Original article

Molecular basis of serological weak D phenotypes and RhD typing discrepancies identified in the Korean population

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Background - The molecular basis of RhD blood groups differs with race/ethnicity. This study aimed to investigate the molecular basis of serological weak D phenotypes and RhD typing discrepancies in the Korean population.

Materials and methods - The RhD status of 188,852 Korean patients was initially determined using the automated microplate method and manual tile method. In case of no agglutination, weak D testing was further performed using the tube and gel methods. Serologically D-negative samples with C+ and/or E+ were tested using polymerase chain reaction-sequence specific primers for four *RHD* targets and/or exon 9 sequencing. Samples showing a serological weak D phenotype or an RhD typing discrepancy were subjected to full *RHD* gene sequencing.

Results - Of the 32 samples showing a serological weak D phenotype and 191 samples showing a serologically D-negative phenotype with C+ and/or E+, 23 and 50 were genotyped, respectively. Among the weak D samples, the most common alleles were *RHD*15* (n=6), *RHD*13.01* (n=4), and *RHD*01W.25* (n=4), and no variant was found in two samples. *RHD*01EL.01* (n=26) accounted for more than half of the D-negative samples. Of the seven samples that were typed as D-positive using the automated microplate method but showed weak reactivity using the tile method, four were genotyped, and the results were as follows: *RHD*01W.33 (n=2)*, *RHD*01W.43 (n=1)*, and no variant found (n=1).

Discussion - In our cohort, various D variant alleles including *RHD*15* were identified; however, *RHD*01W.1*, *RHD*01W.2*, *RHD*01W.3*, *RHD*09.03.01*, and *RHD*09.04*, accounting for more than 95% of Caucasians with a serological weak D phenotype, were not found. Our study reaffirms that the distribution of D variant alleles differs between East Asians and Caucasians. Our findings also indicate that some D variants including *RHD*01W.33* and *RHD*01W.43* are at risk of being mistyped as D-positive by a highly sensitive RhD typing method such as an automated microplate method.

Keywords: *Rh discrepancy, serological weak D phenotype, RHD genotyping, Asia type DEL.*

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INTRODUCTION

The D antigen (RH1) is highly immunogenic as indicated by the fact that approximately 20-30% of D-negative patients develop anti-D alloantibodies when transfused with

D-positive red blood cells (RBCs)[1-3]. Anti-D alloantibodies can cause severe haemolytic transfusion reactions and haemolytic disease of the fetus and newborn[4]. Therefore, it is standard practice to avoid transfusing D-positive RBCs to D-negative patients, especially women of childbearing age.

A "serological weak D phenotype" shows weak reactivity in direct agglutination testing or agglutination only in weak D testing[5,6]. In direct agglutination testing, weak reactivity is usually defined as ≤2+ agglutination on a 0 to 4+ scale[5,6]. However, weak D samples, particularly those with a relatively high D antigen density, might show ≥3+ agglutination and thus appear as D-positive when using a highly sensitive RhD typing method such as an automated microplate method using a potent anti-D reagent[6]. When using a less sensitive RhD typing method such as the manual tube method, ≤2+ agglutination might be observed in the same samples[6]. With advances in molecular methods and growing knowledge of the molecular basis of serological weak D phenotypes, *RHD* genotyping has emerged as a powerful tool to resolve RhD typing discrepancies.

The frequency of *RHD* alleles encoding serological weak D phenotypes varies among different populations. More than 95% of Caucasians with a serological weak D phenotype harbour the *RHD*01W.1* (weak D type 1), *RHD*01W.2* (weak D type 2), *RHD*01W.3* (weak D type 3), *RHD*09.03.01* (weak D type 4.0), or *RHD*09.04* (weak D type 4.1) allele[7], whereas these alleles were not observed in previous studies conducted on Chinese[8-16] and Japanese[17,18] populations. The molecular basis of serological weak D phenotypes has been studied in the Korean population[19-21], but not extensively. In the present study, we investigated the molecular basis of serological weak D phenotypes and RhD typing discrepancies identified in Korean patients in a tertiary hospital. Based on the study findings, we developed an effective RhD typing strategy for East Asian patients.

MATERIALS AND METHODS

Study samples and DNA extraction

This retrospective study included a total of 188,852 Korean patients whose blood samples were submitted to the transfusion service at Samsung Medical Center, Seoul, South Korea, for routine RhD typing between January 2017 and June 2019. The study protocol was approved by the Institutional Review Board (IRB) of Samsung Medical Center (IRB n. SMC 2019-06-128). For the purpose of this study, patients of non-Korean ethnicity were excluded on the basis of their nationality and surname. Genomic DNA was extracted from whole blood using a spin column method (High Pure PCR Template Preparation Kit, Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions.

