REVIEW

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Clinical, Biochemical and Molecular Characteristics of Congenital Adrenal Hyperplasia Due to 21-hydroxylase Deficiency

Sevinç Odabaşı Güneş[1], Havva Nur Peltek Kendirci[2], Edip Ünal[3], Ayşe Derya Buluş[4], İsmail Dündar[5], Zeynep Şıklar[6]

[1]University of Health Sciences Turkey, Gülhane Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey
[2]Hitit University Faculty of Medicine, Department of Pediatric Endocrinology, Çorum, Turkey
[3]Dicle University Faculty of Medicine, Department of Pediatric Endocrinology, Diyarbakır, Turkey
[4]Keçiören Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey
[5]İnönü University Faculty of Medicine, Department of Pediatric Endocrinology, Malatya, Turkey
[6]Ankara University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey

Abstract

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disease caused by the deficiency of one of the enzymes involved in cortisol synthesis. Between 90% and 99% of cases of CAH are caused by 21-hydroxylase deficiency (21-OHD) caused by mutations in *CYP21A2*. Although 21-OHD has been historically divided into classical and non-classical forms, it is now thought to show a continuous phenotype. In the classical form, the external genitalia in females becomes virilized to varying degrees. If the disease is not recognized, salt wasting crises in the classical form may threaten life in neonates. Children experience accelerated somatic growth, increased bone age, and premature pubic hair in the simple virilizing form of classical 21-OHD. Female adolescents may present with severe acne, hirsutism, androgenic alopecia, menstrual irregularity or primary amenorrhea in the non-classical form. Diagnosis of CAH is made by clinical, biochemical and molecular genetic evaluation. In cases of 21-OHD, the diagnosis is based on the 17-hydroxyprogesterone (17-OHP) level being above 1000 ng/dL, measured early in the morning. In cases with borderline 17-OHP levels (200-1000 ng/dL), it is recommended to perform an adrenocorticotropic hormone (ACTH) stimulation test. Genotyping in cases with CAH should be performed if the adrenocortical profile is suspicious or if the ACTH stimulation test cannot be performed completely. After diagnosis, determining the carrier status of the parents and determining which parent the mutation was passed on from will help in interpreting the genetic results and determining the risk of recurrence in subsequent pregnancies.

Keywords: Congenital adrenal hyperplasia, 21-hydroxylase deficiency, children, adolescent, diagnosis

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disease caused by the deficiency of one of the enzymes involved in cortisol synthesis. Between 90% and 99% of cases are caused by 21-hydroxylase deficiency (21-OHD) due to mutations in the *CYP21A2* gene (1,2). Although 21-OHD has been historically divided into classical and non-classical forms, it is now thought to show a continuous phenotype. The classical form is accompanied by absence or severe deficiency in enzyme activity. The most serious form is the classical form of CAH with salt loss and is characterized by adrenal insufficiency with cortisol and aldosterone deficiency and excessive androgen production (2). If this form is not recognized, salt wasting crises (hyponatremia, hyperkalemia, acidosis, hypovolemia and shock) develop in 75% of cases in the first three weeks of life (3).

In the simple virilizing form, enzyme activity is at the level of 1-5%. Cortisol deficiency and androgen excess are prominent (2). Affected children are identified later in

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Address for Correspondence: Sevinc Odabaşı Güneş MD, University of Health Sciences Turkey, Gülhane Training and Research Hospital, Clinic of Pediatric Endocrinology, Ankara, Turkey
E-mail: sevincodabasi@yahoo.com **ORCID:** orcid.org/0000-0001-5979-9206

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childhood because of early pubic hair development and virilization (3).

Milder forms of the disease are defined as 'late-onset' or 'non-classical' CAH (NCCAH), and partial enzyme deficiencies are compensated by elevations in adrenocorticotropic hormone (ACTH) (1). In NCCAH, mild subclinical impairment in cortisol synthesis does not usually lead to adrenal crisis.

