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SHORT REPORT

Comprehensive polymorphism analysis of ABO using allele-specific separation by bead technology and subsequent sequencing

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The ABO system is the most important blood group system in transfusion and transplantation medicine. Over the last decade ABO genotyping was introduced to complement serological analysis but because of the enormous diversity at the ABO locus, DNA-based typing is highly sophisticated. In some genotype combinations and especially when dealing with hybrid genes, cis/trans linkage analysis is necessary. Therefore, we developed a simple and reliable approach to isolate ABO haplotypes. The described method physically separates diploid genomic DNA using probe-dependent allele hybridization followed by magnetic bead separation. After this haplotype-specific extraction (HSE), sequencing based typing (SBT) was performed to analyze coding and non-coding respectively regulatory regions of ABO.

Key words: ABO blood group, haplotype-specific extraction, sequence-based typing, sequencing, SSP-PCR.

Introduction

The currently used and well established ABO genotyping methods are mainly based on sequence-specific primer (SSP)-PCR and restriction-fragment length polymorphism (RFLP) analysis [1,2]. These approaches are limited in their analytical power by the use of sequence motifs and missing the complete sequence information. Therefore, sequencing is an accepted reference method, providing the highest resolution of ABO allele typing. Moreover SBT is a necessity for the annotation of new alleles. However, when generic sequencing is used, ambiguous results are frequently generated since the linkage between the polymorphic sites is not determined. To overcome the cis/trans difficulty in the complex genetic context of ABO [3], we established an automated, fast and effective method to resolve these problems, which usually require DNA cloning. The novel method physically separates diploid genomic DNA into two haploid components. After probe-dependent hybridization and enzymatic elongation of the bound probe with biotinylated nucleotides, the targeted allele is subsequently coupled to streptavidin-coated paramagnetic beads for separation from non-targeted DNA [4,5]. The probes show single-base specificity and provide high capture efficiency of the target DNA. The haplotype-specific extraction using SNP-specific probes for A^2, non-A^2, B, non-B, O^1, non-O^1, O^2 and non-O^2 is automatically performed on a QIAGEN EZ1 instrument (Qiagen, Hilden, Germany). Analysis of the haplotypes of the targeted alleles by SBT leads to a clear and unambiguous allele identification. The presented data show the comprehensive analysis of coding and non-coding regions of the ABO loci using HSE.

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Methods

Genomic DNA was purified from nine selected ethylenediamine tetraacetate (EDTA) blood samples using the BioRobot EZ1 (Qiagen) following the manufacturer's instructions. Optical density was determined with a BioPhotometer (Eppendorf, Hamburg, Germany). Prior, DNA was investigated by real-time SSP-PCR discriminating the groups A^1, A^2, B, O^1 and O^2 using nucleotide position 261, 802, 803, and 1059. Nine out of 15 known allele combinations: O^1O^2 (O01/O03), BO^1 (B101/O01), A^1O^1 (A101/O01), A^2O^1

(A201/O01), BO^2 (B101/O03), A^1O^2 (A0101/O01), A^1B (A101/B101), A^2B (A201/B101), and A^1A^2 (A101/A201) were applied, except homozygous combinations and the infrequent heterozygous A^2O^2. Haplotype-specific probes were designed (Table 1) according to the nucleotide alterations at positions: 261delG, 802A, 802G, 803C, 803G,

Table 1 Oligonucleotides used in this study. Nucleotide positions of primers and probes were assigned relative to the reference sequences AJ531622.1 for exon 2–7 and AC000397 for promoter – exon 2.

