Original Article

موضوع أصيل

NONINVASIVE FETAL RHESUS D GENOTYPE DETERMINATION BY USING CIRCULATING CELL-FREE FETAL DNA (CCFFDNA) IN PERIPHERAL BLOOD OF PREGNANT WOMEN IN SYRIA

تحديد النمط الوراثي الجنيني RHD بطريقة غير راضة باستخدام DNA الحر الجنيني الجائل في الدم المحيطي (ccffDNA) عند النساء الحوامل في سوريا

Ahlam Hameed, MD; Tahani Ali, MD; Rami A.Jarjour, MD

د. أحلام محمود حميد، أ.د. تهاني علي، د. رامي جرجور

ملخص البحث

هدف البحث: تم تحقيق تطور كبير في مجالات التقنيات غير الراضة في التشخيص والعلاج في حالات الداء الانحلالي لدى الأجنة والولدان HDFN، وذلك عبر التنبؤ الدقيق بالنمط الوراثي RHD الجنيني باستخدام DNA الجنيني الحر الجائل في الدم المحيطي للأم، بدلاً من الإجراءات الراضة مثل بزل السلى أو أخذ عينات من الزغابات المشيمية، حيث تمثل هذه الطريقة خطوة هامة في التدبير السريري للنساء سلبيات الزمرة الدموية RHD. تهدف هذه الدراسة إلى تقييم التوافق بين تحديد النمط الوراثي الجنيني RHD بتقنية PCR التقليدية باستخدام ccffDNA من الدم المحيطي للأم، والنمط الظاهري RHD لدى حديثي الولادة.

طرق البحث: أجريت هذه الدراسة في مستشفى التوليد الجامعي في دمشق على 60 امرأة سلبية RhD بين الأسبوعين 7 و38 من الحمل (22.8±6.1 أسبوعاً)، في الفترة الممتدة من كانون الثاني حتى تشرين الأول من العام 2019، كان هنالك 60/14 سيدة حامل محسسات لـ RHD، كما تم استبعاد حالات الحمل التوأمي. تم استخلاص ccffDNA من الدم المحيطي للأمهات الحوامل باستعمال عتيدة QIAamp DSP virus، ومن ثم تم إجراء PCR في مخبر زرع الخلايا الجذعية الدموية H.C.S.T في مستشفى الأطفال بدمشق، لتحديد النمط الوراثي الجنيني RHD عن طريق تضخيم exon10، exon 7. حيث تمت مقارنة النتائج مع النمط الظاهري للزمرة الدموية RhD لحديثي الولادة، الذي حدد باستخدام دم الحبل السري في مشفى التوليد الجامعي.

النتائج: وجدنا أن النمط الوراثي RHD في 4 من 55 من الأجنة (بنسبة 7.3%) كان سلبي RHD، بينما في 51 من 55 حالة (بنسبة 92.7%) كانت الأجنة إيجابية RHD. أظهرت هذه الدراسة توافقاً تاماً بين النمط الوراثي الجنيني لـ RHD باستخدام ccffDNA في كل المراحل الحملية، والنمط الظاهري للزمرة الدموية RhD بعد الولادة على عينة من دم الحبل السري للوليد، حيث بلغت حساسية ونوعية الاختبار 100%، القيمة التنبؤية الإيجابية والسلبية كانت تساوي 100%.

الاستنتاجات: إن إجراء التنميط الوراثي الجنيني RHD بطريقة غير راضة باستخدام ccffDNA يشكل بديلاً مثالياً لطرق التشخيص قبل الولادة الغازية كبزل السلى والزغابات المشيمية، حيث سيكون خطوة مهمة في التدبير السريري للنساء سلبيات الزمرة الدموية RhD.

ABSTRACT

Objective: *Accurate prediction of fetal RHD genotype using circulating cell-free fetal DNA from mother's blood is a great noninvasive progress in the diagnosis and treatment of Hemolytic Disease of*

*Ahlam Hameed, MD, Teaching Assistant, Department of Laboratory Medicine, Faculty of Medicine, Damascus University, Syria. Email:ahlamhameed@gmail.com
*Tahani Ali, MD, Professor of Hematoimmunology and Blood Transfusion, Department of Laboratory Medicine, Faculty of Medicine, Damascus University, Syria.
*Rami A.Jarjour, MD, Principal Researcher, Clinical Genetics Unit, Molecular Biology And Biotechnology Department, Atomic Energy Commission of Syria.

