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Original Article

Genotype-Phenotype Correlation Analysis and Identification of a Novel Variant in Children with Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency

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ABSTRACT

Aim: Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is an autosomal recessive disorder caused by pathogenic variants in the *CYP21A2* gene. Although genotype is generally predictive of clinical phenotype, genotype-phenotype discordance may occur. This study aimed to evaluate genotype-phenotype correlations and to characterize the molecular spectrum of *CYP21A2* variants in children with CAH.

Materials and Methods: In this retrospective single-center study, 22 patients with CAH due to 21-hydroxylase deficiency were included. The patients were classified as salt-wasting (SW), simple-virilizing (SV), or non-classical (NC) according to clinical and hormonal findings. The detected variants were grouped according to their predicted residual enzyme activity, and genotype-phenotype concordance was evaluated.

Results: Eight patients (36.4%) had the SW phenotype, six (27.3%) had the SV phenotype, and eight (36.4%) had the NC phenotype. The most frequent variant was I2 splice (c.293-13C>G) (25%), followed by I172N (c.515T>A; p.Ile172Asn) (14.2%). Overall genotype-phenotype concordance was observed in 20 of the 22 patients (90%), and genotype severity groups were significantly associated with the clinical phenotype (p=0.001). One homozygous I172N patient exhibited an unexpectedly mild NC phenotype despite an anticipated SV phenotype. A previously unreported compound heterozygous variant, P30L(c.92C>T)/p.E321Afs*60 (c.961_962delGA), was identified in one patient with the NC phenotype.

Conclusion: Our findings demonstrate a strong overall genotype-phenotype correlation in CAH, while also highlighting that patients carrying moderate-severity or novel variants may present with unexpectedly mild or variable clinical phenotypes. Integration of molecular findings with detailed clinical, hormonal, and longitudinal follow-up data remains essential for accurate phenotypic prediction.

Keywords: Congenital adrenal hyperplasia, *CYP21A2*, 21-hydroxylase deficiency, genotype-phenotype correlation, novel variant

Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders resulting from enzyme deficiencies in the adrenal steroid biosynthesis pathway (1). These enzyme defects result in decreased cortisol synthesis, which leads to adrenocorticotropic hormone (ACTH) secretion. This causes hyperplasia and the accumulation of steroid precursors in the adrenal cortex (2,3).

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The most common cause of CAH is 21-hydroxylase deficiency, accounting for 90-95% of cases (4,5). 11-beta-hydroxylase deficiency accounts for approximately another 5% of cases.

Although the incidence of CAH varies across populations, studies based on data from approximately 6.5 million newborns screened worldwide indicate that the overall incidence of classic CAH is 1 in 13,000 to 1 in 15,000 live births (2,6). These differences may be related to ethnicity, consanguineous marriage rates, and the effectiveness of newborn screening programs (2,7).

21-hydroxylase deficiency results from pathogenic variants in the *CYP21A2* gene, located on the short arm of chromosome 6 (6p21.3) (8). This disease presents in three main clinical phenotypes: the classic salt-wasting (SW) form, the classic simple-virilizing (SV) form, and the non-classic (NC) (late-onset) form (9). The clinical spectrum ranges from severe neonatal salt-losing crises to mild forms presenting with hyperandrogenism in adolescence or adulthood (3,10).

Numerous variants leading to CAH have been identified, and many have been reported to correlate strongly with the clinical phenotype (2,4,11). Therefore, genotype analysis provides several major contributions, especially in prenatal diagnosis, disease prognosis prediction, and cases which are difficult to classify clinically (2,4,12). In addition, recent advances in molecular genetic techniques have allowed for a better understanding of the genotype-phenotype relationship in the diagnosis and follow-up of CAH (9). Genotype-phenotype mismatches in CAH may be influenced by residual enzyme activity, modifier genes, androgen receptor sensitivity, and epigenetic factors, resulting in variable clinical severity despite identical genotypes (2,12).

This study aimed to evaluate the genotype-phenotype correlation in those patients with CAH due to 21-hydroxylase deficiency and to provide an individualized assessment of rare variants and clinically discordant cases.

