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Original Article

The diagnostic accuracy of non-invasive fetal RhD genotyping by using cell-free fetal DNA in maternal plasma

Maternal plazmadaki hücre dışı serbest fetal DNA kullanılarak girişimsel olmayan fetal RhD genotiplemesinin tanısal doğruluğu

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ABSTRACT

Introduction: The non-invasive prenatal diagnosis of the fetus RhD genotype in RhD incompatibility has a crucial role in the prevention of increased anti-D immunoglobulin therapy for haemolytic diseases in pregnant women carrying RhD negative fetus. It was aimed to detect fetal RhD genotyping by using maternal circulating cell-free DNA in the current study.

Methods: Maternal blood samples were collected in different trimester of pregnancies (12-40 weeks) in 12 D-negative mothers. Cell-free fetal DNA was extracted from 2 ml of maternal plasma by an conventional DNA isolation technique (Qiagen, Hilden, Germany) and real-time PCR was performed for genotyping target RhD exons 7 and 10 and GLO genes. Postnatal serological evaluations were performed and the results were confirmed.

Results: 6 cases (50 %) were determined D positive and 6 cases (50 %) were determined D negative. All results were also confirmed after birth serologically.

Conclusions: In conclusion, the current results showed us the non-invasive target RhD genotyping from cell free fetal DNA from maternal plasma samples have a diagnostic accuracy in RhD incompatibility pregnancies.

Keywords: Cell free fetal DNA, maternal plasma, prenatal screening, real-time PCR

ÖZ

Giriş: Rh uyuşmazlığında fetusun RhD genotipinin girişimsel olmayan prenatal tanısı, RhD negatif fetus taşıyan gebelerin hemolitik hastalıklar için artmış anti-D immünoglobulin tedavisinin önlenmesinde önemli bir role sahiptir. Bu çalışmada maternal plazmadaki hücre dışı DNA kullanılarak fetal RhD genotiplemesinin saptanmasının tanısal doğruluğunu tespit etmek amaçlandı.

Yöntem: Rh uyuşmazlığı bulunan farklı gebelik haftalarında (12-40 hafta), D negatif 12 gebeden periferik kan örnekleri toplandı. 2 ml maternal plazma kullanılarak konvansiyonel bir DNA izolasyon tekniği (Qiagen, Hilden, Almanya) ile hücre dışı DNA izole edildi. Real Time PCR tekniği kullanılarak fetal RhD geni ekzon 7, ekzon 10 ve GLO geninin genotiplemesi yapıldı. Postnatal serolojik değerlendirmeler yapılmış ve sonuçlar doğrulanmıştır.

Bulgular: 6 olgu (% 50) D pozitif, 6 olgu (% 50) D negatif olarak belirlendi. Tüm sonuçlar ayrıca serolojik olarak doğumdan sonra doğrulandı.

Sonuç: Sonuç olarak RhD uyuşmazlığı görülen gebelerde girişimsel olmayan bir yöntem olarak maternal plazmadan elde edilen hücre dışı fetal DNA kullanılarak yapılan RhD genotiplemesinin tanısal doğruluğa sahip olduğu gösterildi.

Anahtar kelimeler: Hücre dışı fetal DNA, maternal plazma, prenatal tarama, real time PCR

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Introduction

Rh(Rhesus) D incompatibility develops during the pregnancy in cases of mother D negative, fetus D positive. This condition causes alloimmunization and risk in future pregnancies [1]. Which results in severe hemolytic disease such as fetal anemia, jaundice, hydrops fetalis and fetal deaths [2, 3, 4]. For this reason, antenatal and postnatal follow-up is important in RhD incompatibility. All D negative mothers who have RhD incompatibility take RhD immunoglobulin prophylaxis for eliminate the risk of alloimmunization. As a result of this application, D negative fetuses are exposed to unnecessary vaccine product [5]. According to the researchers found to D negative pregnant women who took prophylaxis have 40% off D negative fetuses [6].

