A comprehensive investigation of *RHD* polymorphisms in the Chinese Han population in Xi'an

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Background. This study is a comprehensive analysis of *RHD* in D-negative phenotypes in saline, in Xi'an, Shanxi province, central China.

Material and methods. DCcEe in saline was measured for each blood sample from every donor between January 2008 and June 2012 in the Xi'an Blood Centre, China. D-negative results were confirmed by an indirect antiglobulin test and further investigated by adsorption-elution as required. The initial step of molecular analysis was *RHD* zygosity testing. Then *RHD* was detected by a sequence-specific polymerase chain reaction system for *RHD(1227G>A)*, *weak D type 15*, and *RHD(711delC)* alleles for the samples carrying at least one *RHD*. For the remaining non-identified samples, ten *RHD* exons were amplified using a previously widely used *RHD* coding region sequencing method. Some *RHD/RHCE* conversion alleles were identified while those remaining were submitted to direct sequencing.

Results. Overall, 2,493 D-negative samples in saline were detected in a total of 890,403 donors (D-negative rate, 0.28%). Among the D-negative individuals, *RHD* deletion (*d/d*) was assessed in 1685 donors (67.59%). Non-functional *RHD* alleles were detected in 184 donors (7.38%), the most common being the *RHD-CE(2-9)-RHD* and *RHD(711delC)* alleles. Two new alleles were observed and family investigations were performed; *RHD(1227G>A)* DEL was detected in 516 individuals (20.70%), and weak D or partial D variants were identified in 108 donors (4.33%). The most common alleles were *weak D type 15*, D^{VI} *type 3* and D^{V} *type 2*. Four new weak D alleles were noted, and two cases of *RHD(1227G>A)/weak D type 15* heterozygosity were confirmed.

Conclusions. Currently, it seems to be difficult to observe any new *RHD* alleles in the Han Chinese population. D prediction in this population is easier because popular alleles are dominant, accounting for about 99.80% of alleles in D-negative people. Weak D types and partial D variants are rare and occur in approximately 0.01% of the population.

Keywords: Rh blood group, *RHD*, D antigen, allele.

Introduction

The Rhesus (Rh) blood group system is the most important blood group system in haemolytic diseases of the newborn and foetus. In clinical transfusions, the D antigen of this system is the third most relevant behind the A and B antigens of the ABO blood group system. Over the past decade, molecular technologies in combination with serological tests have been used to investigate the *RHD* allele, which encodes the D antigen, in different ethnic groups. Many D antigen variants and *RHD* alleles have been observed and described[1].

According to D antigen density and D epitopes on red blood cells, D can be classified mainly into normal D-positive, partial D, weak D, DEL and D-negative phenotypes. *RHD* alleles for these phenotypes are formed by molecular events, such as mutations, deletions, conversions, or insertions, which are observed in the coding or non-coding regions by comparing these sequences with the *RHD* sequence from a normal Rh(D)-positive individual. There has been an increasing amount of data from *RHD* studies on the Chinese population[2-15]. However, compared with the alleles found in Caucasians, few alleles have been identified in the Chinese Han population, despite this being the largest population in the world. In this study, we performed a comprehensive investigation of a large sample of the population in Shanxi province in central China.

Materials and methods

Samples

Blood samples from every donor were collected between January 2008 and June 2012 and were first screened for D-negativity in saline in 96-well plates in the Shanxi Blood Centre, Xi'an, Shanxi province, central China. D-negative samples in saline were then collected for further serological and molecular analyses. As almost

all of the donors were from the Han population, the major ethnic group in China, we excluded the small number of samples from other minor ethnic groups for further investigations and statistics. Those samples came from three people of Hui nationality, one Manchu person and one individual from the Tujia ethnic minority. The blood donors' ages ranged from 18 to 55 years old. Approximately 55% of the donors were born in the Shanxi Province and the others were from other parts of China. For family investigations or if the original samples were insufficient, the donors and their family members provided informed consent for a second blood collection.

Serology

For Rh blood group typing[16], the C, c, E, and e antigens were assessed in saline (ant-C: MS24, anti-E: MS12, anti-C: MS33 and anti-e: MS16, Immucor Diagnostik GmbH, Rödermark, Germany), and the D was further determined with an indirect anti-globulin test (IAT), using two anti-D regents (IgM+IgG, clones 175-2 and 415 1E4, Dominion Biological Limited, Nova Scotia, Canada, and IgM+IgG, clones TH-28 and MS-26, Millipore Inc., Livingston, UK). The D epitopes were assessed in samples with new *RHD* alleles that were IAT positive with monoclonal anti-D LHM76/58, LHM76/59, LHM174/102, LHM50/2B, LHM169/81, ESD1, LHM76/55, LHM77/64, LHM70/45, LHM59/19, LHM169/80 and LHM57/17 antibodies (ALBAclone, Z293, Edinburgh, Scotland, UK, Lot V059696), as well as anti-human globulin (Novaclone, Lot N1G03401, Dominion Biological Ltd, Dartmouth, Canada). For the samples containing the *RHD* gene that were IAT negative, and had unidentified *RHD* alleles, adsorption/elution tests were performed routinely with elution by heating.

