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Detection of RHD zygosity in China: using Syber Green Ⅰ real-time polymerase chain reaction based on high-resolution melting curve analysis

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ABSTRACT

Due to relatively higher mutation frequencies in Chinese individuals with the RHD-negative phenotype[25% for 1227 G>A RHD elution and 5% for RHD1-RHCE(2–9)-RHD10], Rhesus box analysis is rarely used in China. Here, quantitative real-time polymerase chain reaction (qPCR) with a high–resolution melting curve mode and a matrix mix containing Syber Green Ⅰ were used to sequence specific primers of 1227 G>A and RHD exons 1, 5, and 10 in two families, consisting of two parents and two children per family (n = 8). The samples with RHD gene deletion homozygous/heterozygous, 1227 G>A heterozygous with RHD gene deletion and normal RHD, normal RHD homozygous/heterozygous, and RHD1-RHCE(2–9)-RHD10 homozygous/heterozygous status were all included. All samples were screened using RHD exon genotyping, Sanger sequencing, and Rhesus box analysis. DNA sample quality was maintained at 68~72 ng/μL, and $OD_{260/280}$ at 1.7~1.9. The Tm ratio of RHD exon 1 (87℃) to internal control (77℃) was 2.49~2.67 and 2.09~2.35 in subjects with RHD exon 1 homozygous and heterozygous, respectively; the Tm ratio of RHD exon 10 (81℃) to internal control (77℃) was 5.01~6.11 and 3.34~4.31 in subjects with RHD exon 10 homozygous and heterozygous, respectively; the Tm ratio of RHD exon 5 (83℃) to internal control (77℃) was 3.98~4.75, 3.02~3.45, and 0.03 in subjects with RHD exon 5 homozygous, heterozygous, and deletion homozygous, respectively; the Tm ratio of 1227A (87℃) to internal control (77℃) was 1.11, 0.51, and <0.03 in subjects with 1227A heterozygous, 1227A homozygous (exon 9 deletion), and wild type, respectively. The results suggest that using the primers of Tm ratio in comparison with an internal control is an effective way to detect RHD gene deletion or RHD-RHCE hybrid variant allele carrier. The method can also be used to calculate the mother-newborn RHD phenotype proportion and assist pedigree analysis.

Keywords: RHD allele, high resolution melting curve, heterozygous, qPCR

INTRODUCTION

The D-negative phenotype frequency in Chinese individuals is estimated to be 0.27%~0.59%[1–3]. In contrast, the most common cause of the D-phenotype in people of European origin is homozygous for a complete deletion of RHD. According to reports, the frequency of the RHD variant allele in Chinese individuals with D-phenotype was 74.1%, 20.2%, and 5.6% for RHD gene deletion, 1227 G>A (RHD elution), and RHD1-RHCE(2–9)-RHD10, respectively [4,5].

Rhesus box analysis is useful for detecting RHD

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gene deletion homozygosity or hemizygosity because RHD gene deletion occurs in the Rhesus box[6]. However, Rhesus box analysis only determines 60% of RHD phenotypes. Shao *et al*. demonstrated that the d frequency was 5.6%; D, 94.3%; and Dd heterozygosity, 10.6% through Rhesus box analysis. The adjusted rate of Dd heterozygosity was 9.0% after excluding DEL (IAT-negative)[7,8]. In contrast, we estimate that >15.6% of all RHD variant alleles cannot be determined using Rhesus box analysis. For example, if one parent has the RHD^- phenotype and the other is the RHD^+ phenotype, the proportion of RHD^- in their child could be 50% to 10.6%, with only 3.7% determinable using the Rhesus box.

Our study aimed to develop a cost-effective relative quantitative genotyping method that uses quantitative real-time PCR (qPCR) with a high-resolution melting(HRM) curve mode and sequence-specific primers and a matrix mix containing Syber Green Ⅰ. The study detected prospectively the different allele combinations of RHD gene deletion, RHD1-RHCE (2–9)-RHD10, and 1227 G>A.

MATERIALS AND METHODS

Subjects

Eight participants consisting of two generations of two unrelated families were enrolled in our study; all four parents had the RHD^+ phenotype, while the two sets of children had the RHD^- phenotype in each family. For RHD serotyping and DNA extraction, 2 mL of ethylene diamine tetraacetic acid (EDTA) containing peripheral venous blood was used. Phenotyping involved the use of monoclonal IgG/IgM mixed anti-D antibody(IgM clone: D175–2, and IgG clone: D4151E4, Novaclone, Dominion Biologicals Ltd., Dartmouth, Nova Scotia, Canada). The study was conducted in accordance with the current version of the Declaration of Helsinki.

