

Blood Group ABO Genotyping in Paternity Testing

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Summary

Background: The ABO blood groups result from DNA sequence variations, predominantly single nucleotide and insertion/deletion polymorphisms (SNPs and indels), in the *ABO* gene encoding a glycosyltransferase. The ABO blood groups A₁, A₂, B and O predominantly result from the wild type allele A1 and the major gene variants that are characterized by four diallelic markers (261G>del, 802G>A, 803G>C, 1061C>del). Here, we were interested to evaluate the impact of *ABO* genotyping compared to ABO phenotyping in paternity testing. **Methods:** The major *ABO* alleles were determined by PCR amplification with sequence-specific primers (PCR-SSP) in a representative sample of 1,335 blood donors. The genotypes were compared to the ABO blood groups registered in the blood donor files. Then, the ABO phenotypes and genotypes were determined in 95 paternity trio cases that have been investigated by 12 short tandem repeat (STR) markers before. We compared statistical parameters (PL, paternity likelihood; PE, power of exclusion) of both blood grouping approaches. **Results:** The prevalence of the major *ABO* alleles and genotypes corresponded to the expected occurrence of ABO blood groups in a Caucasian population. The low resolution genotyping of 4 diallelic markers revealed a correct genotype-phenotype correlation in 1,331 of 1,335 samples (99.7%). In 60 paternity trios with confirmed paternity of the alleged father based on STR analysis both PL and PE of the *ABO* genotype was significantly higher than of the ABO phenotype. In 12 of 35 exclusion cases (34.3%) the *ABO* genotype also excluded the alleged father, whereas the ABO phenotype excluded the alleged father only in 7 cases (20%). **Conclusion:** In paternity testing *ABO* genotyping is superior to ABO phenotyping with regard to PL and PE, however, *ABO* genotyping is not sufficient for valid paternity testing. Due to the much lower mutation rate compared to STR markers, blood group SNPs in addition to anonymous SNPs could be considered for future kinship analysis and genetic identity testing.

Schlüsselwörter

ABO-Genotypisierung · Vaterschaftswahrscheinlichkeit ·
Ausschlusspotenz · Vaterschafts-Trios

Zusammenfassung

Hintergrund: Die ABO-Blutgruppen basieren auf DNA-Sequenz-Variationen – vor allem Single-Nucleotide- und Insertion/Deletion-Polymorphismen (SNPs und Indels) im *ABO*-Gen, das eine Glykosyltransferase kodiert. Die ABO-Blutgruppen A₁, A₂, B und O resultieren hauptsächlich aus dem Wildtyp-Allel A1 und den Haupt-Genvarianten, die durch vier diallele Marker (261G>del, 802G>A, 803G>C, 1061C>del) charakterisiert sind. In der aktuellen Arbeit gilt unser Interesse der Frage, welche Bedeutung die *ABO*-Genotypisierung im Vergleich zu ABO-Phänotypisierung bei der Vaterschaftstestung hat. **Methoden:** Die Haupt-*ABO*-Allele wurden mittels PCR-Amplifizierung mit sequenzspezifischen Primern (PCR-SSP) in einer repräsentativen Auswahl von 1335 Blutspendern untersucht. Die Genotypen wurden mit den in den Blutspenderdateien registrierten ABO-Blutgruppen verglichen. Außerdem wurden die ABO-Phäno- und Genotypen in 95 Vaterschafts-Trio-Fällen, die bereits mit 12 Short-Tandem-Repeat(STR)-Markern untersucht worden waren, bestimmt. Die statistischen Parameter (PL = Vaterschaftswahrscheinlichkeit und PE = Ausschlusschance) beider Ansätze zur Blutgruppenbildung wurden verglichen. **Ergebnisse:** Die Prävalenz der großen *ABO*-Allele und -Genotypen korrespondierte mit dem erwarteten Auftreten der ABO-Blutgruppen in einer europäischstämmigen Population. Die niedrig auflösende Genotypisierung von 4 diallelen Markern ergab eine korrekte Genotyp-Phänotyp-Korrelation bei 1331 der 1335 Proben (99,7%). Bei 60 Vaterschafts-Trios mit bestätigter Vaterschaft des vermeintlichen Vaters auf der Basis einer STR-Analyse waren sowohl die PL als auch die PE des *ABO*-Genotyps deutlich höher als die des ABO-Phänotyps. In 12 von 35 Ausschlussfällen (34,3%) schloss der *ABO*-Genotyp auch den vermeintlichen Vater aus, während dies beim ABO-Phänotyp nur in 7 Fällen (20%) möglich war. **Schlussfolgerung:** Bei der Vaterschaftstestung war die *ABO*-Genotypisierung der ABO-Phänotypisierung in Hinblick auf PL und PE überlegen; allerdings war auch die *ABO*-Genotypisierung nicht ausreichend für eine valide Vaterschaftstestung. Aufgrund der weit geringeren Mutationsrate im Vergleich zu STR-Markern könnten Blutgruppen-SNPs zusätzlich zu anonymen SNPs bei zukünftigen Verwandtschaftsanalysen und Testungen der genetischen Identität miteinbezogen werden.

