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ScienceDirect

Transfusion Clinique et Biologique 14 (2007) 568–571

TRANSFUSION CLINIQUE ET BIOLOGIQUE

http://france.elsevier.com/direct/TRACLI/

Original article

Fetal RhD genotyping: A more efficient use of anti-D immunoglobulin

Génotypage fœtal RhD : une utilisation plus efficace des immunoglobulines anti-D

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Available online 23 April 2008

Abstract

The most important application of blood group genotyping by molecular genetics is the prediction of fetal RhD phenotype in pregnant women with anti-D, in order to assess the risk of haemolytic disease of the fetus and newborn. This diagnostic test performed on cell-free fetal DNA in the maternal plasma, is now a routine procedure in some countries. High-throughput modifications of this form of fetal D-typing would be valuable for testing fetuses of all D-negative pregnant women to avoid unnecessary antenatal treatment with anti-D immunoglobulin in the 40% of D-negative pregnant women with a D-negative fetus. The results of trials in Bristol and Amsterdam suggest that such routine testing is feasible and accurate.

Résumé

L'application la plus importante du génotypage des groupes sanguins par génétique moléculaire est la prédiction du phénotype RhD du fœtus chez les femmes enceintes immunisées contre l'antigène D, afin d'évaluer le risque de maladie hémolytique du fœtus et du nouveau-né. Ce test diagnostic effectué sur l'ADN fœtal libre dans le plasma maternel, est désormais une procédure de routine dans certains pays. Le génotypage fœtal RhD par des méthodes à haut débit serait utile pour tester les fœtus de toutes les femmes enceintes de groupe sanguin rhésus D négatif, afin d'éviter les traitements prénatals anti-Ig D inutiles pour 40 % des femmes enceintes de groupe sanguin rhésus D négatif portant des fœtus de groupe sanguin rhésus D négatif. Les résultats des essais de Bristol et d'Amsterdam suggèrent que ces tests de routine sont réalisables et précis.

Keywords: Rhesus; RhD; Genotyping; antenatal diagnosis; Fetal DNA; Maternal plasma

Mots clés : Rhésus ; RhD ; Génotypage ; Diagnostic prénatal ; ADN fœtal ; Plasma maternel

Rh is the largest and most complex of the human blood group systems [1]. Of the 49 Rh antigens, the most important, from a clinical aspect, is D (RH1). D has a frequency of about 85% in white populations, around 95% in black Africans and approaching 100% in eastern Asia. D antibodies which are predominantly IgG1 and IgG3, have the potential to cause severe and fatal haemolytic disease of the fetus and newborn (HDFN).

Following cloning of the genes encoding the Rh blood group proteins, the molecular bases of the Rh blood group polymorphisms and of numerous Rh variants were identified. This information makes possible the prediction of blood group phenotypes from tests on genomic DNA, with a high degree of accuracy. There are many applications of predicting blood group phenotype from molecular genotype, the most important of which is determining fetal D phenotype in pregnant women with anti-D in order to assess whether the fetus is at risk of HDFN.

1. Molecular background to the D polymorphism

The antigens of the Rh system are encoded by *RHD* and *RHC*E, a pair of paralogous genes on chromosome 1. These

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1246-7820/$ – see front matter
doi:10.1016/j.tracli.2008.03.007

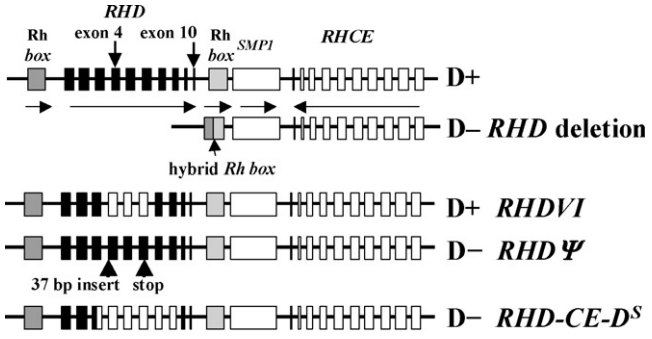

Fig. 1. Diagram of the Rh genes, the *Rh boxes* flanking *RHD* and *SMP1* between *RHD* and *RHCE*, in five haplotypes, two producing D (D+) and three producing no D (D−).

genes each have 10 exons and share 94% sequence identity. They are very unusual for closely linked paralogous genes as they are in opposite orientation (5′*RHD*3′-3′*RHC*E5′), with the sense strand of *RHD* becoming the antisense strand of *RHC*E (Fig. 1) [2]. *RHD* is flanked by 9-kb regions of 98.6% identity, the *Rh boxes*. The Rh genes produce homologous proteins of 417 amino acids that are palmitoylated, but not glycosylated. They traverse the red cell membrane 12 times, with amino- and carboxy-termini in the cytosol and six extracellular loops, the potential sites for antigen activity.

