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SHORT REPORT

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Detection and phenotype analysis of a novel Ael blood group allele

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Abstract

Background and Objectives: The presence of blood subtypes may lead to difficulties in blood group identification; however, third-generation sequencing (TGS) can help in accurately identifying difficult blood groups, and study the serological characteristics and molecular mechanism of Ael subtypes.

Materials and Methods: ABO blood group was identified by the standard serological technique, weak blood group antigen was identified by adsorption-elution experiments, ABH substance in the saliva was determined and glycosyltransferase activity of A and B was detected. The ABO gene full-length sequence and promoter region were amplified by specific primers using single-molecule real-time sequencing, with the amplified products being sequenced directly and analysed in real time.

Results: The patient was serologically identified as Ael subtype, and TGS analysis revealed new intron mutations in Ael patients (c.467C>T; c.29-10T>A).

Conclusion: The discovery of the new allele and the identification of ABO subtypes can be combined with serological characterization and molecular biological methods.

Keywords

ABO blood group, adsorption-elution experiments, Ael subtype, gene sequencing, single-molecule real-time sequencing

Highlights

- The presence of subtypes may lead to difficulties in ABO blood group identification. Serological methods and third-generation molecular sequencing technology can help in identifying them.
- Here we report the discovery of a novel allele of Ael, a group A subtype, using third-generation sequencing.
- The new allele has been submitted to the GenBanK database.

INTRODUCTION

The ABO blood group system, discovered by Karl Landsteiner in 1990, is one of the most important blood group systems in transfusion medicine and organ transplantation [1]. Generally, the ABO blood group system contains four major phenotypes: A, B, AB and O, and the distribution of ABO phenotypes varies among populations and regions [2]. However, because of the polymorphism of the ABO blood group gene, base insertion, deletion, substitution, mutation and splicing errors may affect the activity and specificity of A and B glycosyltransferases, resulting in the weakening or disappearance of A

Cuibi Wang and Yichao Tang contributed to the work equally and should be regarded as co-first authors.

or B antigen expression, which is the reason for the formation of the ABO subtype [3].

The Ael subtype is a relatively rare ABO subtype, in which the red blood cell does not agglutinate with anti-A or anti-AB, and which can be detected only by adsorption-elution experiments [4]. To date, a number of ABO allelic mutations have been identified in individuals, and these mutation sites highlight extensive sequence variations in the coding region of the ABO blood group gene [5]. Here we report a novel mutation c.29-10T>A on the ABO*A1.02 background, which has not been reported in public databases including 1000 Genomes, dbSNP and gnomAD before.

MATERIALS AND METHODS

Study subjects and DNA extraction

The proband is a 30-year-old Chinese man. Informed consent was obtained from the proband for sample collection and related trials, which were approved by the Medical Ethics Committee of Zhongshan Hospital (Xiamen), Fudan University (Approval No: B2022-073). Genomic DNA of the proband was obtained using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). The molecular gene detection part was carried out at the Xi'an Haorui Medical Laboratory Co., Ltd.

Serological tests

The blood type of the proband was detected by routine serological methods for A, B and H antigens and anti-A and anti-B antibodies using anti-A, anti-A1, anti-B, anti-AB and anti-H antibody reagents and A1, B and O red blood cells (Shanghai Blood Biotechnology Co., Ltd., Shanghai, China). Absorption-elution experiments were conducted to detect A and B antigens by thermal elution method using anti-A and anti-B antibody reagents and A1, B and O red blood cells (Shanghai Blood Biotechnology Co.) and bovine serum albumin (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China). For saliva experiments, 5 mL of the proband's naturally flowing saliva was boiled according to the standard method, and the supernatant was retained for further use. Anti-A, anti-B and anti-H reagents (Shanghai Blood Biotechnology Co.) were used for multiple dilution, and the '2+' agglutination strength was considered as the optimal dilution. Detailed procedures for the above two tests can be found in the AABB technical manual [6]. Glycosyl transferase activity was also measured by using uridine 5′-diphospho-*N*-acetylgalactosamine disodium salt (UDP-GalNAc), uridine 5′-diphosphogalactose disodium salt (UDP-Gal) (Sigma, USA), imidazole buffer (Beijing Regan Biotechnology Co., Ltd., Beijing, China), bovine serum albumin (Shanghai Yuanye Biotechnology Co.) and manganese chloride (Shanghai McLean Biochemical Technology Co., Ltd., Shanghai, China). The pooled plasma samples of genotype A, B and O were selected as positive and negative controls (the specific steps can be found in the literature [7]).