Genotyping and Sanger sequencing of RHD exons

DNA was isolated from EDTA-anti-coagulated blood using a commercial kit (magnetic bead whole blood DNA extraction kit, Jiangsu ZhongjiWantai Biological Pharmaceutical Co. Ltd., Jiangsu, China) based on magnetic separation in an automated system (FluoVia Gene Pure Plus, Jiangsu ZhongjiWantai Bio logical Pharmaceutical Co. Ltd.). The quality of DNA samples was maintained at 68~72 ng/μL, and $OD_{260/280}$ at 1.7~1.9.

Due to RHD gene deletion, 1227 G>A, and RHD1–RHCE (2–9)-RHD10 being the major RHD^- phenotypes in Chinese individuals, we used the commercial kit (RH genotyping, Jiangsu ZhongjiWantai Bio logical Pharmaceutical Co. Ltd.) to detect the RHD exons, 1227 G>A, 845 G>A, and RHCE genotyping through qPCR (FluoVia Line Gene 900 plus, Jiangsu Zhongji–Wantai Bio logical Pharmaceutical Co. Ltd.). Rhesus box analysis was used, as per the protocol published previously[6].

Sanger sequencing of RHD exons 1 to 10 was performed using a commercial RHD gene sequencing kit (RHD exon sequence; Jiangsu ZhongjiWantai Biological Pharmaceutical Co. Ltd.). Sanger sequencing of PCR-purified products was performed by Sangon Biotech (Shanghai, China), and the results were analyzed using sequence analysis software (Geneious R9; Auckland, New Zealand).

HRM analysis assay design

High-resolution melting curve analysis assay was performed using the FluoVia Line Gene 900 plus. Primers and matrix mix were processed using a commercial kit(RH genotyping, Jiangsu Zhongji–Wantai Bio logical Pharmaceutical Co. Ltd.). The quality of the DNA samples was maintained at 68~72 ng/μL, and $OD_{260/280}$ at 1.7~1.9, respectively, and the final DNA concentration in the 10 μL matrix mix was 6.2~6.5 ng/μL.

Tm ratio calculating

We used the following formula to calculate the Tm ratio of alleles of RHD exon 1, 5, 10, and 1227A and the internal control to determine homozygosity, hemizygosity, and heterozygosity.

$$\frac{\text{The highest Tm of positive curve area (RHD exon 1 between 86–88℃ or RHD exon 5 between 82–84℃ or RHD exon 10 between 80–82℃ or RHD 1227 between 86–88℃)}}{\text{The highest Tm of internal control area (76–78℃)}}$$

RESULTS

Baseline of subjects

Pedigree analysis was performed by phenotyping, genotyping, and sequencing of two Chinese families. The first family was 1227 G>A carrier and RHD gene deletion carrier in the first generation, while their first child was 1227 G>A/RHD gene deletion, and their second child had normal RHD homozygous. The second family was RHD1-RHCE(2–9)-RHD10 carrier and RHD gene deletion carrier in the first generation, while their first child had RHD1-RHCE(2–9)-RHD10 /RHD gene deletion, and their second child was normal RHD homozygous (***Fig. 1 and Table 1***).

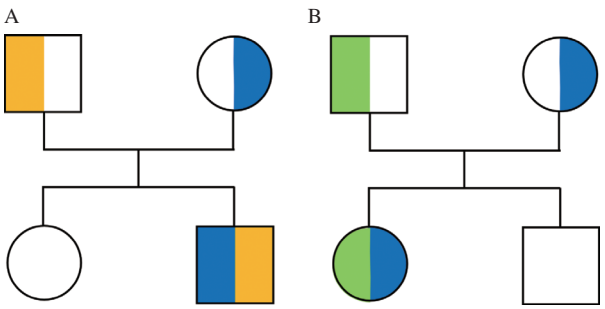

***Fig. 1* Pedigree analysis of RHD$^+$ phenotype parents with RHD$^-$ phenotype children in two Chinese families (A, B).** Yellow, blue, and green geometries indicated 1227 G>A allele, RHD gene deletion, and RHD1-RHCE (2–9)-RHD10, respectively.

