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Original Article

RHD haplotype analysis of a D negative cohort

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ABSTRACT

This study aimed to explore the distribution of the variant *RHD* genotypes in Jiangsu, China. A total of 108 541 blood donor samples and 17 family members were tested from 2020 to 2021. A total of 121 *RHD* variants were found in 414 D negative samples and their families, including seven weak D, four partial, and three Del alleles. *RHD*15* and *RHD*DVI.3* were the prevalent D variant alleles in Jiangsu Province. Heterozygous alleles were identified (c.730G>C, c.1227G>A), and a case of unique intronic mutation was detected (c.802-41_802-38delCTCT). This study suggests that the frequency of *RHD* variants is crucial for establishing specific *RHD* genotyping strategies and can be applied in larger-scale routine tests for blood donors.

Keywords: D variants, *RHD* genotyping, heterozygosity, gene frequency

INTRODUCTION

The Rh blood group system is one of the most complex antigen systems in blood transfusion. In general, the D antigen was reported to exhibit the strongest immunogenicity, which is closely associated with hemolytic disease of the fetus and newborn (HDFN)[1–2]. Rh blood group system antigens are determined by two gene cassettes of *RHD* and *RHCE* located on chromosome 1p34-36[3]. Due to their high similarity, the genes encoding RHD and RHCE proteins are frequently exchanged or hybridized[4]. Furthermore, various *RHD* genotypes are responsible for a weak or incomplete D-epitope expression on erythrocytes. Typically, Del type may be mistyped as D negative, leading to incompatible transfusion to RHD negative recipients, causing possible anti-D immunization[5–6]. Recently, routine RHD confirmation was conducted by three or four monoclonal anti-D reagents involving more than a minimum of 30 different epitopes[7]. However, routine serological tests cannot identify all D epitopes, thus leading to undetected D variants. Therefore, the ethnic specialties of *RHD* alleles and the diversity of *RHD* variants in a target group of D-negative blood donors were explored. Genotyping and DNA sequencing were carried out to reveal the molecular basis of D variants in Jiangsu Province and significant therapeutic implications for clinical transfusion.

MATERIALS AND METHODS

Blood donor samples

A total of 108 541 blood donor samples and 17

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families' samples were collected by the Nanjing Red Cross Blood Center in Jiangsu Province. Informed consents were obtained from all included subjects before sample collection. Routine donor screening for RHD was conducted by Microlab Star BG Machine (SN_905B) with an anti-D reagent. The RHD donor samples initially typed as D negative in routine tests were chosen for subsequent verification.

Serological assays

Four human monoclonal anti-D IgM/IgG blend (D175-2, D415 1E4) and anti-D IgM (RH-1) were bought from DIAGAST, France. Anti-D IgM/IgG blend (TH28, MS26) was bought from Millipore, U.K., and anti-D IgM (MS26) was bought from Shanghai Blood Biopharmaceutical Co., Ltd. These reagents were used for manual phenotyping in suspected samples. The samples with weak (2+ or less) and inconsistent reactions in the immediate spin method and indirect antiglobulin test (IAT) were selected for *RHD* genotyping confirmation. All weak D donors were classified into the RHD positive category.

Genotyping investigation

RHD exon sequencing was performed in this study. Genomic DNA was extracted from 200 μL of peripheral blood (EDTA-K2 anticoagulant) using a Quick Gene-MINI80 DNA extractor, according to the manufacturer's instructions, and the NanoPhotometer Pearl (Implen GmbH, Germany) was used to determine the concentration and purity of DNA. The extracted DNA samples were sent to Jiangsu Zojiwat Biopharmaceutical Co., Ltd. for sequencing analysis of *RHD* gene exon 1 to exon 10 by the Sanger method.

RESULTS

During the one-year period, 0.1% of blood donor samples showed weak or atypical agglutination results in serologic D screening.

Weak D types

Specific allele PCR performed for the identification of weak D types suggested that *RHD*weak partial 15* (*RHD*15*) was the most common variant *RHD* allele in Jiangsu Province (66.7%) followed by *RHD*weak D type 33* (12.5%), *RHD*weak D type 41.01* (4.2%), *RHD*weak D type 54* (4.2%), *RHD*weak D type 72* (4.2%), *RHD*weak D type 95* (4.2%), and *RHD*weak D type 132* (4.2%) (***Table 1***).

