A Novel Mutation in the RPE65 Gene Causing Leber Congenital Amaurosis and Its Transcriptional Expression

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Abstract

The retinal pigment epithelium-specific 65 kDa protein is an isomerase encoded by the RPE65 gene (MIM 180069) that is responsible for an essential enzymatic step required for the function of the visual cycle. Mutations in the RPE65 gene cause not only subtype II of Leber congenital amaurosis (LCA) but also early-onset severe retinal dystrophy (EOSRD). This study aims to investigate a Chinese case diagnosed as EOSRD and to characterize the polymorphisms of the RPE65 gene. A seven-year-old girl with clinical symptoms of EOSORD and her parents were recruited into this study. Ophthalmologic examinations, including best-corrected visual acuity, slit-lamp, Optical coherence tomography (OCT), and fundus examination with dilated pupils, were performed to determine the clinical characteristics of the whole family. We amplified and sequenced the entire coding region and adjacent intronic sequences of the coding regions of the RPE65 gene for the whole family to explore the possible mutation. Our results demonstrate that the patient exhibited the typical clinically features of EOSRD. Her bilateral decimal visual acuity was 0.3 and 0.4 in the left and right eyes, respectively. Spectral-domain optical coherence tomography (SD-OCT) was used to assess the retinal stratification for the whole family. All together, we identified four mutations within the RPE65 gene (c.1056G>A, c.1243+2T>A, c.1338+20A>C and c.1590C>A) in the patient. Among the four mutations, c.1056G>A and c.1338+20A>C had been reported previously and another two were found for the first time in this study. Her mother also carried the novel mutation (c.1243+2T>A). Either a single or a compound heterozygous or a homozygous one mutation is expected to cause EOSRD because mutations of RPE65 gene usually cause an autosomal recessive disease. Therefore, we speculate that the c.1590C>A mutation together with the c.1243+2T>A mutation may cause the patient’s phenotype.

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Introduction

Retinal pigment epithelium-specific 65 kDa protein (RPE65, GenBank accession No. NP000320.1) is an isomerase preferentially expressed in the retinal pigment epithelium (RPE) [1,2]. It is responsible for retinol isomerization and converts all-trans retinyl ester to 11-cis retinol in the visual cycle [3–5]. Previous research demonstrated that retinol isomerization was an essential enzymatic step required for functional vision [6,7]. RPE65 is a microsomal protein encoded by RPE65 gene (MIM 180069), containing 14 coding exons and localizing in chromosome 1p31 [8]. Mutations in RPE65 gene were primarily reported in patients with Leber congenital amaurosis (LCA, MIM204000). Now, 86 mutations have been identified in the RPE65 gene in patients with LCA [9–33]; we summarize these mutations in Figure 1.

LCA, first described in 1869 by Leber T [34], is a severe congenital or early infant-onset form of inherited retinal dystrophy [35]. In general, LCA is defined as blindness within the first two years of life. Based on previous descriptions of patients with LCA, this disease has a wide spectrum of presentation such as early severe visual deficits in childhood, the oculo-digital sign (habitually rubbing or poking the eyes), refractive errors, heterogeneity in retinal appearance, macular atrophy, and optic nerve pallor [36]. In addition, congenital onset and amaurosis by the second year of age. The EOSRD has several names: juvenile and early-onset retinitis pigmentosa, childhood-onset severe retinal dystrophy, and severe early childhood onset retinal dystrophy (SECORD) [37–40]. Many studies indicated that LCA was extremely genetically heterogeneous and was associated with more than 17 genes. Moreover, many mutations associated with the inheritance of...
LCA have been described and used to differentiate the subtypes of LCA1~15, for instance, GUCY2D (LCA1), RPE65 (LCA2), APLI (LCA4), RPGRIP1 (LCA6), CRX (LCA7), and CRB1 (LCA8) (http://www.ncbi.nlm.nih.gov/omim). Among these known diseased-causing genes, RPE65 mutations were first identified and their prevalence ranges from 1.7% to 16% in LCA cohorts in the United States, Canada, Saudi Arabia, Asia, and India [10,41,42]. To date, two cases of RPE65 mutations with LCA have been reported in China [31,32], while most cases occur in Western populations. Notably, homozygous and compound heterozygous mutations in RPE65 gene are associated with subtype II of LCA or EOSRD [37,43]. The description of EOSRD was coined for subtype II of LCA or EOSRD [37,43].

