Received: 10 May 2024 | Revised: 7 February 2025 | Accepted: 13 March 2025

DOI: 10.1111/trf.18223

TRANSFUSION

BRIEF REPORT

Blood Group Genomics

Identification of a novel variant (c.1-111A>G) located in GATA-1 motif of *RHCE* proximal promoter in two Chinese patients with the rare D-- phenotype

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Funding information

Science and Technology Projects in Guangzhou, Grant/Award Numbers: 2023A03J0545, 2023A03J0552; National Natural Science Foundation of China, Grant/Award Number: 82070198; Key Medical Disciplines and Specialties Program of Guangzhou (2025–2027), China

Abstract

Background: D-- is a rare phenotype lacking the expression of the C, c, E, and e antigens and several high-frequency antigens on the red cells. Anti-Rh17 (Hr0) could be developed in individuals with the D-- phenotype to cause hemolytic transfusion reactions (HTR) and hemolytic disease of the fetus and newborn (HDFN). Nuleotide(s) change of the *RHCE* gene and *RHCE-D-CE* hybrid alleles are the common molecular basis of the D-- phenotype.

Study Design and Methods: One D-- Chinese patient detected in routine RhD and RhCE serologic testing and another D-- Chinese patient identified with anti-Rh17 were recruited. Further *RHD*, *RHCE*, and *RHAG* whole gene sequences were analyzed using the PacBio sequencing. A dual-luciferase reporter assay was performed to verify the effect of the variant identified in the promoter of the *RHCE* gene on the transcriptional activity of the reporter gene in vitro.

Results: A homozygous *RHCE*Ce(1-111G)/Ce(1-111G)* genotype and a heterozygous *RHCE*CeN.08/Ce(1-111G)* genotype carried one novel variant (c.1-111A>G) located in the GATA-1 motif of the *RHCE* proximal promoter was identified in two D-- patients, respectively. In the reporter assay, the luciferase transcriptional activity of the mutant *RHCE* promoter [c.1-111G] construct reduced from ~1.0 to 0.28 relative luciferase activity normalized to *RHCE* wild-type, with a ~72% reduction rate.

Conclusion: The novel variant of the GATA-1 motif of the *RHCE* proximal promoter was identified to diminish the binding of the GATA-1 transcription

Abbreviations: 5′-UTR, 5′-untranslated regions; EDTA, ethylene diamine tetraacetic acid; FLU, firefly luciferase; HDFN, hemolytic disease of the fetus and newborn; HEL, human erythroleukemia cell; HTR, hemolytic transfusion reaction; LR-PCR, long-range polymerase chain reaction; RBCs, red blood cells; RLU, renilla luciferase.

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factor and markedly down-regulate the transcription activity of the *RHCE* gene to abolish the expression of RhCE antigens, causing the rare D-- phenotype.

KEYWORDS
anti-Rh17, D-- phenotype, GATA-1 motif, *RHCE* gene

1 | INTRODUCTION

The Rh blood group system is one of the most polymorphic and complicated red cell blood group systems. The RhD and RhCE (C, c, E, and e) antigens with important clinical significance are encoded by the two homologous *RHD* and *RHCE* genes, respectively. Besides, the Rh-associated glycoprotein (RhAG), encoded by the *RHAG* gene, is also essential for the normal expression of RhD and RhCE antigens due to the formation of the Rh complex together on the surface of red blood cells (RBCs).

The D-- blood group is a rare phenotype lacking the expression of C, c, E, and e and several high-frequency antigens, but with usually exalted expression of the D antigen. Nonsense mutation, few nucleotides deletion or insertion, and splicing site mutation of the *RHCE* gene, *RHCE-D-CE* hybrid alleles (Web resources), and some complex gene rearrangments[1] have been described before accounting for the rare D-- phenotype. In the patients with the D-- phenotype, the antibodies against the high-frequency antigens collectively known as anti-Rh17 (-Hr0) could be produced after incompatible transfusion or pregnancy, which could cause hemolytic transfusion reaction (HTR) and severe hemolytic disease of the fetus and newborn (HDFN).[2] In China, since the alloantibodies against RhCE antigens are most commonly distributed among the Chinese patient population,[3] the routine RhCE typing of patients and donors and RhCE-compatible transfusion were increasingly adopted in recent years to avoid anti-RhCE alloimmunization. Consequently, an increasing number of individuals with the rare D-- phenotype were identified.

