Original article

Molecular mechanism of a novel Aweak subtype in Chinese pedigrees: identification and analysis of a large genomic deletion in the promoter upstream distal region of the *ABO* gene

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Background - Accurate ABO typing is essential for transfusion safety, yet weak A/B subgroups continue to present diagnostic challenges. Although most weakened ABO antigen expressions are linked to missense mutations or small indels within coding regions, the contribution of non-coding regulatory variants –especially large structural alterations– remains insufficiently characterized. Standard genotyping methods, including Sanger sequencing and targeted next-generation sequencing, often fail to detect large upstream deletions, leaving some serological-genotypic discrepancies unresolved in donors and patients. Such gaps hinder accurate transfusion risk assessment. To clarify these mechanisms, this study performed detailed ABO blood typing in a donor with an A subgroup and his family and explored the molecular basis for weakened A antigen expression.

Materials and methods - ABO phenotypes of the proband and his parents, spouse, two daughters, and son were determined using serological testing. Full-length *ABO* gene sequencing was then performed using third-generation single-molecule sequencing.

Results - The proband, his father, and his son displayed the Aweak phenotype. All three carried a heterozygous 12,309-bp deletion located upstream of the *ABO* gene promoter, along with a normal ABOA1.02 allele. The remaining family members carried genotypes ABOO.01.01/ABOO.01.01 (mother), ABOB.01/ABOO.01.02 (wife), ABOB.01/ABOO.01.01 (daughter 1), and ABOO.01.01/ABOO.01.02 (daughter 2). No variants were detected in coding regions, introns, or splice sites. Pedigree analysis confirmed that the proband inherited the ABOA1.02 allele with the 12.3-kb deletion from his father and passed it to his son.

Discussion - This study identifies, for the first time, a large 12.3-kb upstream deletion of the ABO promoter responsible for weakened erythrocyte A antigen expression. The finding expands current understanding of regulatory mechanisms underlying weak ABO phenotypes and highlights the value of advanced sequencing for transfusion safety.

Keywords: *ABO gene, promoter, large fragment deletion, pedigree analysis.*

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INTRODUCTION

The ABO blood group system, discovered over a century ago, holds critical clinical significance in transfusion medicine and transplantation due to its potent immunogenicity. Accurate ABO typing is paramount, as errors may trigger life-threatening transfusion reactions. While conventional serology identifies major ABO types, approximately 0.01% of the population exhibits ABO subgroups, characterized by attenuated antigen expression[1]. Molecular studies have elucidated that weakened A/B antigen expression primarily arises from three mechanisms[2-4]: 1) missense variants in the *ABO* gene coding region; 2) regulatory variants affecting transcription in the promoter and first intron (+5.8 kb site); 3) rare heterozygous alleles. The International Society of Blood Transfusion (ISBT) database catalogs over 300 ABO alleles, yet >90% of subgroup mechanisms involve mutations within the glycosyltransferase domain. Notably, no large genomic deletion (>5 kb) in the ABO promoter upstream region has been documented to cause antigen attenuation. This knowledge gap impedes a comprehensive understanding of ABO regulation and limits molecular diagnostic precision.

In this study, we investigated a Chinese pedigree with hereditary Aweak phenotype using serological analysis and third-generation single-molecule sequencing. We report for the first time a 12,309bp deletion variant upstream of the ABO promoter that reduces A antigen expression. This largest regulatory deletion identified to date co-segregated with attenuated A antigen expression across three generations. Our discovery expands the molecular mechanistic spectrum of ABO subgroups and highlights the necessity of screening for non-coding structural variants in cases with aberrant serological results.

MATERIALS AND METHODS

Blood samples

The proband was a 36-year-old male of Hui ethnicity, a voluntary blood donor from Ningxia region with no history of blood transfusion. This was his first participation in voluntary blood donation. Routine ABO blood typing revealed a forward and reverse typing discrepancy. Further molecular biological testing was performed, and family analysis was conducted involving his parents, wife, two daughters, and son. All participants signed informed consent forms.

