Analysis of the Mechanism of T Lymphocytes Promoting Immune Platelet Transfusion Refractoriness by Gene Chip Technique

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Song *et al.*: Analysis of T Lymphocytes in Promoting Platelet Transfusion Refractoriness by Gene Chip Technique

The main objective of this study is to analyze the expression levels of messenger ribonucleic acid and long non-coding ribonucleic acid in patients with platelet transfusion refractoriness and reveal the mechanism of T lymphocytes in immune platelet transfusion refractoriness. The Agilent expression profile chip was used to detect the expression levels; gene ontology and Kyoto encyclopedia of genes and genomes enrichment analysis were performed on the differential genes to determine their main biological functions. Unsupervised hierarchical clustering was used to process differential genes and the differential genes among samples were represented in a heat map. The differentially expressed messenger ribonucleic acids and long non-coding ribonucleic acids in different groups were found as 720 and 1719 in normal control group *vs.* platelet transfusion effective group; 4254 and 12491 in normal control group *vs.* platelet transfusion ineffective group and 1806 and 6216 in platelet transfusion effective group *vs.* platelet transfusion ineffective group. Gene ontology and Kyoto encyclopedia of genes and genomes enrichment analysis were performed on differentially expressed genes, and the results were annotated to be related to T cells. The co-expression of the target gene Ras-related protein 1A and long non-coding transactive response deoxyribonucleic acid binding protein 2 was determined through the national center for biotechnology information database and the interaction between micro ribonucleic acid-4739 and Ras-related protein 1A was predicted using the starBase and TargetScan databases. T lymphocytes play an important role in immune platelet transfusion refractoriness and long non-coding transactive response deoxyribonucleic acid binding protein 2 may affect the differentiation of T lymphocytes and promote the occurrence of immune platelet transfusion refractoriness through micro ribonucleic acid-4739 targeting Ras-related protein 1A gene regulation.

Key words: Platelet transfusion refractoriness, T lymphocytes, gene chip, long non-coding transactive response deoxyribonucleic acid binding protein 2, micro ribonucleic acid-4739

Platelet Transfusion Refractoriness (PTR) has been a common clinical problem in the treatment of bleeding disorders[1,2], which can cause serious consequences for patients and additional economic burdens for families and society. In recent years, it has been recognized that the mechanism of immune PTR is caused by B cells producing platelet antibodies through humoral immune response[3]. But new research has found that about 17 % of patients with PTR and 40 % of patients with Immune Thrombocytopenia (ITP) had no significant platelet antibody[4], indicating that the humoral immune clearance mechanism cannot fully explain the occurrence of PTR. Studies have found that both Cluster of Differentiation 8+ (CD8+) T cells and Cluster of Differentiation 4+ (CD4+) T cells

with antiplatelet reactivity are found in peripheral blood. CD8+ T cells can inhibit B lymphocytes from producing platelet antibodies, while the decrease in the number of CD4+ T cells can make auto reactive B lymphocytes proliferate accordingly, thereby producing some antibodies that promote platelet destruction[5-7], indicating that T lymphocytes can regulate platelet antibody production. The immune response of T lymphocytes may be another mechanism that causes immune PTR, but there are few studies on the regulation mechanism of T lymphocytes on immune PTR. We analyzed the expression levels of messenger Ribonucleic Acid (mRNA) and long non-coding RNA (lncRNA) in patients with PTR by gene chip technology, screened the relevant target genes

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that regulate T lymphocytes in immune PTR, and explored its possible mechanism of action, in order to further explore the role of T lymphocytes in mediating immune PTR. The development mechanism provides a theoretical basis.

MATERIALS AND METHODS

Research objects and specimen collection:

A total of 12 patients admitted to the Sixth Affiliated Hospital of Xinjiang Medical University from August 2021 to March 2022 were selected and their Peripheral Blood Mononuclear Cell (PBMC) samples were included in this study, of which 4 PBMC samples from volunteers included in normal control group, 4 patients included in the platelet transfusion effective group and 4 patients included in the platelet transfusion ineffective (PTR) group. The three groups include 1 male and 3 females, and their ages were 40.25±1.708, 41±2.582 and 41.75±1.708 y. All patients in this study gave informed consent and this study was approved by the Ethics Committee of our hospital.