In this study, two patients with the rare D-- phenotype were identified, in which one was detected during the patients' routine RhCE typing, and another patient was detected due to the identification of anti-Rh17. The PacBio sequencing identified a novel homozygous and heterozygous variant (c.1-111A>G) located on the GATA-1 motif of the promoter region of the *RHCE* gene in two D-- patients, respectively. Further Dual-Luciferase reporter assay was performed to show the novel variant markedly reduced the transcriptional activity of the luciferase reporter gene in vitro, which could cause the D-- phenotype.

2 | MATERIALS AND METHODS

2.1 | Serologic typing

Routine RhD and RhCE serologic typing was performed in the patients ($n = 124{,}249$) from April 2020 to April 2024 in Jiangxi Provincial People's Hospital (Nanchang, China) using Rh blood group card (Jiangyin Libo Co. Ltd, Jiangsu, China). Accidentally, a 55-years-old male patient was identified with a D-- phenotype, who suffered from persistent pain and had no history of blood transfusion recorded. RhCE antigens were retyped using four monoclonal antibodies (anti-C, IgM, clone MS-24; anti-c, IgM, clone MS-33; anti-E, IgM, clone MS-80/MS-258; anti-e, IgM, clone MS-16/MS-21/MS-63, Shanghai Blood Biology Co. LTD, Shanghai, China) with tube method in saline. The antibody screening test was performed with IAT method in Coomb's card (DiaMed GmbH, Cressier, Switzerland).

The second D-- patient was an 8-month-old baby diagnosed with congenital heart disease, who had a need for surgical blood preparation and an unknown transfusion history. The patient was recruited from the RBC reference lab in Guangzhou Blood Center (Guangzhou, China). The ABO, RhD, and RhCE antigens were detected using the tube method in saline or Rh blood group typing card (Jiangyin Libo Co. Ltd). Further antibody screening and identification tests were performed with the tube method in saline and IAT method in Coomb's card (DiaMed GmbH).

Unfortunately, the blood samples of the family members of the two probands were not available for pedigree analysis.

2.2 | Long-read sequencing analysis of *RHD*, *RHCE*, and *RHAG* genes

Genomic DNAs were extracted from the peripheral blood of two D-- patients using the MagNA Pure LC DNA Isolation Kit I (Roche, Basel, Switzerland) and then prepared at a final concentration of 30 ng/μL and stored in Tris-EDTA buffer. Then, sequencing analysis of the full-length *RHD*, *RHCE*, and *RHAG* genes was entrusted to the biological company (Haorui Genomics, Xian, China)

for SMRT (Single Molecule Real Time) sequencing using the PacBio Sequel II platform (Pacific Biosciences, Texas) as briefly reported before.[4] The brief steps were as follows. First, the seven, eight, and four pairs of primers for amplification of the whole sequences of *RHD*, *RHCE*, and *RHAG* genes respectively were designed for long-range PCR (LR-PCR) using KOD FX Neo (TOYOBO, Osaka, Japan). Then, the corresponding LR-PCR products were purified, mixed with the enzyme mixture (NEB, USA), and incubated under specific conditions. The sequencing library was constructed after removing the failed ligation products. The constructed sequencing library was eventually sequenced with the Sequel II platform for 20 h. SMRTLink v10.1.0 (PacBio) and Deepvariant v1.2.0 (Web resources) software were used for data analysis and DNA variant calling. The novel variant identified was confirmed by Sanger sequencing using a pair of primers (*RHD/CE*_5UTRa-F: CCAGGGCTCAGCTCCATTCT, *RHCE*-1b-R: CTAAAGGAAAGCTTACATTGTTGA,[5] 850 bp of length of PCR product) and 2× GoTaq mixture (promega, Madison, WI) in the forward and reverse directions according to the manufacturer's instruction. PCR conditions are 94°C for 5 min; 94°C 30 s, 58°C 30 s, 72°C 50 s, 35 cycles; and plus 72°C for 5 min.

