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Genetics of Congenital Adrenal Hyperplasia

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SYNOPSIS

Congenital adrenal hyperplasia (CAH) refers to a group of autosomal recessive disorders due to single gene defects in the various enzymes required for cortisol biosynthesis. CAH represents a continuous phenotypic spectrum with over 95% of all cases caused by 21-hydroxylase deficiency. Genotyping is an important tool in confirming the diagnosis or carrier state, provides prognostic information on disease severity, and is essential for genetic counseling. The genes for the various variants of CAH are well characterized, and mutation analysis is widely available. Certain ethnic groups have a predilection to certain genotypes, which may have resulted from an ancient founder effect, a hot spot in the gene, unequal crossing over during meiosis or gene conversion of point mutations from a pseudogene. Several pitfalls in the genetic diagnosis of patients with CAH exist. In this article, we provide an in-depth discussion on the genetics of CAH, including genetic diagnosis, molecular analysis, genotype-phenotype relationships and counseling of patients and their families.

Keywords

Congenital Adrenal Hyperplasia; Genetics; Adrenal insufficiency; 21-hydroxylase deficiency; pseudogene; Genetic counseling

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Introduction

Congenital adrenal hyperplasia (CAH) refers to a group of autosomal recessive disorders that impair cortisol biosynthesis. Consequently, overproduction of corticotropin releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) from the hypothalamus and pituitary glands, respectively, results in an increase and accumulation of various steroid precursors proximal to the block. This leads to defective cortisol synthesis, shunting of the accumulated steroid precursors through alternative pathways, and often adrenal gland hyperplasia. The biochemical defects in CAH translate to a spectrum of clinical consequences, which include adrenal insufficiency, genital ambiguity or disordered sex development (DSD), infertility, short stature, hypertension, and an increased risk of metabolic syndrome during adolescence and adulthood. The severity and clinical features of CAH vary depending on the enzymatic defect, its residual activity, age of presentation, and genotype.

CAH represents a continuous phenotypic spectrum (Table 1). Over 95% of all cases of CAH are caused by 21-hydroxylase deficiency (21-OHD). 21-OHD is classified into 3 subtypes according to clinical severity: classic salt-wasting (SW), classic simple virilizing (SV), and nonclassic (NCCAH, mild or late-onset).¹ The classic type affects approximately 1 in 16,000 live births.² NCCAH is one of the most common autosomal recessive disorders in humans and affects approximately 1 in 1,000 individuals.³ The second most common form of CAH, 11-hydroxylase deficiency (11-OHD), occurs in 1 in 100,000 live births in the general population and accounts for approximately 5% of cases.⁴ Other less common forms of CAH include: 3 β -hydroxysteroid dehydrogenase type 2 deficiency, 17 α -hydroxylase deficiency that is more commonly seen in Mennonite descendants of Dutch Frieslander and the Brazilian population; congenital lipoid adrenal hyperplasia that is more commonly seen in the Japanese and Korean populations; side-chain cleavage enzyme deficiency that is most commonly found in Turkey; and cytochrome P450 oxidoreductase deficiency (ORD), the only variant that can manifest with skeletal malformation.

Measurement of 17-hydroxyprogesterone (17-OHP) with or without ACTH stimulation establishes the diagnosis of CAH due to 21-OHD.⁵ Similarly, the rarer forms of CAH are diagnosed based on hormonal testing. Biochemical methods are typically unable to discriminate heterozygotes from the normal population and disease severity. Thus, genotyping is essential in confirming the carrier state, and is useful for genetic counseling.⁶ Moreover, studies of the genetics of CAH have provided insight into the pathophysiology and subtle clinical aspects of the disease, and may provide prognostic information on disease severity. The genes for the various variants of CAH are well characterized, and mutation analysis is widely available. However, several pitfalls in the genetic diagnosis of individuals with CAH exist. In this article, we provide an in-depth discussion on the genetics of CAH, including genetic diagnosis, molecular analysis, genotype-phenotype relationships and counseling of patients and their families.

Genetic forms of congenital adrenal hyperplasia

1. 21-hydroxylase deficiency

21-OHD (OMIM 201910) is the commonest form of CAH, classified into classic (SW and SV) and NCCAH.¹ The classic or severe form is characterized by virilization of the external genitalia in newborn females and by adrenal insufficiency and precocious pseudopuberty due to androgen overproduction in both sexes. Approximately 75% of patients with classic CAH have the SW form. Without neonatal screening, the SW form in males presents with a severe life-threatening salt-wasting crisis in the neonatal period; while patients with the SV form may escape this due to small amounts of aldosterone production. Thus, males with SV CAH, if not detected through neonatal screening, present as toddlers with signs and symptoms of hyperandrogenism. Females with classic CAH are born with ambiguous genitalia, but without proper diagnosis, the majority of females would also experience a life threatening salt-wasting crisis. NCCAH, the mild form, may manifest in childhood or early adulthood with precocious pubarche or a clinical picture resembling polycystic ovary syndrome, and may be asymptomatic.³

