Serologic and molecular characterization of the B(A) blood group in the Chinese population

Z.H. Guo, D. Xiang, Z.Y. Zhu, X. Liu, H.P. Chen, J.L. Wang, D.Z. Liu, and T. M. Zhao

B(A) phenotype individuals have normal B antigen and a small amount of A antigen on the RBCs with anti-A in the plasma. Some highly potent monoclonal anti-A reagents are capable of agglutinating B(A) RBCs, which therefore usually results in a discrepancy between RBC and plasma ABO grouping. To date, five B(A) alleles *(ABO*B(A)01, B(A)02, B(A)03, B(A)04,* and *B(A)05)* have been defined by nucleotide sequences. To get a more complete picture of B(A) phenotypes found in the Chinese population and resolve blood donor typing problems caused by B(A) alleles, a serologic and molecular study of nine unrelated Chinese individuals and three families carrying B(A) alleles was conducted. Allele *B(A)02* with a 700C>G mutation, allele *B(A)04* with a single 640A>G substitution, and allele *B(A)05* with a 641T>C mutation were detected in multigenerational families and unrelated blood donors. Neither the *B(A)01* nor *B(A)03* alleles with 703A>G substitutions were observed in this study. In addition, a polymerase chain reaction with a sequence-specific primer genotyping assay was developed for rapid identification of *B(A)02, B(A)04,* and *B(A)05* alleles using genomic DNA samples. ***Immunohematology* 2007;23:69–74.**

Key Words: ABO subtype, B(A) phenotype, B(A) allele, PCR-SSP genotyping

Owing to the naturally overlapping substrate specificities of the A and B glycosyltransferases, the presence of small amounts of A antigen on certain B group RBCs was observed in the 1980s by using certain highly potent monoclonal anti-A reagents; these were designated as the B(A) phenotype.[1-4] The B(A) phenotype is attributable to specific mutations in the B gene that cause the B transferase to synthesize small amounts of A antigen. B(A) individuals carry normal B antigens and a small amount of A antigens on their RBCs, whereas their sera contain anti-A. The molecular basis of the first B(A) phenotype was determined by Yamamoto et al.[5] To date, five B(A) alleles (*ABO* B(A)01, B(A)02, B(A)03, B(A)04,* and *B(A)05*) have been defined by nucleotide sequences according to the Blood Group Antigen Gene Mutation Database (http://www.ncbi.nlm.nih.gov). Compared with *B101* alleles, the *B(A)01* allele has one silent substitution (657T>C) and one replacement substitution (703A>G), resulting in an amino acid change from serine to glycine at amino acid position 235. The *B(A)02* allele was originally identified in an Asian individual with a single 700C>G substitution, which predicts the alteration of proline to alanine at position 234.[6] The allele *B(A)03* differs from *B(A)01* by only a single silent substitution of 657C>T.[7] Both the alleles *B(A)04* and *B(A)05* were recently found in Chinese populations with 640A>G and 641T>C substitutions, respectively.[8-10] A further analysis of B(A) phenotype found in Chinese individuals was carried out. This study involved nine unrelated $A_{weak}B$ phenotype blood donors who had ambiguous ABO typing results. The study was then extended to three families, followed by identification of three B(A) alleles: *B(A)02, B(A)04,* and *B(A)05.*

Materials and Methods

Sample collection

Nine B(A) samples were collected from unrelated individuals from 2000 to 2005. Five individuals carried the *B(A)02* allele, three carried the *B(A)04* allele, and one carried the *B(A)05* allele. Three samples were obtained from Shanghai city, two from Zhejiang province, and the other four from Sichuan, Fujian, Anhui, and Henan provinces, respectively.

