Fetal RHD genotyping by maternal plasma analysis.

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ABSTRACT: Background and Objective: Determination of the fetal RhD typing using free fetal DNA in maternal plasma, is beginning to gain widespread acceptance in Europe and may allow genetic analysis without the use of invasive techniques. The purpose of this study was to extract DNA from maternal plasma and determine the accuracy of the non-invasive prenatal determination of fetal RhD genotyping with the use of real-time PCR.

Materials and Methods: We analyzed 48 RhD-negative pregnant women in the 16th to 40th weeks of pregnancy using real-time PCR, primers and probes targeting the RHD gene. Results were compared with serologic RhD typing of the newborns.

Results: Among the 48 pregnant women who participated in the study, 9 were in the second trimester of pregnancy and 39 in the third trimester. Twenty nine fetuses genotyped as RhD positive and 19 as RhD negative. Neither false-negative nor false-positive results were observed.

Conclusion: The present report demonstrates that a reliable fetal RHD genotype determination can be achieved with 100% accuracy. It is therefore possible to consider, that such an assay could be systematically proposed to all RhD-negative pregnant women in order to use RhD prophylaxis more effectively.

Key Words: Fetal DNA, Maternal plasma, RHD genotype.

INTRODUCTION

The Rh blood group system is one of the most important, complex and immunogenic systems known in humans because of its involvement in the newborn’s hemolytic disease, the transfusion reactions, and the autoimmune hemolytic anemia. D antigen is highly immunogenic and is the most important blood group antigen in terms of clinical significance ¹,²,³.

Prior to the 1970s, hemolytic disease of the fetus and newborn (HDFN) was a significant cause of fetal and neonatal morbidity and mortality. HDFN is usually caused by immunoglobulin G (IgG) antibodies to the D antigen of the Rh system in a D-negative (D−) mother crossing the placenta and facilitating the immune destruction of D-positive (D+) fetal red cells. Since 1968, with the introduction of immune prophylaxis to prevent Rhesus (Rh)D allo-immunization, the risk of anti-RhD allo-immunization has been markedly reduced (1 to 6 per 1000 live births)⁴,⁵.

However, administration of blood derivatives is not devoid of risk, and every effort should be made to improve techniques that could reduce the number of injections. Therefore, the new possibility of fetal RHD genotyping using polymerase chain reaction (PCR) is a significant advance for clinical purpose. Fetal RHD genotype determination is useful in the management of RhD-negative sensitized women. Genotyping is also useful in RhD-negative pregnant women at risk for RhD immunization in order to avoid unnecessary administration of anti-D immunoglobulin, in the case of an RhD-negative fetus⁶,⁷. In this study, we determined the accuracy of the non-invasive prenatal determination of fetal RhD genotyping by the use of real-time PCR⁸.

MATERIALS AND METHODS

DNA extraction from plasma samples

Forty eight RhD-negative pregnant women in the second and third trimester and six positive RhD samples
as controls elected to participate for noninvasive prenatal determination by maternal plasma analysis. Five mL of blood were collected into EDTA- tubes. Immediately, blood samples were centrifuged for 10 minutes at 3000g, and the plasma was collected in fresh tubes and stored in -80°C until it was used for DNA isolation. DNA was extracted from 200 μl plasma using QIAamp DNA Blood Mini Kit (Qiagen; Hilden, Germany).

**Real-time PCR for the RHD gene in maternal plasma**

The procedure was similar to that previously described. Amplification was carried out in a LightCycler instrument (Roche Biochemicals). PCR reactions were set up in a final volume of 20 μL using the FastDNAMaster Hybridization Probes Kit (Roche Biochemicals), with 0.5 mmol/L of each primer, 0.25 mmol/L of each probe (Proligo, France) (Table), 1 unit of uracil DNA glycosylase (UNG, heat labile) (Biolabs, Saint-Quentin en Yvelines, France), 4.0 mmol/L of magnesium chloride. After an initial 10 minutes denaturation step at 95°C, was followed by an amplification performed for 50 cycles of denaturation (95°C, 10 seconds, ramping rate 20°C/sec), annealing (56°C, 10 seconds, ramping rate 20°C/sec), and extension (72°C, 20 seconds, ramping rate 20°C/sec).

Each sample was treated twice for DNA extraction. The results were compared with those obtained after birth, by RHD serology of the newborn.

**RESULTS**

Among the 48 pregnant women tested for RHD gene presence in their plasma, the status of the fetus was determined in all 48 cases. Among 48 RhD pregnant women, 9 were in their second trimester of pregnancy and the other 39 in third trimester. In 29 cases, RHD sequences had been detected in maternal plasma, and the fetuses could therefore be considered as RhD-positive, while the others (n = 19) were RhD-negative (Figure 1).

Results were in complete concordance, and neither false-negative nor false-positive results were observed. The fetal RHD genotype in all cases matched in 100% with the results obtained from RhD serology typing of the newborns.

