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Molecular basis of weak D in Taiwanese

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Abstract Two genes, RHD and RHCE, encode the antigens of the RH blood group system. The weak D phenotype is caused by many different RHD alleles encoding aberrant RhD proteins, resulting in distinct serologic phenotypes and anti-D immunization. We analyzed seven weak D phenotypes excluding D^{el}, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing methods to detect the changes of all ten RHD exons. The results show that there are four types of weak D in Taiwanese: one case each for CGG to CAG mutation at codon 10, GTG to ATG mutation at codon 174, and GTG to GAG mutation at codon 270, and four cases for GGT to GAT mutation at codon 282. In conclusion, we present the first data of a molecular basis of weak D in Taiwanese, which suggest a clinically relevant potential for anti-D immunization and may improve transfusion strategy in weak D Taiwanese patients.

Keywords Weak D · Mutation · Taiwanese · RHD

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Introduction

The Rh blood group system is, next to ABO, the most clinically significant of human blood groups in transfusion medicine [1, 2]. Consisting of at least 45 independent antigens, it is also the most polymorphic. Two genes, RHD and RHCE, encode the antigens of the RH blood group system. The most clinically important antigen D is determined by the presence of a functional and grossly normal RHD gene [3, 4]. About 18% of Caucasians and 0.5% of Chinese do not express an antigen D (called Rh negative), and most Rh-negative cases are caused by the RHD gene deletion [5, 6, 7]. About 0.2–1% of Caucasians have red blood cells with reduced expression of the D antigen (weak D, formerly D^u) [7]. The molecular basis of weak D in Caucasians has been explored in recent studies [8, 9, 10, 11, 12].

We had explored the molecular basis of a weak D-Rh D^{el} , which is always classified as Rh-negative phenotype, and is caused by a deletion of exon 9 in the RHD gene [13]. Recently, we found seven cases of weak D, which were different from the genotype of Rh D^{el}. Weak D except D^{el} is very rare in Taiwanese, and the molecular basis of this phenotype is still unknown. In this study, we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing methods to analyze the genetic lesion of weak D in Taiwanese.

Materials and methods

Blood samples

Ethylenediaminetetraacetate (EDTA)-anticoagulated blood samples were collected from seven Chinese donors from the Blood Bank of the China Medical College Hospital, Taichung Transfusion Center, and the Blood Bank of Kaohsiung Medical University Hospital. The weak D typing was characterized in accordance with published standards as described [14]. The donors were unrelated, and D^{el} samples were excluded from this study.

Table 1 PCR primers used for specific analysis

Position	Upstream primer	Downstream primer	Restriction enzyme
Exon 1	5'-CTCTAAGTACCCACGGTCTG-3'	5'-ACCTTGATAGGATGCCACGA-3'	Hha I, Hae III
Intron 1	5'-CTTTAAATCTTGGCTGTAGGC-3'	5'-CAGCTTGAGCTCCAGAACG-3'	Taq I
Exon 2	5'-CAGCCTGGGTGACGAGTGAA-3'	5'-CCACCATCCCAATACCTGAA-3'	Xho I
Intron 2	5'-GTGCCACTTGACTTGGGACT-3'	5'-GTGGACCCAATGCCTCTG-3'	Pst I
Exon 3	5'-TGTCGGTGCTGATCTCCG-3'	5'-GATATTACTGATGACCCTCCTCAAG-3'	Bst U, EcoN I
Exon 4	5'-AGACAGACTACCACATGAAC-3'	5'-CCAGCATGGCAGACAAACTG-3'	Cvn I, Bcl I
Exon 5	5'-CCCTCTTCTTGTGGATGTTC-3'	5'-GCTCACCTTGCTGATCTTCC-3'	Taq I, Styl I
Exon 6	5'-TATGTGCACAGTGCGGTGTT-3'	5'-TGTCTAGTTTCTTACCGGCA-3'	Rsa I
Exon 7	5'-TAACCGAGTGCTGGGGGTTC-3'	5'-AGTGACCCACATGCCATTGC-3'	Nla IV, Msp I
Exon 9	5'-TATGCATTTAAACAGGTTTGC-3'	5'-GGAGAAAAATCTTACCTTCCA-3'	BspH I

PCR-RFLP analysis of genomic alterations

DNA was prepared as described previously [13]. Locus-specific assays for exon 1, intron 1, intron 2, intron 4, intron 8, and exon 10 were performed using PCR as described elsewhere [15, 16, 17, 18]. For specific analysis of exons 3, 4, 5, 6, 7, and 9, the PCR primers used are shown in Table 1. The PCR conditions used were as described previously except the annealing temperature was 56°C in this study. Amplified DNA fragments were digested with different restriction enzymes according to the conditions recommended by the manufacturers and were then analyzed on 1.5–4.0% agarose gels after being stained by ethidium bromide.

