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RAPID COMMUNICATION

Detection of the SARS-CoV-2 D614G mutation using engineered Cas12a guide RNA

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Abstract

Detection of pathogens with single-nucleotide variations is indispensable for the disease tracing, but remains technically challenging. The D614G mutation in the SARS-CoV-2 spike protein is known to markedly enhance viral infectivity but is difficult to detect. Here, we report an effective approach called "synthetic mismatch integrated crRNA guided Cas12a detection" (symRNA-Cas12a) to detect the D614 and G614 variants effectively. Using this method, we systemically screened a pool of crRNAs that contain all the possible nucleotide substitutions covering the -2 to +2 positions around the mutation and identify one crRNA that can efficiently increase the detection specificity by 13-fold over the ancestral crRNA. With this selected crRNA, the symRNA-Cas12a assay can detect as low as 10 copies of synthetic mutant RNA and the results are confirmed to be accurate by Sanger sequencing. Overall, we have developed the symRNA-Cas12a method to specifically, sensitively and rapidly detect the SARS-CoV-2 D614G mutation.

KEYWORDS
COVID-19, CRISPR/Cas12a, CRISPR-based detection, D614G, SARS-CoV-2

1 | INTRODUCTION

SARS-CoV-2 causes severe respiratory disease, the coronavirus disease 2019 (COVID-19). The disease has rapidly expanded into a global pandemic with devastating effects.[1,2] As of October 25, 2020, the World Health Organization (WHO) Situation Report has recorded over 42 million confirmed cases of COVID-19, including 1147301 deaths (https://covid19.who.int/). SARS-CoV-2 is highly contagious, and its basic reproduction number (R0) was estimated to vary between 2.2 and 3.9.[3] Mutations have occurred in the viral genome during the

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SARS-CoV-2 evolution.[4] According to the global tracking data, a novel G614 variant, caused by the A23403G substitution in the spike protein coding sequence has become dominant. This strain is more infectious than the wild-type D614 virus, shown in pseudovirus studies.[4,5] Thus, tracking the D614G mutation is critically important for disease monitoring; however, it still lacks a specific, sensitive and rapid detection method.

DNA sequencing is considered as the gold standard for detecting single nucleotide polymorphism (SNP), but is labor intensive and costly.[6] To accurately diagnose and monitor disease-related mutations, many PCR based methods have been developed, including allele-specific detection analysis and melting curve analysis.[7–11] However, combinations of mismatched nucleotides G/A and A/G are refractory to detect by PCR-based methods because the G-A/A-G pair has intermediate energy, compared to the Watson-Crick G-C/C-G base pair that has high energy and G-G/A-A of the non-base-pair with low energy.[12,13] In addition, the PCR-based approaches require expensive instruments and professional operations.

CRISPR/Cas12-based nucleic acid detection methods are sensitive, specific, rapid, simple, and inexpensive, thus offering an attractive alternative to the conventional methods described above.[14,15] The target DNA binds to the Cas12a-sgRNA complex and then triggers the collateral cleavage activity, cleaving ssDNA fluorophore-quencher reporters and generating visible fluorescent signals.[16–22] This crRNA-programmed collateral-cleavage activity has been exploited for detecting a wide range of pathogens, such as Zika virus, dengue virus, papillomavirus (HPV), African swine fever virus (ASFV) and SARS-CoV-2.[17,23–30]

We have previously established Cas12a-based assays for rapid, sensitive, and cost-effective detection of SARS-CoV-2.[26,31] However, we found that the ancestral crRNA-guided Cas12a detection could not distinguish the D614 and G614 variants. To specifically diagnose the D614G mutation, we developed a synthetic mismatch integrated crRNA guided Cas12a detection method, termed symRNA-Cas12a. We screened a total of 24 engineered crRNAs with substitutions of A, T, C, and G from the -2 to +2 position around the A23403G (D614G) site, and obtained a crRNA that could efficiently increase the detection specificity by up to 13-fold and enable the analytical sensitivity of down to 10 copies. The use of this crRNA enables the symRNA-Cas12a to become a powerful assay for monitoring the D614G mutation.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and ethics statement

All clinical samples were collected and treated in strict accordance with the WHO-recommended procedure in the State Key Laboratory of Respiratory Disease and Kingmed Virology Diagnostic & Translational Center (Guangzhou). RNA was extracted from all clinical samples using the QIAamp RNA Viral Kit (Qiagen, Heiden, Germany) in a BLS-2 laboratory.