The purpose of this review is to provide practical clinical points for the diagnosis of 21-OHD in children and adolescents. This evidence-based review with good practice points was developed by the 'Adrenal Working Group' of the 'Turkish Society for Pediatric Endocrinology and Diabetes'. The Adrenal Working Group held meetings online between 2022-2024, at least four times annually. First of all, two subgroup topics (subgroup 1: CAH and other adrenal deficiencies in childhood and adolescence; subgroup 2: Adrenal tumors and Cushing syndrome) were created. A total of 41 researchers, 28 in the first subgroup, 13 in the second subgroup, also organized meetings among themselves. Evidence-based review with good practice points was guided by systematic reviews of evidence and discussion. During the meetings, all comments and suggestions were discussed and implemented as appropriate by the working group.

Good practice points are graded according to the Grading of Recommendations, Assessment, Development, and Evaluation system. When grading, first the strength of recommendation and then the quality of evidence are stated. The recommendations are categorized as 1 (strong recommendation) or 2 (weak recommendation). The quality of evidence behind the recommendations is classified as very low (⊕OOO), low (⊕⊕OO), moderate (⊕⊕⊕O) and strong (⊕⊕⊕⊕) (4).

Epidemiology

Based on newborn screening and national case registries, the frequency of classic CAH has been shown to be 1/14,000-18,000 worldwide (5). In the extended neonatal screening programme for CAH in Turkey, the incidence of classical 21-OHD was determined to be 1:15,067. Of these patients, 75% had salt wasting and 25% had simple virilizing 21-OHD CAH (6). Based on haplotype association studies, the prevalence of NCCAH forms in the white population is estimated to be between 1:500 and 1:1,000, but may be as high as 1:50 to 1:100 in populations with a high rate of consanguineous marriages (5). It has also been reported that the frequency is higher among Ashkenazi Jews, Hispanics, those of Mediterranean origin, those from the Middle East and Inuits (7). In a more recent study, it has been shown that the frequency of NCCAH in the United States population is 1:200 as a result of *CYP21A2* genotype analysis (8). In another study, the frequency of NCCAH was found to be approximately 4% among women presenting with androgen excess symptoms (9).

Clinical Findings

In the classical salt wasting form, affected infants usually present within the first three weeks after birth with poor weight gain, recurrent vomiting, dehydration, hypotension, acidosis, hyponatremia, hyperkalemia, hypoglycemia and shock. With the increase in prenatal androgen production, the external genitalia in females becomes virilized to varying degrees (cliteromegaly, labial fusion, hyperpigmentation, or various degrees of virilization similar to male external genitalia). In the presence of atypical genitalia or bilateral nonpalpable testicles, pelvic ultrasonography may be necessary to determine presence or absence of uterus in females (2,3). External genitalia of male infants with the classical form may present with hyperpigmentation and macrogenitalia (2).

In the mild form of classical CAH (simple virilizing CAH) affected children are identified later in childhood because of early pubic hair development, clitoromegaly, urogenital sinus that was not noticed earlier in life, or both, in females, and with early development of pubic hair, phallic enlargement, or both, in males (3). With androgen excess, these children experience accelerated somatic growth and increased bone age (2,3).

As a result of high androgens and poor hormonal control, central precocious puberty, menstrual irregularities, acne, hirsutism, male pattern hair loss, and masculine body structure in young girls, and decreased fertility are observed. In cases that do not receive appropriate treatment, adult height remains short due to the effect of high androgens (2). Another reason why adult height remains short is the suppression of growth by inappropriate use of high dose steroids for a long time (3). A summary of the clinical findings seen in patients with CAH is shown in Figure 1.

In the non-classical form, the spectrum of symptoms at diagnosis is mostly age-related. While premature adrenarche is the most common complaint (87%) in children under the age of 10 years, female adolescents have been reported to present with severe acne, hirsutism, androgenic alopecia, clitoromegaly (11%), menstrual irregularity (56%) and even with primary amenorrhea (9%) (9,10).