Serologic studies and DNA-based ABO genotyping

Peripheral blood samples were collected using ACD anticoagulant tubes. The participants in this study were all Chinese, and informed consent was obtained. Serologic characteristics were determined by use of hemagglutination and adsorption and elution methods, according to the *AABB Technical Manual,* with the exception of reverse ABO grouping.[11] In our reverse ABO grouping, 1 drop of plasma instead of 2 to 3 drops of serum was added to each tube. The reagents used

for this study were murine monoclonal anti-A (BIRMA-1), anti-B (LB-2), and anti-A,B (ES4+ES15; Serological Corporation, Livingston, UK) and human polyclonal anti-A, anti-B, and anti-A,B (Blood Reference Laboratory, Shanghai, China), in addition to anti-A_1 (*Dolichos biflorus*) and anti-H (*Ulex europaeus*) lectins (Gamma Biologicals Inc., Houston, TX). Genomic DNA was prepared from whole blood using a DNA blood mini kit (QIAamp, Qiagen, Hilden, Germany). Preliminary genotyping for *A101, A2, B,* and *O* alleles was carried out using the duplex polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) method, which allows detection of single-nucleotide polymorphisms (SNP) at nucleotide (nt) 261, nt 467, nt 703, and nt 1096, as described by Olsson and Chester.[12] In brief, a 252-base pair (bp) fragment of exon 6 and an 843-bp fragment of exon 7 were amplified by PCR in the same tube using primer pairs mo57/mo46 and mo101/mo71 (Table 1). Each PCR reaction was carried out with a final volume of 25 µL containing 2.5 µL of PCR buffer, 20 mM $MgCl_2$, 0.2 mM dNTP, 1 U *Pfu* DNA polymerase (Bio Basic Inc., Markham Ontario, Canada), 0.2 µM primers, 10% (vol/vol) glycerol, and 50 ng of genomic DNA. After denaturation for 10 minutes at 95°C, samples were subjected to 35 cycles of PCR in a DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA). The first 10 cycles consisted of 94°C for 60 seconds, 63°C for 90 seconds, and 72°C for 60 seconds. The remaining 25 cycles consisted of 94°C for 60 seconds, 61°C for 90 seconds, and 72°C for 60 seconds followed by a final extension at 72°C for 10 minutes. Ten microliters of PCR product was digested with *Kpn*I and *Msp*I at 37ºC for 2 hours according to the manufacturer's protocols (New England Biolabs, Ipswich, MA). Then, 7 µL of the digestion mixes was separated electrophoretically for 1.5 hours at 150 V on 12% nondenatured polyacrylamide gels. The gel was stained using ethidium bromide.

Cloning and sequencing

The entire exons 6 and 7 of the ABO gene were amplified by PCR using two pairs of primer, mo57/mo46 and mo101/mo71, respectively. The blunt-end PCR products of *Pfu* DNA polymerase were purified using a gel extraction kit (Qiagen). Adenine tails were added artificially. The PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI), and then transformed into competent DH5α *E. coli.* After transformation, four to six colonies were randomly selected, and plasmid DNA was extracted using Wizard Minipreps' DNA purification system (Promega). For each sample, at least two positive clones were sequenced with a BigDye Terminator Cycle Sequencing kit on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

PCR-SSP genotyping

An easy polymerase chain reaction with a sequence-specific primer (PCR-SSP) assay was developed to detect SNP at nt 640, nt 641, and nt 700. This approach relies on the sequences of the primers used in the PCR. When the 3′ nucleotide of a primer perfectly matches the sequence at the site of a given allele, the sample DNA, as a template for PCR, will be amplified. However, when the 3′ nucleotide of the primer is mismatched, amplification will not occur. For the detection of each allele, two pairs of primers are included in the PCR mixes; one is an allele-specific primer to amplify a specific allele and the other is a common primer to amplify a common gene to control the efficiency of the PCR. The sequence-specific oligonucleotide primers were designed according to published sequence data (Table 1). Six PCR-SSP mixes (B640A, B640G, B641T, B641C, B700C, and B700G) were prepared, to detect nt 640A, nt 640G, nt 641T, nt 641C, nt 700C, and nt 700G, respectively. Control primers that amplified a 427-bp fragment of the human growth hormone (HGH) gene were included in all reactions. The initial PCR was carried out with 1 µL of purified DNA sample (0.05 to 0.10 µg), 1 µL of diluted Taq polymerase (0.25–0.33 U), and 8 µL of PCR mix (10 mM Tris-HC1 [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [wt/vol] gelatin, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, 0.2 µM of each internal control primer) in a final 10-µL reaction volume. After denaturation for 5 minutes at 95°C, samples were subjected to 30 cycles of PCR in a DNA thermal cycler. Each cycle consisted of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes, followed by a final extension at 72°C for 5 minutes. PCR products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 µg of ethidium bromide/mL and visualized with ultraviolet transillumination.

