Original Article

# Differential genes expression of immune tolerance induction in hemophilia A: an exploratory RNA-seq test from a Chinese hemophilia comprehensive care centre

**Jialu Zhang[1,2], Zekun Li[1,2], Guoqing Liu[1], Wanru Yao[1], Di Ai[1,2], Zhengping Li[1,2], Zhenping Chen[2], Runhui Wu[1]**

[1]Department of Hematology Center, National Key Clinical Discipline of Pediatric Hematology, National Key Discipline of Pediatrics (Capital Medical University), Key Laboratory of Major Diseases in Children, Ministry of Education, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing, China; [2]Department of Clinical Laboratory Center, National Key Clinical Discipline of Pediatric Hematology, National Key Discipline of Pediatrics (Capital Medical University), Key Laboratory of Major Diseases in Children, Ministry of Education, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing, China

*Contributions:* (I) Conception and design: R Wu, Z Chen; (II) Administrative support: R Wu; (III) Provision of study materials or patients: D Ai, Zhengping Li; (IV) Collection and assembly of data: G Liu, W Yao; (V) Data analysis and interpretation: J Zhang, Zekun Li; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*Correspondence to:* Runhui Wu, MD. Department of Hematology Center, National Key Clinical Discipline of Pediatric Hematology, National Key Discipline of Pediatrics (Capital Medical University); Key Laboratory of Major Diseases in Children, Ministry of Education; Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, No. 56 Nanlishi Road, Xicheng District, Beijing 100045, China. Email: runhuiwu@hotmail.com; Zhenping Chen, MD. Department of Clinical Laboratory Center, National Key Clinical Discipline of Pediatric Hematology, National Key Discipline of Pediatrics (Capital Medical University); Key Laboratory of Major Diseases in Children, Ministry of Education; Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, No. 56 Nanlishi Road, Xicheng District, Beijing 100045, China. Email: chenzhenping@outlook.com.

**Background:** The production of inhibitors is a serious complication that can arise during coagulation factor replacement therapy for hemophilia A (HA). The primary therapeutic strategy to eliminate inhibitors is immune tolerance induction (ITI), which is known to be an extremely challenging, prolonged, and costly treatment. With the widespread use of RNA sequencing (RNA-seq) to analyze differentially expressed genes (DEGs) across various treatment outcomes, there is potential for predicting ITI outcomes. This study aims to use RNA-seq to test differently expressed genes in different outcomes of ITI treatment for HA patients with high-titer inhibitor (HAI), to explore its prediction possibility.

**Methods:** RNA-seq was employed to screen and compare the DEGs between patients in the Success group and those in the Failure group, based on ITI clinical outcomes. DEGs were subjected to Gene Ontology (GO) analysis and enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

**Results:** Thirteen analyzable HAI cases were collected, comprising seven in the Success group and six in the Failure group. Blood samples were taken before and after ITI. RNA-seq was applied to all samples to screen for expressed genes. In the Success group, a total of 4,967 messenger RNA (mRNA) transcripts were differentially expressed between pre-ITI and post-ITI, with 2,865 being up-regulated and 2,102 down-regulated. In the Failure group, 515 mRNA transcripts were expressed either before or after ITI, showing up-regulation in 68.7% (354/515) and down-regulation in 31.3% (161/515).

**Conclusions:** The increased expression of genes which related to immune system activation suggests a possibly favorable therapeutic outcome of ITI. Future studies should test with a larger cohort to validate these findings.

**Keywords:** Pediatrics; hemophilia A (HA); RNA sequencing (RNA-seq); immune tolerance induction (ITI); predict

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## Introduction

Hemophilia A (HA) is a bleeding disease that is inherited in an X-linked recessive manner, resulting from genetic mutations affecting either factor VIII (FVIII). Infusion of exogenous FVIII products is the fundamental choice for the treatment of HA. However, it is estimated that 20% to 30% of individuals with severe HA may develop neutralizing antibodies against exogenous FVIII (1,2), exacerbating bleeding, increasing mortality and disability rates, and significantly affecting the effectiveness of treatment (3). Therefore, the appearance of inhibitors is a significant complication and challenge in the replacement therapy for HA (3). Immune tolerance induction (ITI) remains the primary treatment approach for eliminating inhibitors in hemophilia patients. The overall success rate of ITI ranges from 50% to 80% (4-7), but different studies report varying success rates.