In this study, we report the clinical examinations and genetic analysis of RPE65 gene in a Chinese family. Sanger sequencing was used to analyze all the coding regions of the RPE65 gene along with the adjacent intronic regions. Furthermore, we constructed the RPE65 minigene containing the c.1243+2T>A mutation and investigated the effects of that mutation in in vitro splicing.

Materials and Methods

Clinical data and sample collection

This study was reviewed by the Department of Ophthalmology, Wuhan General Hospital of Guangzhou Military Command on Clinical Investigation and it conformed to the tenets of the Declaration of Helsinki. The seven-year-old girl and her family were referred to the Department of Ophthalmology, Wuhan General Hospital of Guangzhou Military Command on Clinical Investigation. After informed consent was obtained, all participants underwent six ophthalmologic examinations, including best-corrected visual acuity, slit-lamp, and fundus examination with dilated pupils to exclude infection or other diseases. Ophthalmoscopic findings were recorded by color fundus photography. Optical coherence tomography (OCT, Topcon 3D-1000 Mark II, Tokyo Japan) was used to examine the retinal structure. Spectral-domain OCT (SD-OCT) recording with 3D macular protocol was performed with 6-mm single line scans over the fovea. In detail, the 3D macular protocol consists of a radial-scanning composed of 512×128 scan resolution covering an area of 6×6 mm in the macular region. The patient underwent fundus fluorescein angiography (FFA) and visual field examination. Clinical diagnosis was based on the results of the above-mentioned ophthalmologic examinations.

Mutation identification

Blood samples were obtained by venipuncture and genomic DNA was extracted according to the manufacturer’s protocol (TIANamp Blood DNA Kit, Tiangen) as described in our previous report [44]. For sequencing, the entire coding region and adjacent intronic sequences of 14 coding regions of the RPE65 gene were amplified by PCR, using the primers in Table 1. PCR products were purified with the AxyPrep DNA Gel Extraction Kit (Axxygen, CA, USA). All PCR products were bi-directionally sequenced with the dyeoxy nucleotide chain terminator technique. Sequencing was performed on an automated sequencer – ABI 3730XL DNA Analyzer (ABI, USA). The results were assembled and analyzed using the Applied Biosysytems Sequencing Analysis 5.2 software. Sequences were aligned with the published cDNA sequences of RPE65 gene (GenBank accession no. NM_000329). We also assessed the potential functional consequences of nucleotide changes using multiple web servers for mutation analysis such as PolyPhen-2 (Polymorphism Phenotyping, http://genetics.bwh.harvard.edu/pph-2/) [45], SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org/) [46], and Automated Splice Site and Exon Definition Analyses (http://splice.uwo.ca/) with default parameters.

Cell line and cell culture

The 293T cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Wuhan, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 2 mM glutamine, 10% fetal calf serum (FBS, Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. The Cells were maintained at 37 °C in a 5% CO₂ incubator until confluent, then sub-cultured at 1:3 to 1:10 dilutions using trypsin-EDTA.

RPE65 minigene construction

RPE65 mutated minigenes (pCIneo-m65) were constructed by subcloning the c.1243+2T>A mutation from the patient into pCI-neo vector (Promega, Madison, WI). The wild-type minigene (pCIneo-65), without the c.1243+2T>A mutation, was generated by subcloning the genomic DNA of her father. PCR-amplified exons (11, 12, and 13) of the RPE65 gene were inserted at the

Figure 1. LCA related mutations in RPE65 gene. The black arrow indicates the novel mutation discovered in this study. doi:10.1371/journal.pone.0112400.g001
EcoRI/SalI restriction enzyme site in pCI-neo vector. Primers and conditions used in the PCR amplification of the inserts were listed in Table 1. The corresponding DNA inserts were confirmed by sequencing.