Sequencing of the ten RHD exons from genomic DNA

Nucleotide sequencing of genomic DNA for all ten RHD exons and parts of the promoter was performed as described by Wanger et al. [8].

Direct sequencing of PCR products

For isolation of the fragments from PCR products, the products were electrophoresed in 2% low melting agarose gels and were excised after separation. The fragments were then purified using a commercial kit (Qiaex II gel extraction kit, QIAGEN Inc., Chatsworth, Calif., USA), and the purified fragments were directly sequenced using the cycling sequencing method (AmpliCycle Sequencing Kit, Perkin Elmer, Branchburg, N.Y., USA) and ABI 310 sequencer.

Results

PCR-RFLP analysis of weak D

To investigate whether the RHD gene was present in weak D patients, we analyzed seven weak D patients using PCR-RFLP methods. Several RhD-positive and -negative cases were used as positive and negative controls. The results showed that all cases had RHD genes. One case showed an abnormal fragment (145 bp) in the exon 1 region after restriction enzyme Msp I digestion (Fig. 1A). Four cases had an undigested fragment (242 bp) in exon 6 after Rsa I digestion (Fig. 1B). After direct sequencing analysis of these five cases, we found that there were two kinds of mutations. One was a CGG to CAG mutation at codon 10, which resulted in an abnormal exon 1 and thus abolished an authentic Msp I site (Fig. 2A). The other was a GGT to GAT mutation at codon 282, which resulted in an abnormal exon 6 and thus abolished an authentic Rsa I site (Fig. 2B).

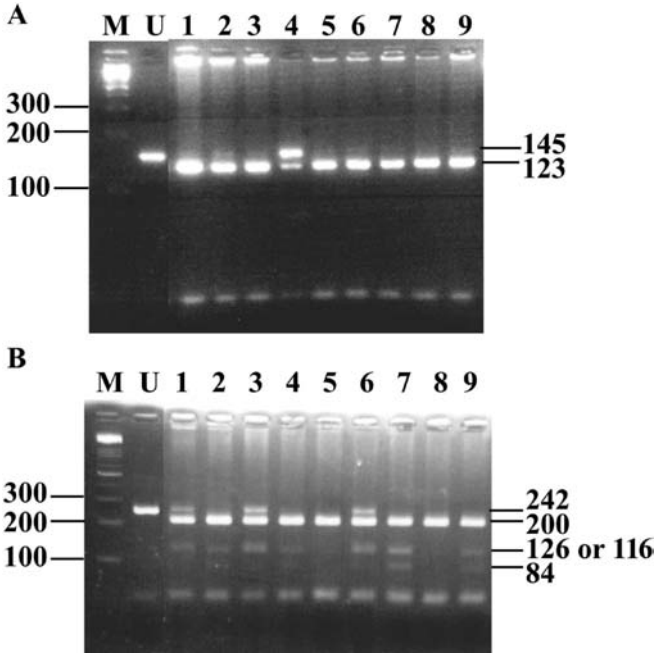

Fig. 1A, B The results of PCR-RFLP analysis of the RH gene for representative cases. **A** The results of PCR-RFLP analysis of exon 1 polymorphism are shown. Case 4 shows an undigested 145-bp fragment and a digested 123-bp fragment, but the other cases are completely digested. **B** The results of PCR-RFLP analysis of exon 7 polymorphism are shown. Cases 1, 3, and 6 show an undigested 242-bp fragment and several digested fragments (200 bp, 126 bp, 116 bp, and 84 bp). *M* 100-bp ladder marker, *U* uncut control. Case 4 in **A** and cases 1, 3, and 6 were subjected to sequencing analysis

