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Genetic polymorphism of RhD-negative associated haplotypes in the Chinese

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Abstract The Rh blood group is the most polymorphic human blood group system, and is clinically significant in transfusion medicine. Individuals are classified as Rh-positive and Rh-negative depending on the presence or absence of the D antigen on the red cell surface. The RhD-negative trait could be generated by multiple genetic mechanisms, which have been shown to be ethnic group-dependent. In this study, we evaluated the status of seven *RHD*-specific exons (exons 3, 4, 5, 6, 7, 9, and 10) and *RH* intron 4 in 119 Chinese blood donors, using the sequence-specific primers polymerase chain reaction (SSP-PCR). Of the 87 individuals who were RhD-negative, 52 with the ce/ce, ce/cE, or Ce/ce genotype (60%) lacked the above seven *RHD* exons; 22 with the Ce/Ce or Ce/ce genotype (25%) had all the *RHD* exons examined; 13 with the Ce/ce genotype (15%) carried at least one *RHD* exon. Antigen association analysis suggested the existence of a novel class of RhD-negative associated haplotypes in the Chinese, tentatively denoted D(nf)Ce. The D(nf)Ce haplotype consisted of a normal *RHCe* allele and a nonfunctional *RHD* gene, which vary depending on the structure of the *RHD* gene. Among the RhD-negative Chinese, the estimated frequencies of the dce, dCe, and D(nf)Ce haplotypes were 0.7500, 0.0465, and 0.2035, respectively. No statistically significant deviation from Hardy-Weinberg equilibrium was observed using this genetic model.

Key words Molecular basis of Rh blood group · Novel RhD-negative haplotypes · Chinese population

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Introduction

The Rh blood group system was discovered 60 years ago in the context of a case of hemolytic disease of the newborn (HDN). It is now understood that the pathogenesis of this disorder involves maternal alloimmnunization by Rh antigens. The Rh blood group is the most polymorphic human blood group system and has served as a genetic marker for studying the evolution of human populations (Cavalli-Sforza et al. 1994; Zhao 1998). With the use of a serological method, 45 Rh antigens have been defined (Daniels 1995). The D, C/c, and E/e antigens are the most clinically important antigens, which are associated with incompatible hemolytic transfusion reactions, HDN, and autoimmune hemolytic anemia.

Individuals are clinically classified as Rh-positive and Rh-negative depending on the presence or absence of the D antigen on the red cell surface. In Caucasians, approximately 85% of individuals are Rh-positive, whereas roughly 15% are classified as Rh-negative. The Rh blood group antigens are carried on two glycoproteins encoded by two highly homologous genes, denoted as *RHD* and *RHCE*. The D, C/c, and E/e antigens are inherited en bloc from one generation to another. There are eight common Rh haplotypes encoded by the *RHCE* and *RHD* genes: Dce, dce, DCe, dCe, DcE, dcE, DCE, and dCE. Although a "d" antigen does not exist, the letter "d" is used to indicate the D-negative phenotype. Both *RHD* and *RHCE* consist of ten exons and exhibit similar exon-intron organization. The two genes are closely linked and tandemly organized on chromosome 1p34.3–p36.1, with *RHD* located 3′ of *RHCE* (Chérif-Zahar et al. 1990, 1991; Colin et al. 1991; Le Van Kim et al. 1992; Mouro et al. 1993). The *RHD* gene encodes the D polypeptide, and no allele has been detected. The *RHCE* gene has four main alleles, *Ce*, *CE*, *ce*, and *cE*. There are wide racial differences not only in the frequencies of the *RH* gene complex but also in the genetic background of the Rh antigens. For instance, about 15% of Caucasoid people are RhD-negative, but only 0.1%–0.4% of the population of most Chinese nationalities exhibit this phenotype (Zhao

1987, 1998). Although early studies indicate that complete deletion of the *RHD* gene can result in the RhD-negative phenotype in Caucasian populations (Colin et al. 1991), the RhD-negative trait could be generated by multiple genetic mechanisms. These mechanisms involve point mutation (Avent et al. 1997), insertion (Singleton et al. 2000), and partial deletion of the *RHD* gene (Hyland et al. 1994; Cartron et al. 1995; Okuda et al. 1997; Andrews et al. 1998). In addition, somatic mutation within the *RHD* gene may cause the shift from RhD-positive to RhD-negative in leukemia patients (Chérif-Zahar et al. 1998). In order to determine the genetic basis of the *RH* gene in RhD-negative Chinese blood donors, we evaluated the status of seven *RHD*-specific exons (exons 3, 4, 5, 6, 7, 9, and 10) and *RH* intron 4, using the sequence-specific primers polymerase chain reaction (SSP-PCR).

