Transfusion Medicine and Hemotherapy

Original Article

Transfus Med Hemother 2013;40:454–458
DOI: 10.1159/000356378

Received: March 26, 2013
Accepted: May 13, 2013
Published online: October 27, 2013

A Novel *ABO* Gene Variant Leads to Discrepant Results in Forward/Reverse and Molecular Blood Grouping

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Keywords
ABO phenotyping and genotyping · *ABO* sequencing
ABO gene variant · PCR-SSP

Summary
Background: Discrepant results in antigen and reverse ABO blood typing are often caused by a variant *ABO* gene. Molecular analysis can help to characterize such variants. Here, we describe the identification of a novel *ABO* gene variant in a patient with aberrant ABO phenotype and discrepant genotyping results. **Methods:** A patient with discrepant results in automated forward and reverse ABO phenotyping was further investigated by serological (gel and tube technique) and molecular (commercial and inhouse PCR-SSP, DNA sequencing) methods. A PCR-SSP system was established to screen the novel mutation in 1,820 blood donors. **Results:** Standard serological tests confirmed blood group O, however, only anti-B isoagglutinins were present. A monoclonal anti-AB antibody detected very weak agglutination in gel technique. Standard *ABO* genotyping using PCR-SSP led to discrepant results (O^1/O^1 or O^1/A) depending on the test system used. *ABO* exon re-sequencing identified a novel missense mutation in exon 6 at position 248A>G (Asp83Gly) in the binding region of PCR-SSP primers for the detection of 261G alleles. Blood donors with regular ABO blood groups were all negative for the 248G allele designated Aw34. **Conclusion:** The novel *ABO* gene variant Aw34 is associated with very weak A antigen expression and absent anti-A isoagglutinins. The mutation is located in exon 6 close to the O^1-specific 261G deletion in the binding region of PCR-SSP primers. Presumably, depending on the primer concentration used in commercial ABO genotyping kits, the mutation could lead to a false-negative reaction.

Introduction

The *ABO* gene is located on the long arm of human chromosome 9 and consists of 7 exons. Exons 6 and 7 encode the major proportion including the catalytic domain of the glycosyltransferases that mediate the expression of A and B antigens [1, 2]. A number of studies have shown a clear genotype-phenotype correlation [3–5], and almost 300 gene variants are listed in the open access database dbRBC at the NCBI website [6]. Most of the gene variants are characterized by one or more SNPs leading to amino acid changes or stop codons. In addition, some variants result from nucleotide insertions or deletions (INDELs) mostly leading to a frame shift of the reading frame and subsequent alteration of the encoded amino acid sequence. The ABO blood groups A_1, A_2, and B result from the wild-type allele ABO*A101 and the major variant alleles ABO*A201, and ABO*B101. The O phenotype is mostly caused by deletional O alleles such as ABO*O01 or ABO*O02 each characterized by the INDEL polymorphism 261delG in exon 6. The most common non-deletional O allele is ABO*O03 with a point mutation in exon 7 also leading to the O phenotype. Weak expression of A or B antigens (Aw, Ax, Ael, Bw, Bx, Bel) is caused by many different alleles with mutations distributed over the entire coding region of the *ABO* gene.

Here, we describe a novel *ABO* gene variant in a patient who showed discrepancies in forward and reverse phenotyping. Depending on the PCR system used, the *ABO* variant also led to discrepant genotyping results.

Material and Methods

Patient

A 62-year-old woman was hospitalized because of peripheral artery occlusive disease. We performed blood group typing because surgical in-

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Table 1 . Tests and sera used in serological ABO typing.

Test	Anti-A (clone)	Anti-AB (clone)
Microplate test (Galileo)	Birma-1	-
ADK Cassette	anti-AB01	-
ID-Card Diaclon AB0/Rh for patients	A5	ES 131 (ES-15) + Birma-1 + ES-4
ID-Card Diaclon AB0/Rh for newborns	LM 297/628 (LA-2)	ES 131 (ES-15) + Birma-1 + ES-4
Tube technique (immuClone)	Birma-1	-

Table 2. Primers for amplification and sequencing of *ABO* exons 1–7.

