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ORIGINAL ARTICLE

The spectrum of *CYP21A2* gene mutations in patients with classic salt wasting form of 2l-hydroxylase deficiency in a Chinese cohort

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Abstract

Background: 21-Hydroxylase deficiency (21-OHD) caused by the *CYP21A2* gene mutations is the most common form of congenital adrenal hyperplasia. It is an autosomal recessive disorder that results in defective synthesis of cortisol and aldosterone. The incidences of various *CYP21A2* gene mutations and the genotype–phenotype correlations vary among different populations.

Materials and Methods: The clinical and molecular data of 22 patients were analyzed in this study. All patients were recruited from the neonatal intensive care unit. Locus-specific polymerase chain reaction and Sanger sequencing were applied to identify gene micro-conversions, and multiplex ligation-dependent probe amplification was used to detect large fragment deletions/conversions. Then, the genotypes were categorized in to Null, A, B, C, and D groups to analyze the relationships between genotypes and phenotypes.

Results: All 22 patients were classified into classic salt wasting form of 21-OHD. Molecular defects were detected in 44 alleles (100%). Micro-conversion mutation IVS2-13A/C>G (70.5%) is most common in our cohort, followed by large gene deletions and conversions (22.7%). The other mutations present were p.R357 W (4.5%) and E6 Cluster (2.3%). Genotypes of 22 patients (100%) were consistent with the predictive phenotypes.

Conclusion: In this study, we identified the mutation spectrum of *CYP21A2* gene in Chinese patients, especially the younger age cohort in pediatrics. Micro-conversions

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were the most popular mutations. Moreover, the genotypes and phenotypes were well correlated in this cohort of salt wasting 21-OHD recruited from neonatal intensive care unit.

KEYWORDS

2 l-hydroxylase deficiency, congenital adrenal hyperplasia, *CYP21A2* gene, genotype, phenotype

1 | INTRODUCTION

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders, caused by the disorder of adrenal steroid synthesis due to the enzyme defects in the steroidogenic pathway (Kirac et al., 2014). It mainly includes 21-hydroxylase deficiency (21-OHD, OMIM#201910), 11β-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase deficiency, and 17α-hydroxylase deficiency (Al, Nordenstrom, & Falhammar, 2019; Bulsari & Falhammar, 2017; Miller, 2018). Among them, the most common is 21-OHD caused by the *CYP21A2* gene mutations, accounting for 95%–99% of all cases (Arlt et al., 2010; Gidlof et al., 2013). The incidence of classic 21-OHD is approximately 1 per 15,000 live births worldwide as an average, and the incidence of nonclassical 21-OHD is 1 per 200 persons in the U.S. Caucasians population (Kirac et al., 2014; Nordenstrom & Falhammar, 2018). In 21-OHD, it results in the defective synthesis of cortisol and aldosterone, whereas the excessive synthesis of androgen. Depending on the various extent of 21-hydroxylase impairment, the disease can be divided into three clinical forms: classic salt wasting (SW) form, classic simple virilizing (SV) form, and nonclassical (NC) form. The most severe type of this disease is SW form. In such patients, complete deficiency of 21-hydroxylase leads to serious deficiency of aldosterone, which results in SW crisis as hyponatremia and hyperkalemia (Speiser et al., 2018).