Name	Specificity	Sequence 5′–3′	Location [Ref.]
Haplotype-specific probes[a]			
O1	261delG	GATGTCCTCGTGGTACC	nt 247–263
non-O1	261G	GATGTCCTCGTGGTGAC	nt 247–263
O2	802A	GATTTCTACTACCTGGGGAG	nt 784–803
non-O2	802G	GATTTCTACTACCTGGGGGG	nt 784–803
B	803C	ACCCCCCGAAGAACG	nt 803–817
non-B	803G	ACCCCCCGAAGAACC	nt 803–817
A2	1059delC	GCGGTCCGGAACCGT	nt 1048–1062
non-A2	1059C	CGGTCCGGAACCCG	nt 1049–1062
Amplification primer[b]			
ABO_mo-12F	Promoter, Exon 1	GGCGCCGTCCCTTCCTAG	5′-UTR nt −188 to −171 [7]
ABO_mo-12R	Promoter, Exon 1	CCTGCGGTAGCGGCTCCT	Intron 1 nt 33–51 [7]
ABO_mo-21F	Exon 2–3	GGTGAGAGAAGGAGGGTGAG	Intron 1 nt 12933–12952 [7]
ABO_E3_R	Exon 2–3	GGTCAAGGCTGACTCCAG	Intron 3 nt 168–185
ABO_E3_F	Exon 3–4	CCAACAGGCAGTCTTCGT	Intron 2 nt 670–687
ABO_E4_R	Exon 3–4	CTGCAGGACAATTCTGTGA	Intron 4 nt 37–55
ABO_E4_F	Exon 4–5	TGTTTCTGGTGGCCTCTG	Intron 3 nt 1314–1331
ABO_mo-57arev	Exon 4–5	GCAAAGAGATAGGAACAAACCC	Intron 5 nt 285–306
ABO_mo-51F	Exon 5–6	TGCATCCCACGCTTTCCATGC	Intron 4 nt 1635–1655 [7]
ABO_mo-46R	Exon 5–6	ACTCGCCACTGCCTGGGTCTC	Intron 6 nt 27–47 [7]
ABO019s	Exon 6–7	AAGCTGAGTGGAGTTTCCAG	Intron 5 nt 453–472 [8]
ABO118as	Exon 6–7	CCTAGGCTTCAGTTACTCAC	3′-UTR nt 103–122 [9]
Sequencing primer[b]			
ABO_mo-12F	Promoter, Exon 1	GGCGCCGTCCCTTCCTAG	5′-UTR nt −188 to −171 [7]
ABO_I2_247as	Exon 2–3	CCAGCCCCAGACTCCACACTTAG	Intron 2 nt 225–247
ABO_I2_186s	Exon 2–3	TGGGCAACAGGCTCATCTCACTCC	Intron 2 nt 187–210
ABO_E3_35as	Exon 2–3	ACCCCCGTTCCAGGCTTCC	Exon 3 nt 133–151
ABO_E3_F	Exon 3–4	CCAACAGGCAGTCTTCGT	Intron 2 nt 670–687
ABO_I3_260s	Exon 3–4	ACCCCTCCTCCACCAGCCATCCT	Intron 3 nt 260–282
ABO_1153as	Exon 3–4	GGCCGGGCAGAGTGGAGTTTCA	Intron 3 nt 1153–1174
ABO_E4_R	Exon 3–4	CTGCAGGACAATTCTGTGA	Intron 4 nt 37–55
ABO_I4_187as	Exon 4–5	GCTCCAGCTCCATCGTG	Intron 4 nt 184–200
ABO_I4_154s	Exon 4–5	GGCCGCTCCTTCCCCAGTCTCTA	Intron 4 nt 124–146
ABO_I4_476s	Exon 4–5	CCGCCTCAGCCTCCCAAAGTG	Intron 4 nt 476–496
ABO_I4_815s	Exon 4–5	CTCTGTCCCCATAAAATAGCAACT	Intron 4 nt 815–838
ABO_I4_1636as	Exon 4–5	GGAAAGCGTGGGATGCAGGTAAGC	Intron 4 nt 1628–1651
ABO_I5_51as	Exon 4–5	AAACCGCCCTCTAATACCTTCA	Intron 5 nt 51–72
ABO_mo-51F	Exon 5–6	TGCATCCCACGCTTTCCATGC	Intron 4 nt 1635–1655 [7]
ABO_I5_156s	Exon 5–6	TCCTCTTTCCCTTTGCTTTCTCTG	Intron 5 nt 157–180
ABO_I6_43as	Exon 6–7	AATGTCCACAGTCACTCGC	Intron 6 nt 42–60
ABO35as	Exon 6–7	ACCTCAATGTCCACAGTCAC	Intron 6 nt 46–65 [10]
ABO032s	Exon 6–7	CAGTTCAGGCTCCAGAACAC	Exon 6 nt 322–341 [10]
ABO037s	Exon 6–7	TCACTGACCAGAGATAGCAG	Intron 6 nt 322–341 [10]
ABO042as	Exon 6–7	GGTGGCCCACCATGAAGTG	Exon 7 nt 418–436 [10]
ABO039s	Exon 6–7	CGTCCGCCTGCCTTGCAG	Intron 6 nt 1035–1052 [10]
ABO053sgeneric	Exon 6–7	AGCTGTCAGTGCTGGAGGTG	Exon 7 nt 506–525
ABO0105as	Exon 6–7	TCCCAGAGCCCCTGGCAG	3′-UTR nt 5–22 [10]

[a]Probes for haplotype-specific extraction were purchased from IDT (Integrated DNA Technologies, Coralville, USA) normalized to 100 μM in TE pH 8.0.

[b]Amplification and sequencing primers were synthesized by Metabion (Metabion GmbH, Martinsried, Germany) normalized to 100 μM in PCR grade water.