Fetus and Newborn (HDFN), rather than invasive procedures such as amniocentesis, or chorionic villus sampling. This study aims to assess the concordance between RHD genotyping by conventional PCR using ccffDNA from mother's peripheral blood, and the newborn RhD phenotype.

Methods: *A prospective cohort study was carried out on 60 RhD-negative pregnant women ranging from 7-38 weeks of pregnancy (22.8±6.1 weeks), who attended Maternity university hospital in Damascus, Syria during the period from January 2019 to October 2019. There were 14 out of 60 (23.3%) RhD alloimmunized women. Twin pregnancies were excluded. ccffDNA was extracted from peripheral blood of pregnant mothers by QIAamp DSP virus kit, and then PCR was done at Hematopoietic Stem Cell Transplant LAB (H.C.S.T) at Pediatric Hospital, Damascus, to determine fetal RHD genotype by amplification of exon 7, exon 10. Results were compared with newborn RHD phenotype done on neonate cord blood.*

Results: *We correctly diagnosed 4/55 cases (7.3%) as RHD negative fetuses, and 51/55 cases (92.7%) as RHD positive fetuses, and they were in different pregnancy trimesters. This study showed high concordance between the results of fetal RHD genotyping using ccffDNA in all pregnancy trimesters, and newborns RhD phenotype. Sensitivity and specificity were both 100%, negative predictive value (NPV) and positive predictive value (PPV) were 100%.*

Conclusions: *A non-invasive prenatal diagnosis of RHD is an appropriate alternative to an invasive prenatal IPD diagnosis, which will be a significant step in the clinical management of alloimmunized RHD women.*

INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is caused by the destruction of fetal red blood cells (RBCs) carrying paternal antigens by maternal antibodies, and leads to mild fetal anemia, hydrops fetalis, ultimately stillbirth and neonatal death, in some cases.[1]

Rhesus D incompatibility between a pregnant woman and her fetus, which occurs in up to 9-10% of pregnancies, based on the race, is the major cause of maternal alloimmunization, as maternal anti-D can cross the placenta to fetal circulation and cause hemolysis of fetal red cells.[2] Without any kind of intervention, about 50% of Rh-positive fetuses will be slightly influenced and may not need a treatment, 25% of cases will develop jaundice and kernicterus that leads to severe neurosensory defects if not treated, but the remaining fetuses 20-25% will become hydropic in utero, and will die in the uterus or during the neonatal period. So without treatment of RhD alloimmunization, the perinatal mortality rate approximated 50%.[3,4]

Since 1960s, introduction of anti-D immunoglobulin (Ig) ASA routine postnatal prophylaxis in the first 72 hours after delivery, has led to a decrease in the incidence of HDFN from 16% to 1-2% of negative pregnant women, and a decline in HDN-associated mortality.[5]

But combination of Routine Antenatal Anti-D Prophylaxis (RAAPD) around 28 weeks, in addition to routine postnatal anti-D prophylaxis could reduce the number of RHD-immunization, compared to only postnatal dose, however; HDFN due to anti-D continues to occur in 0.5% of RHD negative pregnancies, in industrialized countries.[1] According to American Congress of Obstetricians and Gynecologists (ACOG), and Royal College of Obstetricians and Gynecologists guidelines (RCOG), many European countries introduced RAADP based on the result of fetal RHD typing, and only women carrying RhD positive fetus receive prophylaxis, because about 50% of RhD-negative women will carry RhD-negative fetuses (when father is heterozygous for the D-antigen) and be unaffected, therefore no prenatal anti-D prophylaxis will be needed, so-called targeted prophylaxis.[6,7] And meanwhile, anti-D (Ig) is derived from large number of plasma donations from individuals exposed to human red cells, so doubts about its viral safety, and its high cost, makes administration in strictly controlled doses an urgent necessity, and every single effort should be made to develop practices that reduce the number of doses.