**DISCUSSION**

Noninvasive prenatal diagnosis is one of the many major goals in human genetics. The discovery of cell-
free fetal DNA in maternal plasma in 1997 has opened up new possibilities for noninvasive prenatal diagnosis. Circulating fetal DNA has been shown to increase in concentrations with the progress in gestational age and to be cleared rapidly following delivery. With the use of real-time polymerase chain reaction (PCR) methodology, circulating fetal DNA has been detected robustly in the plasma of pregnant women, even early in the first trimester of pregnancy. As a result of these developments, fetal DNA in maternal plasma has been used for the noninvasive prenatal diagnosis of sex-linked disorders and single gene disorders such as beta-thalassemia, congenital adrenal hyperplasia and achondroplasia. In addition, quantitative aberrations of circulating fetal DNA have been also found in various pregnancy-associated disorders, including preeclampsia, preterm labor and fetal trisomy.

One of the first clinical applications of noninvasive detection of fetal DNA in the mother was for rhesus D typing of the fetus. Fetal RHD genotype can be determined with a high level of accuracy by analysis of fetal DNA circulating in maternal plasma and serum. It is therefore possible to consider that such an assay could be included in prenatal care of RhD-negative women.

We performed fetal RHD genotyping using maternal plasma from 48 pregnant women in 16–40 weeks of pregnancy. Serological RhD typing of the newborns showed 100% concordance between genomic and serological typing. Among the women studied, 39.6% carried an RhD-negative fetus. In these cases, no prenatal anti-D prophylaxis is needed and injection of anti-D immunoglobulin can be avoided. Follow-up of pregnancy is therefore made easier, decreasing the patient’s anxiety.

Traditional management for RhD-negative pregnant women at risk for RHD alloimmunization involves determining the RhD status of the fetus through invasive chorionic villus sampling (CVS) or amniocentesis, and administering the prophylactic human derived Rho(D) Immune Globulin during pregnancy and after delivery. Both practices carry their own risks and associated costs. In many European countries, studies showed that fetal RHD genotyping from maternal plasma can save up to 40 percent of the prenatal anti-D injections, because treatment is only limited in women with RhD-positive fetuses.

Hence, the ability to determine this important fetal DNA RHD locus in maternal plasma, in a non-invasive manner using real-time PCR, presents a significant achievement in the application of research to clinical routine.
Εμβρυϊκή RHD γονοτυπική τυποποίηση με ανάλυση μητρικού πλάσματος.

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ΠΕΡΙΛΗΨΗ: Εισαγωγή και Σκοπός: Ο καθορισμός της εμβρυϊκής RHD τυποποίησης χρησιμοποιώντας ελεύθερο εμβρυϊκό DNA από μητρικό πλάσμα έχει αρχίσει να κερδίζει ευρεία αποδοχή στην Ευρώπη και είναι δυνατόν να επιτρέψει τη γενετική ανάλυση χωρίς τη χρήση παρεμβατικών μεθόδων. Ο σκοπός της συγκεκριμένης εργασίας ήταν η απομόνωση εμβρυϊκού DNA από μητρικό πλάσμα και ο καθορισμός της ακρίβειας μιας μη παρεμβατικής προγεννητικής διάγνωσης του εμβρυϊκού RHD γονότυπου με τη χρήση της real-time PCR.

Υλικό και Μέθοδος: Μελετήθηκαν 48 ΡHD αρνητικές έγκυες γυναίκες μεταξύ 16η και 40η εβδομάδας κύησης με τη μέθοδο real-time PCR και τη χρησιμοποίηση αμπελιέων και ανυχισμένων κατάλληλων για το RHD γονότυπο. Τα αποτελέσματα συγκρίθηκαν με ορολογική Rhd τυποποίηση των νεογνών.

Αποτελέσματα: Από τις 48 RhD αρνητικές έγκυες γυναίκες, 9 ήταν στο δεύτερο τρίμηνο της κύησης και οι 39 στο τρίτο τρίμηνο. Είκοσι εννέα εμβρύα τυποποιήθηκαν ως RhD θετικά και 19 RhD αρνητικά. Δεν παρατηρήθηκαν ψευδώς αρνητικά ήταν πειράματα αποτελέσματα.

Συμπόσιο: Με την παρούσα εργασία γίνεται φανερό ότι ο αξιόπιστος RHD γονοτυπικός καθορισμός μπορεί να επιτευχθεί με 100% ακρίβεια. Παρόμοιες μελέτες είναι επομένους δυνατόν να εφαρμοστούν συστηματικά σε όλες τις RHD αρνητικές έγκυες γυναίκες με απότελεσμα να επιτευχθεί ρηματική Rhd προφύλαξη.

Λέξεις Κλειδιά: Εμβρυϊκό DNA, Μητρικό πλάσμα, RHD γονότυπος.

REFERENCES