Girls with NCCAH often present during adolescence and young adulthood with acne, hirsutism, menstrual

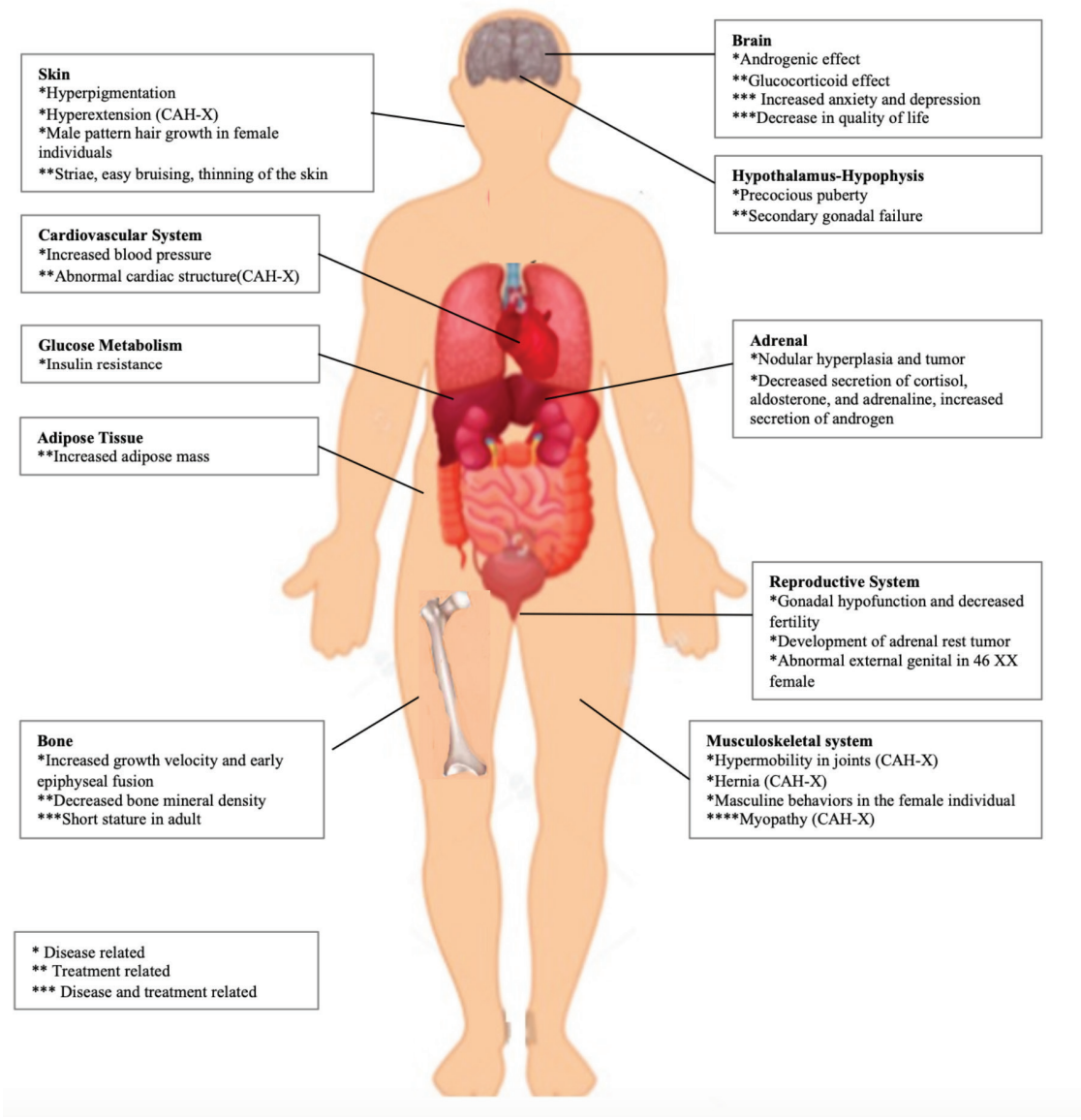

Figure 1. Clinical findings and complications in patients with CAH

CAH: congenital adrenal hyperplasia

abnormalities, or infertility; all of which largely overlaps with polycystic ovary syndrome (PCOS). Male patients with NCCAH are less likely to be admitted to hospital due to symptoms of androgen excess and because of this, they are diagnosed less frequently than girls. Males are mostly diagnosed during genetic screening for pre-pregnancy counseling (9,11). Studies on male patients with NCCAH are thus quite limited. In a study of 45 male cases, it was reported that 29% of the cases presented with premature pubarche (12).

During childhood androgen excess in NCCAH does not cause virilization of the external genitalia in 46, XX fetuses during the prenatal period. The most common findings in patients presenting during childhood may be listed as oily skin, acne or adult-type body odor, and premature pubarche. It has been shown that 20% of children under the age of 10 years have clitoromegaly and acne (10). Increased androgen levels can lead to accelerated growth. However, since androgen excess in the early period does not affect the growth rate, no acceleration in growth is observed before the age of 1-2 years (13,14). In patients with NCCAH, increased 17-OHP and adrenal androgens may convert to estrogens and cause advanced bone age. Although previous studies have reported that the final height of most children with NCCAH reaches the target height range, it is nevertheless suggested that accelerated bone age may negatively affect final height

over time (9,15). In a study conducted among females over the age of 10 years, the most common complaints were: hirsutism (59%); oligomenorrhea (54%); acne (33%); infertility (13%); clitoromegaly (10%); alopecia (8%); primary amenorrhea (4%); and premature pubarche (4%) (10).

Good practice points:

1. CAH is a problem that most commonly occurs as a result of 21-OHD, and in its classical form, cortisol and aldosterone deficiency and androgen excess are observed (1⊕⊕⊕O).

2. Diagnosis is made by clinical, biochemical and genetic evaluation (1⊕⊕⊕O).

