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Original Article

Analysis of clinical manifestations and gene mutations in infants with 21-hydroxylase deficiencies

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Abstract: Objective: The aim of this study was to analyze and summarize the clinical symptoms and genetic characteristics of infants with 21-hydroxylase deficiencies (21-OHD). Methods: A total of 50 infants, diagnosed with 21-OHD deficiencies, were included in the study. Clinical data, including age, sex, chief complaint, history of present illness, family history, past medical history, clinical symptoms, and comprehensive physical examination reports, were collected for analyses. Blood biochemistry parameters, including adrenocorticotropic hormone (ACTH), 17-hydroxyprogesterone (17-OHP), testosterone, progesterone, cortisol, electrolytes, and genetic testing results, were also gathered and analyzed. Results: Of the 50 infants, the number of each of the three types of 21-OHD, including simple virilizing type, salt-wasting type, and non-classic type, were 15, 26, and 9, respectively. Symptoms of the 15 simple virilizing type infants were not apparent. However, their clinical signs were prominent, manifesting as abnormalities of the external genitalia. In contrast, the 26 salt-wasting type infants experienced apparent clinical symptoms, such as anorexia and diarrhea. Some patients also showed signs of external genital abnormalities. Those non-classic type infants had no overt clinical symptoms or signs. The current study found that levels of hormones in infants with 21-OHD were different than normal, with varying degrees. ACTH, 17-OHP, testosterone, and progesterone increased, while serum cortisol decreased, in varying degrees, compared to the normal range. The increase of ACTH, 17-OHP, and progesterone in simple virilizing type or non-classic types was lower than that of the salt-wasting type (both $P<0.05$). However, there were no differences in serum testosterone levels among the three types (all $P>0.05$). Moreover, serum cortisol levels of simple virilizing and non-classic types were lower than those of the salt-wasting type (both $P<0.05$). Predictably, the serum electrolyte panel showed significant differences in hyponatremia and hyperkalemia levels between the salt-wasting type and the other two types (both $P<0.05$). Parents of the 38 infants provided written consent to undergo CYP21A2 gene testing. Sanger sequencing revealed a total of 13 types of point mutations in 76 alleles, of which the intron 2 splice mutation (I2G) was the most common, presenting in 23 alleles and accounting for 30.3%. This was followed by c.518T>A (p. I173N) mutation, which was detected in 9 alleles (11.8%). In 5 patients, only one-point mutation was found in one allele. In the other 4 patients, no gene mutations were detected. Regarding the 5 cases with only one-point mutation and the 4 cases with no-point mutations, multiplex ligation-dependent probe amplification (MLPA) was used for further detection. Results showed that 6 of them had large fragment deletion. Combining those two genetic testing methods could yield a positive rate as high as 92.1%. Conclusion: Early diagnosis of 21-OHD should be based on clinical symptoms, signs, laboratory findings, and genetic tests.

Keywords: 21-hydroxylase deficiency, congenital adrenal hyperplasia, clinical manifestations, gene mutation

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by a lack of related enzymes required for synthesis of adrenal corticosteroids in the human body [1-3]. In 90%-95% of patients, the disease is related to 21-hydroaylase deficiencies (21-OHD) [4, 5]. Abnormal gene coding of 21-hydroxylase is often a result of CYP21A2 gene mutation. Due to the relatively high mutation rate in this gene locus, over 200 types of point mutations have already been identified to be involved in the pathogenesis of 21-OHD [6]. In clinical practice, 21-OHD can be categorized into 3 types: simple virilizing type, salt-wasting type, and non-classic type. Morbidity of 21-OHD, worldwide, is about 1/10,000, with ethnic and regional dif-

ferences. There are no sex differences, however [7]. Previous studies have found that incidence of classic types vary from country to country, with 1/23,000 in Europe and America, 1/27,000 in New Zealand, and 1/6,000 in China and India. However, the non-classical type is relatively more common in clinical practice, with an incidence of 1/1,000 [8-12]. Previous research has demonstrated that the genotype and phenotype are consistent in 80% of 21-OHD patients. However, other reports have shown a remarkable difference [13, 14]. Because of the diverse clinical manifestations, early diagnosis and treatment are particularly important. The present study focused on clinical analysis of 21-OHD infants, aiming to improve the understanding of this disease and improve the accuracy of diagnosis and treatment.