Molecular tests

RHD was analysed in all samples that were D-negative in saline. Genomic DNA was isolated from whole blood samples (Promega wizard genomic DNA extraction kit, Promega Corporation, Madison, WI, USA). *RHD* zygosity was first determined using a published method[17]. Next, the most common *RHD* alleles in the Chinese population, *RHD(1227G>A)*, w*eak D type 15*, and *RHD(711delC)*, were genotyped through three sequence-specific polymerase chain reaction (PCR-SSP) assays for any individuals carrying one or two *RHD* alleles. Some of the primers used for the PCR-SSP were from a previously reported *RHD* genotyping PCR system[3], and some were designed again or modified according to the previous primers (Table I). For the remaining samples that were unidentifiable by the PCR-SSP assays, all ten *RHD* exons were amplified and analysed for *RHD/RHCE* gene conversion, or sequenced if necessary[18].

Family investigations

For each new non-functional *RHD* allele, the related specific PCR-SSP method was designed according to the nucleotide changes from the wild-types to confirm the sequencing results and to decide on family investigations. Samples from family members were assessed for both serology and DNA typing. The PCR primers are listed in Table I. All of the PCR were performed in a total volume of 12.5 µL, each containing 1 µL of genomic DNA, 0.5 U DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA), 200 µM dNTP, primers, and 2.5 mM $MgCl_2$ in a buffer provided by the manufacturer. Thirty-two cycles were programmed on a thermocycler (PE 9700 GeneAmp PCR system; Applied Biosystems) as follows: denaturation at 95 °C for 10 minutes followed by 32 cycles of 10 seconds at 94 °C and 40 seconds at 62 °C. The PCR products were visualised on a 2% agarose gel.

Tests for *RHD1227A/G* and *RHD845A/G*

Two samples were both *1227A* and *845A* positive, as determined by PCR. The sequencing results demonstrated both *1227A/G* and *845A/G* heterozygotes (Figure 1). For further confirmation, two zygosity PCR tests were designed with two new primers (Table I) and the samples were assessed with the latest PCR-SSP conditions.

Results

Summary of alleles

From January 2008 through June 2012, there were 890,403 Chinese Han blood donors in the Shaanxi province blood centre (repeat blood donors excluded). Of these, 2,493 donors were D-negative in saline (0.28%). The IAT confirmed that 2,385 of these samples were D-negative, for a rate of 0.27%, while 108 samples were positive (weak D and partial D types), for a rate of 4.33% among saline D-negative individuals and 0.01% in the general population.

The results of *RHD* zygosity testing demonstrated that 1,685 out of 2,493 individuals had both *RHD* deletions (*d/d*) in a ratio of 67.59%. The remaining 808 individuals had one or two *RHD* genes (32.41%). Among them, 757 were heterozygous (*D/d*), for a rate of 93.69%, and 51 cases were homozygous (*D/D*), for a rate of 6.31%. Of those, 49 cases were mainly *RHD(1227G>A)/RHD(1227G>A)* DEL homozygotes. Sequencing analysis did not detect any *RHD* nucleotide heterozygosities. In the other two *D/D* individuals, sequencing revealed both 1227G/A and 845G/A heterozygosities (Figure 1H). The PCR-SSP confirmed the 1227G, 1227A, 845G and 845A positive detections (Figure 2).