Biochemical Diagnosis

To reiterate, 21-OHD should be kept in mind in an infant with symptoms of dehydration, acidosis, hyponatremia, hyperkalemia, hypoglycemia or shock. There is relative renal tubular resistance to aldosterone in the early period of the disease (2).

Analysis of steroid hormones is based either on immunoassay principles or chromatographic methods combined with mass spectrometry (MS). In the immunoassay method, the antibody used must be specific to prevent cross-reactivity with other metabolites. Moreover, organic extraction will ensure the removal of cross-reacting substances, such as steroid sulfates (2).

Today, MS is the method that provides the most accurate and versatile results in steroid measurement. Applying liquid chromatography (LC) or gas chromatography (GC) initially increases specificity. A significant portion of the steroids is excreted in the urine. Thus urinary steroid analysis is one of the methods with high diagnostic value. LC-MS is a newer technique than GC-MS, and extra filtering by tandem MS (MS/MS) further improves the separation ability of LC. Today, measurement of steroid hormones in plasma or serum by LC-MS/MS is the most appropriate method (2).

In cases of 21-OHD, the diagnosis is based on the 17-OHP level being above 1000 ng/dL (30 nmol/L). However, in most infants the level is above 5000 ng/dL (150 nmol/L). Although random measurements are informative for diagnosis, corticotropin stimulation testing is necessary to confirm the diagnosis and exclude other rare disorders of steroidogenesis. High 17-OHP levels can be seen in 11β-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase (3β-HSD) deficiency and P450 oxyreductase deficiencies (3). Since the 17-OHP level is closely related to the circadian rhythm of ACTH, incidentally measured 17-OHP levels must be checked early in the morning (before 08:00) (1). For menstruating women, steroid measurements should be made during the follicular phase, as fluctuations in 17-OHP levels occur during the luteal phase of the menstrual cycle. Sometimes it may be difficult to distinguish between classical and non-classical forms of CAH that do not cause salt loss (1,5).

