology, vol. 94, no. 1, pp. 13–22, 2015.

[10] J. Liao, L. Zhang, X. Ren et al., "Multifaceted characterization of the signatures and efficacy of mesenchymal stem/stromal cells in acquired aplastic anemia," Stem Cell Research & Therapy, vol. 11, no. 1, p. 59, 2020.

[11] Y. X. Sun, H. Li, Q. Feng et al., "Dysregulated miR34a/ diacylglycerol kinase ζ interaction enhances T-cell activation in acquired aplastic anemia," Oncotarget, vol. 8, no. 4, pp. 6142–6154, 2017.

[12] K. Hosokawa, P. Muranski, X. Feng et al., "Identification of novel microRNA signatures linked to acquired aplastic anemia," Haematologica, vol. 100, no. 12, pp. 1534–1545, 2015.

[13] Y. Q. Song, H. Y. Dong, M. L. Ge et al., "Differential expression profiles of microRNAs between de novo and complete response severe aplastic anemia," Zhongguo Shi Yan Xue Ye Xue Za Zhi, vol. 26, no. 1, pp. 213–218, 2018.

[14] T. R. Mercer, M. E. Dinger, and J. S. Mattick, "Long non-coding RNAs: insights into functions," Nature Reviews Genetics, vol. 10, no. 3, pp. 155–159, 2009.

[15] J. L. Rinn and H. Y. Chang, "Genome regulation by long non-coding RNAs," Annual Review of Biochemistry, vol. 81, no. 1, pp. 145–166, 2012.

[16] M. K. Atianand, D. R. Caffrey, and K. A. Fitzgerald, "Immunobiology of long noncoding RNAs," Annual Review of Immunology, vol. 35, no. 1, pp. 177–198, 2017.

[17] D. B. Uthaya Kumar and A. Williams, "Long non-coding RNAs in immune regulation and their potential as therapeutic targets," International Immunopharmacology, vol. 81, p. 106279, 2020.

[18] S. Dahariya, I. Paddibhatla, S. Kumar, A. Pallepati, and R. K. Gutti, "Long non-coding RNA: classification, biogenesis and functions in blood cells," Molecular Immunology, vol. 112, pp. 82–92, 2019.

[19] J. C. W. Marsh, S. E. Ball, J. Cavenagh et al., "Guidelines for the diagnosis and management of aplastic anaemia," British Journal of Haematology, vol. 147, no. 1, pp. 43–70, 2009.

[20] X. Jiao, B. T. Sherman, D. W. Huang et al., "DAVID-WS: a stateful web service to facilitate gene/protein list analysis," Bioinformatics, vol. 28, no. 13, pp. 1805–1806, 2012.

[21] S. Jiang, M. Xia, J. Yang et al., "Novel insights into a treatment for aplastic anemia based on the advanced proliferation of bone marrow-derived mesenchymal stem cells induced by fibroblast growth factor 1c," Molecular Medicine Reports, vol. 12, no. 6, pp. 7877–7882, 2015.

[22] M. Guttman, I. Amit, M. Garber et al., "Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals," Nature, vol. 458, no. 7235, pp. 223–227, 2009.

[23] P. Riquelme, G. Armodio, C. Macedo et al., "DHRS9 is a stable marker of human regulatory macrophages," Transplantation, vol. 101, no. 11, pp. 2731–2738, 2017.

[24] S. Mommet, R. Ratz, H. Stark, R. Gutzmer, and T. Werfel, "The histamine H4 receptor modulates the differentiation process of human monocyte-derived M1 macrophages and the release of CCL4/MIP-1β from fully differentiated M1 macrophages," Inflammation Research, vol. 67, no. 6, pp. 503–513, 2018.

[25] C. M. Y. Chui, K. Li, M. Yang et al., "Platelet-derived growth factor up-regulates the expression of transcription factors NF-E2, GATA-1 and c-Fos in megakaryocytic cell lines," Blood, vol. 126, no. 24, pp. 2561–2569, 2015.

[26] T. Möroy, L. Vassen, B. Wilkes, and C. Khandanpour, "From cytopenia to leukemia: the role of GFI1b in blood formation," Blood, vol. 126, no. 24, pp. 2561–2569, 2015.

[27] K. Kitamura, Y. Okuno, K. Yoshida et al., "Functional characterization of a novel GFI1B mutation causing congenital macrothrombocytopenia," Journal of Thrombosis and Haemostasis, vol. 14, no. 7, pp. 1462–1469, 2016.

[28] T. Vidotto, F. P. Saggioro, T. Jamaspishvili et al., "PTEN-deficient prostate cancer is associated with an immunosuppressive tumor microenvironment mediated by increased expression of IDO1 and infiltrating FoxP3+ T regulatory cells," Prostate, vol. 79, no. 9, pp. 969–979, 2019.

[29] M. Fayyad-Kazan, M. Najar, H. Fayyad-Kazan, G. Raicevic, and I. Lagneaux, "Identification and evaluation of new immunoregulatory genes in mesenchymal stromal cells of different origins: comparison of normal and inflammatory conditions," Medical Science Monitor Basic Research, vol. 23, pp. 87–96, 2017.