CYP21A2 (previously called *P450c21B*, *CYP21B* or *CYP21*) encodes 21-hydroxylase, a cytochrome P450 type II enzyme of 495 amino acids.^{7,8} The *CYP21A2* gene and its duplicated pseudogene (*CYP21A1P*), a nonfunctional by-product of the functional gene that was formed from selective pressure during evolution, are located 30kb apart in the human leukocyte antigen (HLA) class III region in the major histocompatibility (MHC) locus on the short arm of chromosome 6 (band 6p21.3), and share approximately 98% sequence homology (Figure 1 and 2).⁹ The *CYP21A1P* gene is inactive due to the presence of approximately 11 deleterious mutations in its coding region (Figure 2). Both genes are arranged in tandem repeat with the *C4* (*C4A* and *C4B*) genes encoding the fourth component of complement; *C4/CYP21A2* is flanked by telomeric *RP* (*RP1* and *RP2*) and centromeric *tenascin* (*TNXA* and *TNXB*) genes. This “odd” arrangement was probably caused by an ancestral duplication of 30kb encompassing the *CYP21A2* and *C4* genes and represents a region with a high frequency of genomic recombination. *RP1* encodes a serine/threonine nuclear protein kinase, *C4* encodes for the immune effector protein complement component, and *TNXB* encodes tenascin-X, an extracellular matrix protein. With the exception of *C4*, each of the other functional genes (*RP1*, *CYP21A2* and *TNXB*) has a corresponding highly homologous pseudogene (*RP2*, *CYP21A1P* and *TNXA*). Together with the *RP* and *TNX* genes, the *C4* and *CYP21* genes compose the RCCX module, and most chromosomes are bimodular (*RP1-C4-CYP21A1P-TNXA-RP2-C4-CYP21A2-TNXB*)¹⁰ (Figure 1) with a *CYP21A1P* gene in the telomeric position and the *CYP21A2* gene in the centromeric position. Monomodular and trimodular haplotypes are also found in most populations.^{10–12} These modules are characterized by modular duplication or deletion events in which each duplicated or deleted module usually covers a *CYP21A1P-TNXA-RP2-C4* unit. Copy number variation (CNV) of *C4*, *CYP21*, and *TNX* is widely found in humans; in patients with CAH, a greater *C4* CNV with mutation-specific associations has been shown to possibly confer protection for autoimmune disease.¹³

Due to the high degree of sequence homology between *CYP21A2* and its pseudogene, recombination occurs. Approximately 70% of *CYP21A2* disease causing mutations are pseudogene derived variants due to gene conversion, the transfer of deleterious pseudogene mutations to the active *CYP21A2* gene,¹⁴ and approximately 25–30% are chimeric genes due to large deletions.^{15,16} Misalignment during meiosis can result in a 30kb gene deletion, which produces a nonfunctioning chimeric *CYP21A1P/CYP21A2* gene.^{17–19} Depending on the breakpoints, such genetic alterations result in different types of chimeras between the homologous genes in the RCCX modules; nine different *CYP21A1P/CYP21A2* chimeras have been reported, which have a CH-1 through 9 designation.²⁰ These chimeras are numbered chronologically, based on when the junction sites were reported (Figure 3). Approximately 1–2% of cases are caused by *de novo* mutations not carried by either parent due to the high variability of the *CYP21A2* locus.⁹ This results in complex genetic variation and unusual haplotypes, which can be detected in one in 10³ to 10⁵ sperm cells.²¹ Uniparental disomy of chromosome 6 as a cause of CAH is a rare occurrence with an unknown prevalence.²²

Genotype-phenotype Correlation

CAH due to 21-OHD represents a spectrum of phenotypic heterogeneity, with a direct genotype-phenotype correlation seen in approximately 90% of cases.^{23–26} Since most patients with CAH are compound heterozygotes carrying two different disease-causing mutations, the phenotype is defined by the mutation retaining the most enzyme activity.⁶ Generally, the disease severity in childhood can be accurately predicted by genotypes predicted to result in the SW and NCCAH forms; while the most phenotypic variability is observed with the SV type.^{6,17,23,25,26} Females are readily diagnosed clinically with classic CAH because of the genital ambiguity. However, with neonatal screening, classic CAH cases ascertained are equal between males and females.²⁷ Moreover, females are more likely to be found to have the p.V281L mutation and NCCAH because males may be asymptomatic.²⁸ Genotype is not a good predictor of the development of long-term health outcomes in adults with classic CAH, such as cardiovascular disease or osteoporosis, possibly due to treatment related effects on co-morbidities.^{28,29}

Based on *in vitro* data, *CYP21A2* mutations can be classified into 4 groups (Null, A, B, C) according to their severity (Table 2).⁹ Deletions or nonsense mutations that affect critical enzyme functions, such as membrane anchoring, heme binding, or alter enzyme stability result in a complete loss of functionality and SW. Missense mutations with 1–2% of normal enzyme activity are found in patients with the SV form and affect the transmembrane region or conserved hydrophobic patches.^{30–32} Mutations in p.V281L, p.P453S and p.P30L cause an interference in oxidoreductase interactions, salt-bridge and hydrogen bonding networks, and produce enzymes retaining 20–60% of normal activity as seen in NCCAH (Group C).^{21,33,34} In general, there is good genotype-phenotype correlation for the NCCAH p.V281L and p.P453S mutations, but phenotypic variability has been described for p.P30L.^{6,24}

Uncommon chimeric genes also account for some genotype-phenotype discrepancy and the junction site of the chimeras that develop as a consequence of genetic rearrangements can be

clinically meaningful (Figure 3).²⁰ Most commonly, the large deletions that are associated with *CYP21A1P/CYP21A2* chimeric genes totally inactivate the *CYP21A2* gene and therefore are associated with a classic SW phenotype. This type of *CYP21A1P/CYP21A2* chimera is referred to as a classic chimera. The classic *CYP21A1P/CYP21A2* chimeras contain the IVS2-13A/C>G mutation and often multiple other pseudogene mutations. The 21-hydroxylase enzyme activity is less severely impaired if the junction site occurs upstream of the pseudogene mutation IVS2-13A/C>G within intron 2. This is referred to as an attenuated chimera. To date, 2 attenuated chimeras have been described: CH-4 and CH-9 (Figure 3). Both have junction sites upstream of the common pseudogene mutation IVS2-13A/C>G, carry a weak but active *CYP21A1P* promoter and a nonclassic mutation, p.P30L (c.92C>T) in exon 1, and result in a SV or NC phenotype.²⁰ In a large cohort study of patients with CAH, 96% of large deletions were classic chimeras and 4% were attenuated chimeras.²⁰

Genotype-phenotype discordance or concordance may exist across all mutation groups. As a general rule, there is a high genotype-phenotype concordance in p.V281L, deletions, 8 bp deletion, p.Q318X, and p.R356W mutations (Table 2). SV disease occurs in approximately 76% of CAH patients who carry the p.I172N mutation on one allele and a severe mutation on the other, while 23% of these patients present with SW CAH, likely due to subtle variations in transcriptional regulation or downstream protein translation.⁶ Moreover, the intron 2 IVS2-13A/C>G splicing mutation results primarily in SW CAH; however, approximately 20% of patients have a SV phenotype.⁶

Erroneous reporting may occur in the presence of gene duplication. For example, the p.Q318X mutation is sometimes associated with *CYP21A2* gene duplication. In this situation, the p.Q318X-mutated allele coexists with a normal *CYP21A2*-like gene within the RCCX module on the same chromosome.⁶ This does not represent a mutated allele because the normal gene is expressed.