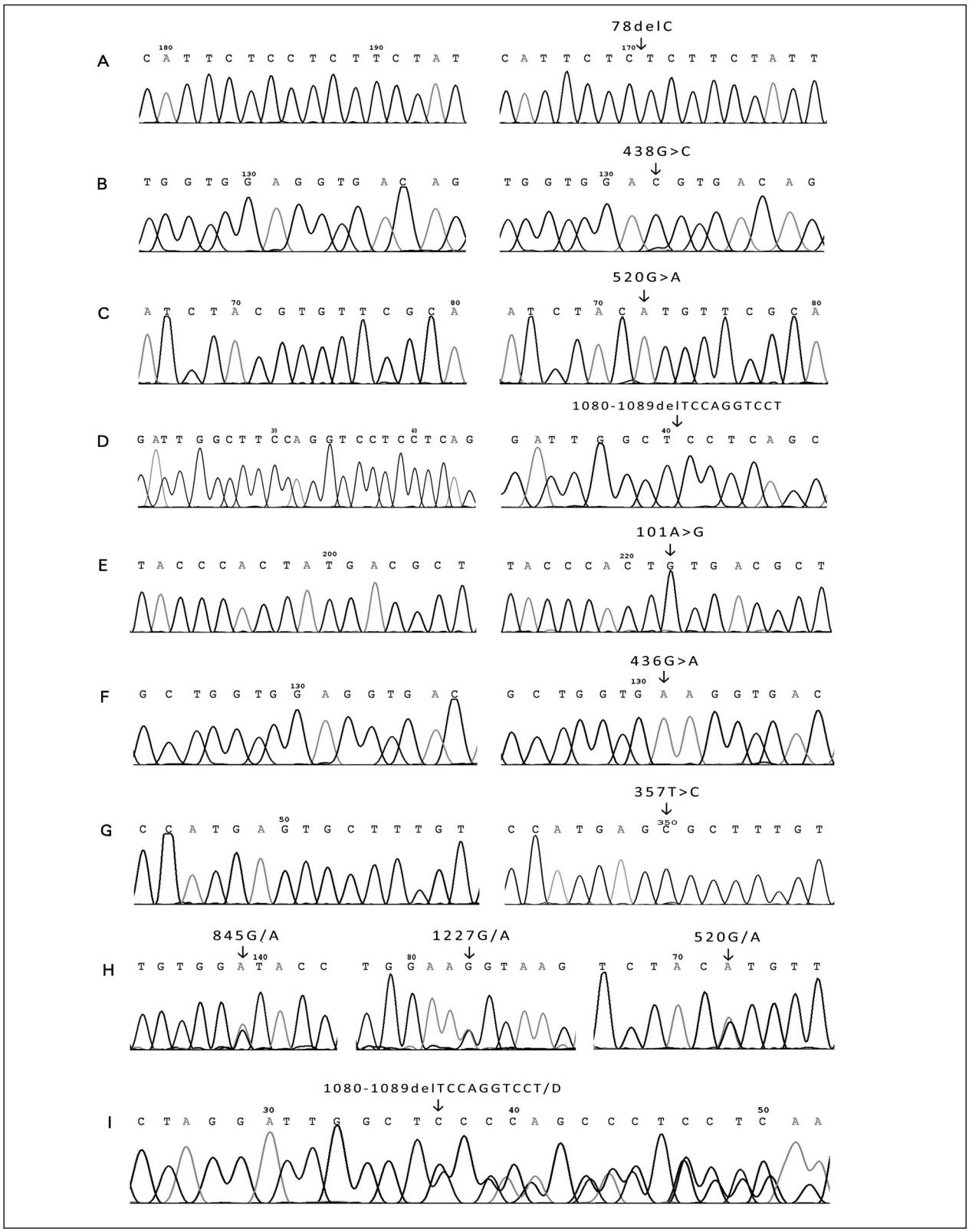

Figure 1 - Partial Sequences of alleles and RHD A-G show partial RHD sequences and nucleotide changes of 78delC, 438C, 520A, 1080-1089delTCCAGGTCCT, 101G, 436A and 357C. H and I show heterozygosities of RHD(1227G>A)/weak D type 15, RHD/RHD(520G>A) and RHD/RHD(1080-1089delTCCAGGTCCT).

Table I - Polymerase chain reaction sequence-specific primers.

Name	Sequences (5'-3')	Positions	Specific
1227A	TGATGACCAAGTTTTCTGGAAA	exon 9, 1206-1227	12227A
1227G	TGATGACCAAGTTTTCTGGAAG	exon 9, 1206-1227	1227G
1227-Lower	AAACAGCAAGTCAACATATACT	intron 9, +85-62	*RHD*
845A	AGGAGGCGTGGCTGTGGA	exon 6, 828-845	845A
845G	AGGAGGCGTGGCTGTGGG	exon 6, 828-845	845G
845-Lower	TCAGCCAAAGCAGAGGAGGT	intron 6, +39-20	*RHD*
710delC	CCAATCGAAAGGAAGAATGCC	exon 5, 691-711	710delC[3]
710-Lower	AGCGCCCTGCTCACCT	intron 5, +14-exon5 800	*RHD*[3]
520A	GAACATGATGCACATCTACA	exon 4, 501-520	520A
520-Lower	TGAACCTGCTCTGTGAAGTGC	intron 4, +194-174	*RHD*
1080-Upper	TTGAGGTCAGGAGTTCGAGATCAC	intron 7, -574-598	*RHD*
1080del10	CCCAATGCTGAGGAGCCAA	eoxn 8, 1104-1076	1080-1089del
1080normal	GCTGAGGAGGACCTGGAAG	exon 8, 1098-1080	*RHD*
78normal	TGGAAGCAGCTCTCATTCTCC	exon 1, 59-79	*RHD*
78delC	TGGAAGCAGCTCTCATTCTCT	exon 1, 80-59	78delC
78-Lower	TCTGTGCCCCTGGAGAACCAC	intron 1, +84-64	*RHD*

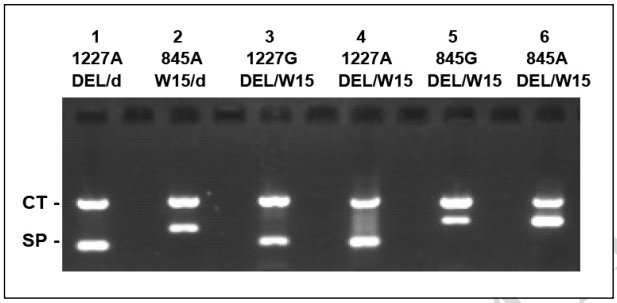

Figure 2 - Detection of an individual with RHD(1227G>A)/weak D type 15 heterozygote.
The land 1 is a DEL control sample, and the land 2 is a weak D type 15 control DNA. The land 3 to 6 detected the individual with *RHD*(1227G>A)/weak D type 15 heterozygote that were found by sequencing. DEL means *RHD*(1227G>A) and W15 means weak D type 15, CT means control and SP means specific bands.

The most frequent alleles

All individuals carrying *RHD* were detected first using a PCR-SSP method for *RHD(1227G>A)*, *weak D type 15*, and *RHD(711delC)* alleles (Table II). The *RHD(1227G>A)* allele was observed in 518 cases including two 1227A/845A individuals. We excluded these two donors with *RHD(1227G>A)/weak D type 15* alleles for the DEL statistical analysis as they were IAT-positive. DEL phenotypes (516 cases) accounted for 63.86% of 808 *RHD*-carrying donors, 21.64% of IAT-confirmed D-negative individuals [516/(2,493-108)], 20.70% of the total 2,493 saline negative individuals, and 0.06% of the total population (516/890,403). Among the DEL individuals, there were 51 who were *D/D* homozygotes (9.88%). With regards to the C antigen, there were 472 positive cases (Cc or CC), which accounted for 91.47%, while the C antigen negative (cc) phenotype was observed in 44 cases (8.53%).