ABO gene full-length sequencing analysis

We analysed the full-length ABO gene sequence using long-read sequencing. All amplicons were purified by magnetic beads, followed by repeat sequencing and analysis using a PacBio RS sequencer and Sequel System [8]. ABO genotypes were assigned according to the nucleotide sequence of the ABO gene polymorphisms. All nucleotide sequences obtained were compared with known ABO gene polymorphisms ('ABO*A1.01' named by the International Society for Blood Transfusion [ISBT] was used as the reference sequence).

Analysis of the ABO gene haplotype sequence

Amplification with allele-specific primers was carefully designed to generate three sets of primers (Figure 1, Table 1) that efficiently amplify the complete ABO gene sequence, followed by direct sequencing and analysis of the amplicons. At the same time, by rolling circle sequencing of a single DNA molecule, the long-range polymerase chain reaction (LR-PCR) technique was performed by KOD FX Neo (TOYOBO). The PCR cycling conditions were set according to the user manual, and a two-step cycle of 10 min each was used for a total of 30 cycles to achieve high accuracy of sequencing data and determine the haplotype of the sample.

TABLE 1 Primer design for ABO haplotype sequences.

Sequence primers	F[a]	R[b]
Primer 1	5′catccctttcaccttggcattt3′	5′agctacattgaccagagagaga3′
Primer 2	5′gcccaccaaaactccctggaa3′	5′ccagttcctgccagggagagga3′
Primer 3	5′gtgtgaaactcatcaaaacc3′	5′cgcagggattgcagtgagg3′

[a]Forward primer.
[b]Reverse primer.

FIGURE 1 Primer sequence diagram of ABO gene haplotypes. The ABO gene with a length of about 23 kb was broken down into three target gene fragments for amplification, and there was an overlap of more than 1 kb between each amplicon of the same gene, and the single-nucleotide polymorphism (SNP) sites were found in this overlap region to distinguish haplotypes. All amplicons are listed in the figure.

Blood group genotyping using third-generation sequencing

At present, the latest third-generation sequencing (TGS) has two major sequencing platforms. We used Pacific Biosciences' single-molecule real-time sequencing (SMRT), which mainly uses zero-mode waveguide holes to detect the fluorescence generated during DNA polymerization and repeat sequencing through a circular DNA library [9]. In brief, the PCR products were qualified by agarose gel electrophoresis and the library was constructed. A reaction master mixture was prepared before use, which contained 10 μL of the reaction mix containing 4 μL of PCR product, 5 μmol/L barcoded adapter (Integrated DNA Technologies), 1× T4 DNA ligase buffer (Enzymatics), 1 mmol/L ATP (New England Biolabs), 200 μmol/L dNTP (New England Biolabs), 2.5 units of T4 polynucleotide kinase (Enzymatics), 0.75 units of T4 DNA polymerase (Enzymatics) and 180 units of T4 DNA ligase (HC) (Enzymatics). And then, the reaction mix was incubated at 37°C for 20 min, 25°C for 15 min and 65°C for 10 min. After that, exonuclease I and exonuclease III (Enzymatics) were added to remove the failed ligation products, and the library was obtained after purification with 0.6× Ampure PB beads. The final library was bound with sequencing enzymes and primers through a Sequel Binding Kit 2.2 (Pacific Biosciences) and Internal Control Kit 1.0 (Pacific Biosciences). The complexes (150 pM DNA–polymerase) were finally loaded and sequenced with the Sequel II platform (Pacific Biosciences) with 20-h movie time. All procedures were performed in strict accordance with the manufacturer's instructions. SnapGene software was used for sequence alignment and analysis using ISBT Names for ABO (ISBT 001) blood group alleles v1.1 171023 as the reference data source.

