Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Investigation and analysis of a 5'UTR single-base insertion mutation leading to enhanced transcriptional activity of the α-1,4-galactosyltransferase gene and potential expression of a novel P1PK phenotype antibody

Xi Qi
Nanjing Medical University
Ziqing Zhu
Yuxuan Sha
Yi Qin
Nanjing Medical University
Yayun Sha
Nanjing Medical University
Xiaoli Zhu
Nanjing Medical University
Junling Han
Nanjing Medical University
Ziang Gao
The First Affiliated Hospital of Soochow University
Lingbao Gao
gaolingbao@njmu.edu.cn

Nanjing Medical University

Article

Keywords: 5' untranslated region, Insertion mutation, α-1,4-galactosyltransferase, P1PK blood group phenotype, Irregular antibody, Gene sequencing

Posted Date: May 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9264891/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Investigation and analysis of a 5′UTR single-base insertion mutation leading to enhanced transcriptional activity of the α-1,4-galactosyltransferase gene and potential expression of a novel P1PK phenotype antibody

Authors

Qi Xi[1], Zhu Ziqing[2], Sha Yuxuan[2], Qin Yi[1], Sha Yayun[1], Zhu Xiaoli[1], Han Junling[1], Gao Ziang[3]*, Gao Lingbao[1]**

Affiliations

1 Department of Transfusion Medicine, The Affiliated Taizhou People's Hospital of Nanjing Medical University, Taizhou 225300, Jiangsu, China

2 College of Health and Nursing, Wuxi Taihu University, Wuxi 214400, Jiangsu, China

3 Department of Otorhinolaryngology, The First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu, China

*Corresponding author: Gao Ziang, E-mail: gaoziang@suda.edu.cn

**Corresponding author: Gao Lingbao, E-mail: gxq1818@163.com

Abstract

Objective To investigate the underlying cause of minor-side incompatibility observed in crossmatching tests performed via the microcolumn gel anti-human globulin card method in a clinical patient. **Methods** Serological and molecular biological assays were conducted to detect the recipient’s red blood cells (RBCs) and the donor’s plasma, respectively. **Results** Minor-side incompatibility was attributed to a blood group-specific antibody in the donor’s plasma that specifically targeted the recipient’s RBCs. This antibody was identified as a mixed IgM/IgG type, and its specificity could not be determined using conventional serological approaches. Random sampling of RBC samples from an in-hospital population was performed with the donor’s plasma, leading to the identification of two additional probands exhibiting identical serological reactivity. Absorption-elution tests confirmed that all three probands expressed an identical novel RBC antigen. Based on rare blood group genotyping results, the antibody was preliminarily excluded as anti-C, -c, -E, -e, -Lw^a,

-Lw^b, -Fy^a, -Fy^b, -Jk^a, -Jk^b, -M, -N, -S, -s, -Mur, -Sc2, -Di^a, -Di^b, -Wr^a, -Wr^b, -K, -k, -Kp^a, -Kp^b, -Co^a, -Co^b, -Do^a, -Do^b, -Lu^a, -Lu^b, -Yt^a or -Yt^b. PacBio third-generation sequencing of the α-1,4-galactosyltransferase (*A4GALT*) gene revealed no genetic variations in the donor, whereas all three probands carried an identical homozygous single-base insertion mutation (-54_-53insG) in the 5′ untranslated region (5′UTR). No mutations were detected in the *B3GALNT1* gene in any of the four subjects. Dual-luciferase reporter assays verified that the mutant *A4GALT* vector exhibited significantly higher binding affinity with transcription factors compared with the wild-type vector. **Conclusion** The donor's plasma contains an uncharacterized blood group antibody, and the three probands with identical serological reactivity share a common molecular basis for the differential expression of the *A4GALT* gene. We hypothesize that this uncharacterized antibody may target a novel phenotype of the P1PK blood group system.

Keywords: 5′ untranslated region; Insertion mutation; α-1,4-galactosyltransferase; P1PK blood group phenotype; Irregular antibody; Gene sequencing

Introduction

Clinical transfusion compatibility testing relies on crossmatching assays to ensure that recipients receive allogeneic donor RBC components free of antigen-antibody reactions, which remain a leading cause of transfusion-related adverse events[1]. As of October 2024, the International Society of Blood Transfusion (ISBT) has characterized 366 human RBC antigens, which are classified into 47 blood group systems and three blood group collections. In China, most clinical RBC transfusions only achieve antigen matching for the ABO blood group system and a small number of antigens in the Rh blood group system. Compatibility assessment therefore remains primarily dependent on the detection of blood group alloantibodies in both recipients and donors.

In clinical practice, we encountered a case of isolated minor-side incompatibility (donor plasma mixed with recipient RBCs) in crossmatching conducted via the microcolumn gel anti-human globulin method. Re-crossmatching with alternative

donors and recipients yielded fully compatible results for both major and minor sides. Meanwhile, the direct antiglobulin test (DAT) of the recipient's RBCs was negative, and initial irregular antibody screening of the donor's plasma also returned negative results; retesting with reagent RBCs from different batches exhibited weak positivity. To clarify the specificity of this blood group antibody and its underlying molecular mechanism, we conducted a comprehensive investigation combining serological, proteomic and molecular biological approaches.

2. Materials and methods

2.1 Clinical data

The recipient was a female patient admitted to the Department of Gynecology with a diagnosis of uterine fibroids and a previous diagnosis of systemic lupus erythematosus. Clinicians requested 2 units of O RhD-positive leukoreduced RBCs for anemia correction. Rechecking in our laboratory confirmed that the recipient's ABO and RhD blood types were consistent with the requested type. A random donor blood bag (code suffix: 113920) was selected for initial crossmatching using the microcolumn gel anti-human globulin card indirect antiglobulin test (IAT), which showed 4+ agglutination indicating minor-side incompatibility. The donor's irregular antibody screening and the DAT of the recipient's saline-washed RBCs were both negative. Re-crossmatching with another donor blood bag showed full compatibility for both major and minor sides, and the blood bag was issued for transfusion. No adverse reactions occurred during transfusion, and transfusion efficacy was evaluated as effective in the medical records.

