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Recent advances in biochemical and molecular analysis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency

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The term congenital adrenal hyperplasia (CAH) covers a group of autosomal recessive disorders caused by defects in one of the steroidogenic enzymes involved in the synthesis of cortisol or aldosterone from cholesterol in the adrenal glands. Approximately 95% of all CAH cases are caused by 21-hydroxylase deficiency encoded by the *CYP21A2* gene. The disorder is categorized into classical forms, including the salt-wasting and the simple virilizing types, and nonclassical forms based on the severity of the disease. The severity of the clinical features varies according to the level of residual 21-hydroxylase activity. Newborn screening for CAH is performed in many countries to prevent salt-wasting crises in the neonatal period, to prevent male sex assignment in affected females, and to reduce long-term morbidities, such as short stature, gender confusion, and psychosexual disturbances. 17α-hydroxyprogesterone is a marker for 21-hydroxylase deficiency and is measured using a radioimmunoassay, an enzyme-linked immunosorbent assay, or a fluoroimmunoassay. Recently, liquid chromatography linked with tandem mass spectrometry was developed for rapid, highly specific, and sensitive analysis of multiple analytes. Urinary steroid analysis by gas chromatography mass spectrometry also provides qualitative and quantitative data on the excretion of steroid hormone metabolites. Molecular analysis of *CYP21A2* is useful for genetic counseling, confirming diagnosis, and predicting prognoses. In conclusion, early detection using neonatal screening tests and treatment can prevent the worst outcomes of 21-hydroxylase deficiency.

Keywords: Congenital adrenal hyperplasia, CYP21A2, 21-Hydroxylase deficiency

Introduction

The term congenital adrenal hyperplasia (CAH) covers a group of autosomal recessive disorders caused by a deficiency of one of the enzymes required for steroid biosynthesis in the adrenal gland[1]. 21-Hydroxylase deficiency (21OHD) is the most common type of CAH, accounting for approximately 95% of cases, and is caused by mutations in the *CYP21A2* gene[2]. 21OHD is clinically divided into classic forms, including salt-wasting (SW) and simple virilizing (SV) forms, and nonclassic forms of disease according to disease severity[3]. Most patients with classic CAH present with SW crisis or ambiguous genitalia in the neonatal period. In contrast, boys do not show ambiguous genitalia and therefore cannot be diagnosed without neonatal screening[4]. Therefore, males with CAH have a higher risk of adrenal crisis in the neonatal period if they were the SW form of 21OHD[5].

The level of 17α-hydroxyprogesterone (17OHP) is used as a marker for 21OHD, as CAH due to 21OHD can be detected in newborn screening programs by measuring the amount of 17OHP in dried blood spots[6]. Newborn screening for CAH is now performed in many

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countries to prevent SW crises in the neonatal period, to prevent male sex assignment in affected females, and to reduce long-term morbidities, such as short stature, gender confusion, and psychosexual disturbances[6].

It is critical to diagnose 21OHD in a timely manner and treat classic and nonclassic CAH to prevent adrenal crises and hyperandrogenism. This review will describe the current knowledge regarding newborn screening and biochemical and molecular genetic diagnoses of CAH due to 21OHD.

Newborn screening for CAH due to 21OHD

The goal of newborn screening for CAH is to detect the SW adrenal crisis; to prevent shock, brain damage, or death and begin presymptomatic treatment; and to prevent or shorten the period of incorrect gender assignment in affected females[7,8]. The benefit of newborn screening is to identify boys with the SW form of CAH, as they do not have ambiguous genitalia at birth[9]. For effective newborn screening, a careful clinical follow-up is critical to confirm diagnosis and early treatment[4]. Neonatal screening tests were first performed for congenital hypothyroidism and phenylketonuria in Korea in 1997. The measurement of 17OHP to screen for CAH has been included in the newborn screening program in Korea since 2006[10]. The incidence of CAH due to 21OHD in the Korean population detected by newborn screening is 1 in 22,700 (http://helpline.cdc.go.kr/).