In Syria, despite application of postnatal anti-D prophylaxis, the most severe cases of HDFN arecaused by anti-D, and RHD-HDFN still one of the most

prevalent diseases of pregnancy, as it is associated with significant neonatal morbidity and mortality. In this study about 23.3% were found D-alloimmunized, and RHD alloimmunization remains the most common cause of fetal anemia, as about 10% of newborn needed blood transfusion due to RHD incompatibility at the Pediatrics' Hospital in 2005.[26]

Fetus RHD genotyping was determined for many years by fetal blood sampling through amniocentesis ,or chorionic villous biopsy, or even fetal venipuncture. But discovery of circulating cell-free fetal DNA) ccffDNA (in 1997,[8] made it possible to give better support to RHD-immunized women, as the fetal RHD genotype can be determined from maternal blood noninvasively, instead of invasive procedures that may worsen the alloimmunization status, or even have risk to fetal loss estimated at 0.5-1%.[9]

This study aimed to assess the concordance between RHD genotyping by conventional PCR using ccffDNA from the mother's peripheral blood, with the newborn RhD phenotype.

METHODS

This prospective cohort study was carried out at University Maternity Hospital in Damascus, while laboratory work was done at Hematopoietic Cell Transplantation (H.S.C.T) Lab, at University Pediatric Hospital, Damascus.

Fetal RHD genotyping was performed on 60 RhD-negative women, who attended the prenatal clinic in the hospital between January and October 2019, after receiving informed consent from pregnant women. The gestational age ranged from 7 to 38 (22.8±6.1) weeks of pregnancy, based on the last menstrual period (LMP). Five women (8.30%) were in the first trimester, 40 (66.7%) were in the second trimester and 15 (25%) in the third trimester. Twin pregnancies were excluded. They were phenotyped as RHD negative using the standard monoclonal anti-Dreagent (IgM+IgG) (Tulip Diagnostics Ltd, India), and indirect anti-globulin test (IAT) was done using gel cards containing polyclonal anti (IgG+C3d) linked to gel within wells (Matrix co, India), according to IAT they were 46/607%.76 () RhD non-immunized, while 14/60 (23.3%) were RhD-alloimmunized.

Neonate's RhD phenotypes were determined using serological techniques on the cord blood sample after birth.

Sample preparation and ccffDNA extraction from plasma: Five ml of maternal peripheral blood was collected using EDTA anticoagulant-coated vacutainer tubes as recommended, immediately aliquot, and processed within six hours. After centrifuging at 1,600 g for 10 min, plasma was carefully removed and centrifuged at high speed 16,000 for 3 min. The supernatant was collected and stored at −20°C until further processing.[11]

DNA extraction: ccffDNA was extracted manually from 500 µl of maternal plasma using the QIAamp DSP Virus Kit (Qiagen, Hilden, Germany) as described.[12,13] DNA was extracted from the blood sample of genomic human positive RHD, and negative RHD non pregnant women, using QIAamp DNA blood mini kit (QIAGEN, Germany) according to the instructions from blood or body fluids.

Primers A1 and A2 from (Inno Train-Kornberg, Germany) amplify a fragment of 135 bp, at exon 7 of both RHD, RHCcEe genes, so we can use it as a internal control.

And primers A3 and A4 were used to amplify gene RHD by amplifying) a fragment of 186 base-pair (bp) at exon 10, which is specific for the RHD gene. Primer A4 is deduced from the sequence of the 3 noncoding region of the RHD gene ′3 UTR,[2,3-8] Table 1.

Polymerase Chain Reaction: PCR was performed in a total volum of 25 ul, in the thermal recycling system (Applied Biosystem, Foster City, CA, USA) with 8-10 ul ccff DNA, 12.5 ul of (Go Taq green Master Mix 2X Promega, USA) (PCR master mix-ready to use, contain Taq DNA polymerase, dNTPs, MgCl2, in addition to the buffer), and the amount of 2 p.mole of each of of the primers. We amplified RHD and RHCE genes independently.