Subjects and methods

Thirty-two RhD-positive and 87 RhD-negative unrelated blood donors were studied. All individuals investigated were of Chinese Han nationality, living in Mainland China. The Rh phenotypes were determined by standard serological methods, according to the manufacturer's instructions (Gamma Biologicals, Houston, TX, USA; Ortho Diagnostic Systems, Raritan, NJ, USA). Genomic DNA was isolated from whole blood by the salting-out method (Miller et al. 1988). *RHD* and *RHCE* genotyping was performed by SSP-PCR, as described below. The *RH* gene-specific primers (Table 1) were designed from the published sequences (Chérif-Zahar et al. 1990, Le Van Kim et al. 1992, Mouro et al. 1993; GenBank accession nos M34015, X63094, X63097) to amplify the *Ce*, *CE*, *ce*, and *cE* alleles; *RH* intron 4; and *RHD* exons 3, 4, 5, 6, 7, 9, and 10. Primers 5F and 5R were used to identify *RHD* and *RHCE* intron 4, depending on the size of the PCR products. The positions of the PCR primers used for the detection of *RH* gene fragments are shown in Fig. 1.

Primers HGHF (5′-GCCTTCCCAACCATTCCCTT-3′) and HGHR (5′-TCACGGATTTCTGTTGTGTTTC-3′) were included in all PCR reactions to amplify an internal positive control PCR product, a 427-bp fragment from the human growth hormone (HGH) gene (GenBank accession no. M13438). The initial PCR was carried out with 0.1–0.5 μg of genomic DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM dNTPs, 1 μM of each forward and reverse primer, and 0.5 units of Taq DNA polymerase (PE Biosystems, Foster City, CA, USA) in a 10-μl reaction volume. After denaturation for 5 min at 95°C, the samples were subjected to 30 cycles of PCR in a DNA Thermal Cycler (PE Biosystems, Foster City, CA, USA). Each cycle included 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min, followed by a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Results

The Rh phenotyping of all 119 randomly chosen blood donors was performed by a serological method. *RHCE* and

Table 1. Primer sequences used for detection of *RHCE* alleles and *RHD* exons

Detection of	Name of primer[a]	Nucleotide sequence (5′ → 3′)	Position (exon)[b]	PCR product size (bp)
RHC (Ce, CE)	1F	GCTGCCTGCCCCTCTGC	32 to 48 (1)	117
	1R	CTTGATAGGATGCCACGAGC	148 to 129 (1)	
RHc (ce, cE)	2F	TTGGGCTTCCTCACCTCAAA	184 to 203 (2)	142
	2R	TGATGACCACCTTCCCAGG	325 to 307 (2)	
RHE (cE, CE)	3F	CTGGCCAAGTGTCAACTCTC	657 to 676 (5)	145
	3R	CATGCTGATCTTCCTTTGGG	801 to 782 (5)	
Rhe (Ce, ce)	4F	CTGGCCAAGTGTCAACTCTG	657 to 676 (5)	145
	4R	CATGCTGATCTTCCTTTGGG	801 to 782 (5)	
RH-Intron 4	5F	CGATACCCAGTTTGTCTGCC	608 to 627 (4)	*RHD*: 478
	5R	AGAACATCCACAAGAAGAGGG	658 to 638 (5)	*RHCE*: 1,126
RHD-Exon 3	6F	TCGGTGCTGATCTCAGTGGA	364 to 383 (3)	112
	6R	TACTGATGACCATCCTCAGGT	475 to 455 (3)	
RHD-Exon 4	7F	TACCACATGAACATGATGCACA	493 to 514 (4)	105
	7R	ATCTTTATCCTCCGTTCCCTC	597 to 577 (4)	
RHD-Exon 5	8F	CAATCGAAAGGAAGAATGCCG	692 to 712 (5)	60
	8R	TCACCACGCTGACTGCTAC	751 to 733 (5)	
RHD-Exon 6	9F	CTTATGTGCACAGTGCGGTG	803 to 822 (6)	130
	9R	GTACTTGGCTCCCCCGAC	932 to 916 (6)	
RHD-Exon 7	10F	CCCACAGCTCCATCATGGG	968 to 986 (7)	98
	10R	GCCGGCTCCGACGGTATC	1065 to 1048 (7)	
RHD-Exon 9	11F	AAATATGGAAAGCACCTCATGA	1172 to 1193 (9)	53
	11R	CCAGAAAACTTGGTCATCAAAAT	1224 to 1202 (9)	
RHD-Exon 10	12F	TTGGATTTTAAGCAAAAGCATCC	1244 to 1266 (10)	186
	12R	ATTCTCCTCAAAGAGTGGCAG	1429 to 1409	