Several factors may be responsible for the genotype-phenotype variability in patients with CAH. Apart from the *CYP21A2* mutations, other genes that may impact phenotype by modifying steroid action or salt balance include: the length of the CAG repeats of the androgen receptor;^{35,36} the highly polymorphic enzyme P450 oxidoreductase; splice mutations variants in RNA splicing factors;^{37,38} and other genes encoding proteins other than cytochrome P450 type II enzyme that have 21-hydroxylase activity.^{39,40} A wide spectrum of phenotypes occurs and genotype-phenotype discordance requires further research.

CAH-X Syndrome

Some deletions that cause CAH due to 21-OHD also cause hypermobility type Ehlers-Danlos syndrome (EDS) due to the monoallelic presence of a chimera extending into the *TNXB* gene, encoding *tenascin-X*, an extracellular matrix protein. This contiguous gene deletion syndrome, involving both the *CYP21A2* and *TNXB* genes, is termed CAH-X syndrome.^{41,42} The first study of the genetic spectrum of CAH-X in a mixed population of CAH patients showed a contiguous *CYP21A2* deletion that extended into *TNXB* in 13% of patients (n=12/91) carrying a *CYP21A2* deletion, and a *TNXB* premature stop codon in a

single patient, with an overall 7% prevalence of CAH-X amongst CAH patients. Additional chimeric genes have subsequently been identified and overall 9% of patients with CAH due to 21-OHD are estimated to have CAH-X, a connective tissue dysplasia, in addition to having CAH.⁴³

The deletions associated with CAH-X are *TNXA/TNXB* chimeras (Figure 4) and 3 types of chimeric genes have been identified to date, designated CAH-X CH-1, CAH-X CH-2, and CAH-X CH-3.^{41,43,44} CAH-X CH-1 involves the 120bp deletion in the exon 35 region of *TNXB* and leads to haploinsufficiency with reduced *tenascin-X*; CAH-X CH-2 involves the c.12174C>G (p.Cys4058Trp) variant in exon 40 of *TNXB*, which does not alter *tenascin-X* expression but rather causes a dominant-negative effect due to the loss of a critical disulfide bond in the *tenascin-X* fibrinogen-like domain; and CAH-X CH-3 involves a cluster of three variants and also causes a dominant-negative effect.

CAH-X patients may present with CAH and joint hypermobility, joint pain and multiple joint dislocations.⁴¹ Other clinical features include midline defects and major cardiac structural abnormalities.⁴² Thus, clinicians involved in the care of patients with CAH, particularly those harboring a *CYP21A2* deletion, should provide a comprehensive evaluation for a connective tissue dysplasia.

Population Genetics and Ethnic diversity of CYP21A2 genotypes

Millions of newborns screened in the general population for the classic form of CAH have demonstrated an overall incidence of 1:13,000 to 1:16,000 live births.^{45,46} The prevalence in specific populations varies, including 1:10,000 - 1:23,000 in the United States and Europe,⁴⁷ 1:21,000 in Japan,⁴⁸ 1:27,000 in New Zealand,⁴⁹ and 1:6,000 in China⁵⁰ and India⁵¹. NCCAH is one of the most common autosomal recessive disorders in humans and affects approximately 1 in 1000 individuals.³ Certain ethnic groups have a predilection to certain genotypes (Table 3),^{9,23,24,52–58} which may have resulted from an ancient founder effect, unequal crossing over during meiosis or gene conversion of point mutations in the pseudogene.⁵⁹

In the United States, the allele frequency of the most common mutations might vary considerably between different ethnicities. For instance, the exon 7 p.V281L (c.1685G>T) mutation associated with NCCAH is very frequent in the Ashkenazi Jewish (AJ) population of New York (estimated in one study to be 1 in 27, with 1 in 3 being heterozygous), which has not been observed in the Asian and Native American populations.⁶ However, this mutation also occurs in other populations. Among compound heterozygotes in the AJ population, the prevalent mutations are large deletion/p.V281L (c.1685G>T) and IVS2-13A/C>G (c.293-13A/C>G)/p.V281L (c.1685G>T).⁵⁹

CAH is rare in Asian, African American, and East Indian populations of New York, while a large gene deletion is observed in Native Americans.⁶ Conversely, the IVS2-13A/C>G genotype and p.V281L are more prevalent in the Middle Eastern population; the common p.Q318X mutation is by far the most frequently found in the Tunisian population.⁶⁰ In the Yupik speaking Eskimos of Western Alaska and Iranians, the IVS2-13A/C>G (c.293-13A/C>G) mutation predominates.⁵⁹ One study found at least half of all patients with 21-OHD in

Finland were due to 3 most common founder mutation-haplotypes, and only one-sixth of the haplotypes represented single cases.⁶¹ Of the chimeras, CH-7 was first identified in a Czech population and represents the most frequent allele.²⁰ Thus, knowledge about the ethnic diversity and specificity of the *CYP21A2* mutations might help guide clinicians toward a diagnosis of 21-OHD prior to molecular testing.