Detection of differential expression of lncRNA and mRNA in PBMC:

Agilent Human lncRNA V6 (4×180K, Design ID: 084410) chips were used to detect the expression levels of mRNA and lncRNA. Agilent Human lncRNA V6 (4×180K, Design ID: 084410) chip was used, NanoDrop® ND-2000 (Thermo Scientific) was used for quantitative analysis of sample RNA and RNA integrity was determined by Agilent Bioanalyzer 2100 (Agilent Technologies). After the qualification of RNA in the quality inspection, the labeling of the sample, hybridization of the chip and the elution refer to the chip standard process was initiated. After the total RNA was reverse-transcribed into double-stranded complementary Deoxyribonucleic Acid (cDNA), Cyanine-3-CTP (Cy3) was used to label the synthesized complementary RNA (cRNA), the labeled cRNA was hybridized with the chip and after elution, the original image was obtained by scanning with Agilent Scanner G2505C (Agilent Technologies).

Data analysis:

The original image was processed by feature extraction software (version 10.7.1.1, Agilent Technologies) to obtain the original data. The data were then subjected to quantile normalization and subsequent processing using GeneSpring software (version 14.8, Agilent Technologies). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed on the differential genes to determine the biological functions and pathways in which the differential genes were mainly involved. Further combine the queries of National Center for Biotechnology Information (NCBI), StarBase and TargetScan databases to screen out key target genes and target pathways. Unsupervised hierarchical clustering is used to process differential genes and the expression status of differential genes between different samples is displayed in a heat map.

RESULTS AND DISCUSSION

Differential expression and co-expression analysis of lncRNA and mRNA in peripheral blood PBMC of patients with PTR was explained here. Agilent expression profiling chips were used in order to detect the changes in expression levels of mRNA and lncRNA in T lymphocytes of patients with PTR. The results showed that there were a total of 720 differentially expressed mRNAs and 1719 differentially expressed lncRNAs in the normal control group *vs.* platelet transfusion effective group, a total of 4254 differentially expressed mRNAs and 12491 differentially expressed lncRNAs in the normal control group *vs.* platelet transfusion ineffective group. A total of 1806 differentially expressed mRNAs and 6216 differentially expressed lncRNAs were found in the platelet transfusion effective group *vs.* platelet transfusion ineffective group. The analysis results are shown in Table 1, fig. 1 and fig. 2.

Through GO enrichment analysis, the differentially expressed genes related to the regulation of T lymphocyte differentiation and platelet apoptosis were annotated in three aspects of biological process, cellular components, molecular functions, p21-Activated Kinase 2 (PAK2), Ras Protein Specific Guanine Nucleotide Releasing Factor 2 (RASGRF2), Colony-Stimulating Factor-1 (CSF1), Ras-related protein 1A (Rap1A), Arrestin Beta 1 (ARRB1) and Mitogen-Activated Protein Kinase Kinase Kinase 7 (MAP3K7), etc. were initially identified. The gene was used as a candidate gene and the co-expression relationship between the target gene Rap1A and lnc-Transactive Response Deoxyribonucleic Acid

Binding Protein 2 (lnc-TARDBP-2) was determined by combining the NCBI database gene function query, literature query and lncRNA-mRNA co-expression analysis. The analysis results are shown in Table 2 and Table 3.

GO enrichment analysis was performed on the differentially expressed genes in terms of biological process, cellular components and molecular functions. The Z_vs._D differentially expressed gene GO enrichment analysis results annotated T Cell Receptor (TCR) complexes, negative regulation of TCR signaling pathways, TCR Variable, Diversity and Joining (VDJ) recombination, alpha-beta T cell differentiation and modulation of the immune response, etc. where Z represents the normal control group and D represents the platelet transfusion effective group, as shown in fig. 3.

