Chimerism and mosaicism are important causes of ABO phenotype and genotype discrepancies

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Discrepancies between blood group genotype and RBC phenotype are important to recognize when implementing DNA-based blood grouping techniques. This report describes two such cases involving the ABO blood group in the Korean population. Propositus #1 was a 22-year-old healthy man undergoing pretransfusion testing for minor surgery. Propositus #2 was a 23-year-old male blood donor. RBCs from both propositi were determined to be group AB and demonstrated unusual agglutination patterns on forward typing, which were inconsistent with their *ABO* genotype determined by allele-specific (AS) PCR. RBCs from propositus #1 demonstrated mixed field agglutination with both anti-A and -B, while RBCs from propositus #2 demonstrated mixed field only with anti-A reagents. Both had *B/O* genotypes by AS-PCR. Cloning and sequencing of *ABO* exons 6 and 7 revealed three alleles in both propositi: propositus #1: *A102/B101/O04;* propositus #2: *A102/B101/O01.* A panel of nine short-tandem repeat (STR) loci was tested on DNA extracted from blood, buccal mucosal cells, and hair from the propositi and on DNA isolated from their parents' blood. In all tissues tested from propositus #1, three loci demonstrated a double paternal and a single maternal DNA contribution, indicating that he was a chimera or a mosaic; in those from propositus # 2, one STR locus demonstrated a double paternal DNA contribution, indicating that he was a tetragametic chimera. Chimerism and mosaicism are uncommon but important causes of ABO genotype and phenotype discrepancies. The evaluation of patients and donors with unusual or unexpected serology in pretransfusion testing and consensus ABO alleles may include the evaluation of STR loci to detect these phenomena. ***Immunohematology* 2006;22:183–187.**

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Chimeric individuals result from the fusion of two or more zygotes. They differ from mosaic individuals whose cells carry different complements of DNA derived from a single zygote.[1,2] Chimeric individuals generally are healthy, but often feature differentially pigmented patches of skin or eyes of different colors. Both of these genetic variants can give rise to ABO discrepancies if RBCs with different ABO groups are produced or if a naturally occurring ABO antibody is unexpectedly absent, as revealed on reverse typing. Resolving an ABO discrepancy caused by weak or unusual agglutination on forward typing can require an investigation into the patient's transfusion history and clinical diagnosis and potentially a molecular evaluation of the *ABO* gene. With the identification of the *ABO* alleles by Yamamoto et al.,[3] it became possible to determine a blood donor's or recipient's ABO blood group at the DNA level. The molecular bases of numerous ABO subtypes in several populations have been elucidated.[4] In Korea, the overall frequency of A and B subtypes among blood donors was reported to be 0.1 percent with *cis-AB01* among the most commonly identified subtype alleles.[5] This allele can produce various phenotypes including A_1B_3, $A_{int}B_3$, A_2B_3, and A_2B and its presence should be considered when evaluating an ABO discrepancy, especially in Korean and Japanese populations, where this allele is most commonly found.[5] A detailed description of the *cis*-AB phenotype can be found in reference 6. On the basis of common *ABO* gene polymorphisms in the Korean population, allele-specific (AS) PCR primers to detect *A, B, O,* and *cis-AB01* alleles were designed and used in resolving ABO discrepancies.[7] *ABO* genotyping is also used as an initial means of resolving disputed paternity claims, which are not infrequent because of the relatively high prevalence of the *cis*-AB blood group in Korea.[5] We have recently discovered two instances where the results of AS-PCR have not been in accord with the patients' RBC phenotypes obtained by serologic methods. Both of these cases were shown by further molecular testing to be chimeras or mosaics.

Materials and Methods

Blood group serologic tests

Forward typing was carried out by the manual tube method using murine monoclonal anti-A, anti-B, anti-H, and anti-AB (Biotest AG, Dreieich, Germany) according to standard protocols. Reverse typing was also performed by tube methods using A_1 and B RBCs (Diamed, Cressier sur Morat, Switzerland). The forward and reverse typings were repeated using gel cards containing monoclonal anti-A and anti-B in cases where ABO subtypes were suspected (Diamed).

Preparation of DNA

EDTA-whole blood, buccal mucosal cells, and hair follicles were obtained from the propositi. Genomic DNA extraction was performed using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI) on EDTA-blood and the QIAamp DNA micro kit (Qiagen, Hilden, Germany) on buccal mucosal cells and hair follicles. Informed consent was obtained from the study participants before their samples were drawn.

Sequence based typing for HLA-A, -B, and -DR

DNA sequencing-based typing (SBT) for HLA class I and II alleles was performed with commercially available AlleleSEQR HLA -A, -B, and -DRB1 SBT reagents (Atria Genetics, San Francisco, CA) according to manufacturer's instructions. The sequence results were analyzed with Assign-SBT (Conexio Genomics, Applecross, Western Australia, Australia) software.

