Molecular Basis of ABO Variants Including Identification of 16 Novel ABO Subgroup Alleles in Chinese Han Population

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Keywords

ABO variants · Molecular basis · Novel ABO allele · Sequence-based typing

Abstract

Introduction: The characteristic of ABO blood subgroup is crucial for elucidating the mechanisms of such variant phenotypes and offering useful information in blood transfusion. **Methods:** In total, 211 ABO variants including part of available family members were investigated in this study. The phenotypes of these individuals were typed with serologic methods. The full coding regions of ABO gene and the erythroid cell-specific regulatory elements in intron 1 of them were amplified with polymerase chain reaction and then directly sequenced. The novel alleles were confirmed by cloning and sequencing. Phylogenetic tree was made using CLUSTAL W software. 3D structural analyses of the glycosyltransferases (GTs) with some typical mutations were performed by PyMOL software. **Results:** Forty-eight distinctly rare ABO alleles were identified in 211 Chinese variant individuals, including 16 novel ABO alleles. All of the alleles were categorized as 5 groups: 16 ABO*A alleles, 23 ABO*B alleles, 4 ABO*BA alleles, 4 ABO*cisAB alleles, and 1 ABO*O alleles. ABO*A2.08 and ABO*BA.02 were the relatively predominant A and B subgroup alleles, respectively. According to the phylogenetic tree, 28 alleles (5 common alleles and 23 alleles identified in our laboratory) were classified into 3 major allelic lineages. The struc-

tural analysis of 3D homology modeling predicted reduced protein stability of the mutant GTs and may explain the reduced ABO antigen expression. **Conclusions:** The molecular basis of ABO variants was analyzed, and 16 novel ABO alleles were identified. The results extended the information of ABO variants and provided a basis for better transfusion strategies and helped to improve blood transfusion safety.

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Introduction

ABO is considered one of the most complicatedly and clinically relevant blood group system in the fields of both transfusion and transplantation medicine [1]. More than 300 different ABO alleles have been characterized to date. Except for the 5 common ABO alleles (ABO*A1.01, ABO*A1.02, ABO*B.01, ABO*O.01.01, and ABO*O.01.02) [2], numerous subgroup allele-related variants with a differential expression of the A or B antigens on the red blood cells have also been found in different populations [3–5]. ABO variant prevalence is rare, with a rate of approximately 0.015% in China [6]. The molecular genetic basis of the ABO system had been known since the ABO cDNA corresponding to the mRNA was cloned in 1990 [7]. Many ABO variants caused by the mutation in the coding region had been identified, although the molecular mechanisms underlying some variants were still not

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understood completely. Several genetic events, including substitution, splice site mutation, base insertion, deletion, and hybrid alleles, have the potential to affect the A or B glycosyltransferase (GT) activity and then cause changes in ABO phenotypic expression [8, 9]. Besides the changes of the exons, the erythroid cell-specific regulatory elements of the *ABO* gene located in the 5'- and 3'-flanking sequence and the first intron 1 were found to affect the transcription efficiency [10–13]. The distribution and diversity of ABO variants were crucial for elucidating phenotype exactly and offering useful information for blood transfusion. In this study, some apparent ABO variants including part of available family members were investigated for the serology characterization by serological agglutination reaction method, and then the molecular basis was further analyzed by polymerase chain reaction sequence-based typing (PCR-SBT) method and 3D structural prediction. The following report described the characteristics of ABO variants in Chinese population.

Materials and Methods

Study Samples

A total of 211 ABO variant samples were collected, including the volunteer blood donors in Blood Center of Zhejiang Province, China, the patients in the hospitals of Hangzhou City in Zhejiang Province, and part of the related family members. All of the individuals were collected from unselected population and are not the same. All individuals were found with discrepancies in ABO red cell grouping and serum grouping, or weak A and/or B antigen expression.

Venous blood of these ABO variant samples was collected. Genomic DNAs were extracted from blood cells using a commercial DNA whole blood kit with a nucleic acid isolation device (Quick-GeneMini80, FujiFilm Corporation, Tokyo, Japan) according to the manufacturer's instruction.

