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Amplicon Sequencing-Based Noninvasive Fetal Genotyping for *RHD*-Positive D Antigen-Negative Alleles

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BACKGROUND: To avoid hemolytic disease of the fetus and newborn resulting from maternal alloantibodies against fetal Rh antigens, anti-D immunoglobulin is routinely administered to RhD-negative pregnant women in Japan. Fetal *RHD* genotyping using cell-free DNA may prevent unnecessary antibody administration; however, current PCR-based methods, which detect *RHD* deletion, do not address the higher rates of *RHD*-positive D antigen-negative alleles in nonwhite populations without additional inspections.

METHODS: We developed an amplicon-sequencing method that could estimate the type of paternally inherited fetal *RHD* allele from 4 major *RHD* alleles in the Japanese population: the D antigen-positive allele (*RHD*01*, 92.9%) and 3 D antigen-negative alleles (*RHD*01N.01*, 6.6%; *RHD*01EL.01*, 0.3%; *RHD*01N.04*, 0.1%) using cell-free DNA obtained from the blood plasma of pregnant women.

RESULTS: The method correctly determined the fetal RhD type even when RhD-negative pregnant women possessed an *RHD*-positive D antigen-negative allele: *RHD*01EL.01* or *RHD*01N.04*.

CONCLUSIONS: This method is a reliable noninvasive fetal *RHD* genotyping method for Japanese and other East Asian populations. The genotyping principle of amplifying 2 different regions using the same primer pair and distinguishing them by their sequence difference during the subsequent mapping procedure is also theoretically applicable to *RHD*-positive D antigen-negative alleles prevalent in Africans. Therefore, this method offers an opportunity to consider targeted administration of anti-D immunoglobulin to RhD-negative pregnant women in East Asian and African countries and to increase the specificity of the fetal *RHD* genotyping implemented nationwide in several European countries.

The Rh blood group system is the most polymorphic among the human blood groups and the second most clinically significant in transfusion medicine next to ABO *(1)*. It is also clinically important in obstetrics because alloantibodies against Rh antigens constitute the main cause of hemolytic disease of the fetus and newborn. Among Rh antigens, D antigen is the most highly immunogenic. D antigen-negative phenotype (RhD-negative)[7] frequency is approximately 15% in whites, 8% in Africans, and <1% in East Asians *(2)*. RhD-negative individuals do not produce D antigen and, therefore, produce anti-D antibody (Anti-D) upon encountering RhD-positive red blood cells after transfusion or carrying an RhD-positive fetus *(3)*. Notably, severe hemolytic disease from Anti-D can lead to fetal death *(4)*.

Worldwide introduction of Anti-D prophylaxis via postnatal and antenatal Anti-D Ig administration has dramatically reduced D antigen alloimmunization in pregnant RhD-negative women *(3)*. Although demonstrated to be safe, as a human-derived pooled product, Anti-D Ig is accompanied by theoretical infection risk, and its production depends on donor availability *(5)*. Because D antigen alloimmunization does not occur when RhD-negative women carry an RhD-negative fetus, fetal *RHD*[8] genotyping may prevent unnecessary Anti-D administration.

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[7] Nonstandard abbreviations: RhD-negative/positive, D antigen-negative/positive phenotype; Anti-D, anti-D antibody; cffDNA, cell-free fetal DNA; NIPT, noninvasive prenatal testing; cfDNA, cell-free DNA; NGS, next-generation sequencing; NCCHD, National Center for Child Health and Development; wt, wild-type; var, variant.

[8] Human genes: *RHD*, Rh blood group D antigen; *RHCE*, Rh blood group CcEe antigens; *SMP1*, transmembrane protein 50A.

Rh antigens are encoded by the evolutionarily ancestral and duplicated genes *RHD* and *RHCE*, located in tail-to-tail orientation within a 200-kb genomic interval on chromosome 1p36.1 *(1)*. Both genes comprise 10 exons and have a highly homologous sequence. The 5′ upstream and 3′ downstream *RHD* gene regions, defined as the upstream and downstream *Rhesus box*, show 98.6% sequence identity *(6)*. Among whites, the most frequent molecular cause of the RhD-negative phenotype is *RHD* gene deletion with *RHD*-positive D antigen-negative alleles being very rare (e.g., 0.6% in the German population) (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue10) *(7)*. However, such alleles, consisting of *RHD-CE-D* hybrid and variant alleles, are more prevalent in African and East Asian populations. Among 177 African RhD-negative individuals, *RHD* deletion, *RHD*psi (pseudogene), and *RHD-CE-D* hybrid frequencies were 43%, 43%, and 15%, respectively (see Table 1 in the online Data Supplement) *(8)*. In East Asians, along with the *RHD* deletion, a single nucleotide variant (c.1227G>A) and an *RHD-CE-D* hybrid allele exhibit 12%, 22%, and 25% total frequency in the Japanese *(9)*, Korean *(10)*, and Chinese *(11)* populations, respectively (see Table 1 in the online Data Supplement).

The discovery of cell-free fetal DNA (cffDNA) in the maternal blood plasma during pregnancy *(12)* allowed noninvasive prenatal testing (NIPT) development and practical use *(13)*. Fetal *RHD* genotyping using maternal plasma cffDNA was subsequently demonstrated by detecting *RHD* sequences in the blood plasma of RhD-negative women lacking the gene *(14)*. cffDNA-based NIPT is feasible at a large scale and concluded to be diagnostic by a recent meta-analysis *(15)*. Several European countries have already introduced NIPT-based *RHD* genotyping nationwide *(16–20)*. However, this method detects fetal *RHD* gene presence or absence in RhD-negative women by quantitative PCR based on the assumption of homozygous *RHD* deletion, thereby yielding false-positive results for nondeletion *RHD*-positive RhD-negative alleles unless extra efforts, such as PCR detection of additional exons *(21)* and allelic discrimination using Taqman probes, are made *(22)*. Currently, no simple, reliable, noninvasive fetal *RHD* genotyping method exists for RhD-negative pregnant women with African or East Asian genetic backgrounds because of the higher nondeletion *RHD* allele frequencies in these populations. This may underlie in part the lack of nationwide fetal *RHD* genotyping in nonwhite populations.