The efficacy of ITI is influenced by multiple factors, including race, type of *F8* gene mutation, inhibitor titer, treatment regimen, and immune regulatory mechanisms (4,5,8). Exploring differently expressed genes before treatment in patients with different treatment responses can help predict outcomes. RNA sequencing (RNA-seq) is a method to accurately identify genes related to the phenotype of human specific diseases. It has a broad prospect for identifying specific gene maps related to detection, prognosis and chemical sensitivity of other diseases (9,10). Co-analysis can clarify the complex causes of human diseases and related regulatory mechanisms (11).

In this study, we divided patients into two groups based on whether ITI was successfully implemented. Using RNA-seq technology, we systematically analyzed the transcriptional changes in peripheral blood cells before treatment in both groups. By comparing the differentially expressed genes (DEGs) between the two groups, we tried to explore predictive markers of ITI outcome. Then we also analyzed the transcriptional changes in peripheral blood cells after treatment in both groups with RNA-seq, trying to explore potential targets for realizing precision therapy.

### Highlight box

**Key findings**

- The increased expression of genes which related to immune system activation suggests a possibly favorable therapeutic outcome of immune tolerance induction (ITI).

**What is known and what is new?**

- The efficacy of ITI is influenced by multiple factors, including race, type of *F8* gene mutation, inhibitor titer, treatment regimen, and immune regulatory mechanisms.
- Exploring differently expressed genes before treatment in patients with different treatment responses can help predict outcomes.

**What is the implication, and what should change now?**

- It was found that there were significant differences in the expression of messenger RNA in peripheral plasma of hemophilia A children with high-titer inhibitor between different outcome groups, showing that upregulation of genes involved in activation of the immune system may indicate favorable therapeutic response. The larger cohort should be tested in the future.

## Methods

### *Patient cohort and blood sample collection*

This exploratory cohort study, conducted at a single center, involved children with HA who had high-titer inhibitors and took place from April 2019 to October 2020 at Beijing Children's Hospital. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Review Committee of Beijing Children's Hospital (No. 2017-k-49), and the informed consent form was acquired from the guardians before participation in the study.

The inclusion criteria were as follows: (I) patients with an established diagnosis of HA (12); (II) with high-titer inhibitors [titers of ≥5 Bethesda units/mL (BU/mL) on at least one occasion from our medical records]; (III) received ITI strategy and followed for more than 24 months, which could gain the final outcomes about ITI.

Patients were divided into two groups by ITI outcome. Patients in Success group achieved inhibitor elimination, while those in Failure group not.

### *Clinical data collection*

Clinical and laboratory information for the patients was extracted from the medical records at the Hemophilia

Comprehensive Care Center, which included baseline FVIII:C levels, age, inhibitor titer at the initiation of ITI, ITI treatment regimens, follow-up data, and outcomes of ITI.

### *RNA sequencing*

Peripheral blood mononuclear cells from patients with HA were separated from 2 mL of EDTA-K2 anticoagulated venous blood through density gradient centrifugation. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) for transcriptomics. RNA integrity was examined using a Fragment Analyzer (Agilent, Santa Clara, USA). The RNAs were fragmented and subsequently reverse transcribed into cDNAs with the use of random primers for the purpose of constructing a library. Following this, sequencing was performed on the prepared library. All raw sequencing reads generated underwent filtering to produce clean reads, which were then saved in FASTQ format. The alignment of these clean reads to the reference gene and genome was carried out using Bowtie2 and HISAT, respectively. Gene expression levels [fragments per kilobase of transcript per million fragments mapped (FPKM)] were computed through RSEM, while read counts for each gene were obtained via the SubRead package. Normalization along with differential expression analysis was executed using edgeR software. In terms of RNA sequencing (RNA-seq) analysis, genes that displayed $|\text{Log}_2$ fold change$| \geq 2.5$ and a false discovery rate (FDR) <0.01 were classified as DEGs.