Serology

All samples submitted for routine RhD typing were subjected to direct agglutination testing using two serological methods: the manual tile method and the automated microplate method. In the tile method, undiluted and unwashed EDTA blood was mixed with SIHDIA anti-D (human monoclonal IgM/IgG blend, clones TH-28/MS-26, Shinyang Diagnostics, Siheung, South Korea) on an acrylic plate. In the automated microplate method, either NEO (Immucor, Norcross, GA, USA) with Novaclone anti-D (human monoclonal IgM/IgG blend, clones D175-2/D415 1E4, Immucor) or QWALYS 3 (DIAGAST, Loos, France) with ABD-Lys containing monoclonal anti-D (human monoclonal IgM, clone P3X61, Diagast) was used. Samples showing no or equivocal agglutination in initial testing were further tested by the immediate spin tube method using BioClone anti-D (human monoclonal IgM/polyclonal blend, clone MAD2; Ortho Clinical Diagnostics, Raritan, NJ, USA). In case of no agglutination, weak D testing was performed by the gel method (ID-Card LISS/Coombs, BIO-RAD, Hercules, CA, USA) and tube method using BioClone anti-D and SIHDIA anti-D.

RhCE phenotypes were determined using the immediate spin tube method employing anti-C, -E, -c, and -e reagents (Ortho Clinical Diagnostics), and antibody screening tests were performed using either NEO with a two-cell panel (Capture-R Ready-Screen, Immucor) or QWALYS 3 with a three-cell panel (HEMASCREEN, DIAGAST). In the automated microplate method, tube method, and gel method, agglutination was graded as 0, w+, 1+, 2+, 3+, or 4+, whereas in the tile method, it was graded as negative, weakly positive, or positive. Weak reactivity was defined as w+ to 3+ agglutination in the automated microplate method and as weakly positive agglutination in the tile method. A "serologically D-negative phenotype" was defined as one displaying no agglutination in both direct agglutination testing and weak D testing. A sample was

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categorised as having an "RhD typing discrepancy" when the sample was typed as D-positive with one method but showed weak or no reactivity with the other method or when the current RhD type was discordant with the historical RhD type.

RHD genotyping

Samples classified as having a serological weak D phenotype or an RhD typing discrepancy were analysed by full *RHD* gene sequencing upon request from clinicians. All ten exons and flanking intronic regions of the *RHD* gene were amplified using the gene-specific primers described in our previous study[20]. Polymerase chain reaction (PCR) amplification was conducted in a thermal cycler (T100, BIO-RAD) with the following conditions: an initial denaturation step at 94 °C for 5 min, then 32 cycles of denaturation at 94 °C for 30 s, annealing at 64-68 °C (depending on the primer pair used) for 30 s, and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. The PCR products were purified with magnetic beads (MagneSil GREEN, Promega, Madison, WI, USA) and sequenced using a cycle sequencing kit (BigDye Terminator v3.1, Thermo Fisher Scientific, Waltham, MA, USA) on an automated DNA sequencer (3730xl DNA Analyzer, Thermo Fisher Scientific). The sequencing primers used were the same as those used for PCR amplification.

Upon request from clinicians, serologically D-negative samples with a C+ and/or E+ phenotype were tested using PCR-sequence specific primers (PCR-SSP) for the presence of *RHD*-specific sequences of the *RHD* promoter, intron 4, exon 7, and exon 10 according to our previously described method with minor modifications[20]. PCR amplification was carried out in a thermal cycler (T100) under the following conditions: an initial denaturation step at 94 °C for 5 min, 32 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Samples positive for all four targets were subjected to PCR amplification and sequencing of the *RHD* gene exon 9 and flanking intronic regions as described in our previous study with minor modifications[20]. The PCR conditions were as follows: an initial denaturation step at 94 °C for 5 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 7 min. The PCR products were purified and sequenced using the same primer pair as for PCR amplification.