The *weak D type 15* allele (weak D type 15) was detected in 64 cases, accounting for 7.92% of 808 individuals carrying *RHD*, 2.68% of IAT-confirmed D-negative individuals [64/(2,493-108)], 2.57% of the 2,493 saline negative individuals, and 0.007% of the total population. All the individuals with weak D15 were E positive except for two cases of ee phenotypes. Two weak D15 samples were homozygous D/D. Both of these people carried the *RHD(1227G>A)* allele and *weak D type 15* (Figure 1F and Figure 2). These individuals were *RHD(1227G>A)/weak D type 15* heterozygotes with CcEe phenotypes.

The *RHD(711delC)* allele was found in 19 cases. All of these individuals were E antigen positive and accounted for 2.35% of 808 individuals with *RHD*, 0.80% of the IAT-negative samples [19/(2,493-108)], 0.76% of the 2,493 saline D-negative individuals and 0.002% of the total population.

RHD-CE hybrid alleles

All together, 163 cases of *RHD-CE(2-9)-RHD* were observed by testing ten *RHD* exons. These individuals accounted for 20.17% of 808 donors carrying *RHD*, 6.83% of IAT-confirmed D-negative individuals [163/(2,493-108)], 6.54% of the 2,493 saline-negative individuals, and 0.02% of the total population (163/890,403). All of the *RHD-CE(2-9)-RHD* carriers were *D/d* heterozygotes. Previously, most *RHD-CE(2-9)-RHD* carriers were observed to be C-positive[3,19] and a few people had cc phenotypes[20-22]. We assessed that

Table II - RHD alleles in 2,493 samples D-negative in saline.

RHD	CcEe (n)							n (%)	GenBank
	ccee	Ccee	CCee	ccEe	CcEe	ccEE	CCEe		
d/d	1315	243	19	98	9	1		1,685 (67.59%)	
RHD-CE(2-9)-RHD/d	56	91	7	4	5			163 (6.54%)	
RHD(711delC)/d				15	1	1	2	19 (0.76%)	
RHD(78delC)/d		1		-				1 (0.04%)	GQ477180
RHD(520G>A+1080-1089delTCCAGGTCCT)/d		1						1 (0.04%)	GU362076
RHD(1227G>A)/d	24	329	51	20	41		2	467 (18.73%)	
RHD(1227G>A)/RHD(1227G>A)		34	15					49 (1.97%)	
RHD(1227G>A)/weak type 15					2			2 (0.08%)	
Weak D type 15/d		2		46	7	6	1	62 (2.49%)	
Weak D type 24/d				1				1 (0.04%)	
weak D type 25/d				1				1 (0.04%)	
Weak D type 33/d			1	1				2 (0.08%)	
RHD(438G>C)/d		1						1 (0.04%)	JN007073
RHD(101A>G)/d				1				1 (0.04%)	JF830785
RHD(357T>C)/d, DBO-2				1				1 (0.04%)	JF274263
RHD(436G>A)/d	1							1 (0.04%)	JQ937026
D^{VI} type 3/d	5	23	2		1			31 (1.24%)	
D^{V} type 2/d		1		3				4 (0.16%)	
RHD(697G>A)/d, DHK		1						1 (0.04%)	
Total	1401	727	96	191	66	8	5	2,493 (100%)	

103 of 163 people were C antigen positive, and 60/163 cases were C antigen negative (cc phenotype).

Partial D type *D^{VI} type 3* was assessed in 31 cases, which accounted for 3.84% of 808 donors carrying *RHD*, 1.30% of IAT-confirmed D-negative individuals [31/(2,493-108)], 1.24% of the 2,493 saline-negative individuals, and 0.003% of the total population (31/890,403). All of those donors had the *D/d* haplotype, and one case was E-positive.

We also detected four cases of *D^{V} type 2*. These cases formed 0.50% of 808 donors carrying *RHD*, 0.17% of IAT-confirmed D-negative individuals [4/(2,493-108)], 0.16% of the 2,493 saline-negative individuals, and 0.0004% of the total population (4/890,403). All of these cases were also *D/d* heterozygotes.

Rare alleles

RHD sequencing of the remaining 11 samples identified one case of *weak D type 24* [*RHD(1013T>C)/d*], one of *weak D type 25* [*RHD(341G>A)/d*], two cases of *weak D type 33* [*RHD(520G>A)/d*] and one of partial D type *DHK*[23] [*RHD(697G>A)/d*]. Six novel *RHD* alleles were found in the other six individuals (Figure 1A-G).

Three weak D type alleles *RHD(438G>C), RHD(101A>G)* and *RHD(436G>A)* were observed in three donors (GenBank JN007073, JF830785 and JQ937026). The mutations resulted in E146D, Y34C and E146K amino acid substitutions. All of the samples were D/d heterotypes, and the Rh phenotypes were DCcee, DccEe and Dccee, respectively (Table I). The red blood cell D antigen tested negative in saline and positive in IAT for all of the samples. The D epitopes were assessed in each sample; monoclonal anti-D LHM76/58, LHM76/59, LHM174/102, LHM50/2B, LHM169/81, ESD1, LHM76/55, LHM77/64, LHM70/45, LHM59/19, LHM169/80 and LHM57/17 antibodies reacted positive with each red blood cell sample. These cases seem to reveal three new weak D types.

