CLINICAL STUDY

Non-Invasive Determination of Fetal c and E Allele of RHCE Gene Via Real-Time PCR Testing of Extracellular DNA Extracted from Maternal Plasma Samples Using QIAamp DSP Virus Kit

Ilona **HROMADNIKOVA**[1], Klara **VESELA**[1], Jindrich **DOUCHA**[2], Katerina **NEKOVAROVA**[4], Daniela **DUSKOVA**[5], Radka **SCHROLLOVA**[3], Pavel **CALDA**[4]

2nd Medical Faculty [1]Cell Biology Laboratory, [2]Clinic of Obstetrics and Gynecology, [3]Blood Bank; 1st Medical Faculty [4]Clinic of Obstetrics and Gynecology, [5]Department of Transfusion Medicine; Charles University, Prague, Czech Republic

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Abstract

Objective: This is the first prospective study showing the possible future implementation of non-invasive fetal RHCE genotyping from maternal plasma into a clinical diagnostic algorithm for following anti-c and anti-E alloimmunized pregnancies at risk of haemolytic disease of the newborn.

Materials and Methods: We carried out the non-invasive determination of fetal c allele and E allele of RHCE gene via real-time PCR analysis of DNA extracted from maternal plasma samples using QIAamp DSP Virus kit. We analysed plasma samples of 48 pregnant women within 12th and 36th week of pregnancy and correlated the results with the serological analysis of cord blood after the delivery.

Results: Non-invasive prenatal fetal RHCE genotyping analysis of maternal plasma samples was in complete concordance with the analysis of cord blood, in all pregnant women delivering 13 Rh E positive, 8 Rh E negative, 21 Rh c positive and 6 Rh c negative newborns.

Discussion: Reliable non-invasive detection of Rhc and RhE negative foetuses in anti-c and anti-E alloimmunized pregnancies may exclude the risk of haemolytic disease of newborn.

Keywords: fetal DNA, maternal plasma, real-time PCR, RHCE gene, haemolytic disease of the newborn

Özet

Maternal Plazma Örneklerinden Elde Edilen Ekstraselüler DNA'nın Gerçek Zamanlı PCR Testi Aracılığıyla RHCE Geni Fetal c ve E Alelinin QIAamp DSP Virüs Kiti Kullanılarak Non-invazif Saptanması

Amaç: Bu çalışma, anti-c ve anti-E alloimmünize olan ve neonatal hemolitik hastalık riski bulunan gebeliklerin takibinde maternal plazmadan tanısal amaçlı non-invazif fetal RHCE gen tiplemesinin gelecekteki olası algoritmik uygulamalarını gösteren ilk prospektif araştırmadır.

Materyal ve Metot: RHCE geninin fetal c alel ve E alellerinin non-invazif olarak belirlenmesi için maternal plazmadan QIAamp DSC virüs kiti kullanılarak elde edilen DNA'nın gerçek zamanlı PCR analizi yapıldı. Gebeliklerinin 12.-36. haftalarında olan 48 kadının plazma örnekleri analiz edildi ve doğum sonrası kordon kanı, kadının serolojik sonuçlarıyla karşılaştırıldı.

Sonuçlar: Doğum yapan tüm kadınlarda (13 Rh E pozitif, Rh E negatif, 21 Rh c pozitif ve 6 Rh c negatif yenidoğan) maternal plazma örneklerinin non-invazif prenatal fetal RHCE genotipleme analizi, kordon kanı analiziyle tam bir uyum içinde bulunmuştur.

Tartışma: Anti-c ve anti-E alloimmünize gebeliklerde, Rhc ve RhE negatif fetüslerin non-invazif yöntemle güvenilir bir şekilde belirlenmesi, yenidoğanda hemolitik hastalık riskini ortadan kaldırabilir.

Anahtar sözcükler: fetal DNA, maternal plazma, gerçek zamanlı PCR, RHCE geni, neonatal hemolitik hastalık

Corresponding Author: Dr. Ilona Hromadnikova
2nd Medical Faculty, Charles University
V Uvalu 84, 150 18 Prague 5, Czech Republic
Phone : +420 224 43 20 22
Fax : +420 224 43 20 21
E-mail : ilona.hromadnikova@lfmotol.cuni.cz

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Introduction

Rh blood group system is the most polymorphic of the human blood groups, consisting of at least 45 independent antigens and, next to ABO, is the most clinically significant in transfusion medicine. Haemolytic disease of newborn (HDN) is in 50% of cases caused by maternal anti-D (IgG) antibody crossing the placenta, binding to fetal red blood cells followed by their destruction causing anaemia. Alloimmunisation due to anti-K and anti-c is in clinical importance next to anti-D (1,2). Although, anti-E were reported to produce rarely clinically significant alloimunization (1,2), we observed in several patients, who had high titres of anti-E alloantibodies, severe HDN followed by fetal loss.