Materials and Methods

This retrospective single-center study included 22 patients diagnosed with CAH due to 21-hydroxylase deficiency and a molecularly confirmed *CYP21A2* gene mutation. We retrospectively reviewed patient data via their pediatric endocrinology outpatient clinic records and the hospital information management system. We included in this study those patients who had complete clinical, hormonal, and genetic data. We excluded those cases with other adrenal steroidogenesis disorders and those with incomplete follow-up data from this study.

We obtained demographic characteristics, age at presentation, age at diagnosis, gender, presenting complaints, physical examination findings, height, weight, body mass index (BMI), bone age, stage of puberty, Prader stage in female patients, and the type and dosage of treatment from the patient file records.

Height, weight, and BMI standard deviation scores (SDS) were calculated according to age and sex. Bone age was assessed using the Greulich and Pyle atlases, and the bone age/chronological age ratio was calculated (13). In the laboratory evaluation, baseline levels of 17-hydroxyprogesterone (17-OHP), ACTH, cortisol, and total testosterone were recorded at the time of diagnosis and at the last follow-up.

The patients were divided into three phenotypic groups based on their clinical and hormonal characteristics: SW type, SV type, and NC type. The SW phenotype was defined as those cases meeting the criteria for hyponatremia, hyperkalemia, dehydration, and elevated plasma renin activity. The SV phenotype was defined as those patients with prenatal or postnatal androgen-excess findings, without mineralocorticoid deficiency. The NC phenotype included patients presenting with late-onset hyperandrogenism, premature pubarche, or hirsutism (14).

CYP21A2 gene variant analyses of the patients were performed using reverse dot blot analysis, Sanger sequencing, and multiplex ligation-dependent probe amplification (MLPA). Variant nomenclature was reported according to the Human Genome Variation Society recommendations. Novel variants were interpreted according to American College of Medical Genetics and Genomics (ACMG) variant classification criteria.

The detected variants were grouped into four categories based on the literature classification of residual 21-hydroxylase enzyme activity:

Group 1: null mutations (gene deletion, conversion, 8-bp deletion, E6 cluster, Q318X (c.955C>T;p.Gln319Ter),R356W(c.1066C>T;p.Arg356Trp),

Group 2: I2 splice (c.293-13C>G) homozygous or compound heterozygous variants with severe mutations,

Group 3: I172N (c.515T>A; p.Ile172Asn) homozygous or compound heterozygous mutations with severe variants,

Group 4: mild mutations V281L (c.844G>T; p.Val282Leu), P30L (c.92C>T; p.Pro31Leu), P453S (c.1358C>T; p.Pro453Ser).

According to this grouping, the expected phenotypes were SW, SW/SV, SV, and NC, respectively. Genotype-phenotype concordance was evaluated by comparing the expected and observed phenotypes.

Ethical Consideration

Ethical approval for this study was obtained from the Non-Interventional Research Ethics Committee of İzmir Katip Çelebi University Faculty of Medicine on September 29, 2016, with decision number 220.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). Due to the small sample size, the distribution of continuous variables was evaluated using non-parametric methods, and data are presented as median (minimum-maximum) or mean ± standard deviation, as appropriate.

The Kruskal-Wallis test was used to compare continuous variables across the three phenotype groups, and the Wilcoxon signed-rank test was used for intragroup comparisons between the diagnostic and last follow-up measurements. Categorical variables are expressed as numbers and percentages. The association between genotype severity groups and observed phenotypes was evaluated using Fisher's exact test. A p value <0.05 was considered statistically significant.

Results

A total of 22 patients with genetically confirmed CAH due to 21-hydroxylase deficiency were included in this study. Eight patients (36.4%) had the SW phenotype, six (27.3%) had the SV phenotype, and eight (36.4%) had the NC phenotype. The SW group consisted of 5 boys and 3 girls, the SV group consisted entirely of girls, and the NC group consisted of 5 girls and 3 boys.

Age at diagnosis differed significantly between the phenotype groups (p=0.006). The median age at diagnosis was 1.6 months (0.5-4.0) in the SW group, 5.6 months (0.3-72.0) in the SV group, and 76.8 months (1.0-198.0) in the NC group. Those patients with the SW and SV phenotypes were diagnosed at significantly earlier ages than those in the NC group.