PCR was conducted in accordance with the following conditions; the first cycle of the PCR was performed at 95°C for 15 minutes, then 50 cycles for one minute at 92°C, and then annealing at 49° for one minute, then primers extension at 72°C for one minute, final step of 9 minutes at 72°C to complete the extension. And specimens were preserved after that at 4°C.[2]

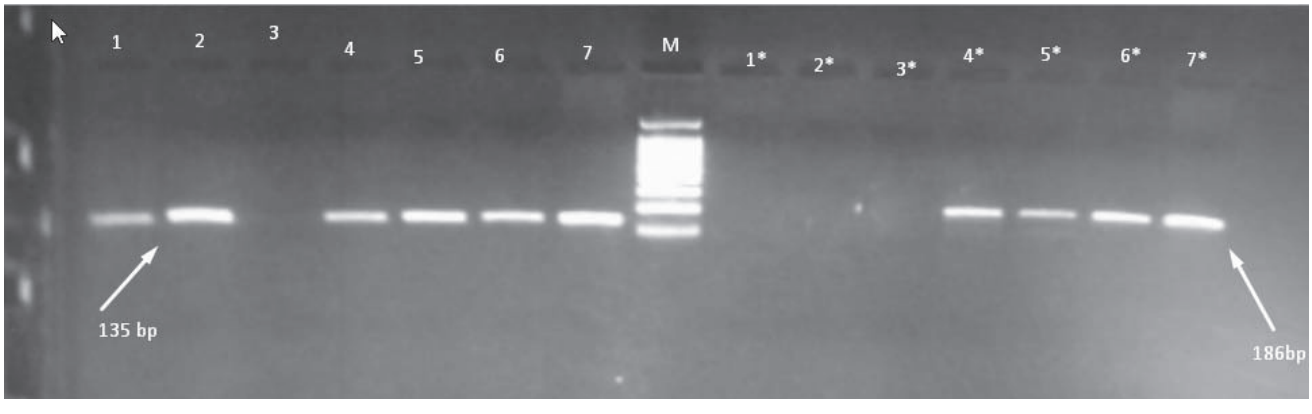
Each experiment included a positive control, and also a negative control, both containing human DNA isolated from a positive RhD, and negative Rhd non-pregnant women, in addition to a negative contol containing distilled water instead of DNA. Sample were electrophresed on 2% agarose gel in TBE 1X solution, 10 microliter of DNA were loaded in the designated wells within the gel and a ladder (bp100) was added, photographes were taken using the camera (Hero Lab Trans illuminator UVT-28M, Germany).

Observed two DNA bands at the specified length of 135 and 186 bp, indicates an RHD positive fetus, while one band of 186 bp, indicates RHD negative fetus, Figure 1.

Statistical study: After data collection was coded and entered into the computer using Excel 2016, IBM-SPSS 25.0 (IBM). Chi-square test was used in the study with confidence intervals at 95%. Diagnostics measures were used as sensitivity, sepcificity and the predictive values/accuracy.

RESULTS

Regarding the fetal RHD genotyping by conventional PCR, 54/60 cases (90%) showed amplification of the RHD exon 10 and exon 7, and considered RHD positive fetuses, and 6/60 (10%) showed amplification of only RHD exon 10, and they were considered negative RHD fetuses, unfortunately there were (5 women) unreachable so the genotyping results were not compared with outcome of the delivery. Out of 55 samples, 51/55 (92.7%) fetuses were RHD positive genotype, and only 4/55 (7.3%) fetuses were negative. In all 55 (100%) cases we observed a full concordance between the results of fetal RHD genotyping and newborns RhD phenotype, Table 2. The sensitivity



M: 100 base pairs ladder; Lanes 1 and 2 indicate Rhesus negative fetuses;
Lanes 4, 6, 5 indicate Rhesus D positive fetuses; Lanes 3: water blank. Lane 7 is PCR positive control.

Figure 1. Polymerase chain reaction (PCR) detection of fetal Rhesus D.