Introduction

After the discovery of the Mendelian inheritance of the ABO blood group system by von Dungern and Hirschfeld in 1910 [1], blood group phenotyping including further systems has been introduced to paternity investigation [2]. For more than 60 years investigation of blood group systems and the HLA system represented the standard in forensic genetics. This serological era was then replaced by molecular genetics through the introduction of DNA fingerprints first on the basis of minisatellite (i.e. variable number of tandem repeat; VNTR) polymorphisms, later by the analysis of microsatellite (i.e. short tandem repeats; STR) polymorphisms [3]. Recent efforts led to the introduction of single nucleotide polymorphism (SNP) typing for human identification [4].

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 [5, 6]. A number of studies have shown a clear genotype-phenotype correlation [7–9], and more than 240 gene variants are listed in the open access database dbRBC at the NCBI website [10]. 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 A₁, A₂, 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 261G>del 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 (table 1).

In the present study we determined the frequencies of the major ABO alleles in a representative German population by using PCR with sequence-specific primers (PCR-SSP). We then used serologic and genetic ABO blood grouping in paternity trios (child, mother, alleged father) in order to compare paternity likelihood (PL) and power of exclusion (PE) of both blood grouping approaches.

Table 1. DNA sequence characteristics in exon 6 and 7 of the major *ABO* alleles

Allele	Phenotype	Exon 6		Exon 7								
		261	297	467	526	657	703	796	802	803	930	1,061
A101 (wt)	A ₁	G	A	C	C	C	G	C	G	G	G	C
A201	A ₂	–	–	T	–	–	–	–	–	–	–	del
B101	B	–	G	–	G	T	A	A	–	C	A	–
O01	O	del	–	–	–	–	–	–	–	–	–	–
O03	O	–	G	–	G	–	–	–	A	–	–	–

– = No difference to the wild type (wt) allele *A101; italic letters/numbers indicate the gene polymorphisms used for *ABO* blood group genotyping in the present study.

Material and Methods

Anonymous DNA Bank 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 1,335 blood donors with a mean age of 46.8 ± 15.3 years (range 18.0–68.8 years) and 1:1 gender distribution. Data about ABO blood groups were taken from the blood donor files. All donors had regular ABO blood groups. Donors gave written consent to provide blood samples for research purposes. The DNA bank was established with anonymous sample coding without the possibility of de-coding. The anonymous DNA bank was approved by the ethics committee of the Heidelberg University, Medical Faculty Mannheim.

DNA Isolation and Standard ABO Genotyping by PCR-SSP

DNA was isolated from buffy coats of CPD-anticoagulated whole blood donations using a commercial system (FlexiGene DNA Kit; Qiagen, Hilden, Germany). For ABO blood group genotyping the ABO gene polymorphisms 261G>del (for deletional O alleles), 802G>A (for the non-deletional O allele *O03), 803G>C (for B alleles), and 1061C>del (for A₂ alleles) were analyzed in all samples by PCR with sequence specific primers (PCR-SSP) as described previously [11]. The alleles were detected by 8 separate reactions each with allele-specific and internal control primers. According to an optimized protocol only 5 ng DNA were used per reaction [12]. In this low-resolution genotyping approach rare variants with mutations at other sites of the *ABO* gene are not recognized. Exclusion of the four variant alleles 261del, 802A, 803C, and 1061del indicated the A₁ blood group.