2.2 | Expression and purification of Cas12a proteins

LbCas12a was expressed and purified as previously described[25] and details were listed in the supporting information.

2.3 | Nucleic acid preparation

The crRNAs were synthesized by GenScript, and the core sequences were listed in Table S1. The *E* and *S* gene fragments were cloned with IVT primers and transcribed using the MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific). Then the RNAs were purified with the MEGAclear Kit (Thermo Fisher Scientific). The RNAs were aliquoted and stored at -80°C until use. The primers used for in vitro transcription were listed in Table S2.

2.4 | Isothermal amplification coupled Cas12a-mediated nucleic acid detection

According to the previous report, the detection assays were performed with minor modifications[25] and details were described in the supporting information.

2.5 | DNA sequencing

The RNAs from clinical samples were reverse transcribed with the kit from Thermo Fisher (catalog number: 18091050) according to the manufacturer's manual. Then 1 μL of cDNA sample was used for cloning the D614G site fragment with primers S614-F and S614-R, and the PCR product was subjected to Sanger sequencing (BGI-Shanghai).

2.6 | Statistical Analysis

All experimental results were shown as Mean ± SEM unless stated otherwise. When only two groups were compared, statistical significance was assessed with an unpaired Student's t-test. Significance was indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Statistical analyses were carried out with GraphPad Prism 8.0.

3 | RESULTS AND DISCUSSION

Our current system for detecting SARS-CoV-2 is composed of LbCas12a, a fluorescence-quenched single-stranded DNA (ssDNA) reporter and CRISPR RNA (crRNA). Cas12a is recruited via crRNA to the target, where its nuclease is activated, resulting in ssDNA cleavage and fluorescence signal emission. However, this assay can hardly distinguish between D614 and G614 using the ancestral crRNA (Figure S1).