HRM analysis

The Tm ratio of RHD exon 1 (87℃) to internal control (77℃) was 2.49~2.67 and 2.09~2.35 in subjects with RHD exon 1 homozygous and heterozygous, respectively; the Tm ratio of RHD exon 10 (81℃) to internal control (77℃) was 5.01~6.11 and 3.34~4.31 in subjects with RHD exon 10 homozygous and heterozygous, respectively; the Tm ratio of RHD exon 5 (83 ℃) to internal control (77℃) was 3.98~4.75 and 3.02~3.45 in subjects with RHD exon 5 homozygous and heterozygous, respectively, and 0.03 in subjects with RHD exon 5 deletion homozygous; the Tm ratio of 1227A (87 ℃) to internal control (77℃) was 1.11, 0.51, and <0.03 in subjects with 1227A heterozygous, 1227A homozygous (exon 9 deletion), and wild type (***Table 2 and Fig. 2***).

***Table 1* Result of RHD phenotyping, genotyping, and sequencing in the members of two Chinese families**

ID	RHD phenotype	RHD exon genotyping	RHD exon sequence	Rhesus box	Allele
Father1	RHD$^+$	RHD exon 1–10 exist, 1227G>A	1227G>A heterozygous	RHD$^+$/RHD$^+$	Normal RHD/1227A
Mother1	RHD$^+$	RHD exon 1–10 exist	Normal RHD	RHD$^+$/RHD$^-$	Normal RHD/RhD gene deletion
Daughter1	RHD$^+$	RHD exon 1–10 exist	Normal RHD	RHD$^+$/RHD$^+$	Normal RHD/ Normal RHD
Son1	RHD$^-$	RHD exon 1–10 exist, 1227G>A	1227G>A homozygous	RHD$^+$/RHD$^-$	1227A/RhD gene deletion
Father2	RHD$^+$	RHD exon 1–10 exist	Normal RHD	RHD$^+$/RHD$^+$	Normal RHD/ RHD1–RHCE (2–9)–RHD10*
Mother2	RHD$^+$	RHD exon 1–10 exist	Normal RHD	RHD$^+$/RHD$^-$	Normal RHD/ RHD gene deletion
Daughter2	RHD$^-$	RHD exon 1,10 exist; RHD exon 2–9 absent	RHD exon 1,10 normal, RHD exon 2–9 absent	RHD$^+$/RHD$^-$	RHD1-RHCE(2–9)-RHD10 / RhD gene deletion
Son2	RHD$^+$	RHD exon 1–10 exist	Normal RHD	RHD$^+$/RHD$^+$	Normal RHD/ Normal RHD

• Depending on the pedigree analysis of his daughter with RHD1-RHCE (2–9)-RHD10/RHD gene deletion.

***Table 2* Consistency between genotyping and monoclonal antibody typing in 23 samples with or without transfusion history**

RHD alleles	ID	RHD exon 1			RHD exon 5			RHD exon 10			RHD 1227A		
		IC77℃	GP87℃	Tm ratio	IC77℃	GP83℃	Tm ratio	IC77℃	GP81℃	Tm ratio	IC77℃	GP87℃	Tm ratio
Normal RHD / Normal RHD	Daughter1	108.02	287.92	2.67	93.18	370.86	3.98	90.42	453.32	5.01	479.1	11.54	0.02
Normal RHD / Normal RHD	Son2	97.82	249.88	2.55	97.6	435.22	4.46	55.08	336.4	6.11	460.06	9.38	0.02
Normal RHD / RHD gene deletion	Mother1	132.06	275.5	2.09	116.04	361.36	3.11	116.52	389.32	3.34	511.88	17.04	0.03
Normal RHD / RHD gene deletion	Mother2	120.24	275.46	2.29	114.88	347.24	3.02	102.14	439.94	4.31	449.34	9.06	0.02
Normal RHD /1227A	Father1	96.98	262.16	2.70	83.72	397.44	4.75	77.82	416.16	5.35	138.26	153.74	1.11
1227A/RHD gene deletion	Son1	111.58	262.14	2.35	105.28	363.74	3.45	99.44	419.58	4.22	175.98	90.16	0.51
Normal RHD / RHD1-RHCE(2–9)-RHD10	Father2	93.89	234.16	2.49	105.28	347.24	3.30	78.43	394.52	5.03	483.35	14.57	0.03
RHD1-RHCE(2–9)-RHD10 / RHD gene deletion	Daughter2	117.58	264.37	2.25	456.83	13.54	0.03	98.58	410.83	4.17	502.81	8.94	0.02