The pharmacological dose currently used for testing is 0.25 mg cosytropin intravenously (iv), which maximally stimulates the adrenal cortex (in very low birth weight infants, the dose may be reduced to 0.125 mg). Blood samples should be taken at baseline and 60 minutes after iv cosyntropin administration (5). There are studies showing that the intramuscular cosyntropin test is safe and effective in the diagnosis of adrenal insufficiency and CAH if the iv form is not available (16,17,18).

For the test to be informative at least cortisol and 17-OHP should be measured. In order to evaluate all enzyme defects that may cause CAH, it is appropriate to measure 17-OHP, cortisol, 11-deoxycorticosterone, 11-deoxycortisol, 17-OH-pregnenolone, dehydroepiandrosterone and androstenedione in blood samples by LC-MS/MS after the stimulation test (5).

It is reported that in a case with suspected CAH, if the 17-OHP level is found to be lower than 80 ng/dL (2.5 nmol/L) in children and 200 ng/dL (6.0 nmol/L) in adults, the diagnosis of CAH should be excluded (19,20,21,22). It has been suggested that only 10% of patients with NCCAH due to 21-OHD had basal 17-OHP levels lower than 200 ng/dL (6 nmol/L) (23). A 17-OHP measurement for 21-OHD deficiency being greater than 200 ng/dL (6 nmol/L) suggests the diagnosis of NCCAH. Typically, this value is greater than 10,000 ng/dL (300 nmol/L) in patients with classical 21-OHD. Patients with moderately high 17-OHP levels (6-30 nmol/L or 200-1,000 ng/dL) should undergo an ACTH (cosyntropin) stimulation test. If the stimulated 17-OHP result is <1000 ng/mL (<30 nmol/L), 21-OHD will be excluded (Figure 2) (5).

The threshold value for the 17-OHP level needs to be evaluated according to the test methodology. When the threshold value of 17-OHP is accepted as >200 ng/dL (>6 nmol/L), the diagnosis may be missed in some patients with NCCAH (24). Therefore lower threshold values have been recommended, especially when using the MS method (2). False-positive 17-OHP screening results are even more common, especially when immunoassay methods are used and blood samples are taken in the luteal phase.

