The spectrum of CYP21A2 gene mutations in patients with 2l-hydroxylase deficiency -induced congenital adrenal hyperplasia in a Chinese cohort

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Research article

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Abstract

Background 21-hydroxylase deficiency (21-OHD) caused by the CYP21A2 gene mutations is the most popular form of congenital adrenal hyperplasia. It is an autosomal recessive disorder results in the defective synthesis of cortisol and aldosterone. The incidences of various CYP21A2 gene mutations and the genotype-phenotype correlations vary among different populations. Therefore, the aim of current study was to identify the spectrum of CYP21A2 gene mutations of patients from northern China and analyze the genotype-phenotype correlation.

Methods The clinical and molecular data of 22 patients were analyzed in this study. Locus-specific polymerasechain reaction and Sanger sequencing were applied to identify gene micro-conversions, and multiplex ligation-dependent probe amplification as an alternative to Southern blotting 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 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.R357W (4.5%) and E6 Cluster (2.3%). Genotypes of 22 patients (100%) were consistent with the predictive phenotypes.

Conclusions In this study, we collected 22 21-OHD patients' clinical and mutations data in Chinese population, especially in northern China. Micro-conversions were the most popular mutations and the frequencies were consistent with other cohorts. Moreover, thegenotypes and phenotypes in 21-OHDwere well correlated. This studyidentifiedthe mutation spectrum of CYP21A2gene and conduced to genetic counseling and prenatal diagnosis.

Background

Congenital adrenal hyperplasia (CAH, OMIM# 201910) is a group of autosomal recessive disorders, caused by the disorder of adrenal steroid synthesis due to the enzyme defects in the steroidogenic pathway. It mainly includes 21-hydroxylase deficiency (21-OHD), 11β-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase deficiency and 17α- hydroxylase deficiency. Among them, the most popular is 21-OHD caused by the CYP21A2 gene mutations, accounting for 90%-95% of all cases. The incidence of classic 21-OHD is approximately 1 per 15,000–20,000 live births worldwide, and the incidence of non-classical 21-OHD is 1 per 100 persons in the general Caucasian population [1–2]. 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 non-classical (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 salt losing crisis as hyponatremia and hyperkalemia.

The CYP21A2 gene, along with the pseudogene CYP21A1Pwhich 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 [3].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 [4–5]. 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. Polymerasechain reaction (PCR) and direct sequencing are necessary for point mutations detection. Southern blotting was used to identify 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 [6–7]. The spectrum of mutations of 21-OHD has been established in many areas of world [8–13], but the frequency of the mutations varies in different regions and races.

Hence, the purpose of the present study was to identify the spectrum of CYP21A2gene mutation of patients from northern China and study the correlation between genotypes and phenotypes, which could provide scientific basis for clinical diagnosis, prenatal diagnosis and genetic counseling for 21-OHD patients. 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.

Methods

Patients

A total of 22 unrelated Chinese CAH patients in Tianjin Children's Hospital were recruited in this study from 2011 to 2019. 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 withelectrolyte 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.Informed consent was obtained from all patients(or their parents) and in accordance with the Ethics Committee of Tianjin Children's Hospital.

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 is 100ul, concentration up to 10ng/uL 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 reverseprimers were P3andP4. 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 [14]. 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.

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[14]. Restriction was carried out in the final volume of 10uL, containing 10×Buffer 1uL, PCR amplicon 6uL and *Eco*RI enzyme 0.5uL. Each product was digested for 2 hours at 37℃. Digestion products were separated by 1% agarose gel electrophoresis.

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 8 probes for *CYP21A2* gene (exons 1, 3, 4, 6 and 7), 4 probes for *CYP21A1P* gene (exons 1, 3, 4 and 7), 6 probes for TNXB gene, 1 probe for ATF6B gene and 8 reference probes. The procedure was carried out according to the manufacturer's instructions. Original volume of DNA was 5ul (100ng). 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 was analyzed by using Coffalyser software (MRC Holland).

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 formwas 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 [15-16]. 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 may

be correlated with SV form, and that of group C may be related with NC form. However, phenotypes in group D could not be predicted.

Results

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 ofpatients ranged from 4 hours to 53 days. The most common clinical manifestations of SW were vomiting, feeding intolerance, diarrhea and growth retardation.Thelaboratory 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).

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%). In addition, 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 (6 alleles) and conversions (3 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, 2 cases were point mutations, 6 cases harbored point mutations and gene deletion or chimera, and 1case 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.

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 2 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.

Discussion

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 the22 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[15, 17], but different from the results in France, Spain and Indian [8-10].It may suggest the existence of racial differences. 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.

The clinical data of 22recruited children were analyzed firstly.It found that although most of the children were detected in the neonatal period, the initial diagnosis time of somepatients was still delayed. A total of14neonatal patients (4 hours~ 30 days) were diagnosed and treated, the remaining 8 cases confirmed age are reaching or more than 1 month,the latest is 53 days.This may be due to various family condition, the parents did not pay enough attention, or not immediately go to the hospital when symptoms appeared. It is reported that the screening for newborn made for early diagnosis of 70% of 21-OHD children before clinical symptoms appeared [18]. 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, we intend to popularize neonatal disease screening to avoid death or growth and intellectual development delays caused by irreversible damage to critical organs.

The geneticdiagnosis 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 [19]. 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 [15, 17, 20-24]. 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 (Table 5).The small number of patients recruited or the composition of the patients 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.

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. Thecorrelation between genotypes and phenotypes of 22 patients were analyzed in this study. It was showed that 2 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 A group 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 [17, 20].

Conclusions

In summary, we analyzed the spectrum of CYP21A2 gene mutations of 22 unrelated Chinese children with classical CAH cased by 21-OHD. 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 thatgenotypes 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 newborn screening and provide assistance to relevant experimenters and clinicians.

Abbreviations

CAH:Congenital adrenal hyperplasia; 21-OHD:21-hydroxylase deficiency; SW:salt wasting; SV:simple virilizing; NC:non-classical; PCR:Polymerasechain reaction; MLPA:Multiplex ligation-dependent probe amplification; I2G:IVS2-13A/C > G mutation; ACTH:Adrenocorticotropic hormone.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Tianjin Children's Hospital and informed consent was obtained from all patients(or their parents).

Consent for publication

Notapplicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from thecorresponding author on reasonable request.

Competing interests

The authors declare that theyhave no competing interests.

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Authors' contributions

Y L, JB S andCQ C participated in the conception of the study and acquisition of data. Y L and J Z drafted of the manuscript. J Z, XW X, XJ Z, Y Z andGX Lcollected 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 finalmanuscript.

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Tables

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