Serologic ABO Blood Grouping

ABO blood groups A₁, A₂, B and O were determined using lectins and monoclonal antisera (Optima GmbH, Bammatal, Germany) in standard hemagglutination tests.

Statistical Analysis

Basic statistical analysis including observed heterozygosity, polymorphic information content (PIC), power of discrimination (PD), PE, and Hardy-Weinberg equilibrium test was performed using the PowerStatsV12 Excel workbook template developed and provided by Promega Corp. (Promega GmbH, Mannheim, Germany) and the statistical software package SPSS (Version 12.0; SPSS Inc., Chicago, IL, USA).

Results

PCR-SSP-based genotyping for the ABO polymorphisms 261G>del, 802G>A, 803G>C and 1061C>del was performed on 1,335 samples of an anonymized blood donor DNA bank. Unambiguous results could be obtained for all samples (table 2). Each of the 4 polymorphisms fulfilled the Hardy-Weinberg

Table 2. Observed *ABO* allele frequencies and resulting statistical parameters in the study cohort of 1,335 German blood donors

Gene variant	Deduced allele	Numbers of alleles (n = 2,670)	Allele frequency
261del	O01	1,590	0.5955
802A	O03	57	0.0213
803C	B	268	0.1004
1061del	A2	170	0.0637
wt*	A1	581	0.2176
Various	Var	4	0.0015

*Wild type (wt) allele A1 was present when no variant allele was identified.

Table 3. Phenotype-genotype correlation for the low resolution *ABO* genotyping

ABO phenotype	<i>ABO</i> genotypes	Numbers of genotypes (n = 1,335)	Percentage of genotypes*
A (n = 555; 41.6%)	O01/A1	335	60.4
	O01/A2	93	16.8
	A1/A1	66	11.9
	A2/A1	47	8.5
	O03/A1	6	1.1
	O03/A2	6	1.1
	A2/A2	2	0.4
	all	555	100.0
O (n = 523; 39.2%)	O01/O01	484	92.5
	O01/O03	34	6.5
	O03/O03	1	0.2
	all	519	99.2
	<i>O01/A1</i>	4	0.8
B (n = 176; 13.2%)	O01/B	156	88.6
	B/B	11	6.3
	O03/B	9	5.1
	all	176	100.0
AB (n = 81; 6.1%)	A1/B	61	75.3
	A2/B	20	24.7
	all	81	100.0

*Given as percentage of the corresponding phenotype; italic letters indicate samples with discrepancy between phenotype and genotype.

equilibrium. The 261del variant characteristic for the majority of O alleles was the most prevalent variant of the *ABO* gene (1,590 of 2,670 alleles; 59.6%). Absence of all four variants indicated the presence of the wild type A1 allele. However, mutations at other sites of the *ABO* gene cannot be excluded by the low-resolution genotyping.

In order to determine the specificity of the low-resolution genotyping approach we compared the ABO blood group phenotype (A, B, AB, or O) registered in the blood donor files with the determined genotype. Blood groups A, B, and AB revealed a 100% phenotype/genotype correlation with the different genotypes (table 3). For 519 of the 523 samples (99.2%) with blood group O, we could determine a corre-

sponding blood group O genotype. In the remaining 4 samples the low-resolution genotyping approach indicated blood group A (O01/A1). It is assumed that in these samples rare variants (Var), i.e. O alleles with mutations at other sites of the *ABO* gene, are present. We therefore classified the genotype of the 4 samples as O01/Var, and the frequency of Var was 0.15% (4 of 2,670 alleles) in our cohort. In summary, 1,331 of 1,335 (99.7%) blood groups were correctly determined from the low-resolution *ABO* genotype. Based on the observed allele frequencies (O01, 0.5955; A1, 0.2176; B, 0.1004; A2, 0.0637; O03, 0.0213; Var, 0.0015) we calculated statistical parameters: i) observed heterozygosity = 0.5775; ii) PIC = 0.5380; iii) PD = 0.7803; iv) PE = 0.2648.