PCR, Polymerase chain reaction

[a] F and R indicate forward and reverse primers, respectively

[b] Numbering of nucleotides starts at ATG codon according to the *RHD* sequence (GenBank accession no. X63094)

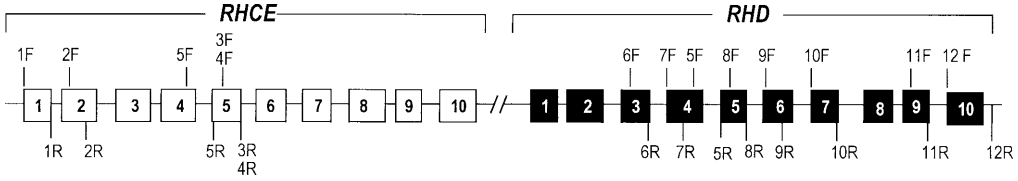

Fig. 1. Organization of exons and introns of *RHCE* and *RHD* is shown. Each *RH* gene is represented as *ten boxes*, each box representing an exon; *RHCE* is shown as *white*, and *RHD* as *black*. The positions of polymerase chain reaction primers used for the detection of *RH* gene fragments are indicated by the primer name (see Table 1). F and R indicate forward and reverse primer, respectively

Table 2. Detection of *RHD* exons and *RH* intron 4 in Chinese donors

Rh phenotype	Number of subjects examined	*RHD* exon[a]							Intron 4[a]	
		3	4	5	6	7	9	10	*RHD*	*RHCE*
RhD-negative ccee	43	−	−	−	−	−	−	−	−	+
ccEe	1	−	−	−	−	−	−	−	−	+
Ccee	8	−	−	−	−	−	−	−	−	+
Ccee	10	−	−	−	−	−	−	+	−	+
Ccee	2	+	+	−	−	−	−	+	−	+
Ccee	1	+	+	+	+	−	+	+	+	+
Ccee	14	+	+	+	+	+	+	+	+	+
CCee	8	+	+	+	+	+	+	+	+	+
RhD-positive	32	+	+	+	+	+	+	+	+	+

[a] −, Absence; +, presence

Table 3. Estimation of Rh haplotype frequency among RhD-negative Chinese[a]

Genotype[b]	Phenotype	No. observed	No. expected	χ^2
dce/dce	ccee	43	48.33	0.60
dce/dCe	Ccee	8	6.00	0.67
dce/D(nf)Ce	Ccee	27	26.25	0.02
dCe/dCe	CCee	0	0.18	0.18
dCe/D(nf)Ce	CCee	8	5.19	1.52
D(nf)Ce/D(nf)Ce				
		86	86.00	$\Sigma\chi^2 = 2.99$ $P_{(2)} > 0.20$

[a] Rare alleles *CE* and *cE* [frequency 0.0003 and 0.0128, respectively, in the Chinese (Zhao 1987)] are not included in this analysis. Haplotype frequency, estimated by gene counting, is as follows: *dce*, 0.7500; *dCe*, 0.0465; *D(nf)Ce*, 0.2035. "*d*" indicates complete deletion of the *RHD* gene. *D(nf)* represents those haplotypes consisting of a normal *Ce* allele and a nonfunctional *RHD* gene, which contains at least one *RHD* exon
[b] Determined by the DNA-based sequence-specific primers polymerase chain reaction typing method described in the text

RHD genotyping was carried out using DNA-based SSP-PCR. All *RHCE* and *RHD* genotyping results were correlated with the serological results. *RHD* and *RHCE* intron 4 and *RHD* exons 3, 4, 5, 6, 7, 9, and 10 were present in all 32 Rh D-positive donors. Of the 87 RhD-negative donors, 44 individuals with the ce/ce or ce/cE genotype (51%), and 8 with the Ce/ce genotype (9%) had neither *RHD* intron 4 nor the above seven *RHD* exons; 22 donors with the Ce/Ce or Ce/ce genotype (25%) had intron 4 and all *RHD* exons examined. Among the 13 remaining RhD-negative donors with the Ce/ce genotype, 10 (15%) were missing *RHD* exons 3, 4, 5, 6, 7, and 9; 2 lacked exons 5, 6, 7, 9; and 1 lacked exon 7 (Table 2). Complete deletion of the *RHD* exons examined was observed in all ce/ce and some Ce/ce individuals, suggesting the presence of two common haplotypes, denoted dce and dCe, respectively.

