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Original Article

A new *RHD* testing process: combining serological weak D confirmation testing and genotyping

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ABSTRACT

The aim of the study is to establish a rapid, effective, and cost-efficient strategy for identifying RhD-negative (D−) blood types in China. Among the 125 samples analyzed, D− samples accounted for 76.0% (95/125), of which 70 cases (73.7%) were ccee, all characterized as *RHD*01N.01/RHD*01N.01*, while 25 cases (26.3%) were non-ccee, including 15 *RHD*01N.01/RHD*01N.01* and 10 *RHD–RHCE* hybrid variants. Samples that were positive in weak D confirmation testing represented 24.0% (30/125). Since confirmation testing did not show significant diagnostic efficacy for *RHD*01EL.01*, *RHD* molecular analysis appeared to be a more accurate detection method. Based on molecular findings, the paper recommends using only five genotyping targets for simple *RHD* genotyping: *RHD* exons 1, 5, and 10, and the *845G>A* and *1227G>A* variants, which effectively detected most samples (29/30; 96.7%). In conclusion, this study recommends performing weak D confirmation testing alongside routine RhD typing for negative samples, thereby eliminating the need for further molecular analysis in over 70% of cases and conserving time and laboratory resources. For samples that test positive in weak D confirmation, simple *RHD* genotyping effectively identifies Asian-type DEL variants, *RHD*15*, and *RHD–RHCE* hybrid alleles, while follow-up molecular analysis facilitates cost-efficient detection of other rare *RHD* variants.

Keywords: Asian-type DEL variant, serological weak D testing, *RHD* genotyping

INTRODUCTION

RhD is the most immunogenic blood group antigen in transfusion medicine. Alloimmunization against the RhD antigen in RhD-negative (D−) individuals can lead to severe transfusion reactions and hemolytic disease of the fetus and newborn (HDFN)[1]. The RhD antigen is the second most clinically significant blood group antigen after the ABO system in transfusion and maternal–fetal immunology. Compared with Caucasians (D−: 15%–17%) and Black populations (D−: 3%–10%), the D− phenotype is rare among East and Southeast Asians (0.3%–0.4%), making the sourcing of D− blood particularly challenging in Asia. Additionally, a substantial proportion of individuals in China who exhibit a serological D− phenotype

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actually carry the D-elution (DEL) phenotype (9%–33%) and are not D−[2–3].

Individuals with the DEL phenotype express a limited amount of RhD antigen on their red blood cells (RBCs). The *RHD*1227A* (*RHD*01EL.01*) variant, commonly referred to as the "Asian-type DEL", is the predominant molecular basis of this phenotype in East and Southeast Asian populations (> 95%). As these individuals do not exhibit agglutination in routine RhD typing, they are typically classified serologically as D− phenotype[4–5]. Multicenter studies have demonstrated that Asian patients with the DEL phenotype do not form alloanti-D antibodies following exposure to RhD-positive (D+) RBCs during transfusion or pregnancy. It is recommended to transfuse D+ RBCs to Asian patients with the DEL phenotype (including pregnant women), along with discontinuation of prophylactic anti-D immunoglobulin administration[6].

The integration of serologic and molecular methods for *RHD* detection is essential for improving diagnostic accuracy and ensuring timely transfusion decisions. Establishing a reliable, cost-effective, accurate, and timely workflow for RhD testing holds significant clinical implications for transfusion safety and treatment efficacy[7]. In this study, we analyzed routine RhD testing samples using automated serologic weak D testing, RBC antibody detection, *RHCE* typing, *RHD* genotyping, and *RHD* sequencing based on routine IgM anti-D blood typing. From these data, we propose a comprehensive testing strategy integrating serologic and molecular findings for weak D testing.

MATERIALS AND METHODS

Study participants

A total of 125 Han ethnic samples were obtained from a 11-month routine follow-up at Hebei Handan Hospital. During this period, 35 174 patients underwent routine RhD testing (with a D− frequency of 0.35%), with duplicate patient results excluded. Blood samples were collected from 125 patients undergoing routine IgM RhD testing, and all results showed D− status (non-agglutination with IgM anti-D). Each sample was subjected to *RHCE* typing, RBC antibody screening, and automated serologic weak D testing. The EDTA-anticoagulated samples were simultaneously utilized for genomic DNA extraction, followed by *RHD* genotyping and sequencing analysis. The study was reviewed and approved by the Ethics Committee of Handan First Hospital, China (Approval No. 2023-L-032).

