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Spectrum of *CYP21A2* Variants: A Retrospective Single-center Study from a Tertiary Healthcare Center in Türkiye

Üçüncü Basamak Sağlık Merkezi Genetik Laboratuvarında *CYP21A2* Genine Yönelik Test Yapılan Olguların Retrospektif Olarak Değerlendirilmesi

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ABSTRACT

Objective: Congenital adrenal hyperplasia due to 21-hydroxylase deficiency is a common autosomal recessive disorder caused by pathogenic variants in the *CYP21A2* gene. The spectrum of variants differs significantly among populations because of complex genetic recombination events involving the neighbouring *CYP21A1P* pseudogene. This study aimed to evaluate the variant distribution, genotype frequencies, and clinical indications in a large Turkish cohort referred for *CYP21A2* genetic analysis.

Methods: A total of 252 pediatric patients who underwent *CYP21A2* gene sequencing and multiplex ligation-dependent probe amplification at a single tertiary center were retrospectively analyzed. Genotype and allele frequencies of classical point variants, small indels, large deletions, and pseudogene-derived micro-conversions were calculated and correlated with clinical referral indications.

Results: Among the 252 patients, 133 (52.8%) were, by both sequencing and copy number analyses, found to carry no reportable variants. The most frequent pathogenic variant identified was c.844G>T p.(Val282Leu), found in the homozygous state in 20 patients (7.9%) and in 77 alleles in total. Other prevalent, severe variants included the intron 2 splice-site variant c.293-13A/C>G and the pseudogene-derived p.(Gln319*) variant. Structural rearrangements, including exon-specific micro-conversions (most notably involving exon 4), large multi-exon deletions, and complex allele combinations such

ÖZ

Amaç: 21-hidroksilaz eksikliğine bağlı konjenital adrenal hiperplazi, *CYP21A2* genindeki patojenik varyantların neden olduğu, sık görülen bir otozomal resesif hastalıktır. Mutasyon spektrumu, komşu *CYP21A1P* psödogeni ile meydana gelen karmaşık genetik rekombinasyon olayları nedeniyle popülasyonlar arasında önemli ölçüde farklılık göstermektedir. Bu çalışma, *CYP21A2* genetik analizi için yönlendirilen geniş bir Türk kohortundaki varyant dağılımını, genotip frekanslarını ve klinik endikasyonları değerlendirmeyi amaçlamıştır.

Yöntemler: Tek bir üçüncü basamak merkezde *CYP21A2* gen dizilemesi ve çoklu ligasyon bağımlı prob amplifikasyonu uygulanan toplam 252 pediatrik hasta geriye dönük (retrospektif) olarak analiz edilmiştir. Klasik nokta mutasyonlarının, küçük indellerin (insersiyon/delesyon), büyük delesyonların ve psödogen kaynaklı mikro-konversiyonların genotip ve alel frekansları hesaplanmış ve klinik sevk endikasyonları ile ilişkilendirilmiştir.

Bulgular: Toplam 252 hasta arasında 133'ü (%52,8) hem dizileme hem de kopya sayısı analizleri için raporlanabilir bir varyanta sahip değil olarak saptanmıştır. Tespit edilen en sık patojenik varyant, 20 hastada (%7,9) homozigot durumda ve toplamda 77 alelde bulunan c.844G>T p.(Val282Leu) varyantı olmuştur. Diğer yaygın varyantlar arasında intron 2 splicing bölgesi mutasyonu c.293-13A/C>G ve psödogen kaynaklı p.(Gln319*) varyantı yer almıştır. En belirgin şekilde ekzon 4'ü içeren ekzona özgü mikro-konversiyonlar ve büyük çoklu ekzon

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ABSTRACT

as CAH-X, were detected in 21 patients (8.3%). Hirsutism, abnormal menstruation, and precocious puberty were the leading indications for genetic testing in adolescent cohorts, whereas scrotal/genital hyperpigmentation and abnormal neonatal screening predominated in infancy.

