Noninvasive fetal *RHD* genotyping using cell-free fetal DNA incorporating fetal *RASSF1A* marker in RhD-negative pregnant women in Korea

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Purpose: Conventional methods for the prenatal detection of fetal RhD status involve invasive procedures such as fetal blood sampling and amniocentesis. The identification of cell-free fetal DNA (cffDNA) in maternal plasma creates the possibility of determining fetal RhD status by analyzing maternal plasma DNA. However, some technical problems still exist, especially the lack of a positive control marker for the presence of fetal DNA. Therefore, we assessed the feasibility and accuracy of fetal *RHD* genotyping incorporating the *RASSF1A* epigenetic fetal DNA marker from cffDNA in the maternal plasma of RhD-negative pregnant women in Korea.

Materials and Methods: We analyzed maternal plasma from 41 pregnant women identified as RhD-negative by serological testing. Multiplex real-time PCR was performed by amplifying *RHD* exons 5 and 7 and the *SRY* gene, with *RASSF1A* being used as a gender-independent fetal epigenetic marker. The results were compared with those obtained by postnatal serological analysis of cord blood and gender identification.

Results: Among the 41 fetuses, 37 were RhD-positive and 4 were RhD-negative according to the serological analysis of cord blood. There was 100% concordance between fetal *RHD* genotyping and serological cord blood results. Detection of the *RASSF1A* gene verified the presence of cffDNA, and the fetal *SRY* status was correctly detected in all 41 cases.

Conclusion: Noninvasive fetal *RHD* genotyping with cffDNA incorporating *RASSF1A* is a feasible, reliable, and accurate method of determining fetal RhD status. It is an alternative to amniocentesis for the management of RhD-negative women and reduces the need for unnecessary RhIG prophylaxis.

Key words: Prenatal diagnosis, RhoD antigen, *RASSF1A* gene, Cell-free fetal DNA.

Introduction

Hemolytic disease of the fetus and newborn (HDFN) has been a major obstetric problem despite the widespread use of immunoprophylaxis. Isoimmunized pregnancies are at high

risk of HDFN, which can cause fetal anemia, hydrops, and intrauterine fetal death. Babies born with HDFN are at significant risk of neonatal morbidity and mortality [1,2]. Prenatal detection of the fetal RhD status in a pregnant woman with RhD-negative provides useful information for predicting the risk of HDFN and

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for subsequent management of the pregnancy. In 1997, Lo and colleagues [3] demonstrated the presence of cell-free fetal DNA (cffDNA) in the plasma and serum of pregnant women. This made the development of new techniques in prenatal diagnosis possible without fetal risk. Lo et al. [4] were the first to determine the fetal *RHD* genotype using cffDNA, and noninvasive assays have now been developed to determine fetal *RHD* genotype using cffDNA. Noninvasive fetal RHD genotyping (NIPD RHD) offers significant potential for changing the way RhD-negative women are managed during pregnancy. For example, special needs could be addressed and monitoring could be initiated at an early stage in alloimmunized pregnant women at risk of HDFN due to the fetus being positive for the respective antigen. Awareness of a RhD-negative fetus being carried by a RhD-negative woman would also eliminate unnecessary antenatal RhIG prophylaxis [5].