The differentially expressed lncRNAs and mRNAs were analyzed for co-expression. If the p value of the correlation coefficient of the expression values of lncRNAs and mRNAs was p≤0.05 and the absolute value of the correlation was ≥0.8, it was considered to be correlated. The expression values were used for unsupervised cluster analysis to draw heat map. For each differentially expressed lncRNAs, the co-expressed coding genes were calculated and according to GO and KEGG pathway enrichment and the application of NCBI database, it was predicted that Rap1A and lnc-TARDBP-2 had a co-expression relationship, as shown in fig. 4 and fig. 5.

TABLE 1: STATISTICS OF DIFFERENTIALLY EXPRESSED GENES

Group name	Type	Up-regulated	Down-regulated	Total
comp1_Z_*vs._*D_FC2P0.05	Coding	432	288	720
comp1_Z_*vs._*D_FC2P0.05	lncRNA	892	827	1719
comp2_Z_*vs._*B_FC2P0.05	Coding	2501	1753	4254
comp2_Z_*vs._*B_FC2P0.05	lncRNA	5059	7432	12491
comp3_D_*vs._*B_FC2P0.05	Coding	990	816	1806
comp3_D_*vs._*B_FC2P0.05	lncRNA	1369	4847	6216

Note: Z represents the normal control group, D represents the platelet transfusion effective group; B represents the platelet transfusion ineffective group (PTR) and FC: Fold Change
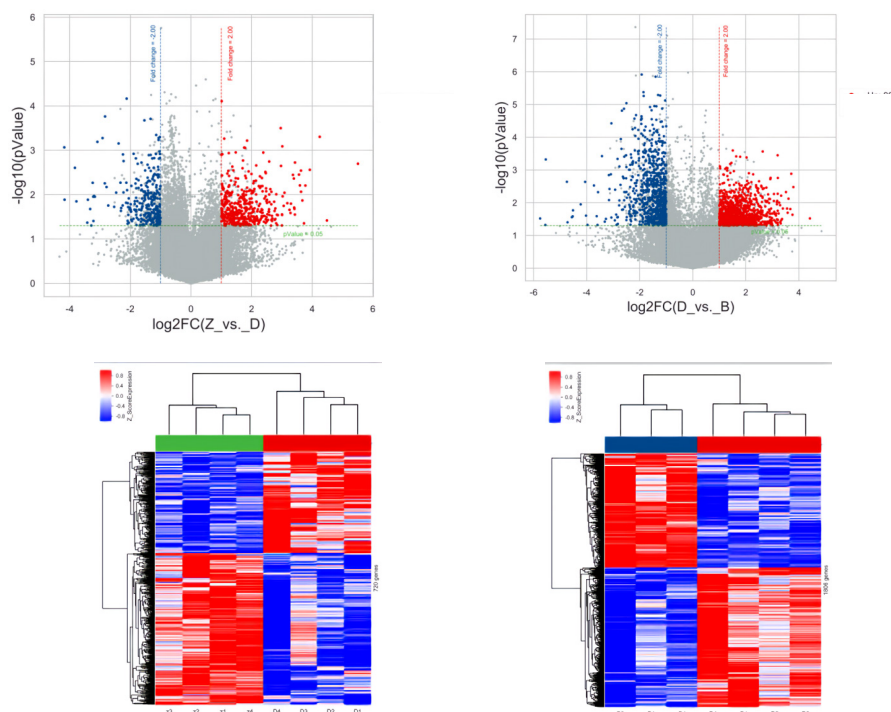