Instruments and reagents

- ABO forward typing reagents: Shanghai Blood Center (Batch No.: 20230301);
- ABO reverse typing reagents: Shanghai Blood Center (Batch No.: 20240507);
- Human whole blood genomic DNA extraction kit: Tiangen Biotech Co. Ltd., Beijing, China (Batch No.: A0321A);
- Red blood cell genotyping detection kit - single-molecule real-time sequencing: Xi'an Haorui Gene Technology Co. Ltd., Shaanxi, China (Batch No.: 20240803);
- Quality control reagent - Qubit dsDNA BR Assay Kit: Thermo Fisher Scientific, Waltham, MA, USA (Batch No.: 2896576);
- Library preparation reagents - Sequel II Binding Kit 3.2 and Cleanup Beads: PacBio, Menlo Park, CA, USA (Batch No.: 033315);
- Sequencing reagents - Sequel II Sequencing Kit: PacBio (Batch No.: 131032);
- Sequencing chip - SMRT Cell 8M Tray: PacBio (Batch No.: 420402);
- PCR amplification reagent - PrimeSTAR GXL DNA Polymerase: Takara Bio Inc., Shiga, Japan (Batch No.: R050Q);
- PCR Thermal Cycler: Thermo Fisher Scientific (Model: A47394);
- Gel Imaging System: Bio-Rad, Hercules, CA, USA (Model: Gel Doc 76S);
- Sequencing Instrument - PacBio Sequel II System: PacBio (Model: 64066).

Serological tests

ABO forward/reverse typing and adsorption-elution tests were performed in strict accordance with the National Clinical Laboratory Procedures (4th Edition) operational standards.

Cell grouping (forward typing): monoclonal anti-A, anti-B, anti-A,B, and anti-H reagents (Shanghai Blood Biopharmaceutical Co. Ltd., Shanghai, China) were used. Testing was performed by the tube method in saline medium. Agglutination strength was recorded on a semi-quantitative scale from 0 to 4+.

Serum typing (reverse grouping): commercial A1, B, and O group reagent red cells (Shanghai Blood Biopharmaceutical Co. Ltd.) were used. Testing was performed by the saline tube method.

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Adsorption-elution test: this test was conducted on the proband and pedigree members with the Aweak phenotype to confirm weak A antigen expression. Briefly, an equal volume of titer-standardized human anti-A serum was added to washed, packed red blood cells for adsorption at 4°C for 1 hour. Following extensive washing, antibody elution was performed at 56°C for 10 minutes. The resulting eluate was then tested against A1 and O red cells via the indirect antiglobulin test.

Genomic DNA extraction

Genomic DNA was extracted from 300 µL of whole blood using the Blood Genomic DNA Extraction Kit (Cat. No. 70423-10 [Suzhou Beaver, Suzhou Industrial Park, China]).

ABO gene amplification

Designed 4 pairs of primers to amplify the full length of the *ABO* gene and its promoter upstream region. Multiplex PCR amplification was performed using PrimeSTAR GXL DNA Polymerase (R050Q [Takara Bio Inc.]) on a PCR thermal cycler under the following program: 94°C for 2 min (1 cycle); 98°C for 12 sec, 68°C for 12 min (27 cycles); 68°C for 10 min (1 cycle). This yielded the full-length *ABO* gene sequence and its distal promoter upstream sequence (**Table I**, **Figure 1**). The PCR products underwent concentration measurement using the Qubit dsDNA BR Assay Kit (Q32854 [Thermo Fisher Scientific]) and quality control via agarose gel electrophoresis. Qualified samples proceeded to library construction.

Library construction

The library construction system contained 4 µL PCR products. 2µL SMRTBELL ADAPTER INDEX PLATE 96A (102009200 [Pacbio]), 1 µL DNase/RNase-Free Deionized Water, 0.5 µL 100 mM ATP (NE, N0440 [New England Biolabs, Ipswich, MA, USA]), 0.5 µL 10 mM dNTP (NEB, N0447 [New England Biolabs]), 0.5 µL (5U) T4 Polynucleotide Kinase (NEB, M0236 [New England Biolabs]), 0.5 µL (1.5U) T4 DNA Polymerase (NEB, M0203 [New England Biolabs]) and 1 µL (400U) T4 DNA Ligase (NEB, M0202 [New England Biolabs]), a total of 10 µL reaction mixture. The reaction mixture was subjected to adapter ligation under the following conditions: 37°C for 20 min; 25°C for 15 min; 65°C for 10 min. Each sample was ligated with a unique barcode adapter for specific identification. Subsequently, 1.5 µL (7.5 U) Exonuclease I (Cat. No. M006 [Novoprotein, Beijing, China]) and 0.5 µL (50 U) Exonuclease III (Cat. No. M007 [Novoprotein]) were added to digest unligated products at 37°C for 60 min. The reaction was purified with 0.45× VAHTS DNA Clean Beads (Cat. No. N411-01 [Vazyme, Nanjing, China]) to obtain the final library.

