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ORIGINAL ARTICLE

RHD Genotyping by Molecular Analysis of Hybrid *Rhesus box* in RhD-Negative Blood Donors from Iran

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Abstract D antigen is the most important and immunogenic antigen of the Rh blood group. The RhD-negative phenotype has different genetic backgrounds with variable distribution in different populations. Hybrid *Rhesus box*, resulting from *RHD* gene deletion, is used in genotyping studies of the Rh blood group as a marker to identify the *RHD* gene deletion. This study for the first time identified genetic mechanisms for the occurrence of RhD-negative phenotype among the Iranian population. 200 RhD-negative blood donors were randomly selected from Tehran Blood Transfusion Center. The phenotype of D, C, E, e and c antigens was serologically identified, and DNA was extracted from buffy coat. The molecular analysis of hybrid *Rhesus box* was performed by PCR-SSP and PCR-RFLP. Moreover, the presence of different exons of *RHD* gene was investigated by real-time PCR on extracted DNA. Hybrid *Rhesus box* was detected in all samples, and PCR-RFLP confirmed that 198 (99%) were *homozygous* for an *RHD* gene deletion and 2 were *heterozygous* for hybrid *Rhesus box* in which one (0.5%) had a weak D type 11 and the other one (0.5%) had a *RHD-CE (2-9)-D_2* hybrid allele. Similar to Caucasians, the frequency of *RHD* gene deletion was high among the Iranian population studied in this investigation, so hybrid *Rhesus box* can be used as an efficient marker to detect *RHD* gene deletion in our population.

Introduction

The antigen D is the most important and immunogenic antigen of the Rh blood group [1, 2]. The proper identification of RhD antigen has a high clinical significance, and can play an important role in preventing the occurrence of hemolytic anemia in patients and newborns [2]. Serology is the standard method for detection of RhD antigen, but has limitations. Genotyping by molecular techniques is a complementary tool to overcome these limitations. Some of the clinical applications of these methods are determination of *RHD* zygosity in RhD-positive fathers, assessment of RhD status in the fetus and multi-transfused patients, resolving the discrepancies in RhD typing in blood donors and patients with auto or alloantibodies [3, 4]. Knowing the prevalence of alleles responsible for the phenotype in a special population, and the establishment of appropriate molecular methods for detection of alleles are required for genotyping and its application in the clinic [5]. Whereas in Caucasians deletion of the entire RHD gene is the most common cause of the RhD-negative phenotype, *RHD*ψ (66%), *RHD-CE-D* hybrid gene (15%) and *RHD* gene deletion (18%) are the most prevalent causes of the RhD-negative phenotype among black Africans [1, 6]. Studies have shown that the D-negative phenotype is present in about 10.08% of the Iranian population [7], but there are not any reports identifying the molecular background of D-negative phenotypes in our country. This study for the first time identified the genetic mechanisms of RhD-negative phenotype among the Iranian population. The hybrid *Rhesus box* is a genomic segment with an

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approximate length of 9000 base, resulting from the *RHD* gene deletion and the integration of two segments of upstream and downstream *Rhesus boxes* that flank the *RHD* gene. This hybrid sequence was used as a marker to identify the *RHD* gene deletion [1]. Regarding the similarity of RhD-negative frequency among Caucasians and Iranian population and high prevalence of *RHD* gene deletion in Caucasians, we decided to evaluate the presence of hybrid *Rhesus box* genomic segment by PCR-SSP and PCR-RFLP. In addition, we performed real-time PCR to investigate the presence of *RHD* gene exons in our samples as a confirmatory test for gene deletion.

Materials and Methods

Sample Collection

Blood samples obtained into K3EDTA-containing tubes from 200 RhD-negative randomly selected donors at the Tehran Blood Transfusion Center. This study was approved by the Ethics Committee of the Institute for Educational and Research in Transfusion Medicine.