The *RHD(357T>C)/d* mutation was first observed in a Chinese Tibetan individual[24] and was named DBO-2 with normal D expression. However, there are no further serological data on this mutation. The sample D antigen in this study tested negative in saline and positive in IAT. All of the tested D epitopes were positive in IAT. These results led us to believe that *RHD(357T>C)* may result in a weak D phenotype (JF274263).

RHD(78delC)/d and *RHD(520G>A+1080-1089delTCCAGGTCCT)/d* non-functional alleles were observed in two individuals. A C nucleotide deletion at position 78 (78delC) occurred in exon 1 of *RHD*, which caused a frame shift by the addition of a new stop codon TAG at position 112 (GQ477180), resulting in a failure to synthesise normal RhD. We have submitted a brief report to *Vox Sang*[15] about this allele. In this study we

performed a family investigation. In another donor, both a single nucleotide mutation (520G>A) and a short fragment deletion (10 nucleotides TCCAGGTCCT were deleted in *RHD*1080-1089) were observed (GU362076). The 520G>A at *RHD* exon 4 is a *weak D type 33* polymorphism. This point mutation resulted in an M174E amino acid substitution of the RhD polypeptide in the transmembrane domain. Unfortunately the short fragment deletion caused a frame shift with a new TAG stop codon at exon 8 position 372, causing a missing normal RhD or even weak D type 33 protein. The samples were D-negative in IAT and even in the adsorption/elution test, which revealed two novel non-functional *RHD* alleles.

Family investigations

The propositus was tested for *RHD(520G>A+1080-1089delTCCAGGTCCT)* and supposed *DCe/dce* genotypes (Figure 3A). The propositus has no siblings, and his parents were all normal Rh D-positive. His father had a DCcEe phenotype and was a *D/d* heterozygote. We assumed him to have a *DcE/dce* genotype. His mother had *D/D* and DCcEe phenotypes; however PCR-SSP showed that she was 520A positive. Thus, we presumed her *RH* genotype to be $D^{(520A)}Ce/DcE$, which suggests that the *dce* haplotype in the propositus come from his father, and the mutated allele haplotype came from his mother ($D^{(520A)}cE/dce$). To get confirmation of our supposition, primer pairs were designed specifically for the *RHD(1080-1089delTCCAGGTCCT)* deletion and normal *RHD* at the same position. The PCR results (Figure 3A) demonstrated that the propositus was positive for the 1080-1089delTCCAGGTCCT deletion and negative for normal *RHD*. His mother was positive for both *RHD* and the fragment deletion while his father had opposite results. The mother's *RH* genotype should, therefore, be $D^{(520G>A+1080\text{-}1089delTCCAGGTCCT)}Ce/DcE$; she is thus the heterozygous carrier of the mutated allele. Because the propositus inherited a *dce* haplotype from his father, he carried the non-functional *RHD*. The sequencing results revealed a G/A superimposed peak at the *RHD* 520 position (Figure 1F) and continuous