Classification and nomenclature

The *ABO* allele names used in this paper conform to the unofficial nomenclature used in the Blood Group Antigen Gene Mutation Database.[13]

Table 1. Primers used for B(A) blood group genotyping by PCR-SSP

Primer*	Sequence (5′-3′)	Nucleotide position	GenBank accession no.	Primers: PCR product size (bp)
mo57(F)	CGGGATCCATGTGGGTGGCACCCTGCCA	20069–20089	AY268591	mo57/mo46:252
mo46(R)	CGGAATTCACTCGCCACTGCCTGGGTCTC	20286–20308	AY268591	
mo101(F)	CGGGATCCCCGTCCGCCTGCCTTGCAG	21292–21311	AY268591	mo101/mo71:843
mo71(R)	GGGCCTAGGCTTCAGTTACTC	22108–22127	AY268591	
640A(F)	GTGTGCGTGGACGTGGACA	622–640	AF134412	640A/703A:100
640G(F)	GTGTGCGTGGACGTGGACG	248–266	DQ124679	640G/703A:100
641T(F)	TGTGCGTGGACGTGGACAT	623–641	AF134412	641T/703A:99
641C(F)	TGTGCGTGGACGTGGACAC	249–267	DQ124678	641C/703A:99
703A(R)	GGCTGCTTCCGTAGAAGCT	6142–6160	AJ536135	
526G(F)	CTGTCAGTGCTGGAGGTGG	893–912	AJ536135	
700C(R)	TGCTTCCGTAGAAGCTGGG	1319–1298	F134412	526G/700C:215
700G(R)	TGCTTCCGTAGAAGCTGGC			526G/700G:215
HGH(F)	GCCTTCCCAACCATTCCCTT	5947–5965	M13438	
HGH(R)	TCACGGATTTCTGTTGTGTTTC	700–718	M13438	HGHF/HGHR:427

*(F) indicates forward primer; (R), reverse primer. Primers mo57, mo46, mo101, and mo71 were prepared according to Olsson and Chester, 1995. The sequences underlined are artificial. Primer 700G was modified from the primer originally reported by Yu et al.[6] Primer pair 640A/703A detects nt 640A and nt 703A; 640G/703A detects nt 640G and nt 703A; 641T/703A detects nt 641T and nt 703A; 641C/703A detects nt 641C and nt 703A; 526G/700C detects nt 526G and nt 700C; 526G/700G detects nt 526G and 700G.