Short-tandem repeat analysis

We studied short tandem repeats (STR) on autosomal chromosomes using commercially available kits for the following loci: vWA, FGA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, and D21S11 (AmpFlSTR Profiler Plus PCR Amplification Kit, ABI, Perkin Elmer/Applied Biosystems, Foster City, CA). Analysis was performed using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer/Applied Biosystems).

Karyotyping

Peripheral blood specimens from both propositi and their parents were studied. Approximately 0.5 mL of whole blood was cultured in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Invitrogen Corporation, Grand Island, NY) with 10% fetal bovine serum and 50 U of penicillin-streptomycin for 72 hours. The cultures were then stimulated with PHA and 20 metaphase cells stained by G-staining methods were karyotyped. The karyotypes were described according to the International System for Cytogenetic Nomenclature 2005.

Molecular analysis of ABO *gene*

1. Allele-specific PCR

AS-PCR at nucleotides (nt) 261 (exon 6) and at nt 526 and 803 (exon 7) of the *ABO* gene to discriminate between the *A, B, O,* and *cis-AB01* alleles was performed using previously described methods.[7] The primers were designed using a modified amplification refractory mutation system. In this technique, the primers' penultimate base was mismatched to increase the specificity of the PCR

PCR was performed in a reaction mixture of 50 µL with 0.25 µM of each primer pair, 300 ng of genomic DNA, 0.2 µM (each) dNTP, 1X PCR buffer [10mM Tris-HCl (pH 8.8), 1.5mM MgCl2, 50 mM KCl, 0.1% TritonX-100], and 2 U DynaZyme (Finzyme Inc., Finland). Thirty cycles of amplification were performed by manual hot-start PCR in a MJ Research PTC-100 thermocycler (MJ Research INC., Watertown, MA) after an initial denaturation at 95°C for 5 minutes. Each cycle consisted of a denaturation step at 94°C for 30 seconds, an annealing step at 64°C for 30 seconds, and an extension step at 72°C for 1 minute. An additional primer extension at 72°C for 10 minutes followed the last cycle. PCR products were separated at 100v for 40 minutes on 1.8% agarose gel prestained with ethidium bromide (0.5 µg/mL).

2. Cloning and sequencing of exons 6 and 7

Genomic DNA was extracted from peripheral WBCs with a DNA isolation kit (SolGent, Daejeon, Korea). The coding and flanking intronic sequences of exons 6 and 7 were amplified, cloned, and sequenced.[5] For the cloning, PCR products (2080 bp) were directly inserted into the PCR 2.1–TOPO vector and transformed into TOP 10 host cells using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). The sequencing reaction was performed in a 10 µL mixture containing 5X sequencing buffer, 4 µL of BigDye, 200 ng of purified DNA, 2 µL of primer (1.6 pmoles/µL). Sequence analysis was performed using SEQUENCHER (Gene Codes Corp, Ann Arbor, MI) software. Alleles were named according to the unofficial nomenclature used in the Blood Group Antigen Gene Mutation Database.[4]

Table 1. Summary of the serologic blood group determinations, sequence-based HLA typing, and karyotypes of both propositi and their parents

Propositus	Age, Sex	Antigen system	Father	Mother	Propositus
1	22, M	ABO phenotype	AB	O	$A_{mf}B_{mf}$
		Genotype (sequencing)	*A102/B101*	*O02/O04*	*A102/B101/O04*
		AS-PCR	*(A/B)*	*(O/O)*	*(B/O)*
		Rh	DcEe	DCe	DCcEe
		HLA-A	A*3303 A*3303	A*0201 A*3303	A*0201 A*3303
		HLA-B	B*4403 B*5801	B*3501 B*5801	B*3501 B*4403
		HLA-DRB1	DRB1*0701 DRB1*1302	DRB1*0403 DRB1*1302	DRB1*0403 DRB1*0701
		Karyotype	46,XY	46,XX	46,XY
2	23,M	ABO phenotype	A	B	$A_{mf}B$
		Genotype (sequencing)	*A102/O01*	*B101/O01*	*A102/B101/O01*
		AS-PCR	*(A/O)*	*(B/O)*	*(B/O)*
		Rh	DCe	DCe	DCe
		HLA-A	A*0201 A*3303	A*2402 A*3002	A*0201 A*3002
		HLA-B	B*2704 B*4403	B*0702 B*1401	B*1401 B*2704
		HLA-DRB1	DRB1*0405 DRB1*1302	DRB1*0101 DRB1*0802	DRB1*0405 DRB1*0802
		Karyotype	46,XY	46,XX	46,XY