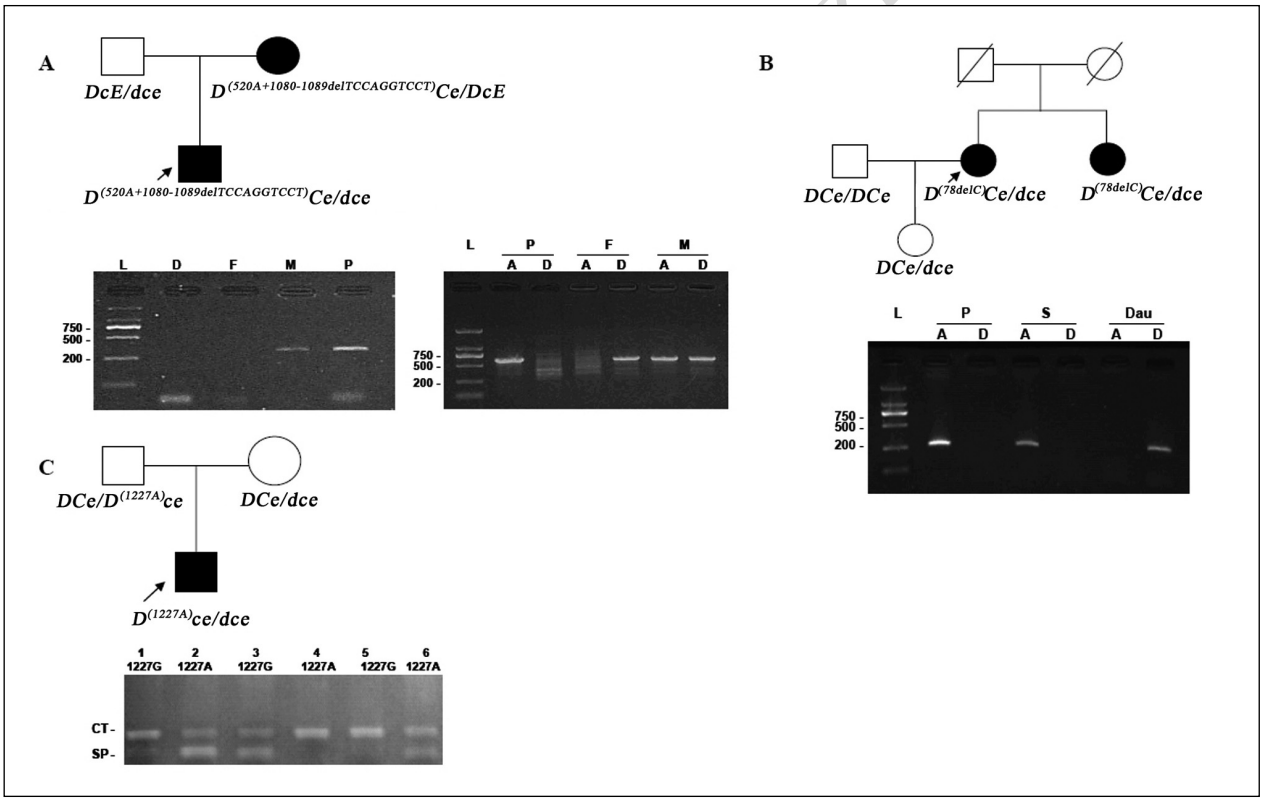

Figure 3 - Family investigations A) The propositus inherited the allele RHD(520A+1080-1089delTCCAGGTCCT) in the haplotype DCe from his mother who carries both a mutated allele and a normal RHD. It was confirmed in the electrophoresis that the left picture detected 520A and the right one detected both RHD(1080-1089delTCCAGGTCCT) and normal RHD. B) The allele of RHD(78delC) was observed in both propositus and her sister. But it is not inherited from the propositus to her daughter which was confirmed by the PCR-SSP specific for both 78delC and 78C. C) A family of a DEL donor with Dccee phenotypes. The lands 1, 3 and 5 detect 1227G, and the lands 2, 4 and 6 are for 1227A. The lands 1 and 2 tested father, 3 and 4 tested mother, and the lands 5 and 6 tested the propositus. The Letter L means DNA ladder, D means RHD, F means father, M means mother, P represents propositus, A means allele, S means sister and the abbreviation Dau means daughter. CT and SP in the part C mean control and specific band respectively.

overlapped peaks starting from the 1080 position (Figure 1G), which is in accordance with the PCR-SSP analysis.

We collected blood samples from the sister of the *RHD(78delC)* proposita, as well as her husband and daughter (her parents were dead). Her sister was D-negative, C+c+E-e+ and *D/d*, which was the same as the proposita. Both of their genotypes may be DCe/dce. Her husband was D-positive, C+c-E-e+ and *D/D*. Her daughter was D-positive, D+C+c+E-e+ and was a *D/d* heterozygote. Thus, their genotype may be *DCe/DCe* and *DCe/dce*, respectively. PCR-SSP detected the *RHD(78delC)* allele in the sister, but not in her husband or daughter (Figure 3B). *RHD* sequencing analysis of the sister demonstrated the presence of *78delC*. Thus, one of the parents of the proposita passed the mutated allele to her and her sister because both were $D^{(78delC)}Ce/dce$ genotypes. However, her daughter had not inherited her non-functional allele.

In total, 44 *RHD(1227G>A)* DEL individuals with cc phenotypes (Rh C antigen negative) were observed in the 516 DEL donors in this study (8.53%). Previous studies demonstrated that most Chinese DEL cases with the *RHD(1227G>A)* allele were Rh C antigen positive (CC or Cc). Only a few $D^{el}ce$ haplotype samples were observed[7,21]. The $D^{el}Ce$ haplotype is, therefore, usually presumed[3,14]. We performed a family investigation of a DEL (*RHD(1227G>A)*) individual with a ccee phenotype (Figure 3C). His father was *D/D* and both 1227A and 1227G positive. His mother was a *D/d* heterozygote, and no *RHD(1227G>A)* allele was detected. Because the propositus had the $D^{el}ccee$ phenotype and was a *D/d* heterozygote, it was clear that his *dce* haplotype had been inherited from his mother while his $D^{(1227A)}ce$ haplotype was from his father and that his father was a recessive *RHD(1227G>A)* allele carrier with a rare $DCe/D^{(1227A)}ce$ genotype.

Discussion

After a brief period of using PCR-SSP for *RHD* studies in China, sequencing analysis technology was gradually adopted for *RHD* research. In 2001, the first *RHD* DEL allele *RHD(1227G>A)* (AF390110) was described in the Chinese through this sequencing method. Over the last decade, a total of 39 *RHD* variants, including five partial D alleles, 13 weak D alleles (including IAT-positive *RHD(1227G>A)*), nine DEL alleles and 12 silent *RHD* alleles were observed in the Chinese Han population (Table III). This is a very small number compared to the hundreds of *RHD* variants found in Caucasians. However, it is quite difficult to discover a new *RHD* allele in the Han Chinese unless an investigation is performed on a very large sample. In this study, samples from nearly one million people from the Han population were investigated (excluding other ethnicities). We found only five new *RHD* alleles, including three weak D alleles and two non-functional alleles.

We also learned that although the number of *RHD* alleles is small, very few *RHD* alleles in the Han Chinese can be observed in the second-level laboratory investigations. Common ones were the partial D alleles D^{VI} *type 3* and D^{V} *type 2*, the weak D allele *weak D type 15*, the DEL allele *RHD(1227G>A)*, and the non-functional alleles *RHD*-CE(2–9)-RHD (D-negative) and *RHD(711delC)* (D-negative). In our study, only 11 rare *RHD* alleles were observed in 2,493 saline D-negative donors if we excluded the common *RHD* variants. Those 11 rare alleles were observed in ten individuals which means that if we include d (*RHD* complete deletion), the common Chinese *RHD* alleles account for as many as 99.78% ([2,493×2−11]/2,493×2) of the *RHD* alleles.