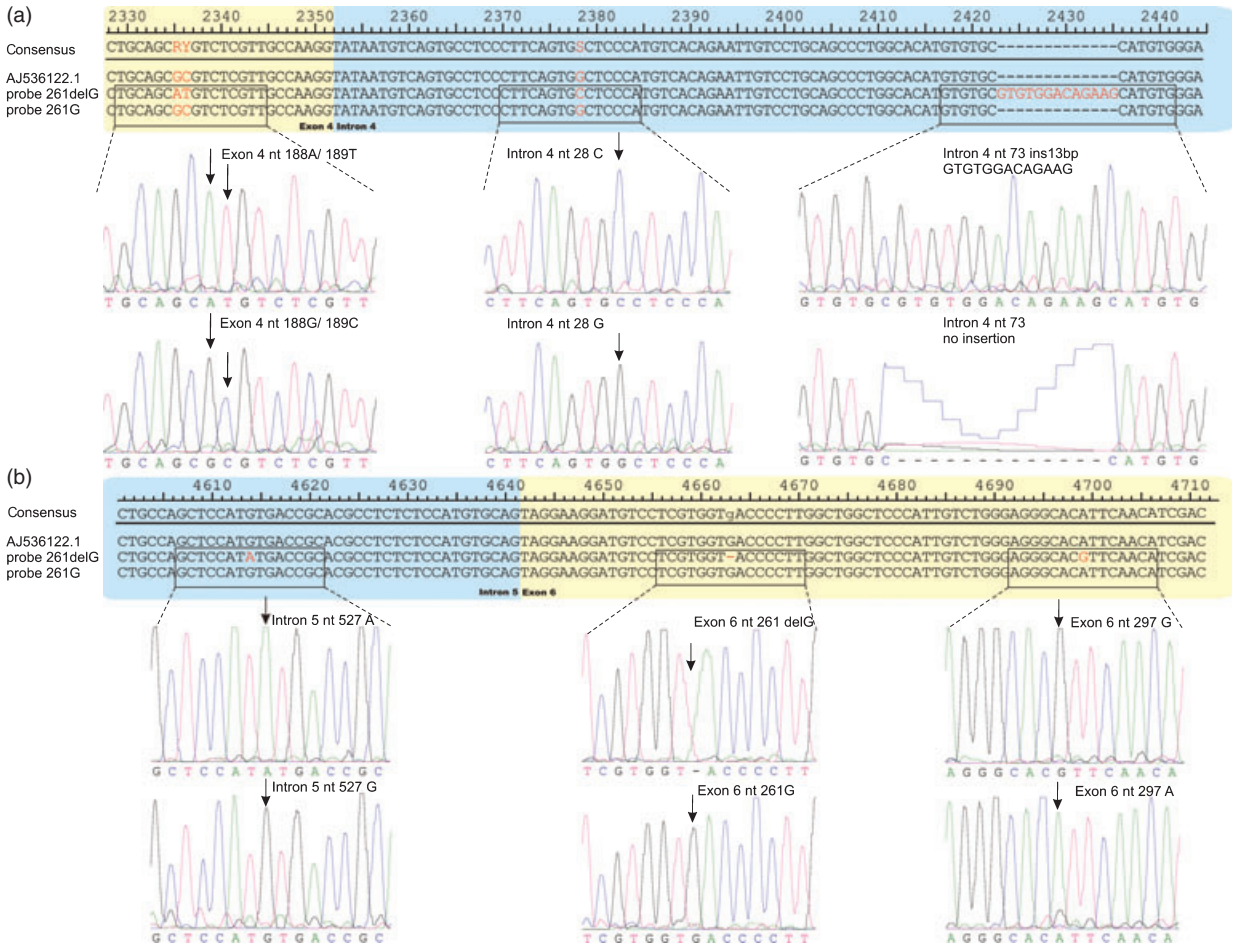

Fig. 1 Sequencing results from a patient sample after HSE with probes specific for O^1 (261delG) und nonO^1 (261G). Seven known polymorphic sites, in exon 4/intron4 (panel A) and intron 5/exon 6 (panel B) are shown. Next to the characteristic 261delG of the O^1 allele, a 13bp insertion occurring in only five O-alleles, further five SNPs are framed and electropherograms are depicted for the polymorphic sites including neighbouring bases to demonstrate exact discrimination of the allele specific nucleotide alterations. No haplotype contamination can be observed indicating the high specificity of the probes. AJ531622.1 was used as reference for identification of polymorphisms. Exonic sequences are coloured in light yellow, intronic sequences in light blue. Alignments were performed with DNAStar Lasergene 8.