The *CYP21A2* gene, along with the pseudogene *CYP21A1P* which is apart from *CYP21A2* 30 kb, are located on chromosome 6p21.3. Both of the *CYP21A2* gene and *CYP21A1P* gene contain 10 exons, 98% of which is homologous in exon region and 96% in intron (White & Speiser, 2000). *CYP21A2* is located in the coding region of human leukocyte antigen with frequent genome recombination effect, which may lead to partial or complete deletions and conversions of *CYP21A2* owing to meiotic unequal crossover and conversion. It is reported that about 70%–80% of 21-OHD cases are caused by micro-conversion or intergenic recombination, and 20% of which are owing to unequal crossover during meiosis (Balsamo, Baldazzi, Menabo, & Cicognani, 2010; Krone & Arlt, 2009). The interference of pseudogene must be eliminated in clinical gene diagnosis for the high homology of *CYP21A2* and *CYP21A1P*. Genetic diagnosis is important for families with clinical symptoms and abnormal hormone levels. Polymerase chain reaction (PCR) and direct sequencing are necessary for point mutations detection. Southern blotting was used to identify the gene deletions/ conversions, but it is limited in detecting *CYP21A1P*/ *CYP21A2* chimeras and it is time-consuming and laborious. Multiplex ligation-dependent probe amplification (MLPA) can avoid the shortcomings and can be an alternative to Southern blotting (Concolino, Mello, Minucci, Zuppi, & Capoluongo, 2011; Concolino et al., 2009). The spectrum of mutations of 21-OHD has been established in many areas of world (Barbat et al., 1995; Friaes et al., 2006; Khajuria, Walia, Bhansali, & Prasad, 2017; Krone, Braun, Roscher, Knorr, & Schwarz, 2000; Loidi et al., 2006; Stikkelbroeck et al., 2003), but the frequency of the mutations varies in different regions and races. In addition, it is not clear whether there are differences in the mutation spectrums among different age groups.

Hence, the purpose of the present study was to identify the spectrum of *CYP21A2* gene mutation of infants admitted to neonatal intensive care unit (NICU) and study the correlation between genotypes and phenotypes, which could provide scientific basis for pediatric clinical diagnosis, prenatal diagnosis, and genetic counseling for 21-OHD children. Locus-specific PCR and Sanger sequencing were applied to identify point mutations, and MLPA as an alternative to Southern blotting was used to detect large fragment deletions/conversions.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

The study and procedures were in accordance with the Ethics Committee of Tianjin Children's Hospital. Informed consent was obtained from all patients (or their parents).

2.2 | Patients

A total of 22 unrelated Chinese CAH patients in our NICU were recruited in this study from 2011 to 2019. This cohort had no neonatal screening for CAH. The diagnoses of 22 patients were confirmed by molecular genetic testing. The classification of patients was based on a retrospective review of

patients' clinical manifestations together with electrolyte and hormonal levels. All patients (including 18 males and 4 females) came from 22 unrelated families, whose parents were unconsanguineous. The age of onset ranges from 4 hours to 53 days. Total 22 patients presented with classical forms and were all classified as SW form.

2.3 | Locus-specific PCR and direct sequencing

Genomic DNA of patients and their parents were extracted from peripheral blood using Blood Genomic DNA Mini Kit (Cowin Bio, Beijing, China) according to the instruction. The volume of DNA was 100 μl, concentration up to 10 ng/μl above, stored at −20°C. Four primers were synthesized to amplify *CYP21* genes listed in Table 1. The *CYP21A2* gene forward and reverse primers were Pl and P2, respectively, and the pseudogene *CYP21A1P*-specific forward and reverse primers were P3and P4. Amplicon of primers P3 and P2 was *CYP21A1P*/ *CYP21A2* chimeric gene, and the amplicon of P1 and P4 was *CYP21A2*/ *CYP21A1P* rearrangement product. The procedure setting of PCR was described in (Keen-Kim et al., 2005). The amplicons were detected by 1.5% agarose gel electrophoresis to confirm the PCR quality and distinguish the gene deletion/conversion mutations. The DNA product was purified from agarose gel using Gel Extraction Kit (Cowin, Beijing, China) and sent to GENEWIZ company (Beijing, China) for Sanger sequencing.

2.4 | Restriction endonuclease analysis

To ensure the locus-specificity of each reaction, the four amplification products above were performed by *Eco*RI enzyme based on the different cleavage sites of *CYP21A2* and *CYP21A1P* genes (Keen-Kim et al., 2005). Restriction was carried out in the final volume of 10 μl, containing 10×Buffer 1 μl, PCR amplicon 6 μl, and *Eco*RI enzyme 0.5 μl. Each product was digested for 2 hours at 37°C. Digestion products were separated by 1% agarose gel electrophoresis.

