Chaopeng Shao · Wen Xiong · Wei Wang

Whole exon 5 and intron 5 replaced by *RHCE* in D^{Va}(Hus)

Received: 22 September 2003 / Accepted: 10 November 2003 / Published online: 9 January 2004
© The Japan Society of Human Genetics and Springer-Verlag 2004

Abstract The D^{Va}(Hus) was previously investigated through cDNA analysis, which revealed an *RHD-CE(5)-D* hybrid allele. However, the 5' and 3' breakpoints remain unknown. In this article, gene recombinations between the *RHD* and *RHCE* alleles were investigated by a combination approach of a sequence-specific primer PCR (PCR-SSP) and an *RHD* full-length coding region sequencing method on two Chinese subjects with weak D phenotypes. The hybrid *Rhesus box* of each individual was also investigated through an established PCR-based method. As a result, two partial D phenotypes, D^{Va}(Hus) and D^{VI} type III, were identified, each carrying one hybrid *RHD-CE-D* allele. The two samples were also serotyped with Rh phenotypes of DccEe and DCcee, respectively. Other sequencing analyses of the D^{Va}(Hus) sample showed that the sequence of intron 4 is identical with *RHD*, whereas the whole sequence of exon 5 and intron 5 is identical with *RHCE* except for seven polymorphisms in the intron 5. We may conclude that in the case of this Chinese D^{Va}(Hus), the whole exon 5 and complete intron

5 of a total segment of 1801 nucleotides were replaced by *RHCE* suggesting that the breakpoints of the replaced region are the 5' end of the exon 5 and the 3' end of the intron 5.

Keywords *RHD* · *RHCE* · D^{Va} · D^{VI} · Sequencing · Chinese

Introduction

The Rhesus blood group (Rh) is the most complex of the blood group systems. Rh antigens are encoded by two closely linked homologous genes, *RHD* and *RHCE*, encoding the antigens of D and CcEe, respectively. A normal D antigen serologically comprises a mosaic of at least nine antigenic epitopes (Lomas et al. 1989; Legler et al. 2000). The lack of expression of some of the epitopes at the surface of red blood cells defines the D category, or partial D phenotype. Based on the reactivity with anti-D sera from immunized partial D carriers or differential monoclonal anti-D antibodies, partial D phenotypes have been classified into six distinct categories—D^{II} to D^{VII} (Tippett et al. 1996). Molecular analysis of the partial D phenotype individuals revealed that the gene conversion events between *RHD* and *RHCE* and also the point mutations in *RHD* are the mechanisms for the observed D variants. Due to the complete or partial replacements of *RHD* exon 5 by the corresponding region of the *RHCE* exon 5, the D^{Va} was classified at least into D^{Va}(Kou), D^{Va}(Hus), D^{Va}(TO), D^{Va}(SM), and D^{Va}-like(YH) types (Rouillac et al. 1995; Omi et al. 1999; Hyodo et al. 2000). D category^{VI} is subdivided into type I, type II, and, recently, type III (Wagner et al. 1998).

Subjects and methods

Two donors (samples-a and -b) from the Shenzhen Blood Center, Shenzhen, China, were detected Rh D-negative in saline (human

C. Shao (✉) · W. Xiong
Shenzhen Blood Center and Shenzhen
Institute of Transfusion Medicine, Meigang South Street,
Nigang West Road, 518035 Futian, Shenzhen, China
E-mail: cpshaos@hotmail.com
Tel.: +86-755-83242567
Fax: +86-755-83244188

W. Wang
Department of Biology, Graduate School,
Chinese Academy of Sciences, Beijing, China

W. Wang
Peking University Shenzhen Hospital
and Medical Centre of Peking University,
Hong Kong University of Science & Technology,
Shenzhen, China

W. Wang
School of Public Health, Peking University Health Science Centre,
Beijing, China

W. Wang
Centre for Human Genetics, Edith Cowan University,
Perth, Western Australia, Australia

monoclonal IgM + IgG blend reagent, IgM: clone 175-2, IgG: Clone 415 1E4, Dominion Biologicals Limited, Nova Scotia, Canada) but positive by indirect antiglobulin test (IAT). The two samples were then defined as weak D phenotypes. Rh C, c, E, and e phenotypes were tested through IgM monoclonal antibodies derived from human/murine heterohybridoma cell lines (anti-C MS24, anti-E MS12, anti-c MS33, anti-e MS16, Immucor Diagnostik GmbH, Roedermark, Germany).