Libraby preparation

The sequencing primer (Binding Kit 3.2 and Cleanup Beads, Cat. No. 102-333-300 [PacBio]) was added to the library, followed by incubation at 20°C for 30 min to allow primer annealing to the adapter sequences. Subsequently, the

Table I - *Primers for third-generation single-molecule sequencing of the ABO gene*

	Forward primer	Reverse primer
Primer 1	5'-TACATAGACAGTATCGGGGTTGCGCTGG-3'	5'-GAGGAGACAGCAATTCCAATCTTACAGAGAT-3'
Primer 2	5'-CCTCCGCAGTCATTCCCCATTCCCACC-3'	5'-TCCCTGCTGTCTCAGAGGCTCATGTTCT-3'
Primer 3	5'-TCCCTACAGCCAAACGATCTACCAACTACA-3'	5'-CCTGCGGGAAGAGTCACTCCAGTCC-3'
Primer 4	5'-ATTGTGCTATCTAACGGTTTTGAGTCTTGGG-3'	5'-GAGGAGACAGCAATTCCAATCTTACAGAGAT-3'

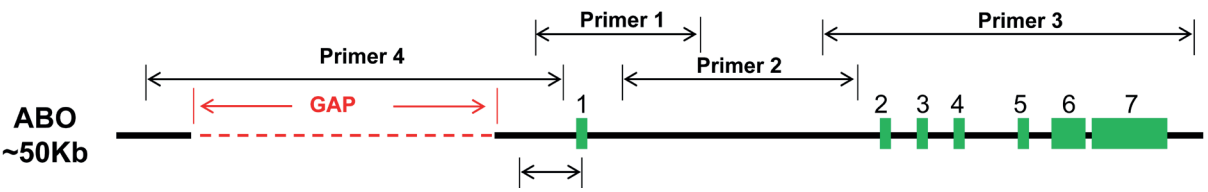

Figure 1 - Primer sequence diagram of *ABO* gene haplotypes

sequencing enzyme (Binding Kit 3.2 and Cleanup Beads, Cat. No. 102-333-300 [PacBio]) was introduced, and the mixture was incubated at 30°C for 30 min to immobilize the enzyme onto the primers. The final sequencing-ready library was obtained via magnetic bead purification.

Library sequencing

The sequencing-ready library was loaded onto a SMRT Cell and sequenced using the PacBio Sequel II high-throughput sequencing platform. The SMRT Cell contains 8 million zero-mode waveguide (ZMW) nanostructures, each designed to capture a single circular DNA molecule. Within each ZMW, DNA templates bound with sequencing primers and immobilized enzymes are anchored. During sequencing, fluorescently labeled dNTPs (each nucleotide tagged with a distinct fluorophore) are introduced. The sequencing enzyme incorporates complementary dNTPs into the nascent strand through base pairing. Upon incorporation of each nucleotide, the attached fluorophore is excited by a specific laser wavelength, emitting a characteristic fluorescence signal. These signals are detected in real time by the instrument and computationally translated into base calls, generating the final DNA sequence.

Data analysis

Sequence alignment analysis was performed using SnapGene software (Dotmatics, Boston, MA, USA) against the reference sequence *ABO*A1.01* to identify mutation sites. The detected variants were then classified according to the Blood Group Antigen Gene Mutation Database (maintained by the International Society of Blood Transfusion, ISBT) to determine the blood group phenotype.

RESULTS

Serological results

In forward typing, the red blood cells of the proband, his father, and his son all exhibited a mixed-field agglutination appearance (2+mf) with both anti-A and anti-A,B reagents. No agglutination was observed with anti-B, while a strong (4+) reaction was seen with anti-H. The reverse typing results were consistent with type A, and the positive adsorption-elution tests confirmed the specific presence of weak A antigens on the red cells of all three individuals, confirming their Aweak subtype. His mother and daughter 2 were both blood group O. His wife and daughter 1 were both blood group B (**Table II**).