RhD and RhCE phenotyping and automated weak D confirmation testing

Routine RhD typing was performed using an IgM anti-D microcolumn agglutination assay (Jiangsu LIBO Medicine Biotechnology Co., Ltd., Jiangyin, Jiangsu, China) to detect D, C, c, E, and e antigens. Samples yielding negative or weakly positive results (agglutination titer ≤ 2+) underwent automated weak D confirmation testing using the Capture-R Select Platform (Immucor Inc., Norcross, GA, USA) and the Novaclone Anti-D IgM+IgG Monoclonal Blend (Dominion Biologicals Ltd., Dartmouth, Nova Scotia, Canada) on an automated blood typing instrument (Galileo NEO, Immucor Inc.)[8].

Blood genomic DNA extraction, *RHD* genotyping, and sequencing analysis

Genomic DNA was extracted from 200 μL of EDTA-anticoagulated whole blood using an automated nucleic acid extractor and a magnetic bead-based extraction kit (VN-32P, Vanzyme, Nanjing, Jiangsu, China). Peripheral white blood cells were isolated *via* magnetic bead separation.

RHD and *RHCE* genotyping, as well as exon analysis, were performed using commercially available kits based on sequence-specific primer (SSP) technology. The detection targets included *RHD* exons 1, 7, 9, and 10, RH box, and the *845G>A* and *1227G>A* variants, which facilitated screening for common Asia-type DEL and *RHD–RHCE* hybrid alleles in Asian populations. All polymerase chain reaction (PCR) amplifications were performed according to the manufacturer's instructions (SYBR *RHD* and *RHCE* Genotyping Kit; Jiangsu ZoJiWat Bio-Pharmaceutical Co., Ltd., Jiangyin, Jiangsu, China).

The amplification was performed using specific sequencing primers, which have been validated in previous publications and were designed to amplify each exon along with at least 50 base pairs of its flanking intronic regions. Each PCR tube contained a total volume of 20 μL, containing 10 μL of 2 × high-purity heat-resistant DNA polymerase mix (Vanzyme), 0.4 mM of each amplification primer, and 100 ng of genomic DNA template. The PCR cycling conditions were as follows: initial denaturation at 96 ℃ for 3 minutes; 5 cycles of 96 ℃ for 20 seconds and 68 ℃ for 60 seconds; 10 cycles of 96 ℃ for 20 seconds and 66 ℃ for 50 seconds; 22 cycles of 95 ℃ for 20 seconds, 62 ℃ for 50 seconds, and 72 ℃ for 45 seconds; and a final extension at 72 ℃ for 2 minutes. PCR amplification was conducted using a Life ECHO

thermocycler (Bioer Technology Co., Ltd., Hangzhou, Zhejiang, China).

Sanger sequencing was applied to analyze the PCR-amplified genomic products. To eliminate the unreacted primers and PCR mixture components, the amplified DNA was treated with ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730 Genetic Analyzer (Thermo Fisher Scientific Inc.) with specific sequencing primers in both forward and reverse directions[9]. The resulting. ab1 sequencing files were analyzed for mutation points using Geneious Prime Software (version 2023.1.2; Dotmatics, Boston, MA, USA). Reference templates were derived from the International Society of Blood Transfusion (ISBT) database for blood group alleles (NG_007494.1).

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (version 30.0.0, IBM Corp., Armonk, NY, USA). Differences between Rh phenotyping results and IgG anti-D confirmatory weak D testing in IgM anti-D-typed samples were assessed using the chi-square test. The distribution of *RHD*01N.01/RHD*01N.01* versus non-*RHD*01N.01* genotypes was compared for ccee distribution and weak D confirmation frequency using the chi-square test. A similar comparative analysis was performed between the *RHD*01EL.01* and non-*RHD*01EL.01* groups.