The *RHD* and *RHCE* were genotyped through a PCR-SSP method that detects exons 3–7 and 9–10 of *RHD*, exons 1 and 2 of *RHCE*, and exon 5 of *RHEe* (Lan et al. 2000). Full-length *RHD* coding region was sequenced according to a published method (Legler et al. 2001). Briefly, ten exons of *RHD* were amplified separately with ten pairs of *RHD*-specific primers that target the complementary sequences in introns. The PCR products were purified using Microcon-PCR Kit (Millipore, Bedford, MA, USA) and submitted to direct sequencing through a BigDye Terminator Cycle Standard method (ABI Prism 3100 DNA Sequencer, Applied Biosystems, Foster City, CA, USA). In addition, the numbers of the *RHD* gene in each individual were determined through a PCR-based method by specific amplification of the hybrid Rhesus boxes (Perco et al. 2003).

The entire length of intron 4 and intron 5 on sample-a was also direct sequenced. By reference to the *RHD* coding sequence (AJ299023 and AJ299024), a pair of primers was chosen to amplify the intron 4 of *RHD/RHCE* (Sense primer, 5'-AACGATACCC AGTTTGCTG-3', position 606–625 of *RHD*; antisense primer, 5'-ATGACCCTGAGATGGCTGT-3', position 769–751). Intron 5 was amplified with primers D5b-U2 and D6-L (Shao et al. 2002). PCRs were performed in a volume of 25 μ l with 50–100 ng genomic DNA, 1 U of DNA polymerase, 200 μ M dNTPs, 6.25 pmol of each primer, and 1.5 mM $MgCl_2$ in a buffer provided by the manufacturer (AmpliTaQ Gold, Applied Biosystems, Foster City, CA, USA). Thirty cycles for intron 4 amplification or 40 cycles for intron 5 amplification were performed on a thermocycler PE 9700 (GeneAmp PCR system, Applied Biosystems, Foster City, CA, USA) under the following conditions: denaturation at 95°C for 10 min, following cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 2 min (for intron 4 amplification) or 5 minutes (for intron 5 amplification), last extension at 72°C for 5 min.

Results

The samples were phenotyped D + C-c + E + e + (sample-a) and C + c + E - e + (sample-b), respectively. In PCR-SSP, sample-a was detected negative for exon 5 of *RHD*, whereas all other exons (exons 3, 4, 6, 7, 9, 10) tested positive. Sample-b was negative for exons 3–6 and positive for exons 7, 9, and 10 of *RHD*. The *RHCE* detections in both samples showed a consistent result with serological tests. For sample-a, the sequencing results demonstrated that the whole exon 5 was identical with the exon 5 of *RHCE* (*RHE*, 676C), while all other exons (exons 1–4 and 6–10) were the same as normal *RHD*. Therefore, combined with the result of the *RHD* zygosity test, sample-a was a partial D phenotype D^{Va} (Hus) with *RHD-CE(5)-D* allele in *cDE* haplotype assuming the *RHCE* and *RHD* in *cis* (*cDE/cde*). For sample-b, the sequences of detected exons (exons 1–2 and 7–10) were the same as *RHD*, which were determined both serologically and genotypically as category D^{VI} type III carrying a hybrid allele *RHD-CE(3–6)-D* (*CDe/cde*).

The D^{VI} type III was previously investigated through both cDNA and genomic DNA. The D^{Va} (Hus),

Fig. 1 Schematic representation of the genomic structures of D^{Va} (Hus) (*cDE/cde*). Whole exon 5 and intron 5 of *RHD* were replaced by an equivalent of *RHCE*. The exon and intron of *RHD* are symbolized by black and gray squares, respectively. The white boxes indicate substituted exon or intron of *RHCE*. Broken lines represent polymorphisms compared with intron 5 of *RHCE* (AY330698). Arrowheads mark the 5' and 3' breakpoints of the hybrid allele

however, was only analyzed with mRNA. To elucidate the breakpoints of the hybrid allele in this D category, whole intron 4 and intron 5 were sequenced. The results showed that the sequence of intron 4 was identical to *RHD*, while all *RHD/RHCE* polymorphisms (a total of 28) in intron 5 were the same as *RHCE* (GenBank AB035197) but with seven novel polymorphisms (EMBL/GenBank/DDBJ nucleotide sequence database accession number AY330698): 23–25(GCA)₂, 98G > A, 168–169insG, 205–206insT, 494–495insA, 1256–1257 insC, and 1347G > T, which distribute to the whole length of intron 5. Our results indicated that the whole exon 5 and complete intron 5 of a total segment of 1,801 nucleotides were replaced by *RHCE* in D^{Va} (Hus), suggesting that the breakpoints of the replaced region are the 5' end of exon 5 and the 3' end of intron 5 (Fig. 1).