Molecular analysis of *CYP21A2* mutations

The genetic diagnosis of CAH is complex due to the high variability of its 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. Molecular analysis of *CYP21A2* should be extensive and performed in a certified laboratory with adequate quality controls and experience in the analysis of CAH genotypes. Care should be taken to prevent genotyping the pseudogene because genetic results can be complicated by the duplication, deletion, and recombination of *CYP21A2* in the chromosome 6q21.3 region. Moreover, mutant alleles must be segregated in the parents to investigate their presence in different alleles, to determine if they are in the *cis* or *trans* configuration, and to verify *de novo* mutations. Targeted site-directed mutation analysis of the 12 most common mutations fails to identify mutations in approximately 10% of patients and rare mutations are identified more often than expected with whole coding region sequencing.²³ Thus, *CYP21A2* sequencing is important in determining an accurate genotype, especially if genotype does not correspond to phenotype or if only one mutation is present in a patient with clinical and hormonal evidence of CAH.

The *CYP21A2* gene consists of ten exons and relatively short introns, which allows for the amplification of the entire exon–intron region. Traditionally, the complete characterization of the locus regarding number of RCCX modules and their specific gene content required Southern blotting with probes for the different genes and using different combinations of restriction endonucleases.⁶² However, given its labor intensity, the need for radioactivity, and the requirement for relatively large amounts of good-quality DNA, Southern blotting has been largely replaced by real-time quantitative polymerase chain reaction (PCR)⁶² and multiplex ligation-dependent probe amplification (MLPA)^{63,64} methods for detecting large gene deletions and duplications. A well-established protocol for sequencing the *CYP21A2* gene is the use of long-range PCR that amplifies an 8.5kb fragment (the CYP779f/Tena32F amplicon) encompassing the full *CYP21A2* gene and part of the *TNXB* gene.²⁰ Since the forward primer is common for both *CYP21A2* and *CYP21A1P*, and while the reverse primer anneals the unique (non-homologous) sequence of the *TNXB* gene, an accurate analysis of the *CYP21A2* gene is guaranteed even when the *CYP21A2* gene is replaced by the pseudogene, either partially or totally. Therefore, the junction site of chimera genes can be automatically determined and MLPA is not needed for detecting large deletions.²⁰ Compared with the targeted common mutation analysis approaches, whole gene (coding regions and canonical splicing sites) sequencing can reveal both common and rare mutations. This 8.5kb CYP779f/Tena32F amplicon is able to reveal *TNXA/TNXB* chimeras and has been utilized to study CAH-X patients.^{41,43,44}

Both the PCR and MLPA-based strategies have limitations. First, allele dropout (i.e. only one allele is amplified and sequenced) is a general limitation of PCR-based Sanger sequencing. If no heterozygotes are found, parental study should be performed to rule out the possibility of allele dropout. Second, the long-range PCR amplicon for sequencing the *CYP21A2* gene alone is not able to detect *CYP21A2* duplication allele simultaneously. Another well-designed long-range PCR amplicon is required to detect *CYP21A2* duplication specifically. Third, MLPA is unable to distinguish attenuated chimeras CH-4 and CH-9 from classic chimera CH-6, owing to the lack of a probe for IVS2-13A/C>G, which is the crucial site for classifying chimeric *CYP21A1P/CYP21A2* genes into the classic and attenuated types.²⁰ Thus, an experienced laboratory in dealing with the *CYP21A2* gene analysis is highly advised for the correct molecular diagnosis of CAH due to 21-OHD. Genotyping for the presence of *TNXA/TNXB* chimeras should be performed in patients clinically suspicious for CAH-X.

2. 11 β -hydroxylase deficiency

11 β -hydroxylase deficiency (11-OHD) (OMIM 20210) is the second commonest variant of CAH (approximately 5–8% of all cases) with an incidence of 1 in 100,000–200,000 live births.⁶⁵ 11-OHD is caused by mutations in the 11 β -hydroxylase gene (*CYP11B1*), with the highest prevalence among Moroccan Jews (approximately 1 in 5000–7000 live births), an association that is partially explained by the founder mutation p.R448H.^{66,67} The CYP11B1 enzyme is a P450 type I mitochondrial enzyme, responsible for the conversion of 11-deoxycortisol to cortisol, and 11-deoxycorticosterone (DOC) to corticosterone; its defect results in hypertension from elevated DOC and possibly other steroid precursors, and hyperandrogenism from shunted precursors into the androgen synthesis pathway. Patients do not present with adrenal insufficiency due to the glucocorticoid effects of excess corticosterone. 11-OHD is divided into the classic form, which presents as virilization of the external genitalia in 46,XX newborn females, and precocious pseudopuberty in both sexes. The nonclassic form is extremely rare and presents with hyperandrogenism during childhood.⁶⁸

The *CYP11B1* gene is located on chromosome 8q21, and consists of nine exons that are approximately 40kb from the highly homologous aldosterone synthase gene (*CYP11B2*).⁶⁹ Over 50 *CYP11B1*-inactivating mutations that are distributed over the entire coding region exist, with the majority being missense and nonsense, but splice-site mutations, small deletions, small insertions, and complex rearrangements exist and result in absent or very little 11 β -hydroxylase activity.⁷⁰ These mutations (Table 2) cause the classic form and tend to cluster in exons 2, 6, 7, and 8.⁷¹ Rarely, unequal crossing-over between the *CYP11B2* and the *CYP11B1* genes produces a chimera that is under the control of angiotensin II and potassium, rather than ACTH,⁷² resulting in glucocorticoid-remediable aldosteronism (familial aldosteronism type 1) which is not considered a type of CAH. Rare chimeric *CYP11B2/CYP11B1* genes can also result in CAH due to 11-OHD.⁷³ A variety of milder mutations that result in nonclassic 11-OHD exist (Table 2).^{70,74} Novel mutations for both subtypes are being discovered routinely.^{70,75,76}