Serologic Typing

ABO phenotypes were determined by agglutination and adsorption-elution tests and serologic diagnostic classification according to standard methods and procedures described in AABB technical manual [14]. The following commercially available reagents were used: monoclonal anti-A, anti-B, anti-AB (Shanghai Hemo-pharmaceutical Biological Company, Shanghai, China), Anti-A1 (Dolichos Biflorus lectin), and Anti-H (Ulex europeus; Dominion Company, Dartmouth, Canada).

PCR Amplification and Sequencing for Full Coding Region of *ABO* Gene

The full coding regions of exons 1–7 of *ABO* gene were amplified with 3 pairs of primers according to our previous reports [15, 16]. PCR amplification products were purified with double enzymes digestion and sequenced bidirectionally using a Bigdye Terminator Cycle version 3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) [17]. The sequence data were analyzed by Seqscape2.5 software (Applied Biosystems, Foster City, CA, USA) and assigned for the *ABO* genotype according to the reference sequence of *ABO**A1.01 gene (AC000397) polymorphism positions [4].

Haplotype Analysis of Novel *ABO* Alleles

To confirm the novel *ABO* alleles, PCR amplified fragments containing the novel mutation were directly ligated and cloned into the plasmid pCR4-TOPO (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Plasmid DNA was extracted from each recombinant colony with 3S Spin Plasmid Miniprep Kits (Shenergy Biocolor, Shanghai, China) and used as the template for sequencing reactions according to our previous reports [15, 17].

PCR Amplification and Sequencing for the 5.8-kb Region in Intron 1

The individuals without any subgroup alleles were analyzed for the nucleotide sequences of the partial intron 1 covering the +5.8-kb region. The method of the amplification and direct sequencing is referred to in our previous reports [10]. The nucleotide sequences were compared with standard *ABO* polymorphism site sequences (NT_035014).

Nomenclature of Mutation and *ABO* Alleles

The new allele sequences have been submitted to the GenBank Database, and part of *ABO* alleles have been named according to the nomenclature by ISBT (<http://www.isbtweb.org/working-parties/red-cellimmunogenetics-and-blood-group-terminology/>) [3].

Dendrogram Construction

The sequences were aligned and phylogenetic tree was constructed using CLUSTAL W software. Phylogenetic trees showing the phylogenetic relationship between the alleles were constructed according to the neighbor-joining method [18].

Modeling of Some Typical Novel Alleles

To understand the effect of mutations on the structure or activity of GTs produced from the novel alleles, 3D molecular models of some typical novel mutations were generated from the template structure of wild-type A glycosyltransferases (GTAs) (PDB code, 1LZI) and B glycosyltransferases (GTBs) (PDB code, 1LZ7) by the PyMOL software.

Results

The Serological Characteristic of *ABO* Variant Phenotypes

A total of 211 ABO variant phenotypes could be fit into the following categories: A₂, A₂B, A_x, A_xB, A_mB, A_{el}, A_{el}B, B₃, B_x, AB_x, B_m, B_{el}, AB_w, and O. Reducing of A or B antigen was found in most cases. Meanwhile, part of individuals with normal antigen expression had lack or superfluous amount of antibody in the serum. Some individuals were just classified as A_w/A_wB or B_w/AB_w because of the discrepancy between the serological characteristic and standard. The detail serologic characterizations of these variants were listed in Table 1.

Molecular Characterization of *ABO* Variants

In all, 48 rare *ABO* alleles responsible for the ABO subgroup phenotypes were found in 136 variants by the di-

Table 1. The serologic characterization of samples with rare ABO subgroup alleles found in this study