Third-generation single-molecule sequencing results

Sequencing analysis of the proband and his family revealed that one haplotype allele of the *ABO* gene in the proband, his father, and his son carried a 12309-bp deletion upstream of the promoter region on the basis of the normal *ABO*A1.02* allele. The other haplotype harbored a normal O allele (*ABO*O.01.01** or **ABO*O.01.02**). The genotypes of his mother, wife, daughter 1, and daughter 2 were (**Table III**, **Figure 2**):

- Mother: *ABO*O.01.01/ABO*O.01.01* (homozygous),
- Wife: *ABO*B.01/ABO*O.01.02*,
- Daughter 1: *ABO*B.01/ABO*O.01.01*,
- Daughter 2: *ABO*O.01.01/ABO*O.01.02*.

No variants were detected in the coding regions, introns, or splice sites of the *ABO* gene. The locations and nucleotide changes in key variant sites are shown in **Table IV**.

Table II - *Serological reaction patterns of the proband and his family members*

Sample	Forward				Reverse				Adsorption and elution		Phenotype
	Anti-A	Anti-B	Anti-AB	Anti-H	A_1c	Bc	Oc	Self	A_1c	Oc	
Proband	$2+^{mf}$	-	$2+^{mf}$	4+	-	4+	-	-	2+	-	Aweak
Father	$2+^{mf}$	-	$2+^{mf}$	4+	-	4+	-	-	2+	-	Aweak
Mother	-	-	-	4+	4+	4+	-	-	/	/	O
Wife	-	4+	4+	1+	4+	-	-	-	/	/	B
Daughter 1	-	4+	4+	1+	4+	-	-	-	/	/	B
Daughter 2	-	-	-	4+	4+	4+	-	-	/	/	O
Son	$2+^{mf}$	-	$2+^{mf}$	4+	-	4+	-	-	2+	-	Aweak

Notes: 1-4+: Graded agglutination strength; - : No agglutination; mf: mixed-field appearance; W: weak agglutination; / : the adsorption-elution test was not performed.

Table III - *Gene sequencing results of the proband and family*

Sample	Haplotype 1		Haplotype 2	
	Allele name1	Mutation 1	Allele name 1	Mutation 2
Proband	ABO*A1.02	c.467C>T Promoter upstream: c.1-13204_c.1-896del	ABO*O.01.01	c.261delG
Father	ABO*A1.02	c.467C>T Promoter upstream: c.1-13204_c.1-896del	ABO*O.01.01	c.261delG
Mother	ABO*O.01.01	c.261delG	ABO*O.01.01	c.261delG
Wife	ABO*B.01	c.297A>G;c.526C>G;c.657C>T;c.703G>A;c.796C>A;c.803G>C;c.930G>A	ABO*O.01.02	c.106G>T;c.188G>A;c.189C>T;c.220C>T;c.261delG;c.297A>G;c.646T>A;c.681G>A;c.771C>T;c.829G>A
Daughter 1	ABO*B.01	c.297A>G;c.526C>G;c.657C>T;c.703G>A;c.796C>A;c.803G>C;c.930G>A	ABO*O.01.01	c.261delG
Daughter 2	ABO*O.01.01	c.261delG	ABO*O.01.02	c.106G>T;c.188G>A;c.189C>T;c.220C>T;c.261delG;c.297A>G;c.646T>A;c.681G>A;c.771C>T;c.829G>A
Son	ABO*A1.02	c.467C>T Promoter upstream: c.1-13204_c.1-896del	ABO*O.01.02	c.106G>T;c.188G>A;c.189C>T;c.220C>T;c.261delG;c.297A>G;c.646T>A;c.681G>A;c.771C>T;c.829G>A

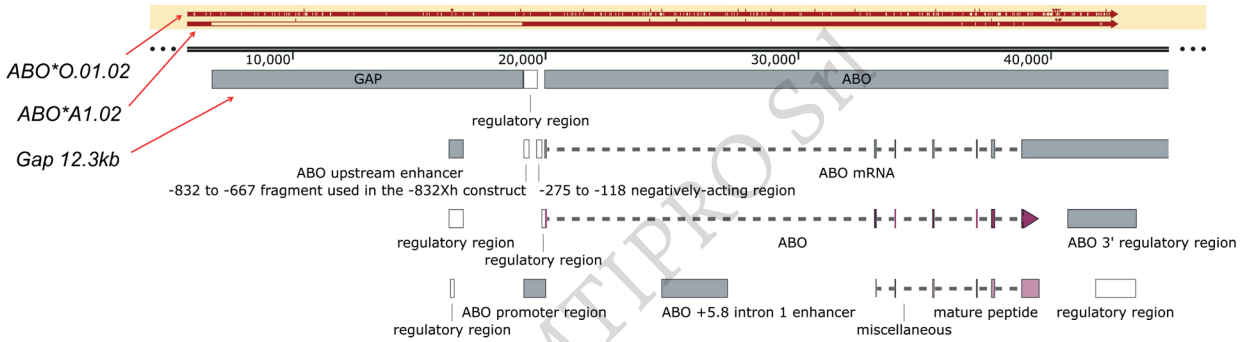

Figure 2 - Sequencing chromatogram showing the 12.3-kb deletion upstream of the promoter in the ABO*A1.02 haplotype allele.