RESULTS

D− sample testing results

Among the 125 samples routinely serologically typed as IgM anti-D-negative, weak D confirmation testing, Rh phenotyping, *RHD* genotyping, and Sanger sequencing were performed, as shown in ***Table 1***. Molecular analysis of the *RHD* alleles identified the following genotype combinations: *RHD*01N.01/RHD*01N.01* (n = 85, 68.0%), *RHD*01EL.01/RHD*01N.01* (n = 27, 21.6%), *RHD*01N.03/RHD*01N.01* (n = 2, 1.6%), *RHD*01N.04/RHD*01N.01* (n = 8, 6.4%), *RHD*03.06/RHD*01N.01* (n = 1, 0.8%), and *RHD*15/RHD*01N.01* (n = 2, 1.6%).

All *RHD*01N.01/RHD*01N.01* samples tested negative in weak D confirmation testing, showing *RHD* exon genotyping negativity and RH box positivity. All *RHD*01EL.01/RHD*01N.01* samples were positive in weak D confirmation testing, with amplification across *RHD* exons 1–10, presence of the *RHD 1227G>A* variant, and RH box positivity. The *RHD–RHCE* hybrid samples included two *RHD*01N.03/RHD*01N.01* samples that were negative in weak D confirmation testing, showing amplification for *RHD* exons 1 and 10 only, negative results for *RHD 845G>A* and *RHD 1227G>A* variants, and positivity for *RHD* exons 1 and 10 and the RH box. Similarly, eight *RHD*01N.04/RHD*01N.01* samples were negative in weak D confirmation testing, with amplification for *RHD* exons 1, 2, and 10, negative results for *RHD 845G>A* and *RHD 1227G>A*, and positivity for *RHD* exons 1, 2, and 10 and the RH box. One *RHD*03.06/RHD*01N.01* sample was collected, which tested positive in weak D confirmation, exhibiting amplification for *RHD* exons 1, 2, 7, 9, and 10, negative results for *RHD 845G>A* and *RHD 1227G>A*, and positivity for *RHD* exons 1, 2, 7, 9, and 10 and the RH box. Additionally, both *RHD*15/RHD*01N.01* samples were positive in weak D confirmation, showing amplification across *RHD* exons 1–10, presence of the *RHD 845G>A* variant, and RH box positivity.

Distribution of *RHCE* typing and weak D confirmation testing among *RHD* allele combinations

Given the significantly higher frequency of the ccee phenotype among *RHD*01N.01/RHD*01N.01* cases, comparisons of ccee versus non-ccee distributions (χ^2 = 66.64, P < 0.001) and weak D confirmation testing frequencies (χ^2 = 83.88, P < 0.001) between the *RHD*01N.01/RHD*01N.01* and non-*RHD*01N.01* groups were statistically significant. The χ^2 value for weak D confirmation testing was significantly greater than that for ccee and non-ccee distributions. The Ccee distribution between *RHD*01EL.01* and non-*RHD*01EL.01* groups also showed a statistically significant difference (χ^2 = 36.86, P < 0.001), although weak D confirmation testing did not demonstrate statistical significance (P = 0.934).

Negativity in the weak D confirmation test exhibited stronger diagnostic power for detecting *RHD*01N.01* compared to ccee distribution; however, it did not provide good diagnostic power for *RHD*01EL.01*, as shown in ***Table 2***.

Proposed *RHD*-testing process

By combining Rh phenotyping and weak D confirmation testing, a comprehensive analysis was performed on routinely tested D− samples (***Fig. 1***). Confirmation-negative samples accounted for 68.0% (85/125), of which 70 (82.4%) were ccee and were all genotyped as *RHD*01N.01/RHD**01N.01, while 25 (26.3%) were non-ccee, including 15 *RHD*01N.01/*

Table 1 **Sequence of *RHD* alleles, Rh phenotyping, weak D confirmation testing, and *RHD* genotyping among 125 routine RhD-negative samples**