2.3 | Transfection and dual-luciferase assay analysis

Dual-Luciferase reporter assay (Promega, Madison, WI) was performed for functional analysis of the novel variant identified in the promoter region of the *RHCE* gene on transcription activity. There were four FLU pGL3 reporter plasmids used in this study, including the pGL3-basic promoterless plasmid (lacking of any enhancer or promoter) (Promega) as a negative control, pGL3-CMV plasmid (contained the CMV promoter and enhancer) as a positive control, pGL3 *RHCE* wild-type (wt) plasmid (carried the wt *RHCE* promoter sequences (238 bp) from c.1-1 to c.1-238), and pGL3 *RHCE* mutant plasmid (carried the mutant *RHCE* promoter sequences (238 bp) containing the c.1-111A>G mutation). The pGL3 *RHCE* wt and mutant plasmids were prepared by inserting wt or mutant PCR fragments of the *RHCE* promoter, as described before,[6] into the basic pGL3 luciferase reporter vector, respectively. Following the instructions of the Dual-Luciferase reporter assay, the four FLU pGL3 reporter gene plasmids were transiently co-transfected with the RLU pRL-CMV reporter plasmid into HEL cells (Erythroleukemia cell line from Kunming Cell Bank, Kunming, China) using the FuGENE HD Transfection Reagent (Promega). The cells were cultured in RPMI 1640 Medium (Gibco) supplemented with 10% fetal bovine serum. After 24 h of culture, relative light units in cell lysates were measured in triplicate using an Infinite 200 PRO Plate-Reading Luminometers (Tecan, Switzerland). Two independent assays were performed. Variations of transfection efficiency were normalized by the activities of RLU expressed by the co-transfected RLU pRL-CMV reporter plasmid. By calculating the ratio of RLU detection value to FLU detection value in different groups, the effect of the *RHCE* mutant promoter on FLU transcriptional activity could be evaluated.

3 | RESULTS

3.1 | Serologic phenotyping results

In the patients ($n = 124{,}249$) for routine RhD and RhCE serologic typing in the Jiangxi Provincial People's Hospital, the male patient (no. 1 patient) (1/124,249) was identified with B type and the rare D-- phenotype. The RhCE antigens were not detected using the Rh blood group card and also the monoclonal anti-C, anti-c, anti-E, and anti-e antibodies in saline with the tube method. No alloantibody was identified in the serum of the patient.

The second patient (no. 2 patient) was detected with blood group A and the rare D-- phenotype. A pan-agglutinated alloantibody (3+) with the reagent cells for antibody screening and identification was detected in the serum using the IAT method in Coomb's card but not detected in saline by tube method while having a negative result for self-control. The blood sample was sent to the RBC reference lab of Shanghai Blood Center (Shanghai, China). The stored A, Rhnull RBCs (Shanghai Blood Center, Shanghai, China) and O, D-- RBCs (IBGRL, UK), and the O, D + C + c + E + e + control RBCs were tested with the serum of the patient. The serum did not agglutinate with the rare A, Rhnull and O, D-- RBCs in saline and IAT with tube method, as well as the O, D + C + c + E + e + control RBC in saline with tube method, but positively agglutinated (2+) with the O, D + C + c + E + e + control RBCs in IAT with tube method. It was concluded that anti-Rh17 was identified in this patient.