These data demonstrate that D allele prediction is much easier in the Chinese population than in whites because popular alleles are dominant in D-negative individuals. For example, a PCR-SSP system that had been designed specifically for Chinese individuals would only require *weak D type 15, RHD(1227G>A)*, *RHD(711delC)* and 697G detection for partial D^{VI} and D^{V}. The experimental design and PCR manipulation would be both easy and efficient. For more accurate or un-serotyped sample genotyping, the system may only need additional reactions to detect 845G, 1227G and 710C, as well as one specific reaction for *RHD* exon 1. In this system, of total of eight reactions can accurately identify common *RHD* alleles and their heterozygosities. False-positive or false-negative rates may be less than 0.15% in the Chinese population. In the case a result cannot be determined, sequencing analysis should be performed.

As in previous DEL alleles studies in Chinese populations, all of the observed DEL alleles in this study were *RHD(1227G>A)*. Among these, approximately 10% of cases were homozygotes (1227A/A). The DEL phenotype rate that we observed was also similar to that in past reports. There were 21.64% of IAT-confirmed D-negative individuals. In China, the high DEL donor rate is a challenge in clinical Rh-negative transfusions because DEL red blood cells may lead to a delayed haemolytic transfusion reaction in a truly Rh-negative blood recipient[25-27]. Conversely, there may be a chance to save rare Rh-negative blood because DEL patients may be transfused D-positive red blood cells safely[28]. For Rh(D)-related mother-foetus alloimmunisation, a high DEL rate may be beneficial because an antenatal anti-D test may not be possible for pregnant woman with the DEL phenotype[29].

Table III - *RHD* alleles observed in Han Chinese.

N.	Alleles	Variants	Descriptions
1	*RHD*DVI.3*	RHD-CE(3-6)-RHD	Common partial D[3]
2	*RHD*DVI.4*	RHD-CE(2-5)-RHD or RHD-CE(3-5)-RHD	Unpublished data
3	*RHD*DV.2*	RHD-CE(5)-RHD	Common partial D[3]
4	*RHD*DV.1*	667T>G, 697G>C	Reference[8]
5	*RHD*DV.4*	697 G>C	Reference[8]
6	*RHD*DEL1*(IAT-positive)	1227G>A	Common weak D[3]
7	*RHD*weak D type* 1	809 T>G	Found only in Taiwan[4]
8	*RHD*Weak D type 6*	29 G>A	Found only in Taiwan[4]
9	*RHD*Weak D type 12*	830G>A	Reference[10]
10	*RHD*Weak D type 15*	845G>A	Common weak D[3]
11	*RHD*Weak D type 33*	520 G>A	Found only in Taiwan[4]
12	*RHD*Weak D type 51*	594A>T, 602C>G	Reference[11]
13	*RHD*Weak D type 52*	92T>C	Reference[11]
14	*RHD*Weak D type 53*	740T>G	Reference[11]
15	*RHD*Weak D type 71*	29 G>C	Reference[12]
16	*RHD*Weak D type 72*	1212 C>A	Reference[12]
17	*RHD*Weak D type 73*	1241C>T	Reference[12]
18	*RHD*DEL*1	1227G>A	Common DEL[3]
19	*RHD*DEL*1	1227A, IVS7 +152A	Reference[3]
20	*RHD*DEL3*	53T>C	Reference[14]
21	*RHD*DEL6*	251T>C	Reference[14]
22	*RHD*DEL*2	3G>A	Reference[14]
23	*RHD(28C>T)*	28C>T	Reference[14]
24	*RHD*DEL7*	410C>A,	Reference[14]
25	*RHD-RHCE(4-9)-RHD*	RHD-RHCE(4-9)-RHD	Reference[13,14]
26	*RHD- RHCE(10)*	RHD- RHCE(10)	Reference[14], Suspected
27	*RHD-RHCE(2-10)*	RHD-RHCE(2-10)	Reference[6]
28	*RHD*01N.03*	RHD-RHCE(2–9)-RHD	Common allele[3]
29	*RHCE(1-5)-RHD-RHCE(7-10)*	RHCE(1-5)-RHD-RHCE(7-10)	Reference[5]
30	*RHD*01N.16*	711delC	Common allele[3]
31	*RHD*01N.10*	270A	Reference[3]
32	*RHD*01N.27*	IVS6+1–4delGTAA, 904–905insGGCTT	Reference[3]
33	*RHD(78delC)*	78delC	Reference[15]
34	*RHD*01N.19*	933C>A	Reference[9]
35	*RHD*01N.05*	RHD-RHCE(2-7)- RHD	Reference[13]
36	*RHD*01N.28*	970delCGC,976del TCCATCATGGGCTACA	Reference[13]
37	*RHD*01N.11*	325delA	Reference[11]
38	*RHD*01N.25*	IVS2 -1G>A	Reference[11]

DEL individuals were reportedly mostly C-positive[3,7,14,20]; however, in this study, 44 individuals had cc phenotypes (C antigen negative), which accounted for 8.53% of 516 DEL donors. A family investigation was performed for a cc phenotype DEL individual (Figure 3). We confirmed that the father of the propositus was a $D^{I227A}ce$ recessive carrier, and that the propositus inherited the $D^{I227A}ce$ haploid from his father and the *dce* haploid from his mother.