RESULTS

Serological weak D phenotypes

Of a total of 188,852 samples sent for routine RhD typing, 188,358 (99.74%) were typed as D-positive in direct agglutination testing using both the manual tile method and the automated microplate method. Only 454 samples (0.24%) showed no agglutination in both direct agglutination testing and weak D testing (serologically D-negative phenotype). Seven samples (0.004%) exhibited weak reactivity in direct agglutination testing and 25 samples (0.013%) showed agglutination only in weak D testing. Of the 32 samples with a serological weak D phenotype, 23 samples were sent for full *RHD* gene sequencing. The serological and molecular results of these 23 samples are summarised in **Table I**. In 21 samples, 11 distinct D variant alleles were identified, whereas in the remaining two samples no variant was found in the *RHD* gene. The most frequently observed allele was *RHD*15* (n=6), followed by *RHD*13.01* (n=4), and *RHD*01W.25* (n=4). Interestingly, compound heterozygosity was observed in two samples: one sample carried the *RHD*01W.25* and *RHD*01EL.01* alleles, and the other harboured the *RHD*01W.43* and *RHD* (c.413A>C) alleles.

Serologically D-negative phenotypes

The RhCE phenotypes of the 454 serologically D-negative samples were as follows: CCee (11, 2.4%), Ccee (105, 23.1%), CcEe (14, 3.1%), ccEe (59, 13.0%), ccEE (2, 0.4%), and ccee (263, 57.9%). Of the 191 samples typed as C+ and/or E+ by serological testing, 50 underwent PCR-SSP. As a result, ten samples (20%) were classified as having the *RHD* deletion (negative for all four targets), and 13 samples (26%) were classified as having the *RHD-CE-D* hybrid allele (positive for promoter and exon 10 but negative for intron 4 and exon 7). The 27 samples (54%) positive for all four targets were analysed by direct sequencing of exon 9. Among these 27 samples, 26 (96.3%) were found to carry the *RHD*01EL.01* allele, and the remaining sample (3.7%) was found to harbour the *RHD*01EL.10* allele.

RhD typing discrepancies

One sample showed no agglutination in either direct agglutination testing or weak D testing and was thus typed as D-negative, but the historical RhD type of the

Table I - *Results of RhD and RhCE phenotyping and RHD genotyping in 23 Korean patients with a serological weak D phenotype*

No. of patients	*RHD* allele	Nucleotide change	Amino acid change	RhD phenotype		RhCE phenotype
				Direct agglutination	Weak D	
6	*RHD*15*	c.845G>A	p.Gly282Asp	Neg	Positive	ccEe
4	*RHD*13.01*	*RHD-cE(5)-D*		Neg	Positive	ccEe
3	*RHD*01W.25*	c.341G>A	p.Arg114Gln	Neg	Positive	ccEe
1	*RHD*01W.25/RHD*01EL.01*	c.341G>A/c.1227G>A	p.Arg114Gln/p.Lys409=	Neg	Positive	CcEe
1	*RHD*01W.43*	c.605C>T	p.Ala202Val	Weak	NT	Ccee
1	*RHD*01W.43/RHD*(c.413A>C)	c.605C>T/c.413A>C	p.Ala202Val/p.Gln138Pro	Weak	NT	CCee
1	*RHD*01W.24*	c.1013T>C	p.Leu338Pro	Neg	Positive	Ccee
1	*RHD*01W.72*	c.1212C>A	p.Asp404Glu	Weak	NT	CcEe
1	*RHD*01W.102*	c.73A>T	p.Ile25Phe	Weak	NT	ccEe
1	*RHD*06.03.01*	*RHD-CE(3-6)-D*		Neg	Positive	Ccee
1	*RHD*(c.634+5G>A)	c.634+5G>A	Intronic variant	Neg	Positive	ccEe
1	No variant was found in the *RHD* gene			Weak	NT	Ccee
1	No variant was found in the *RHD* gene			Weak	NT	CCee

Neg: negative; NT: not tested.

patient from whom this sample was obtained was a serological weak D phenotype (despite employing the same serological methods and anti-D reagents used in this study). The patient's genotype was *RHD*01W.15*. Seven samples displayed 4+ agglutination using the automated microplate method but weakly positive agglutination using the tile method. Four of the seven discrepant samples were genotyped. As a result, *RHD*01W.33* (n=2) and *RHD*01W.43* (n=1) were identified, and no variant was found in one sample (**Table II**). Notably, one sample with the *RHD*01W.33* allele was also found to harbour the *RHD*01EL.01* allele *in trans* (**Table II**). All samples with a serological weak D phenotype or an RhD typing discrepancy displayed a C+ and/or E+ phenotype, and none of these samples contained anti-D alloantibodies.