Table 2. Serologic characteristics and genotypes of B(A) phenotype

			Reaction of RBCs with					Reaction of plasma with		
Samples*	Phenotype	Genotype	Anti-A	Anti-B	Anti-A,B	Anti-A_1	Anti-H	A Cells	B Cells	O Cells
Family A										
1	B†	*B(A)04/B*	0	12	12	0	5	5	0	0
2	O†	*O/O*	0	0	NT	NT	10	8	10	0
3	B†	*B/O*	0	12	12	0	5	8	0	0
4	B(A)	*B(A)04/O*	8	12	12	0	8	8	0	0
5	B(A)	*B(A)04/O*	5	12	12	0	8	8	0	0
6	A	*A/O*	12	0	NT	NT	8	0	12	0
7	B(A)	*B(A)04/O*	8	12	12	0	10	8	0	0
Family B										
1	B(A)†	*B(A)05/O*	5	12	12	NT	10	5	0	0
2	O†	*O/O*	0	0	NT	NT	12	8	8	0
3	B(A)	*B(A)05/O*	8	12	12	0	12	8	0	0
4	B(A)	*B(A)05/O*	8	12	12	0	12	8	0	0
5	B(A)†	*B(A)05/O*	5	12	NT	NT	8	NT	NT	NT
Family C										
1	O†	*O/O*	0	0	NT	0	12	10	10	0
2	B†	*B(A)02/B*	0	12	NT	0	5	10	0	0
3	B(A)	*B(A)02/O*	8	12	NT	0	8	8	0	0
4	B(A)	*B(A)02/O*	8	12	NT	0	8	10	0	0
5	B	*B/O*	0	12	NT	0	5	10	0	0
6	B(A)	*B(A)02/O*	10	12	NT	0	8	8	0	0
7	B	*B/O*	0	12	NT	0	5	8	0	0
8	B(A)	*B(A)02/O*	8	12	NT	0	8	8	0	0
Unrelated										
1	B(A)	*B(A)02/O*	10	12	NT	0	12	8	0	0
2	B(A)	*B(A)02/O*	10	12	NT	0	12	8	0	0
3	B(A)	*B(A)02/O*	12	12	NT	0	12	10	0	0
4	B(A)	*B(A)02/O*	12	12	NT	0	8	10	0	0
5	B(A)	*B(A)04/O*	8	12	12	0	12	12	0	0
6	B(A)	*B(A)04/O*	5	12	12	0	12	12	0	0

*Sample numbers are the same as used in Figure 2.
† No. 5 in family B is a baby. All others marked with dagger are older than 60 years of age.
NT = not tested.

Results and Discussion

Some monoclonal anti-A reagents used in our routine donor blood grouping are capable of agglutinating B(A) RBCs; therefore, this results in a discrepancy between RBC (forward) and plasma (reverse) ABO grouping. In Table 2, a discrepancy between the forward and reverse ABO grouping was observed in nine blood donors (Family A No. 7, Family B No. 4, Family C No. 6, and six unrelated individuals from No. 1 to No. 6). B(A) RBCs showed a trace reaction with human polyclonal anti-A and stronger agglutination with murine monoclonal anti-A. Some samples carrying the *B(A)02* allele scored as high as 12 when they were reacted with monoclonal anti-A. In addition, these sera contained only anti-A antibody. Our observation is in concordance with the study performed by Yu et al.[6] The presence of A antigens on the RBCs was confirmed by adsorption and elution methods, whereas only B and H substances were detected in their saliva (data not shown). The phenotype could be serologically distinct from classic cis-AB. In classic cis-AB, the B antigen on RBCs is weakly expressed, and most of them are represented serologically by A_2B_3.[14] The preliminary genotypes of all nine B(A) donors were B/O heterozygous,