### *Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses*

The analysis of all differentially expressed messenger RNAs (mRNAs) was conducted using the GO enrichment and KEGG pathway databases to elucidate the biological significance of these transcripts. GO terms and KEGG pathways were deemed significantly enriched if their corrected P values were less than 0.01.

### *Statistical analyses*

Statistical evaluations were performed utilizing IBM SPSS version 26.0 for Windows (IBM Corp., Armonk, NY, USA). Continuous variables, represented as the median (range), were analyzed using the Mann-Whitney *U* test for comparison. Categorical variables, expressed as frequency and percentage, were compared by Fisher's exact test. Differences were considered statistically significant when P<0.05. The statistical analysis was carried out using R software version 4.1.3, with P values and FDR q values less than 0.01 considered to be statistically significant.

## Results

### *Clinical characteristics*

There were thirteen cases enrolled, the Success group with seven cases and the Failure group with six cases. The clinical characteristics with the cooperabilities between two groups were presented in *Table 1* (P>0.05).

### *Differentially expressed transcripts in Success group (Figure 1)*

There were 4,967 mRNA transcripts expressed transcripts meeting the threshold of $\text{Log}_2$ fold change $\leq -2.5$ or $\geq 2.5$ with 57.7% (2,865/4,967) mRNAs were upregulated, and 42.3% (2,102/4,967) were downregulated. Following the GO and KEGG enrichment analyses, the five most significantly enriched GO terms for the upregulated differentially expressed mRNAs were identified in relation to biological processes (BPs), cellular components (CCs), and molecular functions (MFs).

The DEGs were predominantly associated with cellular BPs in the context of BP, with three out of five GO terms linked to DNA replication. The remaining terms pertained to the transition phases of the mitotic cell cycle and organelle fission.

Five top DEGs were enriched in the mitochondrial matrix, chromosomal region, transferase complex, transferring phosphorus-containing groups and condensed chromosome spindle in terms of CC. In terms of MF, the five top DEGs were enriched in the Adenosine triphosphate (ATP) hydrolysis activity, single-stranded DNA binding, coreceptor activity, helicase activity and kinase regulator activity.

The five most significantly enriched GO terms for the downregulated differentially expressed mRNAs, in relation to BP, CC, and MF, were associated with processes such as xenobiotic metabolism, small molecule catabolism, striated muscle contraction, general muscle contraction, and vascular transport within the BP category.

Five top DEGs were enriched in the apical plasma membrane, endocytic vesicle lumen, apical part of cell, myofilament and hemoglobin complex in terms of CC. Three of the five DEGs in terms of MF were related to

 

**Table 1** Clinical characteristics of two groups

| Variables | Success (n=7) | Failure (n=6) | P value |
|---|---|---|---|
| Age at inhibitor diagnosis, years | 4.4 (0.5–7.9) | 4.6 (1.0–5.9) | 0.84 |
| Eds of inhibitor development, days | 28 (4–58) | 37 (15–92) | 0.53 |
| Titer at inhibitor diagnosis, BU | 9.6 (0.7–140.2) | 47.5 (6.2–65.8) | 0.37 |
| Historical peak inhibitor titer, BU | 39.0 (7.5–185.6) | 98.6 (57.6–217.4) | 0.07 |
| Interval-time, months | 2.9 (0.3–32.7) | 17.6 (0.2–57.1) | 0.63 |
| Pre-ITI inhibitor titer, BU | 23.0 (7.5–185.6) | 66.4 (21.8–153.6) | 0.45 |
| Peak inhibitor titer during ITI, BU | 28.5 (4.7–114.6) | 98.3 (11.0–271.4) | 0.18 |
| Treatment regimen | | | |
| ITI alone | 3 (42.9) | 0 (0.0) | 0.19 |
| ITI-IS | 4 (57.1) | 6 (100.0) | |

Data are presented as median (Q1–Q3) or frequency (percent). Eds, exposure days; BU, Bethesda units/mL; Interval-time, interval time from inhibitor diagnosis to ITI start; ITI, immune tolerance induction; IS, immunosuppressant.

potassium channel regulator activity, while the rest were related to the haptoglobin binding and action potential repolarization.