DISCUSSION

The prevalence of serological weak D phenotypes differs with race/ethnicity. In the Caucasian population, the prevalence of serological weak D phenotypes is estimated to be relatively high, ranging from 0.2% to 1.0%[22]; however, it is much lower in the Chinese population (0.01-0.02%)[10,23]. In this study, 32 of 188,852 Korean patients (0.017%) were found to have a serological weak D phenotype, and this estimated prevalence is comparable to that in a previous study conducted on 80,430 Korean blood donors (0.014%)[24]. In addition, different RhD typing methods could result in substantially different estimates for the prevalence of serological weak D phenotypes[6]. If the automated microplate method had not been used in this study, seven samples would have reported

Table II - *Serological and molecular results of four D variant samples mistyped as D-positive by either of the two automated microplate methods*

No. of patients	RhD phenotype			RhCE phenotype	RHD allele	Nucleotide change	Amino acid change
	Direct agglutination						
	Microplate	*Tile*	*Tube*				
1	4+ (Neo)	WP	w+	Ccee	*RHD*01W.33*	c.520G>A	p.Val174Met
1	4+ (QWALYS 3)	WP	1+	CCee	*RHD*01W.33/RHD*01EL.01*	c.520G>A/c.1227G>A	p.Val174Met/p.Lys409=
1	4+ (QWALYS 3)	WP	2+	Ccee	*RHD*01W.43*	c.605C>T	p.Ala202Val
1	4+ (Neo)	WP	w+	CcEe	No variant was found in the *RHD* gene		

WP: weakly positive.

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discrepancies between the automated microplate method and the manual tile method (0.004%), and would have been classified as having a serological weak D phenotype. Thus, the estimated prevalence of serological weak D phenotypes would have been increased from 0.017% to 0.021%. This finding indicates that the use of sensitive RhD typing methods can underestimate the prevalence of serological weak D phenotypes.

The prevalence of DEL phenotypes is estimated to be 17-30% among serologically D-negative East Asians, and more than 95% of East Asians with a DEL phenotype are estimated to carry the *RHD*01EL.01* allele, also known as the "Asia type DEL"[25,26]. The *RHD*01EL.01* allele is invariably associated with the Ce or cE haplotype[19,20,27-32]. Determining the presence of the *RHD*01EL.01* allele is clinically important, as it encodes a complete D antigen with all epitopes intact, albeit of very low quantity[33-35], and D-negative patients carrying this allele are not at risk of developing anti-D alloantibodies when exposed to D-positive RBCs[36-38]. Therefore, it is recommended that *RHD* genotyping be performed in D-negative East Asian patients with a C+ and/or E+ phenotype, and patients found to harbour the *RHD*01EL.01* allele should be managed as D-positive. Owing to the relatively high frequency of the *RHD*01EL.01* allele in East Asians, this allele is often found *in trans* with another D variant allele[11,13,16,35]. In two Korean patients in our study, the *RHD*01EL.01* allele was found *in trans* with the *RHD*01W.25* or *RHD*01W.33* allele. The patients' RBCs are predicted to contain D antigens with all epitopes intact encoded by the *RHD*01EL.01* allele, as well as D antigens encoded by the *RHD*01W.25* or *RHD*01W.33* allele. Our group has recently shown that RBCs from individuals with a D variant allele *in trans* with the *RHD*01EL.01* allele possess complete D antigens with all epitopes intact on their surface[35]. Hence, as seen in D-negative patients carrying the *RHD*01EL.01* allele, weak D patients harboring the *RHD*01EL.01* allele could also be safely managed as D-positive. Therefore, *RHD* genotyping should be extended to East Asian patients with a serological weak D phenotype or an RhD typing discrepancy.