3. 17 α -hydroxylase deficiency

17 α -hydroxylase deficiency (17-OHD) (OMIM 202110) results from a defective *CYP17A1* and accounts for approximately 1% of all CAH cases.⁷⁷ The *CYP17A1* enzyme is a microsomal P450 type II enzyme that catalyzes two different enzymatic reactions; 17- α hydroxylation of pregnenolone and progesterone, and the conversion of 17-hydroxypregnenolone to dehydroepiandrosterone and, with lesser efficiency, of 17-OHP to androstenedione through the 17,20 lyase reaction. Thus, 17-OHD results in glucocorticoid and sex steroid deficiency, and impairs both adrenal and gonadal function.⁷⁸ The accumulation of the mineralocorticoid precursors corticosterone and DOC exert glucocorticoid and mineralocorticoid activity respectively and lead to hypertension with hypokalemia. Thus, adrenal insufficiency is not a characteristic feature of 17-OHD. Patients present with hypertension, hypokalemia and sexual infantilism. The classic presentation of severe 17-OHD is a phenotypic female (46,XX or 46,XY) with hypertension and absence of secondary sexual characteristics. In partial 17-OHD, 46,XY patients may present with undervirilization and present as infants with ambiguous genitalia. Isolated 17,20 lyase deficiency that results in amino acid substitutions located within the area of the *CYP17A1* molecule that interacts with the electron-donating redox partner, P450 oxidoreductase (POR), is a rare disorder that manifests with impaired sex steroid biosynthesis only.⁷⁹ These patients may present as a male with undervirilization and gynecomastia or a female with delayed pubarche and oligomenorrhea. Isolated 17,20 lyase deficiency is not considered a type of CAH.

The *CYP17A1* gene consists of 8 exons encoding a 508-amino-acid protein and is located on chromosome 10 (10q24.3).⁸⁰ Over 70 *CYP17A1*-inactivating mutations exist, with no evidence of a hot spot in most large populations. Therefore, sequencing of the entire coding region is usually necessary for molecular genetic diagnostics. However, certain ethnicities are at higher risk of 17-OHD, including Canadian Mennonites and Dutch Frieslanders,⁸¹ Japanese,⁸² East Asians, and the the Brazilian population,⁸³ where 82% of mutant alleles are due to two mutations, suggesting a founder effect.

4. 3 β -hydroxysteroid dehydrogenase type 2 deficiency

3 β -hydroxysteroid dehydrogenase type 2 deficiency (HSD3B2) (OMIM 201810) is a rare autosomal recessive variant of CAH with an unknown incidence. 3 β -hydroxysteroid dehydrogenase exists in two isoforms, type 1 and type 2, each encoded by genes (four exons, of which exons 2–4 are translated) on chromosome 1.⁸⁴ *HSD3B2* is highly expressed in the adrenal glands and gonads, while *HSD3B1* is expressed in the placenta and peripheral tissues. HSD3B2 is responsible for the conversion of the pregnenolone, 17-hydroxypregnenolone and dehydroepiandrosterone to progesterone, 17-OHP and androstenedione, respectively. Similar to 17-OHD, both gonadal and adrenal hormone synthesis is affected. Paradoxically, both male and female infants can be born with genital ambiguity, depending on the severity of the enzyme deficiency. A “nonclassic” form of HSD3B2 deficiency that causes hirsutism and menstrual irregularity in young adult women was previously suggested,⁸⁵ based on exaggerated 17-hydroxypregnenolone production after ACTH stimulation.⁸⁶ However, the *HSD3B2* gene was subsequently found to be normal in these patients, and they most likely have a form of polycystic ovary syndrome.⁸⁷ Thus, the

diagnosis of a mild or “nonclassic” form is questionable and should not be considered unless the diagnostic laboratory parameters are at least six standard deviations above normal.⁸⁸ The genetics of HSD3B2 deficiency was first ascertained in a 46,XY patient with ambiguous genitalia and a homozygous combined missense/frameshift mutation in exon 4.⁸⁹

5. P450 oxidoreductase deficiency

P450 oxidoreductase (POR) deficiency (OMIM 613571) is a rare variant of CAH of unknown incidence, and manifests as apparent combined *CYP17A1* and *CYP21A2* deficiency.⁹⁰ The POR gene is located on the long arm of chromosome 7 (7q11.2), consists of 15 translated exons spanning a region of approximately 32.9 kb, and serves as an electron donor enzyme for CYP17A1, CYP19A1 (aromatase) and CYP21A2.⁹⁰ Both sexes present clinically with severe sexual ambiguity; boys present with severe undervirilization and girls present with severe virilization. Reduced CYP19A1 activity induces androgen accumulation in the placenta, leading to virilization of the female fetus and sometimes the pregnant mother. Adrenal insufficiency is present in the majority of patients.⁹¹

Craniofacial malformations may also be present and overlap with the Antley-Bixler syndrome (OMIM 201750), a rare craniosynostosis syndrome characterized by radiohumeral synostosis presenting in the perinatal period.⁹⁰ The majority of patients with mild to moderate skeletal malformations are compound heterozygous for missense mutations, whereas nearly all patients with severe malformations carried a major loss-of-function defect on one of the affected alleles.⁹¹ Postnatally, females with POR deficiency have low androgens, and virilization does not progress. POR-inactivating mutations, including missense, frameshift and splice site mutations, are scattered throughout the gene with no apparent hot spots.^{92,93} The most frequent mutation in Caucasians is p.A287P,⁹² while p.R457H is the most frequent mutation in Japanese populations.^{94,95} In one study of 30 patients with POR deficiency from 11 countries, 23 POR mutations including a deletion and a partial duplication were detected by MLPA, and only 22% of unrelated patients carried homozygous POR mutations.⁹¹ POR deficiency may present as a form of apparent 17,20 lyase deficiency due to mutations in the POR gene rather than the *CYP17A1* gene.⁸²

6. Lipoid Congenital Adrenal Hyperplasia

Lipoid CAH (OMIM 201710) is the most severe disorder of adrenal and gonadal steroidogenesis and is due to a defect in a transport protein, steroidogenic acute regulatory protein (StAR) that regulates cholesterol transfer within the mitochondria. This step is the rate-limiting step in the production of steroid hormones. In classic congenital lipoid CAH, there is complete or near complete absence of all steroid hormones. Lipoid CAH is caused by homozygous or compound heterozygous mutations in the gene encoding StAR on chromosome 8p11.⁹⁶ The pathophysiology involves an initial genetic loss of steroidogenesis that is StAR-dependent and a subsequent loss of steroidogenesis that is StAR-independent, due to cellular damage from accumulated cholesterol esters in the adrenal and gonadal glands.⁹⁷ Affected patients are phenotypic females with severe SW that is fatal if not treated in early infancy due to glucocorticoid and mineralocorticoid deficiency. This rare disease is most common amongst Japanese, Korean, and Palestinian Arab populations.⁹⁸ Several

mutations in the StAR gene have been described and are mostly located in exons 5, 6, or 7: p.Q258X in Asians, and p.R182L in Palestinian Arabs.⁹⁷