Fig. 1: Z_*vs._*D and D_*vs._*B Differentially expressed genes (mRNA) volcano map and cluster analysis heat map
Note: In Z_*vs._*D, (•) 432 up regulated genes and (•) 288 down regulated genes and in D_*vs._*B, (•) 990 up regulated genes and (•) 816 down regulated genes are present, where Z represents the normal control group, D represents the platelet transfusion effective group and B represents the platelet transfusion ineffective (PTR) group; FC: Fold Change
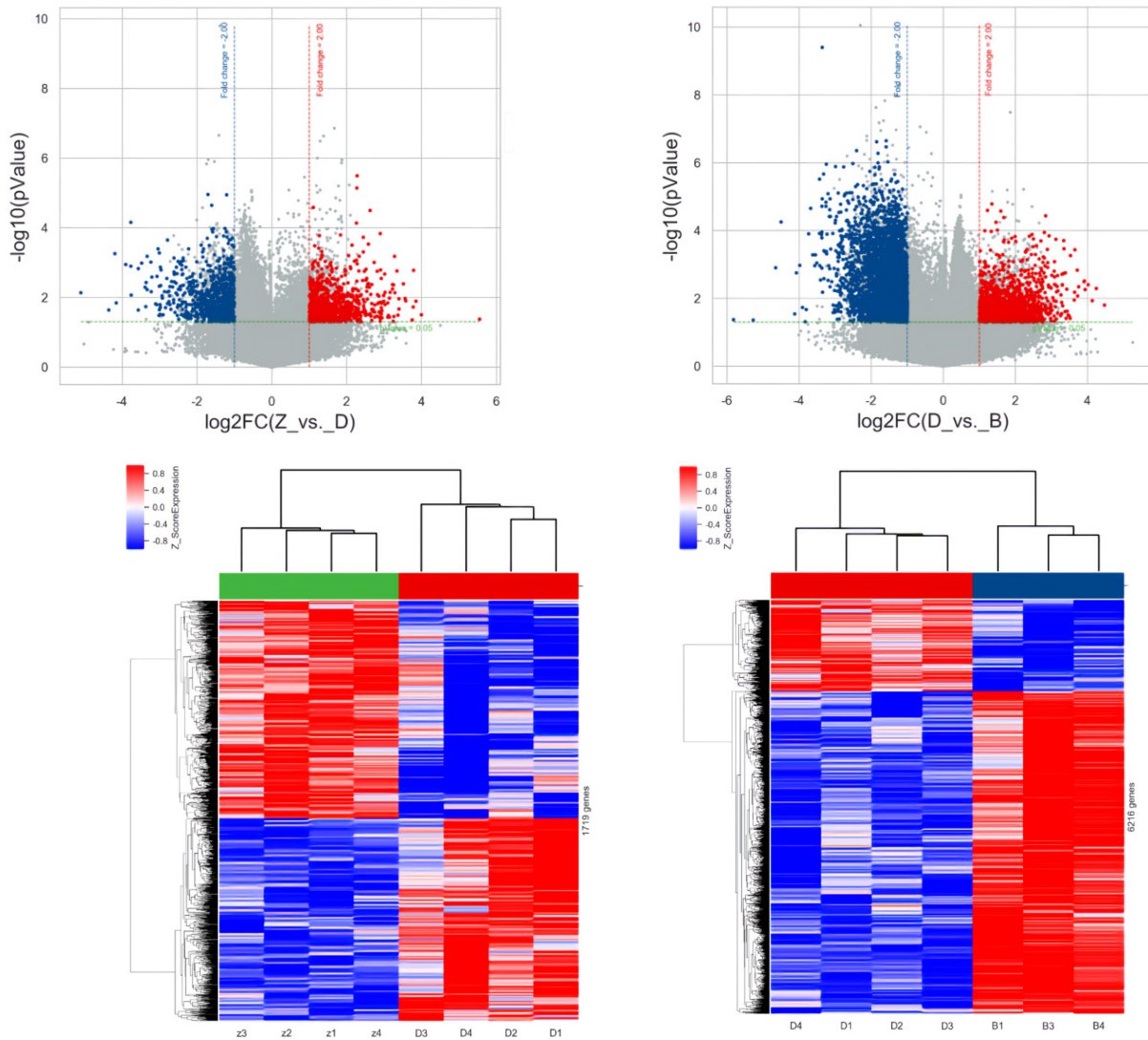
Fig. 2: Z_*vs.*_D and D_*vs.*_B Differentially expressed genes (lncRNA) volcano map and cluster analysis heat map
Note: In Z_*vs.*_D, (•) 892 up regulated genes and (•) 827 down regulated genes and in D_*vs.*_B, (•) 1369 up regulated genes and (•) 4847 down regulated genes are present