mf = mixed field agglutination

Case Studies and Results

Propositus #1 was a healthy 22-year-old man. Routine pretransfusion testing prior to minor surgery revealed an unusual blood group: approximately one half of his RBCs showed strong (4+) agglutination with anti-A and anti-B sera on forward typing while the remaining RBCs were unagglutinated (mixed field agglutination). His serum did not react with A_1 or B RBCs. Furthermore, his blood group was unusual because his father was group AB and his mother was group O (Table 1, Fig. 1). Propositus #2 was a healthy 23-year-old blood donor whose RBCs demonstrated strong and uncomplicated agglutination on forward typing with anti-B reagents but mixed field agglutination with anti-A reagents (Table 1, Fig. 1). His serum did not react with A_1 or B RBCs.

Both propositi were morphologically normal, had no known twins, and denied ever receiving a blood transfusion or a stem cell or solid organ transplant. Samples from these propositi were to detect a *cis-AB* allele in propositus #1 and to elucidate the molecular basis of the mixed field agglutination in propositus #2. By AS-PCR performed on DNA extracted from peripheral blood, the *ABO* genotype of both propositi was *B/O.* Cloning and direct sequencing of *ABO* exons 6 and 7 revealed three alleles in both propositi: propositus #1:

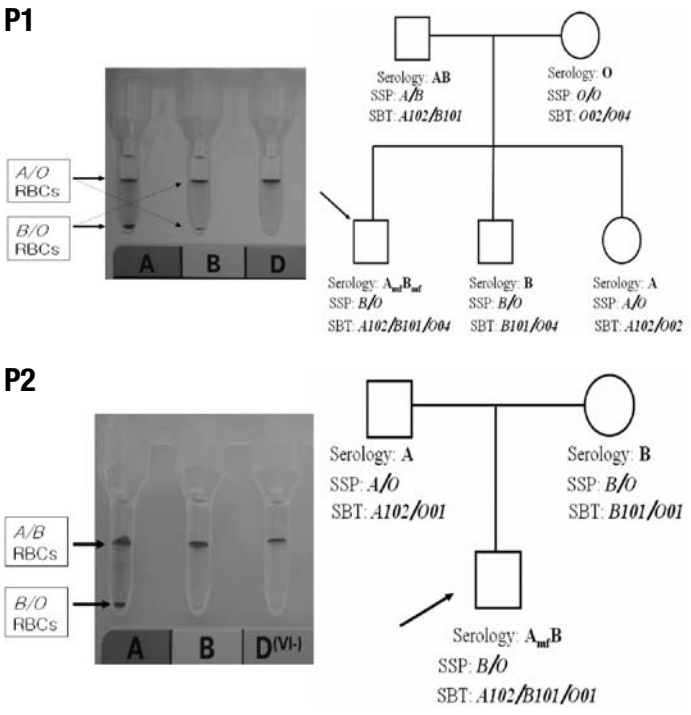

Fig. 1. In propositus #1 (P1, top), mixed field agglutination was observed with anti-A and anti-B sera indicating the simultaneous presence of two distinct RBC populations. This propositus' *A* allele was not detected until exons 6 and 7 were cloned and sequenced. In propositus #2 (P2, bottom), a mixed field agglutination pattern was observed with anti-A because of the presence of A and B RBCs. SSP = sequence-specific primer, SBT = sequence based typing.

Table 2. STR analysis of both propositi and their parents. Only the informative loci are shown for each propositus.

Propositus	DNA polymorphism	Father (blood)	Mother (blood)	Propositus (blood)	Propositus (buccal cells)	Propositus (hair)
1	D8S1179	*10,14*	**12,15**	*10,***12,***14*	*10,***12,***14*	*10,***12,***14*
	vWA	*16,19*	**15,18**	**15,***16,19*	**15,***16,19*	**15,***16,19*
	FGA	*22,23*	**17,22**	**17,***22,23*	**17,***22,23*	**17,***22,23*
2	D7S820	*9,10*	**8,12**	**8,***9,10,***12**	**8,***9,10,***12**	**8,***9,10,***12**

Italicized alleles indicate paternally derived alleles.

Bolded alleles indicate maternally derived alleles.

Underlined alleles indicate those that are shared by mother and father and are uninformative in the propositus.