A milder, “nonclassic” form of lipoid CAH was first described in 2006,⁹⁹ and is caused by mutations that retain about 20–30% of normal StAR activity. The majority of these patients carry the StAR mutation p.R188C, which has been found in patients from Canada, Jordan, India, Pakistan and Thailand; however, other StAR mutations causing non-classic lipoid CAH have also been described.

7. Cholesterol side-chain cleavage (SCC) enzyme deficiency

For years, it was thought that deficiency of cholesterol side-chain cleavage (SCC) enzyme was incompatible with life. This idea was reinforced with the discovery that StAR mutations cause lipoid CAH. However, 28 patients have now been described with *CYP11A1* mutations causing SCC deficiency (OMIM 118485).^{100,101} Most of the reported cases are from Eastern Turkey and are homozygous for the missense mutation p.R451W.¹⁰² Variable phenotype has been described, presumably due to mutation specific variable effects on enzyme activity.¹⁰¹

Genetic counseling of patients with congenital adrenal hyperplasia

All forms of CAH are transmitted as autosomal recessive and the determination of disease risk is dependent on the carrier status of the patient’s parents. Genetic counseling should focus on the future risks of having an affected child. Having one normal gene and one gene carrying a CAH mutation, results in a carrier state and carriers for CAH do not show symptoms. If both parents are carriers, then the risk of having an affected child, a child who is a carrier, and an unaffected child is 25%, 50%, and 25%, respectively (Figure 5). However, if one parent is a heterozygous carrier and the other is affected with CAH, each child has a 50% chance of inheriting one mutation and a 50% chance of being affected. The risk is the same for males and females.

For CAH due to 21-OHD, the incidence of classic CAH is approximately 1:10,000–1:20,000, with a carrier rate in the general population of approximately 1:50–1:71 (median 1:60).^{3,45,103} The chance that a patient with classic CAH will have a child with classic CAH is 1 in 120 (chance of partner being a carrier $[1/60] \times 1/2$) if the carrier status of the partner is unknown. For NCCAH, about two thirds of patients are compound heterozygotes, carrying one allele that causes classic CAH and one that causes NCCAH. The chance that a patient with NCCAH will have a child affected with classic CAH is 1 in 360 (chance of partner being a carrier $\times$ chance of non-classic patient being a carrier of classic mutation $\times 1/4 = 1/60 \times 2/3 \times 1/4$) if the carrier status of the partner is unknown.²⁸ This risk in reality may be higher; a retrospective study of NCCAH women reported a 2.5% risk of a NCCAH woman having a child with classic CAH.¹⁰⁴ When compared to an individual with two normal *CYP21A2* alleles, a heterozygote carrier may have slightly elevated 17-OHP levels when stimulated with ACTH, but hormonal testing does not accurately predict the carrier state. Thus, genetic testing is essential to determine the presence of mutations.

A successful patient counseling model for CAH would incorporate patient’s values and attitudes toward their disease, underscoring the risks and benefits of genetic screening and

counseling, psychosocial interventions and service delivery. Other aspects of a successful counseling model include a thorough personal medical and family history (with a detailed family pedigree), education regarding the genetics of the condition, and discussions on preventing and screening options. Counseling should be carried out by an experienced genetic counselor.

When offering genetic counseling to individuals at risk of or with CAH, several important factors should be considered. First, genetic testing may miss a mutant allele and should be carried out in a reliable laboratory and interpreted by an experienced individual. Second, the optimal time for determination of genetic risk, carrier status, or treatment is before pregnancy. Third, testing of reproductive partners of probands for carrier status is recommended when one or both disease-causing mutations have been identified in the proband. Testing of other family members should be considered prior to reproduction, but is not recommended on a routine basis, especially for children. Fourth, certainties and uncertainties about treatment, prognosis, morbidity, mortality, or the impact of a positive genetic screen on the individual or their families should be discussed in detail with the patient or their surrogate before testing.

Prenatal diagnosis of congenital adrenal hyperplasia

Prenatal diagnosis is an invasive approach that uses fetal DNA, obtained by amniocentesis or chorionic villus sampling, to establish a diagnosis based on genotype.⁶⁵ Determination of the fetal sex could allow for prenatal therapeutic decisions of 46,XX fetuses with virilizing CAH, although administration of dexamethasone to the pregnant mother to reduce masculinization of the genitalia in a fetus at risk for CAH is controversial and no longer recommended outside of a research setting.¹ Concerns include the need to start therapy before disease status is known, thus exposing unaffected fetuses to unknown risk, and the unknown long-term effects of *in utero* glucocorticoid exposure.^{105,106} With the discovery of cell free fetal DNA in maternal circulation,¹⁰⁷ it has become possible to determine the sex of the fetus by the 7th week of gestation. Thus, this new technology partially addresses some of the ethical concerns associated with prenatal therapy and, if used, would potentially avoid treatment of male fetuses and unaffected female fetuses.

Future therapies: Gene therapy and Stem-cell transplantation

Conventional therapy for CAH includes glucocorticoid and mineralocorticoid replacement. However, since CAH is a disorder caused by a single gene defect, and given the rapid molecular understanding of its pathogenesis, investigations into the use of gene therapy or stem-cell approaches for the treatment of CAH are ongoing. The first gene therapy approach for CAH were reported in H-2aw18 mice with 21-OHD.¹⁰⁸ Other studies have examined the selective adenotropism for adenoviruses using a single intra-adrenal injection of adenoviral vector encoding *CYP21A*.^{109,110} These approaches restore the impaired adrenocortical function in 21-hydroxylase knockout mice and bovine adrenocortical cells.^{110–112} Gene or stem-cell transplantation therapies could be a feasible option for treatment of CAH in the future.