Recent studies have reported on fetal *RHD* genotyping in RhD-negative mothers with high accuracy [6-8]. Noninvasive determination of the Rh genotype relies on the amplification of fetal *RHD* gene sequences from DNA present in the mother's blood. Thus, when *RHD* sequences are amplified, they are assumed to arise from fetal DNA, and the fetus is deemed to be RhD-positive. On the other hand, a false-negative result on *RHD* genotyping has always raised concerns about whether fetal DNA is present in the sample. *RHD* genotyping laboratories have certain technical problems, especially a lack of a positive control for the presence of fetal DNA in the maternal sample. To address this problem, most labs have relied on the amplification of control targets, typically Y-chromosome-specific sequences (e.g., the *SRY* gene). However, Y-specific sequences are only present in males, making it difficult to demonstrate the presence of fetal DNA when the fetus is both RhD-negative and female. To this end, gender-independent fetal identifiers have been used, including the *maspin* gene, the hypermethylated *RASSF1A* promoter, panels of common polymorphic short tandem repeats, and single-nucleotide polymorphism (SNP) markers [9-12]. However, the use of SNP markers is expensive, time-consuming, labor-intensive, and not particularly informative [12]. Another attempt at a universal test for the presence of fetal DNA using differential methylation, which is present in the *maspin* (*SERPINB5*) promoter, has been reported [9]. However, the preferential detection of an unmethylated fetal sequence is more tedious and less sensitive than the detection of methylated material [13].

Fortunately, in the *RASSF1A* promoter, it is the fetal DNA that is preferentially hypermethylated and the maternal DNA that

is unmethylated [10]. Therefore, using methylation-sensitive restriction enzymes, hypomethylated maternal sequences can be digested, leaving only hypermethylated fetal sequences available for amplification by real-time PCR (RT-PCR). In this study, we present an NIPD RHD technique for RhD-negative pregnant women using cffDNA with a multiplex RT-PCR assay that is applicable to the detection of hypermethylated *RASSF1A* promoter sequences in all pregnancies, and we demonstrate the diagnostic performance and feasibility of NIPD RHD in RhD-negative pregnant women.

Materials and Methods

1. Maternal blood samples

Peripheral blood samples were obtained from 41 RhD-negative pregnant women with singleton pregnancies who received prenatal care at the obstetrics and gynecology clinic. The samples were requested for routine prenatal care and sent to the blood bank for ABO and RhD typing, as well as testing for unexpected antibodies. All of the women gave informed consent. Approval for the study was provided by the Seoul Clinical Laboratories institution's ethical committee (SCL-IRB-201303). The gestational age at the time of the blood collection was confirmed by routine ultrasound in all cases.

2. DNA extraction and digestion by a methylation-sensitive restriction enzyme

Maternal blood was collected into 2×5 mL tubes containing ethylenediaminetetraacetic acid as an anticoagulant and centrifuged at 1,600×g for 10 min, and the plasma fraction was transferred to a 2 mL centrifuge tube and re-centrifuged at 16,000×g for 10 min. The cell-free plasma fraction was stored at -20°C. Cell-free DNA was extracted from 2 mL of plasma using the QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions and resuspended in 50 µL of the kit's elution buffer for genomic DNA preparation buffer. Next, 2 ng of maternal blood cell DNA were digested with 10 U of *BstUI*, a methylation-sensitive restriction enzyme, at 60°C for 16 h.

3. Enzyme digestion of unmethylated *RASSF1A* and *beta-actin* sequences

To investigate the similarities in the enzyme-digestion efficiencies of un-methylated *RASSF1A* and β -actin gene (*beta-actin*) sequences, 1 µg aliquots of maternal buffy coat were digested with 100 U of *BstUI* enzyme for different time spans

(from 15 min to 16 h). The concentrations of *RASSF1A* and *beta-actin* sequences were measured in each sample after the enzyme digestion.

4. Multiplex real-time PCR

RT-PCR assays were established for the detection of *RHD*, *SRY*, *RASSF1A*, and *beta-actin* sequences. The *RHD* genotype was determined by real-time amplification of 2 regions located at exons 5 and 7 of the *RHD* gene, as recommended by the

Special Non-Invasive Advances in Fetal and Neonatal Evaluation (SAFE) *RHD* workshops [14]. Multiplex RT-PCR assays were established for the detection of 3 targets (*RHD* exon 5, *RHD* exon 7, and *SRY*), which were studied in a single run (Fig. 1). In addition, a duplex assay for the simultaneous amplification of *RASSF1A* and *beta-actin* was developed to confirm the presence of cfDNA. The sequences of the primers and probes are listed in Table 1. The *RASSF1A* gene was targeted using the primers and probes recommended by Chan et al. [10], and the contents