To further evaluate the impact of the *ABO* genotype in kinship analysis, we performed the low-resolution *ABO* genotyping and ABO phenotyping (A₁, A₂, B, and O) in 95 paternity trio cases (child, mother, alleged father). Previously, paternity of the alleged father was determined on the basis of STR analysis including 12 markers (D3S1358, D7S820, D13S317, D16S539, D18S51, D21S11, CSF1PO, FGA, PentaE, PentaD, TH01, TPOX). Paternity was excluded in 35 cases and confirmed in 60 cases. In 23 of the 35 excluded paternities (65.7%) neither the *ABO* genotype nor the ABO phenotype enabled exclusion of the alleged father. In 7 cases (20%) both *ABO* genotype and phenotype confirmed exclusion of the alleged father. In the remaining 5 cases (14.3%) the *ABO* genotype, but not the ABO phenotype, enabled exclusion of the alleged father (table 4).

In the 60 cases with confirmed paternity, both PL and PE of the *ABO* genotype was significantly higher than those of the ABO phenotype (table 5). Only in three cases where the child's ABO phenotype was B and the mother's phenotype was A or O the PE of the *ABO* genotype was slightly lower compared to the ABO phenotype (80.9% vs. 84.8%). In summary, the *ABO* genotype was superior to the ABO phenotype in all 95 paternity trio cases.

Discussion

In our study we determined the prevalence of the major *ABO* gene variants in a population of German blood donors. Four diallelic markers were determined by PCR-SSP with optimized sensitivity in order to reduce DNA consumption [12]. The *ABO* genotype could be obtained from less than 50 ng genomic DNA. However, the impact of DNA degradation on *ABO* genotyping by PCR-SSP has not been investigated so far. The genotypes were used to deduce the corresponding ABO blood group phenotype, and we found 99.7% genotype-phenotype correlation for our low-resolution genotyping approach. The frequency of the different blood groups in our cohort corresponded to the expected frequencies (A 43%; B 9%; AB 4%; O 44%) for Caucasian populations [13]. Thus, we conclude that our study cohort is a representative sample

Table 4. Five paternity trio cases in which the *ABO* genotype but not the *ABO* phenotype enabled exclusion of the alleged father

Case	<i>ABO</i> phenotype		Essential paternal feature (PE%)*	<i>ABO</i> genotype	Essential paternal feature (PE%)
1	C	A ₁	all (0%)	A1/A1	A1 (61.22%)
	M	A ₁		O01/A1	
	AF	O		O01/O01	
2	C	A ₁	all (0%)	A1/A1	A1 (61.31%)
	M	A ₁ B		A1/B	
	AF	O		O01/O01	
3	C	A ₁	all (0%)	A1/A1	A1 (61.22%)
	M	A ₁		O01/A1	
	AF	O		O01/O01	
4	C	A ₁	all (0%)	A1/A1	A1 (61.22%)
	M	A ₁		O01/A1	
	AF	O		O01/O01	
5	C	A ₂	A ₂ (54.65%)	O01/A2	A2 (87.61%)
	M	O		O01/O01	
	AF	A ₁		O01/A1	

C = Child; M = mother; AF = alleged father.
 *PE, power of exclusion; in cases with the dominant phenotype A₁ for child and mother all paternal blood groups are possible (no exclusion).

Table 5. Paternity likelihood and exclusion power of the *ABO* phenotype and the *ABO* genotype in 60 trios with confirmed paternity

	<i>ABO</i> phenotype	<i>ABO</i> genotype	p value*
Paternity likelihood			
Range	38.2–90.0%	38.1–96.0%	
Median	61.1%	62.7%	
Mean ± SD	61.4 ± 13.2%	64.8 ± 12.4%	0.0214
Power of exclusion			
Range	0–84.8%	3.5–95.8%	
Median	4.5%	16.4%	
Mean ± SD	21.8 ± 28.9%	35.3 ± 29.5%	<0.0001

*Statistical significance of the differences between phenotype and genotype calculated by pairwise t-tests.

of the German population. In 4 of 1,335 samples (0.3%) with O phenotype but *O01/A1* genotype we proposed rare variant alleles. Sequence analysis of the entire *ABO* gene confirmed rare alleles such as Aw13 and O51 (data not shown). Most of the alleles stored in the dbRBC database represent rare *ABO* alleles characterized by point mutations in different exons of the *ABO* gene. Many variant alleles have a phenotypic equivalent such as weak expression of A or B antigens or blood group O. The genotype-phenotype correlation could be enhanced by investigating more of the rare variants.