Materials and methods

Patients

This study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University. A total of 50 infants, diagnosed with 21-OHD, admitted from October 2012 to October 2017, were included in the study. All patients were under 6 months of age, including 26 males and 24 females, with an average age of 50.90±16.18 days.

Inclusion criteria: Patients that met the diagnostic criteria for 21-OHD [15]. Simple virilizing type: Female infants can manifest as ambiguous genitalia with female gonads. Male infants can show pseudo-precocious puberty and an enlarged penis with normal-sized testicles. There are also signs of pigmentation, hirsutism, and early growth spurts in both sexes. Apart from the above symptoms, the salt-wasting type usually presents with vomiting, diarrhea, and dehydration. Blood electrolyte panels show hyperkalemia, hyponatremia, and hypochloremia. The non-classical type usually does not come with apparent clinical manifestations. However, patients may be accompanied by pigmentation, rapid growth, and abnormalities detected by related laboratory indicators.

Exclusion criteria: Patients with incomplete clinical data.

Methods

This retrospective case-control study was conducted to analyze patient clinical data, including age, sex, chief complaint, history of present illness, past medical history, family history, clinical symptoms, and comprehensive physical examination reports. Venous blood samples were collected at 8 a.m. in fasting and awake conditions. Auxiliary laboratory tests were performed to determine serum levels of serum cortisol, adrenocorticotropic hormone (ACTH), 17-hydroxyprogesterone (17-OHP), testosterone, progesterone, and blood electrolytes. Karyotype analysis was performed on patients with abnormalities of external genitalia.

Genetic testing

Genetic testing was conducted after obtaining written consent from the parents of the infants. Sanger sequencing was used for genetic testing in this study. Briefly, 5 mL of venous blood was collected from the patients at 8 a.m., from which DNA was extracted and subject to PCR to amplify CYP21A2 gene exons and related regions. Gene sequencing results were compared with normal controls to show if any mutations existed in the CYP21A2 genes. If a gene mutation was detected, then more targeted gene sequencing was performed to the patient's family members to determine the origin of the mutation. If a large fragment deletion or duplication was suspected, further detection using multiplex ligation-dependent probe amplification (MLPA) was conducted. This test was to depict the details of gene deletion or duplication, analyzing peak patterns, peak height, peak area, and fragment lengths, using kit software.

Treatment

Hormone replacement therapy was administered in a timely manner to patients with confirmed diagnosis. Patients of the severe salt-wasting type were rehydrated with intravenous fluids. Hydrocortisone (Tianjin Pharmaceutical Group Co., Ltd.) 50-100 mg/m^2/d was given 2-3 times per day via IV infusion, with appropriate supplementation of sodium chloride and reduction of intake of potassium. Once patient conditions were controlled, hydrocortisone was reduced to a maintenance dose of 10-15 mg/m^2/d, administered orally 2-3 times a day. If hyponatremia or hyperkalemia was refractory, oral fludrocortisone (Haupt Pharma Amareg Gmhh Co., Ltd.) 0.05-0.1 mg/d was given twice a day. Patients diagnosed with the simple virilizing type and non-classic type were treated with oral hydrocortisone 15 mg/m^2/d only,

Table 1. General information (n, %)

Groups	Simple virilizing type 15 (30.0)	Salt-wasting type 26 (52.0)	Non-classic type 9 (18.0)	χ^2	P
Age of onset					
≤1 month	4 (26.7)	5 (19.2)	1 (11.1)	3.537	0.308
1-2 months	9 (60.0)	11 (42.3)	7 (77.8)		
>2 months	2 (13.3)	10 (38.5)	1 (11.1)		
Sex					
Male	2 (13.3)	19 (73.1)	5 (55.6)	13.385	0.001
Female	13 (86.7)	7 (26.9)	4 (44.4)		
Adrenal crisis	0 (0.0)	22 (84.6)	0 (0.0)	35.538	<0.001
Family history	2 (13.3)	3 (11.5)	1 (11.1)	0.036	0.982

