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ABO GENOTYPING IN ITALIAN BLOOD DONORS

Antonietta Villa, Francesca Drago, Raffaela Mistò, Fernanda Morelati, Francesca Poli, Girolamo Sirchia

Centro Trasfusionale e di Immunologia dei Trapianti, IRCCS Ospedale Maggiore, Milano, Italy

ABSTRACT

Background. **Traditional AB0 blood group serology is based on the immunoreactivity of antisera with the carbohydrate A, B and H antigens. Progress in the molecular biology of the AB0 system has recognized the molecular basis of the red cell (RBC) antigens and has provided a genetic model for AB0 polymorphism at the molecular level. Recently, this genetic model was tested in a large number of individuals.**

Materials and Methods. **In this study we applied DNA analysis to determine the frequency of AB0 genotypes in a group of blood donors for whom the AB0 type was known. Two hundred and fifty healthy Italian blood donors were analyzed using polymerase chain reaction (PCR) to amplify two different regions of genomic DNA, each of which contained a different nucleotide polymorphism. The amplified product was digested with 4 restriction enzymes that revealed differences among A, B and 0 individuals. To analyze the genes at polymorphic sites 261 and 703 we used the restriction enzymes BstE II and Kpn I, and Hpa II and Alu I and compared the PCR determined genotypes to serologically determined phenotypes.**

Results and Conclusions. **The results were consistent for all unrelated individuals; however, 2 of 100 individuals with the 0 phenotype carried one allele that differed from the proposed genetic model. This novel 0 allele, termed 0^2 by Yamamoto *et al.*, was found in our series with a frequency of 1%. The blood group AB0 genotype of 250 healthy Italian blood donors was: 13 AA/$A0^2$, 37 $A0^1$, 11 BB, 39 $B0^1$, 50 AB, 98 0^10^1 and 2 0^10^2. This method should be applicable not only in forensic medicine but also in immunohematology when serology fails.**

Key words: antigen, AB0 system, polymerase chain reaction, forensic DNA analysis

The genes encoding for group A and B glycosyltransferases and for a non functional 0 transferase have recently been cloned and sequenced.[1-3] The cDNAs encoding for group A and group B transferases both consist of 1062 base pairs that result in a protein of 353 amino acids. The nucleotide sequences of the A and B genes are highly homologous (99%); the A and B transferase genes differ in 7 base substitutions (297, 526, 657, 703, 796, 803 and 930), but only 4 of these (526, 703, 796, 803) determine an amino acid change, while the substitutions 297, 657, 930 are silent. The 0 and A transferase genes are identical except for a single cytosine deletion at position 261.[2,3] The loss of this single nucleotide disrupts the normal triplet sequence in which base pairs are read and results in a frame shift of a premature stop codon, leading to a truncated, enzymatically inactive protein of 115 amino acids.[4]

Three of the 4 nucleotide differences which cause amino acid differences create allele-specific cleavage sites for the following restriction enzymes: BssH II, Hpa II and BstN I (A allele) and Nar I, Alu I and Nla III (B allele). The single nucleotide deletion in the 0 allele also creates a Kpn I site and eliminates the BstE II site. DNA fragments amplified by PCR can be tested for the presence of these sites.

Yamamoto *et al.*[2] used this approach to

Correspondence: Antonietta Villa, D. Sc. Centro Trasfusionale e di Immunologia dei Trapianti, IRCCS Ospedale Maggiore, via Francesco Sforza 35, 20122 Milan, Italy. Tel. international +39.2.55012558. Fax international +39.2.5458129.

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describe the molecular basis for the AB0 blood group system. Using two pairs of primers, they were able to amplify two regions of DNA including the four effective nucleotide substitutions. The PCR fragments were then digested with different restriction enzymes that revealed the differences among A, B, and 0 individuals.

Chang and Lee[5,6] and O'Keefe *et al.*[7] corroborated the molecular genetic model proposed by Yamamoto *et al.* by employing similar simplified techniques but on a limited number of individuals.