3.2 | Identification of a novel c.1-111A>G mutation located in the GATA-1 motif of the *RHCE* proximal promoter

In the first D-- patient (no. 1 patient), no variants of the *RHCE* gene located in the coding regions and the adjacent sequence were identified, while an *RHCE*Ce/Ce*

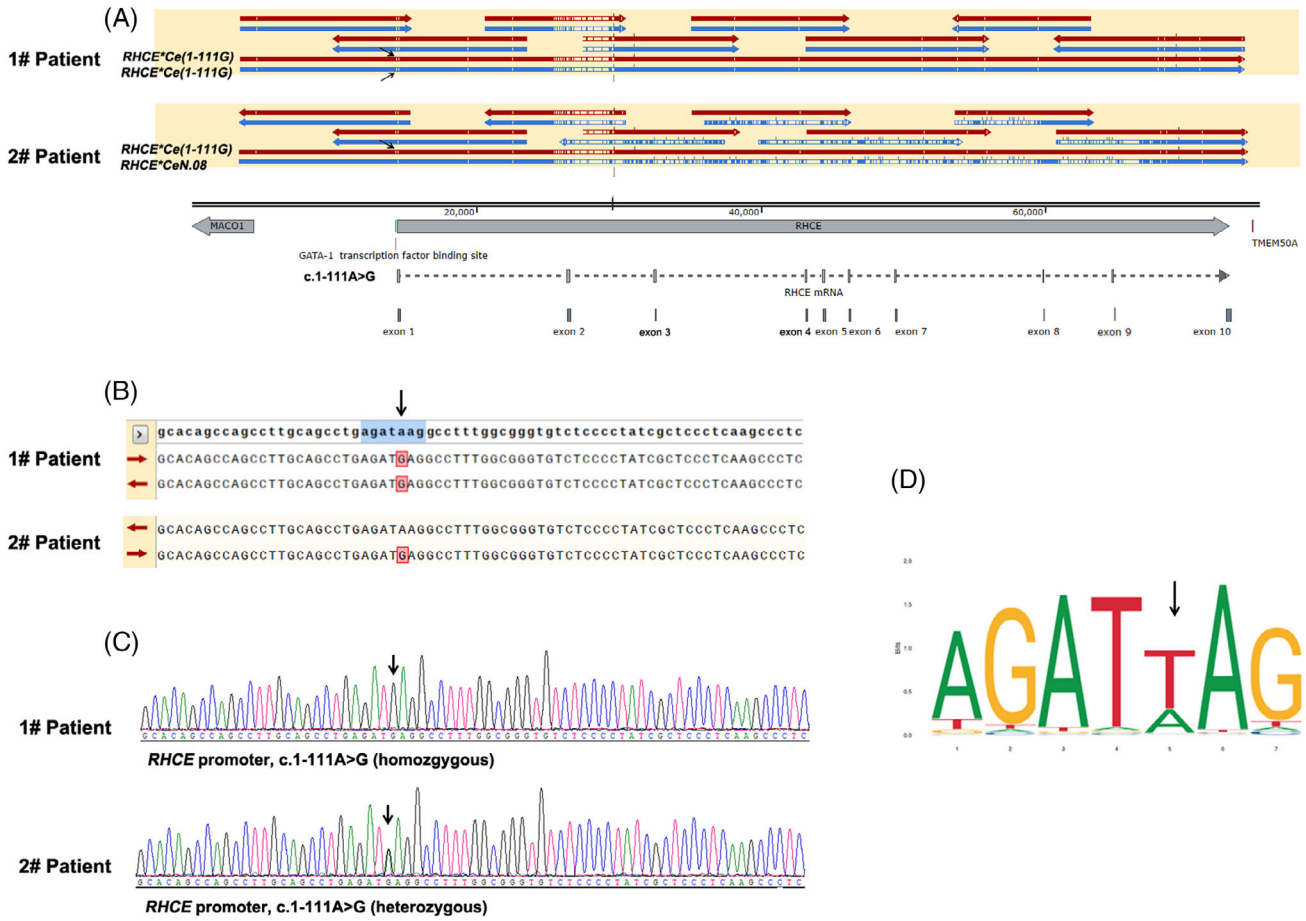

FIGURE 1 PacBio and Sanger sequencing results of the *RHCE* gene. (A) The schematic diagram of PacBio sequencing of the *RHCE* gene. The eight pairs of primers were designed for amplification of the full-length sequences of the *RHCE* gene. The PCR products were sequenced using SMRT sequencing on the PacBio platform. The assembled sequence of the two alleles was shown. The genotypes of two D-- patients (1# patient and 2# patient) were *RHCE*Ce*(*1-111G*)*/Ce*(*1-111G*) and *RHCE*CeN.08/Ce*(*1-111G*), respectively. (B) The novel homozygous and heterozygous mutation (c.1-111A>G) identified in two D-- probands (1# patient and 2# patient) by PacBio sequencing was analyzed using the SnapGene software (Boston, MA). (C) The novel homozygous and heterozygous (c.1-111A>G) mutation identified in two D-- probands (1# patient and 2# patient) was confirmed by Sanger sequencing in forward and reverse directions. But only the reverse sequences were shown. (D) The sequence logo of the transcription factor GATA-1 binding profile, which was downloaded online (Web resource). The position of the c.1-111A > G mutation identified in the GATA-1 motif of the *RHCE* gene was indicated with the arrow. [Color figure can be viewed at wileyonlinelibrary.com]