Table 2. Clinical manifestations (n, %)

Groups	Simple virilizing type (n=15)	Salt-wasting type (n=26)	Non-classic type (n=9)
Clinical Symptoms			
Fever	0 (0.0)	4 (15.4)	1 (11.1)
Anorexia	0 (0.0)	17 (65.4)	1 (11.1)
Diarrhea	1 (6.7)	12 (46.2)	1 (11.1)
Vomiting	1 (6.7)	5 (19.2)	0 (0.0)
Abdominal distention	1 (6.7)	5 (19.2)	1 (11.1)
Coma	0 (0.0)	2 (7.7)	0 (0.0)
Convulsions	1 (6.7)	2 (7.7)	0 (6.7)
Clinical signs			
Pigmentation	3 (20.0)	23 (88.5)	7 (77.8)
Penile enlargement	2 (13.3)	1 (3.8)	0 (0.0)
Hypospadias	0 (0.0)	1 (3.8)	0 (0.0)
Clitoromegaly	13 (86.7)	4 (15.4)	0 (0.0)

given 2-3 times a day. Dosages were subject to adjustment depending on serum levels of corticosteroids and electrolytes.

Statistical analysis

All data were analyzed with SPSS 17.0 statistical software. Quantitative data are expressed as mean ± standard deviation ($\bar{x}$ ± sd). Enumeration data are expressed as number/percentage (n/%). Differences between groups were compared using χ^2 test. *P* values less than 0.05 indicate statistical significance.

Results

General information

There were no significant differences in age of onset and number of cases with family history among the three groups (P>0.05). However, significant sex differences among the three groups were detected (P<0.05). In addition, cases of adrenal crisis showed significant differences (P<0.05). See **Table 1**.

Comparison of clinical symptoms and signs

In the three groups of infants, the predominant symptoms were fever, anorexia, diarrhea, vomiting, abdominal distension, comas, and convulsions. Major clinical signs were pigmentation, penile enlargement, hypospadias, and clitoromegaly. See **Table 2** and **Figures 1**, **2**.

Comparison of laboratory indicators

Levels of hormones in infants with 21-OHD were different than normal, with varying degrees. Serum ACTH, 17-OHP, testosterone, progesterone, and aldosterone increased, while serum cortisol decreased, compared with the normal range. The increase of ACTH, 17-OHP and progesterone in the simple virilizing type or non-classic type was lower than that of the salt-wasting type (P<0.05). However, there were no differences in serum testosterone levels among the three types (P>0.05). Moreover, serum cortisol levels of the simple virilizing type and non-classic type were lower than those of the salt-wasting type (P<0.05). In addition, the serum electrolyte panel showed significant differences in hyponatremia and hyperkalemia levels between the salt-wasting type and the other two types (P<0.05). See **Table 3**.

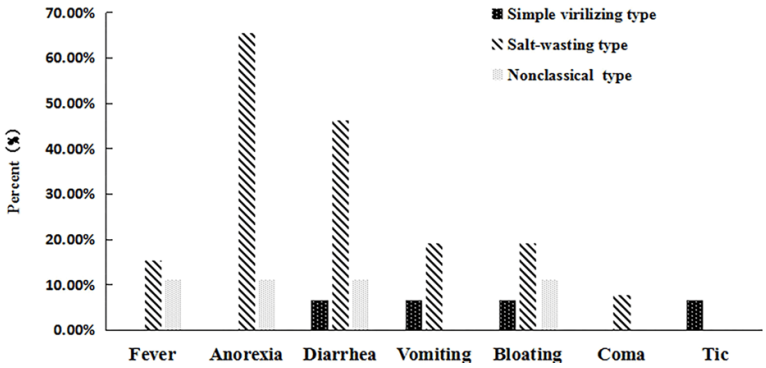

Figure 1. Comparison of clinical symptoms.