The biochemical marker for the diagnosis of 21OHD is elevated 17OHP, the main substrate for the enzyme lying just upstream of the block[4,11]. Using filter paper cards, newborn screening programs measure 17OHP in dried blood spots obtained via a heel puncture performed between 2 and 4 days after birth[1,12]. Because the circadian rhythm is influenced by cortisol[13], an early morning 17OHP level is appropriate for screening[4]. The 17OHP level in normal newborns is <1 ng/mL (3 nmol/L)[1]. After the neonatal period, a cutoff value of an early morning 17OHP <0.8 ng/mL (2.5 nmol/L) in children and <2 ng/mL (6.0 nmol/L) in adults has been suggested to exclude CAH[14]. A random blood sample with 17-OHP >100 ng/mL (300 nmol/L) measured by radioimmunoassay (RIA) is diagnostic of classic 21OHD, while 1–100 ng/mL (30–300 nmol/L) in adults indicates nonclassic 21OHD[2]. In menstruating women, the samples for 17OHP should be measured in the follicular phase, as the 17OHP level usually increases in the luteal phase in about half of normal females with a level of >2 ng/mL (6.0 nmol/L)[15].

Various techniques for measuring 17OHP are available, including RIAs, enzyme-linked immunosorbent assays, and time-resolved fluoroimmunoassays. The RIA was the first method used, but the dissociation-enhanced, lanthanide fluorescence immunoassay is now more common[6]. Each method has advantages and disadvantages in terms of specificity and sensitivity.

One of the disadvantages of newborn screening for 21OHD is the considerably high false positive rate, leading to high rate of repeated tests that cause patient distress and result in significant costs for follow-up. The false positive results are attributed to several factors, such as prematurity, sickness, stress, and low specificity of antibodies for 17OHP due to the cross-reactivity with 17-hydroxypregnenolone and immature adrenal steroid production[4,16-19].

Recent advances in biochemical analysis of adrenal steroid profiles

Primary screening results for CAH using a 17OHP assay must be confirmed by second tier tests, including biochemical or molecular genetic analyses[6]. In addition, because of the relatively high false positive rate of the first tier screening using immunoassays, there is a need for highly specific 17OHP screening methods to select patients for further diagnostic approaches.

Tandem mass spectrometry (MS/MS) has been recently developed to improve the positive predictive value and is more specific than the immunoassays[20]. Second-tier strategies using MS/MS measure a ratio of 17OHP plus androstenedione to cortisol[20,21]. However, they are time-consuming to carry out and are not appropriate as first-tier screening methods[20].

Liquid chromatography linked with MS/MS (LC-MS/MS) is a revolutionary method to measure steroid hormones in various body fluids to improve sensitivity and specificity in highly automated systems[22]. LC-MS/MS allows for rapid, targeted steroid hormone analysis of multiple analytes[23]. It is currently the technique of choice for confirming CAH, as molecular genetic analysis is time-consuming and expensive[24]. The issues of cross-reactivity and specificity can be alleviated by the use of LC-MS/MS[22]. However, the problem of false-positive results in premature or stressed newborns cannot be completely overcome simply by determining 17OHP alone. LC-MS/MS can measure 17OHP levels as well as other compounds, such as androstenedione, 11-deoxycortisol, 21-deoxycortisol, and cortisol[25,26]. The use of analyte ratios (precursor/product), such as (17OHP+21-deoxycortisol)/cortisol, can effectively decrease the possibility of the false positive results in neonates with high 17OHP levels due to stress, sickness, or prematurity[6,27]. In nonclassic CAH, analyte levels are only slightly different compared to those in healthy newborns, but analyte ratios demonstrate more pronounced alterations than analytes themselves[6]. Therefore, the specificity and sensitivity of LC-MS/MS and the use of analyte ratios are expected to facilitate the diagnosis of mild, nonclassic CAH.

A urine steroid profile can be performed using a spot urine sample and is an additional CAH diagnostic tool that helps to differentiate between the different forms of CAH[28]. Gas chromatography-mass spectrometry (GC-MS) is used for urine steroid profile analysis, which is a helpful noninvasive diagnostic test used for confirmatory diagnosis[29]. As markers for 21OHD, urinary 17OHP metabolites, such as 17α-hydroxypregnanolone, pregnanetriol, and 15β,17α-dihydroxypregnanolone, and urinary 21-deoxycortisol metabolites, such as pregnanetriolone, have been studied using GC-MS[25,30]. However, both mass

spectrometric techniques are complementary tools for diagnosing CAH, and further study is still required to improve reproducibility between laboratories[23].