Transfection, RNA isolation, and RT-PCR
To analyze the effects of the c.1243+2T>A mutation on splicing in vitro, 293T cells were transfected with RPE65 mini-genes. Prior to transfection, cells were seeded with a density of 4.0×10^5 cells per well in a six-well plate and grown to approximately 80% confluence. RPE65 minigene used in this study was transfected into cells with Lipofectamine 2000 reagent (Invitrogen) by using 2 μg of DNA per well. After 24 h transfection, cells were serum starved for another 24 h before harvest. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. DNase I (Promega) was used to treat the RNA extract to eliminate DNA contamination. 1.0 μg of treated RNA was used as a template for reverse transcription using the First Strand cDNA Synthesis Kit (TOYOBO). The exogenous RPE65 minigene transcript was amplified using F1 and R1 primers (Table 1) and the fragment sizes of the wild and mutant PCR products were detected by 2% agarose gels electrophoresis and stained with ethidium bromide. To confirm the nucleotide sequences, the wild and mutant PCR products were purified from the gel and sent for sequencing.

Results
Clinical features
In this Chinese family, only the seven-year-old girl reported the typical characteristics of EOSRD. She was a primary school student. When she was three years old in 2008, her parents found she could not see toys in the dark, which meant her visual acuity was markedly decreased in dimmer conditions. Moreover, when she went inside from outdoor sunlight, her dark adaption took nearly two hours. In 2011, her eyesight problem made it difficult for her study at school. She was diagnosed as EOSRD by a full ophthalmologic examination in 2012. Her fundus examination demonstrated mildly attenuated retinal vessels, some whitish dots, and numerous grayish deposits in the mid-peripheral retina (Figure 2). However, no whitish dots or grayish deposits were observed on the fundi of her parents.

The SD-OCT scanning with 3D macular protocol was used to examine the retinal stratification. Compared with foveal SD-OCT recordings of her parents’ eyes, the patient’s SD-OCT images of both eyes showed several characteristics of altered retinal stratification including extremely thinned outer nuclear layer (ONL), heavily thinned retinal pigment epithelial (RPE) layer, altered photoreceptor layers including external limiting membrane (ELM), inner segment (IS), outer segments (OS), and inner segment ellipsoid (ISe) (Figure 3a and 3b). In her patients’ grayscale SD-OCT recording (Figure 3 c-2, 3d-2, 3e-2, and 3f-2), the boundaries of the photoreceptor layers (ELM, IS, ISe, and OS) were not well demarcated. The enlargement of grayscale SD-OCT recordings at the fovea of her patients’ eyes (Figure 3 c-3, 3d-1, 3e-3, and 3f-1) showed that the ELM, IS, ISe and OS layers were not continuous and could not be clearly identified, and the ISe layer previously named IS/OS junction could hardly be shown. In addition, the boundaries of OS/RPE and RPE-Bruch’s membrane complex could not be discerned. But her parents’ foveal SD-OCT recordings showed that the boundaries of the retinal layers were well demarcated and the retinal layers could be clearly identified

Table 1. Primers used in the study.

| Exons | Primers | Sequencing (5’→3’) |
|-------|---------|-------------------|
| 2     | RPE65–2F | GCAGGAGTGAAACGCTTTTG |
|       | RPE65–2R | AGAGACCCCAAGGAATAGGAA |
| 3     | RPE65–3F | GAGGCTGGAGATGAAGAAATC |
|       | RPE65–3R | ACATTGTGAGAGAAATGCTT |
| 4/5   | RPE65–4S | GTCTACCCCAAGGAATAGGAG |
|       | RPE65–5R | GGATTTGAAACTTAATGTGCTC |
| 6     | RPE65–6F | AACTCAAGGGTCAAAGGCTAGG |
|       | RPE65–6R | AGAGAAGCTGCACTTCACTC |
| 7–9   | RPE65–7S | GGAGAAAAATGAAAATACACCCT |
|       | RPE65–9R | GAGTGCAGCAGCTTGAATA |
| 10    | RPE65–10F | GAAATTAGAACAGGGAGCCACT |
|       | RPE65–10R | TTGTCTTGGCTAGTCAACGTA |
| 11–13 | RPE65–11F | CCTCCCTGTCAAGTGACCT |
|       | RPE65–13R | GCCTCATCGTGACACCAAAT |
| 14    | RPE65–14F | ATGCCAGGTGTTACAAGGCTA |
|       | RPE65–14R | TGCTCAACTCTAGTGCTTCTGTA |
| F1*   | GCAGGAGTGAAACGCTTTTG |
|       | RPE65–2R | AGAGACCCCAAGGAATAGGAA |
| R1*   | GTCTACCCCAAGGAATAGGAG |
|       | GCAGGAGTGAAACGCTTTTG |
| F2*   | CCTCCCTGTCAAGTGACCT |
|       | GCCTCATCGTGACACCAAAT |
| R2*   | TCTCTAGATCATCTCTCTTGACGGCCTTG |