Conclusion: The genetic architecture of *CYP21A2* in Türkiye is characterized by a high prevalence of the non-classic c.844G>T p.(Val282Leu) variant alongside a diverse spectrum of severe pseudogene-mediated conversions and deletions. These findings underscore the clinical necessity of using parallel sequencing and copy number detection strategies to achieve accurate molecular diagnosis and genetic counselling in the Turkish population.

Keywords: Congenital adrenal hyperplasia, *CYP21A2* variants, population genetics, Turkish population

Öz

delesyonları dahil olmak üzere yapısal düzenlemeler, CAH-X gibi karmaşık alel kombinasyonları da dahil olmak üzere 21 hastada (%8,3) tespit edilmiştir. Hirsutizm, anormal menstrüasyon (adet düzensizliği) ve prekoks puberte (erken ergenlik), kohortta ileri yaş grubu için genetik test için önde gelen endikasyonlar iken; skrotal/genital hiperpigmentasyon ve anormal yenidoğan taraması süt çocukluğu (infant) döneminde baskın olmuştur.

Sonuç: Türkiye'deki *CYP21A2* genetik yapısı, klasik olmayan c.844G>T p.(Val282Leu) varyantının yüksek prevalansının yanı sıra, şiddetli psödogen aracılı konversiyonlar ve delesyonlardan oluşan çeşitli bir spektrum ile karakterizedir. Bu bulgular, Türk popülasyonunda doğru moleküler tanı ve genetik danışmanlık sağlamak için paralel dizileme ve kopya sayısı tespit stratejilerinin birlikte kullanılmasının klinik gerekliliğini vurgulamaktadır.

Anahtar Sözcükler: Konjenital adrenal hiperplazi, *CYP21A2* varyantları, popülasyon genetiği, Türk popülasyonu

INTRODUCTION

Congenital adrenal hyperplasia (CAH) encompasses a group of autosomal recessive enzymatic disorders characterized by impaired steroid hormone biosynthesis in the adrenal cortex. Over 95% of CAH cases result from 21-hydroxylase deficiency, which leads to a systemic accumulation of cortisol precursors (such as 17-hydroxyprogesterone) and a subsequent shift toward excessive adrenal androgen production (1). Depending on the residual enzymatic activity, the clinical spectrum ranges from severe, life-threatening classic forms (salt-wasting and simple virilizing) manifesting in infancy to milder, non-classic (late-onset) forms that present during childhood or adolescence with signs of hyperandrogenism, premature adrenarche, hirsutism, or menstrual irregularities (2).

The *CYP21A2* gene, which encodes the active 21-hydroxylase enzyme, is located within the highly complex major histocompatibility complex class III region on chromosome 6p21.3 (3). It spans approximately 3.4 kb across 10 exons and lies adjacent to a highly homologous, non-functional pseudogene, *CYP21A1P*, with which it shares approximately 98% exonic sequence identity, complicating testing and analysis. This tandem arrangement and structural homology predispose the locus to frequent intergenic recombination events. These include unequal crossing-over leading to large deletions or duplications (such as the CAH-X chimeric gene fusion with *TNXB*) and gene conversion events that transfer deleterious point variants from the inactive pseudogene to the active *CYP21A2* locus (4).

More than 200 pathogenic variants have been described in *CYP21A2*, although most disease-causing variants originate from a limited number of deleterious sequences transferred from the highly homologous pseudogene *CYP21A1P* through gene conversion and unequal recombination events. Large 30-kb deletions account for approximately 20-30% of classical CAH alleles and are typically associated with severe loss of 21-hydroxylase activity, although breakpoint location may occasionally result in milder phenotypes (5). Most affected individuals are compound heterozygotes carrying different variants on each allele, with disease severity generally determined by the less severe variant. In addition, approximately

10% of patients harbor CAH-X syndrome, a contiguous gene deletion involving both *CYP21A2* and *TNXB*, which combines features of CAH with connective tissue manifestations such as joint hypermobility, recurrent dislocations, hernias, and cardiovascular abnormalities (6).