Table IV - *The nucleotide position in the promoter upstream region*

Genotype	Location	Nucleotide change
ABO*A1.02	promoter upstream	c.1-13204_c.1-896del

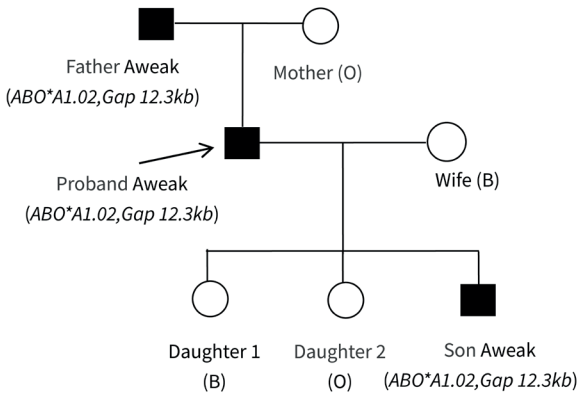

Figure 3 - Pedigree of the proband's family

Pedigree analysis of the proband

The *ABO*A1.02*allele carrying the 12.3-kb deletion was inherited by the proband from his father and subsequently transmitted to his son, as demonstrated in **Figure 3**.

DISCUSSION

ABO antigen expression is indirectly controlled by three principal alleles (A, B, and O), which determine the specificity and density of antigens on red blood cell surfaces, thereby establishing distinct blood group phenotypes. In 1990, Yamamoto *et al.*[5] successfully cloned the cDNA sequence of the A101 allele, providing the first molecular-level explanation of the ABO blood group system. Subsequently, in 1994, Yamamoto et al. identified that the *ABO* gene mutation c.1054C>T reduces glycosyltransferase activity, elucidating the molecular mechanism underlying attenuated antigen expression in subgroups[6]. Inheritance of the ABO blood

group gene follows Mendelian principles. The *ABO* gene spans approximately 19.5 kb, comprising 7 exons and 6 introns. Its 1065-bp coding sequence primarily resides in exons 6 and 7 (823 bp), which encode the core catalytic domain of the glycosyltransferase protein. Notably, 98% of pathogenic mutations cluster within exons 6 and 7[7]. Additional regulatory elements include the 5' and 3' regulatory regions and intronic regions (e.g., intron 1). The phenotypic variation observed in ABO subgroups is primarily attributed to single nucleotide polymorphisms (SNPs)[8].

This study identifies for the first time an Aweak subtype in a Chinese blood donor family, resulting from a large 12.3 kb distal deletion upstream of the *ABO* gene (*ABO***A1.02*, c.1-13204_c.1-896del). The congruence was exact between the serological profile (weak agglutination, strong H, positive adsorption-elution) and the molecular data (intact coding region but the upstream deletion). This deletion co-segregated perfectly with the phenotype in three family members and was absent in those with normal typing. With long-read sequencing excluding other potential variants in the coding and regulatory regions, this distal deletion is confirmed as the direct cause of the attenuated A antigen expression.

Our findings carry dual significance for transfusion safety. First, they elucidate the etiology of a novel category of problematic blood groups. When serology presents as Aweak with positive adsorption-elution tests, yet conventional molecular screening of the coding and core promoter regions reveals no abnormalities, the presence of a large upstream deletion such as this should be strongly suspected.