The donor blood bag with initial minor-side incompatibility was labeled as O RhD-positive, collected by the local central blood station from a healthy young parous female voluntary blood donor. After re-compatibility testing, this blood bag was issued to another inpatient in our hospital with fully compatible results; no transfusion adverse reactions occurred, and transfusion efficacy was also evaluated as effective.

2.2 Serological assays

Irregular antibody screening was performed using three sets of antibody screening RBC kits of different batches from the same manufacturer (Shanghai Blood Bioengineering Co., Ltd.). Antibody specificity identification was conducted using three sets of panel RBC kits of different specifications and batches from two manufacturers, including two batches of 10 reagent RBCs each (Shanghai Blood Bioengineering Co., Ltd.) and one set of 16 reagent RBCs (Jiangyin Libo Biotech Co., Ltd.). The expressed blood group antigens included C, c, E, e, Le^a, Le^b, Jk^a, Jk^b, M, N, S, s, Mur, Fy^a, Fy^b, K, k, Kp^a, Kp^b, Co^a, Co^b, Do^a, Do^b, Lu^a and Lu^b.

ABO and RhD blood typing were performed using microcolumn gel cards for ABO/RhD and standard ABO RBC kits (Changchun Boshun Biotech Co., Ltd.). Rh subtyping (C/c/E/e) was conducted using microcolumn gel Rh antigen typing cards (Jiangyin Libo Biotech Co., Ltd.). P1/P2 serotyping was performed using an IgM monoclonal anti-P1 reagent (Shanghai Blood Bioengineering Co., Ltd.).

The donor's plasma (blood bag 113920) was treated with 2-mercaptoethanol (2-Me, Shanghai Blood Bioengineering Co., Ltd.) and then reacted with the recipient's RBCs in microcolumn gel anti-human globulin (anti-IgG+C3d) cards (Jiangyin Libo Biotech Co., Ltd.). Absorption (37 °C for 1 h) and acid elution (Shanghai Blood Bioengineering Co., Ltd.) tests were performed using the recipient's saline-washed RBCs to treat the donor's plasma. The DAT of the recipient's RBCs after absorption was detected using microcolumn gel anti-human IgG/C3d typing cards (Jiangyin Libo Biotech Co., Ltd.), and irregular antibody screening and identification were performed on the eluate. The donor's plasma before and after absorption was sent for proteomic analysis (Beijing Bio-Tech Pack Technology Co., Ltd.).

2.3 Screening for probands with identical serological reactivity

Residual clinical EDTA-K2 anticoagulated whole blood samples from O RhD-positive inpatients in our hospital were randomly sampled. Saline-washed RBCs were prepared, and DAT-negative samples were confirmed using microcolumn gel anti-human globulin (anti-IgG+C3d) cards (Jiangyin Libo Biotech Co., Ltd.), followed by reaction with the donor's plasma. Samples with identical serological

reactivity to the original recipient were sent for fluorescent PCR-based rare blood group genotyping (Jiangyin Libo Biotech Co., Ltd.) and PacBio third-generation blood group gene sequencing (Xi'an Haorui Biotech Co., Ltd.).

2.4 Molecular biological assays

Fluorescent PCR-based genotyping was used to detect rare blood group antigens, including C/c/E/e, Lw^a/Lw^b, Fy^a/Fy^b, Jk^a/Jk^b, M/N/S/s, Mur, Sc2, Di^a/Di^b, Wr^a/Wr^b, K/k, Kp^a/Kp^b, Co^a/Co^b, Do^a/Do^b, Lu^a/Lu^b and Yt^a/Yt^b. Genomic DNA was extracted from the peripheral blood leukocytes of the donor and probands using a commercial kit. The *A4GALT* and *B3GALNT1* genes were amplified by PCR, and PacBio third-generation sequencing was performed to analyze the full-length gene sequences. SnapGene software was used for sequence alignment and open reading frame (ORF) prediction.

2.5 Dual-luciferase reporter assay

Wild-type (WT) and mutant (Mut) fragments of the *A4GALT* gene 5′UTR were amplified and cloned into the pGL3-basic luciferase reporter vector. HEK-293T cells were co-transfected with the recombinant vectors and the pRL-TK renilla luciferase vector (used as an internal reference). At 48 h post-transfection, firefly and renilla luciferase activities were detected using a dual-luciferase reporter assay kit (Wuhan Boyuan Biotech Co., Ltd.). Relative luciferase activity was calculated as the ratio of firefly luciferase activity to renilla luciferase activity.

3. Results

3.1 Serological findings

Both the donor and the recipient were serotyped as O RhD-positive, CcEe and P1-positive (P1+). Retesting of the recipient’s plasma with irregular antibody screening RBCs of different batches from the same manufacturer showed negative results in all IAT assays. Retesting of the donor’s plasma showed weak agglutination with only one reagent RBC in one set of screening kits. Antibody identification with panel RBCs of different manufacturers and specifications showed distinct weak positive reaction patterns, and the antibody specificity could not be determined by

comparison with the antigen profile of the panel RBCs. Enhancing the reaction at 4 °C using the test tube method or treating panel RBCs with dithiothreitol (DTT) slightly increased the agglutination titer (0.5+/1+) of positive cells but did not alter the original reaction pattern.