Currently, invasive and noninvasive methods are used to determine fetal RhD profile. Invasive methods and carry miscarriage risk of 0.5-1% [7]. Indirect coombs test (ICT) and direct coombs test (DCT) are used for indirect detection of the fetal RhD profile, but their sensitivity is not as accurate as invasive methods; for this reason other noninvasive methods are needed. The presence of cell free fetal DNA in plasma and serum of pregnant women revealed by Lo et al in 1997 [8]. Cell free fetal DNA represents 5-10% concentration of all maternal plasma DNA and it can be detected after gestational day 18 by real-time polymerase chain reaction (RT-PCR) [9, 10]. Cell free fetal DNA concentration in maternal blood increases as pregnancy week increases [11]. Maternal DNA and cell free DNA consist of short fragments and cell free fetal DNA fragments shorter than maternal cell free DNA fragments [12, 13].

Isolation of cell free DNA from maternal plasma and amplified with PCR technique used a noninvasive method for prenatal diagnosis and determined fetal RhD status. With this method, prenatal diagnosis is possible without the risks of invasive techniques. Fetal RhD genotyping from cell free fetal DNA routinely used in many countries including the United States and European countries [14-21,30]. RhD pseudogene (RhDΨ) is not common in Caucasian population because most of the D negative Caucasians completely lack the RhD gene [31]. For this reason analyzing exon 7 and exon 10 is enough for determining the fetal RhD genotype in Caucasian population.

In our study, cell free DNA were isolated from maternal plasma and RhD genotype of the fetus was determined by RT-PCR method, used specific primers and probes to the exon 7 and exon 10 regions of the RhD gene. The obtained data were compared with serologic postnatal results and corrected.

Methods

The plasma samples were collected from 12 D negative women with RhD incompatibility who applied to Canakkale Onsekiz Mart University (COMU), faculty of medicine, outpatient clinic. Six blood samples were taken at 12-13 weeks, 4 samples at 17-23 weeks and 2 samples at ≥28 weeks of gestation. All participants were healthy without any serious pregnancy complications and voluntarily joined the study. Their partners were orally reported serologically D positive. Ten ml peripheral blood sample with EDTA were used for cell free DNA isolation for the current study. Blood samples were immediately taken to the laboratory and centrifuged for 10 min at 2500 rpm. The supernatant was transferred to a new tube and centrifuged at 3000 rpm for 10 min. The cell free DNA was isolated from plasma samples and by using specific primers and probes for determinated to fetal RhD profile by real-time PCR method and absolute quantification analysis.

Ethical procedure

Our project was initiated with the permission of Canakkale Onsekiz Mart University, Ethics Committee of Clinical Applications (Number EK-2013-163) and supported by Canakkale Onsekiz Mart University Scientific Research Projects Commission with the project number of TYL-2014/203.

DNA isolation from maternal plasma

We used two different techniques for the cell free DNA isolation. One was automatic the other one was a manual technique. Both techniques using a 400 µl plasma sample, the amount of elution buffer was determined to be 50 µl. Manuel DNA isolations were done QiAMP DNA Blood Mini Kit according to the protocol from the manufacturer's blood and body fluids and was used by spin column method (Qiagen, Hilden, Germany). Automatic DNA isolation were done in the direction of total nucleic acid plasma protocol with MagNA pure nucleic acid isolation kit (Roche, Germany).

Real - Time PCR

RT-PCR was performed on Roche LightCycler 2.0 (Roche, Germany). DyNAmo Flash Probe qPCR Kit (Thermo Scientific, Lithuania) was used to determine the fetal *RhD* status and *GLO* gene. Exon 7 and exon 10 were analyzed for fetal *RhD* genotyping. *GLO* gene confirmed the presence of total (fetal and maternal) DNA in each sample. *GLO* gene and *RhD* gene primers and probes selected from the literature [2, 22]. Primer and probe sequences were shown in Table 1 (Table 1).