Direct sequencing of all ten RHD exons

Although PCR-RFLP had detected five of seven cases, there were still two cases left unknown. In order to solve these problems, we sequenced all ten RHD exons to analyze all the coding region of the RHD gene. After direct sequencing analysis of the cases, two new mutations were found. The one was a GTG to ATG mutation at

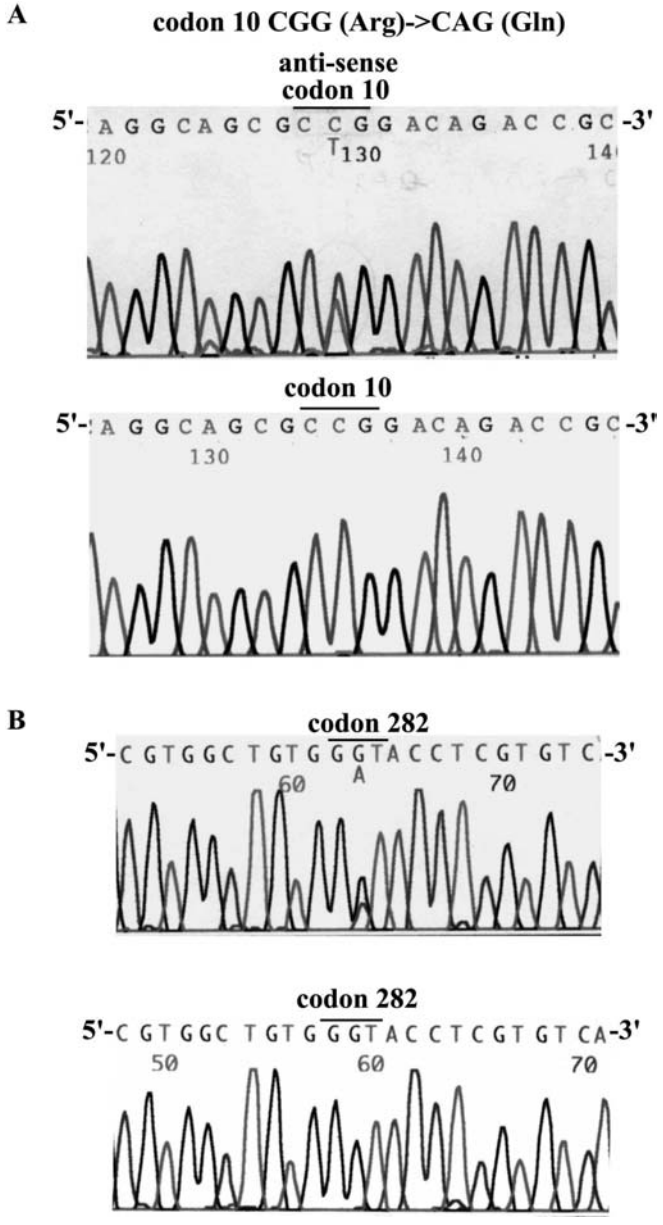

Fig. 2A, B The results of sequencing analysis of abnormal cases in Fig. 1. **A** The results of sequencing analysis of exon 1 for case 5 of Fig. 1A show a G to A substitution at the second base of codon 10 (CGGCAG) (*upper panel*), the normal control is shown in the *lower panel*. **B** The results of sequencing analysis of exon 7 for cases 1, 3, and 6 of Fig. 1B show a G to A substitution at the second base of codon 282 (GGTGAT) (*upper panel*). The normal control is shown in the *lower panel*

codon 174 (Fig. 3A), and the other was a GTG to GAG mutation at codon 270 (Fig. 3B).

Discussion

The weak D phenotype is a group of RHD^+ genotypes with altered RhD proteins and a reduced RhD expression on the red blood cells' surface. Several studies have