The donor's plasma before and after 2-Me treatment (incubated at 37 °C for 40 min) was reacted with the recipient's saline-washed, DAT-negative RBCs in microcolumn gel anti-human globulin cards and saline cards. In the anti-human IgG/C3d typing cards, the agglutination titer was 3+_dp/4+_dp (dp = double population), confirming that the antibody was a mixed IgM/IgG type (Fig. 1).

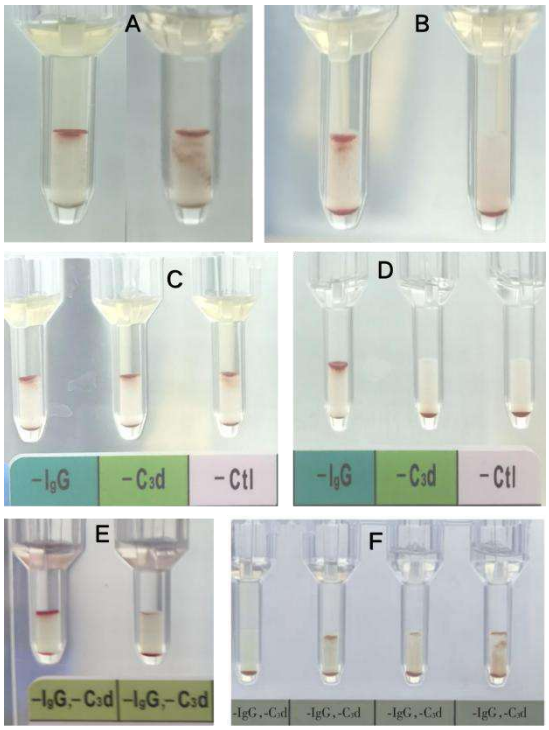


Figure 1. Serological Testing

（A） Reactions in anti-human globulin cards. The left tube shows an agglutination titer of "4+" before 2-mercaptoethanol (2-Me) treatment, and the right tube shows an agglutination titer of "3+" after 2-Me treatment. （B） Reactions in saline cards. The left tube shows an agglutination titer of "3+ and dcp" before 2-Me treatment, and the right tube shows a "negative" agglutination titer after 2-Me treatment. （C） Donor's plasma with recipient's triple-washed RBCs at room temperature; （D） Recipient's triple-washed RBCs after absorbing donor's plasma; Ctl, Quality control. （E） Left tube, Agglutination titer of the eluate with recipient's triple-washed RBCs was 4+dp; Right tube, Agglutination titer of the eluate with standard O-type RBCs was 0+dp; dp was considered to be derived from RBC fragments. （F） 1 and 2, Reaction results of eluates from Proband 2 and 3 RBCs with Proband 1 RBCs, respectively; 3 and 4,

Reaction results of eluates from Proband 2 and 3 RBCs with Proband 1 RBCs after application of low-ionic strength enhancer, respectively.

After absorption of the donor's plasma with the recipient's RBCs at 37 °C for 1 h, only the anti-human IgG reaction well was positive (3+_dp) in the anti-human globulin IgG/C3d typing cards, and all other wells were negative (Fig. 2).

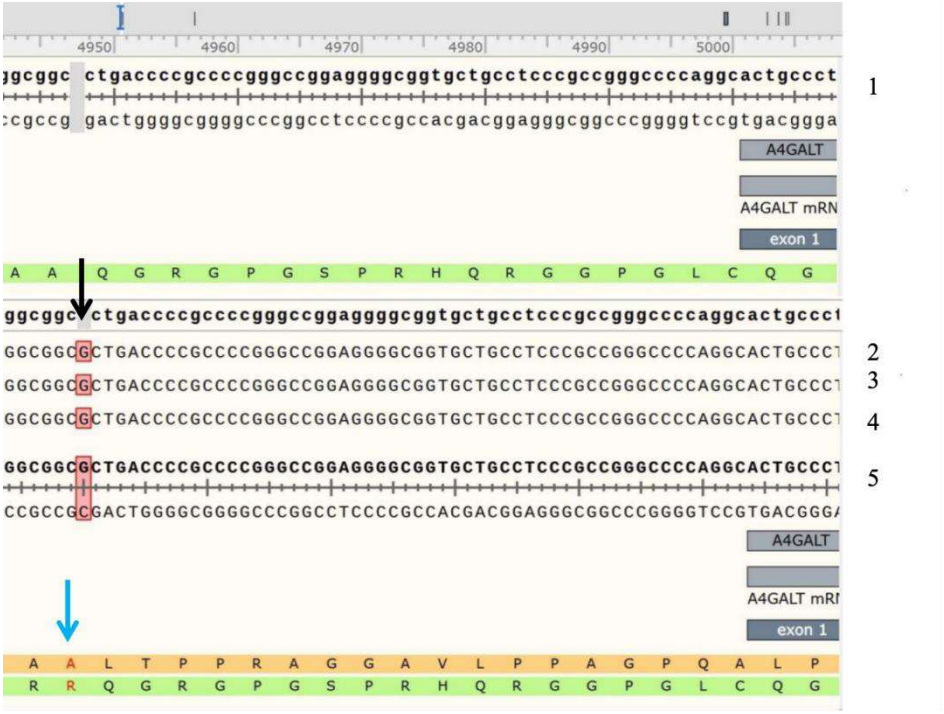


Figure 2. Predicted open reading frame (ORF) changes caused by the identical 5′UTR variation in Probands 1, 2 and 3 via sequence alignment and analysis with Snapgene software.