genotype was detected by PacBio sequencing (Figure 1A). Surprisingly, a homozygous variant was identified in the 5′-UTR of the *RHCE* gene (c.1-111A>G) (Figure 1B), which is located in the GATA-1 motif of the *RHCE* promoter region (Figure 1B,D). In the second D-- patient (no. 2 patient), one *RHCE*Ce* allele carried the same novel variant (c.1-111A>G) (Figure 1A,B) and another reported *RHCE-D-CE* hybrid null allele (*RHCE*CeN.08*, *RHCE-D*(*3–9*)*-CE*) (Figure 1A) was identified by PacBio sequencing. The novel variant identified in the two D-- patients was confirmed by Sanger sequencing in both forward and reverse sequences (only the reverse sequence showed in Figure 1C). The sequence has been submitted to GenBank, and the accession number is PQ179715. The sequence logo and matrix profiles of the GATA-1 motif

3.3 | c.1-111A>G in the GATA-1 motif of the *RHCE* promoter region resulted in a reduction of promoter transcriptional activity in vitro

To determine whether the variant (c.1-111A>G) of the GATA-1 motif in the *RHCE* promoter region affected the

transcription activity of the luciferase reporter gene in vitro, Dual-Luciferase reporter assays were performed in HEL cells. The plasmids carried the wild-type *RHCE* promoter (pGL3-*RHCE* wild-type) and the mutant *RHCE* promoter (pGL3-*RHCE*[c.1-111A>G]) spanning 5′-UTR sequence from −1 to −238 bp were constructed. When obtaining the highest relative light units in FLU pGL3-CMV positive control plasmid and taking promoter-less pGL3-Basic construct as baseline, the mutant pGL3-*RHCE*[c.1-111A>G] construct reduced ∼1.0–0.28 relative luciferase activity normalized to *RHCE* wild-type with ∼72% reduction rate ($P < .01$) (Figure 2). The results illustrated that a single nucleotide variant of the GATA-1 motif of *RHCE* promotion region markedly declined 72% of the transcriptional activity of the *RHCE* promoter.