Phenotype	NO ^a	Cell group					Reverse (serum) grouping			Absorption and elution test	
		anti-A	anti-B	anti-AB	anti-A1	anti-H	Ac	Bc	Oc	Ac	Bc
A2	9	4+ ^b	0	4+	0	4+	0~3+	4+	0	/ ^c	/
A2B	20	4+	4+	4+	0	1+~4+	0~3+	0	0	/	/
A _x	4	+	0	1+~3+	0	2+~4+	1+	4+	0	/	/
A _x B	7	1+	4+	4+	0	2+	1+~2+	0	0	/	/
A _{el}	9	0	0	0	0	4+	1+~2+	4+	0	3+~4+	0
A _w	3	±~2+	0	1+~2+	0	3+~4+	0~1+	4+	0	/	/
A _w B	21	2+~3+	4+	4+	0	2+~4+	0~1+	0	0	/	/
B3	6	0	mf	mf	/	4+	4+	0	0	/	/
B _x	7	0	1+~2+	1+~2+	/	4+	4+	0	0	/	/
AB _x	5	4+	1+~2+	4+	/	1+~2+	0	0	0	/	/
B _{el}	3	0	0	0	/	4+	4+	2+~3+	0	0	3+~4+
B _m	2	0	1+	1+	/	4+	4+	0	0	/	/
B _w	20	0	1+~3+	2+~3+	/	4+	4+	±~1+	0	/	/
AB _w	19	4+	1+~3+	4+	4+	2+	0	±~3+	0	/	/

^a The number of the sample with rare ABO subgroup alleles identified in this study. ^b The “+” denotes the agglutination strength of serology. ^c The “/” denotes that the reaction has not been done.

Table 2. Sixteen novel ABO subgroup alleles identified in our laboratory in this study

Allele ^a	Critical nucleotide ^b	Main amino acid change(s)	Number (R) ^c	Phenotype	GenBank number
<i>ABO</i> *A2.10	268T>C; 467C>T	W90R; P156L	1	A ₂	– ^e
<i>ABO</i> *A2.11	266C>T; 467C>T	P89L; P156L	3	A ₂ B	–
<i>ABO</i> *A2.13	467C>T; 742C>T	P156L; R248C	3	A ₂ B, A _x B	FJ998203
<i>ABO</i> *A2.new5 ^d (A222)	1054_1055 insA	R352Qfs*39	1	A ₂	KJ631752
<i>ABO</i> *A3.new6 ^d (A312)	280A>T; 467C>T	I94F; P156L	1	A _x B	KM921804
<i>ABO</i> *A _w .new7 ^d (Ax22)	389T>C	L130P	1	A _x	KC866362
<i>ABO</i> *AEL.08	467C>T, 804dupG	P156L; F269Vfs*124	6 (3)	A _{el}	HQ843789
<i>ABO</i> *B _w .new9 ^d (B118)	840C>T	L280L	1	A ₂ B	HQ843788
<i>ABO</i> *B _w .new11 ^d (B120)	484delG	V162OPA	1	B _{el}	KC526949
<i>ABO</i> *B3.07	410C>T	A137V	1	B ₃	–
<i>ABO</i> *B _w .new12 ^d (B _w 27)	910A>G	S304G	1	B _x	JQ692625
<i>ABO</i> *B _w .new14 ^d (B _w 35)	737A>G	Y246C	1	B _w	KM362861
<i>ABO</i> *B3.new15 ^d (B314)	3_4delG	M1Mfs*18	1	AB _x	KC960560
<i>ABO</i> *B3.new16 ^d (B315)	928C>G	L310V	1	B _w	KR021361
<i>ABO</i> *B _w .new17 ^d (B _w 38)	518T>C	L173P	1	AB _w	KP245739
<i>ABO</i> *B _w .new18 ^d (B _m 03)	98G>C	G33A	2	B _m	KC960559
<i>ABO</i> *cisAB.05	297A>G; 526C>G; 657C>T; 703G>A; 796C>A; 930G>A	R176G; G235S; L266M	1	AB _w	–

^a The part of novel alleles of A and B firstly identified in our laboratory were named by ISBT, which were cited from ErythroGene (<https://doi.org/10.1182/bloodadvances.2016001867>). ^b The main nucleotide and amino acid changes of the *ABO**A, *ABO**cisAB, and *ABO**O alleles were compared to the consensus *ABO**A1.01; *ABO**B alleles were compared to *ABO**B.01 allele. ^c The number of the sample identified in this study. R, number of related family member. ^d The novel alleles firstly found in our laboratory are not named by ISBT currently, but have been named according to the nomenclature used in the dBRBC before [4]. ^e The sequence was not submitted to the NCBI before.