Data regarding the comprehensive spectrum of *CYP21A2* alterations in Turkish populations, particularly in pediatric cohorts, remain limited. Given the marked genetic heterogeneity of the *CYP21A2* locus and the clinical importance of accurate molecular diagnosis, population-specific data are essential for improving diagnostic strategies. Therefore, this study aimed to characterize the distribution of disease-causing *CYP21A2* variants and structural rearrangements in a large referral cohort of 252 pediatric subjects evaluated at a tertiary medical genetics center in Türkiye, and to assess the additional diagnostic value of multiplex ligation-dependent probe amplification (MLPA) used alongside conventional Sanger sequencing. Characterization of the variational landscape in such large clinical series may contribute to the optimization of molecular diagnostic algorithms and future screening approaches.

MATERIALS AND METHODS

Study Population

This retrospective cohort study evaluated 252 pediatric patients who underwent molecular genetic screening for suspected 21-hydroxylase deficiency before the age of 18 (when the testing procedure was implemented) at the Medical Genetics Laboratory of University of Health Sciences Türkiye, Ankara Etlik City Hospital between 2022 and 2026. Patients were referred for a wide range of indications, including premature adrenarche or pubarche, precocious puberty, hirsutism, severe acne, polycystic ovary syndrome (PCOS) refractory to standard management, ambiguous genitalia, scrotal or labial hyperpigmentation, unexplained neonatal adrenal insufficiency, and positive or borderline neonatal screening results. Patients were divided into seven categories based on these symptoms: Definitive clinical diagnosis; disorders of sexual development' (ambiguous genitalia, scrotal/labial hyperpigmentation); puberty-related problems (menstrual problems, premature adrenarche or

pubarche, precocious puberty, hirsutism, severe acne, PCOS, short stature); fertility problems; abnormal screening tests; adrenal insufficiency; and family history. Ethics committee approval for the study was received from the Scientific Research Evaluation and Ethics Committee of University of Health Sciences Türkiye, Ankara Etlik City Hospital (approval number: AEŞH-BADEK1 2026-434/date: 06.05.2026). Informed consent forms for publication were obtained from the adult patients and from the legal guardians of the pediatric patients.

Genetic Analysis

Genomic DNA was isolated from peripheral blood samples using standard protocols. Following amplification, the 10 coding exons and flanking intronic regions were sequenced using automated Sanger sequencing (Seqline-EYS Medikal, İstanbul, Türkiye/ABI 3500xl DNA Analyzer). Sequence annotations were completed relative to the reference sequence NM_000500.5. To complement sequencing and systematically evaluate copy-number variations, large deletions, duplications, and gene conversions, MLPA was performed using the SALSA MLPA P050 kit (MRC Holland, Amsterdam, Netherlands). Detected variations were curated and classified according to the joint consensus guidelines of the American College of Medical Genetics and Genomics (7). Patients with biallelic compound variants and complex rearrangements, for whom parental Sanger sequencing and MLPA analyses could not confirm that the variants were in trans or were causative, were excluded from the study. Conversions were reported only if the findings were consistent across Sanger sequencing and MLPA analysis and if the results of the two tests correlated.

Statistical Analysis

Descriptive statistics were applied to summarize demographic features, genotype frequencies, and allele occurrences. Quantitative metrics are expressed as means and interquartile ranges, whereas categorical parameters are presented as absolute counts and percentages. This study focused mainly on genetic frequency profiles, excluding biochemical and clinical parameters. Cumulative variant prevalence was evaluated using reference genomic data sourced from three primary repositories: The Turkish Genome Project (TGP, v3.1; accession: June 2026), the Turkish Variome on the Franklin Genoox platform (accession: June 2026), and the Genome Aggregation Database (gnomAD v4.1.1; accession: June 2026).