In order to assess the validity of this molecular genetic model on a larger group of serologically defined AB0 phenotypes, Grunnet *et al.*[8] analyzed 300 healthy Danish blood donors using a PCR-based diagnostic restriction enzyme assay of positions 0_{261}, B_{526} and B_{703}. In all A, B and AB cases at least one allele of the predicted status was found, but in 0 phenotype individuals a novel allele called 0^2 was discovered with a frequency of 3.7% in their study.

Since restriction enzymes and oligonucleotide primers detect differences in only one region of genes, when unexpected variations in the A, B and 0 transferase genes exist, it may be necessary to analyze these genes in several regions. This issue was recently addressed by Stroncek *et al.*[9] They modified the reported methods and used a panel of selected restriction enzymes to analyze differences at nucleotides 261 and 526. This method allowed them to determine the genotypes of several individuals as well as in selected families. Group A was differentiated from group B by analysis of the polymorphism at position 526. Among the 60 individuals tested, two were identified by serology as group A but had a group B nucleotide sequence at position 526. Further studies of nucleotide 703 and 796 showed that these individuals were indeed group A. This finding, recognized previously by Yamamoto *et al.*,[3] demonstrates that analysis of the AB0 genes at nucleotide 526 does not always lead to accurate gene identification.

The aim of this study was to evaluate the applicability of the rapid genotyping method described by Chang *et al.*[5] to the analysis of the genes at nucleotides 261 and 703, utilizing four restriction enzymes (BstE II, Kpn I, Hpa II and Alu I) instead of two. We used this method to determine the AB0 genotype of 250 Italian blood donors and compared the PCR determined genotypes with the phenotypes obtained by standard AB0 typing.

Materials and Methods

Blood samples

We collected 7 mL of EDTA blood from 100 group 0, 50 group A, 50 group B and 50 group AB healthy Italian blood donors.

Techniques

PCR

DNA was isolated from buffy coats using the salting out method described by Miller and colleagues.[10] Two DNA fragments of the AB0 gene, each containing a different nucleotide polymorphism, were amplified by PCR using two pairs of primers.

Nucleotides 261 and 703 were used to distinguish A, B, 0 alleles by restriction enzyme digestion of the PCR products. The primers and restriction enzymes employed, previously described by Yamamoto *et al.*,[2,11] are shown in Table 1.

Five µL of DNA were amplified in 100 µL of reaction mixture which contained a pair of oligonucleotide primers (fy 121/122 [PCR 1] or fy 29/47 [PCR 2], 20 pmol/µL each), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1.2 mM $MgCl_2$ (fy 121/122) or 1.8 mM $MgCl_2$ (fy 29/47), and 2.5

Table 1. Specific primers used to amplify DNA, transferases differentiated and restriction enzymes employed.

*Primers**	*Polymorphic site (nucleotide)*	*Transferases differentiated*	*Restriction enzymes*
PCR 1 fy 121-122	261	0 versus A or B	Kpn I, BstE II
PCR 2 fy 29-47	703	B versus A or 0	Hpa II, Alu I

**sequence of oligonucleotide primers:*
fy-121:5'-CGGAATTCCATGTGACCGCACGCCT-3'
fy-122: 5'-CGGAATTCTCTACCCTCGGCCACC-3'
fy-29:5'-CGTTCTGCTAAACCAAG-3'
fy-47:5'-TCCTGGAGGTGCGCGCCTAC-3'

units of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), using the GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA).

PCR was performed as described by Yamamoto *et al.*[2,11] but with the following modification: in PCR 1 the reaction mixture was incubated at 94°C for 5 minutes before the 45 reaction cycles in which denaturation was conducted at 94°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 30 seconds. The reaction mixture was then incubated for an additional 6 minutes and 30 seconds at 72°C. In PCR 2, there were 35 reaction cycles with denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 2 minutes. After 35 cycles, the reaction mixture was incubated for an additional 6 minutes and 30 seconds at 72°C. Amplified samples were analyzed on 2% agarose gel electrophoresis (BioRad Laboratories, Hercules, CA, USA).