It was noted, however, that the genotypes of the individuals who carried at least one *RHD* exon were either Ce/Ce or Ce/ce. This implied the existence of another class of RhD-negative associated haplotypes in the Chinese, tentatively denoted D(nf)Ce, which carried a normal *Ce* allele and a nonfunctional *RHD* gene. Haplotype D(nf)Ce may vary depending on the structure of the *RHD* gene. Among 27 RhD-negative, phenotype Ccee Chinese individuals, who had *RHD* exon 10, four different D(nf)Ce haplotypes were identified (Table 2). According to the above hypothesis, we estimated the frequencies of the dce, dCe, and D(nf)Ce haplotypes in RhD-negative Chinese to be 0.7500, 0.0465, and 0.2035, respectively. In this population analysis, no statistically significant deviation from Hardy-Weinberg equilibrium was observed ($P > 0.20$; Table 3) when using this genetic model.

Discussion

The structure of the *RH* locus was first established in Caucasian populations (Colin et al. 1991), and early studies confirm the observation that RhD-negative Caucasians,

almost invariably, lack the *RHD* gene (Daniels 1995). This finding not only explains finally why no postulated Rh d antigen has ever been shown, but also makes it possible to determine the RhD status of fetuses at risk for HDN, using a DNA-based SSP-PCR technique (Lo et al. 1993; Denomme et al. 1999). Later studies, however, have revealed that RhD-negative individuals may have different genetic backgrounds. Hyland et al. (1994) identified three unrelated Rh D gene polymorphisms among Rh D-negative CCee phenotypes: one lacked the D gene, one had a partial deletion, and the remaining individual appeared to have a normal D gene. Avent et al. (1997) found an RhD-negative CCee phenotype individual who had full-length *RHD* transcripts, which have a single point mutation, that generates an in-frame stop codon, and this abolished the expression of Rh D antigen. A novel *RHD* gene with a four-nucleotide deletion was detected in a Caucasian RhD-negative CCee phenotype patient (Andrews et al. 1998). Daniels et al. (1997) reported the differences between RhD-negative Africans and RhD-negative Europeans. Of 11 African RhD-negatives, 3 had exons 2, 3, 7, and 10 and intron 4 of *RHD*, 2 had none of these exons or intron 4 of *RHD*, and 6 had exon 10, but no exon 3 or 7 or intron 4 of *RHD*. In some RhD-negative Australian Aborigines and Africans, a fragment of the *RHD* gene or an *RHD* pseudogene (RHDpsi) were present (Cartron et al. 1995; Singleton et al. 2000). In Oriental populations, in whom the RhD-negative phenotype is rare, the *RHD* gene may either be intact or show partial deletion (Okuda et al. 1997).

In this study, the individual exons 3, 4, 5, 7, 9, 10 and intron 4 of *RHD* were determined, because exon 8 of *RHCE* and *RHD* are of identical sequence and their origins are not possible to define by SSP-PCR. Our data indicate that about 40% of RhD-negative Chinese carry both a nonfunctional *RHD* gene and a *Ce* allele, suggesting the existence of a novel class of RhD-negative associated haplotype D(nf)Ce. Population study data support this hypothesis. It appears that the genetic polymorphism of Rh haplotypes determined at the DNA level is more complex than that defined by serological typing. As described above, the association between the nonfunctional *RHD* gene and the CCee or Ccee phenotypes was also observed in Japanese, Caucasians, and other ethnic groups; thus, the D(nf)Ce haplotype may be present in world populations, but differ in its frequency.

Although the exact pathway of the evolution of the D(nf)Ce haplotype is not yet clear, it appears that it may arise from the DCe haplotype, based on an analysis of short tandem repeat (STR) polymorphism in *RH* gene intron 8 (Fujiwara et al. 1999). In addition, our data show that the existing PCR-based methods for prenatal determination of fetal RhD status from DNA samples (Lo et al. 1993) are not suitable for testing the Chinese population. The same situation was also observed in other populations, in which some RhD-negative mothers were found to carry a nonfunctional *RHD* gene (Okuda et al. 1997; Denomme et al. 1999). It is necessary to establish the structure of the *RH* locus for different ethnic groups before DNA-based RhD genotyping is performed.