In the Japanese population, based on the 0.5% frequency of serologically RhD-negative individuals *(23)* and its recessive mode of inheritance, RhD-positive and -negative allele frequencies are estimated as 93% and 7%, respectively, with estimated RhD-positive allele (*RHD*01*) homozygote and heterozygote frequencies of 86.4% and 13.1%, assuming Hardy–Weinberg equilibrium. Three major RhD-negative genotypes were identified from the *RHD* genotypes of 3526 serologically RhD-negative Japanese individuals: *RHD*01N.01/RHD*01N.01* (87.7%), *RHD*01EL.01/RHD*01N.01* (9.0%), and *RHD*01N.04/RHD*01N.01* (2.9%), along with several minor genotypes (total, 0.4%) *(9)*. The most frequent, *RHD*01N.01*, lacks the entire *RHD* gene owing to recombination between identical 902-bp sequences within the upstream and downstream *Rhesus boxes (1, 6, 24)*. The remaining and fused *Rhesus box* portions are termed the hybrid *Rhesus box (6)*. The second and third most frequent D antigen-negative alleles contain deleterious genomic alterations. The *RHD*01EL.01* allele, termed "Asia type" DEL *(25)*, contains a single nucleotide variant at the last nucleotide of exon 9 (c.1227G>A), which likely disrupts normal splicing *(26)*. In the *RHD*01N.04* [*RHD*D-CE(3–9)-D* hybrid] allele, exons 3 to 9 of *RHD* are replaced with those of *RHCE (26)*.

In this study, using the Japanese as a model for populations in which the *RHD*-positive RhD-negative allele frequencies among RhD-negative individuals are high (see Table 1 in the online Data Supplement), we aimed to develop a noninvasive fetal *RHD* genotyping strategy using maternal cell-free DNA (cfDNA) that could accurately estimate the fetal RhD phenotype regardless of whether the mother or fetus carry *RHD*-positive D antigen-negative allele(s). Next-generation sequencing (NGS)-based amplicon sequencing has been applied to noninvasive prenatal genotyping of various targets such as causal genes for monogenic disorders *(27)*, platelet antigen alleles *(28)*, and the KEL1/2 single-nucleotide polymorphisms for the Kell blood group system *(29)*. We used NGS technology to distinguish 4 major *RHD* alleles in the East Asian populations.

Materials and Methods

ETHICS STATEMENT

The study protocol, including participant recruitment, sample collection and preparation, and genetic data analyses, was approved by the Research Ethics Committee of the National Center for Child Health and Development (NCCHD) (approval number: 699,1545) and Showa University (approval number: 233).

BLOOD SAMPLE COLLECTION AND DNA EXTRACTION

Whole blood samples for *RHD* genotyping were provided by the Japanese Red Cross Society according to its guideline for using donated blood for research and development. The residuals of blood donated by individuals living in Japan were provided, including 100 serologically RhD-negative and 10 serologically RhD-positive

blood types. Serologically RhD-negative pregnant women attending the NCCHD (Tokyo, Japan) between April 2014 and March 2018 (n = 24) were recruited as donors of blood for cfDNA preparation. Genetic counseling was performed by clinical geneticists before collection. Written informed consent was obtained from all participants. The DNA extraction procedure is described in the Methods file in the online Data Supplement.

GENOTYPING OF RhD-NEGATIVE AND -POSITIVE INDIVIDUALS

PCR genotyping was designed to detect 3 major RhD-negative genotypes—*RHD*01N.01/RHD*01N.01*, *RHD*01.04/RHD*01N.01*, and *RHD*01EL.01/RHD*01N.01*—expected in the Japanese population based on a previous large-scale study *(9)*, and 2 RhD-positive genotypes: *RHD*01/ RHD*01* and *RHD*01/ RHD*01N.01*. The PCR primers were reported previously *(9)* or newly designed (see Table 2 in the online Data Supplement). PCR conditions and the Sanger sequencing method are described in the Methods file in the online Data Supplement.

ADAPTOR LIGATION FOR PCR AMPLICON AND NGS BY MiSeq

The detail of the amplicon library preparation is described in the Methods file in the online Data Supplement. Briefly, amplicons were obtained separately from 1000 pg of cfDNA as template DNA using either RHbox_KN_F1/R1 primers or RHD/RHCE_exon_9_KN_F1/R1 primers (Fig. 1) with Phusion High-Fidelity PCR Master Mix with HF Buffer. The obtained amplicons from the same cfDNA sample were pooled and subjected to adaptor ligation and PCR amplification (6 cycles). The resulting libraries were subjected to paired-end sequencing (151 bp × 2) on a MiSeq platform (Illumina).

DATA ANALYSIS (QUALITY CONTROL, MAPPING, AND READ COUNTS)

MiSeq Reporter Software version 2.3.32 (Illumina), samtools version 1.6, and the Integrative Genomics Viewer were used for the sequence analysis procedures. The details are described in the Methods file in the online Data Supplement.