Table 1. The primer and probe sequences that used to amplify the target *RhD* and *GLO* genes in the current study

Gene	Primer	Prob
RhD Exon 7	F-5'-CTC CAT CAT GGG CTA CAA-3' R-5'-CCG GCT CCG ACG GTA TC-3'	5'-(FAM) AGC AGC ACA ATG TAG ATG ATCTCT CCA (TAMRA)-3'
RhD Exon 10	F-5'-CCT CTC ACT GTT GCC TGC ATT-3' R-5'-AGT GCC TGC GCG AAC ATT-3'	5'-(FAM) TAC GTG AGA AAC GCT CAT GACAGC AAA GTC T (TAMRA)-3'
GLO	F-5'-GTG CAC CTG ACT CCT GAG GAG-3' R-5'-CCT TGA TAC CAA CCT GCC CAG- 3'	5'-(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)-3'

Total reaction volume was 20 µl. Primers and probes final concentrations were 100 pmol/µl. PCR reactions were shown in Table 2 (Table 2).

Table 2. The optimized PCR conditions for the target RhD exon 7, exon 10 and GLO genes amplification

PCR Amplification Parameters	GENES		
	***RhD* Exon 7**	***RhD* Exon 10**	***GLO***
Forward Primer	0.4 µl	0.5 µl	0.4 µl
Reverse Primer	0.4 µl	0.5 µl	0.4 µl
Prob	0.4 µl	0.2 µl	0.2 µl
Master Mix	12.5 µl	12.5 µl	12.5 µl
ROX	0.2 µl	0.2 µl	0.2 µl
Water	1.1 µl	1.1 µl	1.3 µl
DNA	5 µl	5 µl	5 µl

Cycling conditions consisted of UNG step, denaturation step and cycling step. UNG step consisted of one cycle 2 minutes at 50°C followed by denaturation step consisted of a one cycle 10 minutes at 95°C followed by cycling step consisted of fifty cycles 15 seconds at 95°C and 1 minute at 60°C.

Each sample was run three times with RT-PCR method. Positive and negative controls were performed at each reaction. Samples without amplification on *RhD* exon 7 and exon 10 were evaluated as *RhD* negative (Figure A). Amplification occurred in two of the three runs on *RhD* exon 7 or exon 10 were evaluated the samples *RhD* positive (Figure B). The cycle threshold (Ct) values of 26-40 were considered *RhD* positive.

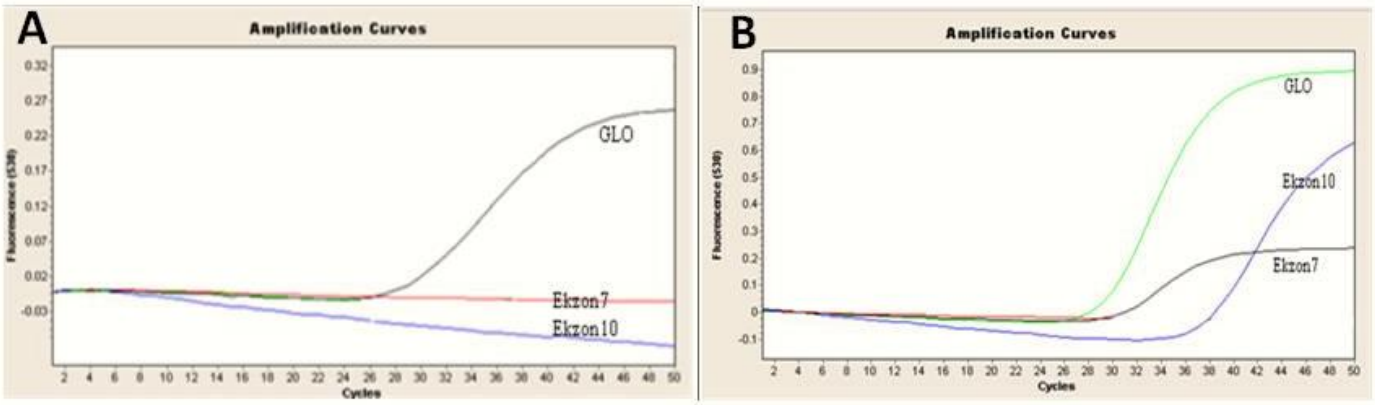

Figure A-B. Shows the negative (A) and positive (B) fetal RhD genotypes in different two pregnant mothers after RT-PCR analyses from cell free fetal DNA. In the D negative fetus only *GLO* gene was amplified, in the D positive fetus exon 7 and 10 were amplified for *RhD* genes

RESULTS

Information about pregnancy week, age, weight, previous pregnancy history, blood transfusions (BT) or bone marrow transplantation (BMT) and anti-D prophylaxis parameters of pregnant women who participated in this study were shown in table 3 (Table 3).