Current experimental non-invasive methods for the prenatal diagnosis of fetal genetic characteristics using free extracellular fetal DNA circulating in maternal peripheral blood seems to be a promising non-invasive alternative for the determination of fetal gender, RhD status and Mendelian genetic disorders involving point mutations (3-19). In this study, we carried out the non-invasive determination of fetal c allele and E allele of RHCE gene via real-time PCR analysis of DNA extracted from maternal plasma samples using QIAamp DSP Virus kit. This is the first prospective study showing the possible future implementation of fetal RHCE genotyping into a clinical diagnostic algorithm for following anti-c and anti-E alloimmunized pregnancies at risk of haemolytic disease of the newborn.

Material and Methods

Forty-eight pregnant women including those alloimunized at risk of HDN at a gestational stage ranging from 12th to 36th weeks were recruited for the study.

The Local Ethics Committee approval and informed consent was obtained for all patients in the study.

To minimise the risk of contamination, plasma preparation, DNA extraction from maternal plasma and preparation of real-time PCR reaction were performed in laminar air-flow and aerosol resistant tips were used.

DNA extraction from maternal plasma samples

Ten ml of maternal peripheral blood from 48 pregnant women was collected into EDTA containing tubes and processed within a few hours (maximally 24 h). In details, blood samples were centrifuged firstly at 1200 g (5,19) for 10 minutes, than plasma samples were re-centrifuged again and the supernatants were collected and stored at –80°C until further processing. DNA was extracted from 1 ml plasma using QIAamp DSP Virus Kit (Qiagen, Hilden, Germany) according to the modified manufacturer's instructions.

DNA extraction from maternal plasma samples by using QIAamp DSP Virus kit

Lower amount of QIAGEN Protease (Qiagen, Hilden, Germany) (20 µl) was used for the degradation of the proteins. Residual contaminants were removed by wash buffers AW1, AW2 and ethanol (600 µl AW1, 750 µl AW2, 750 µl ethanol)

Table 1. Primers and TaqMan probes for RHD, RHCE, SRY and GLO real-time PCR

Gene	Primer sequences	Probe sequence	References
RHD exon 10	5´- CCT CTC ACT GTT GCC TGC ATT - 3´ 5´- AGT GCC TGC GCG AAC ATT - 3´	5´- (FAM) TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC (TAMRA) - 3´	4, 21-23
RHD exon 7	5´- CTC CAT CAT GGG CTA CAA - 3´ 5´- CCG GCT CCG ACG GTA TC - 3´	5´ - (FAM) AGC AGC ACA ATG TAG ATG ATC TCT CCA (TAMRA) - 3´	20-23
RHCE exon 5 E allele	5´- TGG CCA AGT GTC AAC TCT C-3´ 5´- TCA CCA TGC TGA TCT TCC T - 3´	5´ - (FAM) AAG AAT GCC ATG TTC AAC ACC TAC TA TG (TAMRA) - 3´	20,21,23
RHCE exon 2 c allele	5´- TCG GCC AAG ATC TGA CCG - 3´ 5´- ATG ACC ACC TTC CCA GG- 3´	5´ - (FAM) CTT CCT CAC CTC AAA TTT CCG GAG A (TAMRA) - 3´	20
SRY	5´- TGG CGA TTA AGT CAA ATT CGC - 3´ 5´-CCC CCT AGT ACC CTG ACA ATG TAT T- 3´	5´-(FAM) AGC AGT AGA GCA GTC AGG GAG GCA GA (TAMRA) - 3´	8,19
GLO	5´- GTG CAC CTG ACT CCT GAG GAG A - 3´ 5´- CCT TGA TAC CAA CCT GCC CAG - 3´	5´- (FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA) - 3´	8,19,21-23

The primers for c allele and E allele are specific for SNP in exon 2 and exon 5 of RHCE gene.