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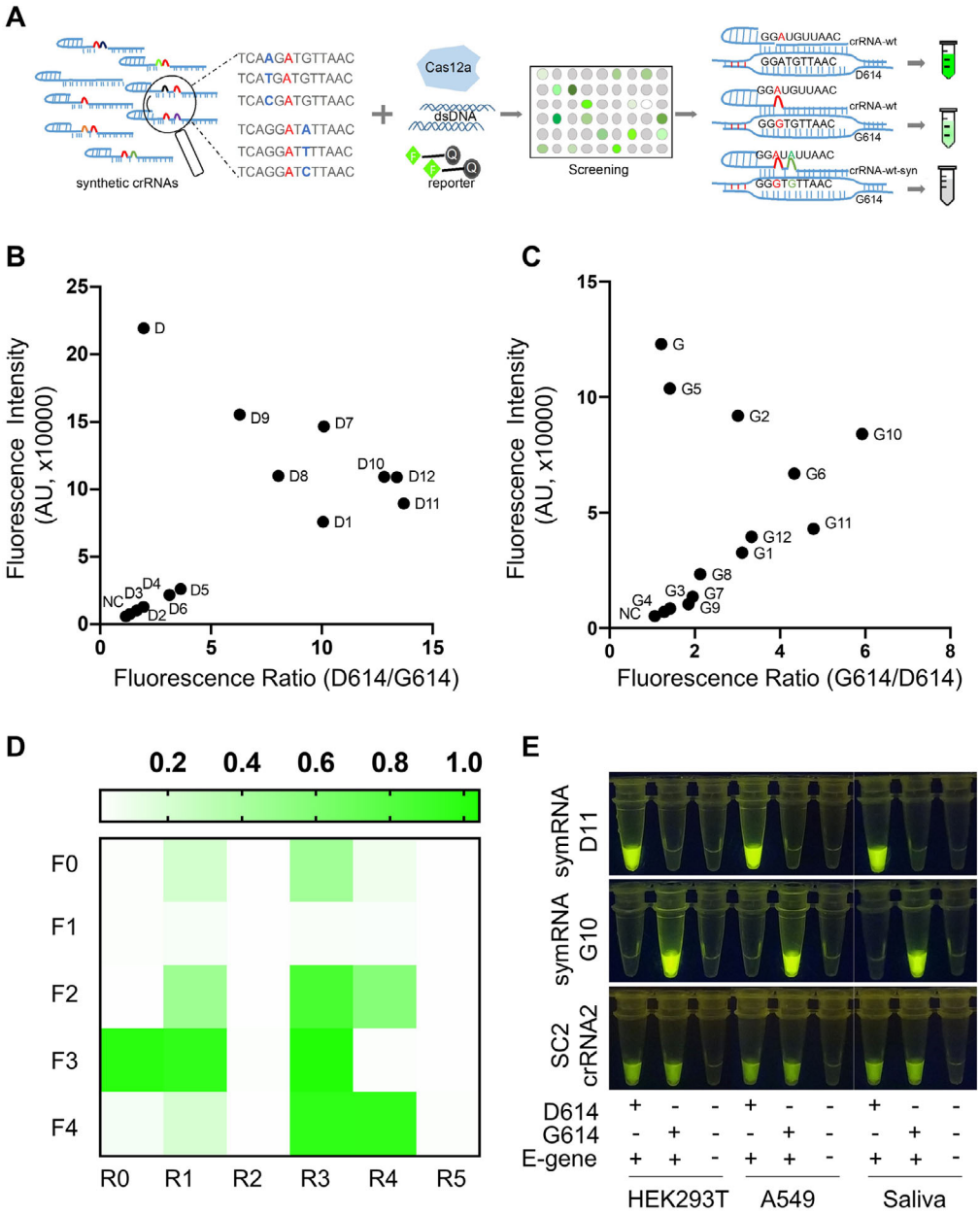
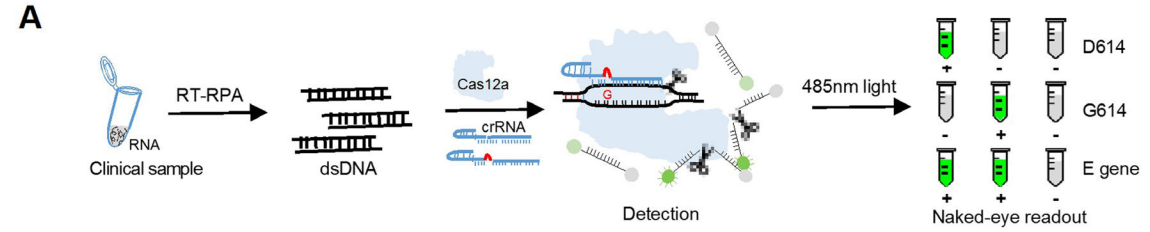
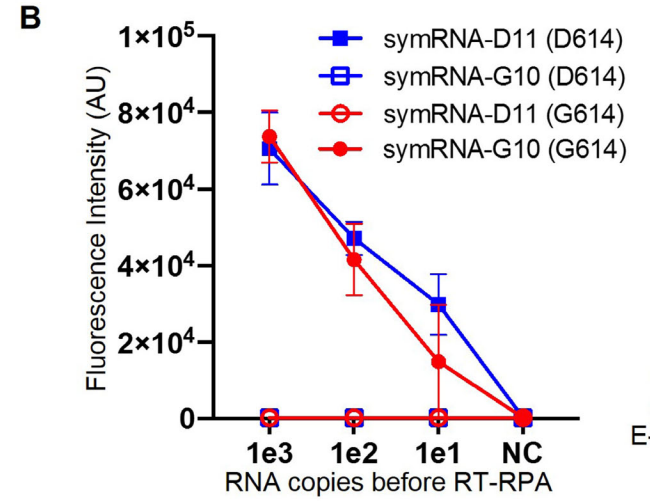