TABLE 2: DIFFERENTIALLY EXPRESSED mRNA SCREENING

Differentially expressed genes	Gene ID	p-value (D_*vs.*_B)	FC (D_*vs.*_B)	p-value (Z_*vs.*_B)	FC (Z_*vs.*_B)
PAK2	5062	0.00987	2.59682	0.00119	3.21288
RASGRF2	5924	0.00698	-5.1452	0.02928	-2.1382
CSF1	1435	0.00136	-8.4982	9E-05	-9.7822
RAP1A	5906	0.03472	2.89331	0.00234	3.45761
ARRB1	408	0.0111	2.83555	0.02006	2.155
MAP3K7	6885	0.00441	2.89151	0.00018	3.40379

TABLE 3: LncRNA AND mRNA CO-EXPRESSION ANALYSIS

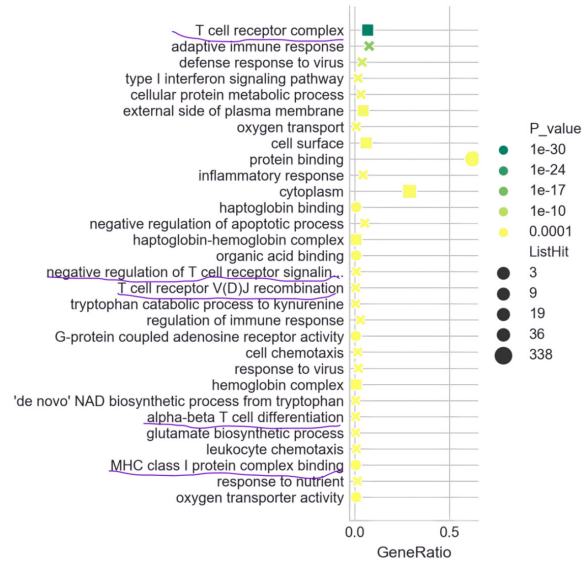| LncRNA	mRNA	Co-expression	p value	Primary ID
lnc-TARDBP-2	Rap1A	0.918	0.0035	lnc-TARDBP-2

Fig. 3: Z_vs._D GO function annotation of top 30 entries, (●) Function; (✱) Process and (■) Component
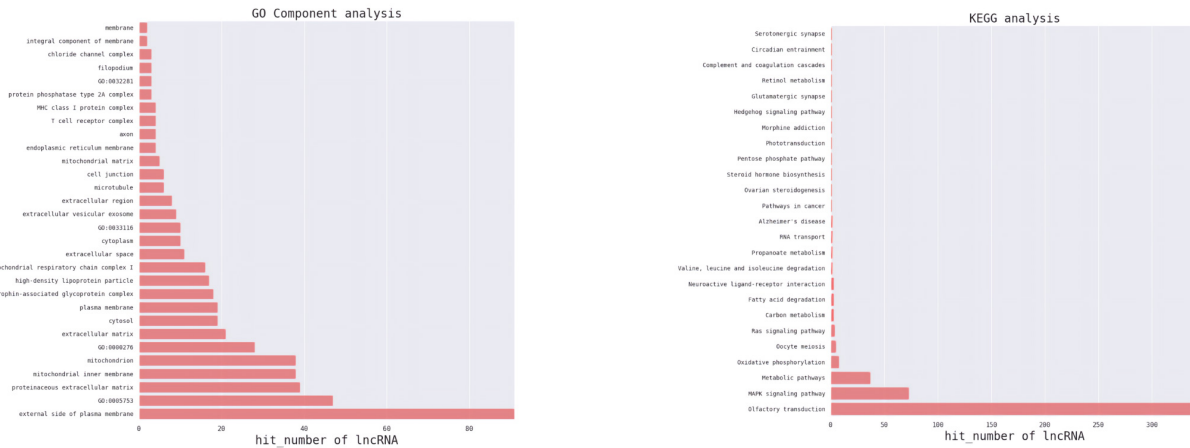