RESULTS

Serological characteristics of the ABO phenotype

Table 2 shows the agglutination status of the proband's red cells with anti-A, anti-A1, anti-B, anti-AB, anti-H and serum containing known groups A, B and O red cells. All individual RBCs showed anti-H agglutination of 3+ or 4+ intensity. The results of absorption-elution experiments confirmed the presence of weak A antigen on the patient's RBCs; the proband was secretory type, and substance H was detected in the saliva. A-transferase was not detected in the proband by glycosyl transferase activity assays. According to the above serological characteristics, the proband could be classified as an Ael subtype variant.

ABO gene full-length sequencing analysis

By direct sequencing of the entire ABO coding region, no mutation was found in exons 1–5 and intron 6 of the ABO gene in the proband. There were c.261delG and c.467C>T in exons 6 and 7 and c.29-10T>A heterozygosity in intron 1 of the ABO gene (Figure 2, Table 3).

Analysis of the ABO gene haplotype sequence

Haplotype analysis of the ABO gene showed that the ABO genotype of the proband was ABO*A1.02/ABO*O.01.01 and there was a heterozygous mutation of T>A at position 29–10 in intron 1 (Figure 2, Table 3), which was not included in the ISBT database. This mutation

TABLE 2 The blood group serologic results.

	Forward						Reverse			Adsorption-elution				
Sample	Anti-A	Anti-B	Anti-A1	Anti-AB	Anti-H	Anti-D	Ac	Bc	Oc	Ac	Bc	Oc	Secretor status	Phenotype
Proband	0[a]	0	0	0	3+[b]	4+	0	4+	0	3+	0	0	H	Ael

[a]'0' denotes the serology did not agglutinate.
[b]'+' denotes the agglutination strength of serology.

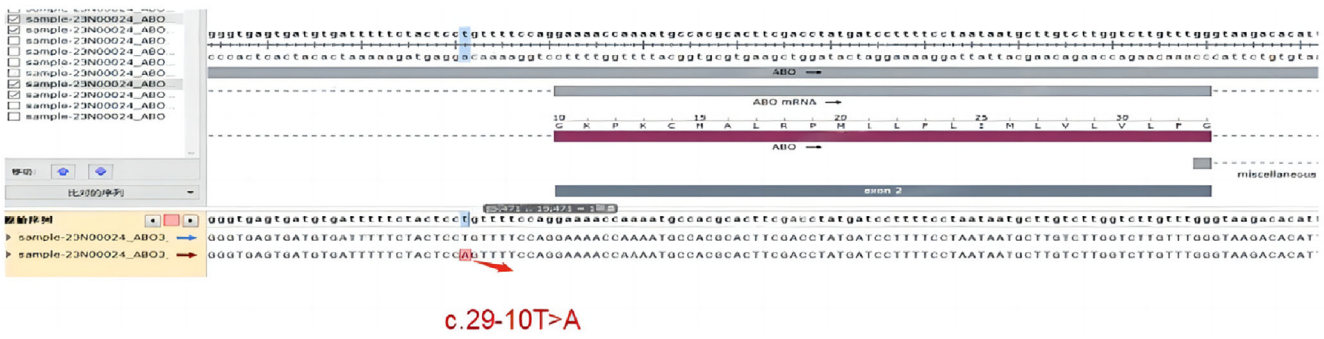

FIGURE 2 ABO gene sequencing results of the proband. *Homo sapiens* ABO, alpha 1-3-*N*-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase (ABO), NG_006669.1 was used as the reference allele sequence.