Target	Primer binding region	Direction	Sequence (5'–3')
Exon amplification			
Exon 1	exon 1	sense	CCGTCCCTTCCTAGCAGG
	intron 1	antisense	GTGCTGAAAATAGCAGCTCATGG
Exon 2–5	intron 1	sense	GGACCATCTTGGCAGATGAAGG
	exon 6	antisense	GCTCGTTGAGGATGTCGATGTTGA
Exon 6	intron 5	sense	GATTTGCCCGGTTGGAGTCG
	intron 6	antisense	GCTGCATGAATGACCTTTCCC
Exon 7	intron 6	sense	TCGCTGGGAAGAGGATGAAG
	exon 7	antisense	GCCTAGGCTTCAGTTACTCACAACAG
Sequencing			
Exon 1	exon 1	sense	GTGTTCGGCCTCGGGAAG
	intron 1	antisense	CTGAAGCCTCGCAGCCCTG
Exon 2	intron 1	sense	GGGTGTGATGCCTGAATTAC
	intron 2	antisense	CCTTAGGACCCTGATAACTG
Exon 3	intron 2	sense	CCTTGCAGCTTCACCGGGAACTC
	intron 3	antisense	AGGCTGACTCCAGAGGTATC
Exon 4	intron 3	sense	CATCGCCACAGTGATGGTTGTTC
	intron 4	antisense	ACTGAAGCTCCAGCTCCATC
Exon 5	intron 4	sense	CCCTGCTTACCTGCATCCC
	intron 5	antisense	CCCCAACTGAGATTTACATC
Exon 6	intron 5	sense	TCGCATTTGCCTCTGGTTGG
	intron 6	antisense	CTACCCTCTGGGAGGACAAG
Exon 7 (part I)	intron 6	sense	CTGCTCTAAGCCTTCCAATG
	exon 7	antisense	AGTAGAAATCGCCCTCGTCC
Exon 7 (part II)	exon 7	sense	AAGGAAACAGAGTTTACCCG
	exon 7	antisense	GAGGTGGATTACCTGGTGTG

tervention was planned for the patient. The tests showed discrepant results in antigen typing (blood group O) on the one hand and in reverse blood group typing (blood group A) on the other hand. As there was no massive transfusion or blood stem cell transplantation in the patient's medical history, further serological diagnostic and molecular tests had to be followed.

DNA Samples of Blood Donors

The geographical origin of blood donors of our transfusion service is the southwestern part of Germany. A DNA bank was established as a representative sample of our blood donor cohort and encompasses 1,340 blood donors with a mean age of 46.8 ± 15.3 years (range 18.0–68.8 years) and 1:1 gender distribution [7]. Data about ABO blood groups were taken from the blood donor files. All donors had regular ABO blood groups: 555 A (41.4%), 525 O (39.2%), 177 B (13.2%), 83 AB (6.2%). Donors gave written consent to provide blood samples for research purposes. This DNA bank was approved by the ethics committee of the Heidelberg University, Medical Faculty Mannheim. Another cohort of 480 blood donors was collected by the Institute of Transfusion Medicine and Immunohematology Magdeburg from a similar geographical region as the present case. These donors also had regular ABO blood groups: 228 A (47.5%), 179 O (37.3%), 72 B (15.0%), 1 AB (0.2%).

Serologic ABO Blood Grouping

Routine ABO blood typing was performed on the Galileo automated system (Immucor GmbH, Rödermark, Germany) using monoclonal reagents for antigen typing and A1, A2, B and O red cells for reverse typing. The aberrant phenotype was confirmed in column agglutination testing (CAT; ADK Cassette and Reverse Diluent Cassette; Ortho GmbH, Neckargemünd, Germany; and ID-Card Diaclon AB0/Rh for patients, ID-Card Diaclon AB0/Rh for newborns; Bio-Rad Laboratories GmbH München, Germany) and tube technique (immuClone; Immucor). All testing sera included monoclonal antibodies (table 1). A polyclonal anti-A serum was not available.

