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Non-invasive prenatal diagnosis of fetal RhD by using free fetal DNA

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Summary

Objective: Anti-D immunoglobulin is applied to all pregnant women having RhD incompatibility to prevent hemolytic disease of the newborn. The aim of this study is to determine fetal RhD status in the Rh incompatible pregnancies with an non-invasive technique; free fetal DNA isolation from maternal circulation. In the case of Rh incompatibility especially with a history of previous fetal anemia, it can be beneficial to know Rh status antenatally in terms of monitoring fetuses with Rh positive [RhD(+)] status consciously. *Materials and Methods:* Total free DNA was isolated in 50 Rh negative [RhD(−)] pregnant women, who had RhD alloimmunisation with their husbands. The gene in isolated DNA was investigated with TagMan prob and real time PCR by using primers belonging to exon 7 of the RhD gene. *Results:* The authors analyzed 50 RhD(−) women by using quantitative real time PCR technique. Five of them were RhD(−) and the rest of them were found to be RhD(+). After birth one of the infants who were analyzed as RhD(+) were found to be RhD(−). *Conclusion:* The detection of fetal RhD status by using a non-invasive method from maternal circulation was found to be possible. Assessing fetal RhD status non-invasively by using free fetal DNA in maternal blood will be cost-efficient, avoiding unnecessary indirect Coombs test and unnecessary Rhogam applications that is used in RH incompatible pregnancies. This study will throw a fresh light on prenatal diagnosis.

Key words: Prenatal diagnosis; Fetal DNA; RhD gene.

Introduction

Hemolytic disease of the newborn which causes fetal anemia, neonatal icterus, and even death is caused by rhesus (Rh) group incompatibility between maternal and paternal blood groups [1].

Anti-D alloimmunisation has been reduced with the implementation of prophylactic anti D treatment in all pregnancies with Rh incompatibility [2] It is known that in a predominantly white population, however about 38% of this women would be carrying an RhD negative [RhD(−)] fetus and would receive treatment unnecessarily [3]

In 1997 a new era in prenatal diagnosis was introduced to science by Lo *et al.*, non-invasive fetal RhD genotyping from maternal plasma was considered a valuable tool in the identification of pregnancies at risk of hemolytic disease of the fetus and newborn [4].

The Rh blood system is a very polymorphic system, RhD and RhCE are located in the region of p36.12-p34.3 on chromosome 1, and they are 97% homologous to each other. Each of these genes consist of ten exons and they contain 69 kb of DNA. The regions of exon 7 and exon 10 within the RhD gene are the areas of focus [5]. The present study is based on detection of exon 7 region to determine the presence of RhD gene.

Materials and Methods

Peripheral five ml blood sample was taken from 51 Rh(-) pregnant women at 5-40 weeks of gestation, having Rh incompatibility with their husbands who admitted for pregnancy follow up at the Istanbul Bilim University, Medical Faculty Obstetrics Department. Blood was centrifuged 15' at 4,100 g, and upper part, plasma was stored at -80°C until the DNA isolation day. The total free DNA isolation was performed according to free DNA isolation procedure of High Pure PCR template preparation kit. The samples that were saved at -80°C were first centrifuged at 13,000 rpm for 10' and upper fluids were taken, 200 µl "binding buffer" and 40 µl "proteinase K" were placed on each sample, and they were placed at 70°C in a water bath for 10'. Isopropanol in the amount of 100 µl was also added to them and the mixtures were taken to strainer eppendorf and centrifuged until all of the liquid passed to the bottom; 500 µl " inhibitor buffer" was added to eppendorfs and centrifuged. Two centrifuged were then also formed by adding 500 µl "wash buffer" each time. Next 50 µl " elution buffer" was added to strainer eppendorfs, the sub-tube which had free DNA after centrifuge was taken to -20 °C until the day of Real time PCR. Real time PCR was then performed under conditions as shown in Tables 1 and 2. Specific primers (5'-CTC CAT CAT GGG CTA CAA-3', 5'-CCG GCT CCG ACG GTA TC-3') for region exon 7 which belongs to RhD and TaqMan prob (5'-FAM AGC AGC ACA ATG TAG ATG ATC TCT CCA TAMRA-3'); FAM [6 carboxyfluorescein] and TAMRA [6 carboxytetramethylrhodamine] were the fluorescent reporter dyes and quencher dye was used. The tubes which contained RhD genes were used for

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Table 1. — *Components of amplification mixture.*

Component	Volume(μ)	Final amount
TagMan Probes	0.6	25 nM
Forward Primer	3	300 nM
Reverse Primer	3	300 nM
dH2O	13.4	-
Template DNA	5	20 ng
PCR Reaction Buffer	25	-

Table 2. — *Cycling parameters.*

	Segment 1	Segment 2
Temperature (°C)	95	95 60
Time	10 min	15 min 1 min
Cycles	1	50

positive control, and the tubes that were known not to contain RhD gene were used for negative control. The tubes containing the NTC (PCR mixture without DNA) reaction mixture was used for the determination of contamination.

