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Case Report

*RHD*509G* Allele Associated with an RhD Variant Phenotype: A Case Report

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ABSTRACT: This study aims to investigate an unusual RhD variant phenotype in a pregnant Chinese woman. Serological methods were utilized to confirm RhD-negative status, perform CE serotyping, and screen for unexpected antibodies in a pregnant woman who was initially identified as D-negative. RHD gene-coding regions were amplified and sequenced, and RHD zygosity was also detected using PCR. The impact of an identified missense mutation was predicted using MutationTaster software. The case was identified serologically as a weak D phenotype with a CE type of Ccee. No unexpected antibodies were detected. The hybrid Rhesus box was identified as RHD^{+}/RHD^{-} heterozygote, and the haplotype was hypothesized to be $CD^{w}e/cde$. The sequencing results revealed a novel missense mutation, c.509T>G, in exon 4 of *RHD* gene, leading to the amino acid change p. Met170Arg. Allele *RHD*509G* was found to be a new RhD variant, and the sequence was submitted to GenBank under accession number MW923730.

Keywords: RhD variant; Novel allele; Genotype; Hybrid Rhesus

1. Introduction

The Rh blood group system (ISBT004) is the most complex and polymorphic blood group known in humans, which is essential for transfusion medicine. The RhD antigen is highly immunogenic, and anti-D antibodies can cause hemolytic transfusion reactions and severe fetal neonatal hemolytic disease [1]. In addition to the common RhD-positive and RhD-negative types, RhD variants also exist in the Chinese population.They are broadly categorized into weak D, partial D, and Del according to differences in the amount and nature of D antigen expression. Alloimmune status differs for RhD variants formed by different RHD alleles, and genetic testing is an effective tool for revealing the molecular mechanisms and signatures underlying RhD variants [2,3]. In this study, we investigated a case of a pregnant woman with a weak D phenotype. DNA sequencing identified c.509 T>G variant, leading to a missense mutation in exon 4 of *RHD* gene.

2. Case Presentation

A 28-year-old pregnant Chinese woman with a maternal history of G1P0A0L0 (G, P, A, and L, representing pregnancy, delivery, miscarriage, and surviving child, respectively) was presented to our center for RhD testing and unexpected antibody screening. The patient was healthy and had no history of blood transfusion. After obtaining informed consent, approximately 3 mL of EDTA-anticoagulated and non-anticoagulated blood was collected.

Serologic Rh-CcDEe typing was performed using anti-D (Runpu, Shanghai, China), anti-C, anti-c, anti-E, and anti-e antibodies (Shanghai Hemo-Pharmaceutical and Biological, Co., Ltd., Shanghai, China). Confirmatory tests for Rh negativity using a tube test and the indirect antiglobulin test (IAT) method were performed with three other anti-D reagents (Reagent 1: IgM + IgG, Sanquin, Netherlands; Reagent 2: IgM + IgG, Dominion, Dartmouth, Germany; Reagent 3: IgG, Shanghai Hemo-Pharmaceutical and Biological, Inc., Shanghai, China). Additionally, unexpected antibodies were screened using RBC antibody screening reagents (Shanghai Hemo-Pharmaceutical and Biological Co., Ltd., Shanghai, China).

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Genomic DNA was extracted from peripheral blood using a commercially available extraction kit (TaKaRa). RHD zygosity analysis was performed following amplifying the *RhD* exon 1 and hybrid *Rhesus box* regions using the specific sequence primer-polymerase chain reaction (SSP-PCR) method described previously [4]. Exons 1–10, including the adjacent flanking intron regions, were amplified *via* PCR using previously designed primers [5]. The PCR procedure was as follows: 95 ℃ for 5 min; 35 cycles at 95 °C for 30 s, 61 ℃ for 30 s and 72 ℃ for 1 min; and then 72 ℃ for 10 min. Both strands of the PCR products were sequenced using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Soft Sequencing Analysis 6 and SeqScape v3.0 were used for the analysis. All sequences were compared with reference sequences (GenBank accession No. NG007494). The online bioinformatics software MutationTaster (https://www.mutationtaster.org/) was used to predict the impact of missense mutations on RhD protein function [6].