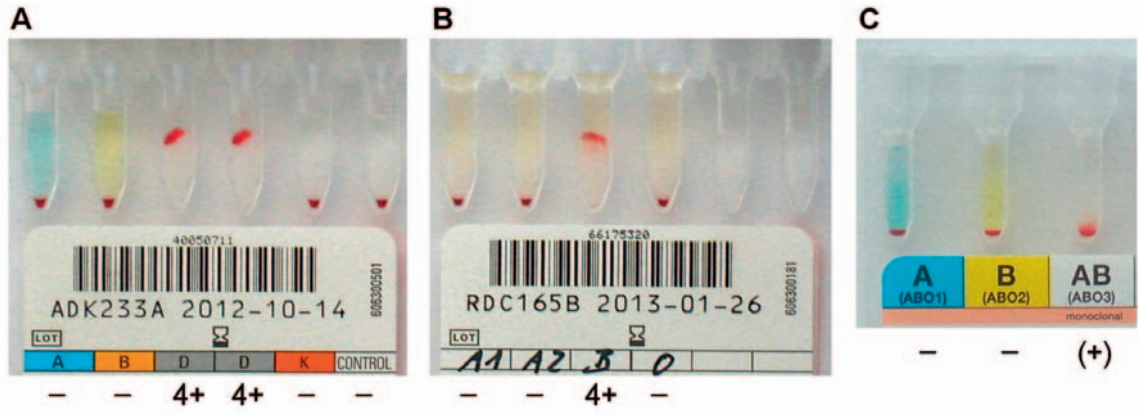

Fig. 1. Results from **A** forward and **B** reverse *ABO* typing of the patient using the gel technique. A and B antigens were clearly absent in antigen typing. Reverse typing failed to detect anti-A1 or anti-A2 isoagglutinins in the patient's serum. Anti-B isoagglutinins were normal (4+). C Antigen typing using further monoclonal reagents including an anti-AB monoclonal antibody led to very weak agglutination reaction detectable by a faint smear of erys in the gel.