Notes: * denotes primers for amplification of the wild and mutant fragments of exons 11, 12, and 13 of RPE65 gene. Bold and underlined sequences are restriction enzyme sites.

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(Figure 3c-2, 3d-2, 3e-2, and 3f-2). The aforementioned abnormalities were not presented in her parents' grayscale foveal SD-OCT recordings (Figure 3c-2, 3d-2, 3e-2, and 3f-2). Meanwhile, we also measured the thickness of IS, ISe, and RPE-choroid of the patient and her parents' right eyes (Figure 3g, 3h, and 3i). Her father/mother's results were 15 µm/15 µm (Figure 3h-2 and 3i-2), 16 µm/16 µm (Figure 3h-3 and 3i-3), and 40 µm/35 µm (Figure 3h-4 and 3i-4). Because the boundaries of IS, ISe, OS, and RPE are not clear, we cannot measure the thickness of ISe and RPE. Therefore, we measured the noncontiguous area of ISe layer, RPE-choroid and ISe-choroid, and the results were 239 µm (Figure 3g-2), 24 µm (Figure 3g-3), and ISe-choroid (Figure 3g-4), respectively.

**RPE65 gene mutation analysis**

Based on the complete sequence analysis of the coding and adjacent intronic regions of RPE65 gene, four mutations were detected in this family. These mutations included one missense, one silent, and two intronic changes. All mutations are listed in Table 2. All four mutations were present in the heterozygous state.
The father carried only one point mutation (c.1338+20A>C, Figure 4), and the mother carried two point mutations (c.1243+2T>A and c.1338+20A>C, Figure 4). The daughter carried four mutant points: c.1338+20A>C (from her parents), c.1243+2T>A (from her mother), c.1056G>A, and c.1590C>A (Figure 4).

Meanwhile, we also used multiple web servers including PolyPhen, SIFT, and Automated Splice Site Analyses to perform the mutation analysis (Table 2). The loss of the splice site was predicted for the c.1243+2T>A mutation by Automated Splice Site Analyses; the c.1056G>A (rs12145904) mutation is predicted to be “benign” and “neutral” according to the results of PolyPhen and SIFT; the c.1590C>A (F530L) mutation is predicted to be “possibly damaging” and “deleterious” by PolyPhen and SIFT; the c.1338+20A>C (rs12564647) mutation cannot be evaluated by Polyphen and SIFT. The c.1338+20A>C (rs12564647) mutation is listed in the SNP database of GenBank (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=12564647).

Transcriptional expression of RPE65 minigenes in 293T cells

To identify the effects of the c.1243+2T>A mutation in intron 11 of RPE65 gene on splicing, we constructed two minigenes: the c.1243+2T>A mutation in RPE65 gene (pCInco-m65) and the wild type (pCInco-65), and transfected them into 293T cell line. Total RNA was isolated from 293T cells transfected with the minigene constructs (Figure 5), and then used for RT-PCR to amplify the exons of 11, 12, and 13 of RPE65 gene. PCR products indicating variations in splicing, were resolved and analyzed by 2% agarose gel electrophoresis (Figure 6b). The RT-PCR product of the wild type showed a 264 bp DNA band, as the expected normal transcript (Figure 6a), but the mutant type produced a
358 bp band (Figure 6a). It is 94 bp longer than the normal transcript of 264 bp (Figure 6b), indicating an insertion of the complete intron 11 (Figure 6a). Sequence analysis of the product revealed that 94 bp were indeed inserted at the 3' end of exon 11. The sequence of the 94 bp insertion perfectly matched that of intron 11 in RPE65 gene containing the c.1243+2T>A mutation. Interestingly, there was another smaller size DNA band (about 200 bp) in wild-type (Figure 6b) not present in mutant. This could be caused by mispriming. These results show that the novel mutation (c.1243+2T>A) may completely inactivate the original splice-donor site.