RESULTS

Demographic and Clinical Characteristics

The study comprised 252 individuals aged 0 months (neonates) to 18 years. The sex distribution showed a female predominance: 150 females (59.5%) versus 102 males (40.5%), reflecting the broader clinical presentation of non-classic phenotypes during adolescence and young adulthood. The median age was 7.9 years (interquartile range, 0-13 years). Clinical referral indications varied markedly by age group: adolescent female patients presented primarily with hirsutism, abnormal menstruation, acne, or PCOS, whereas pediatric patients presented with precocious puberty, premature pubarche/adrenarche, or short stature. Infants and neonates presented with

scrotal or labial hyperpigmentation, ambiguous genitalia, clinical adrenal crisis, or abnormal neonatal dried-blood spot screening tests.

Regarding referral indications, an abnormal screening test was the primary reason for clinical admission, accounting for 23.8% (n = 60) of the cohort and substantially contributing to the high proportion of infant evaluations. Among adolescent and adult presentations, hyperandrogenism-related symptoms predominated; hirsutism was documented in 17% (n = 43) of all patients, and menstrual irregularities (abnormal menstruation) were reported in 12.7% (n = 32). A preliminary clinical diagnosis of CAH, requested by the referring physicians and based on consistent clinical and biochemical findings, was the indication in 13.2% (n = 34) of cases. Other less frequent referral pathways included a positive family history, PCOS phenotypes, isolated short stature, and overt adrenal insufficiency (Supplementary Material).

Genetic Findings

The analysis revealed a broad spectrum of molecular variants; however, no diagnostic pathogenic or likely pathogenic alterations were detected in a significant proportion of patients (n = 133, 52.8%). Among the molecularly confirmed cases, the most prevalent point variant was the c.844G>T p.(Val282Leu), detected in 77 alleles and accounting for a cohort allele frequency of 0.152. This variant was observed in both homozygous and heterozygous states (isolated or compound) across various clinical indications. Other frequently observed pathogenic variants included the intron 2 splice-site variant c.293-13A/C>G (n = 25, allele frequency: 0.049) and the missense variant c.1360C>T p.(Pro454Ser) (n = 13, allele frequency: 0.025). Notably, severe classical variants were identified in patients exhibiting classical CAH phenotypes: The nonsense variant c.955C>T p.(Gln319*) was found in 11 alleles (allele frequency: 0.021), while the missense alterations c.392G>A p.(Gly131Asp) (n = 8, allele frequency: 0.015) and c.518T>A p.(Ile173Asn) (n = 5, allele frequency: 0.009) were well represented in the classical presentation subgroup. In 43 patients, only one disease-causing variant was detected. In addition to pathogenic or likely pathogenic variants, 4 variants of unknown significance that were thought to be causative (as a result of phasing, biochemical profile, etc.) were reported.

Beyond single nucleotide variants, MLPA analysis identified complex structural rearrangements and copy number variations in 21 individuals. These included heterozygous or homozygous micro-conversions of exon 4, exon 4-8 and exon 4-9 micro-conversions, and various multi-exon deletions (e.g., deletion of exons 1-3). Heterozygous or homozygous micro-conversions in exon 4 were identified in 12 alleles, corresponding to a cohort frequency of 0.023. Complete gene deletions, large homozygous deletions characterizing the CAH-X syndrome, and total gene conversion events were also identified in a small subset of subjects (Supplementary Material).

DISCUSSION

This study provides a comprehensive overview of *CYP21A2* sequence variants and structural rearrangements in a Turkish pediatric referral cohort. The findings demonstrate genetic heterogeneity and confirm the importance of using complementary molecular techniques for accurate diagnosis.