TABLE 3 ABO genotyping results.

Sample	Haplotype 1			Haplotype 2		
	Phenotype 1	Allele 1	Mutation 1	Phenotype 2	Allele 2	Mutation 2[a]
Proband	O	ABO*O.01.01	c.261delG	A1	ABO*A1.02	c.467C>T c.29-10T>A

[a]The new alleles first identified in our laboratory were not included in International Society for Blood Transfusion but were submitted to the GenBank database.

is located near the splicing region of exon, which may affect the normal splicing of gene products, resulting in the phenotype Ael of the haploid carrying this mutation. Then, we submitted the nucleotide sequence of this new allele of the proband to GenBank database (accession number: BankIt2672115 Seq1 OQ414473).

Blood group genotyping using TGS

According to the sequencing results in Table 3, by using real-time SMRT assay covering the full-length sequence and promoter region of the ABO gene, again we found this novel heterozygous mutation (c.29-10T>A) in intron 1 of the ABO gene. ABO gene-specific primers and PacBio RS sequencing confirmed that the intronic variant was located on the ABO*A1.02 allele. No variant gene was detected in the promoter region and +5.8-kb site of the ABO gene.

DISCUSSION

By December 2022, a total of 44 blood group systems had been recognized by the ISBT. The ABO blood group system is one of the earliest and most clinically significant blood group systems. The majority of subtype A is caused by the inheritance of rare alleles in ABO locus. Clinically, subtype A is divided into A1, A2, A3, Ax, Aend, Am and Ael according to decreasing number of A antigens [10], among which Ael subtype contains the least number of A antigen on the red blood cells. The reactivity of Ael red blood cells can be detected only by absorption-elution experiments, and its serum does not agglutinate or weakly agglutinate with type A red blood cells, that is, A elution type [11–13].

It had been reported that there was no substance A in the secretory saliva of Ael but only substance H. The serum of Ael individuals often contains anti-A1 but can also contain antibodies capable of agglutination with A2 cells [14, 15]. No A-transferase had been detected in the serum or erythrocyte membrane of Ael [16–18]. In this study, the proband was confirmed by absorption-elution experiments because there was weak A antigen on red blood cells and substance H in saliva but no A-transferase was detected on red blood cell membrane, which was consistent with the serological performance of Ael subtype individuals. However, the genotyping of the proband could not be accurately determined, so ABO gene-sequencing analysis was performed. Genotyping results showed that there was C>T mutation at position 467 and a heterozygous T>A mutation at position 29–10 in the intron 1 (which was not included in the ISBT database). The mutation was located near the exon splicing region, which may affect the splicing error during the transcription of gene products, leading to the mutation before and after the variable indirect recognition site and resulting in the weak expression of the antigen and forming the Ael subtype.

The majority of mutations reported so far in ABO subtypes were located in exons 6 and 7. However, ABO subtype pathogenic variants may also occur in regions other than ABO exons 6 and 7 (including exons 1–5 and 6 introns and the regulatory region of the ABO gene), which may have important but rare implications for gene expression [19–22]. In addition, the discovery and genotyping of SNPs in introns are key to the study of intronic gene polymorphisms, and introns are also considered important in assessing genetic diversity [2]. Here, a novel heterozygous mutation in ABO intron 1 (c.29-10T>A) was detected by the third-generation SMRT method for blood group genotyping of the proband. However, it is not certain whether the mutation will cause splicing abnormality, so functional verification is needed. Therefore, it is necessary to use molecular detection techniques to screen all these regions to avoid the phenomenon of weak antigen missed detection, leading to transfusion in the case of blood group discordance.

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C.W. and Y.T. carried out formal analysis, data curation and writing the original draft, P.Z. carried out the validation, L.X. was involved in funding acquisition and did language modification of the manuscript, W.C. carried out the main investigation and X.L. administered the project, mobilized the resources and contributed to the methodology, conceptualization and review and editing of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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