（1）Reference sequence of the *A4GALT* gene; （2） *A4GALT* gene sequencing fragment of Proband 1 (only one haplotype); （3~4） *A4GALT* gene sequencing fragments of Proband 2 (two different haplotypes with the same single-base insertion mutation); （5）*A4GALT* gene sequencing fragment of Proband 3 (only one haplotype). The black large arrow indicates a single-base insertion mutation (-54_-53insG), and the blue small arrows indicate an additional ORF (orange) and nucleotide substitutions (red letters) in the original ORF (green) compared with the ORF of the reference sequence.

In the anti-human globulin cards, the eluate from the absorbed RBCs reacted with the recipient's saline-washed RBCs with an agglutination titer of 4+_dp, while no reaction was observed with standard O RhD-positive RBCs (Fig. 3).



Figure 3. Different ORF changes caused by the same single nucleotide variant (SNV) in different haplotypes of Proband 2 via sequence alignment and analysis with Snapgene software.

ORFs predicted from two different haplotypes of Proband 2; （A）Corresponding to 3 in Figure 2; （B）Corresponding to 4 in Figure 2.

The irregular antibody screening and identification results of the eluate were essentially consistent with those of the donor's plasma, and the specificity remained undetermined. These results indicated that the IgG fraction of the uncharacterized antibody could be absorbed and eluted by the recipient's RBCs, and the eluted antibody exclusively reacted with the recipient's RBCs expressing the novel antigen.

3.2 Proteomic analysis

Differential protein analysis was performed on the donor's plasma before (BF) and after (AF) absorption with the recipient's RBCs. A total of 366 proteins and 3941 peptides were identified, with 123 proteins downregulated in AF compared with BF. Gene Ontology (GO) functional enrichment analysis showed that these differentially expressed proteins were significantly enriched in biological processes including the immune system process, immune response, humoral immune response and blood coagulation—consistent with the characteristics of antigen-antibody reactions.

3.3 Identification of additional probands with identical serological reactivity

A total of 50 residual clinical blood samples from O RhD-positive inpatients (30 samples from healthy pregnant women for blood preparation and 20 samples from clinical immune hemolysis screening) were randomly sampled and reacted with the donor's plasma. Two additional probands with identical serological reactivity to the original recipient were identified. The agglutination intensity of the three probands with the donor's plasma in anti-human globulin cards was ranked as Proband 1 (original recipient) > Proband 3 > Proband 2. Proband 2 was a middle-aged female patient with moderate anemia, a history of childbirth, no transfusion history, and a previous diagnosis of chronic myeloid leukemia with the BCR-ABL1 fusion gene t(9;22)(q34.1;q11.2). Proband 3 was an elderly male patient with moderate anemia, no transfusion history, and a diagnosis of viral hepatitis cirrhosis. Serotyping with an anti-P1 reagent showed that Proband 2 was P1-positive and Proband 3 was P2-negative (P1-).

Absorption (4 °C overnight) and heat elution (56 °C) were performed on the donor's plasma using the saline-washed, DAT-negative RBCs of Proband 2 and Proband 3, respectively. The eluates were reacted with the saline-washed RBCs of Proband 1, and positive reactions were only observed in microcolumn gel anti-human globulin cards (Fig. 4).

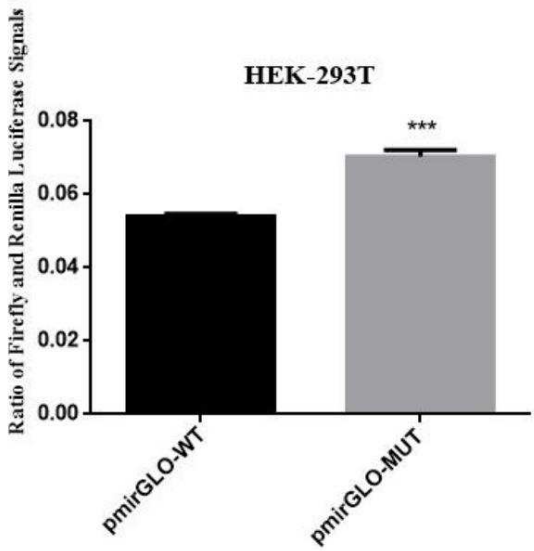


Figure 4. Comparison of fluorescent signal expression in HEK-293T cell lines between wild-type and mutant vectors constructed by the dual-luciferase reporter assay.

The ordinate represents the ratio of firefly luciferase to Renilla luciferase signals;

P<0.05 compared with the wild-type vector.

3.4 Rare blood group genotyping results

Rare blood group genotyping was performed on the donor, Proband 1 and Proband 2 (Proband 3 was not screened at this stage and thus not tested). The results showed that all tested antigens were positive for the donor and the two probands, with no antigen deficiency detected (Table 1).

Table 1. Rare blood group genotyping results of the donor and Probands 1 and 2

	C	c	E	e	Lwa	Lwb	Fya	Fyb	Jka	Jkb	M	N	S	s	Mur	Sc2	Dia	Dib	Wra	Wrb	K	k	Kpa	Kpb
D*	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+	-	+	-	+	-	+
P1	+	+	+	+	+	-	+	+	+	+	-	+	-	+	-	-	-	+	-	+	-	+	-	+
P2	+	+	-	+	+	-	+	-	-	+	+	+	-	+	-	-	-	+	+	+	-	+	-	+

Note： + indicates positive antigen genotyping, - indicates negative antigen genotyping.