Table 2. Fetal *RHCE* genotyping (E allele and c allele) on extracellular DNA isolated from maternal plasma samples by using QIAamp DSP Virus kit

No.	UPN	Week of gestation alloantibodies	The number of posive wells/ The number of replicates	Rh phenotype cord blood
		Fetal RHCE genotyping (E allele)		
1	1156	28 anti-D 1: 512 IAT anti-C 1:8 IAT	0/6 -	RhE neg.
2	1186	12 no	6/6 +	RhE pos.
3	1196	28 anti-E 1:32 IAT	13/14 +	RhE pos.
4	1229	12 Evans syndrome	0/14 -	RhE neg.
5	1357	26 anti-E 1:4000 IAT anti-C 1:2 IAT	12/14 +	RhE pos.
6	1403	19 no	6/6 +	RhE pos.
7	1418	14 anti-D 1:512 IAT	3/3 +	RhE pos.
8	1420	12 no	3/3 +	RhE pos.
9	1443	17 anti-D 1:256 IAT anti-C 1:16 IAT	5/6 +	RhE pos.
10	1498	12 anti-E 1:32 IAT	6/6 +	RhE pos.
11	1520	27 anti-D 1:4000 IAT anti-C 1:16 IAT	0/6 -	RhE neg.
12	1553	18 no	5/6 +	RhE pos.
13	1554	20 anti-D 1:512 IAT	3/3 +	RhE pos.
14	1589	12 no	6/6 +	RhE pos.
15	1658	30 anti-c 1:16 IAT	0/9 -	RhE neg.
16	1765	27 anti-E 1:512 IAT	6/6 +	RhE pos.
17	1800	14 anti-E 1:8 IAT	0/12 -	RhE neg.
18	1871	19 anti-E 1:8 IAT	0/12 -	RhE neg.
19	1906	19 anti-c 1:256 IAT anti-E 1:4 IAT	0/12 -	RhE neg.
20	1928	27 anti-c 1:8 IAT anti-E 1:8 IAT	0/9 -	RhE neg.
21	1992	21 anti-E 1:128 IAT	6/6 +	RhE pos.
		Fetal RHCE genotyping (c allele)		
1	860	12 no	3/3 +	Rhc pos.
2	965	12 no	3/3 +	Rhc pos.

Table 2. continued

No.	UPN	Week of gestation alloantibodies	The number of posive wells/ The number of replicates	Rh phenotype cord blood
3	978	12 no	3/3 +	Rhc pos.
4	989	12 no	3/3 +	Rhc pos.
5	993	12 no	0/3 -	Rhc neg.
6	998	33 no	6/6 +	Rhc pos.
7	1072	12 no	0/3 -	Rhc neg.
8	1142	36 no	3/3 +	Rhc pos.
9	1208	12 no	3/3 +	Rhc pos.
10	1218	12 no	3/3 +	Rhc pos.
11	1235	28 anti-c 1:16 IAT	0/21 -	Rhc neg.
12	1419	35 anti-c 1:4 IAT	8/12 +	Rhc pos.
13	1428	12 no	0/3 -	Rhc neg.
14	1456	12 no	5/6 +	Rhc pos.
15	1472	12 no	4/6 +	Rhc pos.
16	1498	12 anti-E 1:32 IAT	6/6 +	Rhc pos.
17	1521	12 no	3/3 +	Rhc pos.
18	1625	12 no	0/3 -	Rhc neg.
19	1631	12 no	3/3 +	Rhc pos.
20	1658	30 anti-c 1:16 IAT	6/6 +	Rhc pos.
21	1693	17 anti-c 1:32 IAT	3/3 +	Rhc pos.
22	1699	25 anti-c 1:16 IAT	3/3 +	Rhc pos.
23	1765	27 anti-c 1:128 IAT	3/3 +	Rhc pos.
24	1812	27 anti-c 1:64 IAT	5/6 +	Rhc pos.
25	1866	29 anti-c 1:2 IAT	0/12 -	Rhc neg.
26	1906	19 anti-c 1:256 IAT anti-E 1:4 IAT	10/12 +	Rhc pos.
27	1928	27 anti-c 1:8 IAT anti-E 1:8 IAT	6/6 +	Rhc pos.

and by using QIAvac 24 Plus vacuum system and vacuum pump (Qiagen, Hilden, Germany). DNA was eluted in 60 µl Buffer AVE and 15 µl was used as a template for the PCR reaction (SAFE NIPD recommendation for standardization of non-invasive RHD testing).