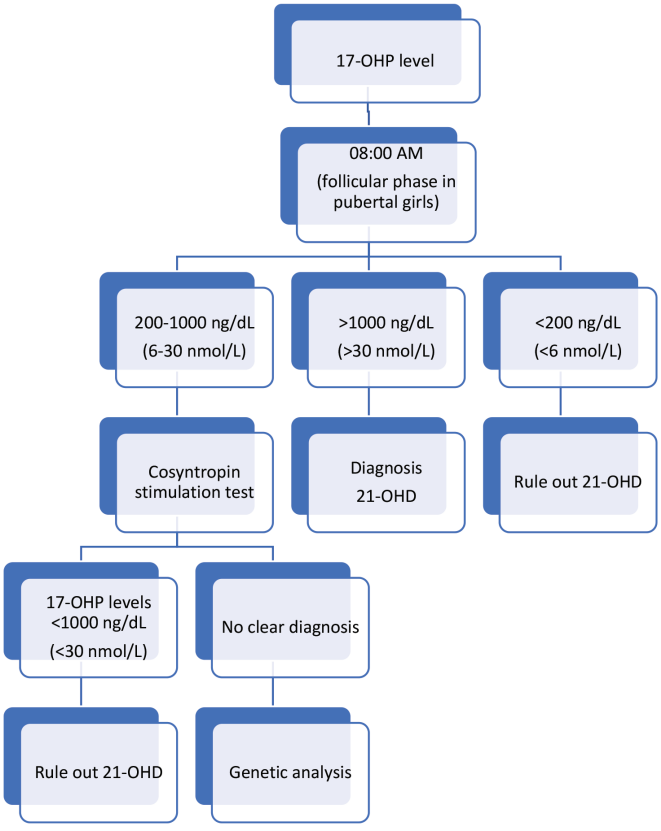

Figure 2. 17-OHP levels for 21-OHD diagnosis

17-OHP: 17-hydroxyprogesterone, 21-OHD: 21-hydroxylase deficiency

NCCAH and PCOS may have similar clinical appearance. Moderate elevation of 17-OHP has been reported in 25% of women with PCOS (25,26). Furthermore, some individuals with adrenal incidentalomas may have 17-OHP levels above >1000 ng/mL (30 nmol/L) without a genetically confirmed carrier state or NCCAH (27). Most patients with NCCAH have normal ACTH levels. In cases with 21-OHD, it results in an increase in 17-OHP levels, independent of the increase in ACTH, due to the kinetics of the enzymatic steps. Increased 17-OHP levels results in overproduction of adrenal androgens and their precursors. These androgens include testosterone (T) and androstenedione, as well as metabolites called 11-oxyandrogens (28). Usually, a high androstenedione/T ratio suggests adrenal androgen excess. However, while women with CAH often develop PCOS, the production of adrenal androgens is also increased in the majority of patients with PCOS. This makes the situation more complicated (1).

The most common symptom of NCCAH in childhood is premature adenarche. In a study including 59 cases diagnosed with premature adrenarche, basal and ACTH stimulated 17-OHP levels were examined. In addition, DNA sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis were performed to identify mutations in *CYP21A2*. In this study, twelve girls were diagnosed with NCCAH (29). In a study with 238 patients with premature pubarche, the frequency of NCCAH was 4% with ACTH stimulation test (20). In another study including 111 patients, six cases were diagnosed with NCCAH and the investigators also concluded that a basal 17-OHP level of >200 ng/dL (>6 nmol/L) was a useful screening test (30).

A total of 126 patients with premature pubarche, hirsutism, or PCOS were studied by Binay et al. (31). Among them, six patients (4.7%) were diagnosed with NCCAH based on mutational analysis. NCCAH was diagnosed in 4.2% of cases with premature pubarche and in 3.8% of cases with PCOS.

Good practice points:

1. LC-MS/MS method should preferably be used in adrenal steroid measurement. However, if LC-MS/MS is not available, immunoassay methods using specific antibodies are used (1⊕⊕⊕O).

2. In symptomatic cases after infancy, screening is performed in the early morning hours (before 08:00) with the basal 17-OHP level measured by the LC-MS/MS method (1⊕⊕OO).

3. In cases with borderline 17-OHP levels (6-30 nmol/L or 200-1000 ng/dL), it would be appropriate to perform an ACTH stimulation test and check a complete adrenocortical hormonal profile to distinguish 21-OHD from other enzyme defects (1⊕⊕OO).

4. NCCAH should be considered in the differential diagnosis in all adolescent girls with a PCOS-like phenotype. In addition, NCCAH should be excluded in all pediatric cases presenting with premature adrenarche (2⊕⊕OO).

Molecular Genetic Diagnosis

In suspected cases, the diagnosis should be confirmed with genetic tests and genetic counseling should be provided with a treatment decision accordingly (10). Interpretation of the steroid profile may be difficult in some cases; slight elevations of 17-OHP may be observed in some cases with heterozygous mutation. In some cases with homozygous mutations, the same mutation (such as c.293-13C>G) may result in simple virilization in some patients or salt wasting in others. Genetic analysis may be necessary to provide genetic counseling and make a definitive diagnosis (5). In addition, an accurate and reliable genotype-phenotype association

in 21-OHD will help in therapeutic management. In fact, genetic mutation analyzes of *CYP21A2* are recommended by some authors as one of the first options, as the use of genetic techniques is increasing, they are becoming cheaper, and the time to get results is shortening. It is considered a practical and economical diagnostic modality by some authors (32).