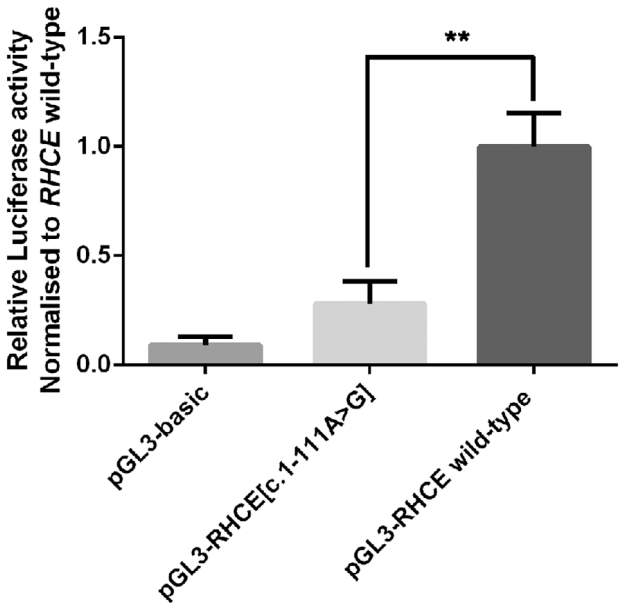

FIGURE 2 Results of the Dual-Luciferase reporter assay to assess the effect of c.1-111A>G of the *RHCE* promoter on the transcriptional activity in vitro. The upstream 238 bp wild type (wt) and mutant sequences from the translation start site of the *RHCE* gene were amplified and inserted into the pGL3 vector, respectively, to obtain the pGL3-*RHCE* wild-type and pGL3-*RHCE* [c.1-111A>G] constructs. Human erythroleukemic (HEL) cells were transiently transfected with the luciferase gene constructs (pGL3 basic as negative control, pGL3-*RHCE* wild-type, and pGL3-*RHCE* [c.1-111A>G], and pGL3-CMV as positive control). The single point mutation (c.1-111A>G) at the *RHCE* promoter region remarkably decreases the transcriptional activity of the report gene, while the pGL3-CMV positive control gets the highest relative light units (not shown). ** indicates $P < .01$.

4 | DISCUSSION

The D-- blood group is a rare phenotype, with very low frequency in African American (0.005%), Japanese (0.001%), and Sweden (0.0005%).[2] After immunization of the common RBCs with normal RhCE phenotypes during transfusion or pregnancy, the recipients or pregnant women with D-- phenotype could produce anti-Rh17 (anti-Hr0) against the high-prevalence antigens expressed by all common Rh haplotypes. Anti-Rh17 could cause a hemolytic transfusion reaction and severe disease of fetuses and newborns (HDFN).[7] Due to the extremely rare distribution, D-- blood has been declared as one of the most difficult types of rare blood to be obtained by many countries. Therefore, the routine screening and storage of D-- blood is very crucial for the guarantee of blood supply in D-- patients and the newborns suffering from severe HDFN due to anti-Rh17. In China, with the increasing application of routine RhCE typing, more D-- patients lacking alloimmunization were identified,[8] such as the first patient described in this study.

Concerning the molecular basis of the D-- phenotype, the *RHCE*CE-D-CE* hybrid alleles resulting from gene rearrangement between *RHD* and *RHCE* genes, such as *RHCE*CeN.07* (*RHCE*CE-D(3–8)-CE*),[9,10] *RHCE*CeN.08* (*RHCE*CE-D(3–9)-CE*),[9] and *RHCE*CE-D(2–6)-CE*,[11] are commonly encountered. In this study, the *RHCE*CeN.08* allele was detected in the second D-- patient with the *RHCE*CeN.08/Ce(1-111G)* genotype. Besides, other types of genetic changes, including nonsense mutation, few nucleotides deletion or insertion, and splicing site mutation, also have been reported to cause the D-- phenotype (Web resource). Recently, a complex inversion, insertion, and recombination in *RH* genes (*RHCE*Ce(1–2)-D(3–10)-Ce(10–8)-Ce*(3–10)) was also identified in one Chinese D-- proband to abolish the expression of RhCE antigens.[1]

In this study, a novel variant (c.1-111A>G) located in one of the GATA-1 motifs of the *RHCE* promoter region was first identified in two Chinese D-- probands, in which three GATA-1 motif sequences of the *RHCE* promoter have been described before.[12] In a previous study, only one variant (c.1-83G>T) located in another GATA motif of the *RHCE* promoter region was identified in Black individuals to show variable expression of the e antigen in the context of the genetically related *RHCE*ceBI* and *RHCE*ceSM* variant alleles.[13] The reporter assay showed this *RHCE* variant (c.1-83G>T) reduced the expression of the reporter gene in vitro, which indicated it could reduce the expression of the *RHCE* gene to contribute to the RhCE variant phenotype.