1059delC and 1059C. Before separation, the ABO-SSP result is required for appropriate probe selection. HSE was done with the generic EZ1 HaploPrep Kit (Qiagen) on the BioRobot EZ1 (Qiagen) running the Haploprep protocol version 2.0. The hybridization reaction was carried out in a final volume of 30 µl using the following mixture: 15 µl HaploPrep Hybridization Buffer, 5 µl PCR grade H_2O, 1.5 µl specific probe (100 µM) and 8.5 µl genomic DNA (30 ng–150 ng/µl). The mixture was denaturated in an external incubator for 15 min at 95°C. During denaturation the EZ1 instrument was started to reach the 64°C hybridization temperature. After quick sample transfer into the machine the hybridization process and subsequent magnetic separation was started. Finally, 45 µl DNA-coated beads were available for downstream application.

PCR amplification for all primer combinations described in Table 1 was carried out in an ABI 9700 machine (Applied Biosystems, Foster City, CA, USA) including an incubation step at 93°C for 3 min and 40 cycles of 93°C 15 s, 62°C 30 s and 68°C 3 min and a final extension for 10 min at 68°C. The PCR mixture contained 1× LongRange PCR Buffer (Qiagen), 2.5 U LongRange PCR Enzyme Mix (Qiagen), 1× MasterAmp™ PCR Enhancer (EPICENTRE, Madison, USA), 500 µM dNTPs (Qiagen), 500 nM of each oligonucleotide (Table 1) and 7.5 µl haploseparated DNA adjusted to a final volume of 25 µl. Amplicons were verified on a gel and the product used for cycle sequencing. Standard PCR clean-up was done by Montage PCR centrifugal filter devices (Millipore, Schwalbach, Germany) to remove primers and dNTPs, according to the

manufacturer's instructions. Exon 1 PCR was performed with 5% glycerine instead of MasterAmp PCR Enhancer. Direct cycle sequencing was performed in a 9800 Fast Thermal Cycler (Applied Biosystems) using the BigDye Fast program: hold at 96°C for 1 min and 25 cycles of 93°C 10 s, 50°C 5 s and 60°C 75 s. The cycle sequencing reaction mixture was as follows: 1.5 μl BigDye v 1.1 (Applied Biosystems), 1× sequencing buffer, and 500 nM of the respective sequencing primer described in Table 1, 3 μl purified amplicon in a final volume of 20 μl. Post-sequencing reaction purification was performed using MultiScreen plates (Millipore) and Sephadex G50 (Sigma-Aldrich, Steinheim, Germany). The sequencing device was an ABI 3100 DNA sequencer and generated sequences were analyzed with SeqScape V2.5 (Applied Biosystems).

Results and discussion

In recent years sequencing improved and more sequence information of ABO is available, leading to an unavoidable continuous increase of new alleles. The accurate prediction of the ABO phenotype with established genotyping methods is challenging, especially in case of non- deletional O alleles, hybrid genes and infrequent alleles [6]. Sequence-based typing is one of the most comprehensive methods utilized for ABO notably in case of new alleles. However, generic sequencing of Exon 6 and 7 leads often to ambiguous typing results. To resolve the two main types of ambiguities it is firstly necessary to extend the observed regions of ABO and furthermore it is required to resolve the cis/-trans linkage of the polymorphism. Commonly used methods to determine the sequences of haplotypes are cloning followed by sequencing or allele-specific sequencing. Nevertheless, these methods have some drawbacks as cloning is a labour-consuming procedure and allele-specific sequencing can only phase polymorphisms when there is an overlapping SNP for each amplicon. Hence, we developed a straightforward approach to define the phase of polymorphic positions by HSE and extended sequencing. For the nine selected study samples, haplotype separation and sequence analysis was performed for exon 1–7 including the promoter and intron 2–6. Because of the long distance (~24 kb) of the enhancer to the binding site of the haplo-specific probes, we were not able to separate the enhancer sequences most likely because of physical limits as a result of strand breaks. This described method is easy, automated and provides large fragments of haploid DNA and by the use of relatively low number of hybridization probes the technique is advantageous for high-throughput haplotype analysis of ABO. Using this method, the identification of phased sequence information of almost 6.7 kb of the ABO gene was possible suggesting haplotype-specific sequencing as a dedicated approach for ABO genotyping when extended sequence information is needed. However, haplotype separation by HSE can also be combined with various downstream techniques including SSP-PCR, real-time PCR, RFLP analysis and is an applicable method to overcome conventional cloning procedures or extensive family pedigree analysis. HSE is a promising tool for ABO genotyping to improve the cis/trans resolution of exon polymorphisms and moreover, combined with intron sequencing, it is possible to define evolutionary relationships, and complex unequal and reciprocal recombination events in the ABO gene.

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