BMI SDS showed an increasing trend in all phenotype groups during the follow-up period. This increase was more pronounced in the classic phenotype patients, and at the last follow-up, BMI SDS values had moved into the positive range in all groups. The individual clinical presentations, phenotypes, and genotypes of the patients are presented in Table I.

No significant difference was found between the phenotype groups in terms of their baseline 17-OHP and ACTH levels (p>0.05). However, cortisol levels were higher in the SW and NC groups when compared to the SV group (p=0.028). Total testosterone levels were significantly higher in the SV group in comparison to the other two groups (p=0.026). There were no significant differences among the phenotype groups in terms of their follow-up hormone values.

Table I. Patients' presenting symptoms, phenotypes, and detected genetic mutations

Patient	Gender	Presenting symptoms	Phenotype	Allele 1	Allele 2	Allelic interpretation
1	Male	Vomiting	SW	R356W (c.1066C>T; p.Arg356Trp)	R356W (c.1066C>T; p.Arg356Trp)	Homozygous
2	Female	Vomiting, ambiguous genitalia	SW	I2splice (c.293-13C>G) + Q318X (c.955C>T; p.Gln319Ter)	Q318X (c.955C>T; p.Gln319Ter)+ E6 cluster + R356W (c.1066C>T; p.Arg356Trp)	Complex alleles
3	Female	Ambiguous genitalia, hyponatremia	SW	I2 splice (c.293-13C>G)+ Q318X (c.955C>T; p.Gln319Ter)+ E6 cluster + R356W (c.1066C>T; p.Arg356Trp)	R356W (c.1066C>T; p.Arg356Trp)	Complex allele
4	Male	Vomiting	SW	I2 splice (c.293-13C>G)	I2 splice (c.293-13C>G)	Homozygous
5	Male	Vomiting, diarrhea	SW	Exon 1-7 conversion	Exon 1-3 conversion	Compound heterozygous conversion
6	Female	Ambiguous genitalia	SW	Q318X (c.955C>T; p.Gln319Ter)	Q318X (c.955C>T; p.Gln319Ter)+ E6 cluster + R356W (c.1066C>T; p.Arg356Trp)	Complex allele
7	Male	Vomiting	SW	I2 splice (c.293-13C>G)	I2 splice (c.293-13C>G)	Homozygous

Table I. Continued

Patient	Gender	Presenting symptoms	Phenotype	Allele 1	Allele 2	Allelic interpretation
8	Male	Vomiting	SW	No mutation detected	No mutation detected	Mutation-negative clinically confirmed case
9	Female	Clitoromegaly	SV	I172N (c.515T>A; p.Ile172Asn)	I2 splice (c.293-13C>G)	Compound heterozygous
10	Female	Ambiguous genitalia	SV	I2 splice (c.293-13C>G)	I2 splice (c.293-13C>G)	Homozygous
11	Female	Clitoromegaly	SV	I172N (c.515T>A; p.Ile172Asn)	I172N (c.515T>A; p.Ile172Asn)	Homozygous
12	Female	Ambiguous genitalia	SV	I172N (c.515T>A; p.Ile172Asn)	I2 splice (c.293-13C>G)	Compound heterozygous
13	Female	Ambiguous genitalia	SV	I2 splice (c.293-13C>G)	I2 splice (c.293-13C>G)	Homozygous
14	Female	Ambiguous genitalia	SV	P30L (c.92C>T; p.Pro31Leu) + I2 splice (c.293-13C>G) + 8-bp deletion	P30L (c.92C>T; p.Pro31Leu)+I2 splice c.293-13C>G	Complex alleles
15	Male	Premature pubarche	NC	I172N (c.515T>A; p.Ile172Asn)	I172N (c.515T>A; p.Ile172Asn)	Homozygous
16	Female	Premature pubarche	NC	Q318X (c.955C>T; p.Gln319Ter) + V281L (c.844G>T; p.Val282Leu)	V281L (c.844G>T; p.Val282Leu) + I172N (c.515T>A; p.Ile172Asn)	Complex alleles
17	Female	Premature pubarche	NC	V281L (c.844G>T; p.Val282Leu)	P30L (c.92C>T; p.Pro31Leu)	Compound heterozygous
18	Male	Premature pubarche	NC	P30L (c.92C>T; p.Pro31Leu)	c.961_962delGA (p.Glu321Alafs*60)	Compound heterozygous novel variant
19	Female	No symptoms	NC	P453S (c.1358C>T; p.Pro453Ser)	P453S (c.1358C>T; p.Pro453Ser)	Homozygous
20	Male	Premature pubarche	NC	p.R340H c.1019G>A (p.Arg340His) + P453S (c.1358C>T; p.Pro453Ser)	P453S (c.1358C>T; p.Pro453Ser)	Complex allele/ compound heterozygous
21	Female	Premature pubarche	NC	P453S (c.1358C>T; p.Pro453Ser)	P453S (c.1358C>T; p.Pro453Ser)	Homozygous
22	Female	Virilization	NC	V281L (c.844G>T; p.Val282Leu)	I172N (c.515T>A; p.Ile172Asn)	Compound heterozygous

