Genotyping of Samples Lacking Expected Antibodies in ABO Blood Group

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We report nine donations with ABO inconsistency in reverse typing caused by partly or entirely missing antibodies. A and B antigens and antibodies were examined by serological blood typing, and ABO deoxyribonucleic acid (DNA) analyses were performed by sequence-specific priming and sequencing. A *B101* allele was demonstrable in a case with O phenotype. The molecular mechanisms in deficiency of natural ABO antibody could be partly clarified. The ABO genotyping

technique is an accurate method for determining the blood samples involved in ABO grouping discrepancies and is a valuable complement to serology for correct determination of donor blood status. The mechanisms involved in the absence of potent natural antibodies directed against A and B antigen lacking on an individual's own red cell membranes remain to be further investigated. *J. Clin. Lab. Anal.* 21:363–366, 2007. © 2007 Wiley-Liss, Inc.

Key words: ABO; antibody; ABO allele; genotype

INTRODUCTION

ABO antibodies are among the most predominant naturally-occurring antibodies. Antibodies are regularly present in serum of individuals who do not express the corresponding ABO antigens on their red cells. Type A persons possess anti-B antibodies in their plasma. Type B persons display anti-A antibodies. Type O individuals exhibit both anti-A and anti-B antibodies (1). The physiological importance of natural antibodies remains unclear. However, it is widely accepted that natural ABO antibodies were produced for nonspecific antibacterial defense and self-regulation of the immune system. When a healthy individual is exposed to ABO trisaccharides, which are widely expressed on bacteria of the intestinal flora, as a result, he produces antibodies to allo-ABH antigens although he remains unresponsive to autologous ABH antigens (2). The ABO antibodies mainly consist of immunoglobulin M (IgM), while immune antibodies of the immunoglobulin G (IgG) isotype occur after alloimmunization in pregnancy or ABO-incompatible transfusions (3). ABO antigens and the corresponding antibodies are maintained through the lifespan following birth with no specific stimuli (4). With the exception of newborn infants, the absence of expected antibodies is extremely rare.

For these characteristics of the ABO blood group, routine ABO blood typing includes a forward ABO antigens test and a corresponding reverse antibody test. Through assaying ABO blood group type, discrepancy samples were occasionally reported. That is, using routine serological tests, the ABO phenotype of some samples cannot be correctly typed.

In the present study, we collected some exceptional samples to characterize the ABO antigen and antibodies and to explore the conundrum of ABO type.

MATERIALS AND METHODS

A total of nine samples were collected with consent by our blood group reference laboratory in the ShenZhen Blood Center from March 2006 to August 2006. These samples were all from healthy Chinese blood donors

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including eight male donors and one female donor whose age ranged from 18 to 35 years. Venous blood samples were collected according to standard blood banking practice: 5 mL of whole blood (no additives in tube) for ABO blood grouping, 5 mL of ethylenediaminetetraacetate (EDTA)- or citrate-anticoagulated whole blood for the adsorption-elution test, and deoxyribonucleic acid (DNA) preparation.

ABO typing tests including forward red blood cell (RBC) and reverse serum blood grouping procedures, and additionally, adsorption-elution tests were performed. Antisera used included monoclonal anti-A and anti-B (Dominion, Darmouth, Canada), polyclonal anti-A, anti-B (ShuBao, Chengdu, China), anti-A1 from *Dolichus biflorus* (Dominion), monoclonal anti-AB (Immucor, Houston, TX), and polyclonal anti-AB (blend of human serum). Lectins were used for anti-H from *Ulex europaeus* (Brother, ChangChun, China). Type A, B, and O RBCs for reverse blood tests were prepared from freshly drawn venous blood of healthy volunteers. All routine serological tests were carried out according to the AABB Technical Manual (14th edition; Bethesda, MD).

DNA was prepared from the buffy coats by using a salting-out method (5). We utilized an ABO genotyping reagent kit, which consisted of four polymerase chain reactions with sequence specific primers (PCR-SSPs) designed specially to differentiate the common A, B, O, A201 alleles only (6). Four SSP pairs were designed specific according to the single nucleotide polymorphisms (SNPs) at nucleotide(nt) positions 526, 803, 261, 1060 of exon 7 of the ABO gene, respectively. The following primers were designed and synthesized by our laboratory: 5'-gga agg atg tcc tgc agg tg-3' and 5'-tga gga tgt gga tgt tga at-3'; 5'-ctg tca gtg ctg gac gtg g-3' and 5'-tcc acg cac acc agg taa tc-3'; 5'-gga agg atg tcc tcc tgg ta-3' and 5'-ctt gat ggc aaa cac agt taa c-3'; and 5'-cag gcc aac ggc atc gag3' and 5'-ctg gca gcc gct cac ggt-3'. PCR reaction conditions were as follows: the initial denaturing step occurred at 95°C for 5 min, followed by 30 rounds of a temperature cycle at 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min. PCR products were run via electrophoresis on 4% agarose gel and visualized by ethidium bromide staining.