Discussion

This is the first report of partial D phenotypes D^{Va} and D^{VI} in the Chinese population. The samples were screened from 83,722 donors in southern China, which gives a very low frequency in this population. However, as the numbers of the partial D antigen sites may be different in different individuals with the same D category, some might be detected as D-positive with the currently sensitive IgM + IgG anti-D reagents in saline. Mostly, a D^{Va} is produced by a D^{Vace} haplotype in blacks and D^{VaCe} in whites and Japanese (Tippett et al. 1996; Wagner et al. 1998; Legler et al. 2000). Interestingly, in our case, D^{Va} was expressed in a D^{Vace} haplotype (indicating gene conversion happened *in cis*), which is rare in other populations.

The 5' hybrid site of the *RHCE*-derived genomic sequences of the D^{VI} type III allele, *RHD-CE(3–6)-D*, was located in the middle of intron 2, while the 3' breakpoint was located in intron 6 between +360 bp and +963 bp (Wagner et al. 1998). Our results demonstrated that the 5' hybrid point of the replaced exon-5-intron-5 region in D^{Va} (Hus) was between position –246 of intron 4 (the first *RHD/RHCE* polymorphism from the 3' end, AB041536 and AB041535) and 667 of *RHD* exon 5, and the 3' hybrid site would be between

+ 1497 of intron 5 (the first *RHD/RHCE* polymorphism from the 3' end, AB035198 and AB035197) and 916 of *RHD* exon 5, which suggests a replaced region of the 5' end of exon 5 to the 3' end of intron 5.

References

- Hyodo H, Ishikawa Y, Kashiwase K, Ogawa A, Watanabe Y, Tsuneyama H, Toyoda C, Uchikawa M, Akaza T, Fujii T, Kozuma S, Taketani Y, Juji T (2000) Polymorphisms of RhD(Va) and a new RhD(Va)-like variant found in Japanese individuals. *Vox Sang* 78:122–125
- Lan JC, Chen Q, Wu DL, Ding H, Pong DB, Zhao T (2000) Genetic polymorphism of RhD-negative associated haplotypes in the Chinese. *J Hum Genet* 45:224–227
- Leader KA, Kumpel BM, Poole GD, Kirkwood JT, Merry AH, Bradley BA (1990) Human monoclonal anti-D with reactivity against category DVI cells used in blood grouping and determination of the incidence of the category DVI phenotype in the DU population. *Vox Sang* 58:106–111
- Legler TJ, Wiemann V, Ohto H, Matuda I, Obara T, Uchikawa M, Kohler M (2000) D(Va) category phenotype and genotype in Japanese families. *Vox Sang* 78:194–197
- Legler TJ, Maas JH, Kohler M, Wagner T, Daniels GL, Perco P, Panzer S (2001) RHD sequencing: a new tool for decision making on transfusion therapy and provision of Rh prophylaxis. *Transfus Med* 11:383–388
- Lomas C, Tippet P, Thompson KM, Melamed MD, Hughes-Jones NC (1989) Demonstration of seven epitopes on the Rh antigen D using human monoclonal anti-D antibodies and red cells from D categories. *Vox Sang* 57:261–264
- Omi T, Takahashi J, Tsudo N, Okuda H, Iwamoto S, Tanaka M, Seno T, Tani Y, Kajii E (1999) 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* 254:786–794
- Perco P, Shao CP, Mayr WR, Panzer S, Legler TJ (2003) Testing for the D zygosity with three different methods revealed altered Rhesus boxes and a new weak D type. *Transfusion* 43:335–339
- Rouillac C, Colin Y, Hughes-Jones NC, Beolet M, D'Ambrosio AM, Cartron JP, Le Van Kim C (1995) Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D proteins associated with alteration of D epitopes. *Blood* 85:2937–2944
- Shao CP, Maas JH, Su YQ, Kohler M, Legler TJ (2002) Molecular background of Rh D-positive, D-negative, D^{el} and weak D phenotypes in Chinese population. *Vox Sang* 83(2):156–161
- Tippet P, Lomas-Francis C, Wallace M (1996) The Rh antigen D: partial D antigens and associated low incidence antigens (Review). *Vox Sang* 70:123–131
- Wagner FF, Gassner C, Muller TH, Schonitzer D, Schunter F, Flegel WA (1998) Three molecular structures cause rhesus D category VI phenotypes with distinct immunohematologic features. *Blood* 91:2157–2168