The top three most enriched KEGG overrepresented upregulated pathways were cell cycle pathway, T cell receptor signaling pathway and human T-cell leukemia virus 1 infection pathway. The top three most enriched KEGG overrepresented downregulated pathways were cytoskeleton in muscle cells pathway, glycine, serine and threonine metabolism pathway and serotonergic synapse pathway.

### *Differentially expressed transcripts in Failure group (Figure 2)*

Differentially expressed transcripts between the sample of before-treatment and after-treatment in the Failure group were first analyzed (*Figure 2A*). In total, 515 mRNA transcripts were identified as differentially expressed based on the criteria of $Log_2$ fold change $\leq$–2.5 or $\geq$2.5. Among these, 354 mRNAs were upregulated, and 161 mRNAs were downregulated.

GO and KEGG enrichment analyses were conducted, and *Figure 2B* displays the five most significantly enriched GO terms for the upregulated differentially expressed mRNAs across BP, CC, and MF. The DEGs were primarily associated with cellular BPs in the context of BP. Among the five GO terms, three were connected to coagulation and hemostasis, while the others pertained to wound healing and the regulation of body fluid levels. DEGs were mainly enriched in platelet and hemoglobin in terms of CC. In terms of MF, the five top DEGs were mainly enriched in the oxygen transport.

*Figure 2C* illustrates the five most significantly enriched GO terms for the downregulated differentially expressed mRNAs concerning BP, CC and MF. Five top DEGs were mainly enriched in the meiotic cell cycle and innate immune response in terms of BP. Five top DEGs were enriched in the specific granule lumen, specific granule, Golgi lumen, CMG complex and primary lysosome in terms of CC. Five top DEGs were enriched in the bitter taste receptor activity, taste receptor activity, polyamine transmembrane transporter activity, serine-type endopeptidase activity and cysteine-type deubiquitinate activity in terms of MF.

The findings from the KEGG enrichment analysis are presented in *Figure 2D*,*2E*. The three most significantly enriched upregulated pathways identified were the malaria pathway, complement and coagulation cascades pathway, and hematopoietic cell lineage pathway. The top three most enriched KEGG overrepresented downregulated pathways were cytoskeleton in asthma pathway, staphylococcus aureus infection pathway and allograft rejection pathway.

### *DEGs between the two groups (Figure 3)*

Based on the results of the above analysis, we further screened for DEGs that showed opposite trends between the Success group and the Failure group. There were 328 mRNAs upregulated in the Success group after ITI while downregulated in the Failure group, seventeen of these were related with immune system. And there were 605 mRNAs which were upregulated in the Failure group

 