In 2015, the American Association of Blood Banks (AABB)-College of American Pathologists (CAP) Working Group recommended that *RHD* genotyping be performed in patients with a serological weak D phenotype, as patients carrying any of the three most prevalent alleles in Caucasians with a serological weak D phenotype (*RHD*01W.1*, *RHD*01W.2*, and *RHD*01W.3*) can be safely managed as D-positive[5]. This practice allows optimal allocation of scarce D-negative RBCs and prevents unnecessary administration of Rh immune globulin (RhIG)[5]. Very recently, members of the AABB-CAP Working Group have updated their recommendations that patients with the *RHD*09.03.01* (weak D type 4.0) or *RHD*09.04* (weak D type 4.1) allele also be managed as D-positive (except pregnant women with the *RHD*09.03.01* allele)[39]. However, the *RHD*01W.1*, *RHD*01W.2*, *RHD*01W.3*, *RHD*09.03.01*, and *RHD*09.04* alleles were not found in our cohort, suggesting that these alleles are very rare, if found at all, in the Korean population, as seen in Chinese[8-16] and Japanese[17,18] populations. In the Caucasian population, approximately 5-10% of serological weak D phenotypes are estimated to be partial D phenotypes[22]. But this study reveals that partial D phenotypes account for a substantial proportion of serological weak D phenotypes in the Korean population, as two of the three most common alleles, *RHD*15* (weak D type 15)[40] and *RHD*13.01* (DBS-1)[41-43], are known to encode partial D phenotypes. *RHD*15* is a common D variant allele in Chinese[8-11,13-16], Japanese[17,18], and Korean[19] individuals with a serological weak D phenotype; however, this allele is not common in Caucasians with a serological weak D phenotype[44-48]. Given the rare frequency of D variant alleles which can be safely managed as D-positive (*RHD*01W.1*, *RHD*01W.2*, *RHD*01W.3*, *RHD*09.03.01*, and *RHD*09.04*) in the East Asian population, genotyping strategies targeting these alleles are of limited utility in East Asian patients.

Most serological weak D phenotypes result from sequence variants in exons and flanking intronic regions of the *RHD* gene, which can be detected using our *RHD* sequencing assay. However, in two patients with a serological weak D phenotype and one patient with an RhD typing discrepancy, we found no variant in the *RHD* gene. In these cases, sequence variants in deep intronic regions of the *RHD* gene or in genes responsible for membrane expression of Rh proteins, such as *RHAG* and *ANK1*, might result in weak D expression. One study has recently shown that a missense variant of the *RHAG* gene could cause a serological weak D phenotype without affecting RhCE expression[49]. Further studies are needed to identify the

molecular causes of weak D expression in these patients with an apparently intact *RHD* gene (*RHD*01*).

Automated immunohaematology analysers have been developed to reduce the workload of laboratory technicians and to improve precision and accuracy by minimising manual procedures, and more and more laboratories are adopting these[50]. Despite these advantages, it is important to recognise that ABO and RhD groups can be misclassified using automated immunohaematology analysers. Our group previously revealed that some cisAB samples were mistyped as group AB using the automated microplate method[51]. Likewise, this study revealed that some D variant samples including *RHD*01W.33* are at risk of being mistyped as D-positive by the automated microplate method. This finding suggests that when using an automated immunohaematology analyser alone in direct agglutination testing, patients with a D variant phenotype could be classified and managed as D-positive, possibly leading to an increased incidence of anti-D alloimmunisation. Hence, when using an automated immunohaematology analyser, it may be useful to use a less sensitive manual method to double-check the patient's RhD status. The manual tube method has traditionally been considered as the gold standard, but this is labour-intensive, imposing a significant burden on clinical laboratories. The manual tile method is simpler and less time-consuming to perform than the manual tube method and is thus being used as a routine RhD typing method in many clinical laboratories in South Korea.

Based on our study findings, an effective RhD typing strategy for East Asian patients has been developed (**Figure 1**). Although this study only included Korean patients, we believe that the RhD typing strategy proposed in this study is also applicable to other East Asian patients, and we and many other studies[8-11,13-20,28-32] suggest that the distribution of D variant alleles including *RHD*01EL.01* in Koreans is comparable to that in other East Asians. With this strategy, East Asian patients are screened for the presence of the *RHD*01EL.01* allele when weak reactivity or discordant results are observed in direct agglutination