Results

GENOTYPING OF RhD-NEGATIVE AND -POSITIVE JAPANESE INDIVIDUALS

To confirm the *RHD*-negative genotype frequencies among the Japanese population and identify individuals with the 3 major RhD-negative genotypes, we genotyped 100 serologically RhD-negative individuals using a subset of reported primers *(9)* and newly designed primers (see Table 2 in the online Data Supplement). The individuals harbored 87 *RHD*01N.01/RHD*01N.01* (deletion/deletion), 9 *RHD*01EL.01/RHD*01N.01* (c.1227G>A/deletion), and 4 *RHD*01N.04/RHD*01N.01* [*RHD*D-CE(3–9)-D* hybrid/deletion] genotypes, consistent with reported frequencies *(9)*. We also genotyped 3 RhD-positive individuals, identifying 2 with *RHD*01/RHD*01* (wild/wild) and 1 with *RHD*01/RHD*01N.01* (wild/deletion).

SELECTION OF GENOMIC SEQUENCES FOR DISTINGUISHING D ANTIGEN-POSITIVE AND -NEGATIVE ALLELES

In addition to the high *Rhesus box* sequence similarity (98.6%) *(6)*, the *RHD* and *RHCE* genomic sequences are 96% identical and considered as having duplicated during primate evolution *(30)*. Such high sequence similarities of duplicated regions frequently hamper PCR amplification of a particular genomic interval but allow the design of primers perfectly matching the 2 homologous regions to amplify both regions simultaneously. To apply NGS-based amplicon sequencing to such PCR amplicons from the *RHD/RHCE* locus, we designed the PCR primers, RHbox_KN_F1/R1, whose sequences perfectly matched both the upstream and downstream *Rhesus boxes* and amplified 105-bp amplicons from both regions. The individual amplicons could be distinguished via a single nucleotide difference at the 49th base, corresponding to hg19 nucleotide positions chr1:25,592,628 (G, upstream) and chr1:25,662,955 (A, downstream) (Fig. 1). We also designed PCR primers, RHD/RHCE_exon_9_KN_F1/R1, which amplified 148-bp amplicons from both the *RHD* and *RHCE* loci wherein the forward and reverse primer sequences perfectly matched the exon 9 and intron 9 sequences of both genes, respectively. These target intervals contained 2 nucleotide differences at the 23rd and 119th bases of the amplicons: A at chr1:25,648,419 and T at chr1:25,697,015, and A at chr1:25,648,515 and G at chr1:25,696,896. The *RHD* locus target interval also contains the *RHD*01EL.01* allele single nucleotide variant site (c.1227G>A) at the 54th base, corresponding to chr1:25,648,453 (Fig. 1). Using Sanger sequencing, we confirmed that the obtained PCR products contained amplicons from 2 loci, judged by the expected mono or mixed peaks at the nucleotide positions for allelic discrimination (see Fig. 1 in the online Data Supplement). NGS of these 2 PCR products and mapping of the resulting reads to the reference genome were expected to distinguish 4 major RhD-positive and 3 major RhD-negative genotypes *(9)* (Table 1) by their mapped patterns and the mapped read number ratio (see Fig. 2 in the online Data Supplement). By considering all possible 20 combinations of 4 paternally inherited alleles and 3 maternal RhD-negative genotypes (5 possible maternally inherited patterns) (see Fig. 3 in the online Data Supplement), we ascertained that the same strategy could also apply to determine the fetal *RHD* genotype using cfDNA of pregnant RhD-negative women.