The predominance of p.(Val282Leu) observed in this cohort is consistent with its recognized status as one of the most common *CYP21A2* variants associated with milder or non-classical forms of CAH. The frequent detection of this variant among individuals presenting with hirsutism, menstrual irregularities, and hyperandrogenic symptoms highlights its clinical relevance in endocrine referral populations. This variant has traditionally been associated with the non-classical form of CAH and is known to retain partial 21-hydroxylase activity, providing a biochemical basis for delayed diagnosis. This finding was consistently observed in our cohort, where carriers of this variant predominantly presented with milder, later-onset clinical manifestations. Consequently, as shown in Table 1, this variant exhibits a considerably higher frequency in population genomic databases compared with most other disease-causing *CYP21A2* alleles. Consistent with its relatively high carrier frequency, homozygosity for this variant was also observed in a substantial proportion of affected individuals within our cohort.

The recurrent identification of c.293-13A/C>G, p.(Gln319*), p.(Pro454Ser) further highlights the strong predominance of well-established pathogenic *CYP21A2* variants within our cohort. Overall, our findings demonstrate a clear dominance of a limited set of

approximately ten classical pathogenic variants that have been repeatedly reported in previous studies and are known to account for a substantial proportion of disease alleles in CAH populations (8). These variants have been consistently associated with markedly reduced enzymatic activity and are typically enriched among patients with classical forms of CAH.

In addition to this well-defined variant spectrum, we identified several rare variants that are currently classified as variants of uncertain significance according to ACMG criteria and may represent population-specific alleles or reflect enrichment in the Turkish cohort, such as c.865C>T p.(Leu289Phe), c.1352C>T p.(Thr451Met), c.86A>C p.(His29Pro). To date, there is limited large-scale clinical or functional evidence regarding the pathogenicity and genotype-phenotype correlations of these variants, and they are either absent from or only sparsely represented in genome databases (Table 1). Therefore, further investigation in larger, well-characterized cohorts, ideally supported by functional studies, will be essential to clarify their clinical relevance and refine genotype-phenotype interpretation in CAH.

Compared with neighbouring countries, the variant spectrum appears to resemble that of western neighbours more closely

Table 1. Allele frequencies of identified *CYP21A2* variants compared with reference datasets.

Variant (NM_000500.5)	Allele count (total n=504)	Cohort allele frequency	TGP frequency	TV frequency	gnomAD frequency
c.844G>T p.(Val282Leu)	77	0.152	0.018/14*	-	0.005515/2*
c.293-13A/C>G	25	0.049	0.0029	-	0.002420/7*
c.1360C>T p.(Pro454Ser)	13	0.025	0.0059/2*	-	0.006282/3*
c.955C>T p.(Gln319*)	11	0.021	0.012	-	0.0008961
c.392G>A p.(Gly131Asp)	8	0.015	0.000097	-	0
c.518T>A p.(Ile173Asn)	5	0.009	0.00058	-	0.0005579/3*
c.1447C>T p.(Pro483Ser)	5	0.009	0.0026	0.0022	0.0004815/20*
c.92C>T p.(Pro31Leu)	4	0.0079	0.00049	-	0.0001412/2*
c.961G>A p.(Glu321Lys)	2	0.0039	-	-	-
c.1069C>T p.(Arg357Trp)	5	0.009	0.000097	-	0.00003537
c.1019G>A p.(Arg340His)	2	0.0039	0.00068	0.001537	0.000009918
c.850A>G p.(Met284Val)	2	0.0039	0.000097	-	0.00004836
c.865C>T p.(Leu289Phe)	2	0.0039	0.000097	-	0.000009921
c.1352C>T p.(Thr451Met)	1	0.0019	-	-	0.00004909
c.397C>T p.(Arg133Cys)	1	0.0019	0.000097	0.000377	0.00006701/1*
c.86A>C p.(His29Pro)	1	0.0019	-	-	0.00000062
c.1174G>A p.(Ala392Thr)	1	0.0019	0.00078	-	0.007159
c.188A>T p.(His63Leu)	1	0.0019	0.000097	-	0.02901

*: Number of homozygous individuals. Structural changes were excluded, TGP: Turkish Genome Project, TV: Turkish Variome.

than that of eastern neighbours. Total sample sizes vary across studies, which may influence these observations. Moreover, when reports from geographically similar populations in the literature are collectively evaluated, a consistent theme is the presence of a distinct, country-specific genetic landscape, underscoring the unique population-specific structure of each cohort (9,10).