We demonstrated that PL and PE of the *ABO* genotype is superior to the *ABO* phenotype. The inclusion of additional

ABO alleles could even strengthen this finding; however, analysis of further SNPs would be necessary to identify such alleles. The *O02* allele is a common variant of the *O01* allele carrying the indel polymorphism 261G>del in exon 6 and nine additional nucleotide changes in exons 2–7. It was shown that the *O02* allele accounts for approximately 40% of *O* alleles [14]. The differential typing for *O01* and *O02* alleles would further enhance the statistical power of *ABO* genotyping, but was not performed in this study. Using a theoretical allele frequency of 0.41 of the *O01* and 0.18 of the *O02* allele in our population and assuming Hardy-Weinberg equilibrium for all alleles we re-calculated the statistical parameters. Discrimination of *O01* and *O02* alleles further increased the observed heterozygosity to 0.6856 (before 0.5775), PIC to 0.7025 (before 0.5380), PD to 0.8925 (before 0.7803), and PE to 0.4064 (before 0.2648). These values are similar to those reported in *ABO* genotyping of a small (n = 60) Italian cohort [15]. We assume that discrimination between the *O01* and *O02* alleles is reasonable and could be performed by typing a further SNP such as 646T>A or 681G>A in exon 7 of the *ABO* gene.

Typing of highly polymorphic STR markers is the current worldwide standard in kinship analysis and forensic case work. The high PD and PE for each STR marker enable a reliable determination of paternity. Other marker systems such as blood groups, HLA, or RFLPs became uncomely due to the lower statistical power and higher labor input. However, the reasonable mutation rates of up to 1% of each STR

marker could hamper result interpretation [16]. The mutation rate per meiosis of diallelic markers including SNPs is estimated to be 100,000 times lower compared to STRs. Therefore, the reliability of SNP-based kinship analysis is higher and could probably replace STR-based analysis in future. By using ABO blood grouping in paternity trio cases we could show that ABO genotyping is superior to phenotyping with regard to statistical parameter such as PL and PE. In 12 of 35 trios (34.3%) where paternity was excluded by at least 5 of 12 STR markers, the ABO genotype also excluded paternity. The ABO phenotype could exclude paternity in only 20% of the cases. However, we must point out that neither ABO genotyping nor ABO phenotyping is sufficient for valid paternity testing. It should be regarded as an supplementary and optional approach to add further value to paternity cases based on STR systems.

Since the discovery of SNPs as the genetic basis of the ABO and of many other blood groups, SNP typing could probably bring back blood grouping to paternity testing on a molecular genetic basis. For this purpose the genotype-phenotype correlation is an important prerequisite and has been demonstrated for many different blood group systems including ABO, Rh, Duffy, MNS, Kidd, and Kell. Identification of individuals using anonymous SNPs on different chromosomes such as the SNPforID markers [4] could be combined with SNPs of different blood group systems. In selected cases and in terms of control purposes, i.e. to match a blood sample with a DNA sample, the phenotypes of blood groups on different chromosomes could provide useful information. Phenotyping and genotyping of HLA antigens could be used as well.

The application of SNP typing in a routine laboratory such as clinical diagnostics or paternity testing requires proof of reliability of the method and standardization of the procedure. In

our laboratory, ABO phenotyping and genotyping is validated for clinical diagnostics and paternity testing, and the procedure is defined in standard operation protocols. The same is true for other blood group systems such as Duffy, MNS, Kidd, and Kell. Low-resolution ABO genotyping by PCR-SSP method including agarose gel electrophoresis is a very time- (less than 2 h; many samples simultaneously) and cost-effective method (less than EUR 2.– per DNA sample). In paternity cases or any other forensic case work DNA samples for STR analysis can also be used for SNP typing. Using PCR-SSP with optimized sensitivity [12], genotyping requires less than 10 ng DNA per SNP and can be performed on DNA extracted from buccal swabs. Other SNP genotyping methods including multiplex PCR with capillary electrophoresis [17] or other procedures [18] would require even lower DNA amounts.

Finally, for the development of systems for genetic identity testing based on diallelic markers, we would recommend to combine anonymous SNPs and SNPs associated with an easy-to-determine phenotype such as blood groups or HLA. This could open a new field of clinical application of genetic identity testing, e.g. where the identity of a hematopoietic stem cell transplant shipped from the donation site to the transplantation center is rechecked.

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Disclosure Statement

The authors declare no conflict of interest.

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