Table 3. Some clinical characteristics such as; gestational week, age, weight, pregnencies number, BT or MT, and Anti-D prophylaxis parameters for the current study

Case No	Gestation Week	Age (Year)	Weight (kg)	Number of pregnancies	BT or BMT	Anti-D prophylaxis
1	12	29	50	G1P0A0	-	-
2	16	37	-	G6P2A3	-	-
3	12	36	64	G4P1A2	-	.
4	12	25	51	G1P0A0	-	-
5	13	27	60	G1P0A0	-	+
6	23	40	-	G6P2A3	-	+
7	20	20	-	G1P0A0	-	-
8	12	41	81	G2P1A0	-	-
9	35	26	87	G2P1A0	-	-
10	12	-	-	G2P1A0	-	-
11	20	-	-	-	-	-
12	40	40	91.4	G2P0A1	-	+

The ages of the participated study were 20-41 (average 32.1) and their weight was 50-92 (average 69.2). The case number five, a concomitant ectopic pregnancy has occurred with normal pregnancy. After the ectopic pregnancy was terminated anti-D prophylaxis was performed. The case number six and twelve were taken anti-D prophylaxis because of indirect coombs test positive.
Maternal DNA samples were obtained by buffy coat. These DNA samples were used to confirm the maternal *RhD* profiles and optimization of the RT-PCR method. The case 1 maternal *RhD* profile was identified D positive. After the interviews with the patient 1, and after recurrent serology we determined the patient knows the blood group wrong. The case 1 joined the study individually. All maternal *RhD* profiles shown in table 4 (Table 4).

Table 4. The pre and postnatal comparison of RT-PCR results of target genes for each patient in the presented current study

Case No	Maternal RhD Profile		Fetal RhD Profile		Postnatal RhD Profile	All PCR Reactions
	RhD Gene Exon 7	RhD Gene Exon 10	RhD Gene Exon 7	RhD Gene Exon 10	Rh Profile	GLO Gene
1	+	+	+	+	+	+
2	-	-	+	+	+	+
3	-	-	-	-	-	+
4	-	-	-	-	-	+
5	-	-	+	+	+	+
6	-	-	-	-	-	+
7	-	-	+	+	+	+
8	-	-	-	-	-	+
9	-	-	-	+	+	+
10	-	-	-	-	-	+
11	-	-	-	-	-	+
12	-	-	+	+	+	+

The *GLO* gene amplification has shown all PCR reactions and all DNA samples as a positive control shows the optimising of the PCR conditions. This proved DNA presence and that PCR reactions were working. The cell free DNA samples obtained by maternal plasma. After RT-PCR technique was used to determine the fetal *RhD* profiles. Fetal *RhD* profiles shown in table 4 (Table 4). The case number 9 cell free fetal DNA samples amplification of the *RhD* gene was detected in exon 10 but amplification in exon 7 was not detected. After three times running with RT-PCR method the same result were obtained. We have evaluated number nine fetal *RhD* genotype D positive. As a result of the fetal *RhD* analysis, it was determined that 6 cases were *RhD* positive and 6 cases were *RhD* negative. The fetal *RhD* profiles that detected in the present results were indicated in table 4.

The case numbers two and six were done amniocentesis independent of Rh incompatibility because case number two had advanced maternal age and case number six had in 1/250 risk of Down syndrome in triple screening test results. After QF-PCR analysis we used residual DNA samples from amniocentesis to fetal *RhD* genotyping by RT-PCR. The results obtained were 100% compatible with fetal *RhD* genotyping with cell free fetal DNA. All postpartum *RhD* profiles were shown in Table 4 (Table 4). Our fetal *RhD* profile and postnatal *RhD* profile were 100% compatible.