Fig. 1. Detection of fetal-derived *RHD* exons 5 and 7 in an RhD-negative pregnant woman by real-time PCR. Representative amplification curves are presented, showing real-time monitoring of fluorescence intensity for PCR *RHD* exon 5 and exon 7 as a function of the number of cycles. *RHD*+ control, plasma sample from an RhD-positive woman; *RHD*+ fetus, plasma sample from a pregnant woman carrying an RhD-positive fetus; *RHD*- control, plasma sample from an RhD-negative man; *RHD*- fetus, plasma sample from a pregnant woman carrying an RhD-negative fetus.

Table 1. Sequences of PCR primers and probes for real-time PCR assays

Target	Sequence 5' to 3'	Primer/probe
<i>RHD</i> exon 5	CGCCCTCTTCTTGATG	Primer
<i>RHD</i> exon 5	GAACACGGCATTCTTCCTTC	Primer
<i>RHD</i> exon 5	TCTGGCCAAGTTTCAACTCTGCTCTGCT	Probe
<i>RHD</i> exon 7	GGTGTGTAAACGAGTGCTG	Primer
<i>RHD</i> exon 7	ATGCCATTGCCGGCTCCGA	Primer
<i>RHD</i> exon 7	FAM-GGGCTACAACCTCAGCTTGCTGG-TAMRA	Probe
<i>RASSF1A</i>	AGCCTGAGCTCATTGAGCTG	Primer
<i>RASSF1A</i>	GGACCAGCTGCCGTGTGG	Primer
<i>RASSF1A</i>	FAM-GTGCCAACGCGCTGCGCATC-TAMRA	Probe
<i>SRY</i>	GAGAATCCCAGAATGCGAAA	Primer
<i>SRY</i>	TTCCGACGAGGTCGATACTT	Primer
<i>SRY</i>	JOE-CAGAAATTACAGGCCATGCACAG-TAMRA	Probe
<i>Beta-actin</i>	GCGCCGTTCCGAAAGTTGC	Primer
<i>Beta-actin</i>	CGGCGGATCGGCAAAGGC	Primer
<i>Beta-actin</i>	JOE-CACCGCCGAGACCGCTCCG-TAMRA	Probe

and thermal profiles of the assays were identical apart from the different primers and probes. A total of 4 μ L of digested DNA was added to a 20 μ L RT-PCR kit containing 300 nM of forward and reverse primers and 100 nM of probe and 1 $\times$ TaqMan[®] Universal PCR master mix (Life Technologies, Carlsbad, CA, USA). Products were amplified using a Rotor-Gene[®] 6000 (Qiagen) with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 55 cycles at 95°C for 15 sec, and a final cycle at 60°C for 60 sec. All of the reactions were run in triplicate for each exon (Fig. 1). To safeguard against false-negative results, we concurrently incorporated the *RASSF1A* system to confirm the presence of cffDNA in *RHD*-negative and *SRY*-negative samples (Fig. 2). The *Bst*UI enzyme removes the maternal *RASSF1A* gene, and the fetal gene can then be detected by RT-PCR. Amplification was performed in tandem with *beta-actin*, which served as an internal control to test for the completeness of the digestion. The positive control for the *RHD* and *SRY* genes was an RhD-positive male, and the negative control for both of these genes and for *RASSF1A* was an RhD-negative nonpregnant woman

(Fig. 2).