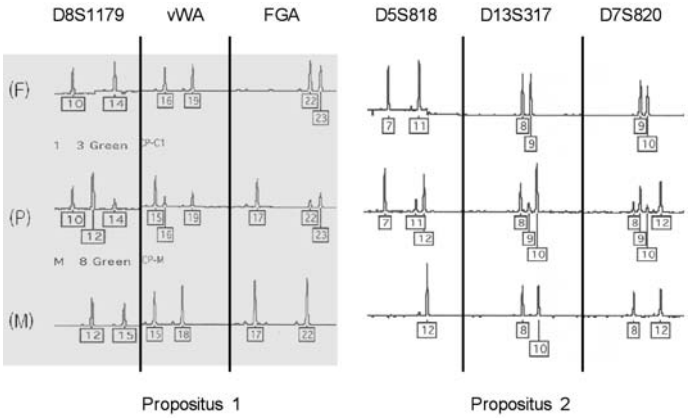

Fig. 2. STR analysis in the father (F), propositus (P), and mother (M) on DNA isolated from blood. Fragment size is indicated below each peak. In propositus #1, two STR loci reveal a double paternal DNA contribution, while only a single maternal DNA contribution is detectable. In propositus #2, one STR locus demonstrated a double maternal and double paternal contribution thus confirming this propositus as a tetragametic chimera. Identical STR results were found in the DNA isolated form buccal swabs and hair cells from both propositi.

A102/B101/O04 (likely genotype: *A102/O04* plus *B101/O04*); propositus #2: *A102/B101/O01* (inferred *ABO* genotype from serological results: *A102/B101* and *B101/O01*) (Table 1). The brother and sister of propositus #1 demonstrated uncomplicated B and A phenotypes and genotypes, respectively. A panel of nine STR loci was tested on DNA extracted from blood, buccal mucosal cells, and hair from both propositi, and on DNA isolated from blood from their parents. In propositus #1, three loci demonstrated a double paternal and a single maternal DNA contribution from all tissue samples studied (Table 2, Fig. 2). The FGA locus was uninformative in the propositus as his parents shared an allele. In propositus #2, one STR locus (D7S820) demonstrated a double parental DNA contribution, while other loci were uninformative in that they revealed either double maternal or paternal DNA contributions with only a single contribution from the other parent, or else it was not possible to determine if the propositus' extra allele was maternal or paternal owing to shared alleles between the parents (Table 2, Fig. 2). Both propositi had normal male (46, XY) karyotypes. DNA-sequence based HLA typing revealed only single parental DNA contributions in both propositi.

Discussion

Propositus #1 demonstrated one maternal and two paternal DNA contributions in all tissues tested. There are two embryologic explanations for this finding: he might be a mosaic who arose from one zygote with three pronuclei instead of the normal two, i.e., the zygote was formed from one ovum containing a haploid nucleus fertilized by two haploid sperm nuclei (dispermy) or one ovum plus one diploid sperm nucleus (diplospermy). Dispermy is a frequent event (1% of human conceptions) and results in a triploid zygote.[8] As the zygote divides, some or all daughter cells become diploid.[9] Thus, if one zygote with three pronuclei survived as an embryo, it would be composed of different cell populations.[9] Equally plausible, however, is that propositus #1 is a dispermic chimera resulting from the parthenogenetic division of the ovum and its subsequent fertilization by two spermatozoon carrying different paternal DNA—one with a 23,Y chromosome complement and another spermatozoon with a different 23,Y DNA complement.[10] This would explain the apparent single maternal and double paternal DNA contribution. Propositus #2 is a tetragametic chimera because one STR locus clearly demonstrated the presence of two maternal and two paternal alleles. The finding of only a single parental HLA type in these individuals was unexpected but a similar finding has been previously reported.[11] The detection of only one parental HLA haplotype in this tetragametic chimera probably relates to the relatively lower sensitivity of HLA typing methods compared with STR techniques.

In this study, both propositi were initially found to have ABO genotype and phenotype discrepancies; both had group A and group B RBCs detectable by serologic methods, but only demonstrated *B* and *O* alleles by AS-PCR, i.e., an *A* allele was not initially detected in either

propositus using this technique, probably because of the relative insensitivity of this test and the small quantity of DNA containing an *A* allele present. In fact, the *cis*-AB phenotype was initially suspected in propositus #1. The failure, by AS-PCR, to detect an allele known to encode the *cis*-AB phenotype led us to perform the STR testing and gene sequencing which revealed the cause of his unusual forward typing. When propositus #2 was an embryologic explanation for his genotype and phenotype discrepancy was sought. The mixed field agglutination pattern of RBCs was thus the result of the simultaneous presence of separate group A and group B RBCs in propositus #1 and of separate group AB and B RBCs in proprositus #2. As these two cases suggest, chimerism and mosaicism can confound *ABO* genotyping such that if blood group genotyping becomes more commonly used, more cases might be identified in attempts to explain similar genotype and phenotype discrepancies.[11,12] Our approach to the evaluation of patients with unusual serology on forward typing and consensus *ABO* alleles now includes the evaluation of STR loci in the propositus and available relatives to determine if chimerism or mosaicism is responsible for the unusual ABO phenotype.

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