GATA-1 and GATA family member GATA-2 are expressed in erythroid and megakaryocytic lineages, in which they play a crucial role in cell maturation and differentiation.[14] GATA-1 regulates the transcription of many specific and nonspecific erythroid genes by binding to the DNA of target genes at the consensus sequence

WGATAR.[15] It is well known that the variant of the GATA motif located in the promoter region of the *FY* gene causes the Fy(a-b-) phenotype in the Black population, which abrogates the expression of Fy^a or Fy^b antigens.[16] Recently, the variants of the GATA motif located in the regulatory elements of *ABO* intron 1 may lead to Bm and Am phenotypes with decreased expression of A and B antigens on RBCs.[17,18] Besides, one variant (c.1-115A>C) in the GATA-1 motif of the *RHD* promoter was also identified in one individual with a mixed-field agglutination tested with anti-D,[13] while another variant (c.1-110A>C) identified in one individual with the Del phenotype.[19]

In the Dual-Luciferase reporter assays analysis in this study, the promoter transcriptional activity of the reporter gene carried the mutant *RHCE* promoter sequence decreased to 28% of the reporter gene carried the wt *RHCE* promoter sequence. The results indicated that a small amount of transcriptional activity might remain and therefore the possibility of weak expression of RhCE antigens still could not be excluded. By chance, recently, a similar variant (c.1-110A>C) located in the promoter region of the *RHD* gene was identified to be responsible for the Del phenotype.[19] In their study, the Dual-reporter luciferase assay was also conducted and ~70% of relative luciferase activity normalized to *RHD* wild-type existed. It indicated that a 30% reduction in transcriptional activity of the *RHD* gene could result in the significant decrease of RhD antigen expression. In comparison, only 28% of relative luciferase activity normalized to the *RHCE* wild-type promoter was left in this study, which might not be enough to initiate the transcription of the *RHCE* gene. Besides, in our study, the production of anti-Rh17 in the second D-- patient was detected; that indicated the existence of a null phenotype carried two *RHCE* null alleles including the reported *RHCE*CeN.08* and the novel *RHCE*Ce(1-111G)* alleles. Therefore, we concluded that the novel variant (c.1-111A > G) in the GATA-1 motif of *RHCE* should result in the D-- null phenotype through markedly reduced transcription of the *RHCE* gene.

In conclusion, a novel variant of the GATA-1 motif of the *RHCE* promoter region was first identified in two Chinese probands with the rare D-- phenotype. The observation suggests that the nucleotide substitution of the GATA-1 motif of the *RHCE* proximal promoter could diminish the binding of the GATA-1 transcription factor and down-regulate the transcription activity of the *RHCE* genes to abolish the expression of RhCE antigens to cause the rare D-- phenotype.

5 | WEB RESOURCES

RHCE allele table, the International Society of Blood Transfusion (ISBT) Working Party on Red Cell Immunogenetics and Blood Group Terminology: https://www.isbtweb.org/resource/004rhce.html

JASPAR, the open-access database of transcription factor binding profiles: GATA-1 binding profiles, https://jaspar.elixir.no/matrix/MA0035.5/?revcomp=1

Deepvariant v1.2.0 software for data analysis of PacBio sequencing: https://github.com/google/deepvariant/blob/r1.2/docs/deepvariant-quick-start.md

FUNDING INFORMATION

This work was supported by the Science and Technology Projects in Guangzhou (2023A03J0545 and 2023A03J0552), China, the National Natural Science Foundation of China (No. 82070198), and the Key Medical Disciplines and Specialties Program of Guangzhou (2025–2027), China.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest relevant to the article submitted to TRANSFUSION.

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How to cite this article: Zhang R, Wen J, Tang C, Jia S, Chen Q, Ji Y. Identification of a novel variant (c.1-111A>G) located in GATA-1 motif of *RHCE* proximal promoter in two Chinese patients with the rare D-- phenotype. Transfusion. 2025. https://doi.org/10.1111/trf.18223