### Conclusion and Discussion

In this study, we report the case of a girl clinically diagnosed as EOSRD in her family. This Chinese family were clinically and genetically characterized. The girl had obvious clinical characteristics of EOSRD; however, these characteristics were absent in the fundi of her parents. Clinically, OCT is an important auxiliary diagnosis method to provide in vivo visualization of intraretinal stratification. Disorders of retinal stratification is generally considered to be related with disease. Segmentation of retinal layers is important for diagnosis and analysis of disease [47,48]. In the grayscale foveal SD-OCT recordings, differences of retinal stratification were very extinct (Figure 3). Based on the method of retinal layers segmentation reported by Ehnes A et al [49], the patient's SD-OCT recordings at the foveal showed altered photoreceptor layers (ELM, IS, ISe, and OS). The boundaries of photoreceptor layers could not be clearly discerned (Figure 3a-2, 3a-3, 3b-1, and 3b-2), and the ELM, IS, ISe and OS layers were not continuous. The ISe, previously named IS/OS junction, could hardly be shown in the OCT. Therefore, we cannot measure the thickness of Ise and RPE; instead, we measured the noncontiguous area of Ise layer (Figure 3g-2), RPE-choroid (Figure 3g-3), and ISe-choroid (Figure 3g-4).

In addition, abnormalities of retinal stratification also included extremely thinned ONL and heavily thinned RPE layer (Figure 3a and 3b). These abnormalities proved the aforementioned relation of disease and retinal stratification disorder. But in her parents' foveal SD-OCT recordings, the retinal layers could be clearly identified (Figure 3c-2, 3c-3, 3d-1, 3d-2, 3e-2, 3e-3, 3f-1, and 3f-2), and meanwhile the thickness of ISe,RPE, and RPE-choroid could be measured (Figure 3h and 3i). This may be caused by autosomal recessive inheritance (Figure 4). It has been verified that the RPE65 mutation causes EOSRD [9,37]. By analysis of mutations in RPE65 gene in this case, we found that both the mother and the daughter have the mutation (c.1243+2T>A) at the consensus sequence of the splice donor. This mutation uniformly results in splicing errors [50] and is disease-causing. However, by ophthalmologic examination, only the daughter has obvious symptoms of EOSRD; we speculate that the daughter’s phenotype may be associated with the combined effects of the c.1243+2T and c.1590C>A mutations.

The entire coding region and adjacent intronic regions of RPE65 gene were sequenced. Four mutations were identified in the patient, two of which were found novel (c.1243+2T>A and c.1590C>A) and have not been reported before (Table 2). According to previous studies, mutations in RPE65 gene could cause EOSRD. There are several mutant types in this study: missense, nonsense, splicing site, deletion, insertion, and indel mutation. Eighty-six mutations of RPE65 gene in patients with LCA have been reported in twenty-four published studies [9–32]. Two cases have been reported in 188 Chinese patients [31,32] containing nine point mutations were identified in RPE65 gene.
These mutations were one insertion mutation (c.1059_1060insG) [50], four missense mutations (c.295G>A, c.997G>C, c.200T>G, and c.1103A>G) [30,31], and four polymorphisms (c.643+22C>T, c.1338+20A>C, c.1056G>A, and c.*726_*727insAG) [31]. In this study, four mutations were found in the patient (Table 2), who carried two reported single nucleotide polymorphisms (SNPs) of c.1338+20A>C (rs12534647) and c.1056G>A (rs12145904) [32]. In the SNP database of GenBank, with benign allele was predicted for both c.1338+20A>C and c.1056G>A mutations.