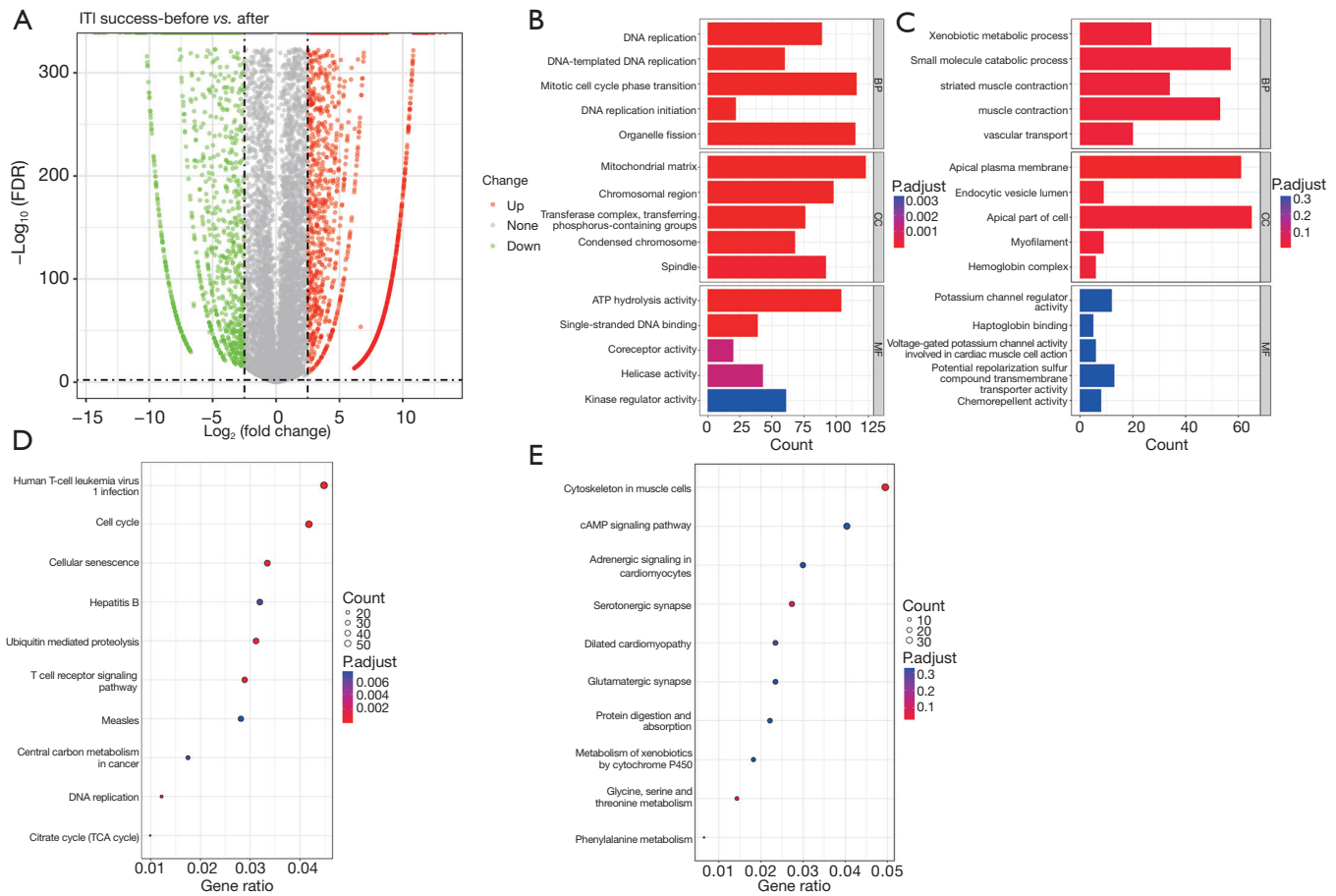


**Figure 1** Transcription profile of patients in the Success group. (A) Volcano plot representing the expression level of mRNAs in terms of the P value (<0.01). (B) mRNAs with up-regulated differential expression acquired through GO enrichment analysis. (C) mRNAs with down-regulated differential expression acquired through GO enrichment analysis. (D) mRNAs with up-regulated differential expression acquired through KEGG enrichment analysis. (E) mRNAs with down-regulated differential expression acquired through KEGG enrichment analysis. mRNAs, messenger RNA; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ITI, immune tolerance induction; FDR, false discovery rate; BP, biological process; CC, cellular components; MF, molecular function.

after ITI while downregulated in the Success group, one hundred of these were related with immune system.

## Discussion

This study made a comparison of the gene expression level among HAI patients having diverse outcomes of ITI. The DEGs were primarily distributed within the domain of immune response regulation, and similar outcomes were identified both in the enrichment analysis of GO terms and KEGG pathways. There are also many studies which have explored the immune regulatory mechanisms underlying ITI (13,14), highlighting the roles of memory B cells, T cells, long-lived plasma cells, and regulatory T cells in promoting tolerance to FVIII (15). This discovery is consistent with the traditional theory that the generation of FVIII inhibitors is a process relying on T cells. Once FVIII epitopes are identified, T cells become activated, thereby promoting the proliferation and differentiation of B cells into plasma cells and ultimately leading to the formation of inhibitors. Evidence suggests that CD4+ T cells play a crucial role in the development of exogenous FVIII antibodies. Additionally, Th17, Th1, and Th2 cells have been regarded as significant elements in the progress of FVIII inhibitor formation (16,17). Apostolou *et al.* (18) indicates that chronic exposure to antigens in the primary