RHD allele	Sample size [*n* (%)]	Rh phenotyping (IgM anti-D)	*RHCE*	Weak D confirmation testing	*RHD* genotyping											
					Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 9	Exon 10	*1227G>A*	*845G>A*	RH box
*RHD*01N.01/RHD*01N.01*	85 (68.0)	−		−	−	−	−	−	−	−	−	−	−	−	−	+
	70 (82.4)		ccee													
	9 (10.6)		Ccee													
	4 (4.7)		ccEe													
	1 (1.2)		CcEe													
	1 (1.2)		CCee													
*RHD*01EL.01/RHD*01N.01*	27 (21.6)	−		+	+	+	+	+	+	+	+	+	+	+	−	+
	19 (70.4)		Ccee													
	4 (14.8)		CCee													
	2 (7.4)		ccee													
	1 (3.7)		CcEe													
	1 (3.7)		ccEe													
*RHD*01N.03/RHD*01N.01*	2 (1.6)	−		−	+	−	−	−	−	−	−	−	+	−	−	+
	1 (50)		Ccee													
	1 (50)		ccEe													
*RHD*01N.04/RHD*01N.01*	8 (6.4)	−		−	+	+	−	−	−	−	−	−	+	−	−	+
	5 (62.5)		Ccee													
	2 (25.0)		CCee													
	1 (12.5)		ccEe													
*RHD*03.06/RHD*01N.01*	1 (0.8)	−	CcEe	+	+	+	+	−	−	−	−	+	+	+	−	−	+
*RHD*15/RHD*01N.01*	2 (1.6)	−	ccEe	+	+	+	+	+	+	+	+	+	+	+	−	+	+

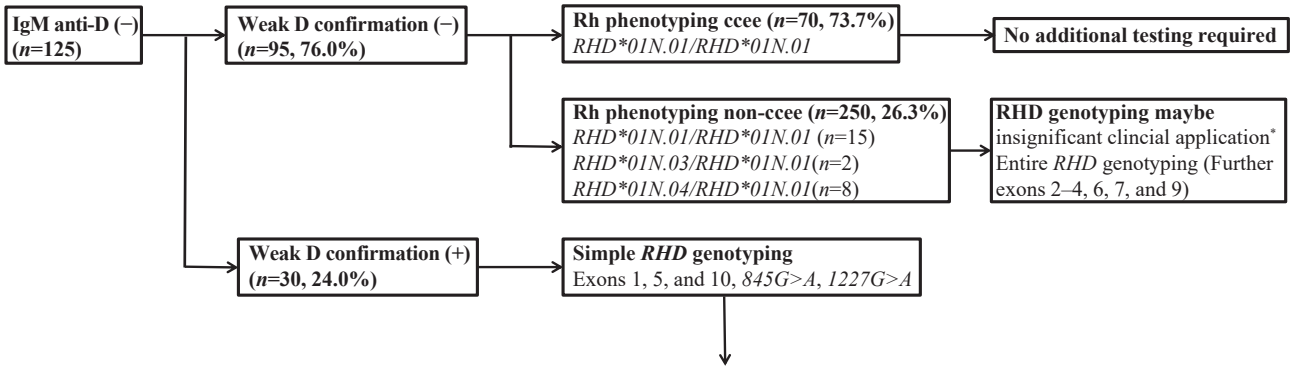
Table 2 **Distribution of *RHCE* typing and weak D confirmation testing among *RHD* alleles**

	RHCE typing			Weak D confirmation		
	ccee	Non-ccee	*P* value	Positive	Negative	*P* value
*RHD*01N.01/RHD*01N.01*	70	15	< 0.001	0	85	< 0.001
Non-*RHD*01N.01* homozygous	3	37		30	10	
*RHD*01EL.01/RHD*01N.01*	2	25	< 0.001	27	85	0.934
Non-*RHD*01EL.01*	71	27		3	10	

*RHD**01N.01 and 10 *RHD–RHCE* hybrid variants (***Table 3*** and ***Fig. 1***). Weak D confirmation-positive samples accounted for 24% (30/125).