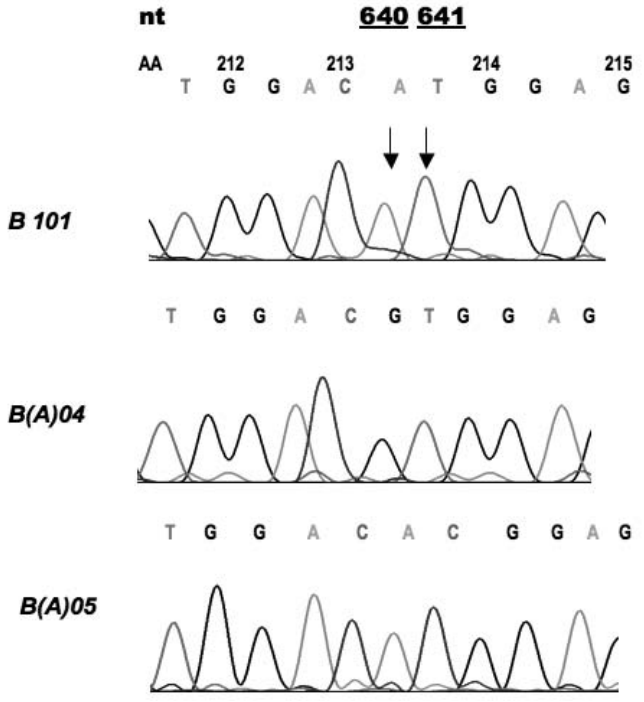

Fig. 1. Sequencing chromatograms. The region from codon 212 to 215 (number on top) in exon 7 of *B101, B(A)04,* and *B(A)05* alleles is shown. The arrows indicate the position for nucleotide 640 and 641, respectively.

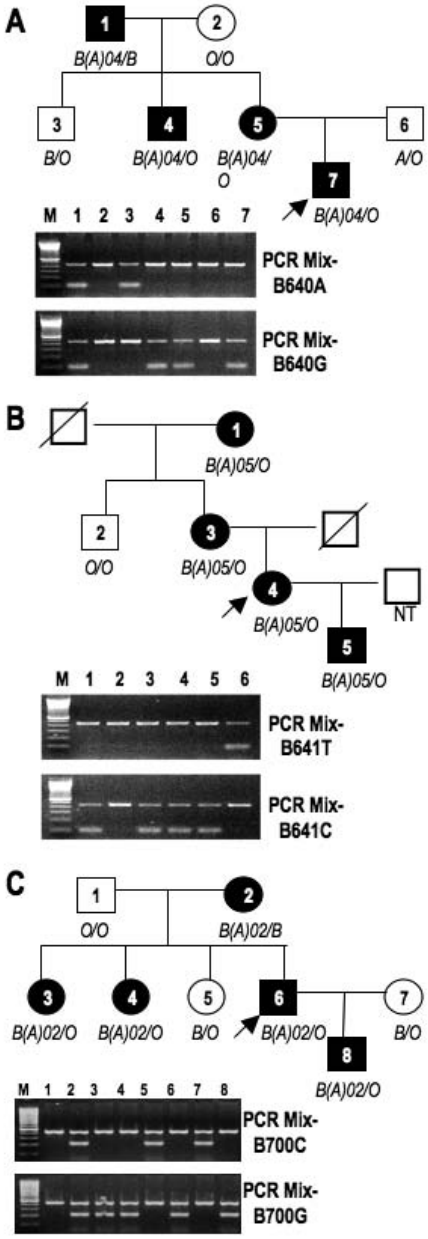

Fig. 2. Family pedigree and detection of SNP by PCR-SSP assay. The arrows indicate the propositi. ABO genotypes are noted in the symbols. Filled squares and circles represent *B(A)02, B(A)04,* or *B(A)05* allele carriers; open squares and circles indicate those family members who do not carry the B(A) alleles; NT = not tested; / = deceased. The numbers used for the family members are the same as those used on the gel electrophoretogram. Lane M shows a 100-bp DNA ladder. The top band is the housekeeping gene (HGH) as an internal positive control. **(A)** Segregation and inheritance of *B(A)04* allele in family A. Genotypes at nt 640 were determined by two PCR mixes, B640A and B640G, in two separate PCR amplifications. **(B)** Inheritance of *B(A)05* allele in family B. Two PCR mixes, B641T and B641C, were used in two separate PCR amplifications to determine genotypes at nt 641. An unrelated donor with genotype *B101/B101* was used (number 6 on the gel) as a positive control for PCR mix B641T. **(C)** Segregation and inheritance of *B(A)02* allele in family C. Two PCR mixes, B700C and B700G, were used in two separate PCR amplifications to determine genotypes at nt 700.