IC: internal control; GP: greatest positive

DISCUSSION

This study was the first to use the HRM mode to analyze the RHD genotype´s allele status. The method may assist gynaecology, medical genetics, and transfusion to estimate the patient´s RHD hereditary status. Especially, in pregnant women with RHD$^-$ phenotype, the proportion of RHD$^-$ phenotype new-borns or foetuses still remain about 10%, even the father has the RHD$^+$ phenotype. This lack of a useful method to predict the RHD typing of new-borns is still a limit for predicting the risk for hemolytic disease of the new–born. Besides, in RHD$^-$ Chinese individuals, only 7% of individuals can be screened using Rhesus box analysis due to RHD gene deletion, while other 3% lack an examination to detect the RHD variant allele. In this study, the use of genotyping Tm ratio to internal control presents a plausible way of detecting relative high-frequency alleles, including 1227A and RHD–RhCE hybrid.

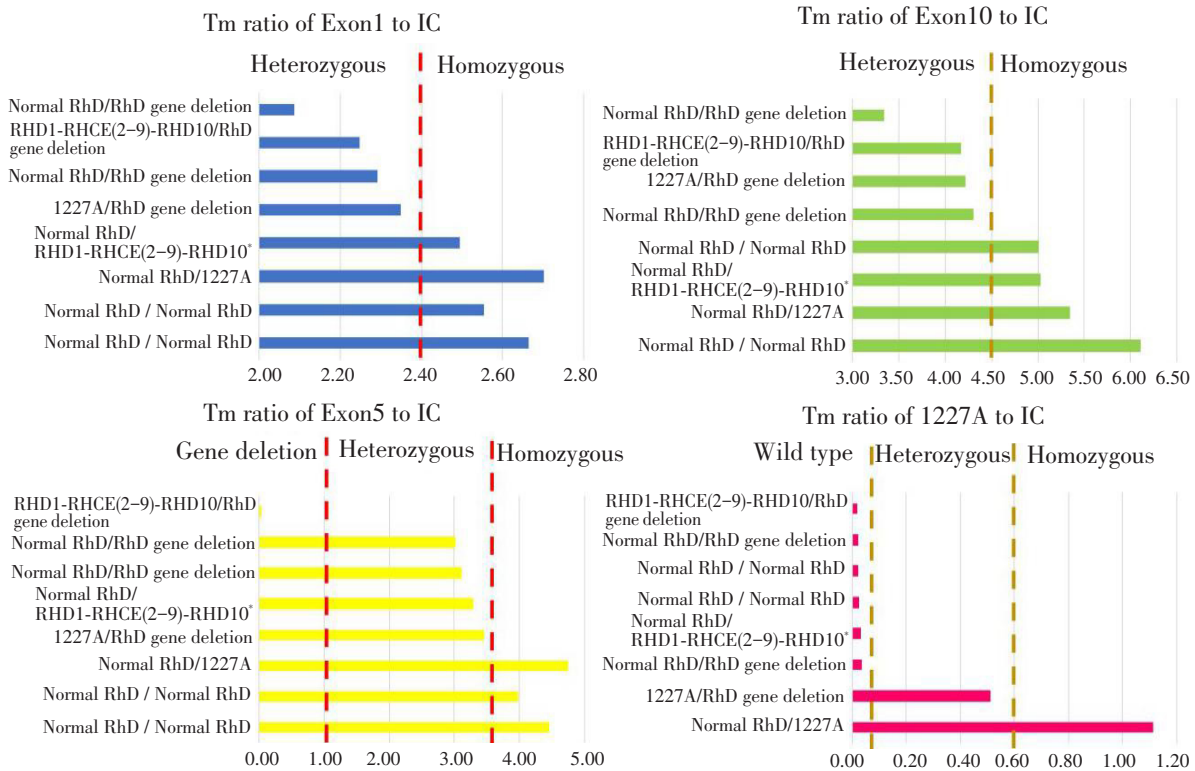

Fig. 2 The Tm ratio of sequence-specific primers of RHD exons 1, 5, 10, and 1227A to internal control in RHD allele homozygous, heterozygous, wild type, and gene deletion cases.