Phenotype Determination

The antigenic phenotype (D, C, E, c, e) of red blood cells were determined for all specimens using a monoclonal antibody (IMMUNDIAGNOSTIKA, GmbH, Germany) by standard serological method. Monoclonal antibody reagents were used to test the following specificities: anti-C (RH2, clone MS24), anti-E (RH3, clone MS80/MS258), anti-c (RH4, clone MS33) and anti-e (RH5, clone MS62/MS69).The anti-D reagent was a blend of both IgG and IgM. Subsequently, weak D test was performed on all samples by indirect antiglobulin test (IAT) according to the standard serological guidelines. Adsorption–elution test was not performed in this study.

DNA Extraction

Genomic DNA was extracted from buffy coat samples using column-based DNA extraction kit (YT 9040, YEKTA TAJHIZ AZMA, IRAN). The extracted DNA was stored at − 20 °C after determining the concentration using the *Nano Drop* (one/one^c, Thermo Fisher Scientific Inc, USA).

PCR-SSP

A 2778-bp fragment was amplified from the hybrid *Rhesus box* gene, causing by deletion the *RHD* gene. This segment flanking the breakpoint region in the hybrid *Rhesus box*

gene comprised of segments from upstream *Rhesus box* (775 bp), identity region (1467 bp) and downstream *Rhesus box* (536 bp) [1]. The segment was amplified by the forward primer (u1-s) and reverse primer (rnb31) (Table 1). PCR reaction was performed in a volume of 25 μl in 35 cycles using thermal cycler (PEQSTAR 2X 95-08002, PEQLAB Biotechnology GmbH, Germany) under the following conditions: initial denaturation at 95 °C for 10 min, denaturation at 92 °C for 20 s, annealing at 64 °C for 30 s, extension at 68 °C for 3 min, final extension at 72 °C for 5 min. The concentration of forward and reverse primers was 0.4 μm. The PCR products were electrophoresed on 0.8% agarose gel.

PCR-RFLP

The PCR-RFLP was used to discriminate the status of homo- or heterozygosity of *RHD* gene deletion allele [1]. The primers were designed so that they could amplify both fragments of the hybrid *Rhesus box* in the deletion of *RHD* gene and also of the downstream *Rhesus box* in the presence of *RHD* gene [1, 8, 9]. The sequences of forward (Rez7) and reverse (rnb31) primers are shown in Table 1. PCR reaction was performed as described for PCR-SSP, but the number of cycles was 30 and annealing temperature was 68 °C. The PCR products were digested with *Pst*I enzyme (Jena Bioscience, GmbH, Germany) for 1 h at 37 °C and electrophoresed on 2% agarose gel.

Real-time PCR

The real-time PCR was used to investigate the presence of three exons 5, 7 and 10 in the *RHD* gene by sequence-specific primers (Table 2). The RHD-positive sample was used as a control for the amplification of exons and melting curve analysis of PCR products. In the presence of *RHD* gene deletion, none of the three exons would be amplified, while in the presence of any *RHD* allele that resulted in RhD-negative phenotype it was expected that one or more of these exons would be amplified based on the type of the allele [10, 11].

Real-time PCR reaction was performed in a volume of 25 μl in 30 cycles using thermal cycler (Rotor-Gene, RG3000, Corbett, Australia) and SYBR Green Master Mix under the following conditions: initial denaturation at 94 °C for 2 min, denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s. The concentration of forward and reverse primers for each exon was 0.4 μm.

RHD-CE-D Hybrid Molecular Analysis

We initially analyzed the presence of exons 3, 4, 6 and 9 of *RHD* gene using the PCR-SSP method [13] in one sample

Table 1 Sequence and specificity of primers comprising segments of hybrid (AJ252313), downstream (AJ252312) and upstream *Rhesus box* (AJ252311) (Reproduced with permission from [1, 8, 9])

Primer	Sequence	Specificity
u1-s	TGAGCCTATAAAATCCAAAGCAAGTTAG	Hybrid and upstream *Rhesus box*
rnb31	CCTTTTTTTGTTTGTTTTTGGCGGTGC	Hybrid and downstream *Rhesus box*
rez7	CCTGTCCCCATGATTCAGTTACC	Hybrid and downstream and upstream *Rhesus box*

Table 2 Primers' sequence of exons 5, 7, and 10 of the *RHD* gene (Reproduced with permission from [12])