rect sequencing of the entire ABO coding region and flanking splicing sites, including 16 novel ABO alleles. All of these alleles were classified as 5 groups: 16 *ABO**A alleles, 23 *ABO**B alleles, 4 *ABO**BA alleles, 4 *ABO**cisAB

alleles, and 1 *ABO**O alleles. *ABO**A2.08, *ABO**BA.02, *ABO**BA.04, *ABO**B_w.03 alleles had a relatively high frequency (see www.karger.com/doi/10.1159/000501862; online suppl. Table S1).

Fig. 1. The phylogenetic tree for 28 alleles (5 common alleles and 23 alleles identified in our laboratory) based on ABO gene sequences of exon 1–7.

Sequence Characteristics of the Novel Alleles

After haplotype sequence analysis of ABO gene, 16 novel alleles were identified in variant samples (Table 2), and part of ABO alleles have been named according to the nomenclature by ISBT. Most of the novel alleles had a nucleotide mutation in exon 6–7, resulting in an amino acid change in the catalytic region. Compared with the reference allele, two naturally occurring ABO*B alleles (ABO*B_W.new11 and ABO*B₃.new15) had a nucleotide deletion and 2 ABO*A alleles (ABO*AEL.08 and ABO*A2.new5) had a single base insertion. All of them led to a frameshift to create a premature terminal codon at different position of GT enzyme. One interesting new mutation, ABO*cisAB.05, had a nucleotide reversion c.803C>G compared with ABO*B.01 allele.

Phylogenetic Tree

A phylogenetic tree was constructed from a neighbor-joining analysis of 28 ABO alleles (the common ABO alleles and the subgroup alleles detected in our laboratory) based on the sequences of exon 1–7 (Fig. 1). Most branch lengths were very short and bootstrap values were small. It was clear that 28 alleles were classified into 3 distinct allelic lineages. Group I consisted of ABO*B-related alleles and ABO*cisAB, while Group II consisted of ABO*A-related alleles and ABO*O.01.01. The ABO*O.01.02 is a separate group.

The Analysis of the 3D Structures of the Mutation GTs from the Typical Novel Allele Mutations

Most of the novel alleles were point mutations located in the catalytic domain (Fig. 2A, B). Thus, 2 point mutations (ABO*A: c.389T>C, p.L130P; ABO*B: c.410C>T, p.A137V) and 1 deletion mutant allele (ABO*B: c.484delG, p.V162OPA) were selected for analysis of the enzyme structure prediction. The overall structures of the point mutant GTs were predicted to be similar to that of the

wild type. However, although the overall shapes of these enzymes were similar, the mutations were predicted to form the different hydrogen bonds with the other surrounding residues (Fig. 2C–F). In the wild-type GTA, amino acid residue L130 was predicted to form 2 hydrogen bonds with 2 surrounding residues (V127 and L134), as indicated in Figure 2C, while in the p.L130P mutant GTA, P130 also formed hydrogen bonds with these 2 amino acids, but the atoms and positions for forming the hydrogen bonds had changed significantly (Fig. 2D). The hydrogen bond distance was elongated and structural stability changed. Meanwhile, compared with the wild-type GTB, the p.A137V mutant GTB reduced one hydrogen bond with the surrounding amino acid residues. Four hydrogen bonds were reduced to 3 bonds, because the V137 mutation did not form a hydrogen bond with F141. The atoms and distances of the hydrogen bonds were different as well (Fig. 2E, F). For the deletion mutation allele, c.484delG formed C-terminally truncated enzymes, which mainly lead to the GTB structural changes and may not form effective active center (Fig. 2G).