3.5 Gene sequencing results

PacBio third-generation sequencing— a recommended high-throughput sequencing technology for clinical blood use molecular research—revealed no mutations in the *A4GALT* gene of the donor, with the predicted genotype *A4GALT*01/*A4GALT*01. Proband 1 harbored a heterozygous c.903C>G mutation in Exon 3 of the *A4GALT* gene, while Proband 2 and Proband 3 both carried a homozygous c.903C>G mutation in Exon 3. In addition, Proband 2 had heterozygous c.109A>G and c.987G>A mutations in Exon 3. Although these mutation sites have been recorded in the National Center for Biotechnology Information (NCBI) database, the haplotype with the coexistence of these three mutation sites has not been reported and was thus predicted as *A4GALT*01.02.NEW—a novel haplotype related to the P1PK blood group system with potential clinical blood use significance. The genotypes of Proband 1, Proband 2 and Proband 3 were predicted as *A4GALT*01/*A4GALT*01.NEW, *A4GALT*01.02.NEW/*A4GALT*02.NEW and *A4GALT*02.NEW/*A4GALT*02.NEW, respectively. No mutations were detected in the *B3GALNT1* gene in any of the four

subjects, with the predicted genotype *GLOB*01/*GLOB*01 for all—indicating the normal expression of the GLOB blood group system gene, which is closely related to clinical blood transfusion compatibility.

Sequence alignment of the *A4GALT* gene using SnapGene software in compliance with molecular data analysis norms revealed that all three probands harbored an identical homozygous single-base insertion mutation (-54_-53insG) in the 5′UTR near Exon 1 (Fig. 5)—a novel mutation site not previously reported in clinical blood use research related to the P1PK blood group system. The sequencing results of the *A4GALT* gene 5′UTR of the three probands were submitted to NCBI with the accession numbers PQ319855, PV577986 and PV630325, respectively, which provides a genetic data basis for subsequent clinical blood use research on this mutation. ORF prediction showed that this single nucleotide variant (SNV) altered the original ORF in the region near the upstream of Exon 1. Proband 2 had two different haplotypes, and the same SNV caused distinct ORF changes in different haplotypes (Fig. 6), which may account for the different agglutination intensities of the three probands with the uncharacterized irregular blood group antibody—a key finding for explaining serological reaction differences in clinical blood use detection.

3.6 Dual-luciferase reporter assay results

Dual-luciferase reporter assays showed that the relative luciferase activity of the mutant *A4GALT* 5′UTR vector was significantly higher than that of the wild-type vector in HEK-293T cells ($P < 0.05$, Fig. 7), indicating that the -54_-53insG mutation in the *A4GALT* gene 5′UTR significantly enhanced the transcriptional activity of the gene by increasing its binding affinity with transcription factors. This molecular mechanism is a new finding in the research on the P1PK blood group system, which provides a theoretical basis for explaining the differential expression of blood group antigens in clinical blood use.

BLAST analysis of the *A4GALT* gene sequencing results of Proband 1 in NCBI in compliance with bioinformatics analysis norms identified a silencer sequence (NG_202031.1) on chromosome 22, described as *Homo sapiens* ATAC-STARR-seq

lymphoblastoid silent region 13842 (LOC130067616). Sequence comparison showed that the identified SNV also altered the ORF of this silencer sequence, which may represent the molecular mechanism underlying the enhanced transcriptional activity of the mutant *A4GALT* gene—this finding enriches the genetic regulatory research of blood group genes in clinical blood use.

4. Discussion

Blood group phenotypes are traditionally defined by the serological reactions of antibodies in accordance with clinical blood group typing norms, which can be naturally occurring (predominantly IgM) or induced by alloimmunization (predominantly IgG) [2]. IgM-type irregular blood group antibodies causing transfusion incompatibility are most commonly found in the ABO, MN(S), P1PK and Lewis blood group systems in clinical blood use practice, whereas IgG-type irregular blood group antibodies are predominantly detected in the Rh blood group system [3]—these are key findings for clinical irregular blood group antibody screening and risk assessment. Clinical laboratories can verify the presence of IgM and/or IgG blood group alloantibodies in blood samples using various serological assays specified in the *Clinical Blood Use Technical Specifications*; however, the sensitivity and accuracy of the same assay vary considerably among different laboratories due to methodological limitations and quality control differences [4,5], which is a key challenge in clinical blood use detection. Combined with the serological characteristics of the uncharacterized antibody (mixed IgM/IgG type irregular blood group antibody), we only sent the P1PK and related blood group genes for sequencing after a review of relevant literature [6] in compliance with clinical blood use research norms, and thus the possibility of missing low-frequency antigens of other blood group systems cannot be excluded—this is a common limitation in single-center clinical blood use research.

The P1PK blood group system includes the P1, Pk and NOR antigens, which are encoded by the *A4GALT* gene; the GLOB blood group system includes the P, PX2 and ExtB antigens, encoded by the *B3GALNT1* gene. These two blood group systems

are closely related and are important research objects of rare blood groups in clinical blood use. The P1, Pk and NOR antigens are all glycosphingolipid antigens, synthesized by the stepwise glycosylation of lactosylceramide (CDH) [7]—the synthetic pathway of these antigens is the molecular basis for their clinical blood transfusion compatibility. The NOR antigen is produced by the p.Q211E mutation of the *A4GALT* gene [8], a known mutation site with clinical rare blood group significance. The P1 antigen was first discovered by Landsteiner and Levine in 1927, the Pk antigen in 1951, and the NOR antigen in 1982; these antigens were reclassified into the P1PK blood group system by the ISBT in 2011 [9], which is the standard classification basis for clinical blood group identification. The expression of blood group antigens in the P1PK and GLOB systems is closely linked, but the genetic and regulatory mechanisms governing *A4GALT* gene expression remain incompletely elucidated [10]—the research on these mechanisms is of great significance for improving clinical blood use detection technology.