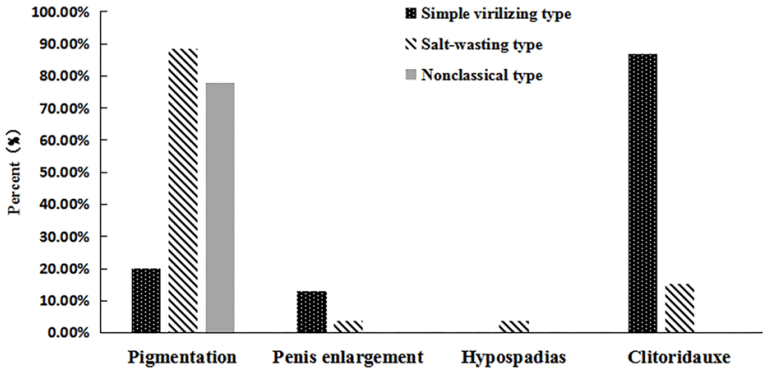

Figure 2. Comparison of clinical signs.

Table 3. Biochemistry indicators

Items	Simple virilizing type	Salt-wasting type	Non-classic type
ACTH (pg/mL)	11.96±8.43*	110.43±88.35	12.06±8.21#
17-OHP (ng/L)	13.95±50.45*	159.15±292.79	13.85±45.23#
Cortisol (μg/dL)	3.55±2.49*	6.62±6.21	3.62±2.13#
Testosterone (ng/dL)	207.41±92.14	156.91±109.39	157.36±91.23
Progesterone (ng/mL)	0.79±1.04*	2.64±4.23	0.76±1.06#
Sodium (ng/dL)	138.93±3.89*	125.03±5.51	137.26±3.65#
Potassium (ng/mL)	4.40±0.42*	6.64±0.43	4.42±0.45#

Note: ACTH: Adrenocorticotropic hormone; 17-OHP, 17-hydroxyprogesterone. Compared with salt-wasting type, *P<0.05; compared with salt-wasting type, #P<0.05.

Genetic testing results

Analysis of CYP21A2 gene using Sanger sequencing: Parents of the 38 patients provided written consent to undergo CYP21A2 gene testing. Sanger sequencing revealed a total of 13 types of point mutations in 76 alleles, of which the intron 2 splice mutation (I2G) was the most common, presenting in 23 alleles and accounting for 30.3%. This was followed by c.518T>A (p. I173N) mutation, which was detected in 9 alleles (11.8%). In 5 patients, only one-point mutation was found in one allele. In the other 4 patients, no gene mutations were detected. Sequencing chromatograms of two common point mutations in patients and their family members are shown in **Figures 3**, **4**.

Analysis of CYP21A2 gene using MLPA: In this study, 5 patients with only one-point mutation and 4 patients with no-point mutation were further tested using MLPA. For the 5 patients with only one-point mutation, 3 cases were detected with large fragment deletion. Similarly, one of the 4 patients with no-point mutation was found to have large fragment deletion. The other 3 patients showed no abnormalities. Sanger sequencing, combined with MLPA, was used to analyze the CYP21A2 gene in 38 patients, with 35 confirmed with CYP21A2 gene mutations. The combination of those two gene testing methods may yield a positive rate as high as 92.1%.

Treatment and prognosis

All 50 patients were treated with hormone replacement therapies. For the 26 patients with the salt-wasting type, intravenous hydrocortisone was administered to correct fluid and electrolyte imbalances. Four of them were given additional fludrocortisone, orally, for refractory hyponatremia and hyperkalemia. Two patients developed heart failure and died after ineffective resuscitation. Fifteen cases of the simple virilizing type and 9 cases of the non-classic type were treated with oral hydrocortisone to alleviate symptoms.