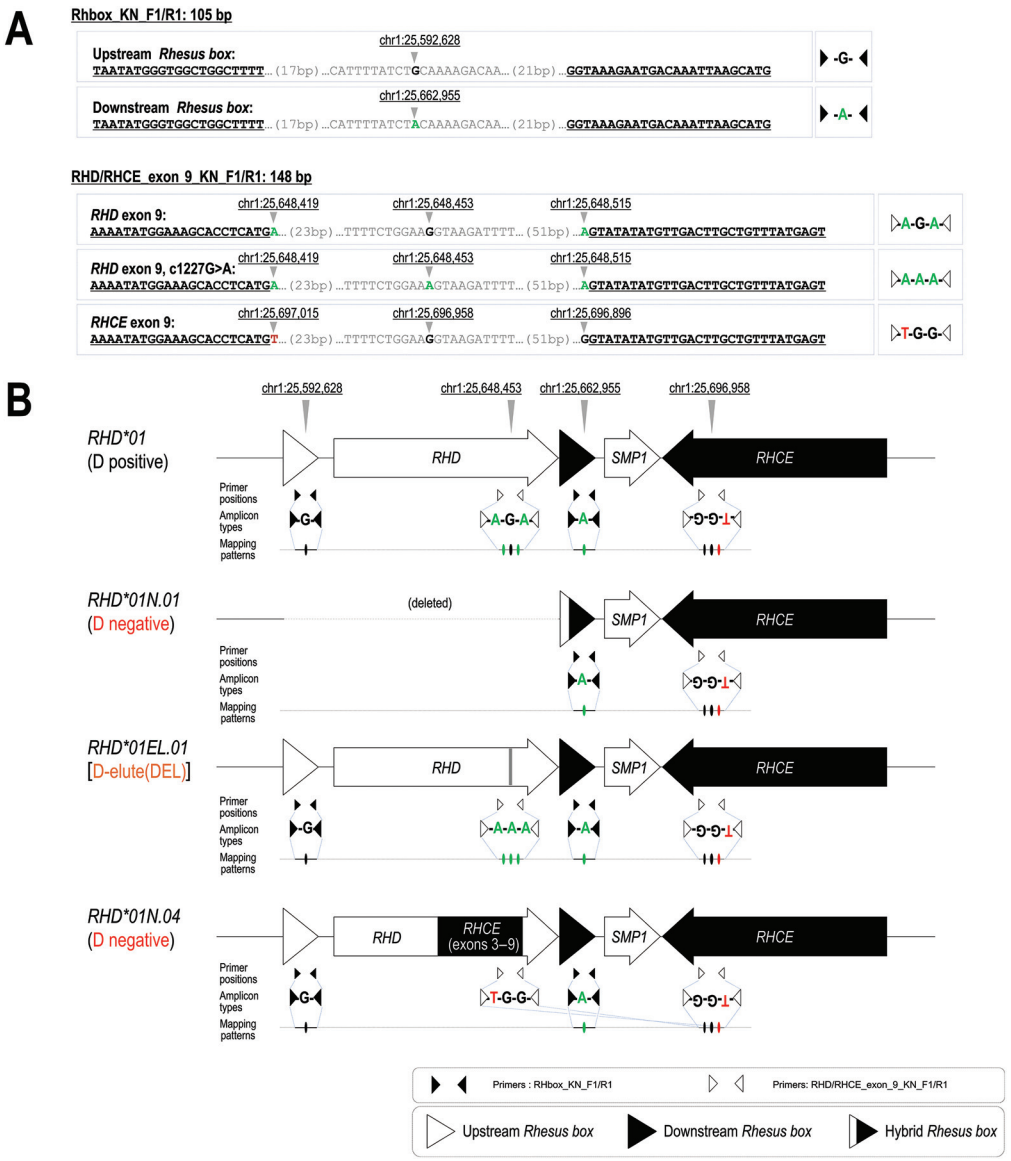

Fig. 1. PCR primers designed to distinguish D antigen-positive and -negative alleles by amplicon sequencing-based genotyping. (A), PCR amplicon sequences amplified by the primers RHbox_KN_F1/R1 (top) and RHD/RHCE_Exon9_ F1/R1 (bottom). The nucleotides corresponding to the positions of forward and reverse primers are underlined and bold. Primers RHbox_KN_F1/R1 amplify 105-bp fragments from both the upstream and downstream *Rhesus boxes*. The 2 types of amplicons can be distinguished by the sequence difference of G and A at the 49th base of the 105-bp products. Primers RHD/RHCE_Exon9_ F1/R1 amplify 148-bp fragments from both *RHD* exon 9 and *RHCE* exon 9. The 2 types of amplicons can be distinguished by the sequence differences at the 23rd and 119th bases of the 148-bp products; A and T, and A and G, respectively. In the RhD-negative *RHD*01EL.01* allele, the 54th base of the 148-bp amplicon from the *RHD* locus is A instead of G in the reference genome owing to the c.1227A>G variation. Diagrams shown at the right represent PCR primers (RHbox_KN_F1/R1, solid triangles; RHD/RHCE_Exon9_ F1/R1, open triangles) and the nucleotide(s) used to distinguish amplicon types. The hg19 coordinates of the nucleotides for distinguishing amplicon types are shown. (B), Schematic representations of the genomic organization of the D antigen-positive allele *RHD*01* and 3 D antigen-negative alleles, positions to which PCR primers are hybridized, amplicon types, and the genomic regions where each type of amplicon is expected to be aligned. *RHD, SMP1,* and *RHCE* genes are shown by arrows. The upstream and downstream *Rhesus boxes* are shown as open and closed arrowheads, respectively. The gray vertical bar in the *RHD* gene of the *RHD*01EL.01* allele indicates the position of the c.1227A>G variation (at chr1:25,648,453).

Table 1. Expected frequencies of *RHD* genotypes among the Japanese population.

		Genotype	Expected frequencies		
			Among the Japanese population, %		Among the RhD-negative individuals, %
RhD-positive genotypes					
	4 major RhD-positive genotypes	*RHD*01/RHD*01* *RHD*01N.01/RHD*01* *RHD*01EL.01/RHD*01* *RHD*01N.04/RHD*01*	86.36 13.14 12.31 0.59 0.19		
	Others			0.05	
RhD-negative genotypes					
	3 major RhD-negative genotypes	*RHD*01N.01/ RHD*01N.01* *RHD*01EL.01/ RHD*01N.01* *RHD*01N.04/ RHD*01N.01*	0.50	0.439 0.045 0.015 0.002	87.7 9.0 2.9
	Others				0.4

COMPARISON OF EXPECTED AND OBSERVED AMPLICON RATIOS FOR DISTINGUISHING RhD-POSITIVE AND -NEGATIVE GENOTYPES

We performed amplicon sequencing on blood DNA samples containing the RhD-positive genotype (*RHD*01/RHD*01*) and 3 RhD-negative genotypes (*RHD*01N.01/RHD*01N.01*, *RHD*01EL.01/RHD*01N.01*, *RHD*01N.04/RHD*01N.01*) and confirmed that the observed read count ratios mapped to the upstream and downstream *Rhesus boxes* and that those mapped to *RHD* exon 9 [wild-type (wt) or variant (var)] and *RHCE* exon 9 were consistent with the ratios expected for each genotype (Fig. 2 and Table 2). We subsequently examined mapped read numbers from genomic DNA mixtures of 2 individuals (A and B) at a 10:1 ratio, which served as approximation models of cfDNA from serologically RhD-negative pregnant women, with "A" corresponding to the mother, comprising 1 of the 3 RhD-negative genotypes and "B" corresponding to the fetus, being any of the 4 RhD-positive or -negative genotypes. Although the genotype of the fetus conceived by an RhD-negative woman cannot be *RHD*01/RHD*01*, we used *RHD*01/RHD*01* rather than *RHD*01/RHD*01N.01* because genomic DNA of the latter genotype was originally unavailable. For the 12 A and B combinations mimicking maternal cfDNA containing 9.1% fetal DNA, we performed amplicon-based *RHD* genotyping and obtained amplicon ratios similar to those expected (see Table 3 in the online Data Supplement). We also assessed the sensitivity and quantitative accuracy of fetal DNA detection in the amplicon-based *RHD* genotyping by obtaining data for the mixtures of genomic DNAs of *RHD*01EL.01/RHD*01N.01* and *RHD*01/RHD*01N.01* with 5 different ratios of the latter (20%, 10%, 5%, 3%, and 1%) (see Table 4 in the online Data Supplement). Amplicons from the *RHD*01* allele were detected in all mixtures. The expected and observed amplicon ratios from the *RHD*01* allele showed high correlation (1%–20%). During the assessment of the feasibility and accuracy of our amplicon sequencing-based noninvasive fetal genotyping method for RhD-positive D antigen-negative alleles, we unexpectedly observed a C to T transition at chr1:25,648,439 within the *RHD* exon 9 amplicons in some of the samples at low frequencies (0.08%–11.9%). However, the frequencies of this nucleotide change were inconsistent between duplicate data sets for the same samples. When we repeated the amplicon sequencing analysis for the same sample set using Ex *Taq*HS (TaKaRa) for adaptor-ligated library amplification rather than the Phusion High-Fidelity PCR Master Mix, C to T transition at chr1:25,648,439 was not observed (data not shown). Therefore, we concluded that the phenomenon was an artifact generated in the library amplification procedure to be disregarded in our subsequent analyses.