Both c.1243+2T>A and c.1590C>A (F530L) are novel mutations and there are no previous reports. The c.1243+2T>A mutation is a point mutation at consensus sequences at the 5’ end of intron 11 of RPE65 gene. It has been reported that mutations at consensus sequences uniformly result in aberrant splicing [50]. To confirm the effects of this mutation on splicing, we constructed RPE65 minigene from three exons, containing either normal or mutant intron 11 sequences (Figure 5) and investigated their transcripts in 293T cell line by RT-PCR. A normal transcript consisting of three exons (264 bp) was obtained from the wild-type minigene (Figure 6a and b). For the mutant, the transcript is 358 bp in length that is longer than the normal transcript.

Sequence analysis showed that the mutant transcript is 94 bp longer than the normal transcript at the 3’ end of exon 11, whose sequence perfectly matches the sequences of intron 11 in RPE65 gene containing the c.1243+2T>A mutation (Figure 6a and b). These results indicate that the c.1243+2T>A mutation results in aberrant splicing, which is consistent with the result of “splicing site abolished” by Automated Splice Site Analyses. Based on the result of RPE65 minigene experiment in vitro, the c.1243+2T>A mutation may be pathogenic. Both the mother and the daughter had this mutation; however, the mother showed no symptoms of EOSRD. Since mutations of RPE65 gene usually cause an autosomal recessive disease, either a single or a compound heterozygous or a homozygous mutation is expected to cause EOSRD. Therefore, the single heterozygous state of the c.1243+2T>A mutation in the mother does not rule out its pathogenicity.

The c.1590C>A change is the second novel mutation in this study. This mutation results in an amino acid transition from phenylalanine to leucine, but it is not detected in patient’s parents. Based on an RPE65 topology diagram, the phenylalanine residue 530 in RPE65 gene is conserved and located within blade VII of the seven-bladed β-propeller motif in the RPE65 protein [52]. Seven mutations found from LCA and RP patients are reported to

![Figure 4. Pedigree of the Chinese family and mutations of RPE65 gene.](https://www.plosone.org/doi/fig/10.1371/journal.pone.0112400.g004)

In the family structure, male and female are represented by squares and circles, respectively. The filled square symbol represents the ESORD-affected daughter (c). One mutation (c.1338+20A>C) is detected in RPE65 gene of her father (a); two other mutations (c.1243+2T>A and c.1590C>A) are detected in her mother (b); four mutation are found in the daughter (a). The red color highlights the novel mutation.

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be located within blade VII. They are L22P, P25L, G40S, R44Q, H68Y, Y79H, and G528V [14,15,17,24]. In our study, F530L (c.1590C>A) is found to be the eighth mutation in blade VII. Moreover, F530L and G528V are in the same sheet of blade VII. Both biochemical and crystal structure studies on RPE65 show that residues in any sheet of each blade of the propeller structure are essential for RPE65 isomerase activity [5,52,53]. In addition, the c.1590C>A (F530L) mutation was also analyzed by the online tools (PolyPhen and SIFT). The potential functional consequence of this nucleotide change was predicted to be “possibly damaging” and “deleterious”. Therefore, the c.1590C>A (F530L) mutation could be responsible for the daughter’s phenotype. It has also been verified to cause the pathogenesis of blade VII in RPE65 gene, based on its isomerase activity and crystal structure [52,53]. If there were no other mutant genes involved in ESORD in this family, it could be concluded that this mutation played an important role in the pathogenesis of blade VII in RPE65 gene.

From the above, we could infer that the c.1590C>A (F530L) mutation together with the c.1243+2T>A mutation is disease-causing mutation. However, RPE65 is not the only gene associated with EOSRD. Although reports about mutations of the RPE65 gene are mainly found in EOSRD patients, it is still difficult to say whether the daughter’s phenotype is definitely caused by the two novel mutations, because the mother has one of these novel mutations and shows no symptoms of EOSRD. Either the c.1590C>A (F530L) mutation or other mutated genes in the daughter may be responsible for her phenotype. Thus, further studies should be performed to confirm if there is a definite genotype-phenotype correlation between RPE65 gene and EOSRD.

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**Author Contributions**
Conceived and designed the experiments: GYM YPS GHY. Performed the experiments: GYM. Analyzed the data: GYM GHY LJB QD. Contributed reagents/materials/analysis tools: GYM ZSC YBL MY. Wrote the paper: GYM YPS GHY.

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