Overall, the detection rate of biallelic pathogenic variants was significantly higher in both the clinically compatible diagnosis group and the puberty-related disorders group, with a cumulative detection rate of 36.8% (35/95). In contrast, the molecular diagnostic yield was substantially lower in groups presenting with isolated menstrual problems or hirsutism and in those referred because of suspicious screening test results, with detection rates of 16.4% (11/67) and 3.3% (2/60), respectively. Among molecularly confirmed cases, monoallelic heterozygous variants were detected in 17.1% of patients, whereas biallelic alterations—comprising homozygous (16.7%) and compound heterozygous (13.5%) genotypes—comprised the remainder of the cohort (Table 2).

CAH is included in the neonatal screening program in Türkiye, and, correspondingly, an abnormal screening result constitutes a substantial proportion of the indication criteria for inclusion in our cohort (23.8%, n = 60). Additionally, the higher frequency of females in the cohort may be attributed to shunting of accumulated biochemical materials toward androgen production in cases of 21-hydroxylase deficiency. This leads to more frequent and more pronounced clinical presentations in females and naturally drives these individuals to seek medical attention.

Table 2. Cumulative distribution of CYP21A2 genotypes according to their zygosity in the cohort.

Genotype (Sanger sequence/MLPA)	Number of patients (n)	Percentage (%)
Wild-type (no pathogenic variant detected)	133	52.8
Monoallelic genotype [heterozygous only]	43	17.1
Biallelic genotype [homozygous]	42	16.7
Biallelic genotype [compound heterozygous]	34	13.5
Total	252	100

MLPA: Multiplex ligation-dependent probe amplification.

Study Limitations

This study has several limitations. First, because this study was a retrospective evaluation conducted at a single tertiary referral center, the cohort may be subject to referral bias and may not fully reflect the general Turkish population. The highly complex genetic architecture of this region, particularly the presence of pathogenic variants involving structural rearrangements, may not always be adequately detected by Sanger sequencing combined with MLPA analysis. In this context, long-read sequencing approaches are likely to provide additional clinical benefit. Additionally, variants considered to have no clear clinical impact—such as gene duplications, exon duplications, or combinations like p.Gln319* with

heterozygous total duplication—which are frequently encountered in analysis were not included in the frequency analyses, as the study was primarily focused on clinically oriented pathogenic variants (5). Because national genomic databases included a limited number of alleles at the time of this analysis, larger multicenter studies are required to establish definitive nationwide carrier frequencies for rare and non-classical structural variants.

CONCLUSION

The CYP21A2 variant landscape in Türkiye is characterized by a high prevalence of the non-classic c.844G>T p.(Val282Leu) variant and a diverse spectrum of severe pseudogene-mediated micro-conversions and macro-deletions. These findings emphasize the clinical importance of combining sequence analysis with copy number methodologies to ensure accurate molecular diagnosis, optimize clinical management, and guide genetic counseling for affected families.

Ethics

Ethics Committee Approval: Ethics committee approval for the study was received from the Scientific Research Evaluation and Ethics Committee of University of Health Sciences Türkiye, Ankara Etlik City Hospital (approval number: AEŞH-BADEK1 2026-434/date: 06.05.2026).

Informed Consent: Informed consent forms for publication were obtained from the adult patients and from the legal guardians of the pediatric patients.

Footnotes

Authorship Contributions

Concept: A.K., Design: A.K., Data Collection or Processing: A.K., F.B., A.B., H.B.E., Ş.S.E., A.Y.T., Analysis or Interpretation: A.K., F.B., H.B.E., Ş.S.E., A.B., Literature Search: A.K., Writing: A.K., F.B.

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