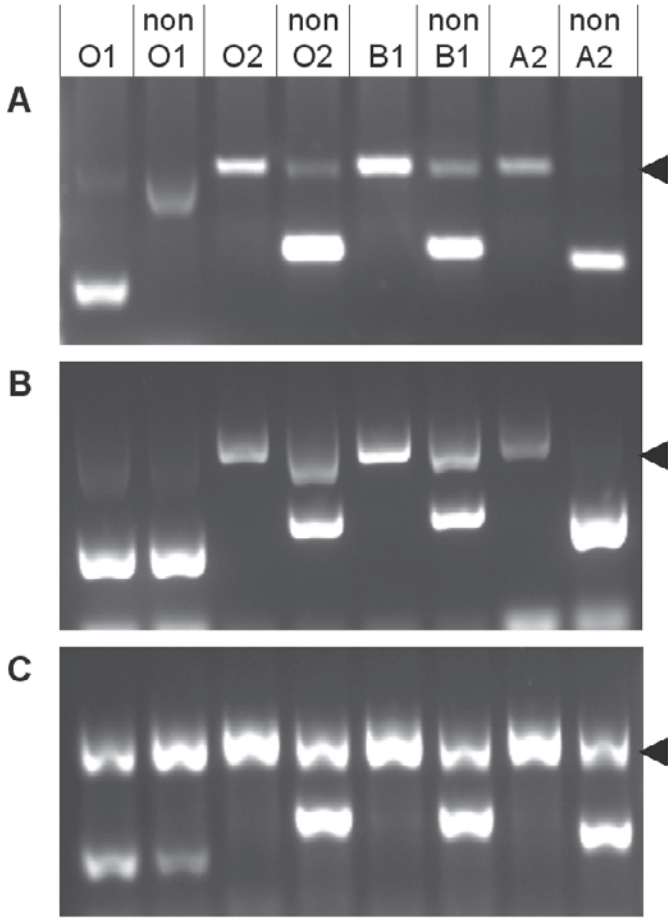

Fig. 2. Results from *ABO* genotyping using different PCR-SSP systems. **A** ABO-Type (BAG Healthcare) indicated genotype O^1/O^1; **B** ABO-SSP (Inno-Train Diagnostik GmbH) and **C** inhouse system both indicated genotype O^1/A. The discrepancy resulted from the second reaction (nonO1) specific for the detection of 261G. The PCR products of internal controls are indicated by arrowheads. Note the weaker PCR band in the nonO1 reaction of the inhouse system.

DNA Isolation and ABO Genotyping

DNA was isolated from EDTA-anticoagulated blood using a commercial system (QIAamp Blood DNA Mini Kit; Qiagen, Hilden, Germany). ABO blood group genotyping was performed using PCR with sequence-specific primers (PCR-SSP) by two commercial kits (ABO-SSP; Inno-Train Diagnostik GmbH, Kronberg, Germany; and ABO-Type variant; BAG Healthcare, Lich, Germany) and an inhouse system. For the inhouse PCR-SSP the *ABO* gene polymorphisms 261delG (for deletional O^1 alleles), 802G>A (for the nondeletional O allele O03), 803G>C (for B alleles), and 1061delC (for A^2 alleles) were analyzed as described previously [8]. The alleles were detected by 8 separate reactions each with allele-specific and internal control primers. The sizes of PCR products in all three systems were very similar, indicating the use of similar if not identical primers for allele-specific amplification. For further analysis of *ABO* alleles we applied another commercial PCR-SSP kit (ABO-Subtype; Inno-Train Diagnostik GmbH).

For genomic DNA sequencing *ABO* exons 1–7 and flanking intron regions were amplified using primers listed in table 2. Sequence data were compared to *ABO* alleles listed in the RBC database [6]. A PCR-SSP system was established for rapid identification of the new 248A>G mutation in exon 6 of the *ABO* gene. Primers for detection of the 248A>G mutation were: 248A-forward 5'-ctccatgtgcagtaggaagga-3'; 248G-forward 5'-tccatgtgcagtaggaaggg-3'; 248AG-reverse 5'-cttgatggcaaacacagttaac-3'. The system was also validated for screening of DNA samples in pools of up to 7 samples.

Results

Automated and CAT-based ABO blood group antigen typing of the patient showed absence of A and B antigen indicating blood group O (fig. 1A). However, reverse typing using A1, A2 and B test cells only showed agglutination of B cells in the automated system. In CAT and tube technique anti-A1 and anti-A2 reactions were also negative (fig. 1B). When using a monoclonal anti-AB antibody, we could see very weak agglutination in CAT (fig. 1C). The absence of anti-A isoagglutinins could indicate a variant *ABO* allele that leads to