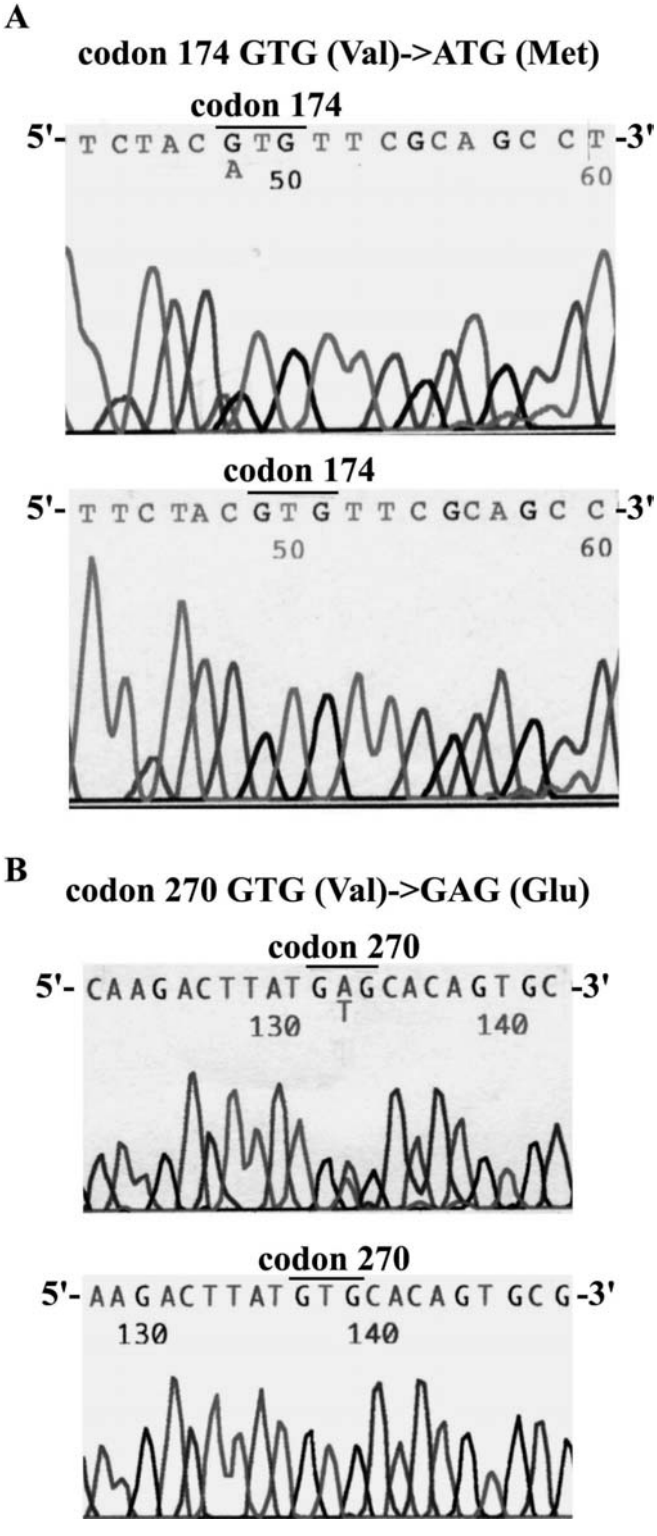

Fig. 3A, B The results of direct sequencing analysis of the RHD gene. Two new mutations are found in two D^u phenotype of Taiwanese. One case has a G to A substitution at the first base of codon 174 (GTGATG). (**A**, *upper panel*). The *lower panel* is a normal control. The other case has a T to A substitution at the second base of codon 270 (GTGGAG). (**B**, *upper panel*). The *lower panel* is a normal control

shown that the molecular defect of weak D is caused mostly by a mutation in the RHD gene [8, 9, 10, 11, 12]. More than 20 types of weak D have been analyzed, and most of them have amino acid substitutions in the intracellular or transmembranous parts of the RhD protein [8, 9, 10, 11, 12]. In this study, we found that there were more than four types of mutations in Chinese weak D patients; they are R10Q, G282D, V174 M, and V270E. The R10Q and G282D were named weak D type 6 and type 15, respectively, while V174 M and V270E have not been reported yet. We found that the G282D mutation was the most common type of weak D (four of seven) in Taiwanese. Shao et al. also found this mutation in Shenzhen of Mainland China [10].

RHD and RHCE genes are highly homologous. Because of their high similarity, it is difficult to design primers to amplify the RHD gene specifically. Wagner et al. developed a specific PCR to amplify the exon of RHD from genomic DNA. In this study, we used the same method to analyze the mutation of weak D in Taiwanese. Our results confirm the efficacy of the method.

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