5. Data analysis

A fetus was assessed as being RhD-positive if 2 of the 3 replicates for each of the *RHD* exons (5 and 7) gave threshold cycle (Ct) values of <42. The *RHD* results of the assays of the 2 exons were identical in all cases. Therefore, the RhD-positive and RhD-negative results are presented (Fig. 1). The presence of *RASSF1A* was determined by positive RT-PCR amplification plots (Ct value <40). Three replicates for each *RASSF1A* restriction digest were analyzed. For the methylation-insensitive digest (the control for complete enzyme digestion of the *RASSF1A* target sequence), complete digestion was assumed to have occurred if no replicates were positive. For the methylation-sensitive digest, 2-3 replicates were required to be positive for confirmation of the presence of hypermethylated fetal DNA. Samples with fewer than 2 positive replicates were considered not to have sufficient cffDNA for a reliable interpretation of the RhD result (Fig. 2). A fetus was considered to be male only if the *SRY* sequences were

Fig. 2. Flowchart for fetal *RHD* genotyping in RhD-negative pregnant women using cell-free fetal DNA from maternal plasma. Fetal DNA, cell-free fetal DNA.

amplified in more than 2 or 3 isolated samples. Concurrently, a diagnosis of a female fetus required the absence of *SRY* amplification in all replicates, along with the amplification of

RASSF1A-BstUI and the absence of *beta-actin-BstUI*. The RhD type of the newborn blood was obtained from all of the samples. The accuracy of the predicted fetal RhD status was assessed in

Table 2. Results of fetal *RHD* real-time PCR genotyping using plasma from RhD-negative pregnant women

Case	<i>Beta-actin-BstUI</i>	<i>RASSF1A</i>	<i>RASSF1A-BstUI</i>	<i>SRY</i>	<i>SRY</i> Ct ^a	<i>RHD</i>	<i>RHD</i> Ct ^a	Interpretation
1	ND	D	D	—		+	31.37	RhD+ female
2	ND	D	D	—		+	35.76	RhD+ female
3	ND	D	D	+	38.60	+	26.14	RhD+ male
4	ND	D	D	—		+	27.85	RhD+ female
5	ND	D	D	+	39.43	—		RhD— male
6	ND	D	D	—		+	24.10	RhD+ female
7	ND	D	D	—		+	25.83	RhD+ female
8	ND	D	D	—		+	34.13	RhD+ female
9	ND	D	D	—		—		RhD— female
10	ND	D	D	+	38.56	+	25.35	RhD+ male
11	ND	D	D	+	36.38	+	24.60	RhD+ male
12	ND	D	D	—		+	28.37	RhD+ female
13	ND	D	D	—		+	31.07	RhD+ female
14	ND	D	D	+	37.40	+	29.66	RhD+ male
15	ND	D	D	+	37.86	+	30.13	RhD+ male
16	ND	D	D	+	34.98	+	39.39	RhD+ male
17	ND	D	D	—		—		RhD— female
18	ND	D	D	—		+	30.52	RhD+ female
19	ND	D	D	—		+	28.88	RhD+ female
20	ND	D	D	+	34.83	+	32.35	RhD+ male
21	ND	D	D	—		+	27.54	RhD+ female
22	ND	D	D	+	38.69	+	31.38	RhD+ male
23	ND	D	D	+	38.80	—		RhD— male
24	ND	D	D	—		+	26.72	RhD+ female
25	ND	D	D	—		+	30.35	RhD+ female
26	ND	D	D	—		+	25.46	RhD+ female
27	ND	D	D	+	38.82	+	28.94	RhD+ male
28	ND	D	D	—		+	35.07	RhD+ female
29	ND	D	D	+	37.55	+	26.76	RhD+ male
30	ND	D	D	+	39.02	+	28.16	RhD+ male
31	ND	D	D	+	39.21	+	33.43	RhD+ male
32	ND	D	D	—		+	32.98	RhD+ female
33	ND	D	D	—		+	31.33	RhD+ female
34	ND	D	D	+	39.51	+	33.06	RhD+ male
35	ND	D	D	—		+	30.37	RhD+ female
36	ND	D	D	—		+	33.83	RhD+ female
37	ND	D	D	+	36.77	+	32.42	RhD+ male
38	ND	D	D	+	36.68	+	26.45	RhD+ male
39	ND	D	D	—		+	32.48	RhD+ female
40	ND	D	D	+	39.51	+	32.21	RhD+ male
41	ND	D	D	—		+	39.02	RhD+ female

^aThe Ct value is the mean of the Ct of positive replicates. Ct, threshold cycle; D, detected; ND, not detected.

a comparison with the infants' RhD serotype determined from cord blood after delivery. All of the samples in this study were analyzed in a blinded fashion.

