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Original Article

Establishment of a real-time fluorescent recombinase polymerase amplification (RT-RPA) assay for *BCR-ABL* fusion gene detection

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ABSTRACT

This study aims to establish a rapid and highly sensitive real-time fluorescence recombinase polymerase amplification (RT-RPA) method for the qualitative and quantitative analysis of *BCR-ABL* fusion genes. The method is based on the design of primers and probes for the *BCR* and *ABL* (*ABL1*/*ABL2*) gene breakpoints, and takes advantage of the high sequence homology of the ABL1/ABL2 binding region to achieve universal reverse primer and probe design for the two fusion genes. Through optimization of primer design, reaction system, and amplification conditions, the study successfully realized specific amplification of *BCR-ABL* fusion genes, with a detection limit as low as 10 copies/μL and a total reaction time of 20 minutes. In comparison to conventional quantitative real-time (qRT-PCR) methodology, RT-RPA circumventes the need for complex thermal cycling equipment, making it suitable for resource-limited settings. These findings demonstrate that this novel method provides efficient technical support for the early diagnosis and therapeutic monitoring of chronic myeloid leukemia (CML).

Keywords: chronic myeloid leukemia, *BCR-ABL* fusion gene, real-time fluorescence recombinase polymerase amplification, rapid detection, isothermal amplification

INTRODUCTION

Chronic myeloid leukemia (CML) is a malignant myeloproliferative neoplasm originating from a clonal expansion of hematopoietic stem cells in bone marrow. It is characterized by leukocytosis and the accumulation of granulocytes and their precursors, accounting for approximately 15% of adult leukemia cases[1–2]. Most CML patients are initially diagnosed in a relatively benign chronic phase, marked by a significant increase in myeloid cells. However, without timely intervention, the disease progresses to a fatal acute leukemia of either myeloid or lymphoid phenotype. The fusion tyrosine kinase BCR-ABL plays a crucial role in the pathogenesis of CML. The advent of small-molecule tyrosine kinase inhibitors (TKIs) targeting the molecular mechanism of CML has brought CML treatment into the stage of molecular targeted therapy, significantly improving patient survival[3–5]. The successful clinical application

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of imatinib, a BCR-ABL kinase inhibitor, has validated the critical dependence of CML cells on BCR-ABL tyrosine kinase activity. Nevertheless, patients often develop primary and acquired resistance, which is associated with the reactivation of BCR-ABL.

The Philadelphia chromosome and the constitutive expression of the fusion protein BCR-ABL represent pathognomonic features of CML cells. The Philadelphia chromosome abnormality, specifically the t(9;22) translocation, results from genetic material exchange between the long arms of chromosomes 9 and 22. This translocation results in the fusion of the *ABL* tyrosine kinase gene on chromosome 9 to the *BCR* gene on chromosome 22, generating *BCR-ABL* fusion genes[6–7]. ABL is a non-receptor tyrosine kinase expressed in most tissues. The ABL protein is distributed throughout both the nucleus and cytoplasm within cells, and is capable of shuttling between these compartments. It transduces signals originating from cell surface growth factors and adhesion receptors to regulate cytoskeletal organization. BCR is also a signaling protein comprised of multiple modular domains. The fusion of *BCR* sequences with *ABL* enhances the tyrosine kinase activity of ABL and introduces novel regulatory domains/motifs to ABL. Several signaling pathways are activated in the presence of BCR-ABL, resulting in decreased apoptosis and accelerated cell proliferation.

The breakpoint region within the *BCR* gene of *BCR-ABL* fusion genes is primarily classified into three types: major-BCR (M-BCR), minor-BCR (m-BCR), and micro-BCR (u-BCR). In the M-BCR region, the *BCR* gene breakpoints are typically confined to a 5.8 kb DNA fragment region, occurring between exons e13 and e14 or exons e14 and e15. Following the break, the upstream *BCR* gene exons remain on chromosome 22 and fuse with exon a2 of the *ABL* gene, resulting in the transcription of e13a2 or e14a2 mRNA, which encodes the P210 fusion protein. This is observed in over 95% of CML cases. In the m-BCR region, the *BCR* gene breakpoints typically occur between exons e1 and a2, leading to the transcription of e1a2 mRNA, which encodes the P190 fusion protein. This is found in 3% of atypical CML cases and in two-thirds of Philadelphia chromosome-positive (Ph+) acute B-lymphoblastic leukemia cases. In the u-BCR region, the *BCR* gene breakpoints are typically located between exons e19 and e20, generating e19a2 mRNA that encodes the P230 fusion protein, commonly observed in chronic neutrophilic leukemia[8–11].