Initial RhD typing showed the sample to be negative for RhD antigens. However, the confirmatory test revealed a strongly positive reaction. The CE serotype was identified as Rh-Ccee, and screening for unexpected antibodies in the pregnant woman's plasma produced negative results (Table 1).

Table 1. Results of serological Rh typing.

	Initial RhD typing	**Confirmatory test for Rh negative**			**Antibody screening**	**Rh-CcEe typing**			
		Reagent 1	**Reagent 2**	**Reagent 3**		**Anti-C**	**Anti-c**	**Anti-E**	**Anti-e**
IS	0	0	0	/	0	4+	4+	0	4+
IAT	/	4+	4+	4+	0	/	/	/	/

IS: immediately spin; IAT: indirect antiglobulin test.

The sample tested positive for exon 1 and hybrid *Rhesus box*, indicating a heterozygous status (Figure 1). This haplotype was hypothesized to be *CD^{w}e/cde*. A missense mutation, c.509 T>G, which results in an amino acid substitution from Met to Arg at residue 170 (p.Met170Arg), was identified in exon 4 (Figure 2). This sequence was deposited in the GenBank database under accession number MW923730. MutationTaster predicted a genetic alteration to be one of four possible types: disease-causing (probably deleterious), disease-causing automatic (known to be deleterious), polymorphism (probably harmless), and polymorphism automatic (known to be harmless). According to MutationTaster, the missense mutation c.509 T>G was predicted to result in an amino acid sequence change, indicating that protein features might be affected, with splice site changes typed as "disease-causing".

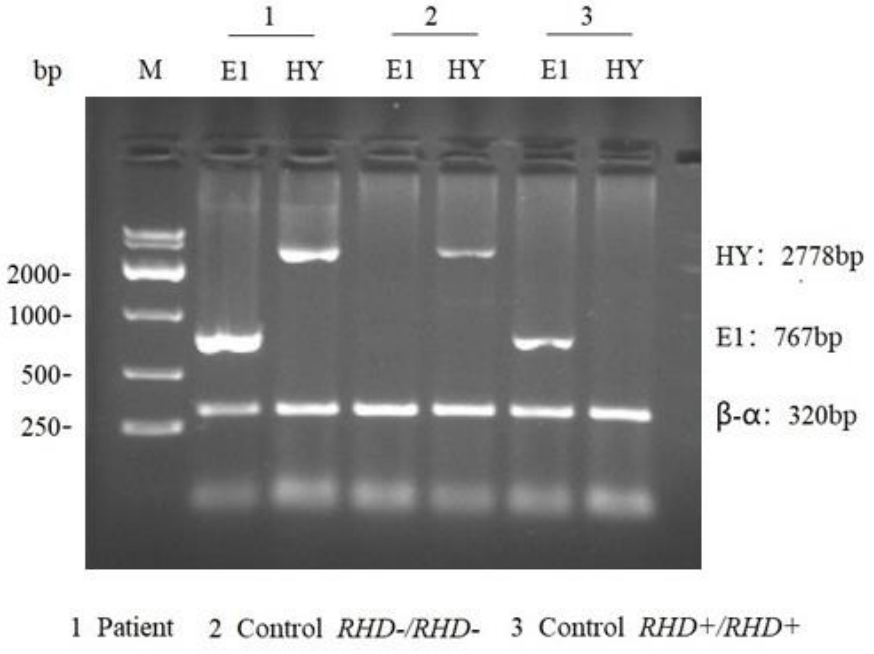

Figure 1. Specific sequence primer-polymerase chain reaction (SSP-PCR) analysis of *RHD* zygosity.