Results

Eighty-two maternal plasma samples, representing 2 separate isolations from each of 41 RhD-negative pregnant women, were subjected to enzyme digestion and subsequently analyzed using the multiplex RT-PCR protocol in order to evaluate this technique's performance in noninvasive fetal *RHD* genotyping from maternal plasma. The maternal ages ranged from 21 to 43 years (mean, 30 years), and the gestational ages at the time of the blood sampling ranged from 11 to 40 weeks. Seven (17.1%) of the samples were from the first trimester of gestation, 29 (70.7%) were from the second, and 5 (12.2%) were from the third.

The *RHD* RT-PCR assay was based on the amplification of exons 5 and 7, and for each group of samples, Ct values were determined. Plasma samples from pregnant women carrying RhD-positive fetuses gave Ct values in the range of 35–40 cycles, whereas no Ct values were observed when the fetus was RhD-negative. The results of the *RHD*-PCR assay of the maternal plasma samples are shown in Table 2. All of the cases showed positive amplification of *RASSF1A* in the undigested and digested samples, indicating that cfDNA had been extracted successfully in all cases. All of the samples showed complete enzyme digestion of *RASSF1A* via methylation-insensitive digest reactions. Thirty-seven cases were RhD-positive and 4 were RhD-negative. Among the 4 RhD-negative cases, 2 (cases 9 and 17) were *SRY*-negative and *RASSF1A*-positive. The positive determination of hypermethylated *RASSF1A* sequences in these cases confirmed fetal DNA, which shows that *RHD*-negative and *SRY*-negative typing are predictive of a female RhD-negative fetus. Eighteen cases showed amplification of *SRY* and were male at delivery, while 23 cases had no detectable *SRY* and were female at delivery (Table 2).

The results of the fetal *RHD* RT-PCR assay of the maternal plasma samples at different gestational trimesters are shown in Table 3. The earliest positive result was obtained at 11 weeks of gestation. A concordance rate of 100% (41/41) was observed between the PCR and the serological typing. The overall sensitivity of the determination of the fetal RhD status and gender from the maternal plasma was 100%, and the specificity was also 100%.

Discussion

The benefits of antenatal testing of the fetal RhD status by analysis of cfDNA in the maternal plasma of RhD-negative pregnant women could be considerable. When the father is heterozygous at the *RHD* locus, the fetus has up to a 40% chance of being RhD-negative and therefore being unaffected [15]. Pregnant women with RhD-negative fetuses would not require anti-RhD immunoglobulin prenatally after potential sensitizing events, such as amniocentesis or other trauma. Recent techniques developed for NIPD RHD using cfDNA have opened new pathways for the management of RhD-negative pregnant women. NIPD RHD by multiplex RT-PCR is particularly appealing because of the feasibility of large-scale screening in the laboratory [16,17]. Multiple reports have been published regarding the accuracy of determination of fetal RhD status with cfDNA [17]. The overall diagnostic accuracy is 96.5%, with the highest rate during the first trimester [18]. This new method was introduced as a routine practice in many European Union countries, including the Netherlands and the United Kingdom, for all fetuses carried by RhD-negative women [19]. We have developed an RT-PCR assay for NIPD RHD and fetal gender determination using cfDNA in maternal plasma for the first time in Korea [20].