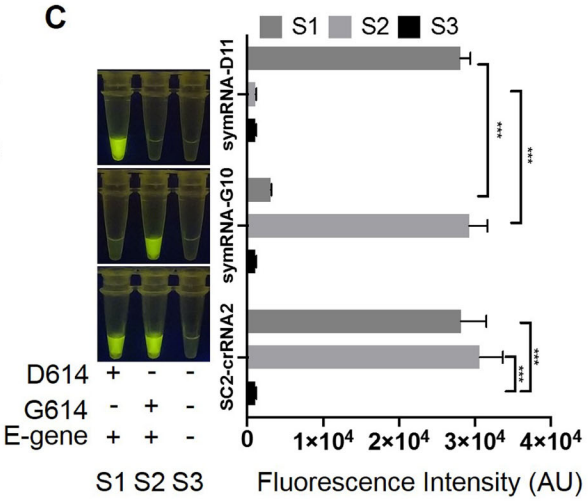
FIGURE 1 SymRNA-Cas12a detection for the D614G (A23403G) mutation in SARS-CoV-2 spike protein. (A) Schematic of symRNA design and optimization strategy for D614G variant. Synthetic crRNAs were introduced with single mismatches covering all possible nucleotide substitutions in the position -2 to +2 around A23403G site. The optimized crRNA can guide Cas12a to generate a specific signal with high fluorescence ratio of that on-target signal over the cross-reaction signal. (B) Optimization of specific symRNA targeting A23403 (D614). Specificity was determined as the ratio of the on-target DNA induced fluorescence intensity over the off-target DNA induced fluorescence intensity. The fluorescence intensity was obtained from symRNA-Cas12a reaction for 30 min reaction measured on PerkinElmer EnSpire reader. The core symRNA sequences were listed in Table S1. (C) Optimization of specific symRNA targeting G23403 (G614). The core symRNA sequences were listed in Table S1. (D) Optimization of efficient RPA primers for amplifies D614G related gene fragment of SARS-CoV-2. The amplification efficiency of thirty pairs of RPA primers was evaluated, and quantification of normalized fluorescent intensity of symRNA-Cas12a biosensing signal at 30 min. (E) Specificity validation of symRNA-Cas12a detection. SARS-CoV-2 RNA fragments containing D614, G614 or *E*-gene fragment were mixed with total RNAs of cell line HEK293T, A549 or saliva, respectively

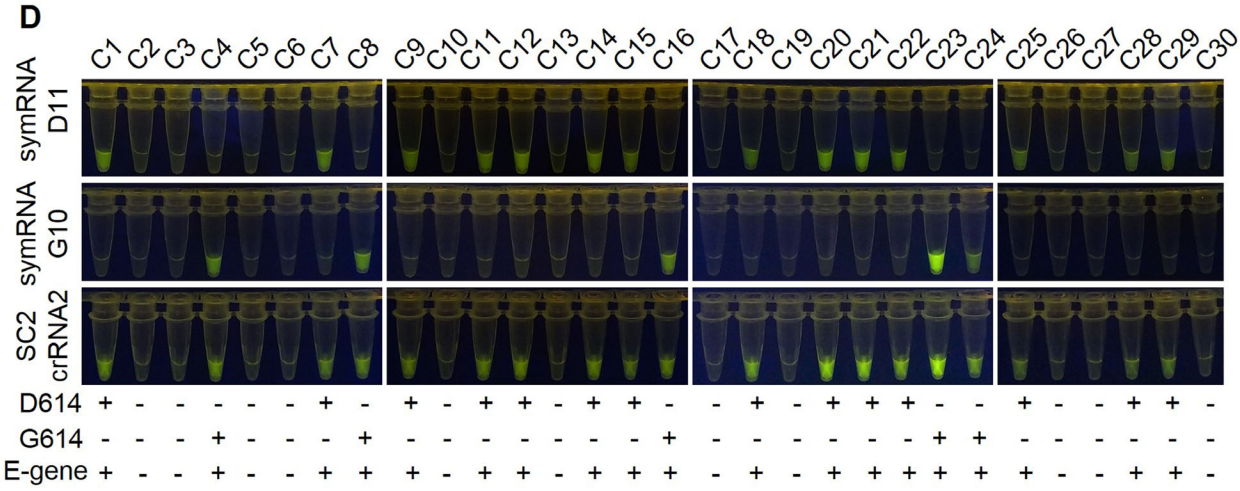
To address this problem, we mutated the crRNAs at a 4-bp region (-2 to +2) flanking the A23403G (D614G) mutation site, creating a total of 24 single-base mismatch mutant crRNAs covering all possible nucleotide substitutions in this region (Figure 1A). We then evaluated the selectivity of these mutant crRNAs (termed synthetic mutant RNA or symRNA) by the ratio of the fluorescence intensity induced by the on-target DNA to that induced by the off-target DNA. As shown in Figure 1B and C, symRNA-G10 and symRNA-D11 induced strong fluorescence intensity and the highest fluorescence ratio for the G614 and D614 sequences, respectively (Figure S2-5). The cleavage