Second, our results provide precise guidance for the clinical transfusion management of such individuals. For the individual as a blood donor: If misclassified as group O and transfused to an O recipient, the donor's red blood cells, which still express trace A antigen, can react with the recipient's anti-A antibodies, potentially triggering a delayed hemolytic transfusion reaction. Therefore, their blood must be labeled as an A subgroup and transfused to type-A recipients (with special notation). In our study, the proband's initial donation was routinely misclassified as O. Discrepancy was identified upon reverse typing, and subsequent adsorption-elution testing and molecular diagnosis confirmed the Aweak subtype, thereby averting a mistransfusion risk. This case underscores that for blood donors exhibiting weak antigen expression or forward/reverse typing discrepancies, it is imperative to supplement with adsorption-elution tests and employ long-read sequencing for full-gene ABO analysis, establishing a combined "serology + molecular" identification protocol. For the individual as a patient: their blood group must be accurately identified as an A subgroup. When transfusion is required, the safest choice is either O-type washed red blood cells or blood of the identical A subgroup to avoid hemolytic reactions from high-titer anti-A in donor plasma. Additionally, since their serum may contain low-titer anti-A1 antibodies, extreme caution is warranted when transfusing plasma-rich components like A-type platelets; in such cases, AB-type blood products are the preferred choice.

ABO gene expression is under the precise control of complex upstream regulatory elements. The core promoter is responsible for basal transcription, and its activity is distally regulated by elements such as enhancers and silencers[9,10]. These elements fine-tune transcription through chromatin remodeling and three-dimensional genome architecture, notably via enhancer-promoter looping. The large deletion identified in our study likely removes several cis-regulatory elements that are critical for high-level ABO expression in erythroid cells. These may include[11-14]:

1. tissue-specific enhancer(s);
2. clusters of binding sites for key erythroid transcription factors such as GATA1 and KLF1;
3. regions that maintain an open chromatin state;
4. elements that insulate the ABO promoter from the influence of adjacent repressive chromatin domains;
5. alterations to the three-dimensional chromatin structure. By disrupting the function of these elements and/or altering the local chromatin environment, this large deletion ultimately leads to a significant reduction in the transcriptional level of the *A* gene in erythroid cells, thereby manifesting as weakened A antigen expression on the red blood cell membrane.

Currently, the mechanisms known to cause weak A antigen expression are primarily concentrated on missense mutations, small insertions/deletions, or splice-site mutations within the *ABO* gene coding region[15-18],

which directly impair the structure and function of the A transferase. Recent studies have also identified point mutations in the core promoter region that can diminish promoter activity, leading to weakened antigen expression[19,20].In contrast, the mechanism identified in our study is distinctly different: the variant is located distally upstream of the promoter (>10 kb), is a rare large genomic deletion, and its mode of action involves the long-range disruption of the gene regulatory network, rather than a direct effect on the enzyme protein or the core promoter. This finding provides novel insight into the molecular genetic basis of ABO subgroups.

The success of this study is attributed to the application of third-generation single-molecule long-read sequencing. Its ultra-long reads enable seamless spanning of complex structural variation regions, and its unbiased whole-genome sequencing strategy ensures the accurate detection of large deletions hidden distally upstream of the gene[21,22]. This highlights the unique value of this technology in resolving challenging serological cases that are intractable with conventional methods. Our findings have clear implications for transfusion safety. For individuals exhibiting an A subgroup phenotype with discordant routine serological and genotyping results, screening for large deletions upstream of the *ABO* gene should be considered to achieve a precise diagnosis.

We acknowledge that this study has limitations, primarily the lack of in vitro functional validation. Future research directions include: constructing reporter gene vectors to quantitatively assess the impact of this deletion on transcriptional activity; utilizing gene-editing technologies to validate its function in an endogenous model; and screening for the frequency of this variant in larger cohorts of individuals with A subgroups.

CONCLUSIONS

This study not only reports a novel mechanism for weakened A antigen expression caused by a large 12,309 bp deletion upstream of the ABO gene, but more importantly, by integrating detailed serological characterization with third-generation sequencing technology, we successfully resolved a challenging case with serological and genotypic discrepancies and established a clear clinical pathway for the precise transfusion management of such individuals. This deepens our understanding of the complex regulation of the *ABO* gene and highlights the significant value of translating advanced genomic discoveries into transfusion safety practice. This finding expands the spectrum of variants causing weakened ABO antigens from the traditional coding region and proximal promoter to distal regulatory regions, deepening the understanding of the complex regulatory network governing *ABO* gene expression and providing a new scientific basis for precise blood typing and transfusion safety.

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AUTHORS' CONTRIBUTIONS

JL was responsible for experimental design, third-generation single-molecule sequencing, data interpretation, and manuscript drafting. FS conducted critical review and final approval of the manuscript. YM, YB, JY and JH were responsible for phenotypic analysis.

The Authors declare no conflicts of interest.

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