ABO Subgroup Phenotypes with the Other Molecular Basis

Another 75 variants were considered to have an unclear molecular basis because only normal sequences were detected in the analysis regions. Among these individuals, 32 samples were identifiable for infection, hematologic disorders, and other diseases. Thus, we presumed that the antigen or antibody changes in these individuals might be concerned with the diseases. Seven ABO variants were concerned with the erythroid cell-specific regulatory element in the first intron of ABO gene. The molecular genetic analyses were shown in our recently report [10]. The other 36 samples (including 4 individuals with pregnancy) without any diseases or other molecular basis might have an unclear molecular basis.

Discussion

In this study, 211 ABO variants associated with discrepant ABO phenotype were investigated. However, there were only 136 individuals driving from the rare alleles with mutations distributed over the entire coding region of the ABO gene by PCR-SBT method. Thus, 48 distinctly rare ABO alleles, including 16 novel subgroup alleles, were characterized. The detection of alleles was not only an important contribution for each individual case, but also a development of the blood group genetic factors. The availability of these rare ABO alleles will present researchers with more information regarding the structure of ABO genes. The sequences can be used to design new primers and oligonucleotide probes to aid

Fig. 2. Ribbon diagrams of the wild-type GTs (GTA: PDB code, 1LZI and GTB: PDB code, 1LZ7) and the mutant GTs. **A** Structures of the wild-type GTA and the amino acid residue position with the novel point mutation. **B** Structures of the wild-type GTB and the amino acid residue position with the novel point mutation. **C** In the wild-type GTA, the side chain of L130 can form 2 hydrogen bonds with 2 surrounding residues (V127 and L134) indicated by the red dotted lines. **D** In the p.L130P mutant GTAs, the side chain of P130 can form different hydrogen bonds with these residues. **E** In the wild-type GTB, the side chain of A137 can form 4 hydrogen bonds with 4 surrounding residues (F133, H140, F141, and R168) indicated by the red dotted lines. **F** In the p.A137V mutant GTB, the side chain of V137 only can form another 3 different hydrogen bonds with these residues except the F141 amino acid. **G** The structural model of the mutant GTB with *c.484delG* allele. The figures were generated using Pymol software.

more exact *ABO* typing, and the polymorphism data can be used for evolutionary studies as well.

Among these *ABO* variants, 80 samples were screened from about 0.7 million blood donors. However, the frequency of the rare alleles could not be accurately calculated because the samples tested were not collected by large-scale screening from random populations. Meanwhile, only the samples with obvious discrepant phenotype were collected for sequencing, so, many potential samples might be lost. For the low-frequency alleles (only

1 individual was found), the numbers detected also did not represent the true distribution. But according to our previous study, none of these mutations were found in 417 random samples, which suggested that the frequency of these mutations was <0.12% in Chinese population [17].

From the distribution of alleles, *ABO**A2.08 and *ABO**BA.02 were the relatively predominant alleles. The number of rare *ABO**B alleles (including *ABO**BA; 27 *ABO**B alleles) was a little higher than the *ABO**A alleles

(including *ABO*cisAB*; 20 *ABO*A* alleles) in our study. Combining our previous studies on the distribution of *ABO* blood group allele and the molecular basis of A2 subgroup before [15, 17], the type of the rare *ABO* alleles in this study was little different from the results of the others [19]. It clearly indicated that the molecular basis of *ABO* variants had specific characteristics among the different populations. The best blood transfusion practices involving *ABO* variants will be based on the data and experience of specific populations. The amino acid changes had different properties and significant effects on the encoded protein. The nucleotide mutation occurred with high frequency (91.3%) in exons 6 and 7 corresponding to the catalytic domain of GT, which is the crucial function part of the protein [20]. According to the 3D structure analysis, although the overall structure of the point mutant GTs was similar to the wild type, the mutation GTs expected that the amino acid residue replacement may change the numbers and the spatial distance of hydrogen bonds forming and induce the steric hindrance with the neighboring residues, resulting in decreasing the protein stability. Thus, the protein stability reduced by the hydrogen bond change may be a possible explanation of the changes of enzyme activity and properties, leading to the phenotypes of the mutants.