Partial D types

In addition, *RHD* variant alleles associated with partial D, such as *RHD*DV type 2* (4.7%), *RHD*DVI type 3.2* (14.2%), *RHD*DVI type 4* (14.2%), and *RHD*DVI type 3* (66.7%) were identified (***Table 1***).

Del

Moreover, *RHD*01EL.01* (44.7%), *RHD*01EL.20* (6.6%), and *RHD*01EL.32* (48.7%) were found in samples (***Table 1***).

DISCUSSION

Previous studies showed that RHD negative samples may contain multiple alleles and have different genetic backgrounds due to ethnic differences[8–9]. Mutations within the *RHD* gene and individual differences lead to different typing results. RHD is regarded as a type Ⅳ transmembrane protein, which is divided into intracellular, transmembrane, and extracellular regions, relying on its proline structure as a channel switch for regulation[10].

In general, the weak D type is a variant of the RHD protein comprising an amino acid substitution located in the transmembrane or intracellular segments, which may have normal RHD epitopes with only a slight decrease in RHD antigen expression[9]. Weak D antigen is considered to have essentially intact epitopes of the RHD antigen, but with reduced simplicity of the D antigen locus. Thus, weak D types are usually not alloimmunized, except for some weak D types, such as weak D type 15 correlated to the partial D. The possible reason is that *RHD* gene mutations can lead to amino acid sequence changes in the RHD protein's intracellular district and transmembrane area, while having no effect on antigen epitopes except for the efficiency of mRNA recognition[11–13].

The RHD Del type exhibits reduced erythrocyte expression on red blood cells (RBCs)[9]. Del type accounts for a high proportion of the Asian population and can only be identified in adsorption and elution experiments. Some believe that it is caused by differences in gene regulation, while others believe that it is resulted from gene mutations[14–16].

Partial D identification is of clinically significance as carriers usually produce anti-D antibodies upon exposure to normal D antigens[17–18]. Partial D is considered to have a reduction in the number of expressed antigens like weak D, as well as the loss of epitopes, with mutations in the gene occurring mainly in the nucleic acid sequences encoding the extracellular region of the RHD protein. However, the

Table 1 **D variants detected in Jiangsu blood donors based on the Sanger sequencing**

Genotype	Nucleotide change	Rh phenotype	*n*	%
Weak partial D 15	E6: c.845G>A	CcDee	6	4.96
		CCDee	3	2.48
		CcDEe	2	1.65
		ccDEe	3	2.48
		ccDee	2	1.65
Weak D type 33	E4: c520G>A	ccDEe	2	1.65
		CcDee	1	0.83
Weak D type 41.01	E9: c.1170T>C, c.1193A>T	ccDEe	1	0.83
Weak D type 54	E3: c.365C>T	CcDee	1	0.83
Weak D type 72	E9:c1212C>A	ccDee	1	0.83
Weak D type 95/Del	c.730G>C, c.1227G>A	CcDee	1	0.83
Weak D type 132	c.394G>A	ccDEe	1	0.83
DV type 2	RHCE (5)	ccDee	1	0.83
DVI type 3.2	RHCE (3-9)	ccDEe	2	1.65
		CcDee	1	0.83
DVI type 4	RHCE (3-5)	CcDEe	1	0.83
		CcDEe	2	1.65
DVI type3	RHCE (3-6)	CcDee	4	3.31
		CcDee	3	2.48
		ccDEE	2	1.65
		ccDEe	5	4.13
*RHD*01EL.01*	E9: c.1227G>A	CcDee	16	13.22
		CcDEe	11	9.09
		CCDee	4	3.31
		ccDEE	1	0.83
		ccDEe	2	1.65
*RHD*01EL.20*	c.1154-8T>A	CcDee	3	2.48
		CCDee	2	1.65
*RHD*01EL.32*	c.149-29G>C	CcDEe	19	15.70
		CCDee	9	7.44
		CcDee	6	4.96
		ccDEe	3	2.48
Total			121	100

n: number of individuals.

boundary between partial D and weak D is not definitive, and serological techniques or PCR-SSP assays are unable to distinguish them. DNA sequencing is a preferable way to improve immunohematology typing.