In the present study, we have evaluated an RT-PCR assay for fetal *RHD* genotyping based on an analysis of multiple *RHD* exons. The combination of exon 5 and exon 7 prevents amplification of *RHD*-specific sequences when a variant allele

Table 3. Noninvasive fetal *RHD* real-time PCR genotyping compared to cord-blood serological results during different gestational trimesters

Trimester	No.	RhD+ by serological analysis of cord blood (n=37)		RhD– by serological analysis of cord blood (n=4)	
		<i>RHD</i> + by NIPD <i>RHD</i>	<i>RHD</i> – by NIPD <i>RHD</i>	<i>RHD</i> + by NIPD <i>RHD</i>	<i>RHD</i> – by NIPD <i>RHD</i>
First	7	6	0	0	1
Second	29	26	0	0	3
Third	5	5	0	0	0
Total	41	37	0	0	4

NIPD *RHD*, noninvasive fetal *RHD* genotyping.

of *RHD* is present, thus avoiding false-positive results [21,22]. False-positive results could be due to sample contamination, gene variation, atypical mutation, or gene selection [16]. False-positive diagnoses did not induce over-treatment in these patients since the RhD-negative pregnant women received RhIG prophylaxis, which would have been performed using the standard approach. However, false-negative results may be more serious clinically, leading to isoimmunization because of omission of antenatal RhIG prophylaxis, or omission of intrauterine therapy in isoimmunized pregnancy. Failure to amplify *RHD* may mean that the fetus is indeed RhD-negative, but it may also result from technical issues, such as the absence or a low level of fetal DNA in the sample. To minimize false-negative results, we incorporated *RASSF1A* as a universal cffDNA control in addition to existing *SRY* in fetal *RHD* genotyping, improving the diagnostic reliability and excluding false-negative complications (Figs. 2 and 3). A fetal *RHD* genotype is interpreted as RhD-negative when a maternal plasma sample negative for *RHD* is shown to be positive for hypermethylated *RASSF1A*. Any fetal *RHD* genotyping that results in negative detection of enzyme-digestion-resistant *RASSF1A* should be considered as inconclusive, and further testing with another plasma sample should be performed. The internal control system in the present study consisted of an RT-PCR assay targeting the *beta-actin*

gene promoter. As the digestion efficiencies of unmethylated *beta-actin* and *RASSF1A* sequences are similar, undetectable *beta-actin* signals can be deemed to reflect the completeness of the enzyme digestion of unmethylated *RASSF1A* sequences (Figs. 2 and 3). As shown in Table 2, in 2 cases (cases 9 and 17) that were *RHD*-negative and *SRY*-negative, the incorporation of *RASSF1A* to verify the fetal DNA confirmed its existence, thereby avoiding the possibility of a false-negative result.

These results indicate that *RASSF1A*, as a hypermethylated universal marker, could be useful to confirm the presence of cffDNA in *RHD*-negative and *SRY*-negative samples, improving the diagnostic accuracy of NIPD *RHD* and of fetal gender determination. The *RASSF1A* fetal marker was detected in maternal blood with 100% sensitivity and specificity [23]. In present study, the precise detection of fetal RhD status and gender was achieved throughout all gestational stages. Zhao et al. [24] demonstrated a positive correlation between hypermethylated *RASSF1A* levels and the severity of preeclampsia; therefore, this assay can be used for the early prediction of pregnancies at risk for this dangerous condition [25].

An *RHD* DNA genotyping may be instructed by practical considerations specific to a particular laboratory and population. Approximately 15% of Caucasian individuals are RhD-negative,

Fig. 3. Hypermethylated *RASSF1A* testing system to confirm the presence of cell-free fetal DNA (cffDNA) in *RHD*- and *SRY*-negative samples. A multiplex real-time PCR assay of the *RASSF1A* promoter gene was conducted using FAM- and TAMRA-labeled probes and DNA undigested and digested with *Bst*UI. The *beta-actin* gene was used as an internal control to show that enzyme digestion was complete. The data show the signals crossing the threshold for undigested control DNA from a nonpregnant female. Note that after digestion, the signal does not cross the threshold. Undigested patient DNA: this signal represents amplification of *RASSF1A* from plasma-derived cffDNA and maternal DNA. Digested DNA from patient: this signal, above the threshold remaining after *Bst*UI digestion, shows that cffDNA is present, as the fetal gene is hypermethylated and enzyme-resistant. *RHD*- and *SRY*-negative sample types are predictive of a female RhD-negative fetus.