Fig. 4: GO and KEGG pathway enrichment analysis for the prediction of lncRNA function
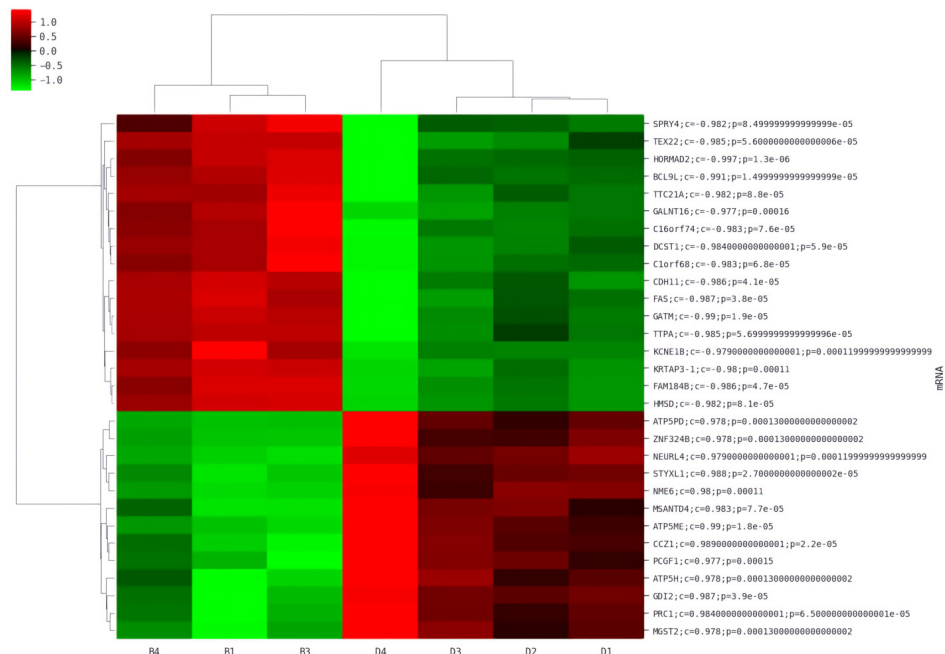

Fig. 5: D_*vs._*B lnc-TARDBP-2:3 co-expressed mRNA cluster analysis of top 30 genes

Prediction analysis of the micro Ribonucleic Acid (miRNA) was reported here. To predict the miRNA interacting with Rap1A StarBase database and TargetScan database was used and to predict the miRNA that can interact with lnc-TARDBP-2 RegRNA 2.0 database was used, and further querying the references corresponding to the miRNA to confirm that miR-4739 is the predicted miRNA.

This study was the first to elucidate the differences in gene expression profiles between PBMCs of patients with immune PTR, healthy people and people with effective platelet transfusion through gene chips and found that there were significant gene differences between patients with immune PTR and the other two groups. Through GO enrichment analysis, the results showed that T cells were closely related to immune PTR. At the same time, PAK2, RASGRF2, CSF1, Rap1A, ARRB1, MAP3K7 and other genes were initially identified as candidate genes and further combined with NCBI database gene function query, literature query and lncRNA-mRNA co-expression analysis to determine the co-expression of target genes Rap1A and lnc-TARDBP-2 relation.

Rap1 plays a key role in TCR signal transduction and is expressed in cells by one of its two isoforms: Rap1A and Ras-related protein 1B (Rap1B)[8]. The expression of Rap1A is required for the fine-tuning of the immune response[9]. Su *et al.* studied the effect of Rap1A deficiency on the number of T cells[10]. When Rap1 was deficient, T cells in Peripheral Lymph Nodes (PLN) decreased significantly and T cells in blood increased. In addition, studies have found that the low expression of Rap1A activity was detected in resting T lymphocytes. When T lymphocyte surface receptors are activated by antigens, the enhancement of Rap1A activity can be clearly detected and Rap1A activation enhances TCR acceptance. Signaling and transduction capabilities further promote the ability of T lymphocyte proliferation and differentiation[11]. In summary, Rap1A has been confirmed to be closely related to the activation of T lymphocytes. At the same time, we found that the expression of Rap1A increased in patients with immune PTR, indicating that Rap1A may play a role in immune PTR by regulating T lymphocytes.