In a white population, almost all D-negative individuals are homozygous for a deletion of *RHD*, the deletion encompassing the whole of *RHD* and part of each of the *Rh boxes* [2]. D-positive individuals may have one or two copies of *RHD*. The D-negative phenotype therefore usually results from an absence of the whole RhD protein from the red cell membrane, although the RhCcEe protein is almost universally present. Consequently, anti-D produced as a result of allo-immunisation by D-positive red cells introduced by blood transfusion or pregnancy comprises antibodies recognising a variety of epitopes on the external loops of the RhD protein.

In black Africans, the situation is different: only 18% of D-negative black Africans are homozygous for an *RHD* deletion. Sixty-six percent of D-negative black Africans have an inactive *RHD*, called *RHDΨ*, that has a 37-bp duplication in exon 4 and a nonsense mutation in exon 6 [3]. In addition, 15% of D-negative black Africans have a hybrid gene, *RHD-CE-D*^{S}, that contains exons 1, 2, part of 3, 9 and 10 from *RHD*, but part of exon 3 and exons 4–8 from *RHC*E [4,5]. This hybrid gene produces no epitopes of D.

There are numerous rare variants of the D antigen [1,6,7]. These are recognised by weakness of expression of D and/or absence of some or many epitopes of D. In some cases, individuals with variant D antigens, if allo-immunised by normal D-positive red cells, can make antibodies to one or more epitopes of D. These D variants result either from single nucleotide polymorphisms (SNPs) in *RHD* encoding amino acid substitutions or from hybrid Rh genes. For example, DVI, the most common D variant associated with production of anti-D, arises from an *RHD–CE–D* gene consisting of exons 1–3 and 7–10 of *RHD*, but exons 4–6 of *RHC*E (Fig. 1) [8].

2. Fetal genotyping in allo-immunised pregnant women

When a pregnant woman has anti-D, it is beneficial to determine the D type of her fetus. If the fetus is D-positive, it is at risk of HDFN and the pregnancy can be managed appropriately; if it is D-negative, it is not at risk and unnecessary interventions can be avoided. D phenotype can be predicted from fetal DNA by polymerase chain reaction (PCR) amplification of one or more regions of *RHD* to determine whether the gene is present. Primers must be designed so that *RHCE* is not amplified and precautions must be taken so that false results do not occur owing to the presence of *RHDΨ*, *RHD-CE-D*^{S}, or *RHD*VI.

Initially, the usual source of fetal DNA was amniocytes, obtained by amniocentesis and occasionally cells of the chorionic villi, obtained by chorionic villus sampling (CVS). The method employed at the International Blood Group Reference Laboratory (IBGRL) in Bristol involved PCR amplification of exons 4 and 7 of *RHD* followed by polyacrylamide gel electrophoresis of the amplified products [3]. The exon 4 primers amplify across the region of the 37-bp duplication in *RHDΨ*, so its presence can be recognised by the different size of the product.

Both amniocentesis and CVS are invasive and are associated with increased risks of spontaneous abortion [9]. In addition, with amniocentesis, there is a 20% risk of transplacental haemorrhage, which could boost the maternal antibody, enhancing the risk of severe HDFN [10]. Consequently, a better source of fetal DNA was required.

In 1998 Lo et al. [11] showed that about 3% of cell-free DNA in the plasma of first-trimester pregnant women is of fetal origin, with this proportion rising to 6% in the third trimester. This fetal DNA is almost certainly derived from the placenta. Numerous methods have been devised for predicting fetal D type from this fetal DNA in the plasma of D-negative pregnant women [12–14]. The fetal DNA cannot be separated from the maternal DNA, but as the allo-immunised women are D-negative, presence of *RHD* predicts a D-positive fetus.

Methods that have proved reliable for determining D phenotype from fetal DNA in maternal plasma utilise real time quantitative PCR (RQPCR), with Taqman chemistry. The method we use at the IBGRL incorporates Taqman primers and probes to detect exons 4, 5 and 10 of *RHD*, but not *RHCE*. The exon 10 reaction also detects *RHDΨ* and *RHCE–D–CE*^{S}, but the exon 4 and 5 reactions do not detect these two ‘D-negative’ genes [15,16].

A problem in all tests on fetal DNA derived from maternal plasma arises from the large quantity of maternal DNA present in the DNA preparation, complicating the inclusion of satisfactory internal controls to test for successful amplification of fetal DNA. Reactions for detecting the Y-linked gene *SRY* can be included in the test, which will provide a positive result when the fetus is male [15,16]. In addition, when a result suggesting a D-negative female fetus is obtained, some laboratories test for polymorphisms that involve insertion or deletion of DNA sequence, in an attempt to obtain a positive