AMPLICON-BASED *RHD* GENOTYPING USING 8 CASES OF cfDNA FROM RHD-NEGATIVE PREGNANT WOMEN

We performed *RHD* genotyping on leukocyte DNA of 24 RhD-negative pregnant women who gave birth at NCCHD between 2014 and 2018, and selected 8 individuals to test fetal *RHD* genotyping on various types of maternal *RHD* genotypes (Table 3). The gestational ages ranged from 9 to 30 weeks. We estimated the maternal and fetal *RHD* genotypes based on the ratios of the reads mapped to the *Rhesus boxe*s (upstream/downstream) and those of wild-type *RHD* exon 9, variant *RHD* exon 9, and

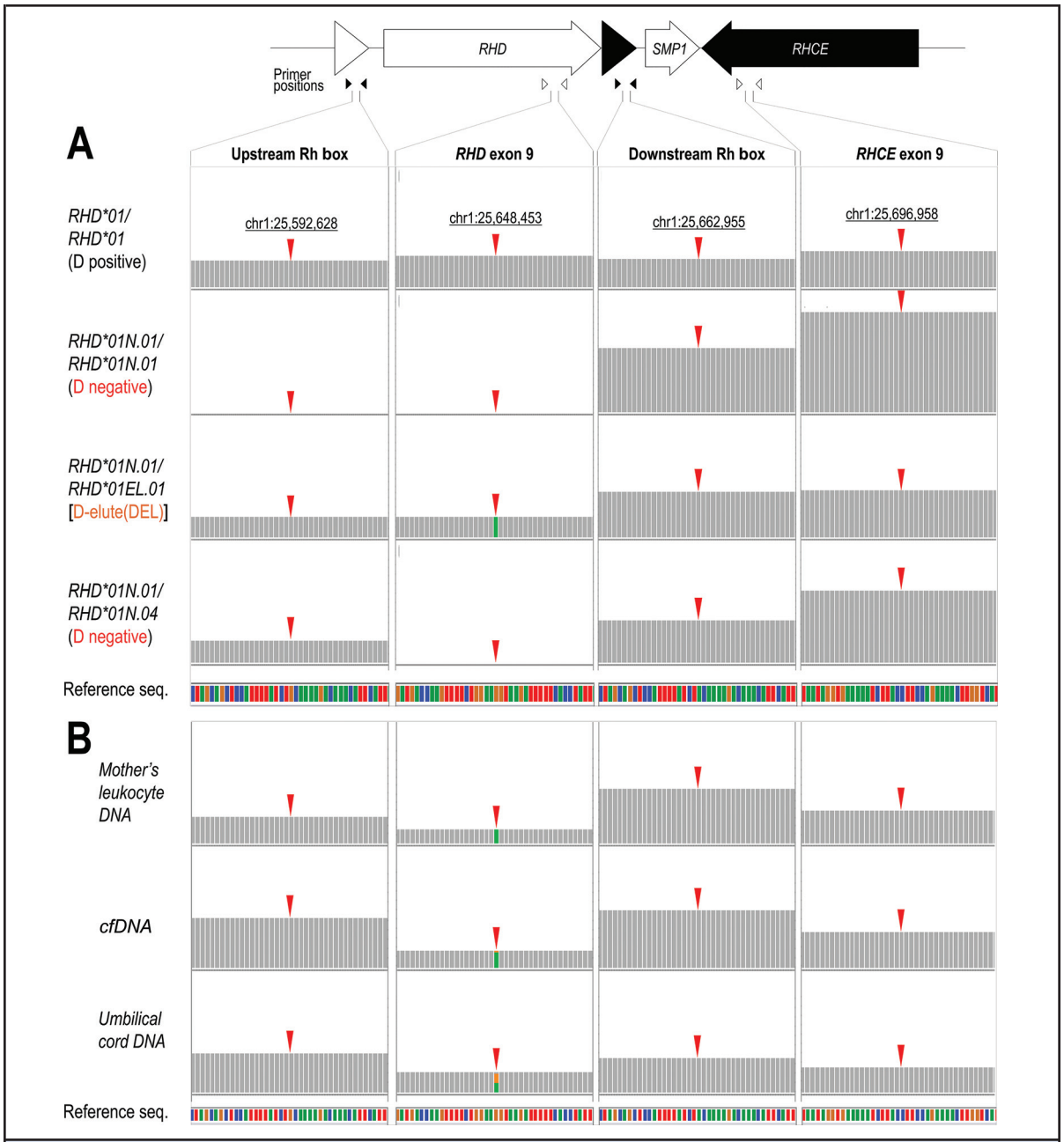

Fig. 2. Examples of mapped read numbers and nucleotides of amplicons from 4 genomic loci–the upstream Rh box, *RHD* exon 9, the downstream Rh box, and *RHCE* exon 9 – corresponding to 4 columns from left to right.