Current primary methods for detecting *BCR-ABL* fusion genes include fluorescence *in situ* hybridization (FISH), quantitative real-time PCR (qRT-PCR), gene chip technology, and PCR sequencing[12]. However, these techniques are limited by their reliance on specialized equipment, high costs, complex experimental procedures, and long reaction time. Recombinase polymerase amplification (RPA) has garnered significant attention due to its rapid, sensitive, specific, efficient, and convenient nature since its development in recent years. It has been widely applied in the identification of viruses, bacteria, and components of animal and plant origin, making substantial contributions to large-scale and rapid species detection[13–16]. Nevertheless, the application of RPA technology for detecting *BCR-ABL* fusion genes in CML remains unexplored in the current literature.

This study established a rapid detection method for both *BCR-ABL1* and *BCR-ABL2* fusion genes based on RT-RPA technology. Capitalizing on the inherent advantages of RT-RPA, this method shows potential for the rapid and convenient detection of *BCR-ABL* fusion genes in the early screening and treatment prognosis of CML patients.

MATERIALS AND METHODS

Materials, reagents, and instruments

The *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 plasmids were designed by our research team and synthesized by GenScript Biotech (Nanjing, China). The RT-RPA nucleic acid amplification reaction unit was purchased from GenDx Biotech (Suzhou, China). RT-RPA primers and probes were synthesized by GenScript Biotech (Nanjing, China). The StepOnePlus Real-Time PCR System was from Applied Biosystems (Foster, CA, USA).

Screening for *BCR-ABL* fusion gene sequences, and design and synthesis of primers and probes

The sequences of the fusion genes *BCR-ABL1* and *BCR-ABL2* were retrieved from the NCBI human genome database. A 400 bp sequence fragment, centered on the fusion point of the *BCR* gene and *ABL* gene, was extracted (200 bp flanking each side of the fusion point), which was served as the target sequence for designing forward and reverse primers, as well as probes, using Primer Premier 5.0. In this method, RPA primers were designed with a length of 30–35 bp, and the fluorescent probe was 46–52 bp in length

(composed of tetrahydrofuran (THF), fluorophore, quencher group, and 3' blocking group). The reverse primers and probes were universal for both *BCR-ABL1* and *BCR-ABL2* fusion gene types. All primers and probes were synthesized by GenScript Biotech. The sequences were shown in ***Table 1***.

Table 1 **Primers and probes for RT-RPA amplification of *BCR-ABL* fusion genes**

Primer/probe	Sequence (5'→3')
BCR-ABL1-F1	CTCTATGGGTTTCTGAATGTCATCGTCCA
BCR-ABL1-F2	AATGTCATCGTCCACTCAGCCACTGGATTT
BCR-ABL1-F3	TGTCATCGTCCACTCAGCCACTGGATTTAA
BCR-ABL2-F1	ATCCGGGAGCAGCAGAAGAAGTGTTTCAGA
BCR-ABL2-F2	CCGGGAGCAGCAGAAGAAGTGTTTCAGAAG
BCR-ABL2-F3	TGTGAAACTCCAGACTGTCCACAGCATTCC
BCR-ABL12-R	TTCCTTAGAGTTCCAACGAGCGGCTTCACT
BCR-ABL12-PB	FAM-AGCCCTTCAGCGGCCTGTAGCATCTGACT(THF)TGTGCCTCAGGGTCT(C3-Spacer)

Establishment of the RT-RPA reaction system

The total reaction volume was standardized to 10 μL. A premix solution was prepared by combining 4 μL of solvent, 0.42 μL of forward primer (10 μM), 0.42 μL of reverse primer (10 μM), 0.12 μL of fluorescent probe (10 μM), and 4.24 μL of ddH_2O. This solution was thoroughly homogenized with the lyophilized fluorescent reagent. Subsequently, 0.4 μL of fusion gene DNA sample and 0.4 μL of activator were added, followed by vortexing and brief centrifugation to eliminate any air bubbles in the PCR reaction tube. The prepared reaction mixture was placed in an ABI StepOnePlus Real-Time PCR System. The reaction temperature was 37 ℃, with fluorescence signal acquisition every 30 seconds for a total of 40 cycles and a reaction time of 20 minutes.