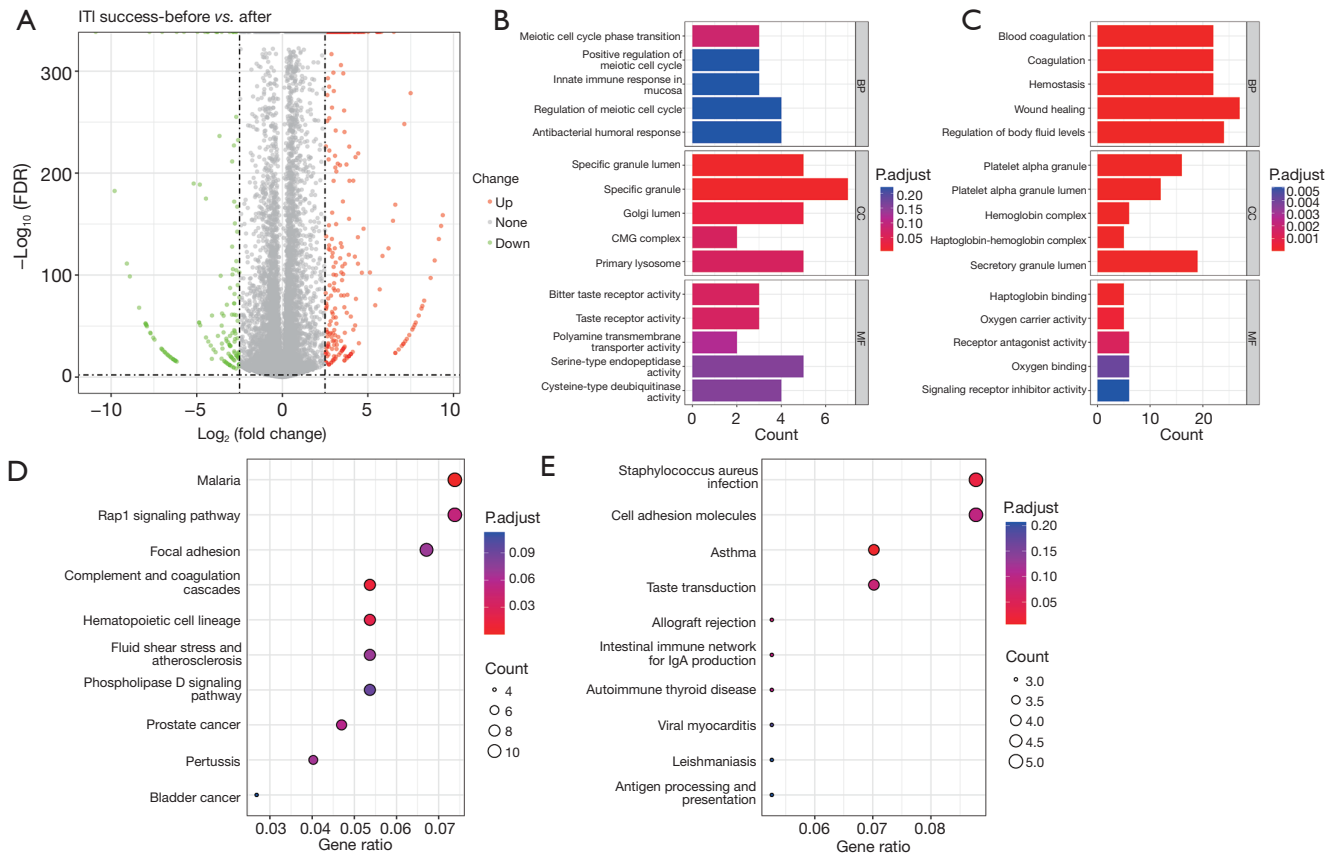


**Figure 2** Transcription profile of patients in the Failure group. (A) Volcano plot representing the expression level of mRNAs in terms of the P value (<0.01). (B) mRNAs with up-regulated differential expression acquired through GO enrichment analysis. (C) mRNAs with down-regulated differential expression acquired through GO enrichment analysis. (D) mRNAs with up-regulated differential expression acquired through KEGG enrichment analysis. (E) mRNAs with down-regulated differential expression acquired through KEGG enrichment analysis. mRNAs, messenger RNA; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ITI, immune tolerance induction; FDR, false discovery rate; BP, biological process; CC, cellular components; MF, molecular function; CMG, CDC45-MCM2-7-GINS.