The images of the bam coverage track for each of the mapping results (.bam files) visualized by the Integrative Genomic Viewer are shown for 4 regions (a 41-bp interval for each amplicon). In the bam coverage track, for each nucleotide position, the ratio of the reference and variant sequences is color-coded: gray for the reference nucleotide and blue (C), green (A), red (T), or orange (G) for a variant nucleotide. The height of the bar at each nucleotide position is proportional to the number of mapped reads. The data range was set from 0 to 50 000 for all panels. The red vertical arrows show the nucleotide positions at chr1:1:25,592,628, chr1:25,648,453, chr1:25,662,955, and chr1:25,696,958, respectively, for each of the 4 columns. Fig. 1 shows the principle used to distinguish 2 different PCR amplicons amplified by the same primer pair based on the sequence difference(s) at these nucleotide positions. (A), Results for the RhD-positive and 3 RhD-negative genotypes. (B), Results for case 4 (in Table 3). The presence of G (orange) in addition to A (green) at chr1:25,648,453 in the cell-free DNA indicates that the fetus inherited the RhD-positive allele from the father and therefore is RhD-positive.

Table 2. Expected and observed ratios of amplicons from individuals with RhD-positive and -negative genotypes.

	Amplicons from *Rhesus boxes* (primers: RHbox_KN_F1/R1)				Amplicons from *RHD/RHCE* exon 9 (primers: RHD/RHCE_exon_9_KN_F1/R1)			
	Copy number/cell				Copy number/cell			
RHD genotype	Upstream *Rhesus box* (G at chr1: 25,592,628)	Downstream *Rhesus box* (A at chr1: 25,662,955)	Expected ratio Upstream/ downstream	Observed ratio (and counts) Upstream/ downstream	*RHD* (wt/ var) (G/A at chr1: 25,648,453)	*RHCE* (G at chr1: 25,696,896) (T at chr1: 25,697,015)	Expected ratio RHD_wt/RHD_var/ RHCE	Observed ratio (and counts) RHD_wt/ RHD_var/RHCE
*RHD*01/RHD*01* (D positive)	2	2	50.0%/50.0%	49.3%/50.7% (11 126/11 423)	2 wt	2	50.0%/0.0%/50.0%	46.1%/0.0%/53.9% (12 698/0/14 818)
*RHD*01N.01/RHD*01N.01* (D negative)	0	2	0.0%/100%	0.02%/99.98% (7/25 695)	0	2	0.0%/0.0%/100%	0.007%/0.0%/99.9% (3/40 184)
*RHD*01N.01/RHD*01EL.01* (D negative)	1	2	33.3%/66.6%	32.2%/67.8% (8653/18 189)	1 var	2	0.0%/33.3%/66.6%	0.01%/31.2%/68.8% (3/8638/19 069)
*RHD*01N.01/RHD*01N.04* (D negative)	1	2	33.3%/66.6%	34.0%/66.0% (8981/17 441)	0	3	0.0%/0.0%/100%	0.0%/0.003%/99.9% (0/1/29 159)

RHCE exon 9 (*RHD*_wt/*RHD*_var/*RHCE*) (Table 3). Six and 2 fetuses were estimated to be RhD-positive and RhD-negative, respectively. When the RhD-negative mother is a compound heterozygote of *RHD*-negative alleles, 2 possible fetal *RHD* genotypes are considered, as shown for cases 1, 4, and 7 (Table 3 and Fig. 2). However, this did not interfere with estimating the fetal RhD phenotype to be positive because of the detection of the "*RHD*_wt" type amplicons, at ratios between 1.3% and 3.1% (Table 3). We also determined the maternal and newborn *RHD* genotypes using blood DNA and cord blood DNA, respectively, by the amplicon-based genotyping in addition to the serological RhD type of the newborns using their peripheral blood. We confirmed that these *RHD* genotypes and RhD types were all consistent with the estimated genotypes/phenotypes derived from the amplicon-sequencing data.

As a limitation of NGS reads, unexpected nucleotide sequences were found in mapped sequences at low frequencies, mostly fewer than 10 per several 10 000 reads (Tables 2 and 3 here plus Table 3 in the online Data Supplement). Such DNA sequencing errors reportedly occur at approximately 1×10^{-3} nucleotides using the Illumina MiSeq platform *(31)*. By excluding reads containing base-quality scores <26, we removed most such sequencing errors. However, other factors need to be considered as potential sources of unexpected nucleotide sequences including PCR replication errors, index hopping, remnants in flow cells, and DNA contamination. We detected 21 unexpected nucleotide sequences at chr1:25,648,453 in the mother's leukocyte DNA of case 4 among 19 905 total reads (Table 3). Because the leukocyte DNA was extracted from the buffy coat of mother's blood obtained during her pregnancy with an RhD-positive fetus carrying the *RHD*01* allele, contamination of cffDNA may underlie this higher number. Our amplicon-based *RHD* genotyping accurately estimated the fetal *RHD* genotype noninvasively in 8 cases of RhD-negative pregnant women.

Discussion

We developed an amplicon sequencing-based noninvasive fetal *RHD* genotyping method that could estimate the paternally inherited fetal *RHD* allele from among D antigen-positive (*RHD*01*) and 3 D antigen-negative alleles (*RHD*01N.01*, *RHD*01EL.01*, and *RHD*01N.04*). Fetal *RHD* genotyping for RhD-negative pregnant women is currently implemented nationwide in several countries to reduce unnecessary Anti-D Ig administration *(32)*. A cost-effectiveness analysis for the UK also concluded that noninvasive fetal *RHD* genotyping is cost-saving compared with providing Anti-D to all RhD-negative pregnant women *(13)*. However, such estimates for the US, Australia, and Canada vary depending on the estimated fetal *RHD* genotyping and Anti-D Ig costs

Table 3. Estimated fetal RhD types by amplicon sequencing-based genotyping using cfDNA of pregnant RhD-negative women, and ratios and read counts for cfDNA and leukocyte genomic DNA from pregnant RhD-negative women and umbilical cord DNA.