Gene	Forward/reverse	Sequence	Product size
Exon 5	F	5′-CGCCCTCTTCTTGTGGATG-3′	82 bp
	R	5′-GAACACGGCATTCTTCCTTTC-3′	
Exon 7	F	5′-CTCCATCATGGGCTACAA-3′	90 bp
	R	5′-CCGGCTCCGACGGTATC-3′	
Exon 10	F	5′-CCTCTCACTGTTGCCTGCATT-3′	74 bp
	R	5′-AGTGCCTGCGCGAACATT-3′	

whose real-time PCR reaction was positive for exon 10. Then, we investigated the presence of introns 1 and 2 of the *RHD* gene by PCR-RFLP [14, 15].

Weak D Molecular Analysis

Weak D molecular diagnostic kit (inno-train, Diagnostic GmbH, Germany) was used for further analysis of the positive sample in which the three exons 5, 7 and 10 were amplified by RT-PCR according to the manufacturer's guidelines. Exon 6 was sequenced (ABI 3130 XL Applied Biosystem, Macrogene, Korea) to confirm the weak D allele type.

Results

Rh Antigen Phenotypes

The phenotype of the antigens D, C, E, c, e was determined on samples of 200 blood donors (Table 3). An indirect antiglobulin test was performed for the elimination of donors with weak D phenotype. The result of indirect antiglobulin test proved negative in all donors.

Hybrid *Rhesus box* Gene Analysis by PCR-SSP

The genomic segment with a length of 2778 bp was amplified using PCR-SSP technique, indicating that all 200 (100%) donors were positive for hybrid *Rhesus box* gene in at least one allele (Fig. 1).

Zygosity Analysis of Hybrid *Rhesus box* Gene by PCR-RFLP

A genomic segment with an approximate length of 3030 bp was amplified by PCR and specific primers and digested with *Pst*I enzyme. The number and size of fragments on a 2% agarose gel showed that 198 (99%) of donors had a hybrid *Rhesus box* genomic segment in both RH gene alleles (homozygote) (Fig. 2a) and the 2 remaining donors (1%) had a hybrid *Rhesus box* in one of the RH gene alleles (heterozygote); in the latter, in addition to hybrid *Rhesus box*, a genomic segment was amplified from downstream *Rhesus box* of another allele (Fig. 2b).

RHD Exons Analysis

In this study, none of the exons 5, 7 and 10 were amplified in 198 (99%) of 200 samples studied by real-

Table 3 Frequency of phenotypes and predicted genotypes

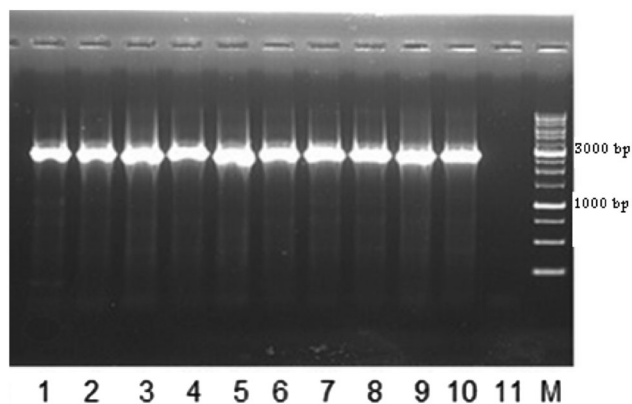| Phenotypes	The most probable genotypes	Number(200 samples)	Frequency (%)
D−C−E−c+e+	cde/cde	184	92
D−C+E−c−e+	Cde/Cde	4	2
D−C+E−c+e+	Cde/cde	11	5.5
D−C−E+c+e+	cdE/cde	1	0.5