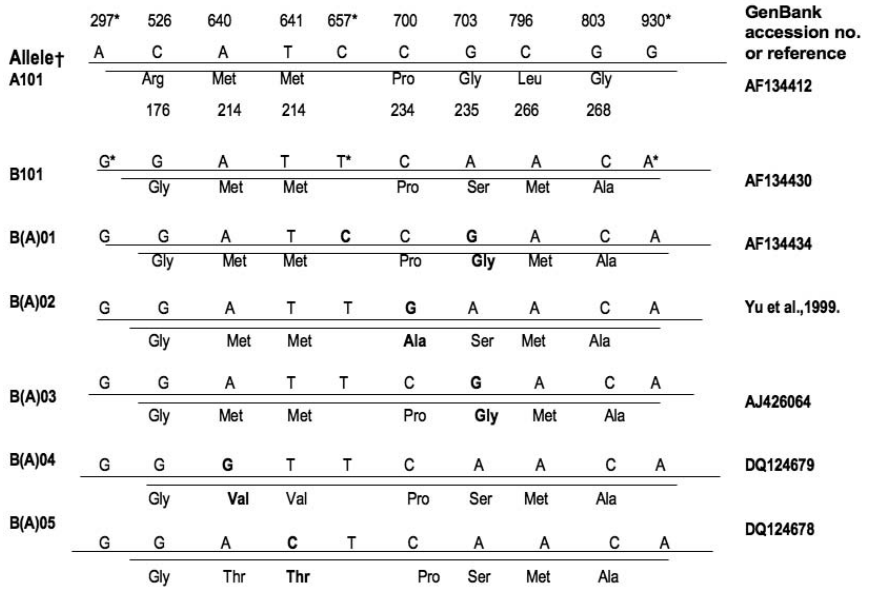

Fig. 3. Variation of nucleotides and deduced amino acid in exon 6 and exon 7 of *ABO* gene. Dagger signifies allele nomenclature according to Blood Group Antigen Gene Mutation Database.[13] The letter marked with an asterisk indicates silent substitution.

determined by performing a PCR–RFLP genotyping assay (Table 2).

The genotypes of the family propositi were determined using sequence analysis. Exons 6 and 7 of the *ABO* gene were cloned and then sequenced in both forward and reverse directions. Nucleotide sequence alignment revealed that the exon 7 sequence of donor No. 7 in family A differs from the *B101* allele by a single 640A>G substitution, which changes codon 214 from methionine to valine (ATG → GTG; Figure 1). Sequence analysis of another donor (No. 4 in family B) indicated a single 641T>C substitution in exon 7 of *ABO* gene, which results in an amino acid replacement of methionine by threonine at codon 214 (ATG → ACG; Figure 1). Donor No. 6 (in family C) was found to carry a 700C>G mutation that had been reported previously as the *B(A)02* allele by Yu et al.[6]

B(A) alleles with 640A>G or 641T>C mutations were designated as *B(A)04* and *B(A)05,* respectively. Family studies indicated that both the *B(A)04* and *B(A)05* alleles were inherited as expected (Figure 2). The nucleotide sequences of these two alleles have been deposited in GenBank with accession number DQ124679 for *B(A)04* and DQ124678 for *B(A)05,* respectively. To rapidly detect *B(A)02, B(A)04,* and *B(A)05* alleles using genomic DNA, an easy PCR-SSP method was developed. As shown in Figure 2, six possible genotypes at nt 640, nt 641, and nt 700 could be clearly distinguished from each other. To check the reliability and specificity of the typing method, a total of 34 DNA samples, including 19 B(A), 5 A, 5 B, and 5 O phenotypes, were repeatedly tested and a concordance rate of 100 percent was observed. In addition, the validity of this method was verified by sequence analysis.

Most ABO variants arise from mutations in exons 6 and 7 of the *ABO* gene. The products of *A* and *B* genes differ by four amino acid substitutions at 176, 235, 266, and 268. It was thought that the amino acid residues at these four positions were critical for determination of the specificities of the A and B glycosyltransferase, and that an overlapping specificity results in the formation of B(A) phenotype.[5,14] Both the *B(A)01* and *B(A)03* alleles have a Ser235Gly substitution, whereas the *B(A)02* allele encodes a Pro234Ala substitution just before position Ser235. As shown in Figure 3, the *B(A)04* and *B(A)05* alleles encode a Met214Val and a Met214Thr substitution, respectively, at the same position 214. This suggests that the residue at 214 might also be involved in the determination of the enzyme specificity.