Indication of cosyntropin stimulation test

Marked elevation of 17OHP levels is characteristic of classic 21OHD. However, equivocal values of 17OHP cannot distinguish nonclassic CAH from heterozygous carriers[31] and require dynamic testing with corticotropin (cosyntropin) stimulation[2]. This test is the gold standard for diagnosing nonclassic CAH[4]. The cosyntropin stimulation test is performed by intravenously injecting cosyntropin at a dose of 0.25 mg and measuring baseline and stimulated levels of 17OHP[1]. Blood samples are obtained at baseline and 60 minutes after the administration of cosyntropin. Cortisol levels should also be measured at baseline and 60 minutes to measure the cortisol level. A basal 17OHP >5 ng/mL (15 nmol/L) and/or peak 17OHP >10 ng/mL (30 nmol/L) traditionally indicates nonclassic CAH[32].

Genetics of 21OHD

The *CYP21A2* gene encodes 21-hydroxylase and requires electrons transferred from NADPH via the electron donor enzyme P450 oxidoreductase. This gene is located in the HLA class III region between the HLA-B and HLA-DR chromosome 6p21.3[1]. This is a highly complicated region including a highly homologous pseudogene, *CYP21A1P*[1]. The functional gene (*CYP21A2*) and a nonfunctional pseudogene (*CYP21A1P*) are located closely adjacent to each other in tandem arrangement with the *C4A* and *C4B* genes encoding for the fourth component of the serum complement. Moreover, these units are located between a telomeric *RP* gene and a centromeric *TNX* gene, comprising the RCCX modules (RP-C4-CYP21-TNX) (Fig. 1)[33]. These genes are located in tandem and in an array (*C4A*, *CYP21A1P*, *TNXA*, *C4B*, *CYP21A2*, and *TNXB*). Genes *C4A*, *C4B*, *CYP21A2*, and *TNXB* all encode functional proteins, while *CYP21A1P*, *TNXA*, and *RP2* genes are pseudogenes that do not encode proteins[34].

The RCCX module shows high homology between the functional genes and the corresponding pseudogenes, leading to a wide variety of genetic rearrangement by unequal crossing over events, such as duplications, deletions, and fusions of the RCCX module[1,35].

To date, more than 200 *CYP21A2* mutations have been discovered (http://www.hgmd.cf.ac.uk), and about 10 common mutations account for approximately 90% of cases[4]. More than 90% of *CYP21A2* gene mutations are caused by gene conversion or unequal crossing over[36-38]. Approximately 70%–75% of 21OHD cases are the result of the microconversion of the mutations in *CYP21A1P* to *CYP21A2*[1,39]. About 20% are caused by unequal crossing over during meiosis, resulting in the deletion of a 30-kb gene segment[38], encompassing the 3' end of the *CYP21A1P*, all of the adjacent *C4B*, and the 5' end of *CYP21A2*, producing the nonfunctioning chimeric *CYP21A1P/CYP21A2* and chimeric *TNXA/TNXB* genes[33]. The remaining 1%–2% of affected alleles are spontaneous mutations not carried by either parent[40].

Molecular analysis of the *CYP21A2* gene

The genetic diagnosis of patients with 21OHD is not straightforward. However, it is a useful adjunct to hormonal measurements in the genetic counseling of parents upon the birth of a CAH child and of adolescents during the transition to adult care[2]. Therefore, molecular diagnosis of *CYP21A2* mutations is recommended for genetic counseling and confirming the diagnosis in patients with the nonclassic form of 21OHD because neonatal screening may miss most cases of nonclassic CAH and adrenocorticotropic hormone stimulated 17OHP levels can be equivocal[2,6,41].

It should be stressed that molecular genetic diagnosis is more complicated for 21OHD than for many other monogenic disorders due to the high variability of the genomic region. This includes the coexistence of two or more mutations on the same allele or the presence of more than one *CYP21/C4* repeat unit on the same chromosome. In addition, care should be taken to prevent genotyping the pseudogene because genetic results can be complicated due to the duplication, deletion, and recombination of *CYP21A2* in the chromosome 6q21.3 region. Therefore, mutant alleles must be segregated in the parents to investigate their presence in different alleles and to verify *de*

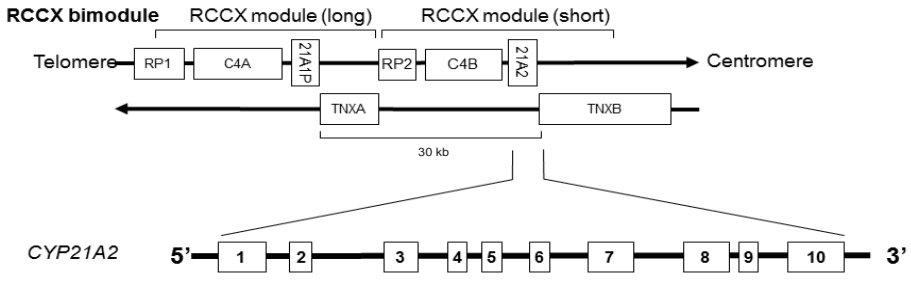

Fig. 1. Chromosomal region of 6p21.3 containing the 21-hydroxylase genes representing the structure of RCCX module.