Except for the alleles with the point mutation, 2 nucleotide insertions c.804 dupG (*ABO*AEL.08*) and c.1054_1055 insA (A222) were identified with Ael and A2 phenotypes, respectively, resulting in a frame shift to p.F269Vfs*124 and p.R352Qfs*39 in GTAs. c.1054_1055insA was added, an additional 39 amino acids, to the enzyme before translation halted, whose features were similar to the c.1061delC (*ABO*A2.01*) [5]. In addition, 2 nucleotide deletion c.3_4delG (*ABO*B3.new15*) and c.484delG (*ABO*B_W.new11*) were detected with ABx and Bel phenotypes, respectively, resulting in a frameshift (p.M1Mfs*18 and p.V162OPA) for a premature termination in GTBs. Premature termination codons were generally considered to form production of a truncated and inactive protein [21]. The 3D structure of the mutant with c.484delG also predicted that these deletion mutations result in the inability to form a complete protein. But very weak antigens were detected in the individual by an adsorption-elution test. Previous studies showed that several *ABO* subgroup alleles predicted to code for C-terminally truncated enzymes had been described in individuals with weak A phenotypes as well [22, 23]. Thus, the reductions of activity in these cases might be explained by partial lack of the catalytically active site. Remarkably, the individual with *ABO*B₃.new15* (c.3_4delG), leading to an N-terminally truncated B transferase (p.M1Mfs*18), showed agglutination strength 1+ with anti-B. A possible explanation

for this result was that another Met downstream of the premature termination codon might be initiated as the translation start codon, being expected to produce an N-terminally shortened polypeptide, which may be capable of producing GTs with reduced enzyme activity [24].

All the genomic sequence data were useful for evaluating the genetic diversity and for lineage definition. Selt-sam et al. reported that the intron-based phylogenetic analysis revealed 5 main lineages: *ABO*A*, *ABO*B*, *ABO*O01*, *ABO*O.02*, and *ABO*O03* [18]. In this study, we constructed phylogenetic tree for the common alleles and the subgroup alleles found in our laboratory according to the exon 1–7 nucleotide sequences. This finding may enrich the knowledge of coding region in the *ABO* evolution. The figure clearly revealed that there were three distinct allelic lineages, *ABO*A*, *ABO*B*, and *ABO*O.01.02*. The *ABO*O.01.01* alleles were included in the A-related group, which indicated that the relationship between A and O alleles was close. The *CisAB* belonged to *ABO*B* allelic lineage, indicating that the similarity between *CisAB* and *ABO*B* was higher than the *ABO*A*. This relationship also indicated that *CisAB* was likely to be derived from the antisense mutation of *ABO*B* allele. However, understanding the evolutionary history of the *ABO* locus is complicated because of the different levels of nucleotide diversity. The phylogenetic analysis may change by using different region sequences and the major allelic lineages were different.

In our study, another 75 variants were considered to have another molecular basis. In the sequence of the entire *ABO* coding region and flanking splicing sites, we did not find any changes by the PCR-SBT method. The phenotype changes of 32 patient samples might be due to the diseases [25, 26]. The erythroid cell-specific regulatory element in the first intron of *ABO* gene also played an important role in the antigen differential expression. But the molecular mechanism underlying the other samples still remains largely unknown. We presumed that the other transcriptional regulatory elements and methylation might affect the transcriptional activity of the *ABO* gene [27, 28]. Further studies will be needed to assess the impact of the other regulatory elements on *ABO* transferase function.

In summary, we investigated the molecular basis of 211 Chinese *ABO* variants. 48 rare *ABO* alleles, including 16 novel alleles, were characterized and their genetic diversity was analyzed. The effect of protein stability change induced by the mutation was a potential cause of the *ABO* subgroup. In the practical work, our study may be beneficial for making more appropriate transfusion strategies for certain populations and thus help to improve the blood safety.

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Statement of Ethics

Informed consent was obtained, and the study was approved by the local Ethics Committee of the Blood Center of Zhejiang Province.

Disclosure Statement

The authors have no conflicts of interest to declare.

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