Acknowledgments

This work was supported by a grant (No. 02ZB14087) from the Shanghai Natural Science Foundation. We are grateful to Li-Yang Xie and Jing Li for collecting blood samples from the B(A) families.

References

1. Beck ML, Hardman JT, Henry R. Reactivity of a licensed murine monoclonal anti-A reagent with group B cells (abstract). Transfusion 1986; 26:572.
2. Beck ML, Yates AD, Hardman JT, et al. Consequences of overlapping substrate specificity of glycosyltransferases (abstract). Transfusion 1987; 27:535.
3. Goldstein J, Lenny L, Hurst, et al. Direct evidence for the presence of A antigen on group B erythrocytes. Blood 1987;70(Suppl. 1):109a.
4. Goldstein J, Lenny L, Davies D, et al. Further evidence for the presence of A antigen on group B erythrocytes through the use of specific exoglycosidases. Vox Sang 1989;57:142–6.

5. Yamamoto F, McNeill PD, Yamamoto M, et al. Molecular genetic analysis of the ABO blood group system: 3. A(X) and B(A) alleles. Vox Sang 1993; 64:171–4.
6. Yu LC, Lee HL, Chan YS, et al. The molecular basis for the B(A) allele: an amino acid alteration in the human histoblood group B alpha-(1,3)-galactosyltransferase increases its intrinsic alpha-(1,3)-*N*-acetylgalactosaminyl transferase activity. Biochem Biophys Res Commun 1999;262:487–93.
7. Seltsam A, Hallensleben M, Kollmann A, et al. Systematic analysis of the ABO gene diversity within exons 6 and 7 by PCR screening reveals new ABO alleles. Transfusion 2003;43:428–39.
8. Guo ZH, Xiang D, Zhu ZY, et al. Molecular study on CisAB and B(A) in China (Abstract). Vox Sang 2004;87(Suppl 3):81.
9. Guo ZH, Xiang D, Zhu ZY, et al. Novel B(A)640 and B(A)641 alleles detected in Chinese individuals (Abstract). Vox Sang 2005;89(Suppl 2):24.
10. Deng ZH, Yu Q, Wu GG, et al. Molecular genetic analysis for Ax phenotype of the ABO blood group system in Chinese. Vox Sang 2005; 89:251–6.
11. Brecher ME. ABO, H, and Lewis Blood Groups and structurally related antigens In: Brecher ME, AABB Technical manual. 14th ed. Bethesda, MD: American Association of Blood Banks, 2002:282.
12. Olsson ML, Chester MA. A rapid and simple ABO genotype screening method using a novel B/O2 versus A/O2 discriminating nucleotide substitution at the ABO locus. Vox Sang 1995; 69:242–7.
13. Blumenfeld OO, Patnaik SK. Allelic genes of blood group antigens: a source of human mutations and cSNPs documented in the Blood Group Antigen Gene Mutation Database. Human Mutation 2004; 23:8–16.
14. Daniels G. ABO, Hh, and Lewis systems. In: Daniels G, Human blood groups 2nd ed. Oxford, UK: Blackwell Scientific, 2002:40–1.

Zhong-Hui Guo, MS (corresponding author), Dong Xiang, BS, Zi-Yan Zhu, BS, Xi Liu, He-Ping Chen, and Jian-Lian Wang, BS, Blood Group Reference Lab, Shanghai Blood Center, 1191 Hong Qiao Road, Shanghai 200051, P. R. China; Da-Zhuang Liu, BS, Shanghai Transfusion Institute, Shanghai Blood Center, Shanghai, P. R. China; Tong-Mao Zhao, PhD, NIAID, National Institutes of Health, Bethesda, MD, USA.