It is currently reported that in 21-OHD cases, *CYP21A2* genotyping with next-generation sequencing and MLPA as a genetic diagnosis method can accurately and reliably confirm the diagnosis (32). Today, the Southern blot analysis method is no longer considered the gold standard method because it requires a large amount of high-quality DNA, is time-consuming, has excessive workload, and is insufficient to detect deletions/duplications. The most commonly used method for determining gene dosage (deletion, duplication, rearrangement, fusion) is MLPA. It is appropriate to use *CYP21A1*-specific primers to prevent pseudogene amplification and allele loss of non-amplified PCR fragments (2). Within the scope of 21-OHD genetic testing, copy number variation (CNV) evaluation is required. Since individuals carrying the p.Gln319Ter variant usually have a duplication in *CYP21A2*, CNV evaluation is always recommended in cases where this mutation is detected (33).

Targeted molecular genetic strategies for frequent mutations are implemented in some laboratories. However, direct sequencing of amplified PCR products and their combination with methods that detect gene deletions/chimeric genes can detect almost all of the mutations (2). In a study using the next-generation sequencing method, 222 of 226 alleles were detected in cases with 21-OHD. Therefore, its diagnostic sensitivity was 98.2% (32).

The gene encoding 21-hydroxylase, *CYP21A2*, is located in the human leukocyte antigen (HLA) class 3 region on chromosome 6 at the 6p21.3 position, with 98% homology to the active gene, but together with the inactive *CYP21A1P* pseudogene. Four genes [serine/threonine kinase 19, complement C4, steroid 21-hydroxylase CYP21, and tenascin X (TNX)] are organized in this region, forming an RCCX module. Mutations that cause 21-OHD mostly occur as a result of intergenic recombinations, microconversion events, gene deletions, chimerism and gene duplications (11,34).

Intergenic recombinations are responsible for 70% of mutations related to 21-OHD. Among intergenic recombinations, approximately 75% occur when mutations in the *CYP21A1P* pseudogene are transferred to the functional *CYP21A2* as a result of microconversion (3,33).

To date, nine different chimeric *CYP21A1P/CYP21A2* genes have been identified. Seven chimeras carry the pseudogene specific mutation c.293-13C>G in intron 2 and this is associated with severe salt-wasting CAH; this chimerism is called classical or general chimerism. If the junction site occurs upstream of the c.293-13C>G variant, 21-hydroxylase activity is less affected and a milder clinical phenotype occurs, termed attenuated chimerism (33).

Clinical severity is determined by the extent of the residual enzyme activity in one or both variant alleles. Mutations that lead to the salt-wasting CAH phenotype that completely abolish 21-hydroxylase enzyme activity are associated with both lack of glucocorticoid and mineralocorticoid production that leads to salt loss. Milder phenotypes are caused by *CYP21A2* variants that result in lesser degrees of enzymic dysfunction/loss. The simple virilizing CAH-associated variants have 2-10% residual function while the enzymatic activity of the milder NCCAH phenotypes has a wide range, between 10% and 75% (11,34,35,36).

The correlation of clinical phenotype with genotype was strong, particularly in salt-wasting and NCCAH disease (37). Variants on *CYP21A2* are classified into four groups, Group 0, A, B and C, according to residual 21-hydroxylase activity. Group 0 and A are associated with the salt-wasting form and the Group 0 (null variants) have 0% enzyme activity. Group A variants carry a minimal (<1%) residual activity, Group B has almost 2% residual enzymatic activity, and Group C variants have 20-50% enzyme activity (32,38).