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References

1) Daniels G. *Human Blood Groups* [M], 2nd edn. Oxford, UK: Blackwell Science Press; 2002. p. 165.
2) Chang JG, Wang JC, Yang TY, et al. Human RhD is caused by a deletion of 1,013 bp between introns 8 and 9 including exon 9 of *RHD* gene. Blood 1998; **92**: 2602-4.
3) Shao CP, Maas JH, Su YQ, et al. Molecular background of RhD-positive, D-negative, D(el) and weak D phenotypes in Chinese. Vox Sang 2002; **83**: 156-61.
4) Lin IL, Shih MC, Hsieh MH, et al. Molecular basis of weak D in Taiwanese. Ann Hematol 2003; **82**: 617-20.
5) Xu Q, Zhang J, Wang Q, et al. *RHD* gene polymorphism among RhD-negative Han Chinese. Chin Med J (Engl) 2003; **116**: 1539-43.
6) Shao CP, Xiong Wen. A new hybrid *RHD* positive, D antigen negative allele. Transfus Med 2004; **14**: 185-6.
7) Xu Q, Grootkerk-Tax MG, Maaskant-Van Wijk PA, et al. Systemic analysis and zygosity determination of the *RHD* gene in a D-negative Chinese Han population reveals a novel D-negative *RHD* gene. Vox Sang 2005; **88**: 35-40.
8) Wu JJ, Hong XZ, Xu XG, et al. Molecular basis of partial D phenotypes in Chinese [in Chinese]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2006; **14**: 587-91.
9) Yan L, Wu J, Zhu F, et al. Molecular basis of D variants in Chinese persons. Transfusion 2007; **47**: 471-7.
10) Sun G, Jing H, Xiong W, et al. A weak D type 12 found in the Chinese. Chin J Blood Transfus 2006; **19**: 14-7.
11) Ye LY, Guo ZH, Li Q, Zhu ZY. Molecular and family analyses revealed two novel *RHD* alleles in a survey of a Chinese RhD-negative population. Vox Sang 2007; **92**: 242-6.
12) Ye LY, Guo ZH, Zhu ZY. Homo sapiens Rhesus blood group D antigen (RHD) gene, RHD-weak D allele, exon 1 and partial cds (EU335051), Homo sapiens Rhesus blood group D antigen (RHD) gene, RHD-weak D allele, exon 9 and partial cds (EF103573) and Homo sapiens Rhesus blood group D antigen (RHD) gene, RHD(A414V) allele, exon 10 and partial cds (EU604751). Available at: http://www.ncbi.nlm.nih.gov/genbank. Accessed on 10/07/2013.
13) Ye L, Yue D, Wo D, et al. Molecular bases of unexpressed *RHD* alleles in Chinese D- persons. Transfusion 2009; **49**: 1655-60.
14) Li Q, Hou L, Guo ZH, et al. Molecular basis of the *RHD* gene in blood donors with DEL phenotypes in Shanghai. Vox Sang 2009; **97**: 139-46.
15) Xu H, Ye SH, Shao CP, et al. A new *RHD*-positive, D antigen negative allele in Chinese. Vox Sang 2010; **98**: 576.
16) The Ministry of Public Health of China. Guidelines of technical operation for transfusion. 2nd edn. Tianjin, China: Tianjin Science and Technology Press; 1998. p. 64.
17) Shao CP, Xiong W. A new PCR method for direct determination of *RHD* zygosity. Natl Med J China 2004; **84**: 736-9.
18) Legler TJ, Maas JH, Kohler M, et al. *RHD* sequencing: a new tool for decision making on transfusion therapy and provision of Rh prophylaxis. Transfus Med 2001; **11**: 383-8.
19) Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. BMC Genet 2001; **2**: 10.
20) Sun GD, Yi ZZ, Zhang YP, et al. RHD gene polymorphism of 118 Han population IAT D-negative individuals [in Chinese]. Chin J Blood Transfus 2007; **20**: 292-4.
21) Zhuo CS, Zhuo X, Guo YJ, et al. RHD gene polymorphism of RhD negative individuals in population of Fujian Province [In Chinese]. J Exp Hematol 2008; **16**: 435-8.
22) Cruz BR, Chiba AK, Moritz E, et al. RHD alleles in Brazilian blood donors with weak D or D-negative phenotypes. Transfus Med 2012; **22**: 84-9.
23) Omi T, Takahashi J, Tsudo N, et al. The genomic organization of the partial D category DVa: the presence of a new partial D associated with the DVa phenotype. Biochem Biophys Res Commun 1999; **254**: 786-94.
24) Qing Wei. Random survey for RH allele polymorphism among 50 native Tibetans. Dissertation for the attainment of the Doctor Degree of Medicine (Dr. med.) at the Faculty of Medicine, University of Ulm. 2006. Available at: http://vts.uni-ulm.de/query/longview.meta.asp?document_id=5775. Accessed on 10/07/2013.
25) Shao CP, Wang BY, Ye SH, et al. Childbearing-aged women and anti-D positive blood recipients prevent DEL transfusion from allosensitization and ineffective treatment in East Asia. J Zhejiang Univ Sci B 2012; **13**: 913-8.
26) Kim KH, Kim KE, Woo KS, et al. Primary anti-D immunization by DEL red blood cells. Korean J Lab Med 2009; **29**: 361-5.
27) Yasuda H, Ohto H, Sakuma S, et al. Secondary anti-D immunization by Del red blood cells. Transfusion 2005; **45**: 1581-4.
28) Shao CP. Transfusion of RhD-positive blood in "Asia type" DEL recipients. N Engl J Med 2010; **362**: 472-3.
29) Shao CP, Xu H, Xu Q, et al. Antenatal Rh prophylaxis is unnecessary for "Asia type" DEL women. Transfusion Clin Bio 2010; **17**: 260-4.

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