which is usually associated with the deletion of *RHD* between the upstream and downstream Rhesus boxes [26]. In contrast to its relatively high prevalence in the Caucasian population, the RhD-negative blood type occurs in only 0.1% of Koreans [27]. Interestingly, a previous study reported that RhD-negative individuals may have a partial or intact *RHD* gene, and that this genotype occurs at different frequencies, depending on ethnicity [27]. One Korean study found that nearly 16% of RhD-negative individuals had an *RHDeI* allele, which is a rare variant of the Rh system, with a grossly intact *RHD* gene [28]. One of these alleles is the 1227G>A mutation, which probably disrupts normal intron-splicing [29]. The most frequent *RHD* allele among Asian individuals, including the Korean population, is the *RHDeI* variant *RHD1227A*, which serves as an important genetic marker in *RHDeI* Asians [30]. Relatively few studies of noninvasive fetal *RHD* genotyping have been performed in the Asian population. It is essential that more studies are carried out to evaluate the validity of *RHD* genotyping by PCR for a particular population. A validity study should be conducted in the near future to develop an improved strategy for the NIPD *RHD* genotyping of common *RHD*-negative alleles in Korean RhD-negative individuals.

The present study demonstrated that NIPD *RHD* incorporating *RASSF1A* as a gender-independent fetal epigenetic marker improves the diagnostic accuracy of the fetal RhD status and the fetal gender determination. NIPD *RHD* allows the avoidance of unnecessary invasive procedures, such as amniocentesis and chorionic villus sampling, in RhD-negative pregnant women carrying RhD-negative fetuses. This is the first successful study in Korea using cfDNA in maternal plasma, which creates a new avenue for clinical applications of noninvasive fetal *RHD* genotyping. The limitations of this study were the small sample size, which could introduce bias into the performance evaluation. Further expansion of the sample size would increase the power for the robust determination of the sensitivity and specificity of NIPD *RHD*. Also, most cases were tested in the second trimester. A validation study should be conducted using more samples from the first trimester. The effectiveness of the *RASSF1A* assay means it may be applicable in other pregnancy-associated disorders, such as X-linked diseases and the early prediction of preeclampsia.

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References

1. Portmann C, Ludlow J, Joyce A, Chan FY. Antecedents to and outcomes of Rh(D) immunization: mater mothers hospital, Brisbane, 1988-1995. *Aust N Z J Obstet Gynaecol* 1997;37:12-6.
2. Moise KJ Jr. Management of rhesus alloimmunization in pregnancy. *Obstet Gynecol* 2002;100:600-11.
3. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485-7.
4. Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998;339:1734-8.
5. Daniels G, Finning K, Martin P, Massey E. Noninvasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenat Diagn* 2009;29:101-7.
6. Wikman AT, Tiblad E, Karlsson A, Olsson ML, Westgren M, Reilly M. Noninvasive single-exon fetal RHD determination in a routine screening program in early pregnancy. *Obstet Gynecol* 2012;120:227-34.
7. Tynan JA, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. Multiplexed analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD testing. *Am J Obstet Gynecol* 2011;204:251.e1-6.
8. Bombard AT, Akolekar R, Farkas DH, VanAgtmael AL, Aquino F, Oeth P, et al. Fetal RHD genotype detection from circulating cell-free fetal DNA in maternal plasma in non-sensitized RhD negative women. *Prenat Diagn* 2011;31:802-8.
9. Chim SS, Tong YK, Chiu RW, Lau TK, Leung TN, Chan LY, et al. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc Natl Acad Sci U S A* 2005;102:14753-8.
10. Chan KC, Ding C, Gerovassili A, Yeung SW, Chiu RW, Leung TN, et al. Hypermethylated *RASSF1A* in maternal plasma: A universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211-8.
11. Pertl B, Sekizawa A, Samura O, Orescovic I, Rahaim PT, Bianchi DW. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. *Hum Genet* 2000;106:45-9.
12. Page-Christiaens GC, Bossers B, van der Schoot CE, de Haas M. Use of bi-allelic insertion/deletion polymorphisms as a positive control for fetal genotyping in maternal blood: first clinical experience. *Ann N Y Acad Sci* 2006;1075:123-9.
13. Grunau C, Clark SJ, Rosenthal A. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res* 2001;29:E65-5.