Real-time PCR analysis

The real-time PCR analysis was performed using 7300 real-time PCR system (Applied Biosystem, Branchburg, New Jersey, USA).

Primer and probe sequences are shown in Table 1 (4,8,19-23). The β-globin (GLO) served as a control to confirm the presence of DNA in each sample (8,19,21-23). Amplicons for β-globin control gene were detected in all analysed samples.

TaqMan amplification reactions were set up in a reaction volume of 50 µl using the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA). Primers and probes were optimized to determine the minimum primer and probe concentrations that give the maximum Rn. The RHCE and β-globin probes were used at concentrations of 100 nM. The PCR primers were used at a final concentration of 300 nM. DNA amplifications were carried out in 8- well reaction optical tubes/stripes (Applied Biosystem, Branchburg, New Jersey, USA). The TaqMan PCR conditions were used as described in TaqMan guidelines using 50 cycles of 95°C for 15s and 60°C for 1-min with 2-min preincubation at 50°C required for optimal AmpErase UNG activity and 10-min preincubation at 95°C required for activation of AmpliTaq Gold DNA polymerase. Each sample was analysed at least in 3 replicate setting. A patient's specimen was considered positive if amplification signal occurred on threshold cycle <40.

Results

In this prospective study, we analysed plasma samples of 48 pregnant women within 12th and 36th week of pregnancy and correlated the results with the serological analysis of cord blood.

Non-invasive prenatal fetal RHCE genotyping analysis of maternal plasma samples was in complete concordance with the analysis of cord blood in all pregnant women delivering 13 Rh E positive, 8 Rh E negative, 21 Rh c positive and 6 Rh c negative newborns. The results are summarised in Table 2.

Discussion

Non-invasive cell-free fetal RHD genotyping has become a routine part of prenatal care in several European countries.

We have routinely determined fetal sex in pregnancies at risk of X-linked disorders and fetal RHD genotyping in alloimmunized pregnancies at risk of haemolytic disease of newborn (HDN) by analysis of DNA circulating in maternal plasma using real-time PCR assay (19,21-23).

Fetal RHCE genotyping may allow the identification of foetuses at risk of haemolytic disease of the newborn. When positive foetuses are identified and anti-c and/or anti-E alloantibodies have already been present than alloantibody titre variations are important to be carefully monitored during the pregnancy in order to perform fetal blood sampling in time to determine the level of fetal haemolysis and start early treatment of sensitised pregnancies (ultraviolet phototherapy, exchange or intrauterine transfusion if required). Invasive procedures are used as a last option in monitoring HDN, because they may cause further leakage of fetal RBCs into the maternal circulation. On the other hand the detection of negative foetuses in the current pregnancy will exclude the need to perform invasive procedures despite the presence of alloantibodies that may be present in maternal circulation from previous pregnancies for a variety of reasons (1,2).

In our previous study, we showed that QIAamp DSP Virus kit (Qiagen, Hilden, Germany) enhanced the recovery of extracellular fetal DNA from maternal plasma that is crucial especially for the detection of those paternally-inherited alleles that differ from maternal alleles only in one nucleotide. Therefore we recommended performing the detection of fetal Rhc allele and/or RhE allele of RHCE gene by analysis of extracellular DNA extracted from maternal plasma samples by using QIAamp DSP Virus kit entirely. Overall, in our retrospective setting the sensitivity of fetal Rhc and RhE allele detection was 98-99%. The primers and probes for the prediction of fetal Rh CcEe phenotype from maternal plasma were firstly described by Legler et al. (20).

This is the first prospective study performed on extracellular DNA extracted from maternal plasma samples by using QIAamp DSP Virus kit showing the possible future implementation of fetal RHCE genotyping into a clinical diagnostic algorithm for following anti-c and anti-E alloimmunized pregnancies at risk of HDN. We are aware that the small number of patients in our study does not yet allow drawing a final conclusion and that this preliminary study needs to be expanded with larger sample size to get appropriate statistical correlates. However, reliable non-invasive detection of negative foetuses in alloimmunized pregnancies may exclude the risk of haemolytic disease of newborn.

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