Most individuals exhibit either the P1 (P1+) or P2 (P1-) phenotype in the P1PK system in clinical population screening; P2 RBCs do not express the P1 antigen, and both P1 and P2 phenotypes express the Pk and P antigens in accordance with clinical serological detection results. In addition, there are three rare autosomal recessive homozygous phenotypes (p, $P1^k$, $P2^k$) and several rare weak variants, among which the p phenotype highly expresses the PX2 antigen [11–13]—these rare phenotypes are key populations for rare blood bank construction in clinical blood use. The NOR antigen-positive phenotype is a polyagglutination phenotype with simultaneous expression of the P1 and Pk antigens [14], which has important clinical transfusion compatibility implications. Naturally occurring irregular blood group antibodies against self-deficient antigens (anti-P1, anti-P, anti-Pk, anti-PX2) can be detected in the serum of individuals with the rare p, $P1^k$ and $P2^k$ phenotypes [15]—these antibodies are important causes of unexplained transfusion incompatibility in clinical blood use. Alloanti-P1 antibodies can be detected in approximately 20% of P2 individuals in clinical screening, mostly as cold-reactive IgM-type irregular blood group antibodies;

most alloanti-P antibodies are mixed IgM/IgG type irregular blood group antibodies, and both the $P1^k$ and $P2^k$ phenotypes produce anti-P antibodies due to P antigen deficiency [16]—these findings are key for clinical transfusion risk assessment of P1PK blood group system variants. The characteristic complex antibody of the p phenotype (P1-Pk-P-, formerly anti-Tj^a) is a naturally occurring polyagglutinating RBC antibody that can cause early neonatal miscarriage or severe hemolytic disease of the fetus and newborn (HDFN) [17]—this antibody is a high-risk irregular blood group antibody in clinical obstetric blood use. In this study, the *B3GALNT1* gene was normally expressed in all three probands and the donor; the donor, Proband 1 and Proband 2 were P1-positive, and Proband 3 was P2-negative, essentially excluding the possibility that the probands exhibit the $P1^k$ or $P2^k$ phenotypes with high Pk antigen expression in accordance with clinical blood group phenotype judgment criteria. Due to the inaccessibility of anti-Pk and anti-P reagents in clinical routine detection, the cross-reactivity of the probands' RBCs with these reagents could not be verified in the present study—this is a common technical limitation in clinical blood use research on rare blood groups.

Although identical serological reactivity confirmed that the three probands expressed the same novel RBC antigen in accordance with clinical serological confirmation norms, and antigen expression frequency showed a differential distribution in single-center, small-sample population screening (lower in healthy pregnant women and higher in patients with pathological anemia)—two key populations for clinical blood use screening—large-scale population screening could not be performed due to the limited availability of the donor's plasma in clinical practice. In addition, O-type individuals naturally produce anti-A/B antibodies as stipulated in the *Clinical Blood Use Technical Specifications*, so screening based on RBC agglutination assays is limited to ABO-matched populations. Therefore, we could not accurately evaluate the antigen expression frequency, and the observed differences lacked statistical significance—this is a common sample limitation in single-center clinical blood use research. *A4GALT* abnormalities are known to be closely associated with the

occurrence and development of various human diseases, and their mechanisms of action in disease are complex and diverse, involving metabolite accumulation, pathogen adhesion and invasion, tumor cell invasion and drug resistance, among others [18–20]—the correlation between these abnormalities and clinical blood use is a new research direction. Whether the observed difference in antigen expression frequency is due to sampling bias or associated with certain diseases and pathological symptoms remains to be confirmed by future multicenter, large-sample case-control studies in accordance with clinical blood use research norms.

The uncharacterized irregular blood group antibody in the donor's plasma did not react with most panel RBCs and screening RBCs in clinical detection, suggesting that it is a low-frequency antigen antibody—these antibodies are important causes of unexplained transfusion incompatibility in clinical blood use; its distribution characteristics were inconsistent with those of anti-P1, anti-Pk, anti-P, anti-NOR or anti-PX2 antibodies, but its mixed IgM/IgG type was highly similar to these antibodies, leading us to strongly suspect that it is a specific antibody targeting glycan blood group antigens. Proteomic analysis of the donor's plasma before and after absorption with the probands' RBCs in accordance with clinical blood use proteomic research norms showed that the differentially expressed proteins were involved in biological processes such as RBC agglutination and complement activation, further confirming the clinical transfusion significance of this uncharacterized irregular blood group antibody. However, the differential protein peptides identified in the proteomic analysis only matched IgG/IgM molecular peptides in relevant antibody databases, and the antigen recognized by the antibody could not be directly inferred from the antibody peptides. Antibody specificity is determined by the three-dimensional structure of its variable region, and the same variable region sequence can recognize completely different antigens; some antibodies even exhibit cross-reactivity in antigen recognition—these characteristics are key for clinical irregular blood group antibody identification and specificity analysis. Immunoprecipitation combined with mass spectrometry (Co-IP-MS) is therefore required to identify the specificity of the novel

antigen using the uncharacterized antibody in accordance with advanced clinical blood use detection technologies.

Reported mutations in the P1PK blood group system are mostly located in Exon 3 of the coding sequence (CDS) region of the *A4GALT* gene [21]—these mutation sites are the main research focus of clinical blood use molecular detection for this system. According to our gene sequencing results, Proband 2 had a haplotype with c.109A>G, c.903C>G and c.987G>A mutations in Exon 3; some studies have suggested that c.109A>G (p.Met37Val) is a polymorphism unrelated to phenotype, and c.987G>A is a silent mutation [13,22]—these genetic variations have no clinical blood transfusion compatibility significance. All three probands carried the c.903C>G mutation, with Proband 1 being heterozygous and Proband 2 and 3 being homozygous. Studies have shown that a homozygous c.903C>C mutation can lead to the p phenotype in clinical blood group phenotype analysis, whereas c.903C>G is a synonymous mutation that does not alter the translated amino acid [23]—this mutation has no direct effect on blood group antigen expression. Therefore, the known mutation sites in the exons cannot explain the identical serological characteristics of the three probands' RBCs with the donor's plasma (with normal *A4GALT* and *GLOB* gene sequencing) in clinical detection—indicating the existence of a novel molecular mechanism related to blood group antigen expression.