Conclusion

CAH is a common disorder with genetic and clinical heterogeneity and is life-threatening in its most severe form. Life-saving neonatal screening programs exist in the United States and many other countries based on hormonal testing; genetic diagnosis of neonates is not being used in screening programs because of deficiencies in accuracy, speed and cost-effectiveness. However, the molecular analysis of CAH is useful in confirming the diagnosis, and provides a powerful tool in genetic counseling. Over the years, the development of comprehensive methodologic approaches has improved our ability to genotype patients. However, several pitfalls and challenges remain in the molecular diagnosis of CAH across its many genotypes. Although the various forms of CAH are monogenic disorders, the large number of possible mutations and the presence of duplicated genes, makes the development of inexpensive, fast and accurate genetic tools particularly challenging. Future research is needed to assist in the molecular pathogenesis of CAH that will assist in the management of affected patients and their family members across all age groups. Advances in genetics are transforming medicine and one day, personalized genetic information that includes CAH genotyping may help guide individualized therapy.

List of laboratories for CAH molecular analysis in the United States

Esoterix Molecular Endocrinology

Calabasas, Hills, California

www.genetests.org/laboratories

Molecular Genetics Laboratory, Emory

Atlanta, Georgia

www.geneticslab.emory.edu

Molecular Genetics Laboratory, Mayo Clinic

Rochester, Minnesota

www.mayoclinic.org

PreventionGenetics

Marshfield, WI

www.preventiongenetics.com

Quest Diagnostics, Nichols Institute

San Juan Capistrano, California

www.education.questdiagnostics.com

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KEY POINTS

- Congenital adrenal hyperplasia (CAH) refers to a group of autosomal recessive disorders due to single gene defects in the various enzymes required for cortisol biosynthesis.
- CAH represents a continuous phenotypic spectrum with over 95% of all cases caused by 21-hydroxylase deficiency. Genotyping is an important tool in confirming the diagnosis or carrier state, provides prognostic information on disease severity, and is essential for genetic counseling.
- The genes for the various variants of CAH are well characterized, and mutation analysis is widely available.
- Certain ethnic groups have a predilection to certain genotypes, which may have resulted from an ancient founder effect, a hot spot in the gene, unequal crossing over during meiosis or gene conversion of point mutations from a pseudogene.
- Several pitfalls in the genetic diagnosis of patients with CAH exist.

Figure 1.

The *CYP21A2* gene and its duplicated pseudogene (*CYP21A1P*) are located 30kb apart in the human leukocyte antigen (HLA) class III region in the major histocompatibility (MHC) locus on the short arm of chromosome 6 (band 6p21.3), and share approximately 98% sequence homology. Both genes are arranged in tandem repeat with the *C4* (*C4A* and *C4B*) genes. *C4/CYP21A* is flanked by telomeric *RP* (*RP1* and *RP2*) and centromeric tenascin (*TNXA* and *TNXB*) genes. *RP1* encodes a serine/threonine nuclear protein kinase, *C4* encodes for the immune effector protein complement component, and *TNX* encodes an extracellular matrix protein. With the exception of *C4*, each of the other functional genes (*RP1*, *CYP21A2* and *TNXB*) has a corresponding highly homologous pseudogene (*RP2*, *CYP21A1P* and *TNXA*). The *RP*, *TNX*, *C4* and *CYP21* genes together compose the RCCX bimodule (*RP1-C4-CYP21A1P-TNXA-RP2-C4-CYP21A2-TNXB*). Enlarged area represents the 10 exons of *CYP21A2*. Active genes are solid colors. Pseudogenes are grey, outlined with the color of the corresponding active gene.

Figure 2.

The high degree of sequence homology between *CYP21A2* and its pseudogene *CYP21A1P* allows for recombination events. Unequal crossing-over through intergenic recombination results in large deletions, and the transfer of deleterious pseudogene sequence to the active gene. The majority of mutations found in CAH due to 21-OHD are pseudogene derived or large 30kb deletions resulting in chimeric genes. The most common pseudogene-derived mutations found in the *CYP21A2* are shown: p.P30L; IVS2-13A/C>G; 8bp deletion; p.I172N; exon 6 cluster (p.I236N, p.V237E, p.M239K); p.V281L; p.Leu307fs; p.Q318X; p.R356W; p.P453S.

Figure 3.

Junction sites of *CYP21A1P/CYP21A2* chimeras. Misalignment during meiosis can result in a 30kb gene deletion, which produces a chimeric *CYP21A1P/CYP21A2* gene. Nine different *CYP21A1P/CYP21A2* chimeras have been reported with unique junction sites. These chimeras are numbered chronologically, CH-1 through CH-9, based on when the junction sites were reported. The location of the junction site is clinically meaningful. Seven chimeras are nonfunctional and are associated with a classic salt-wasting phenotype. Two chimeras, CH-4 and CH-9, carry the p.P30L mutation and along with a weak *CYP21A1P* promoter, only partially impair the gene. These chimera are referred to as attenuated and are associated with a milder phenotype.

Figure 4.

Formation of *CYP21A1P/CYP21A2* and *TNXA/TNXB* chimeras. Due to the high degree of sequence homology between *CYP21A2* and *CYP21A1P*, recombination events occur. The deletions associated with CAH-X syndrome, a condition characterized by having a combination of CAH and a connective tissue dysplasia, are *TNXA/TNXB* chimeras. The formation of a *TNXA/TNXB* chimera results in deletion of the *CYP21A2* gene.

Figure 5.