2.5 | MLPA analysis

MLPA was performed using the SALSA MLPA probemix P050-C1 CAH kit (MRC-Holland, Amsterdam, The Netherlands) to detect large gene deletions and conversions. This P050 kit contains 37 probes, including eight probes for *CYP21A2* gene (exons 1, 3, 4, 6, and 7), four probes for *CYP21A1P* gene (exons 1, 3, 4, and 7), six probes for *TNXB* gene, one probe for *ATF6B* gene, and eight reference probes. The procedure was carried out according to the manufacturer's instructions. Original volume of DNA was 5 μl (100 ng). The PCR products were detected using an ABI 3130 Genetic Analyzer (Applied Biosystems, USA) for capillary electrophoresis detection after multiplex PCR amplification reaction. The raw data were analyzed using Coffalyser software (MRC Holland).

2.6 | Classification of patients based on genotypes

The phenotypic categorization of 21-OHD was mainly based on the degree of decrease of 21-hydroxylase caused by different gene mutations. The enzyme of SW form was completely inactive and that of SV form and NC form was partially and only slightly inactive, respectively. Genotypes were grouped according to the strategy described by Wang et al. and Speiser et al. to predict phenotypes (Speiser et al., 1992; Wang et al., 2016). The patients were divided into five groups, including group Null, group A, group B, group C, and group D. The group Null included patients carrying homozygous deletion, homozygous mutation, or compound heterozygous mutation that could lead to completely inactive 21-hydroxylase. The group A was composed of the patients with homozygous IVS2-13A/C>G (I2G) mutation or a compound heterozygous mutation consisting of I2G and a Null group mutation. Patients with homozygous p.I173N mutation or heterozygous p.I173N combined with a mutation from group Null or group A composed the group B. Group C was consisted of patients harboring homozygous p.P31L and p.V282L mutations (resulting in remaining 20%–60% enzymatic activity) or heterozygous state combined with a mutation from group Null, A, or B. Lastly, patients carrying the uncertain significance mutations were categorized into group D. Genotypes of group Null and A were predicted to be associated with SW form. Genotypes in group B were correlated with SV form, and that of group C may be related with NC form. However, phenotypes in group D could not be predicted.

TABLE 1 Primers for amplification of PCR.

No.	Primer sequence	Nucleotide positions	Gene
P1	5′-GCTTCTTGATGGGTGATCAAT-3′	−216 to −196	*CYP21A2*
P2	5′-CCTCAATCCTCTGCAGCG-3′	3152 to 3169	*CYP21A2*
P3	5′-TCCCCAATCCTTACTTTTTGTC-3′	−840 to −819	*CYP21A1P*
P4	5′-CCTCAATCCTCTGCGGCA-3′	3151 to 3168	*CYP21A1P*

TABLE 2 Genetic and phenotypic features of patients with 21-hydroxylase deficiency.

No.	Sex	AOD	Na^+ (mmol/L)	K^+ (mmol/L)	Cort (nmol/L)	17-OHP (ng/ml)	Test (nmol/L)	ACTH (pg/ml)	Clinical symptoms	Clinical phenotype
1	M	42 h	120.3	7.23	511.34	31	-	41.7	Dropsy, oliguria	SW
2	M	16 d	108.3	6.19	121.62	220.6	8.40	25.3	Diarrhea	SW
3	F	42 d	113.8	7.44	527.92	7.4	-	863	Vomiting, poor weight gain	SW
4	F	52 d	125	6.82	1382	6.7	-	56.5	Vomiting, metabolic acidosis	SW
5	M	53 d	130	6.58	157.55	185.2	3.02	143	Vomiting, weight loss	SW
6	M	40 d	115.5	7.77	251.52	102.1	9.20	128	Severe malnutrition	SW
7	M	31 d	103.7	7.15	464.35	175.9	58.33	128	Vomiting, poor weight gain	SW
8	M	15 d	118.4	13.54	273.64	253.2	-	67.3	Lassitude, metabolic acidosis	SW
9	M	21 d	97.5	8.13	279.16	529	46.20	209	Vomiting, diarrhea, metabolic acidosis	SW
10	M	33 d	106	8.5	326.4	95.7	-	387.7	Poor weight gain, diarrhea	SW
11	F	30 d	112.6	7.82	198.93	11.8	33.59	633.5	Feeding difficulty, ambiguous external genitalia	SW
12	M	45 d	129.6	4.98	254.28	48.6	3.54	125.4	Diarrhea, poor weight gain	SW
13	M	15 d	104.6	7.33	1693.93	26.4	16.28	19.82	Vomiting, metabolic acidosis	SW
14	M	23 d	118.3	10.56	942.03	271.3	29.36	294.7	Feeding difficulty, metabolic acidosis	SW
15	M	4 h	120.8	8.96	256.8	5.5	-	50.57	Vomiting, diarrhea, metabolic acidosis	SW
16	M	21 d	114.6	8.46	299.45	95.8	18.46	280.9	Feeding difficulty, metabolic acidosis	SW
17	M	20 d	131.6	3.89	369.3	124	53.22	1864	Vomiting, metabolic acidosis	SW
18	M	17 d	126	7.93	194.4	21.2	22.13	230.3	Metabolic acidosis	SW
19	M	33 d	111.2	7.64	786.41	142.6	1.96	122.61	Diarrhea, poor weight gain, metabolic acidosis	SW
20	F	5 d	100.9	7.99	207.88	145.4	24.02	230.44	Metabolic acidosis, ambiguous external genitalia	SW
21	M	30 d	111	3.98	1012.01	817.9	37.71	1151.54	Abdominal distension	SW
22	M	27 d	97.3	7.35	750.09	480.1	32.61	776.81	Vomiting	SW