Primer screening

Using *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 plasmid DNA templates at 10^5 copies/μL, we performed RT-RPA amplification with synthesized primers and fluorescent probes in various combinations. The template plasmid copy number was calculated using DNA copy number calculation formula. Based on the time-to-peak and saturation of fluorescence curves resulting from amplification, the optimal amplification primers were selected.

$$\text{Plasmid copy number (copies/μL)} = \frac{\text{Plasmid concentration (ng/μL)} \times 6.022 \times 10^{23}}{\text{Plasmid length (bp)} \times 660\ \text{(g/mol/bp)} \times 1 \times 10^{9}}$$

Sensitivity testing

The recombinant plasmids *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 were diluted tenfold to reach final concentrations ranging from 10 to 10^5 copies/μL. RT-RPA reactions were performed with *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 recombinant plasmids at concentrations of 10 copies/μL, 10^2 copies/μL, 10^3 copies/μL, 10^4 copies/μL, and 10^5 copies/μL as templates, using the two sets of optimal primers. The minimum detectable concentration of the method for recombinant plasmids *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 was confirmed by analyzing the real-time amplification curves, thus establishing the sensitivity of the RT-RPA method.

Specificity testing

To evaluate the presence of specific and non-specific fluorescent amplification curves, RT-RPA detection was performed using the two selected optimal primer sets with 10^5 copies/μL of *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 recombinant plasmids as templates, respectively.

RESULTS

Primer screening

To identify optimal primers for target fusion gene amplification, we evaluated recombinant plasmids *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 (each at a concentration of 10^5 copies/μL) as templates. ***Fig. 1*** illustrates the RT-RPA amplification results for the *BCR-ABL1* fusion gene, using three forward primers (*BCR-ABL1*-F1, *BCR-ABL1*-F2, and *BCR-ABL1*-F3) individually paired with the reverse primer *BCR-ABL12*-R and the fluorescent probe *BCR-ABL12*-PB. The data indicated that the combination of *BCR-ABL1*-F1 with *BCR-ABL12*-R and *BCR-ABL12*-PB yielded typical fluorescence amplification curves with the earliest peak time and the highest amplification efficiency. Similarly, ***Fig. 2*** shows the RT-RPA amplification results for the *BCR-ABL2* fusion gene,

using three forward primers (*BCR-ABL2*-F1, *BCR-ABL2*-F2, and *BCR-ABL2*-F3) individually paired with the reverse primer *BCR-ABL12*-R and the fluorescent probe *BCR-ABL12*-PB. The results demonstrated that the combination of *BCR-ABL2*-F1 with *BCR-ABL12*-R and *BCR-ABL12*-PB produced typical fluorescence amplification curves, displaying the strongest fluorescence signal and the highest amplification efficiency. Therefore, *BCR-ABL1*-F1 and *BCR-ABL2*-F1 were determined to be the optimal forward primers for amplifying the *BCR-ABL1* and *BCR-ABL2* fusion genes, respectively.

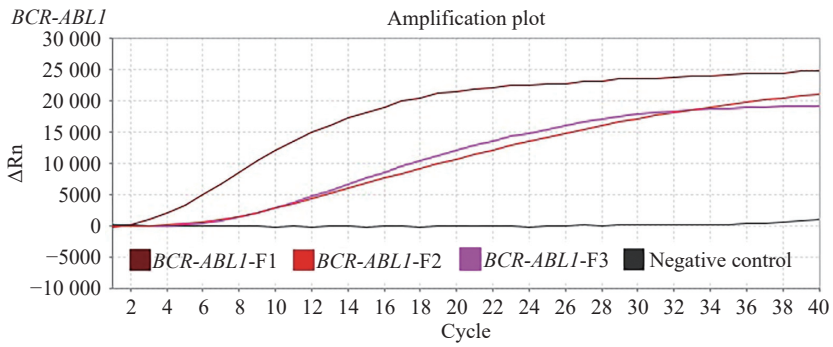

***Fig. 1* Screening results of *BCR-ABL1* primers.** Real-time fluorescence curves of RT-RPA amplification using plasmid *BCR-ABL1*-pUC57 (10^5 copies/μL) as template. Three forward primers (F1, F2, F3) were individually paired with universal reverse primer *BCR-ABL12*-R and probe *BCR-ABL12*-PB. The combination of *BCR-ABL1*-F1 showed the earliest amplification peak and highest efficiency.