PCR product analysis by restriction enzymes

Amplified DNA was purified with phenol-(chloroform:isoamyl alcohol, 24:1), ethanol-precipitated and resuspended in 20 µL of double-distilled H_2O. The product (2 µL) of PCR 1, a DNA fragment of 199 or 200bp containing the polymorphic site at nucleotide 261, was digested with restriction enzymes Kpn I (10U) and BstE II (10U). If nucleotide 261 was deleted (group 0 subjects), the fragment was completely digested by Kpn I; on the other hand, if nucleotide 261 remained, the Kpn I site was not created and DNA was digested by treatment with BstE II, which digests type A or B transferases.

Moreover, the product of PCR 2, a DNA fragment (621bp) containing the polymorphic site at nucleotide 703, was digested with Hpa II (12U) and Alu I (16U) in a final volume of 20 µL. Enzyme Hpa II digests the transferase gene of individuals with type A or 0 transferase and Alu I digests the DNA of individuals with type B transferase. All enzymes were purchased from New England Biolabs, Beverly, MA, USA. Incubation temperatures were 37°C for Hpa II, Alu I and Kpn I, and 60°C for BstE II. DNA was incubated with the enzymes for 2 hours. The digested amplified DNA products were subsequently analyzed on 2% agarose gel electrophoresis or on 12% PAGE. The bands were visualized by ultraviolet light.

The strategy for analyzing AB0 alleles on genomic DNA is illustrated in Figure 1. By examining the digestion patterns of the AB0 genotypes, one of 7 patterns (AA/A0^2, A0^1, BB, B0^1, AB, 0^{1}0^1 and 0^{1}0^2) was possible.[8,11]

Results

AB0 genotyping by restriction enzyme analysis

The results of AB0 genotyping of the 250 individuals studied are shown in Table 2.

In 98 of the 100 type 0 individuals the typing at nucleotide position 261 showed a homozy-

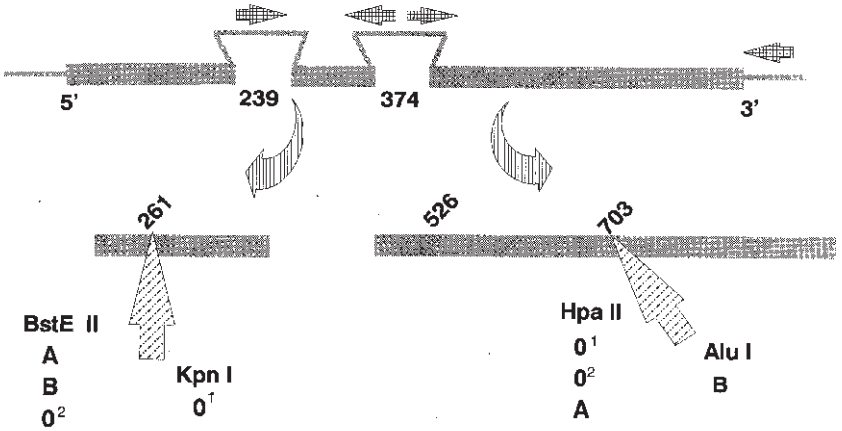

Figure 1. Illustration of the effects of restriction enzymes on PCR products of different AB0 glycosyltransferase genes. To determine the AB0 genotype, the genes are first amplified by PCR and the amplified DNA is treated with restriction enzymes Bst E II, Kpn I, Alu I and Hpa II. Enzyme Bst E II digests the A and B transferase genes, Kpn I the 0 transferase gene, Hpa II the A and 0 transferase genes, and Alu I the B transferase gene.