Abbreviations: 17-OHP, 17-hydroxyprogesterone; ACTH, adrenocorticotropic hormone; AOD, age of diagnosis; Cort, cortisol; d, days; F, female; h, hours; K^+, serum potassium; M, male; Na^+, serum sodium; SW, salt wasting form.; Test, testosterone.

TABLE 3 Detecting result of *CYP21A2* gene mutations.

Structure group	*CYP21A2* mutation	Protein effect	Alleles (n = 44)	Relative frequency (n = 44) (%)
Large gene deletion/ conversion	conversion		3	6.8
	chimera		6	13.6
	deletion		1	2.3
Micro-conversions	c.293-13C>G	I2G	31	70.5
	c.1069C>T	p. R357 W	2	4.5
	c.710 T > A; c.713 T > A; c.719 T > A	E6 Cluster	1	2.3

TABLE 4 Genotype–phenotype in 21-hydroxylase deficiency patients.

Group	Allele 1	Allele 2	Number of patients	Real phenotype			Predicted phenotype
				SW	SV	NC	
Null	conversion	conversion	1	1	—	—	SW
	chimera	conversion	1	1	—	—	SW
A	I2G	I2G	12	12	—	—	SW
	I2G	deletion	1	1	—	—	SW
	I2G	chimera	5	5	—	—	SW
	I2G	p.R357 W	1	1	—	—	SW
	E6 Cluster	p.R357 W	1	1	—	—	SW

3 | RESULTS

3.1 | Clinical evaluation

Based on clinical manifestations and endocrinological evaluation, all of the 22 patients were classified into SW form (n = 22, 100%) containing 18 males and 4 females. Age of onset of patients ranged from 4 hours to 53 days, with 12 patients in neonatal period. Eight of the 22 cases were from rural areas, of which three (37.5%) received treatment within 28 days of birth. However, of the 14 cases from urban areas, nine (64.3%) received treatment within 28 days of birth. The clinical manifestations of 22 patients varied. The most common were vomiting, feeding intolerance, diarrhea, and growth retardation. Some children presented with severe SW crisis. The laboratory examines of SW showed hyponatremia, hyperkalemia, metabolic acidosis and abnormalities of adrenocorticotropic hormone (ACTH), cortisol, and testosterone. The elevated 17-OHP levels were detected in all patients (Table 2). It was suggested that six patients showed elevated cortisol levels.

3.2 | Mutations analysis of the gene

Applying locus-specific PCR, direct sequencing of PCR products combined with MLPA, 44 mutant alleles (100%) were identified in 22 patients. Large gene deletions/conversions were detected on 10 alleles (22.7%). Micro-conversions were detected in 34 alleles (77.3%), including one splice mutation (I2G) and two missense mutations (p.R357W and E6 Cluster). The most common mutation was I2G, which was detected in 31 out of 44 alleles (70.5%), followed by chimeras (six alleles) and conversions (three alleles). The detected mutations and the frequencies were listed in Table 3. Additionally, among the 22 patients, 13 patients (59.1%) were homozygotes, including 12 cases of homozygous I2G and one of homozygous gene conversion. Of the remaining 9 (40.9%) cases carrying compound heterozygous mutations, two cases were point mutations, six cases harbored point mutations and gene deletion or chimera, and one case carried gene conversion and chimera. All of the mutations were inherited from the father or mother, which were consistent with the autosomal recessive inheritance pattern.