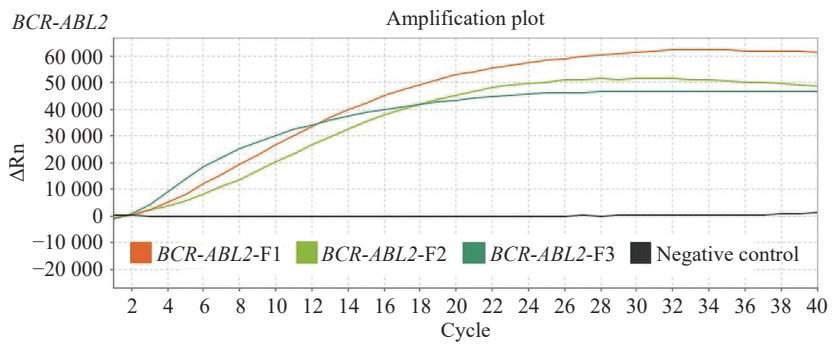

***Fig. 2* Screening results of *BCR-ABL2* primers.** RT-RPA amplification curves of plasmid *BCR-ABL2*-pUC57 (10^5 copies/μL) using three forward primers (F1, F2, F3) with universal reverse primer *BCR-ABL12*-R and probe *BCR-ABL12*-PB. *BCR-ABL2*-F1 exhibited the strongest fluorescence signal and highest amplification efficiency.

Specificity testing

RT-RPA amplification was performed using 10^5 copies/μL of recombinant plasmids *BCR-ABL1*-pUC57 and *BCR-ABL2*-pUC57 as templates, with *BCR-ABL1*-F1 and *BCR-ABL2*-F1 as the forward primers. As shown in ***Fig. 3***, only *BCR-ABL1*-pUC57 demonstrated specific amplification. Similarly, RT-RPA amplification using *BCR-ABL2*-F1 as the forward primer, as illustrated in ***Fig. 4***, showed specific amplification of *BCR-ABL2*-pUC57 only. These findings indicated that the method exhibited favorable specificity.

Sensitivity testing

To assess the sensitivity of the RT-RPA method, serial dilutions of *BCR-ABL1*-pUC57 recombinant plasmids, ranging from 10 to 10^5 copies/μL, were used as templates. As shown in ***Fig. 5***, the RT-RPA method demonstrated good sensitivity for the *BCR-ABL1* fusion gene, with a minimum detection limit of 10 copies/μL. Similarly, a sensitivity test was performed using serial dilutions of *BCR-ABL2*-pUC57 recombinant plasmids, also ranging from 10 to 10^5 copies/μL. ***Fig. 6*** shows that the RT-RPA method exhibited good sensitivity for the *BCR-ABL2* fusion gene, with a detection limit of 10 copies/μL.

DISCUSSION

The *BCR-ABL* fusion gene, a critical biomarker for

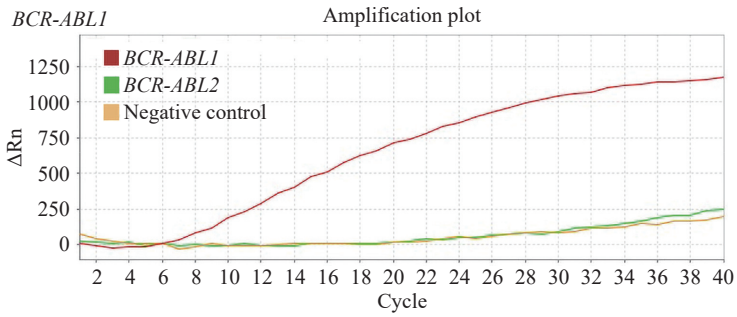

***Fig. 3 BCR-ABL1* specificity test results.** RT-RPA amplification using primer set *BCR-ABL1*-F1/*BCR-ABL12*-R with template *BCR-ABL1*-pUC57 (10^5 copies/μL). Specific amplification was observed only for *BCR-ABL1*, confirming no cross-reactivity with *BCR-ABL2* or negative controls.