Statistical analysis could not performed due to case number was not sufficient.

Discussion

RhD negative genotypes have been most commonly seen in Caucasian population and most of them completely lack the *RhD* gene. It is reported that 15% of the Caucasian population is *RhD* negative and 9% of these pregnancies are Rh incompatible [23,29,31]. This high incidence makes Rh incompatibility and related hemolytic diseases an important health problem. Appropriate follow-up of D negative pregnant women during pregnancy and accurate identification of fetal *RhD* genotype is of great importance. Discovery the cell free fetal DNA in maternal plasma gave us a non-invasive prenatal diagnostic methods identification of fetal *RhD* genotype and aneuploidies [8].

The QIAamp DNA Blood Mini Kit and QIAamp DSP virus kit for manual isolation, MagnaPure Compact Nucleic Acid Isolation Kit for automatic isolation are the leading isolation kits in the literature [24-27]. QIAamp DNA Blood Mini Kit (Qiagen, Germany), Roche manual isolation kit, Roche viral isolation kit (RTA, Turkey) were used for manual isolation methods and MagnaPure Compact Nucleic Acid Isolation Kit I was used for automatic isolation. The same amount of plasma and elution buffer was used in all isolation kits. We performed RT-PCR with the DNA samples obtained from different isolation kits and compared the results for any diference. As a result of the comparison, it was determined that the DNA samples obtained with QIAamp DNA Blood Mini Kit and the MagnaPure Compact Nucleic Acid Isolation Kit I were more reliable than the others. For this reason in our study cell free DNA was obtained by QIAamp DNA Blood Mini Kit and the MagnaPure Compact Nucleic Acid Isolation Kit I for each pregnant woman and RT-PCR was performed in three run for each cell free DNA sample. We compared between manual and automatic isolation technique and didn't determine any diference for results.

Postnatal serologic results were found 100% compatible with fetal DNA analysis from maternal blood. Even though our case count is small but confirming the results of all of the postpartum tests are indicative of the high sensitivity and specificity of the assay performed with this method. When fetal *RhD* genotyping from maternal plasma is diagnostically working, the mother genotype should be confirmed by DNA isolation from

the buffy coat. In case number nine buffy coat study showed that the mother was negative for both exon 7 and exon 10 but cell free DNA was positive for exon 10 and negative for exon 7.

In doubtful situations PCR products could be analyzed an alternative technique by capillary electrophoresis [28]. We used this method in our study for case number nine and control sample PCR products run on capillary electrophoresis (ABI 3100) for 1 hour and were analyzed in the Multiplex ligation-dependent probe amplification (MLPA) program. MLPA program was based on fragment analysis. The exon 7 PCR product peaked at 75 and the exon 10 PCR product peaked at 81. We didn't detect any peak on exon 7 for case number nine like RT-PCR results.

The current results were considered to case number nine *RhD* positive fetal genotype and postpartum serological analysis confirmed that the baby was Rh positive. This may be due to the deletion of the exon 7 in the baby and the father or it may be due to the fact that the sensitivity of the exon 7 to the RT-PCR reaction is lower than that of exon 10. The father and the baby were invited to give samples for genotyping but they were rejected. If analysis results are obtained only by exon 7 should be taken to the possibility of false negative. For this reason at least two exons should be evaluated for fetal *RhD* genotyping.

Limitations

Our study had some limitations, including small sample size and that cause could not performed statistical analysis. We recommend that the study in a larger sample size should be performed in further investigations.

Conclusion

In conclusion, the current results showed the importance and validity of the diagnostic accuracy of non-invasive fetal *RhD* genotyping by using cell free fetal DNA in circulating maternal plasma of D negative pregnant women at risk for haemolytic disease of the newborn. That most accurant test may be contribute the eliminated unnecessary anti-D therapies in D negative pregnancies. We recommend that the study in a larger sample size, should be performed in further investigations.

Conflict of interest: None.

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