Table 2. ABO blood group genotyping of 250 Italian blood donors.*

Phenotype	*# of cases*	*Nucleotide 261 BstE II/Kpn I*	*Nucleotide 703 Hpa II/Alu I*	*Inferred ABO genotype*
A	13	+/–	+/–	$AA/A0^2$
	37	+/+	+/–	$A0^1$
B	11	+/–	–/+	BB
	39	+/+	+/+	$B0^1$
AB	50	+/–	+/+	AB
0	98	–/+	+/–	0^10^1
	2	+/+	+/–	0^10^2

**The symbols minus (–) and plus (+) indicate a negative and positive cutting for each allele, respectively.*

gous pattern with both alleles carrying the 0_{261} deletion (0^10^1). In the remaining two 0 subjects no deletion was found in one of the 2 alleles; therefore the genotype was 0^1 and 0^2.

Of the 50 subjects with serologically defined A blood, 37 where found to express both A and 0 transferase genes and 13 were homozygous for the A transferase gene.

Of the 50 subjects with serologically defined B blood, 39 had both B and 0 transferase genes, while 2 B transferase genes were detected in 11 subjects.

Both A and B alleles were found in all 50 AB individuals.

Discussion

The molecular basis of red cell AB0 group antigens was recently determined.[1-3] The A and B genes are similar; in fact, they only differ in a few single-base substitutions, changing four amino acid residues that may cause differences in A and B transferase specificity. The 0 gene contains a single base deletion which causes a reading frame shift. The nucleotide sequences of the coding regions of different AB0 alleles has led to the identification of variations that determine a change in amino acid sequences which are responsible for the different specificities in the AB0 blood group system.

Traditional blood group serology, which is based on immunoreactivity with the carbohydrate of the A, B and H antigens, does not provide information on AB0 genotypes. These can be determined by using RFLP analysis of the nucleotide positions where the differences which change the amino acid sequence are situated.

In this study we employed a rapid molecular method to determine the AB0 genotype of 250 healthy blood donors, using four restriction enzymes at two nucleotide positions (0_{261} and A/B_{703}).

A similar approach had previously been reported by Yamamoto *et al.*,[2,11] who employed 8 different endonucleases at nucleotide positions 261, 526, 703 and 796. This molecular method was recently applied by Grunnet *et al.*[8] to a larger group of individuals and by Stroncek *et al.*[9] to selected families.

This study demonstrates that using molecular genetic techniques for AB0 genotyping at nucleotide positions 261 and 703 makes it possible to distinguish A, B and 0 alleles. Complete agreement with the proposed molecular genetic model for AB0 blood group polymorphism was found for the A, B and AB individuals. An A or B allele was always identified when appropriate, and in the case of heterozygous A0 or B0 individuals an 0^1 allele with the 0_{261} deletion was found. In the 100 type 0 individuals, we found 2 subjects with unexpected 0 alleles that did not contain the usual deletion at position 261 and were typed as A at position 703. These subjects were defined as 0^10^2. The fact that none of the A or B individuals were found to carry the 0^2 allele may be due to the relatively small number of cases examined. However, in 150 Danish blood donors with 0 type Grunnet *et al.*[8] detected 11 individuals with genotype 0^10^2, while the frequency of the alleles A and B was similar to that found in our Italian blood donor population. Both studies seem to confirm the relative advantage for heterozygous individuals (well known from other genetic studies), as appears from the frequency of $A0^1$ and $B0^1$ in the subjects with type A and B RBCs.

In all cases, analysis of the AB0 genes at nucleotide 703 led to accurate type identification.[9]

Such low-frequency variants may remain important for DNA analysis in forensic medi-

cine. Moreover, similar potential applications exist for the DNA typing of the A_2, A_3 and *cis*-AB genes.[12,13]

The methods used in this study, in addition to other molecular methods previously described that allow the identification of unusual genotypes, represent a useful tool for AB0 typing in difficult situations and for the detection of weakly expressed AB0 antigens when serology fails.

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