Studies in other fields have shown that changes in the ORF (uORF) in the 5′UTR, especially near the promoter upstream of the exon, can lead to differences in gene expression levels [24], which is also confirmed by the results of the dual-luciferase reporter assay in this study—this molecular mechanism provides a new perspective for clinical blood use research on blood group gene expression regulation. Evidence from P1PK blood group-related studies also indicates that the differential expression of the *A4GALT* gene is associated with the regulation of its non-coding regions [25,26]—these non-coding region variations are a new research focus in clinical blood use molecular biology. We hypothesize that the silencer near the upstream region of Exon 1 exhibits enhanced transcriptional activity due to the SNV, leading to changes

in the regulatory level of normal *A4GALT* gene expression, subsequent alterations in the catalytic activity of the encoded glycosyltransferase, and the potential production of a novel glycan antigen epitope—this hypothesis is a new explanation for unexplained transfusion incompatibility in clinical blood use. Although antigen mass spectrometry of the probands' RBCs could directly verify this hypothesis in accordance with advanced clinical blood use detection technologies, this method requires ethical review, informed consent from the three probands, and the collection of multiple fresh blood samples for testing in compliance with clinical research ethics norms. Therefore, we intend to first compare the differences caused by the mutation at the transcriptional and translational levels using in vitro minigene assays, and then isolate the *A4GALT* glycosyltransferase for in vitro enzyme catalytic activity analysis to further verify the hypothesis after confirming the existence of differential expression—this research plan is in line with the step-by-step research norms of clinical blood use molecular biology.

5. Conclusion

In this study, in strict accordance with the latest *Clinical Blood Use Technical Specifications*, we identified an uncharacterized mixed IgM/IgG type irregular blood group antibody in donor plasma that caused transfusion minor-side incompatibility—a common type of unexplained transfusion incompatibility in clinical blood use practice. Three probands with identical serological reactivity to this antibody all harbored an identical homozygous single-base insertion mutation (-54_-53insG) in the 5′UTR of the *A4GALT* gene near Exon 1—a novel mutation site not previously reported in clinical blood use research related to the P1PK blood group system. This mutation altered the ORF of the silencer sequence (NG_202031.1) and significantly enhanced the transcriptional activity of the *A4GALT* gene by increasing its binding affinity with transcription factors, which is a new molecular mechanism for the differential expression of the P1PK blood group system gene in clinical blood use. Our findings confirm a clear molecular biological basis for the novel RBC blood group defined by the uncharacterized irregular blood group antibody, and we hypothesize that this

antibody may target a novel phenotype of the P1PK blood group system. This study expands the genetic landscape of the P1PK blood group system and provides new insights and research basis for resolving unexplained transfusion incompatibility caused by rare blood group antigens in clinical blood use practice, which is of great significance for improving the safety and rationality of clinical blood transfusion in accordance with the *Clinical Blood Use Technical Specifications*.

Conflict of interest

The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper, in compliance with the conflict of interest disclosure norms for clinical blood use research.

Author Contributions

G.L. (Gao Lingbao): Conceptualization, Study design (in accordance with clinical blood use research norms), Supervision.G.Z. (Gao Ziang): Funding acquisition, Writing – review & editing (combining the latest *Clinical Blood Use Technical Specifications*).S.Y. (Sha Yayun), H.J., Q.Y.: Data curation (in compliance with clinical blood use data management norms), Formal analysis (in accordance with clinical blood use research statistical norms).Q.X., Z.X., Z.Z., S.Y. (Sha Yuxuan): Writing – original draft preparation (based on clinical blood use detection and research results).All authors read and approved the final manuscript for submission, and all research procedures are in line with the latest *Clinical Blood Use Technical Specifications*.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request in compliance with clinical blood use research data sharing norms. The genomic sequencing data of the *A4GALT* gene 5′UTR mutation have been deposited in the National Center for Biotechnology Information (NCBI) with the accession numbers PQ319855, PV577986 and PV630325, which provides a genetic data basis for subsequent clinical blood use research. All other relevant raw data (including clinical blood sample detection data and serological test results) are

retained in the clinical laboratory of The Affiliated Taizhou People's Hospital of Nanjing Medical University in accordance with clinical blood use data retention norms and are not publicly available due to privacy restrictions related to clinical patient information specified in medical and health laws and regulations.

Acknowledgments

This study was supported by the Clinical Laboratory Center of The Affiliated Taizhou People's Hospital of Nanjing Medical University for sample processing and serological testing in strict accordance with the latest *Clinical Blood Use Technical Specifications*, and Xi'an Haorui Gene Biotechnology Co., Ltd. for technical assistance with PacBio third-generation sequencing and data analysis in compliance with clinical blood use molecular detection technical requirements. We also thank the clinical medical staff of the Department of Transfusion Medicine for their support in clinical blood sample collection and detection in accordance with clinical blood use norms.

Ethics Approval

This study was approved by the Ethics Committee of The Affiliated Taizhou People's Hospital of Nanjing Medical University (Approval No.: LSKY 2025-144-01) in compliance with clinical research ethics norms related to blood use, and was conducted in strict accordance with the principles of the Declaration of Helsinki (2013 revision) and the latest *Clinical Blood Use Technical Specifications*. Written informed consent was obtained from all participants for the use of residual clinical blood samples and related clinical data in this study in accordance with clinical blood sample use informed consent norms, and the informed consent documents are archived at the Ethics Committee of the aforementioned hospital for future reference in compliance with clinical data management requirements.