Because exons 6 and 7 constituted 77% of the ABO gene, PCR-based gene analysis was performed on these two exons. Primer pairs mol-46/mol-57 and mol-71/mol-101, described in the previous study (7), were used to amplify exons 6 and 7. The PCR fragment size for exons 6 and 7 were 252 bp (251 bp for O1) and 843 bp, respectively. PCR amplification was carried out in a reaction volume of 50 μ L containing 1 $\times$ PCR buffer, 400 μ M of each of the four deoxynucleotide triphosphate (dNTP), 0.2 μ M each primer, 300–500 ng of genomic DNA, and 0.5 U of Taq DNA polymerase

(Promega, Madison, WI). Amplification was carried out under the following conditions: 95°C for 10 min; 10 cycles of: 94°C for 60 sec, 63°C for 90 sec, and 72°C for 60 sec; 25 cycles of: 94°C for 60 sec, 61°C for 90 sec, and 72°C for 60 sec; followed by a final elongation of 72°C for 10 min. The PCR products were purified with Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA) according to the manufacturer's instruction. The purified products (templates) were subjected to sequencing reaction using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and were analyzed by ABI Prism 3100 DNA Sequencer (both from Applied Biosystems, Foster City, CA).

RESULTS

Serologic Phenotype

A total of nine blood samples lacking predictable antibodies were discovered among 25,160 blood donors, owing to the observed discrepancies between forward and reverse typing in routine ABO grouping. Adsorption-elution tests performed by testing A or B RBCs were all negative. In routine ABO serological typing, the serum from all of these nine samples were shown to be entirely or partly deficient of natural antibodies. RBCs of one sample tested by anti-A and anti-B reagents were characterized as O type and his sera sample only contained anti-B activities and wholly lacked anti-A antibody; RBCs of one sample were characterized as O type and his sera sample contained normal anti-A activity but no anti-B activity; RBCs of one female and one male sample were diagnosed as O type and their sera samples simultaneously entirely lacked anti-A and anti-B antibodies. RBCs of three individuals were characterized as A type and their sera samples contained weak part anti-B activities, and RBCs of two samples were characterized as common B type and the sera completely lacked anti-A antibody. Serological results of all the nine individuals are shown in Table 1.

Genotype and Sequence Analysis of the ABO Allele

We defined the ABO allele using the unofficial nomination described by the Blood Group Antigen Gene Mutation Database (<http://www.ncbi.nlm.nih.gov/projects/mhc/xslcgi.fcgi?cmd=bgmute/home>). Initial genotype screening by PCR-SSP and the further direct DNA sequencing results were compared to the consensus sequence of the *A101* allele. Except for a special interesting sample phenotyped as O by serological forward ABO blood grouping (his sera contained normal anti-A activity but no anti-B activity), sequencing analysis demonstrated to be heterozygous as *B101/*

TABLE 1. Results of nine samples with typing discrepancies by serologic tests and ABO genotyping

Sample (Sex)	Anti-A ^a	Anti-B ^a	Anti-A	Anti-B ^b	Anti-AB	A type cell	B type cell	O type cell	Phenotype	Genotype
1 (M)	0	0	0	0	0	0	4+	0	O	<i>O01/O02</i>
2 (M)	0	0	0	0	0	0	0	0	O	<i>O01/O02</i>
3 (F)	0	0	0	0	0	0	0	0	O	<i>O01/Ov(nt1096A>G)</i>
4 (M)	0	0	0	0	0	4+	0	0	O	<i>B01/O01</i>
5 (M)	4+	0	3+	0	4+	0	W+	0	A	<i>A102/O01</i>
6 (M)	4+	0	4+	0	4+	0	1+	0	A	<i>A102/O02</i>
7 (M)	4+	0	4+	0	4+	0	W+	0	A	<i>A101/A102</i>
8 (M)	0	4+	0	4+	4+	0	0	0	B	<i>B101/B101</i>
9 (M)	0	4+	0	4+	4+	0	0	0	B	<i>B101/O02</i>

^aMonoclonal antisera.^bPolyclonal antisera.

M = Male, F = Female.

O01; bolded genotype in Table 1 disagreed with the phenotype; the alignment revealed that the genotyping results by sequencing analysis of other eight samples were consistent with the ABO forward grouping as follows: among three *OO* samples, two of which were determined as *O01/O02* genotype, one was determined as *O01/O* novel allele (nt1096G>A on the background of *O01* allele); three A phenotype samples were determined as *A102/O01*, *A102/O02* and *A101/A102*, respectively; two B phenotype samples were typed as *B101/B101* and *B101/O02*, respectively.