DISCUSSION

The Asian-type DEL variant (*RHD*01E.01*) is the most prevalent DEL allele worldwide, underscoring the need for its routine detection in individuals with weak or partial D phenotypes from East and Southeast Asian populations[7]. Over the past two decades, there has been an ongoing debate regarding transfusion and RhD immunoprophylaxis strategies for pregnant women. Recent multicenter studies from southern China have recommended transfusing ordinary D+ blood to all Asian-type DEL patients, discontinuing RhIG immunoprophylaxis for Asian-type DEL women, and ceasing any anti-D immune monitoring during Asian-type DEL pregnancies[6]. Therefore, efficiently and economically distinguishing *RHD* 01N.01* (approximately 65%–75%) from *RHD* 01E.01* (9%–33%) has become a critical objective in clinical testing. In this study, 125 samples underwent Rh phenotyping, weak D confirmation testing, and genotyping of *RHD* exons 1–7, 9, and 10, the *845G>A* and *1227 G>A* variants, and the RH box, followed by direct *RHD* sequencing. Using the direct sequencing results as the reference, we systematically assessed the concordance of different testing results with *RHD* variants and established an effective and economical testing process.



Exon 1	Exon 5	Exon 10	*1227G>A*	*845G>A*	Result	Frequency [*n* (%)]	Action
+	+	+	+	−	*RHD*01EL.01*	27 (21.6)	No need
+	+	+	−	+	*RHD*15*	2 (1.6)	No need
+	−	+	−	−	*RHD–RHCE* hybrid	10+1 (8.8)[$]	Entire *RHD* genotyping (Further exons 2–4, 6, 7, and 9), and/or sequenced
+	+	+	−	−	SNP mutation		Sequenced

***Fig. 1* Proposed workflow for weak D confirmation testing, Rh phenotyping, *RHD* genotyping, and molecular analysis of *RHD* alleles.** The figure illustrates the diagnostic pathway for 125 samples with IgM anti-D negativity, including weak D confirmation, Rh phenotyping, and *RHD* genotyping strategies. Among 95 samples (76.0%) with negative weak D confirmation, Rh phenotyping identified two groups: ccee and non-ccee. The latter underwent conditional genotyping due to possible hybrid alleles. Thirty samples (24.0%) with positive weak D confirmation were subjected to simple *RHD* genotyping targeting exons 1, 5, and 10, and two single nucleotide polymorphisms (SNPs) (*845G>A* and *1227G>A*). The lower table summarizes exon amplification patterns, corresponding alleles, frequencies, and recommended follow-up actions, including when full *RHD* sequencing is indicated. *: Current studies showed that both *RHD*01N.03* and *RHD*01N.04* could induce anti-D immunization, consistent with *RHD*01N.01*. $: Among the 10 cases of *RHD*01N.03* and *RHD*01N.04* (8.0%), only one case (0.8%) tested positive for weak D confirmation required identification by genotyping and/or sequencing. Although both genotyping and/or sequencing identified *RHD–RHCE* hybrid alleles, genotyping is the faster method.

***Table 3* Correlation between weak D confirmation testing and Rh phenotyping among *RHD* alleles [*n* (%)]**

	*RHD*01N.01/ RHD*01N.01* (*n* = 85)	*RHD*01EL.01/ RHD*01N.01* (*n* = 27)	*RHD*01N.03/ RHD*01N.01* (*n* = 2)	*RHD*01N.04/ RHD*01N.01* (*n* = 8)	*RHD*03.06/ RHD*01N.01* (*n* = 1)	*RHD*15* (*n* = 2)
Confirmation–	85 (100)	0 (0)	2 (100)	8 (100)	0 (0)	0 (0)
Confirmation– and ccee	70 (82.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Confirmation– and non-ccee	15 (17.6)	0 (0)	2 (100)	8 (100)	0 (0)	0 (0)
Confirmation+	0 (0)	27 (100)	0 (0)	0 (0)	1 (100)	2 (100)
confirmation+ and ccee	0 (0)	2 (7.5)	0 (0)	0 (0)	0 (0)	0 (0)
confirmation+ and non-ccee	0 (0)	25 (92.5)	0 (0)	0 (0)	1 (100)	2 (100)

Serological identification of the Asian-type DEL variant requires adsorption–elution testing. However, due to variability in testing standardization and limitations associated with direct antiglobulin test-positive samples, its detection rate and consistency are lower than those achieved through molecular methods[10]. Traditional adsorption–elution testing requires an experienced operator and approximately 3 hours to complete, whereas *RHD* genotyping takes about 4 hours, and additional *RHD* sequencing extends the testing time by over 24 hours[11]. Consequently, methodological and time constraints have limited the detection of Asian-type DEL variants in the past. Previous studies have observed a correlation between *RH* typing with the ccee phenotype and the *RHD*01N.01* allele, thus allowing for additional analysis of non-ccee RhD samples as a convenient and effective detection method. Our findings further confirmed this consistency. However, 17.6% (15/85) of the samples in our study exhibited a non-ccee phenotype.