Results

One patient was excluded because of first trimester abortion. The total DNA of 50 women who were known to have Rh incompatibility was analyzed with positive control, negative control by TagMan prob method. Five samples were found to be RhD(−) and the rest were Rh positive [RhD(+)]. After birth, RhD status of the fetus were corrected; all of the patients who were found to be RhD(−), were RhD(−) and 44 patients who were found to be RhD(+) were RhD(+). Only one patient who was found to be RhD(+) was RhD(−) in fact. Sensitivity was 100%, specificity was 83.3%, positive predictive value was 97.7%, and negative predictive value was 11.3%.

Discussion

Hemolytic disease of the fetus and newborn (HDFN) has been a prevalent pathology of pregnancy and a major obstetric problem, with an important impact on fetal and neonatal morbidity and mortality. The introduction of postpartum and antenatal anti-D immunoglobulin prophylaxis for phenotypic RhD(−) pregnant women, has dramatically improved the risk of the affected fetuses and the incidence of D sensitisation has decreased [6] The current strategy for monitoring RhD(−) pregnant women at high risk for HDFN relies on serial assessment of maternal antibody levels, paternal screening, administration of anti-D prophylaxis for RhD(−) pregnant women, and when necessary, fetal monitoring using ultrasound and Doppler and intrauterine fetal blood sampling [7]. It is known that a kind of human blood product, anti-D immunoglobulin carries risk for blood-borne infections and the supplies are limited worldwide. Availability of non-invasive diagnostic assay of fetal RhD status makes it possible to restrict use of antenatal prophylactic anti-D immunoglobulin. Identification of fetal Rh status will prevent approximately 40% of women having RhD(−) fetus from being vaccinated [8]. The population which were included in the present study had 12% of RhD(−) infants. The difference in ratios of RhD(−) infants between the present study population and general population may be attributed to narrow number of individuals.

Fetal RhD genotype can be determined with a high level of accuracy by analysis of fetal DNA circulating in maternal plasma and serum [9]. If the fetus is known to be RhD(+), especially with a history of anemic or hydropic fetus, close monitoring may be rendered. Further investigations may result as a change in algorithms in management of pregnancies with RhD incompatibility like Rhogam application doses and timing of vaccine.

Several studies examined the prenatal accuracy of fetal RhD genotyping from analysis of circulating cell-free fetal DNA (ccff DNA) in maternal plasma which ranged from 32% to 100% [10]. Non-invasive determination of fetal RhD genotype usually relies on DNA amplification by PCR, and detection of chromosome 1 specific sequences in maternal plasma [4]. The present authors used exon 7 on chromosome 1 to determine Rh status of the infant.

One previous study with a population of 2,000 patients revealed 0.8% rate for the infants being RhD(−) but found to be RhD(+) [11]. The present study had one patient (2%) who was actually RhD(−) but found to be RhD(+). Incidence seems to be high in the present study, and the authors attribute this result to small amount of their study population.

Müller *et al.* highlighted the importance of transport time on sample quality. At the room temperature, after six days even the concentration of fetal DNA remains same, total DNA amount increases. Thus, maternal DNA contamination is higher than average [2]. Rouillac – Le Sciellour *et al.* do not recommend to study with samples older than 48 hours [12]. The present authors did centrifugation and stabilization in hours and they do not relate their false positive result with transport time.

In the Caucasian population deletion of homozygous RhD gene is the main cause of negative phenotype in contrast with black Africans who do not have homozygous deletion but carry one or two variant genes, the RhD pseudogene or RHD-CE-Ds hybrid gene. [13,14] Rouillac-Le *et al.* found 31 patients to have pseudogene or hybrid gene in 893 patients, Chinen *et al.* found two in 102 patients, Gunel *et al.* found two in 40 patients. [12, 15, 16] Multiplex PCR for more than one region should be performed to detect this hybrid or pseudogenes [16]. The present authors performed only exon 7 in this study, not exon 10 therefore one patient with a false positive result may have pseudogene.

It has been previously shown that fetal DNA passage in maternal circulation increases in pathological pregnancies associated with placental abnormalities such as preeclampsia, preterm labor, and pregnancies with karyotypically abnormal fetuses [4]. None of the patients in the present study had abnormality during pregnancy follow up. Only one patient had early first trimester abortion and she was excluded.

Conclusion

Fetal RhD status could be determined non-invasively from maternal circulation. This will obstruct anti immunoglobulin use in pregnant women carrying RhD(−) fetus. Determining RhD(+) of the fetus gives opportunity for timely close monitoring due to hydrops. Fetal DNA isolation from maternal blood will soon provide diagnosis of aneuploidies and single gene disorders non-invasively.

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