Besides, we further divided 22 patients into neonatal period group and over 28-day group. In neonatal period group, the male to female ratio of cases was 11:1. The most common mutation was I2G (18/24, 75%), followed by large gene deletions/conversions (5/24, 20.8%) and p.R357W (1/24, 4.2%). The ratio of male to female in the over 28-day group was 7:3. The variant I2G was detected on 13 alleles (13/20, 65%), followed by large gene deletions/conversions (5/20, 25%), E6 Cluster (1/20, 5%), and p.R357W (1/20, 5%). These results indicated that far more males than females were diagnosed

early and the micro-conversion I2G was the most popular mutation.

3.3 | Correlation between genotypes and phenotypes

The genotypes and phenotypes of 22 patients above were illustrated in Table 4. The genotypes were categorized into Null, A, B, and C groups in present study. The results showed that there were two patients (100%) in the Null group whose clinical phenotypes were consistent with the predicted phenotypes, all of whom were SW form. Mutations in the Null group included gene conversions and chimera that resulted in completely inactive enzyme. A total of 20 patients were assigned to group A, and their phenotypes were predicted to be SW form.

4 | DISCUSSION

The clinical data of 22 recruited children were analyzed first. It found that although most of the children were detected in the neonatal period, the initial diagnosis time of some patients was still delayed. A total of 12 neonatal patients (4 hours to 28 days) were diagnosed and treated, the remaining 10 cases confirmed age are reaching or more than 1 month, the latest is 53 days. Additionally, we analyzed the age of diagnosis in urban and rural families. The results demonstrated that 8 of 22 cases came from rural areas, three (37.5%) of which were treated within 28 days after birth. However, of the 14 cases from the urban areas, nine (64.3%) were treated within 28 days after birth. It suggested that rural and urban areas differ in the early identification of neonatal 21-OHD. This may be due to various family condition, the parents did not pay enough attention, or unbalanced distribution of medical resources.

It is reported that the screening for newborn made for early diagnosis of 70% of 21-OHD children before clinical symptoms appeared (Champion, 2010). The neonatal period screening is considered important because it can decrease neonatal mortality, reduce gender misjudgments, and improve growth and development. As most of the patients in this study were diagnosed in the neonatal period and had no neonatal screening for CAH, we intend to popularize neonatal disease screening to avoid death or growth and intellectual development delays caused by irreversible damage to critical organs. In addition, it has been reported that the frequency of SW is equal to males and females for the existence of sever SW crisis. Many males with SW often died undiagnosed before the introduction of neonatal screening (Gidlof et al., 2013). Females are easily diagnosed in SV due to virilization of external genitalia caused by hyperandrogenemia (New et al., 2013). In our study, the amount of SW type male patients far more than females, which was inconsistent with previous reports. Reviewing the clinical data, we found that nearly a third of the patients were from rural areas. In primary care settings female patients are often more easily identified generally. This inconsistence may be a result of that the females were not diagnosed in the NICU but earlier due to virilization. Additionally, all patients were recruited from the NICU merely, resulting in a small number of enrolled patients, may lead to the gender bias. However, the aim of our study was to focus on the 21-OHD patients from NICU, aiming to study the clinical characteristics and mutant spectrum of such population, in order to provide guidance for clinical work.

It was found that high normal cortisol level was detected in six patients, which was expected to be low. It has been reported that the existence of cross reaction between different steroid precursors give rise to interference in detection (Tuhan et al., 2015). What's more, elevated 17-OHD leads to a high level of 21-deoxycortisol, which can produce 45.4% cross-reactivity level with cortisol in patients with 21-OHD (Boddu & Madhavan, 2017). Although dried blood spot examining of 17-OHP has been used in newborn screening, various neonatal and maternal factors and differences between various measurement techniques may result in false positive values (Anandi & Shaila, 2017; Hayashi et al., 2017). Hence, we should draw attention to genetic diagnosis and constantly improve the mutation spectrum.