In China, several studies have discussed the frequencies of RHD allele in RHD^- or variant phenotype and summarized that RHD gene deletion, RHD-RHCE (2–9)-RHD, and 1227 G>A were the most common alleles that cause the RHD^- phenotype, while 845 G>A (weak D15 or partial D12) was the major allele that causes weak D phenotype[9–11]. However, very few studies have investigated RHD allele combinations in the Chinese population. Lan *et al*. used Rhesus box analysis to present the RHD(+)/RHD(−), and RHD (+)/RHD (+) genotypes accounting for 9.0% and 91.0%, respectively in RHD^+ phenotype individuals. Frequencies of RHD(+)/RHD(−),RHD(+)/RHD(+), and RHD(−)/RHD(−) genotypes were 26.14%, 3.92%, and 69.94%, respectively in RHD^- individuals. However, the result also only showed 3.92% in RHD (+) / RHD (+) and 13.07% (half of 26.14%) in RHD (+) / RHD(−)from the RHD^- phenotype[12]. So, the frequency of RHD variant carriers with RHD^+ phenotype has not yet been estimated. Shao et al. reported on a Chinese Han woman who delivered an RHD^- negative child at a rate of 3.7%~4.5% despite both parents being RHD^+ [1].

The molecular analysis used to determine the RHD variant in RHD^-, or weakly positive phenotype is well documented[13]. However, to identify the allele in RHD^+ phenotype or decipher genotype data without phenotype data were very difficult. For example, in this study the genotype showed 1227 G>A with the presence of RHD 10 exons, for which RHD^+ and RHD^- are both possible phenotypes. Because of the two alleles in the subject being 1227 G>A, the other allele could be RHD gene deletion or RHD-RHCE(2–9)-RHD for RHD^- phenotype or normal RHD for RHD^+ phenotype. The method of our study could be used to estimate the frequency of RHD-RHCE hybrid in RHD^+ Chinese individuals and predict the hazard ratio of RHD phenotype in Chinese parents. Our study also has some limitations: only the RHD-RHCE, 1227 G>A, and weak D15 were detected, and other known (or unknown) mutation alleles cannot be screened. Subsequent research could utilize a larger sample size and include RHD gene deletion homozygous, although sequence-specific primers aren´t able to amplify RHD gene deletion specimens.

Using the Tm ratio of sequence-specific primer to internal control is an effective method to detect RHD gene deletion or an RHD-RHCE hybrid variant allele carrier. The method can also be used to calculate RHD phenotype proportion of parent-newborn and assist in pedigree analysis.

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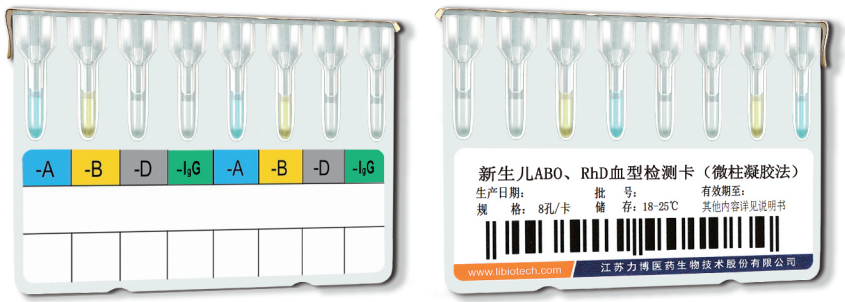
Diagnosis of Hemolytic Disease of the Newborn

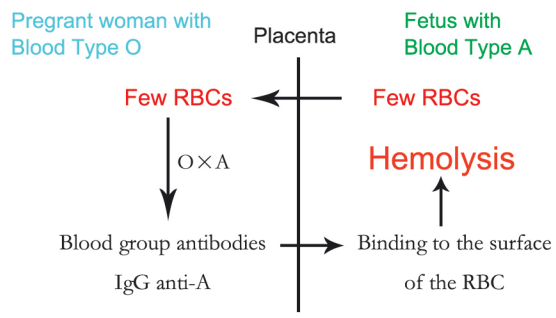
ABO、Rh(D) Antigen Testing Card Newborn

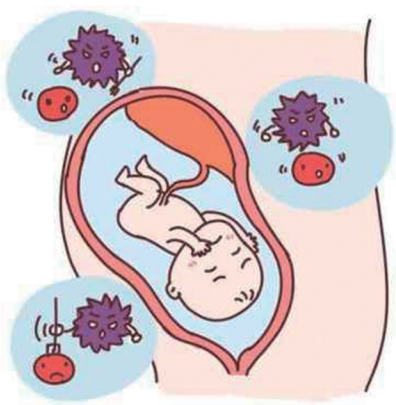


Intended Use：

The product is suitable for clinical tests on ABO and Rh (D) blood typing and direct antiglobulin test (DAT) for newborns and suspected hemolytic disease patients. It is only applicable for clinical test, but not for blood screening.

Case：





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