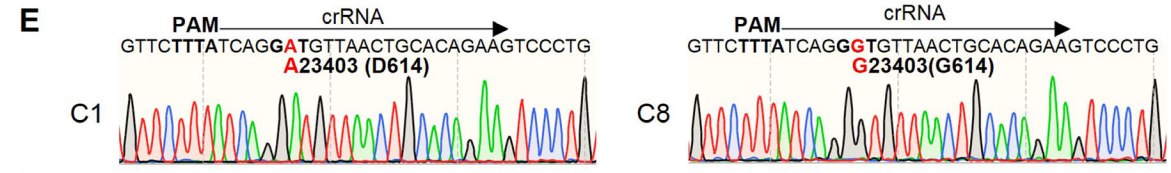
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F

Sanger sequencing				symRNA-Cas12a				Comparison of two methods		
D614	G614	Negative	Total	D614	G614	Negative	Total	Sensitivity (%)	Specificity (%)	Kappa
14	5	11	30	14	5	11	30	100	100	1

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preference was confirmed by the gel analysis of the cleaved DNA fragments (Figure S6).

Having optimized the detection specificity, we next sought to enhance the detection sensitivity. To this end, we optimized RT-RPA-mediated pre-amplification by testing 30 pairs of RPA primers for the D614G site fragment (Figure S7A and Table S2). As shown, the F4 and R3 primer pair had the highest efficiency (Figure 1D and Figure S7B, C).

To validate the symRNA-Cas12a-based assay in clinical scenarios, we prepared simulation samples by mixing the D614 or G614 gene fragments, each with RNA isolated from HEK293T cells, A549 cells or saliva samples. In order to keep the accuracy of the detection, we detected three signals (*E gene* and *S* gene D614 type, and G614). The signal of *E gene* was used as an internal control to confirm the infection of SARS-CoV-2. The intensity ratio of D614/G614 was used to distinguish the mutations. Our results showed that the symRNA combination remained robust and worked very well under these conditions (Figure 1E and Figure S8).

To further improve the assay, we designed a strategy for simultaneous detection of three genes: the *E* gene, and the *S* gene, both D614 type and G614 type (Figure 2A), where the *E* gene was used as an internal control to confirm the SARS-CoV-2 infection, based on a previous study.[26] The crRNAs targeting the *E* gene are responsible for the detection sensitivity. The D614 or G614 crRNA are responsible for distinguishing the mutations. The sample RNAs were pre-amplified with RT-RPA for 30 min before being subjected to symRNA-Cas12a detection for 15 min, and the detection limit was assessed by dilution series of the sample RNAs. We found that this system was able to detect as few as 10 copies of the synthetic D614 and G614 RNA (Figure 2B and Figure S9), which is consistent with the previously reported detection limits using CRISPR.[27,32,33] and is more sensitive than those methods using LAMP and EXO-RPA.[34–36] The PCR-based detections, including TaqMan and Exo-probe, recognize the target sequence merely based on the base pairing between the probe and target sequence, thus performing poor sensitivity, particularly for single-nucleotide variations.[37] Previous studies demonstrated that the sgRNA-Cas12/Cas13 complex could recognize single base mismatch.[16,38] The three-gene assay also proved robust in the simulation test (Figure 2C and Figure S10).