Weak D confirmation testing requires only an additional hour, utilizing the routine IgM/IgG anti-D antibody detection in an automated blood typing

analysis system. This method achieves standardization and automation, reducing reliance on human resources[10,12]. Based on the capture solid-phase agglutination technique, the ratio of antibody to RBC antigen is increased ninefold compared to traditional tube methods, thereby achieving a high sensitivity advantage of adsorption–elution testing[10]. Weak D confirmation testing with negative results effectively identified 76% (95/125) of the samples, including *RHD*01N.01* in 70 ccee cases (73.7%, 70/95) and 15 non-ccee cases (15.8%, 17/95), *RHD*01N.03/RHD* 01N.01* in 2 non-ccee cases (2.1%, 2/95), and *RHD* 01N.04/RHD*01N.01* in 8 non-ccee cases (8.4%, 8/95). These *RHD* alleles have been reported to induce anti-D immunization. Therefore, in our recommended testing process, only 24% (30/125) of patients required additional genotyping. Simple *RHD* genotyping could effectively detect 96.7% (29/30) of the samples, including 90% (27/30) of Asian-type DEL variants. Only 0.8% of *RHD–RHCE* hybrid variants and nucleotide mutations required further genotyping for additional *RHD* exons (2–4, 6, 7, and 9). Furthermore, simple *RHD* genotyping can identify samples that require sequencing analysis. Since confirmation testing showed limited diagnostic power for *RHD*01EL.01*, *RHD* molecular analysis appeared to be a more accurate testing method. Based on the molecular analysis results, we propose a simplified *RHD* genotyping panel consisting of only five targets: *RHD* exons 1, 5, and 10, as well as the *845G>A* and *1227G>A* variants, which can effectively detect most samples (29/30, 96.7%).

This study is limited by the complexity and variety of *RHD* variants. Variants such as *RHD–RHCE* hybrid genes, weak D, and partial D require both weak D confirmation testing and molecular analysis to verify the reliability of the recommended testing process. Scholars in Brazil and Japan tested weak D type 01, weak D type 07, and weak D type 11 samples, all of which tested positive in weak D confirmation assays. The identification of *RHD–RHCE* hybrids may limit the sensitivity of weak D confirmation testing. However, hybrid variants may still pose a risk of anti-D immunization. Therefore, a negative result is not considered harmful, whereas a positive result can effectively guide variant identification through genotyping. Besides, the frequency of D− was 0.35% in the samples we observed. Although 125 D− samples were included in this study, a larger sample size would enhance the statistical power of this process.

In summary, this study recommends performing weak D confirmation testing as part of routine RhD typing for serologically negative samples, thereby eliminating the need for further molecular analysis in these cases. This approach could reduce unnecessary waiting time and resource expenditure in approximately 76% of the samples. For weak D-positive samples, simple *RHD* genotyping can effectively identify Asian-type DEL variants, *RHD*15*, and *RHD–RHCE* hybrid alleles, while follow-up molecular analysis enables cost-effective detection of other rare *RHD* variants.

Author contributions

Conceptualization, Peiyun Dong and Tianshen Tang; Methodology, YuShiang Lin; Software, Jingyan Chang; Validation, YuShiang Lin, Jingyan Chang, and Peiyun Dong; Formal Analysis, Tianshen Tang; Investigation, Tianshen Tang; Resources, Peiyun Dong; Data Curation, Jingyan Chang; Writing – Original Draft Preparation, Tianshen Tang; Writing – Review & Editing, Peiyun Dong; Visualization, Jingyan Chang; Supervision, Jingyan Chang; Project Administration, Peiyun Dong.

Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Handan First Hospital (Approval No. 2023-L-032).

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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CLINICAL TRIAL REGISTRATION

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