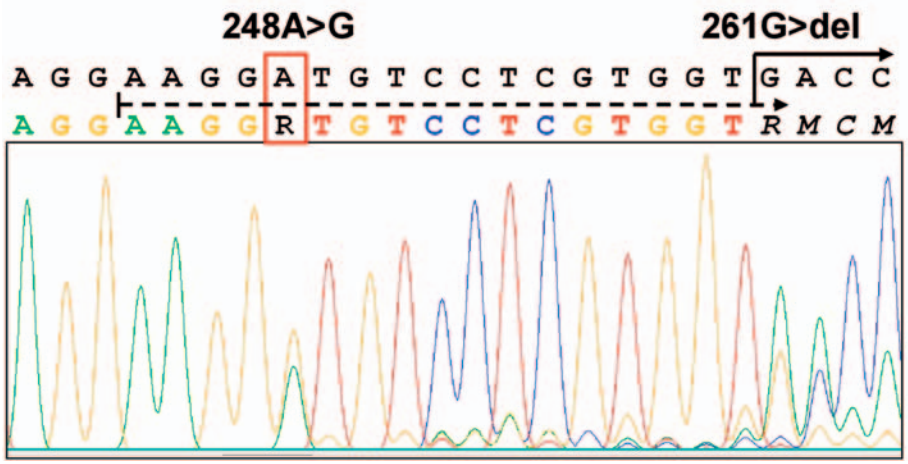

Fig. 3. Sequence analysis of *ABO* exon 6 in the patient's DNA. Heterozygosities were seen at position 248A>G (novel variant) and 261G>del (O^1 allele). The dashed line indicates the binding region of the forward PCR-SSP primer specific for the O1 (261del) and the nonO1 alleles (261G).

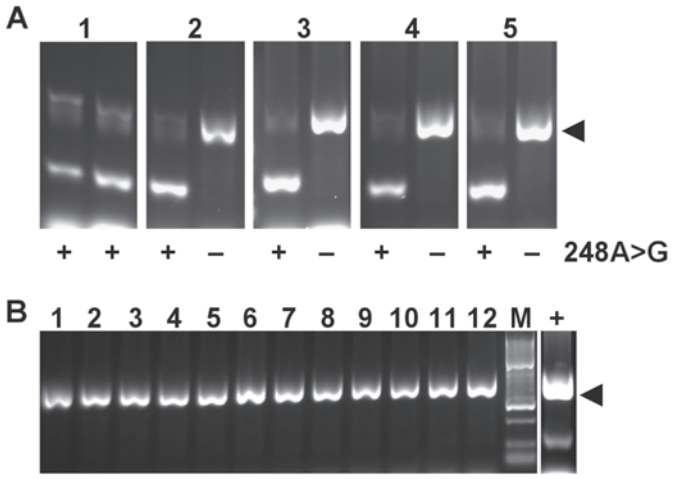

Fig. 4. Genotyping of the 248A>G mutation by PCR-SSP. **A** The 248G-specific reaction was positive (144 bp) in the patient sample (1) but negative in donors with regular ABO phenotype (2–5). **B** Representative result of screening for the 248A>G mutation in blood donors by PCR-SSP. For efficient screening we pooled 7 DNA samples and tested 260 pools. As a positive control (+) we pooled the patient DNA carrying the 248A>G mutation with 6 negative DNAs. The PCR products of internal controls are indicated by arrowheads. None of the pools were positive for the 248G allele (shown for pools 1–12).

very low levels of A-antigens, almost non-detectable by standard agglutination tests.

This prompted us to perform *ABO* genotyping using a commercial PCR-SSP kit (fig. 2A). We found a homozygous O^1 genotype indicating a regular blood group O. This did not confirm the postulated A variant and could not explain the absence of anti-A isoagglutinins. The second commercial kit and our inhouse PCR-SSP system revealed a heterozygous O^1/A genotype (fig. 2B and 2C). Remarkably, using our inhouse system the PCR band in the non-O^1 reaction was weaker compared to the O^1 reaction. This emphasized our hypothesis of a variant A-allele, however, PCR-SSP analysis of several common *ABO* variants was negative (data not shown).

ABO exon re-sequencing confirmed the heterozygous O^1/A genotype because of heterozygous 261G deletion in exon 6. In addition we found a heterozygosity at position 248 in exon 6 (fig. 3). From the PCR-SSP results shown in figure 2 we have clear evidence for the trans constellation of the 248A>G mutation to 261delG of the O^1 allele. The nonO^1(261G)-specific reaction (lane 2) is cleary affected by the mutation, whereas the O^1(261delG)-specific reaction (lane 1) is not. The mutation has to be in cis to the 261G nucleotide and affects binding of the 261G-specific primer. Consequentially, the mutation is in trans to the 261delG mutation and binding of the 261delG-specific primer is not affected. The 248A>G mutation is presumably located on an A-allele and leads to an amino acid change Asp83Gly in the encoded glycosyltransferase. The novel variant was deposited in Genbank (JX519570) and designated Aw34 in the dbRBC.