The genetic diagnosis of 21-OHD is more complicated than that of monogenic diseases due to diversity of mutated sites. More than 200 variants have been reported so far, including some common ones such as p.P31L, I2G, p.I173N, p.R357W, p.Q319X, E6 cluster, 8-bp deletion in exon 3, and large gene deletion. About 70% of *CYP21A2* mutations are due to micro-conversions of pseudogene, and 25%–30% due to large gene deletions and gene chimerism. Only 1%–2% of mutations are novel mutations in the *CYP21A2* gene (Hannah-Shmouni, Chen, & Merke, 2017). In present study, large gene deletions and conversions accounted for 10 alleles (22.7%), while the most common was I2G mutation (70.5%), followed by p.R357W (4.5%) and E6 Cluster (2.3%). Micro-conversions comprised a major portion in current study and I2G is the most popular point mutation, which is the same as other regions in China and other Asian populations (Asanuma et al., 1999; Chan et al., 2011; Hou et al., 2019; Lee et al., 2008; Loke, Lee, Lee, & Poh, 2001; Su et al., 2018; Wang et al., 2016). Two common mutations (I2G and p.R357W) in our research were also predominant in other populations. In addition, the p.I173N mutation was not identified in this study but was frequently detected in other cohorts. The small number of patients recruited, the composition of age of the patients, and/ or the selection of patients (since all were recruited from NICU) may explain the differences. We will further expand the research subjects to obtain a more accurate gene mutation spectrum and provide a basis for rapid screening of *CYP21A2* gene.

In this study, the phenotypes of patients were discussed through biochemical detection, and then, genotype correlation analysis was conducted in combination with phenotype. Among the 22 children in this study, all of the 22 children were SW type but no NC form was found. It has been reported that SW and SV forms accounted for the major part of patients, and the results of this study were similar to other domestic studies (Su et al., 2018; Wang et al., 2016), but different from the results in France, Spain, and Indian (Barbat et al., 1995; Khajuria et al., 2017; Loidi et al., 2006). It may suggest the existence of racial differences. It has been reported that the clinical symptoms of SV and NC patients appear late, symptoms of NC patients may first appear after the age of 60 months (Nordenstrom & Falhammar, 2018). It is also possible that the NC patients are late-onset and have mild symptoms and go to an adult hospital for treatment instead of children's hospital. In our study, all patients were recruited from the NICU merely, the clinical symptoms of SV patients and NC patients may not appear during this period or are overlooked because of gender misjudgment. Therefore, there are no SV and NC patients in our cohort. Based on previous research, there was a correlation between genotypes and phenotypes. In general, the mutations p.R357W, E6 cluster, 8-bp deletion in exon3, and gene deletions are associated with SW form. In this study, it was showed that two patients (100%) in Null group were predicted to be SW type according to genotypes, which was consistent with their true clinical phenotypes. Total 20 children in group A were predicted to be SW type, the concordance in group A was 100% (20/20). The total positive prediction value of 22 cases was 100% (22/22), which was slightly higher than values of southern China (Hou et al., 2019; Su et al., 2018).

In summary, we analyzed the spectrum of *CYP21A2* gene mutations of 22 unrelated Chinese children with classical CAH cased by 21-OHD admitted to NICU. Various micro-conversions, large gene deletions, and large gene conversions were responsible for the disease. The frequencies of the most common mutations were consistent with other cohorts. It is found that genotypes and phenotypes are well correlated. The study of the mutation spectrum is conducive to faster and more accurate diagnosis and the provision of accurate treatment for the local population. In addition, we will further promote the newborn screening and provide assistance to relevant experimenters and clinicians.

5 | ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Ethics Committee of Tianjin Children's Hospital and informed consent was obtained from all patients.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y L, JB S, and CQ C participated in the conception of the study and acquisition of data. Y L, J Z, and N L drafted of the manuscript. J Z, N L, XW X, XJ Z, Y Z, and GX L collected and cleaned the data. JB S and GL L analyzed and interpreted the results. JB S revised and submit the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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