Fig. 1 PCR-SSP for hybrid *Rhesus box* gene. A 2778 bp fragment was amplified by PCR-SSP indicates the presence of hybrid *Rhesus box* gene (lanes 1–10). Lane 11: Negative control. M: Molecular marker 1 kb (YT8507, YEKTA TAJHIZ AZMA, IRAN)

time PCR. Of the two remaining samples (1%), all three exons 5, 7 and 10 were amplified in one sample, and in the other one only exon 10 was amplified. In the positive sample for all three exons 5, 7 and 10 with more molecular analysis by the diagnostic kit, weak D allele type 11 (885 G>T) was detected and confirmed by sequencing of exon 6. In the sample that was positive only for exon 10, the results of PCR-SSP for exons 3, 4, 6 and 9 indicated that these exons were absent in the *RHD* gene in this case. Results of molecular analysis of introns 1 and 2 showed that this sample is a *RHD-CE (2-9)-D₂* hybrid allele. (Table 4).

Discussion

The prevalence of D-negative phenotype is estimated to be between 15 and 17% among Caucasians, 5% in black Africans and less than 3% in Asians [16]. The prevalence of D-negative phenotype in Iran is estimated at approximately 10.08% [7], which is more consistent with the Caucasian population. This study for the first time identified the genetic mechanisms of D-negative phenotype among the Iranian population.

Our results, using the PCR-SSP, showed that all donors were positive for the presence of a 2778 bp genomic segment obtained from the hybrid *Rhesus box* gene. The amplification of this segment implies that all donors have at least one *RHD* gene deletion allele, and another allele of the *RHD* gene may be a deletion or non-deletion allele. Therefore, the PCR-RFLP was used to check the status of the other allele for the presence or absence of *RHD* gene.

The results of PCR-RFLP showed that 198 (99%) of donors in both RH gene alleles had a hybrid *Rhesus box* genomic segment, which means that these cases are homozygote for *RHD* gene deletion. Analysis of exons 5, 7 and 10 using real-time PCR also showed that none of the three exons were amplified in these 198 donors, and the PCR-SSP and PCR-RFLP results were confirmed. Moreover, the PCR-RFLP results showed that 2 (1%) of the RhD-negative donors had a *RHD* gene allele. The analysis of exons 5, 7 and 10 using real-time PCR showed that all three exons 5, 7 and 10 were amplified in one sample, but,
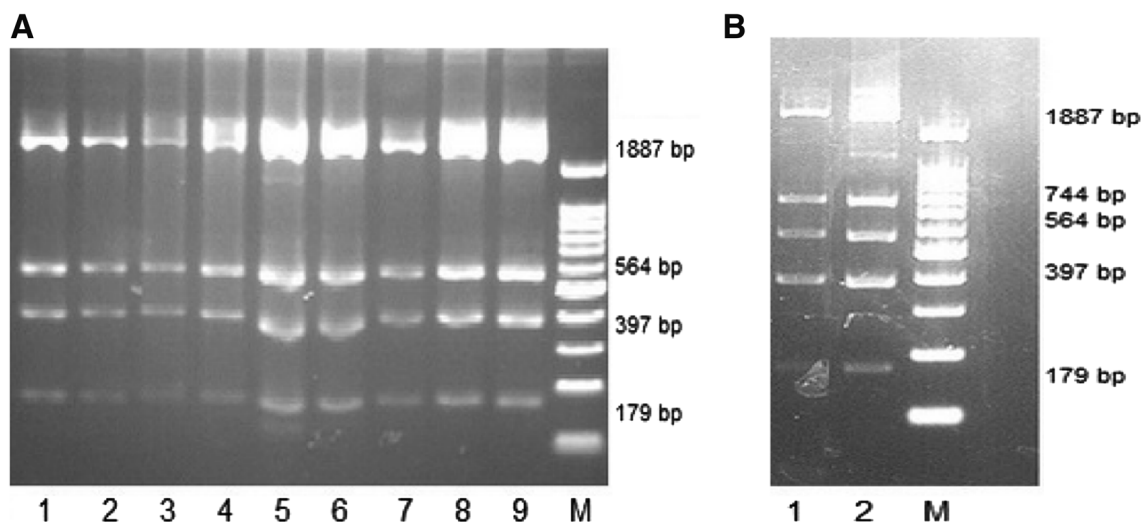