immune system may induce immune tolerance by generating FVIII-specific T cells. These T cells inhibit the activity of anti-FVIII-specific effector T cells. Since anti-FVIII-specific B cells are no longer regulated by effector T cells, their differentiation is hindered, ultimately inhibitors production is hindered and B cells are cleaned. At this point, memory cells, both memory B cells and memory $CD4^+$ T cells, take the lead in the immune response. Memory B cells expressing high-affinity antigen receptors, upon re-exposure to specific antigens, promptly differentiate into plasma cells and generate antibodies. Additionally, high-potency antigen-presenting cells activate and induce the differentiation of FVIII specific memory B cells by stimulating T cells. Exploring pre- and post-treatment differences in gene regulation in patients with different therapeutic responses can help find therapeutic targets.

In our study, the Failure group had longer Interval-time than the Success group [17.6 (range 0.2–57.1) *vs.* 2.9 (range 0.3–32.7)], which shows that persistent suppression leading to a poor prognosis, and mechanisms that modulate long-term immunity may play an important role. We found out that the gene *Bcl6* in the Failure group was significantly up-regulated, which is necessary to establish and maintain T and B cell immune memory. Hausl *et al.* (19) contends that the re-stimulation of antigen specific T cells is capable of potentiating the stimulation and differentiation of FVIII specific memory B cells. The absence of such restimulation leads to the selective inhibition of FVIII-specific memory B cells, which may be an important mechanism. However, there are some different views that suggest that memory B

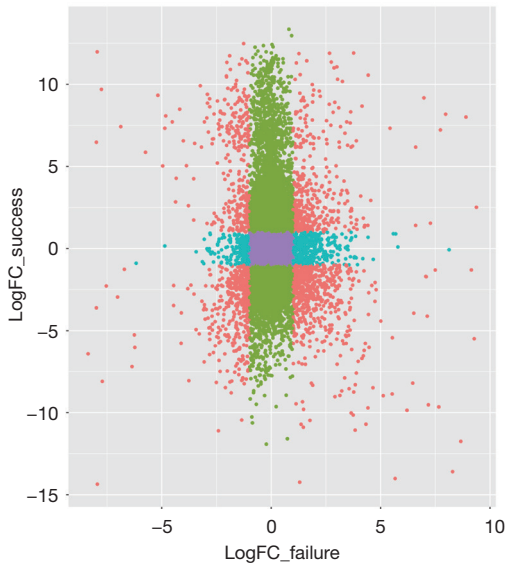


**Figure 3** Different transcription profile of patients between the two groups. FC, fold change.

cells are not the only factor. Since FVIII-specific memory B cells have been shown to be associated with persistent positivity for inhibitors, but not all patients with persistent inhibitors can be detected (20-22).

Research on signaling pathways related to inhibitor production and immune tolerance in hemophilia is less extensive. Some studies suggest that the Toll-like receptor (TLR) and tumor necrosis factor signaling pathways may be associated with the production of inhibitors in HA (23-25). In this study, the genes that negatively regulate the signal transduction of TLR were significantly up-regulated in the successful ITI group after treatment. TLR signal transduction induces upregulation of maturation markers and costimulatory molecules (such as CD80, CD83 and CD86) on dendritic cells (26), these molecules can enhance the stimulation of $CD4^+$ T cells needed to induce memory B cell differentiation. This finding may suggest that down-regulation of TLR signal transduction may play an important role in the reconstruction of immune tolerance.

There are some limitations in this study. We did not classify immune cells and were unable to determine whether the DEGs were significantly enriched in which particular cell types. In the future, it might be feasible to conduct single-cell sequencing to further explore information on key cell types. The type of gene mutation in the patients we selected was not consistent, and previous studies have confirmed that the type of gene mutation is an important predictor of the efficacy of ITI. Additionally, our sample size was relatively small.

## Conclusions

In summary, our study shows that the increased expression of genes which related to immune system activation suggests a possibly favorable therapeutic outcome of ITI. These findings have the potential to reveal novel therapeutic targets for prevention and treatment of inhibitors.

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## Footnote

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-24-300/coif). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Review Committee of Beijing Children's Hospital (No. 2017-k-49), and the informed consent form was acquired from the guardians before participation in the study.

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