Finally, we validated the diagnostic accuracy of symRNA-Cas12a for identifying the D614G mutation in clinical samples. All clinical samples were collected and treated in strict accordance with the WHO recommended operation for COVID-19. Extracted RNA from a total of 30 clinical samples was used for SARS-CoV-2 detection with the symRNA-Cas12a assay and Sanger sequencing. Nineteen samples were determined to be SARS-CoV-2 positive by Cas12a detection of the *E* gene, which was consistent with the clinical diagnosis (Figure 2D). Among these positive samples, 14 samples were identified as the D614 type and 5 samples as the G614 variant, which were confirmed by the Sanger sequencing (Figure 2D, 2E and Figure S11, S12). The results of the symRNA-Cas12a detection method and Sanger sequencing showed perfect agreement, with a kappa value (κ) of 1.0 ($p < 0.001$; Figure 2F), confirming the outstanding performance of the symRNA-Cas12a.

4 | CONCLUSIONS

In conclusion, symRNA-Cas12a can specifically, sensitively, and rapidly detect SARS-CoV-2 and distinguish D614G variants, providing a powerful method for COVID-19 monitoring and epidemiological analyses. It could generally be applied to other CRISPR-based SNP detection systems. CRISPR can contribute ca. 10-fold signal amplification in the current study. The sensitivity of CRISPR detection has been largely enhanced by the performance of nucleic acid pre-amplification, such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). LAMP requires only single enzyme for the exponential amplification, but its working temperature is higher than that of the CRISPR cleavage.[27,39,40] RPA can efficiently amplify the target gene at the same temperature, but the CRISPR detection requires a more complex enzymatic system.[41] The current strategy focused on the optimization of crRNA, which is essential for the CRISPR reaction. It could be further modified and integrated with other nucleic acid amplification and sample preparation methods, such as LAMP, HUDSON, and electric field-driven microfluidics.[33,42,43] to develop simpler, more sensitive and faster methods to identify mutant strains in saliva directly without RNA extraction.

FIGURE 2 Diagnosis of the SARS-CoV-2 D614G mutation with symRNA-Cas12a. (A) The workflow of SARS-CoV-2 D614G mutation detection using symRNA-Cas12a. The sample RNA was amplified with RT-RPA and then subjected to a three-gene parallel-test on *E* gene, D614 type, and G614 type. After 15 min reaction, the green fluorescence was observed by the naked eye under the 485 nm light. (B) The detection limit of the symRNA-Cas12a system. Gradient diluted SARA-CoV-2 RNA fragments containing D614 or G614 were amplified by RT-RPA, then detected by symRNA-D11 and symRNA-G10, respectively. The fluorescence intensity at 15 min was shown. The copy numbers of gradient diluted samples were shown in figures (1e3 = 1000, 1e2 = 100, 1e1 = 10). (C) Simulated diagnostic of SARA-CoV-2 D614G mutation with symRNA-Cas12a for E gene, D614 and G614 variants. The samples contain RNA fragment of *E* gene, D614 and G614 variants were pre-amplified using RT-RPA and then detected by symRNA-D10, symRNA-G10 and SC2-crRNA2, and the fluorescent images at the 15-min of the reaction were shown. The fluorescence intensities were calculated by software ImageJ and visualized with GraphPad. (D) Detection of SARS-CoV-2 D614G mutation in clinical samples with the symRNA-Cas12a assay. The target sequence was amplified by RT-RPA for 30 min and then detected with symRNA-D10, symRNA-G10 and crRNA-E guided Cas12a. The images of Cas12 detection at 15-min were shown. (E) Sanger sequencing chromatographs show the D614G region of clinical samples C1 and C4 in (D). (F) The performance of symRNA-Cas12a for D614G detection in clinical samples compared with Sanger sequencing. Statistical significance between two groups was assessed with an unpaired two-tailed Student's t-test. Significance was considered as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

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AUTHOR CONTRIBUTIONS

Q. M.: Formal analysis; investigation; writing-review & editing. X. W.: Conceptualization; formal analysis; funding acquisition; investigation; project administration; writing-original draft. Y. W.: Formal analysis; resources. L. D.: Formal analysis. X. L.: Formal analysis. X. M.: Formal analysis. T. C.: Project administration; writing-review & editing. X. W.: Formal analysis. Q. Z.: Formal analysis. G. Y.: Conceptualization; project administration. M. L.: Formal analysis; funding acquisition; project administration; resources. X. H.: Conceptualization; funding acquisition; project administration; writing-review & editing. P. M.: Conceptualization; formal analysis; investigation; project administration; writing-original draft.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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