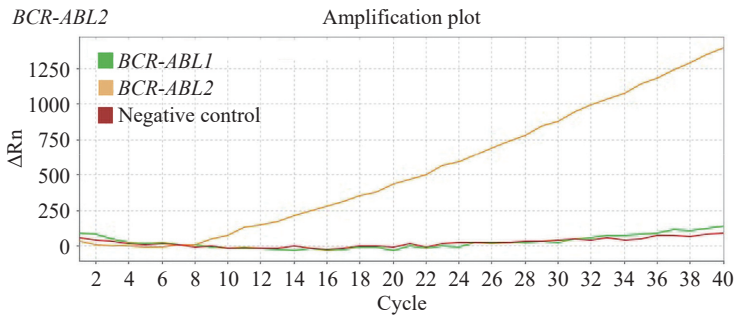

***Fig. 4 BCR-ABL2* specificity test results.** Amplification curves using primer set *BCR-ABL2*-F1/*BCR-ABL12*-R with template *BCR-ABL2*-pUC57 (10^5 copies/μL). Specific signal was generated only for *BCR-ABL2*, demonstrating high specificity.

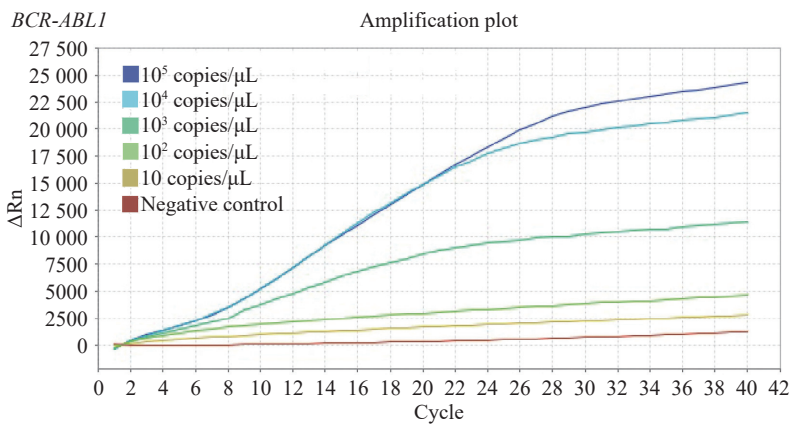

***Fig. 5 BCR-ABL1* sensitivity test results.** Serial dilutions of *BCR-ABL1*-pUC57 plasmid (10–10^5 copies/μL) amplified by RT-RPA. The detection limit was 10 copies/μL.

CML, serves as a prognostic and predictive indicator for CML patients, and its detection has become the "gold standard" for CML diagnosis. In CML, the *BCR-ABL1* fusion gene (e14a2 type) encodes the P210 fusion protein and exhibits a mutation frequency of 52.64%. The *BCR-ABL2* fusion gene (e14a2 type) encodes the P210 fusion protein and shows a mutation frequency of 32.68%. These two fusion genes make up 85.32% of all *BCR-ABL* fusion genes. Current detection methods for *BCR-ABL* fusion genes include chemiluminescent imaging, qRT-PCR, colorimetric method, and fluorescence in situ hybridization[10,17–19]. However, the majority of these methods involve complex procedures, are time-consuming, require stringent temperature conditions, and some necessitate operation by specialized personnel.

RPA, one of the emerging isothermal amplification technologies, has found increasing application in the detection of bacteria, viruses, and genetically modified organisms due to its high sensitivity, rapid processing, and operational simplicity. Its amplification targets involve RNA, miRNA, ssDNA, and