In order to estimate the frequency of the 248A>G variant we screened 1,340 blood donors with regular ABO blood groups from southwestern Germany and 480 blood donors from the patient's geographical origin by using PCR-SSP and pooled DNA samples. None of the samples was positive for the Aw34 allele (fig. 4).

Discussion

In the present case we describe a novel *ABO* gene variant associated with an aberrant ABO phenotype. In routine-based (automated) blood typing we found blood group O, but absence of anti-A isoagglutinins (normal anti-B). By using more sensitive gel techniques and a monoclonal anti-AB antibody a very weak agglutination was seen. Thus, the novel gene variant was classified as an Aweak allele and designated Aw34. Discrepancies between antigen typing and reverse typing could be a good hint for a variant *ABO* gene and often lead to the identification of novel *ABO* gene variants [9, 10].

The novel *ABO* gene variant results from a single base exchange (248A>G) in exon 6 introducing glycine instead of aspartate at amino acid position 83 (D83G) of the ABO glycosyltransferase. The two amino acids have very different prop-

erties and significant effects on the encoded protein are expected. The same amino acid is altered by the 247G>T mutation (D83Y) of the B304 allele identified in one individual with the B3 phenotype [11]. Two mutations in close proximity, 266C>T (P89L) and 268T>C (W90R), were identified as the A210 and A211 alleles, respectively, in Chinese individuals with A2 phenotype. Furthermore, the 278C>T variant (Bw12) leads to the amino acid exchange P93L and is responsible for a Bw phenotype [12]. From our findings with regard to the D83G mutation and from phenotypes described for other alleles we assume that the protein region 83–93 is essential for the glycosyltransferase enzymatic activity and could cause a weak expression or absence of A or B antigens.

In our case the mutation 248A>G is located close to the 261G nucleotide while the 261delG mutation is located in trans. The 261G deletion is the most frequent molecular basis for the O phenotype (59 alleles currently listed in dbRBC). Two commercial and our inhouse PCR-SSP systems for *ABO* genotyping detect the 261G deletion in the O1-specific reaction and 261G in the nonO1-specific reaction [8]. From the PCR product sizes (130 bp in all systems) we assume that the primers used in the three systems are very similar if not identical. The 248A>G mutation is located in the binding region of the 261G-specific primer in all three PCR-SSP systems. The reason for the discrepant genotyping results could be differences in PCR buffer composition (i.e. Mg^{2+} concentration) and primer concentrations that is very crucial for the sensitivity and specificity of PCR-SSP [13]. It could be speculated that the primer concentration is the lowest in the ABO-Type kit (BAG Healthcare) and the highest in the ABO-SSP kit (Inno-Train Diagnostik GmbH). Our inhouse system could have an intermediate concentration (0.5 mmol/l each) of the allele-specific forward and reverse primers. This could explain the different results of the 261G-specific (nonO1) reactions: negative in the ABO-Type kit; regular positive in the ABO-SSP kit; alleviated positive in the inhouse system.

Based on our results on the screening for the Aw34 allele in 1,820 German blood donors, we suggest that the variant allele is very rare if not a private mutation in the patient's family. However, the characterization of the patient's blood group was important for further transfusions and blood group diagnostics. Despite the absence of anti-A isoagglutinins, transfusion of blood group O red blood cell concentrates is recommended in such cases because immunization against A antigens cannot be fully excluded. On the other hand, as blood donors the carriers of such *ABO* variants should be classified blood group A since residual A antigens could lead to immunological reaction in recipients with anti-A isoagglutinins. In summary, the identification of *ABO* gene variants associated with ABO subgroups is an important contribution in each individual case and also adds knowledge to the current understanding of genetic factors that influence blood groups.

Disclosure Statement

The authors declare no conflict of interest.

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