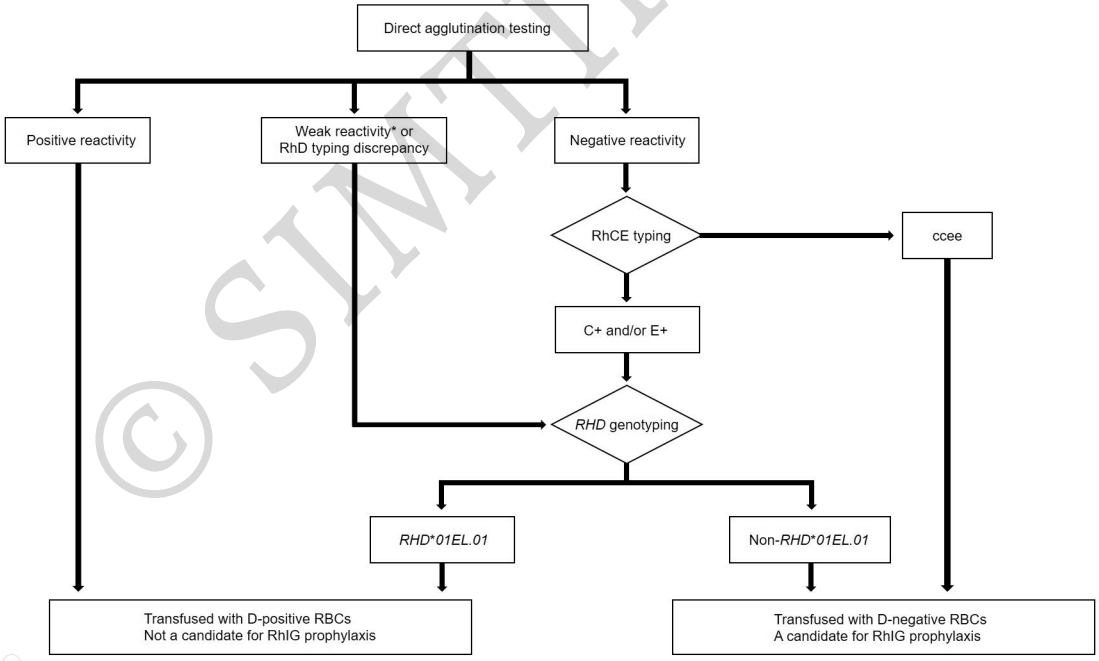

Figure 1 - RhD typing strategy recommended for East Asian patients
Under this strategy, East Asian patients are tested by *RHD* genotyping when weak reactivity or discordant results are observed in direct agglutination testing. As previously suggested by our group[20], D-negative East Asian patients carrying a C+ and/or E+ phenotype are tested by *RHD* genotyping. Patients are classified and managed as D-positive when the *RHD*01EL.01* allele is detected by *RHD* genotyping. *The definition of weak reactivity can vary from laboratory to laboratory, depending on the serological method and anti-D reagent used for direct agglutination testing.

testing. Patients confirmed to have the *RHD*01EL.01* allele are managed as D-positive, whereas patients without the *RHD*01EL.01* allele are managed as D-negative. RhCE typing is used as an initial screening strategy for D-negative East Asian patients, and those with a ccee phenotype are considered as D-negative without the need for *RHD* genotyping, as previously suggested by our group[20]. D-negative East Asian patients with a C+ and/or E+ phenotype are screened for the presence of the *RHD*01EL.01* allele. The D-negative patients found to have the *RHD*01EL.01* allele are managed as D-positive, whereas those without this allele are managed as D-negative. Genotyping assays to rapidly screen for the *RHD*01EL.01* allele have been developed[52,53]. However, it is important to note that RhD typing strategies customised for East Asian patients may not be useful for foreign patients in East Asia. During the study period, one Greek patient with a serological weak D phenotype (not included in the present study) was found by *RHD* sequencing to have the *RHD*01W.3* allele. Using the *RHD* genotyping method targeting only the *RHD*01EL.01* allele suggested in this study, this patient would have been misclassified and mismanaged as D-negative. However, the number of weak D patients misclassified and mismanaged by the genotyping method is thought to be very small in East Asian countries that are relatively racially/ethnically homogeneous.

CONCLUSIONS

To our knowledge, the present study is the first to extensively investigate the molecular basis of serological weak D phenotypes and RhD typing discrepancies in the Korean population. Here, we identified various D variant alleles including *RHD*15*, *RHD*13.01*, and *RHD*01W.25*; however, *RHD*01W.1*, *RHD*01W.2*, *RHD*01W.3*, *RHD*09.03.01*, and *RHD*09.04* alleles, accounting for more than 95% of Caucasians with a serological weak D phenotype, were not found. Our data suggest that the distribution of D variant alleles differs between East Asians and Caucasians. Our findings also confirm that some D variants including *RHD*01W.33* could be mistyped as D-positive by highly sensitive RhD typing methods such as the automated microplate method.

AUTHORSHIP CONTRIBUTIONS

YNC and TYK contributed equally to this work. All authors contributed to the collection, analysis, and interpretation of data. YNC, TYK, and DC drafted the first version of the manuscript. All authors reviewed and edited the manuscript.

The Authors declare no conflicts of interest.

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