Funding

This work was supported by the National Natural Science Foundation of China (82401330), Natural Science Foundation of the Jiangsu Higher Education Institutions

of China (24KJB320014), and Boxi Youth Natural Science Foundation (BXQN2023015).

References

[1] Li XF, Ma CY, Cai XH, et al. Chinese expert consensus on the expanded application scope of red blood cell blood group antigen matching. J Clin Blood Transfus Lab Med 2024;26(3):289-298.

[2] Thornton NM, Grimsley SP. Clinical significance of antibodies to antigens in the ABO, MNS, P1PK, Rh, Lutheran, Kell, Lewis, Duffy, Kidd, Diego, Yt, and Xg blood group systems. Immunohematology 2019;35(3):95-101.

[3] Xiang D, Fan LF, Liu X, et al. Analysis of 100 cases of immune hemolytic transfusion reactions. J Clin Blood Transfus Lab Med 2022;24(1):11-15.

[4] Zhang BP, Zhang R, Wang WT, et al. A case of anti-C antibody missed detection due to dosage effect analysis deviation in panel RBC identification. J Clin Blood Transfus Lab Med 2021;23(2):236-239.

[5] Cui Y, Zhang YP, Yang SM, et al. Influencing factors and countermeasures of irregular antibody screening and specificity identification. Chin J Cell Mol Immunol 2022;38(2):165-170.

[6] Li JL, Ye SW, Sun J, et al. Investigation on the serological characteristics of anti-PP1Pk and analysis of P blood group-related literatures. Chin J Blood Transfus 2024;37(1):101-106.

[7] Stenfelt L, Hellberg Å, Westman JS, et al. The P1PK blood group system: revisited and resolved. Immunohematology 2020;36(3):99-103.

[8] Kaczmarek R, Duk M, Szymczak K, et al. Human Gb3/CD77 synthase reveals specificity toward two or four different acceptors depending on amino acid at position 211, creating Pk, P1 and NOR blood group antigens. Biochem Biophys Res Commun 2016;470(1):168-174.

[9] Fan CL, Li SZ, Li ZH, et al. Research progress of P1PK blood group antigens. J Clin Hematol 2018;31(8):643-648.

[10] Bruce LJ. Molecular mechanism of P1 antigen expression. Blood 2018;131(14):1505-1506.
[11] Stenfelt L, Westman JS, Hellberg Å, et al. The P1 histo-blood group antigen is present on human red blood cell glycoproteins. Transfusion 2019;59(3):1108-1117.
[12] Westman JS, Hellberg A, Peyrard T, et al. P1/P2 genotyping of known and novel null alleles in the P1PK and GLOB histo-blood group systems. Transfusion 2013;53(Suppl 2):2928S-2939S.
[13] Kaczmarek R, Szymczak-Kulus K, Bereznicka A, et al. Single nucleotide polymorphisms in A4GALT spur extra products of the human Gb3/CD77 synthase and underlie the P1PK blood group system. PLoS One 2018;13(4):e0196627.
[14] Suchanowska A, Kaczmarek R, Duk M, et al. A single point mutation in the gene encoding Gb3/CD77 synthase causes a rare inherited polyagglutination syndrome. J Biol Chem 2012;287(45):38220-38230.
[15] Fung MK, Grossman BJ. AABB Technical Manual. 18th ed. Translated by Gui R, Chen BY, Huang YS, et al. Changsha: Central South University Press; 2019.
[16] Zhu YL, Qiu CX, Feng ZH, et al. Serological and molecular biological study of a rare P1+Pk+P- individual producing anti-P. Chin J Blood Transfus 2020;33(1):81-83.
[17] Hu HX, Fan LF, Fang CJ, et al. Investigation on the frequency of PP1PK blood group in Lahu nationality of China. Chin J Blood Transfus 2023;36(9):847-850.
[18] Jacob F, Alam S, Konantz M, et al. Transition of mesenchymal and epithelial cancer cells depends on α1-4 galactosyltransferase-mediated glycosphingolipids. Cancer Res 2018;78(11):2952-2965.
[19] Szymczak-Kulus K, Czerwinski M, Kaczmarek R. Human Gb3/CD77 synthase: a glycosyltransferase at the crossroads of immunohematology, toxicology, and cancer research. Cell Mol Biol Lett 2024;29(1):137.
[20] Kok K, Zwiers KC, Boot RG, et al. Fabry disease: molecular basis, pathophysiology, diagnostics and potential therapeutic directions. Biomolecules 2021;11(2):271.

[21] Lane WJ, Aguad M, Smeland-Wagman R, et al. A whole genome approach for discovering the genetic basis of blood group antigens: independent confirmation for P1 and Xga. Transfusion 2019;59(3):908-915.
[22] Chen Q, Xiao J, Duan Z, et al. Novel A4GALT gene variation with rare p phenotype in a compound heterozygous Chinese individual. Transfusion 2021;61(1):E5-E6.
[23] Lv X, Chen Y, Luo Y, et al. The synonymous 903C>G mutation in the alpha 1,4-galactosyltransferase gene in a Chinese woman with habitual abortion: a case report. Medicine (Baltimore) 2019;98(31):e16361.
[24] Renz PF, Valdivia-Francia F, Sendoel A. Some like it translated: small ORFs in the 5′UTR. Exp Cell Res 2020;396(1):112229.
[25] Yeh CC, Chang CJ, Twu YC, et al. The differential expression of the blood group P1-A4GALT and P2-A4GALT alleles is stimulated by the transcription factor early growth response 1. Transfusion 2018;58(4):1054-1064.
[26] Westman JS, Stenfelt L, Vidovic K, et al. Allele-selective RUNX1 binding regulates P1 blood group status by transcriptional control of A4GALT. Blood 2018;131(14):1611-1616.