In this study, we observed 121 *RHD* variants (28.1%) in D-negative cohorts and their families. Furthermore, 12 variants (2.8%) with an ordinarily serological phenotype were found among them, and 26.3% of the D-negative blood donors in Jiangsu Province had weak serological reactions. We also found that *RHD*15* and *RHD*DVI.3* were the typical D variants in this region. Weak partial D 15 accounted for 66.67% of the total weak D. Single nucleotide mutation (*RHD* c.845G>A) was the most common type in the Chinese population studied, which was consistent with previous studies[19–21]. A particular *RHD* allele frequency may be related to the ethnic composition of the population. The mutation of weak D 15 occurred in the sixth exon and ninth transmembrane region of the *RHD* gene (c.845G>A, p.Gly282Asp), which may affect the membrane's integrity, thereby affecting the synthesis and expression of RHD protein and D antigen. Among 21 cases of partial D phenotype, DVI type 3 accounted for 66.67% of the total number of partial D phenotypes and was generally the predominant type of partial D phenotypes in the Chinese population, which is consistent with the findings from other studies[21–22]. *RHD*DVI.3*, also known as *RHD-RHCE (3-6)-RHD,* was a hybrid allele formed by replacing exons 3 to 6 of the *RHD* gene with the corresponding part of the *RHCE* gene[23].

RHD-RHCE-RHD gene fusion leads to the substituting of single amino acids or multiple amino acids in the extracellular ring, resulting in the loss of

the D epitope or affecting the 3D conformation of the extracellular loop, hence the partial D[24]. The individuals identified as D-positive transfusion recipients may develop antibodies against the missing epitopes, resulting in potential transfusion reactions[25]. In addition, blood products from partial D phenotype donors could be transfused to D-negative individuals and may have a probability of producing anti-D antibodies. Therefore, the detection of partial D gene mutations is of great significance in predicting the potential risk of anti-D alloimmunization in blood transfusion or pregnancy[26–27]. Besides, RHD Del accounted for 18.36% of RHD negative and 62.81% of the D variants. *RHD*01EL.01* and *RHD*01EL*.32 were the dominant types in Del samples. The heterozygous alleles accounted for slightly more numbers than the homozygous types in *RHD*01EL.01* samples. Although presented results suggested that the mutation *RHD* c.149-29G>C was the most common mutation among the 76 Del phenotype individuals, we identified eight blood samples with c.149-29G>C, demonstrating 3+ to 4+ in a serological test for D antigen. Based on our experimental observation, we speculate that the intronic variant *RHD* c.149-29G>C may not cause a Del phenotype.

It is worth noting that the mutation (c.802-41_802-38delCTCT) between intron 5 and exon 6, with four bases missing, was detected in one sample. This mutation, along with c.1157T>A, was reported by International Society of Blood Transfusion (ISBT) and designated *RHD*01N.58*. This mutation may lead to the early generation of termination codon, making the D antigen unable to express. However, compared with the allele *RHD*01N.58*, this sample lacks the c.1157T>A mutation and does not affect overall D antigen expression. Two mutations of c.1157T>A and c.802-41_802-38delCTCT are also known to have coexisted, which might cause weak D expression.

The distribution of D variant alleles observed in Jiangsu Province differs from other regions in China. *RHD*15* is dominant in D variants in Chinese populations, represented by Jiangsu, Shanghai, Zhejiang, Guangdong, and Shaanxi Province[28–32]. *RHD*DVI.3* has high frequency in Guangdong and Shaanxi Province. Besides, the prevalence of *RHD*DEL.01* is mainly in the eastern part of China and higher in Shanghai and Zhejiang Province compared with other populations.

RH polymorphisms were found to be more diverse in the Jiangsu Province, probably because the haplotype sequencing analysis was used. However, the laboratory equipment and time-consuming testing may restrict the application of molecular technologies. A comparative study of D antigen expression by serological screening and RHD molecular properties should be greatly advocated in view of the clinical significance of the RH system in the field of transfusion. The importance of knowing the occurrence of weak and partial RHD variants in populations and regions from different countries worldwide should be stressed. Establishing a haplotype gene pool and developing specified *RHD* genotyping strategies are beneficial for predicting genotypes in transfusion medicine and discovering other unreported heterozygous types in the Chinese population.

In summary, our study provided insights into the *RHD* gene polymorphisms of Jiangsu Province and a comparison with other densely populated regions of China. Specifically, this study renewed knowledge on the distribution of variant *RHD* genotypes in Jiangsu Province. The findings of some rare or concealed genotypes are still dependent on Sanger Sequencing to avoid the probability of missed detection. Moreover, our results suggest that a larger sample cohort is needed to reinforce this observation, which means that large numbers of ethnic minorities and mixed-race areas would affect the minority results. The observation here indicates the potentially important role of understanding the reasons for atypical or discrepant D typing reactions in transfusion medicine to reduce the risk of alloimmunization.

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