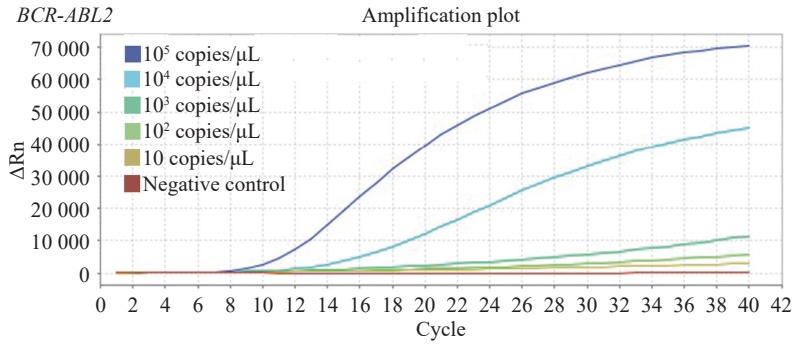

***Fig. 6 BCR-ABL2* sensitivity test results.** RT-RPA amplification of tenfold serial dilutions of *BCR-ABL2*-pUC57 plasmid (10–10^5 copies/μL). The detection limit was 10 copies/μL.

dsDNA from various organisms and samples[20–22]. In contrast to other amplification methods, RPA operates at a relatively constant temperature, typically between 37 ℃ and 42 ℃, and utilizes primers and TaqMan probes with high specificity, resulting in a shorter reaction time. The optimal length for RPA primers is between 30 bp and 35 bp, which promotes the formation of stable nucleoprotein filaments. The RPA amplification cycle commences with the binding of a primer to a recombinase, forming an active nucleoprotein filament capable of rapidly scanning dsDNA libraries and identifying homologous sequences. Following hybridization of the primer, the nucleoprotein filament invades the target dsDNA. Subsequently, a DNA polymerase is loaded onto the 3' end of the primer, initiating primer extension through displacement of the complementary parental strand, ultimately leading to the formation of a new double-stranded DNA containing one parental strand[23]. RPA eliminates the thermal cycling required in PCR by employing recombinase for primer loading. RPA results can be visualized using various methods, including lateral flow assay, agarose gel electrophoresis, and real-time fluorescence assay, the latter of which allows for real-time monitoring of the amplification process.

This study successfully established an RT-RPA method for *BCR-ABL1* and *BCR-ABL2*, the most common *BCR-ABL* fusion gene subtypes in CML. Following optimization of primer conditions within the reaction system and subsequent application testing, the results demonstrated that the method exhibits strong specificity and favorable sensitivity in detecting *BCR-ABL1* and *BCR-ABL2* fusion genes. The minimum detectable limit for DNA samples is 10 copies/μL, offering a novel alternative for the rapid detection of *BCR-ABL* fusion genes.

The primary advantages of this detection method can be summarized in the following four points: (1) Amplification can be performed at a constant temperature of 37 ℃, eliminating the need for high-temperature annealing and thermal cycling for nucleic acid denaturation; (2) Primers and TaqMan probes exhibit high specificity, minimizing non-specific amplification; (3) The fluorescence signal from TaqMan probes can be collected in real time during amplification, providing a direct visualization of the amplification curve for the target sample; (4) The reaction time is short, with the amplification process completed in 20 minutes. The presence or absence and type of *BCR-ABL* fusion gene can provide critical prognostic information. These molecular results enable physicians to develop precision treatment strategies, particularly regarding the use of tyrosine kinase inhibitors (TKIs), including both the indication for TKI therapy and selection of the most appropriate agent. Plasmid templates lack inhibitors in blood/bone marrow samples, and chromatin structure in genomic DNA may reduce target accessibility. This paper can only illustrate the theoretical optimal performance, and in the future, will be validated using real patient samples. We anticipate that this approach will eventually enable rapid and convenient monitoring in resource-limited settings.

Author contributions

Conceptualization, Shuhui Wu and Songbai Liu; Methodology, Shuhui Wu and Jiani Ge; Software, Shuhui Wu and Jiani Ge; Validation, Shuhui Wu and Jiani Ge; Formal Analysis, Shuhui Wu and Jiani Ge; Investigation, Yingxin Yang and Zirui Hu; Data Curation, Yingxin Yang and Zirui Hu; Writing – Original Draft Preparation, Shuhui Wu and Jiani Ge; Writing – Review & Editing, Shuhui Wu, Jiani Ge, and Songbai Liu; Supervision, Rui Liang and Songbai Liu; Project Administration, Rui Liang and Songbai Liu; Funding Acquisition, Rui Liang and Songbai Liu.

Ethics statement

Not applicable.

Informed consent statement

Not applicable.

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Declaration of competing interest

The authors declared no conflict of interests.

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