LncRNA plays an important role and has multiple functions in biological processes such as cell function, growth, development and disease development[12], including the regulation of T lymphocyte immunity and platelet biological processes[13]. Ghafouri-Fard *et al.* found that in stage III immature T cells, Thymic T lymphocyte-natural cytotoxicity triggering Receptor 1 (Thy-ncR1) activates the adjacent surface differentiation antigen CD gene cluster by degrading mRNA-Microfibril Associated Protein 4 (MFAP4), indicating that lncRNAs are involved in the differentiation and maturation of T cells[14]. Liu *et al.* found that lncRNA may affect the activation of T cells and the proliferation of CD4+ and CD8+ cells by inhibiting miRNA[15,16]. Another study found that silencing lncRNA-Antisense Non-coding RNA in the Inhibitor of the cyclin D-dependent kinases (INK4) Locus (ANRIL) can inhibit the activation of T cells[17]. These studies suggest that lncRNAs play an important role in the differentiation and maturation of immune T lymphocytes in the body. Different lncRNAs can regulate the biological process of T lymphocytes through the corresponding target gene mRNA. It is closely related to death and has a regulatory effect on platelets. Vesicle-Associated Membrane Protein 8 (VAMP8) has an important function in the platelet activation pathway and lnc-Ubiquitin Specific Peptidase 39 (lnc-USP39-1) can regulate the protein-coding gene VAMP8 in cis, thereby regulating platelet activation[18]. Moreover, platelets cannot perform physiological functions without the aggregation of actin filaments lnc-Neuroblastoma Breakpoint Family 9-5:1 (lnc-NBPF 9-5:1), lnc-Spindle Pole body Component 25-6:1 (lnc-SPC25-6:1), lnc-Nuclear factor-kappa-B-Repressing Factor-1 (lnc-NKRF-1) and lnc-High Mobility Group Nucleosome binding domain 5-3:1 (lnc-HMGN 5-3:1) closely interact with platelet actin protein filament aggregation and they can delay the activation of platelets and thus help to prolong the life of platelets[19]. As a kind of lncRNA, lnc-TARDBP-2 has been detected to be up-regulated in immune PTR, but its regulatory relationship with T cells and its role in immune PTR needed further study.

In addition, we combined StarBase database and TargetScan database to predict miRNAs interacting with Rap1A. It was determined that miR-4739 may participate in the occurrence of immune PTR by regulating T lymphocytes. A variety of miRNAs have been identified to play key roles in T lymphocyte development, differentiation and function[20]. Dolz *et al.* found that miR-17-92 is closely related to the proliferation of CD4+ T cells[21]. Veiga *et al.* found

that miR-155 is a key positive regulator of regulatory T cells (Tregs)[22]. At the same time, studies have shown that miRNAs play an important role in hematopoietic differentiation, which is related to the cellular immune response leading to ITP[23]. Zuo *et al.* found that miR-4739 significantly increased in ITP[24]. Therefore, miR-4739 may participate in the occurrence of PTR by regulating T cell immunity and miR-4739 may become a new target of PTR biomarkers in the future.

To sum up, this study analyzed the gene expression differences between PBMC of immune PTR population, healthy population and platelet transfusion effective population by gene chip technology, selected a batch of valuable target genes and clarified their related functions. Molecular functions found that T lymphocytes may play an important role in the occurrence of immune PTR. Moreover, in PTR patients, lnc-TARDBP-2 may affect the proliferation and differentiation of T lymphocytes through miR-4739 targeting Rap1A gene regulation to promote disease occurrence. Our study provides a theoretical basis for revealing the mechanism of T lymphocytes in the development of immune PTR and also provides valuable genes and directions for further research on the markers of immune PTR. However, the conclusions proposed in this paper need to be confirmed by further molecular biology experiments.

Acknowledgments:

Conflict of interests:

The authors declared no conflict of interest.

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