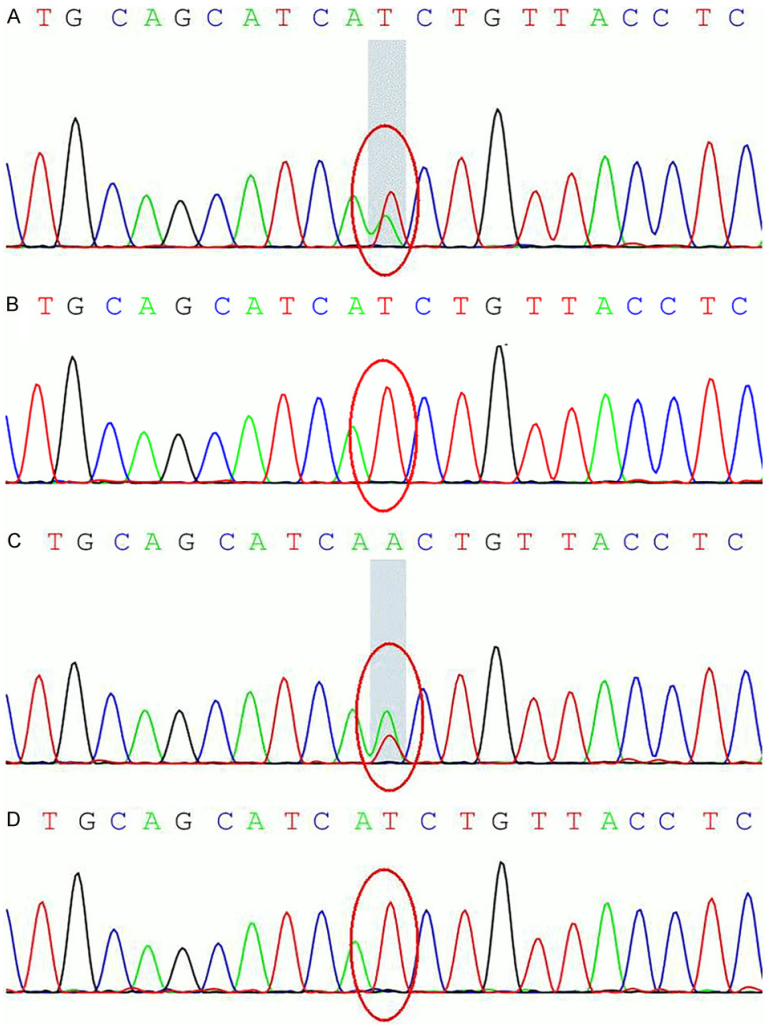

Figure 3. Sequencing chromatogram of a patient with heterozygous mutation c.293-13C>G and the sequencing chromatograms of his family members. A: The patient has heterozygous mutation c.293-13C>G. B: The patient's father has heterozygous mutation c.293-13C>G. C: The patient's mother does not have this mutation. D: The patient's sister does not have this mutation.

Discussion

Based on clinical manifestations, 21-OHD is categorized into 3 types, the simple virilizing type, salt-wasting type, and non-classic type. Previous studies have shown that the most common type is the salt-wasting type, followed by the simple virilizing type. The non-classic type is relatively uncommon. This disease has no apparent sex differences, with incidence rates for males and females very similar [15]. Of the 50 infants included in the study, 26 cases of the salt-wasting type accounted for 52.0%, 15 cases of the simple virilizing type accounted for 30.0%, and 9 cases of the non-classic type accounted for 18.0%. Moreover, there were 26 males and 24 females in the study, with a male to female ratio of 1.08:1, consistent with previous studies. However, the current study showed significant sex differences between the three groups. This may be explained by the fact that female infants of the simple virilizing type were more likely to get an early diagnosis than males, due to the distinct abnormalities of external genitalia.