Case number	Gestational age at sampling	Detected ratio of *RHD*-positive allele in cfDNA (%) and the predicted fetal RhD type	Expected *RHD* genotypes of the mother (M) and the fetus (F) based on the amplicon-seq data using cfDNA	Mother's *RHD* genotype[a]	Neonatal *RHD* genotype[b]	Neonatal serological RhD type
1	9w2d[c]	1.3% (positive)	*M: RHD*01N.01/RHD*01EL.01* *F: RHD*01/RHD*01EL.01 or RHD*01/RHD*01N.01*	*RHD*01N.01/RHD*01EL.01*	*RHD*01/RHD*01EL.01*	Positive
2	12w1d	3.1% (positive)	*M: RHD*01N.01RHD*01N.01* *F: RHD*01/RHD*01N.01*	*RHD*01N.01/RHD*01N.01*	*RHD*01/RHD*01N.01*	Positive
3	24w2d	4.5% (positive)	*M: RHD*01N.01RHD*01N.01* *F: RHD*01/RHD*01N.01*	*RHD*01N.01/RHD*01N.01*	*RHD*01/RHD*01N.01*	Positive
4	21w6d	2.9% (positive)	*M: RHD*01N.01/RHD*01EL.01* *F: RHD*01/RHD*01EL.01 or RHD*01/RHD*01N.01*	*RHD*01N.01/RHD*01EL.01*	*RHD*01/RHD*01EL.01*	Positive
5	10w0d	0.0% (negative)	*M: RHD*01N.01RHD*01N.01* *F: RHD*01N.01RHD*01N.01*	*RHD*01N.01/RHD*01N.01*	*RHD*01N.01/RHD*01N.01*	Negative
6	9w0d	0.0% (negative)	*M: RHD*01N.01RHD*01N.01* *F: RHD*01N.01RHD*01N.01*	*RHD*01N.01/RHD*01N.01*	*RHD*01N.01/RHD*01N.01*	Negative
7	10w2d	3.1% (positive)	*M: RHD*01EL.01/ RHD*01N.04* *F: RHD*01/RHD*01EL.01 or RHD*01/RHD*01N.04*	*RHD*01EL.01/ RHD*01N.04*	*RHD*01/RHD*01EL.01*	Positive
8	30w2d	4.6% (positive)	*M: RHD*01N.01RHD*01N.01* *F: RHD*01/RHD*01N.01*	*RHD*01N.01/RHD*01N.01*	*RHD*01/RHD*01N.01*	Positive

	Rhesus boxes (upstream/downstream)			*RHD/RHCE* exon 9 (RHD_wt/RHD_var/RHCE)		
Case number	cfDNA	Mother's leukocyte DNA	Umbilical cord DNA	cfDNA	Mother's leukocyte DNA	Umbilical cord DNA
1	38.9%/61.1% (21 235/33 311)	36.5%/63.5% (28 230/49 152)	49.3%/50.7% (44 395/45 580)	1.3%/37.9%/60.8% (434/12 271/19 671)	0.0%/30.1%/69.9% (2/8304/ 19 276)	22.3%/22.3%/54.4% (7425/7778/18 163)
2	4.6%/95.4% (2392/49 373)	0.04%/99.96% (15/37 034)	31.5%/68.5% (17 090/37 152)	3.1%/0.01%/96.9% (1062/2/33 001)	0.0%/0.0%/100% (0/0/20 715)	28.5%/0.0%/71.5% (9269/0/23 251)
3	2.9%/97.1% (1124/38 189)	0.06%/99.94% (19/34 420)	34.3%/65.7% (25 338/48 594)	4.5%/0.004%/95.5% (1077/1/22 745)	0.005%/0.0%/99.99% (1/0/22 100)	28.9% /0.01%/71.1% (8052/3/19 853)
4	46.6%/53.4% (20 316/23 305)	32.4%/67.6% (10 732/22 391)	53.7%/46.3% (16 155/13 918)	2.9%/30.8%/66.3% (634/6759/14 550)	0.11%/29.8%/70.1% (21/5680/13 394)	22.1%/22.4%/55.5% (4054/4096/10 177)
5	0.01%/99.99% (1/12 287)	0.09%/99.91% (29/32 729)	0.01%/99.99% (5/35 332)	0.0%/0.0%/100.0% (0/0/6363)	0.0%/0.03%/99.97% (0/6/20 545)	0.0%/0.02%/99.98% (0/3/16 324)
6	0.02%/99.98% (7/37 773)	0.04%/99.96% (15/34 402)	0.01%/99.99% (2/29 585)	0.0%/0.004%/99.99% (0/1/24 843)	0.009%/0.019%/99.97% (2/4/20 034)	0.0%/0.0%/100.0% (0/0/16 429)
7	56.7%/43.3% (24 752/18 920)	50.7%/49.3% (16 408/15 953)	44.9%/55.1% (14 138/17 364)	3.1%/25.9%/71.0% (773/6503/17 844)	0.01%/21.3%/78.7% (2/3995/14 729)	24.8%/23.0%/52.2% (5552/5166/11 694)
8	5.6%/94.4% (1962/33 229)	0.04%/99.96% (18/44 068)	34.9%/65.1% (18 228/33 937)	4.6%/0.01%/95.4% (1637/3/33 652)	0.0%/0.002%/99.99% (0/1/39 560)	32.0%/0.0%/68.0% (8710/0/18 496)

[a] Determined by amplicon-sequencing using leukocyte genomic DNA.
[b] Determined by amplicon-sequencing using cord blood genomic DNA.
[c] w, week; d, day.