DISCUSSION

ABO is the most important blood group in transfusion medicine, and also is one of the major histocompatibility antigens. IgM anti-A and/or anti-B regularly present in human serum constitute a major barrier against ABO-incompatible blood transfusions and organ transplantation. The phenotypes A, B, O, and AB have been clinically examined by hemagglutination for transfusion medicine. Some doubts have been explained that ABO antigens can be weakened for diseases or genetic background, and expected antibodies are lacking for infant or has been related to a twin chimera, dispermy, hypogammaglobulinemia, or old age in adults (8). The individuals in this study were all healthy blood donors and all meet the donor selection criteria, they are from 18 to 35 years old and they were further questioned to exclude the above and any immunodeficiency events.

Mistyping or incompatible red cells transfusion can lead to adverse consequences. Through PCR-based genotyping with SSP for determination of ABO and elucidating the molecular genetic background of the ABO blood group, we can possibly predict the ABO phenotype for the right ABO type and ensure the safety of transfusion and transplantation.

It is well known that the ABO gene locus is located on the long arm of chromosome 9 (9q34.1–q34.2). The

ABO gene was shown to consist of seven exons ranging in size from 28 to 688 base pairs (bp), with most of the coding sequence lying in exons 6 and 7. Since Yamamoto et al. (9) first cloned the complementary DNA (cDNA) of A1 glycosyltransferase in 1990, we know that the blood group *A101* allele DNA with a 1,062-bp nucleotide sequence is predicted to encode a 41 kDa enzyme protein. Continuous nucleotide sequence of other alleles coding the ABO blood group had been studied. *ABO* alleles all have highly-conserved sequences. The five alleles, *A101*, *A102*, *B101*, *O01*, and *O02* are all common in every ethnic group. Compared with the reference *A101* allelic sequence, the DNA sequence of the *B101* allele consistently differed from *A101* by three synonymous nucleotide substitutions and four missense mutations at nt526, 703, 796, and 803 that would result in four amino acid substitutions (Arg176-Gly, Gly235Ser, Leu266Met, and Gly268Ala) in the expressed protein. The blood type *A* allele encodes α 1,3 N-acetylgalactosaminyltransferase that forms the A determinate while the blood type *B* allele encodes an α 1,3galactosyltransferase that forms the B determinate. The *O01* and *O02* alleles had a common single nucleotide deletion, G261- in exon 6, encode functionally inert polypeptides that do not further modify H-active precursors, and therefore represent null alleles at this locus. Simultaneously, the *O02* allele was found to harbor mutations at nt 297, 646, 681, 771, and 829 on the basis of the *O01* allele. For the *A102* allele prevailing in Asians, the 467C>T mutation on the *A101* allele is a well-known polymorphism (10–12).

During our routine laboratory work, difficulties in the demonstration of expected isoagglutinins are a common problem in ABO reverse typing. There is some knowledge regarding the causes of weak isoagglutinin activity. Literature reviewed had showed that specific ABO gene may frequently caused a blood typing problem. Non-deletional *O* alleles are the most frequent cause of weak anti-A isoagglutinin detection in the reverse type of

blood group O donors because the *O* allele without the nt261G deletion can express a weak blood group A phenotype (13,14). Some individuals showed no detectable A antigen in their RBCs by absorption-elution tests, the serum anti-A antibody level was reduced, or they harbored recombinant alleles such as *A110* or *R102* (15). These observations provided evidence that *A110*- or *R102*-possessing individuals express a trace amount of A antigen in RBCs that possibly cannot be detected using common serologic methods. Another case, with the inconsistency that the red blood cells lacked both A and B antigens while the serum showed reactivity with control B-red cells but not with A-red cells, was demonstrated to have an *A* allele in the nail of the individual (16). The amounts of A antigen encoded by these ABO alleles appeared to cause inhibition of anti-A production. The molecular mechanisms in deficiency of natural ABO antibody could be partly clarified. So it is effective to solve difficult cases of ABO typing using genotyping technology. In our report, the fact that the RBCs of one case are O type, while his sera lacked anti-B antibody, and he contained a *B* allele, maybe demonstrates the conclusion above. In another case of our study, we defined a novel allele, nt1096G mutation on the *O01* allele, which lay out of the ABO coding sequences area.

Some weak ABO subgroups can easily be confused with O group without antibody. They have some similar serological characteristics. It may be safe for the individual with O phenotype carrying B allele, as a recipient, to be transfused with O type RBCs; however, it can cause a direct antiglobulin test with A type plasma transfusion. The ABO genotyping technique could be an accurate method to distinguish them. We see that ABO genotyping is a valuable complement to serology technology. It resolves ABO discrepancies and leads to more safety in the course of transfusion.

According to the ABO genotype results, we demonstrated the ABO type of these samples and every whole blood unit was divided into RBC and plasma. The two blood components were transfused to patients of the same ABO types without any indisposed symptom. Also, we asked these nine donors not to give their blood in the future.

A total of seven samples showed no specific nt mutations on the exons 6 and 7 of the ABO gene; we will perform sequencing analysis extended to the whole ABO gene and regulatory mechanisms of the ABO antigen expression and etc., as we can not explain the crisis in our present tests. The mechanisms involved in

the absence of natural antibodies remain for further investigation.

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