Table 1. The mutations in *CYP21A2* and their phenotypes according to their residual activities based on *in vitro* transfection assay

	Group A	Group B	Group C
Phenotype	Salt-wasting	Simple virilizing	Nonclassic
In vitro activity of CYP21A2	0%–1%	1%–10%	20%–60%
Mutation	Gene deletion/conversion	p.I172N	p.P30L
	8 bp deletion E3		p.V281L
	E6 cluster		p.P453S
	F306+t		
	L307insT		
	p.Q318*		
	p.R356W		

novo mutations[4,42].

Several strategies have been developed for molecular analysis of *CYP21A2*, based on polymerase chain reaction (PCR)-driven amplification with allele-specific oligonucleotides to the *CYP21A2* gene, followed by direct sequencing with assessment of the *CYP21A2* gene copy number[43]. Sanger sequencing is the gold standard for detecting point mutations and small sequence variations (indels). However, large gene rearrangements cannot be detected by direct sequencing of PCR-amplified gene fragments[33]. The Southern blot method has traditionally been used to detect large gene deletions/conversions in the RCCX module. However, it is time-consuming, highly labor-intensive, and requires radioactive probes and a large amount of DNA. In addition, the Southern blot method has limitations in detecting chimeric RCCX modules, including *CYP21A1P/CYP21A2* chimeric genes and *TNXA/TNXB* chimeric genes[44].

Therefore, alternative methods have been developed. Recently, Multiplex ligation-dependent probe amplification (MLPA) analysis for the diagnosis of 21OHD has been increasingly used as an easy, simple, rapid, and sensitive tool to detect deletions or duplications of the *CYP21A2* gene[45]. MLPA allows easy and rapid detection of gene copy number variations and the identification of chimerical genes in patients with 21OHD without using radioactive probes[45,46] and is thought to be a valid alternative to Southern blotting[47]. However, false positive results could occur because the mutations or polymorphisms very close to the probe binding regions and the ligation site may prevent probe hybridization and ligation[47].

Prediction of clinical phenotype according to genotype

There is a wide spectrum of clinical features according to the type of mutations in *CYP21A2*. Depending on the residual activity of the mutation based on *in vitro* mutagenesis and expression studies, there is a good correlation between genotype and phenotype[40]. *CYP21A2* mutations can be classified into 3 categories (A, B, C) according to the level of enzymatic activity predicted by *in vitro* transfection studies (Table 1)[1,33,40]. Group A consists of mutations such as deletions or nonsense mutations that totally ablate enzyme activity; these are most often associated with the severely affected SW form. Group B consists of the missense mutations, such as p.I172N, with 1%–2% of normal enzyme activity. These mutations are characteristically found in patients with the SV form. Group C includes mutations such as p.V281L and p.P30L that produce enzymes retaining 20%–60% of normal activity; these mutations are associated with mild, nonclassic CAH[1,40].

Therefore, genotyping provides valuable diagnostic information by predicting of the clinical course of disease, and severe complications can be prevented, particularly in adults[1,4,40]. Approximately 65%–75% of CAH patients are compound heterozygotes with disease-causing mutations. Compound heterozygotes with two different *CYP21A2* mutations usually have a phenotype compatible with the presence of the greater residual activity[40].

Conclusions

Early diagnosis of classic CAH is critical to save lives, and diagnosing nonclassic CAH is important to prevent unnecessary suffering. A baseline measurement of 17OHP levels can be used for screening and diagnosis of 21OHD. LC-MS/MS and GC-MS have recently been developed as highly sensitive and specific methods for targeted steroid hormone analysis. The detection of *CYP21A2* mutations is important for clinical diagnosis because there is a high variability in 17OHP levels. Molecular genetic analysis of *CYP21A2* is useful in confirming the diagnosis, providing genetic counseling, and predicting prognoses. Genotype is well correlated with the clinical severity of 21OHD. However, further research is needed to identify modifier genes in 21OHD, which could explain the phenotypic variability of androgen effects.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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