Primers	Sequence	Position	
Forward primer A1	(5′-TGT GTT GTA ACC GA GT -′3)	(1069-1084)	Exon 7
Reverse primer A2	(5′-ACA TGC CAT TGC CG -′3)	(1203-1190)	
Forward primer A3	(<5-TAA GCA AAAGCATCCAA- 3>)	(1252-1268)	Exon 10
Reverse primer A4	(<5-ATGGTGAGATTCTCCT-3>)	(1437-1422)	

Table 1. Sequence of used primers.

Gestational age (weeks)	Anti-D detection		Total	Fetal genotype		Fetal phenotype	
	Negative	Positive		RHD+	RHD-	RhD+	RhD-
I (7-13)	3	2	5	4	1	4	1
II (14-26)	26	10	36	33	3	33	3
III (27-38)	13	1	14	14	0	14	0
Total	42	13	55	51	4	51	4
	76.40%	23.60%	100.00%	92.70%	7.30%	92.70%	7.30%

Table 2. Results of RHD genotyping and phenotyping of the newborn.

was 100%, specificity 100%, positive predictive value PPV=100% and the negative predictive value of the test was NPV=100%, accuracy was 100% at confidence interval 95%.

DISCUSSION

This study is the first of its kind in Syria, in the field of fetal blood group genotyping using circulating cell-free fetal DNA (ccffDNA) from RHD negative mother's peripheral blood.

Previous studies have shown that ccffDNA concentration increases with gestational age, and its amount increases during pregnancy from 3.4% to 6.2% of the mean total cell-free DNA concentration in maternal plasma, so can be isolated at late pregnancy more easily by suitable extraction method.[11,20]

Our study agreed with many researches that have studied prenatal determination of RHD by analysis of ccffDNA in the mother's plasma using conventional PCR, with accuracy ranging from 80.8-100% with mean 97.9%,21 as we got 100% accuracy, and both PPV, NPV were 100%. The false-positive incidence for RHD genotyping has limited importance when compared to false-negatives, because the occurrence of a false-negative result for the RHD gene could lead to the delay of prophylaxis with the Anti-D Ig, which would lead to possible alloimmunization and HDFN in subsequent pregnancies.

It was possible in our study to extract ccffDNA from maternal peripheral blood, from pregnant women in all three trimersters of pregnancy, and like most of the studies that examined plasma to isolate ccffDNA, majority of women were in the second trimester between (14-25) gestational weeks. While previous studies showed inconclusive results and less sensitivity and specificty especially in the early pregnancy due to the low levels of ccffDNA, in our study we were able to determine fetal D genotype using conventional PCR successfully in the seventh week of pregnancy by extracting ccffDNA, that may beacause of choosing aproppiate kit for extraction ccffDNA, with special modification to the instructions, and full carefullness of avoiding contamination during all work processes.

Due to high complexity of RH system, Chan et al, declared that it is important to test more than one region of the gene RHD, and established that the use of one specific primer set for RHD genotyping may cause false-negative and false-positive results, so several RHD exons should be examined with a careful choice of primers to identify variant RHD alleles and to avoid false-positive results in RHD genotyping, so we examined exons 10 and 7 to have optimal combination for analysis of the RHD cocerning nucleotide differences in the ′3UTR of RHD and RHCE.[22,23]

Early fetal RHD genotyping is recommended in sensitised pregnancies (23.3%), to plan further diagnostic procedure and assist in the management of D immunized women, as accurate management may be made through the serial assessment of the levels of anti-D, and the fetal monitoring using ultrasound and MCA-PSV Doppler after 18th week, and then optimal intervention must be done, that means a big chance for alloimmunized pregnant women to have the proper management, and also for non-immunized

RHD negative pregnant women -if the fetus was determined RHD positive- to administer RAAPD at 28 week (or earlier if invasive event should be done).[24,25]

CONCLUSIONS

Noninvasive prenatal diagnosis (NIPD) of fetal Rhesus (Rh) D status is possible by analyzing ccffDNA from plasma isolated from maternal blood, with a high level of accuracy, instead of using procedures with a high rate of risk to a fetus, as amniocentesis or CVS.

This method should be applied in Syria to be useful in clinical practice as aguide for antenatal anti–D targeted prophylaxis to reduce the rate of alloimmunization incidence, but more cost-benefit analysis would be required for our country to determine the utility of prophylaxis.

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