(32). A work group study by the American Association of Blood Banks recommended that fetal *RHD* genotyping be performed whenever a discordant RhD typing result and/or a serologically weak D phenotype is detected in patients to reduce unnecessary Anti-D Ig injections and increase RhD-negative red blood cell availability for transfusion *(33)*. The routine antenatal Anti-D prophylaxis policy also raises ethical issues regarding unnecessary Anti-D Ig administration to RhD-negative pregnant women carrying an RhD-negative fetus *(5)*. Thus, introducing target antenatal Anti-D prophylaxis to Rh-negative pregnant women in East Asian countries is warranted.

Rather than specifically amplifying certain *RHD* alleles, we amplified different alleles or highly homologous regions using single primer sets and subsequently distinguished them by their 1 to 3 base sequence differences during the mapping procedure. Our method distinguished the D antigen-positive and 3 D antigen-negative alleles (deletion, single nucleotide variant, and hybrid types) in a quantitative manner using cfDNA from preg-

nant women with only 2 primer pairs without paternal genotype information. Owing to the nature of high read depth (several 10 000s of reads/sample) of the amplicon sequencing, this method detected cfDNA in the maternal plasma in a quantitative manner, and also provided *RHD* and *RHCE* exon copy number information. Therefore, as another application, our method could be used to determine *RHD* zygosity.

In compensation for discerning *RHD*-positive RhD-negative alleles, the amplicon sequencing-based method described here has higher costs than those of PCR-based fetal *RHD* deletion allele-based genotyping. However, by collecting a large number of amplicons (e.g., 200 amplicons in total for 100 cfDNA samples) for 1 MiSeq run, the sequencing cost per sample can be <$10 when 20 000 read-pairs are obtained for each amplicon. Although the current library preparation cost by our protocol is approximately $25, this may be reduced to one-fifth or less by introducing 1-step or 2-step PCR methods using custom-designed PCR primers including target-specific, index, and Illumina adaptor sequences. Therefore, at a large scale, our amplicon sequencing-based fetal *RHD* genotyping can be performed at costs reasonable for an NIPT.

The *RHD*01EL.01* allele, the "Asia type" DEL variant, expresses a very weak D antigen with the complete repertoire of D antigen epitopes *(34)*. DEL phenotype individuals are serologically typed as RhD negative and can be detected only by adsorbing and eluting Anti-D from the cell surface of the red blood cell *(2)*. Three studies on Chinese individuals *(35–37)* have suggested that the "Asia type" DEL variant is not at risk of alloimmunization to D antigen, and antenatal Anti-D prophylaxis to DEL pregnant women is unnecessary. If such evidence continues to accumulate for "Asia type" DEL individuals, the current healthcare policy of Anti-D prophylaxis administration to all RhD-pregnant women including "Asia type" DEL individuals adopted in countries such as Japan may change in the future. Despite such a controversy, the *RHD*01EL.01* served as a good model for other point mutation alleles responsible for alloimmunization.

The majority of cffDNA is estimated to be <150 bp in size *(38)*. Although we used primers that amplified a 148-bp amplicon for *RHD/RHCE* exon 9 in this study to include 2 nucleotide positions distinguishing *RHD* and *RHCE* loci, our results of the *Rhesus boxe*s (upstream/downstream) demonstrated that 1 nucleotide difference was enough to accurately map sequence reads to either *RHD* or *RHCE* loci. Therefore, by adopting PCR primers amplifying a shorter amplicon including 1 nucleotide position distinguishing *RHD/RHCE* exon 9, it is likely possible to improve the analytical sensitivity for detecting cffDNA in our method.

Because our current primer sets were designed to detect 2 major *RHD*-positive D antigen-negative alleles, *RHD*01EL.01* (single nucleotide variant) and *RHD*01N.04* [*RHD*D-CE(3–9)-D* hybrid)], our method would falsely detect other minor *RHD*-positive D antigen-negative alleles (0.4% total frequency in the Japanese population) *(9)* as D antigen-positive alleles. Nevertheless, 1 advantage of introducing amplicon sequencing is its flexibility and expandability for additional primer sets for those rare alleles containing nucleotide sequence variation(s) with the RhD-positive allele. Therefore, our amplicon sequencing strategy should be easily applicable to major *RHD*-positive D antigen-negative alleles, such as *RHD*psi in the African population *(8)*, which contains a 37-bp duplicated insertion in exon 4 that introduces a premature stop codon at position 210 and 5 single nucleotide variants (c.609G>A in exon 4, c.654G>C and c.667T>G and c.674C>T in exon 5, c.807T>G in exon 6). A previously reported PCR-based method for fetal genotyping of the *RHD*psi allele used PCR primers matched with the wild-type allele but mismatched with the *RHD*psi allele at the 3′ ends, and a Taqman probe distinguishing the wild-type nucleotide and the missense variant in exon 5 *(22)*. However, examination of multiple exons rather than that of only exon 5 has been recommended for the accurate fetal genotyping of the *RHD*psi allele *(21)*. Differential haplotype amplification using primers with mismatches at 3′ ends has been indicated to lead to misgenotyping *(39)*. By contrast, our sequencing-based method is expected to be able to directly target the 37-bp duplication, which is the primary feature of the *RHD*psi allele. The principle of our strategy is also applicable to distinguish *RHD*-positive RhD-negative alleles observed in white populations such as *RHD*06* alleles *(40)*.

Our fetal *RHD* genotyping method offers the first opportunity for East Asian countries to introduce such a genotyping service for RhD-negative pregnant women and represents a model for other nonwhite countries to establish a genotyping strategy customized to the *RHD*-positive D antigen-negative alleles prevalent in each country. Moreover, our method also has the potential to improve the specificity of nationwide fetal *RHD* genotyping in European countries if introduced as an option for nonwhite individuals in these countries.

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