The current study found that infants of the salt-wasting type had insufficient serum aldosterone and cortisol levels. This was due to their extremely low 21-hydroxylase activity, resulting in symptoms of hyperkalemia, hyponatremia, and dehydration [16]. There were 26 cases of the salt-wasting type in this study, characterized by early onset and notable severity. The most common clinical symptoms of the salt-wasting type were diarrhea, vomiting, anorexia, and adrenal crisis, occurring in 22 patients. Studies have found that adrenal crisis is the leading cause of death in 21-OHD children [17]. However, unfortunately, the symptoms lack specificity in the early stage. Diagnosis of adrenal crisis is considered if various clinical manifestations are accompanied by genital abnormalities. Delayed treatment could lead to serious conditions, such as shock, comas, and even death. Early diagnosis and intervention could make a positive impact on prognosis. Previous studies have shown that a precipitating factor can be identified in 90% of children with adrenal crisis [18]. Gastrointestinal infections are the most common factors among all precipitating factors [19]. In this study, 17 cases of the salt-wasting type had a chief complaint of anorexia, along with 12 cases of diarrhea at the time of admission. Gastrointestinal symptoms were the major cause of misdiagnosis, consistent with the above studies. Re-

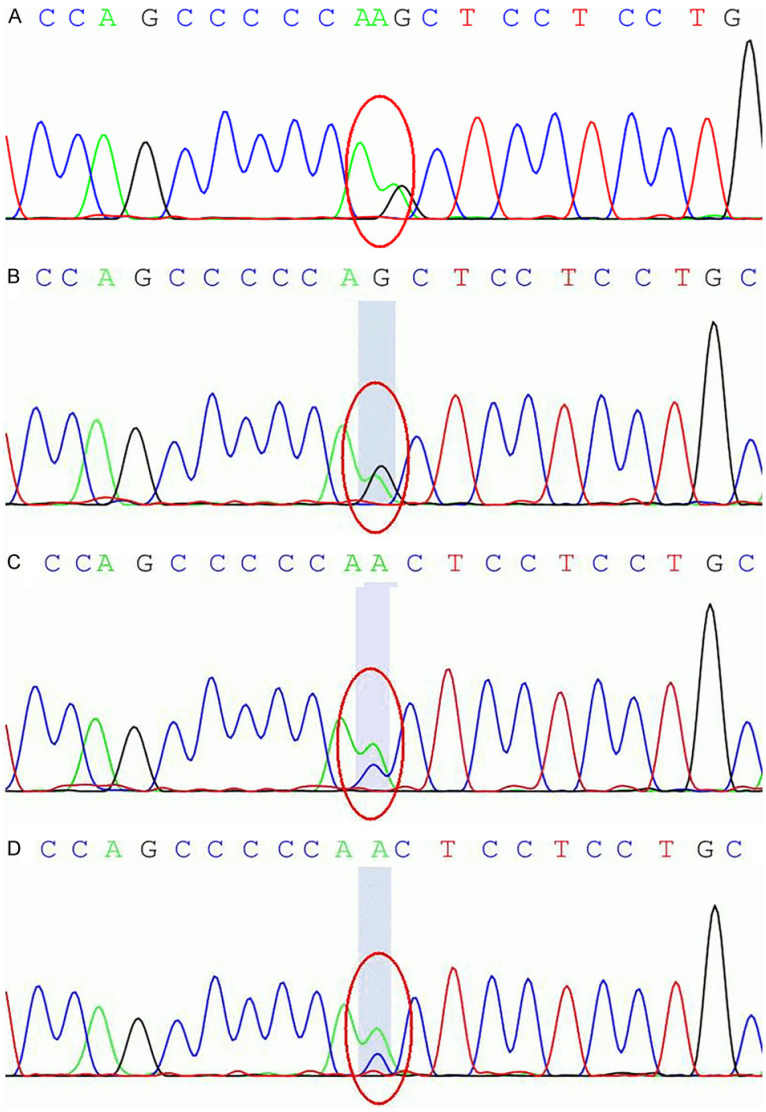

Figure 4. Sequencing chromatogram of a patient with heterozygous mutation c.518T>A and the sequencing chromatograms of his family members A: The patient has heterozygous mutation c.518T>A. B: The patient's father does not have this mutation. C: The patient's mother has heterozygous mutation c.518T>A. D: The patient's sister does not have this mutation.