Group A and included patients who carry homozygous null mutations result in completely inactive enzymes, including gene deletions such as 8bp del, large gene conversion, E6 cluster, p.Arg357Trp, p.Gln319Ter, p.L308Ffs*6, p.Gly111Valfs*21, novel frameshift mutation, and multiple mutations alleles with any of these mutations. Group A variants include those homozygous for the IVS2-13A/C>G mutation or compound heterozygosity with the null variants. Group B include most frequently the mutation p.Ile173Asn and the promoter conversion + Pro31Leu, homozygous or compound heterozygous with a null or Group A mutation. Group C variants include homozygous p.Pro30Leu, p.Pro453Ser, p.Val281Leu mutations (20-50%) or in compound heterozygosis with the former (null, A, or B) mutations. Group B and C variants are related to the simple virilizing and NCCAH form of the 21-OHD, respectively (30,31,32,34,35,36,37,38,39,40). In addition, Group D and Group E genetic variants have been identified. Group D consist of patients with novel mutations or variants whose enzymatic activity impairment had not been assessed, and Group E consist of patients with at least one allele without mutations (38).

In a study of 113 cases, genotype-phenotype correlation was analyzed to determine predictive properties of variant groups of *CYP21A2*. Genotype-phenotype correlation was reported to be 91.5%. A complete genotype-phenotype match was observed in Group 0. It was also proposed that Group A be divided into two subgroups, A1 and A2, and the positive prediction of subgroup A1 was higher than Group A and subgroup A2. Genotypes correctly predicted phenotypes in 79.5% of patients in Group A; 100% in subgroup A1 and 75% in subgroup A2 (32).

In the case of compound heterozygosity, the mutation with the mildest effect on enzymatic activity determines the predicted phenotype. However, in alleles containing multiple mutations, the most deleterious mutation determines the genotype groups (41).

The c.293-13C>G mutation is more deleterious than homozygous genotype when trans with null mutations. While the c.293-13C>G mutation mostly causes salt-wasting CAH, it is also seen in approximately 20% of simple virilizing cases. NCCAH is observed in the majority of cases carrying the p.Pro30Leu mutation, while the classical CAH phenotype can be seen in >30%. While the p.Ile173Asn mutation usually causes a simple virilizing phenotype, it is also detected in 23% of salt-wasting CAH cases (32,34,40).

In different studies from Turkey, c.293-13C>G, large deletions/conversions, p.Arg357Trp and p.Gln319Ter were found to be the most common genetic mutations in the salt-wasting form; c.293-13C>G and p.Ile173Asn were found to be the most common genetic mutations in the simple virilizing form and c.293-13C>G, p.Ile173Asn, p.Pro30Leu, and p.Gln319Ter were found to be the most common genetic mutations in the non-classical form (23,32,42,43,44,45).

In two studies in which children born to women with NCCAH were retrospectively analyzed, the risk of having a child with classical 21-OHD was found to be 1.5-2.5% (46,47). In terms of risk identification, *CYP21A2* genotyping is recommended before planning pregnancy (5).

As a result of complete deletion of *CYP21A2*, the recessive form of Ehler-Danlos syndrome associated with tenascin-X-deficiency occurs. Joint hypermobility, arthralgia, joint dislocation, hernias and midline defects are observed in these cases (2,3,9,33).

Genetic screening of carriers: If possible, determining the carrier status of the parents and determining which parent the mutation was passed on from (especially in case of combined heterozygosity) will help in interpreting the genetic results and determining the risk of recurrence in subsequent pregnancies (2).

Good practice point:

1. Genotyping in cases with CAH is recommended for diagnosis and management in cases where 21-OHD is considered. If the patient has a steroid profile and ACTH stimulation test result that suggests the diagnosis of CAH, or baseline steroid levels may not be readily available, genotyping should be performed (2⊕⊕OO).

Footnotes

Authorship Contributions

Concept: Zeynep Şıklar, Design: Zeynep Şıklar, Data Collection or Processing: Zeynep Şıklar, Analysis or Interpretation: Havva Nur Peltek Kendirci, Zeynep Şıklar, Literature Search: Sevinç Odabaşı Güneş, Havva Nur Peltek Kendirci, Edip Ünal, Ayşe Derya Buluş, İsmail Dündar, Zeynep Şıklar, Writing: Sevinç Odabaşı Güneş, Havva Nur Peltek Kendirci, Edip Ünal, Ayşe Derya Buluş, İsmail Dündar, Zeynep Şıklar.

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