Genetic risk in the offspring of a patient with CAH. A) If the partner of a patient with classic CAH is unaffected, then all of the children will be carriers of classic CAH. B) If the partner of a patient with classic CAH is a carrier of a classic mutation, then there is a 50% chance that a pregnancy will result in a child affected with classic CAH. Phenotype is determined by the most functional allele. C) If the patient has nonclassic CAH, but carries a classic gene, and the partner is a carrier of a classic mutation, then there is a 25% chance of having a child with classic CAH.

Table 1

Types of congenital adrenal hyperplasia.

CAH Type	Causative Gene	Clinical Manifestation
21-hydroxylase deficiency	<i>CYP21A2</i> <i>CYP21A2</i> and <i>TNXB</i>	Classic: 46,XX ambiguous genitalia, adrenal insufficiency, salt-wasting, postnatal virilization. Nonclassic: hyperandrogenism during childhood or early adulthood may be asymptomatic. CAH-X: In addition to the above, joint hypermobility, joint pain, multiple joint dislocations, midline defects including possible cardiac structural abnormalities.
11 β -hydroxylase deficiency	<i>CYP11B1</i>	Classic: 46,XX ambiguous genitalia, postnatal virilization, hypertension. Nonclassic: hyperandrogenism during childhood or early adulthood. May be asymptomatic.
17 α -hydroxylase deficiency	<i>CYP17A1</i>	Classic: female phenotype (46,XX or 46,XY sex reversal), hypertension, pubertal delay with absence of secondary sexual characteristics. Partial: 46,XY variable degrees of genital ambiguity. 46, XX variable development of secondary sexual characteristics.
3 β -hydroxysteroid dehydrogenase type 2 deficiency	<i>HSD3B2</i>	Classic: 46,XX and 46,XY ambiguous genitalia, adrenal insufficiency, salt-wasting.
P450 oxidoreductase (POR) deficiency	POR	46,XX and 46,XY ambiguous genitalia, adrenal insufficiency, severe salt-wasting, possible maternal virilization during pregnancy. Possible skeletal malformations (Antley-Bixler syndrome). Post-natal virilization does not occur.
Lipoid CAH	StAR	Classic: phenotypic female (46,XX or 46,XY sex reversal), adrenal insufficiency, severe salt-wasting Nonclassic: 46,XY variable degrees of genital ambiguity, adrenal insufficiency.
Cholesterol side-chain cleavage enzyme deficiency	<i>CYP11A1</i>	Classic: phenotypic female (46,XX or 46,XY sex reversal), adrenal insufficiency, salt-wasting. Nonclassic: 46,XY variable degrees of genital ambiguity, adrenal insufficiency.

Abbreviations: CAH, congenital adrenal hyperplasia; CAH-X, congenital adrenal hyperplasia with *tenascin-X* impairment; StAR, steroidogenic acute regulatory protein

Table 2
The classification of common *CYP21A2* and *CYP11B1* mutations based on *in vitro* data

21-hydroxylase deficiency ²³⁻²⁵				
Group	Null	A	B	C
Phenotype		SW	SV	NC
<i>In vitro</i> activity of <i>CYP21A2</i>	0%	<1%	1–2%	20–60%
Mutations ^a	30kb deletion ^b 8bp deletion exon 6 cluster ^c p.Q318X p.R356W p.Leu307fs	IVS2-13A/C>G	p.I172N p.I77T	p.V281L p.R339H p.P453S p.P30L

11β-hydroxylase deficiency ^{66,67,70,76}		
Phenotype	Classic ⁷⁰	Nonclassic ⁷⁶
Mutations ^a	p.R448H p.R448C p.W247X p.W116G p.W116X p.W116C p.V129M p.L158P p.A165D p.K254_A259del p.R383Q p.P94L p.L299P p.T318M p.A331V p.R374Q p.F438del	p.P42S p.P42L p.R43Q p.H125R p.P135S p.F139L p.P159L p.A297V p.R332Q p.R383Q p.N133H p.M88I p.R366C p.T401A p.T319M p.F79I p.R138C

11 β -hydroxylase deficiency ^{66,67,70,76}		
Phenotype	Classic ⁷⁰	Nonclassic ⁷⁶
	p.L464dup p.V441G	p.R143W p.T196A

Abbreviations: SW, salt-wasting; SV, simple-virilizing; NC, nonclassic.

^aNomenclature at the protein level is based on conventional codon numbering.

^bAbout 4 percent are attenuated chimeras in Group C.

^cp.I236N, p.V237E, p.M239

Table 3

Frequency of *CYP21A2* mutations across various populations.

Country	p.P30L	IVS2-13A/C>G	8bp del	p.I172N	exon 6 cluster ^a	p.V281L	p.Leu307fs	p.Q318X	p.R356W	p.P453S	Del/Conv
USA ²³	0.8	23.4	0.5	12.6	1	12.6	0.3	3.3	3.6	0.5	30.5
Argentina ⁵⁵	0.7	20.6	0.8	8.2	2	26.2	ND	6.7	4.2	1.4	11.2
Netherlands ²⁵	0.3	28.1	4.3	ND	3	2.2	0.3	3.5	8.4	0.5	31.9
Sweden ⁵²	1.6	27.7	1.2	20.8	0.5	5.4	0.5	0.5	3.8	0.5	29.8
Brazil ⁵⁶	0.6	21.1	1.8	7.5	1.2	26.6	2.2	6.1	5.4	1.4	9
Germany ²⁴	2.6	30.3	1.6	19.7	1	2.9	0.3	4.8	4.5	0.3	27.4
Italy ⁵⁴	2.7	19.9	0.0	6.2	0	11.0	ND	8.2	0.0	ND	ND
France ⁵⁷	ND	20.5	2.7	8.9	5	16.7	1.2	3.9	ND	0	22.9
Spain ⁵⁸	2.6	22.4	3.9	1.3	0	15.8	2.0	3.9	3.9	ND	19.7
Mexico ¹¹³	8.5	47.9	2.1	11.7	0	8.5	1	4.2	7.4	2.1	1.0

Abbreviations: Del, deletions; Conv, conversions; ND, not detected

Nomenclature at the protein level is based on conventional codon numbering

^a p.I236N, p.V237E, p.M239K