garding clinical signs, infants of the salt-wasting type were typically characterized by skin pigmentation, likely related to abnormal hormone levels. However, patients could also present with external genital abnormalities. For infants of the simple virilizing type, the symptoms were not obvious. Clinical signs were more prominent with abnormalities of the external genitalia. Because of excessive secretion of androgens, females showed external genitalia malformations, while males exhibited pseudoprecocious puberty. It is often misdiagnosed because of its atypical clinical symptoms [20, 21]. In this study, all 15 cases of the simple virilizing type infants presented with abnormalities of the external genitalia. In female patients, 21-OHD is also the most common cause of ambiguous genitalia, accounting for nearly 60% [22]. The nonclassic type is more likely to be misdiagnosed due to nonobvious symptoms and clinical signs, consistent with previous studies [15].

It has been shown that serum 17-OHP is an important indicator for diagnosis of 21-OHD [22]. In this study, analysis of serum hormones indicated that all 21-OHD infants had elevated levels of serum 17-OHP, with varying degrees. Despite its high sensitivity, 17-OHP is not considered a specific indicator for diagnosis of 21-OHD [22]. Testosterone is another serum hormone elevated in patients with 21-OHD. The elevation is more pronounced in the simple virilizing type, which could explain the abnormalities of external genitalia caused by excessive androgen secretion. Related studies have also found that 17-OHP and testosterone play an important role in diagnosis of 21-OHD [23]. Due to the impediment of cortisol synthesis caused by the insufficiency of 21-hydroxylase activity, serum ACTH levels increase as compensation for decreased cortisol secretion. This change is more significant in patients of the salt-wasting type [24]. However, in this study, serum cortisol and ACTH levels in infants of the salt-wasting type were higher than those of the simple virilizing type, likely because the circadian rhythms of cortisol secretion are usually established at 6 months after birth. Patients included in this study were below 6 months of age. In addition, due to the lack of 21-hydroxylase in the body, serum progesterone increases lead to hyperprogesteronemia [16]. The current study found that progesterone levels in children with 21-OHD increased to

varying degrees, which is consistent with previous studies. With respect to the serum electrolyte panel, the 22 infants of the salt-wasting type developed dehydration, hyponatremia, and hyperkalemia, as a result of adrenal crisis. Predictably, the salt-wasting type had lower sodium levels and higher potassium levels than the simple virilizing type. Present results are consistent with previous reports [17]. The non-classic type is prone to be misdiagnosed due to its non-prominent clinical symptoms and signs [15].

Regarding genetic testing, this study used Sanger sequencing combined with MLPA. Sanger sequencing can detect gene insertion, deletion, and point mutation, but it lacks sensitivity in the diagnosis of large fragment deletion. The MLPA method can be used as a supplement to Sanger sequencing to detect duplications or deletions of large gene fragments [25]. In this study, 13 types of point mutations were detected by Sanger sequencing, of which the intron 2 splice mutation (I2G) was the most common, presenting in 23 alleles and accounting for 30.3%. This was followed by c.518T>A (p.I173N) mutation, which was detected in 9 alleles (11.8%). For the 5 cases with only one-point mutation and 4 cases with no-point mutation, MLPA was used for further detection. Results showed that 6 of them had large fragment deletion. The combination of two genetic testing methods can yield a positive rate as high as 92.1%.

The main strategy for treatment of 21-OHD is hormone replacement therapy. Salt-wasting type patients can experience severe symptoms like adrenal crisis. In this study, 2 salt-wasting type patients died after developing heart failure caused by adrenal crisis. Simple virilizing type and non-classic type patients responded well to hormone replacement therapy, in agreement with previous reports [17].

The present study analyzed clinical symptoms and genetic characteristics of infants with 21-OHD. However, a limitation of this study was the relatively small number of patients. This may have impeded in-depth statistical analyses of outcomes. A future prospective and multicenter-based study, with a larger sample size, is necessary to confirm present results.

In conclusion, early diagnosis of 21-OHD should be based on clinical symptoms, signs, laboratory findings, and genetic tests.

Disclosure of conflict of interest

None.

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