14. Legler TJ, Liu Z, Mavrou A, Finning K, Hromadnikova I, Galbiati S, et al. Workshop report on the extraction of foetal DNA from maternal plasma. *Prenat Diagn* 2007;27:824-9.
15. Chilcott J, Lloyd Jones M, Wight J, Forman K, Wray J, Beverley C, et al. A review of the clinical effectiveness and cost-effectiveness of routine anti-D prophylaxis for pregnant women who are rhesus-negative. *Health Technol Assess* 2003;7:iii-62.
16. Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 2002;42:1079-85.
17. van der Schoot CE, Hahn S, Chitty LS. Non-invasive prenatal diagnosis and determination of fetal Rh status. *Semin Fetal Neonatal Med* 2008;13:63-8.
18. Geifman-Holtzman O, Grotegut CA, Gaughan JP. Diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood--a meta-analysis. *Am J Obstet Gynecol* 2006;195:1163-73.
19. Hahn S, Chitty LS. Noninvasive prenatal diagnosis: current practice and future perspectives. *Curr Opin Obstet Gynecol* 2008;20:146-51.
20. Yang YH, Han SH, Lee KR. Noninvasive prenatal diagnosis using cell-free fetal dna in maternal plasma: clinical applications. *J Genet Med* 2011;8:1-16.
21. Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. *BMC Genet* 2001;2:10.
22. Grootkerk-Tax MG, Soussan AA, de Haas M, Maaskant-van Wijk PA, van der Schoot CE. Evaluation of prenatal RHD typing strategies on cell-free fetal DNA from maternal plasma. *Transfusion* 2006;46:2142-8.
23. Zejskova L, Jancuskova T, Kotlabova K, Doucha J, Hromadnikova I. Feasibility of fetal-derived hypermethylated RASSF1A sequence quantification in maternal plasma--next step toward reliable non-invasive prenatal diagnostics. *Exp Mol Pathol* 2010;89:241-7.
24. Zhao F, Wang J, Liu R, Yang J, Cui K, Wu Y, et al. Quantification and application of the placental epigenetic signature of the RASSF1A gene in maternal plasma. *Prenat Diagn* 2010;30:778-82.
25. Papantoniou N, Bagiokos V, Agiannitopoulos K, Kolialexi A, Destouni A, Tounta G, et al. RASSF1A in maternal plasma as a molecular marker of preeclampsia. *Prenat Diagn* 2013;33:682-7.
26. Wagner FF, Flegel WA. RHD gene deletion occurred in the Rhesus box. *Blood* 2000;95:3662-8.
27. Hwang YS. Haplotype frequencies of RH gene loci in Koreans. *Korean J Blood Transfus* 1996;7:233-40.
28. Wang XD, Wang BL, Ye SL, Liao YQ, Wang LF, He ZM. Non-invasive foetal RHD genotyping via real-time PCR of foetal DNA from Chinese RhD-negative maternal plasma. *Eur J Clin Invest* 2009;39:607-17.
29. Kim JY, Kim SY, Kim CA, Yon GS, Park SS. Molecular characterization of D- Korean persons: development of a diagnostic strategy. *Transfusion* 2005;45:345-52.
30. Luettringhaus TA, Cho D, Ryang DW, Flegel WA. An easy RHD genotyping strategy for D- East Asian persons applied to Korean blood donors. *Transfusion* 2006;46:2128-37.