References

Andrews KT, Wolter LC, Saul A, Hyland CA (1998) The RhD-trait in a white patient with the RhCCee phenotype attributed to a four-nucleotide deletion in the *RHD* gene. Blood 92:1839–1840

Avent ND, Martin PG, Armstrong-Fisher SS, Liu W, Finning KM, Maddocks D, Urbaniak SJ (1997) Evidence of genetic diversity underlying RhD-, weak D (Du), and partial D phenotypes as determined by multiplex polymerase chain reaction analysis of the *RHD* gene. Blood 89:2568–2577

Cartron JP, Le Van Kim C, Chérif-Zahar B, Mouro I, Rouillac C, Colin Y (1995) The two-gene model of the RH blood-group locus. Biochem J 306:877–878

Cavalli-Sforza LL, Menozzi P, Piazza A (1994) The history and geography of human genes. Princeton University Press, Princeton

Chérif-Zahar B, Bloy C, Le Van Kim C, Blanchard D, Bailly P, Hermand P, Salmon C, Cartron JP, Colin Y (1990) Molecular cloning and protein structure of a human blood group Rh polypeptide. Proc Natl Acad Sci USA 87:6243–6247

Chérif-Zahar B, Mattei MG, Le Van Kim C, Bailly P, Cartron JP, Colin Y (1991) Localization of the human Rh blood group gene structure to chromosome region 1p34.3–1p36.1 by in situ hybridization. Hum Genet 86:398–400

Chérif-Zahar B, Bony V, Steffensen R, Gane P, Raynal V, Goosens D, Laursen JS, Varming K, Jersild C, Cartron JP (1998) Shift from Rh-positive to Rh-negative phenotype caused by a somatic mutation within the RHD gene in a patient with chronic myelocytic leukaemia. Br J Haematol 102:1263–1270

Colin Y, Chérif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP (1991) Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. Blood 78:2747–2752

Daniels G (1995) Human blood groups. Blackwell Science, Oxford

Daniels G, Green C, Smart E (1997) Differences between RhD-negative Africans and RhD-negative Europeans. Lancet 350:862–863

Denomme GA, Akoury H, Sermer M, Kelton JG (1999) RhD status of a fetus at risk for haemolytic disease with a discrepant maternal DNA-based RhD genotype. Prenat Diagn 19:424–427

Fujiwara H, Okuda H, Omi T, Iwamoto S, Tanaka Y, Takahashi J, Tani Y, Minakami H, Araki S, Sato I, Kajii E (1999) The STR polymorphism in intron 8 may provide information about the molecular evolution of RH haplotypes. Hum Genet 104:301–306

Hyland CA, Wolter LC, Saul A (1994) Three unrelated Rh D gene polymorphisms identified among blood donors with Rhesus Ccee (r′r′) phenotypes. Blood 84:321–324

Le Van Kim C, Mouro I, Chérif-Zahar B, Raynal V, Cherrier C, Cartron JP, Colin Y (1992) Molecular cloning and primary structure of the human blood group RhD polypeptide. Proc Natl Acad Sci USA 89:10925–10929

Lo Y-MD, Bowell PJ, Selinger M, Mackenzie IZ, Chamberlain P, Gillmer MDG, Littlewood TJ, Fleming KA, Wainscoat JS (1993) Prenatal determination of fetal RhD status by analysis of peripheral blood of rhesus negative mothers. Lancet 341:1147–1148

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

Mouro I, Colin Y, Chérif-Zahar B, Cartron JP, Le Van Kim C (1993) Molecular genetic basis of the human Rhesus blood group system. Nature Genetics 5:62–65

Okuda H, Kawano M, Iwamoto S, Tanaka M, Seno T, Okubo Y, Kajii E (1997) The RHD gene is highly detectable in RhD-negative Japanese donors. J Clin Invest 100:373–379

Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A, Narter-Olaga EG, Hawthorne LM, Daniels G (2000) The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the RhD-negative blood group phenotype. Blood 95:12–18

Zhao TM (1987) Human blood group genetics. Science Press, Beijing

Zhao TM (1998) The Uyghurs, a Mongoloid-Caucasoid mixed population: genetic evidence and estimates of Caucasian admixture in the peoples living in northwest China. In: Mair VH (ed) The bronze age and early iron age peoples of eastern central Asia. Institute for the Study of Man, Washington DC