Fig. 2 PCR-RFLP analysis for RHD gene deletion. **a** Banding patterns of RHD−/RHD− homozygotes. There are 3 *Pst*I sites in the hybrid *Rhesus box* amplicons. Digestion with this enzyme (*Pst*I) results in 179, 397, 564, 1887-bp fragments (lanes 1–9). **b** PCR-RFLP for RHD+/RHD− heterozygous samples. An additional 744-bp fragment results from downstream *Rhesus box* digestion and indicates the heterozygous situation (lanes 1–2). M: Molecular marker 100 bp (YT8503, YEKTA TAJHIZ AZMA, IRAN)

Table 4 Frequency of *RHD* gene alleles in RhD-negative samples

RHD gene alleles	Number (200 samples)	(%)	Phenotypes	PCR-SSP (for hybrid *Rhesus box*)	PCR-RFLP (for *RHD* gene deletion)	*RHD* exons 5, 7, 10
Gene deletion	198	99	D−C−E−c+e+ (92.92%)	+	Homozygote	−
			D−C−E+c+e+ (0.5%)			
			D−C+E−c−e+ (1.51%)			
			D−C+E−c+e+ (5.05%)			
Weak D type 11	1	0.5	D−C+E−c−e+	+	Heterozygote	+
RHD-CE(2-9)-D_2	1	0.5	D−C+E−c+e+	+	Heterozygote	Only 10 +

in the other one, only exon 10 was amplified. In the first sample, further molecular analysis showed that the donor had a weak D allele type 11 that has not been detected by the weak D serological test. In some cases, the weak D phenotype may not be detected by the conventional serological tests (indirect anti-globulin test) [17]. The 885 G>T mutation associated with the replacement of M295I results in lowered density of D antigen on red blood cells in the weak D type 11 [18]. In the second sample that was positive only for exon 10, the results of further molecular analyses for exons 3, 4, 6 and 9 and introns 1 and 2 of *RHD* gene indicated the presence of the *RHD-CE (2-9)-D_2* hybrid allele. Several studies in East Asia have shown that the most common mechanisms for the D-negative phenotype in this population are the *RHD* gene deletion, the DEL allele (approximately 10–30%) and the *RHD-CE-D* hybrid allele (approximately 10%) [19, 20]. These genetic backgrounds of RhD-negative are different from the Iranian population located in the Middle East, while the frequency of our results is more similar to the European population [21]. The frequency of D-negative phenotype with a nondeletion *RHD* gene allele is approximately 0.6% in Caucasians, 10% in black Africans and 30% in Asians [2, 22]. In our study, non-deletion *RHD* gene allele was found in 2 (1%) D-negative donors, indicating more consistency with the Caucasian population. Genotyping of RhD-negative donors at the blood transfusion centers is very important because some alleles like DEL and weak D may not be detected by conventional serological tests and are considered as RhD-negative. Transfusion of these blood products into RhD-negative individuals, especially for girls and women with childbearing potential, may increase the risk of immunization with anti-D in these individuals. So, the screening of RhD-negative donors by genotyping for identification of *RHD* gene alleles, especially in a population where these alleles are highly prevalent, can be very important [17, 23]. Despite the high prevalence of DEL

allele in East Asia (approximately 10–30% of the RhD-negative population), our results (high frequency of *RHD* gene deletion) imply that the frequency of the DEL allele among the Iranian population should be very low and similar to the Caucasian population [21, 24]. In our study, the *RHD* gene deletion had the highest association with the D−C−E−c+e+ phenotype (92.92%) and 87.5% of the C+/E+ donors had *RHD* gene deletion alleles. The frequency of D-negative phenotype with the presence of *RHD* gene non-deletion alleles in association with C and E antigens has also been reported [2, 11]. Both donors with non-deletion allele in this study were positive for C antigen.

Conclusion

In this study, using PCR-SSP, PCR-RFLP, and real-time PCR to identify the genetic background of D-negative phenotype showed that *RHD* gene deletion is the most common genetic mechanism for D-negative phenotype in Iran. The results of the present study are consistent with other studies performed in Caucasians. Furthermore, hybrid *Rhesus box* molecular analysis could effectively identify this allele.

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