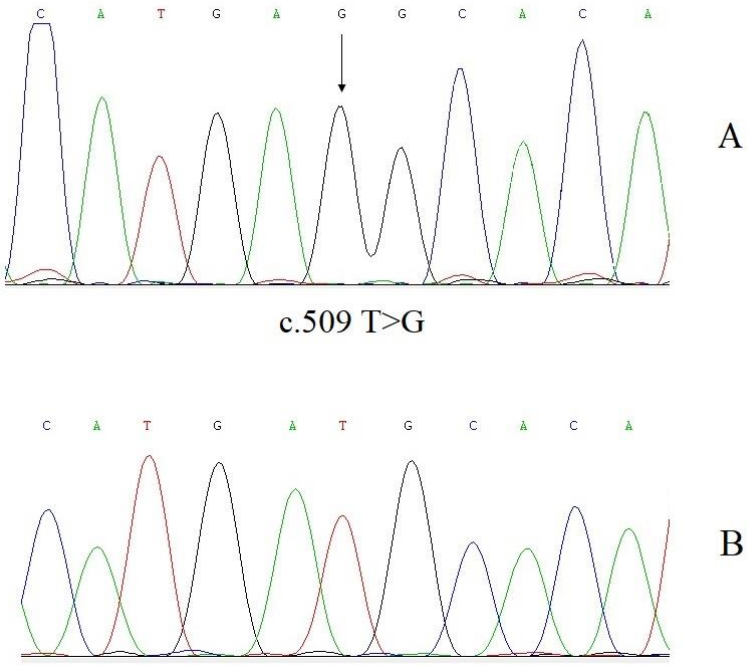

Figure 2. DNA sequencing chromatograms showing the c.509T>G sequence (**A**) and the wild-type sequence (**B**). The arrow shows the location of this mutation.

3. Discussion

We found a weak D phenotype associated with a single nucleotide mutation, c.509 T>G in exon 4, which results in a p.Met170Arg amino acid substitution. Methionine is a hydrophobic amino acid usually located in the core region of proteins, whereas arginine is not. The 170th amino acid is predicted to be located in the 6th transmembrane region rather than on the outer surface of the protein expressed on red blood cells, suggesting that this mutation may cause a reduction in the folding and insertion efficiency of the RhD antigen into the cell membrane and thereby decrease antigen quantity [7]. MutationTaster predicted this mutation to be “disease-causing”.

Ding M et al. also discovered a similar case and published the allele first. She examined the blood sample using a D-screen identification kit [8]. Red blood cells agglutinated from 0 to 4+ with different anti-D antibodies, and the reactivity pattern was similar to that of partial DFR. Therefore, Ding M et al. presumed that the c.509T>G mutation leads to the loss of certain D-specific epitopes and can be classified as partial D.

According to the International Society of Blood Transfusion (ISBT), more than 160 weak D or partial D alleles or both have been found to date [9]. Over 95% of the weak D phenotypes in Caucasians were classified as weak D 1, 2, 3, or 4, whereas the most common weak D type in the Chinese population was weak D15 [10–12]. The belief that weak D solely entailed a reduction in the number of RhD antigens without the involvement of alterations in antigenic epitopes is now considered outdated [13]. Numerous weak D cases were found to express alloanti-D, including weak D 4.2, 11, 15, 21, and 57 [10,13,14], and the term “weak partial D” was proposed. However, it remains unclear if *RH*509* individuals can be immunized to produce anti-D antibodies after exposure to the D antigen. Although the pregnant woman in this study did not produce alloanti-D at the time of this report, she should receive Rh immunoprophylaxis like truly RhD-negative pregnant women and undergo regular antibody testing during pregnancy.

In conclusion, we identified a missense mutation, c.509T>G, in the RhD blood group system. This mutation encodes a p.Met170Arg amino acid substitution associated with an RhD variant phenotype.

Author Contributions

L.M. analyzed the data and wrote the paper. T.L. and R.Z. performed the serological and genetic experiments, respectively. F.Z. and L.S. contributed the sample collection. W.L. reviewed the literature.

Ethics Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Jiangsu Province Blood Center (protocol code 2023002, 21 December 2023).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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Declaration of Competing Interest

The authors declared no conflict of interests.

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