*Traditional *CYP21A2* variant nomenclature is presented together with HGVS nomenclature. Variant nomenclature has been reported according to HGVS recommendations using the *CYP21A2* reference transcript NM_000500.9
SW: Salt-Wasting, SV: Simple-virilizing, NC: Non-classical, HGVS: Human Genome Variation Society

The detailed clinical, auxological, hormonal, and longitudinal follow-up characteristics of the phenotype groups are summarized in Table II. The individual clinical presentations, Prader stages, pubertal characteristics, surgical interventions, and genotypes of the patients are presented in Table III.

Molecular analysis identified a total of 56 variant events representing 17 distinct variants across 44 alleles from 22 patients. As some alleles carried multiple pathogenic changes, the total number of detected variant events exceeded the number of alleles analyzed. One patient exhibited a clinically significant SW phenotype, but no mutation could be demonstrated. The most frequent variant was I2 splice (c.293-13C>G) (25%), followed by I172N (c.515T>A; p.Ile172Asn) (14.2%). These were followed by R356W (c.1066C>T; p.Arg356Trp) (10.7%), Q318X (c.955C>T; p.Gln319Ter) (10.7%), and P453S (c.1358C>T; p.Pro453Ser) (10.7%). The distribution and frequencies of the detected variants according to phenotype groups are summarized in Table IV.

Table II. Clinical, auxological, and hormonal characteristics according to phenotype groups

	Salt-wasting (n=8)	Simple-virilizing (n=6)	Non-classical (n=8)	p value
Age at diagnosis (months)	1.6±1.3	5.6±4.2	76.8±63	0.006
Target height SDS at diagnosis	-0.87±1.4	-0.45±1.17	0.46±1.87	0.2
Target height SDS at last follow-up	0.08±1.15	0.92±0.7	0.30±1.49	0.4
Height SDS at diagnosis	0.17±2	-0.02±1.3	1.4±1.6	0.161
Height SDS at last follow-up	-1.64±0.82	-0.36±1.5	-0.55±2.03	0.2
BMI SDS at diagnosis	-1.67±1.09	-0.82±1.73	-0.45±2.2	0.2
BMI SDS at last follow-up	1.2±1.2	1.39±0.49	1.01±1.3	0.9
Basal 17-OHP at diagnosis (ng/mL)	58.6 (4.4-207)	36.5 (1.9-110)	23.4 (3.2-41.8)	0.5
Basal 17-OHP at last follow-up (ng/mL)	27.83 (0.08-128)	20.9 (1.08-27)	9.7 (0.40-32.1)	0.2
ACTH at diagnosis (pg/mL)	42.8 (18-70)	153.5 (13-338)	48.3 (29-100)	0.5
ACTH at last follow-up (pg/mL)	19.6 (2-55)	152.3 (3-347)	30.4 (2-138)	0.2
Basal cortisol at diagnosis (µg/dL)	17.07 (1-50)	10.7 (4.9-18)	21.2 (14.2-27)	0.028
Basal cortisol at last follow-up (µg/dL)	8.63 (0.5-34)	9.07 (3-14.8)	10.1 (1-33.8)	0.7
Testosterone at diagnosis (ng/dL)	136.9 (0.4-1014)	494.7 (23.4-1600)	40.3 (0.17-107)	0.026
Testosterone at last follow-up (ng/dL)	32.2 (1.2-163.10)	58.7 (15-161)	34.1 (0.12-115)	0.23

Data are presented as mean±standard deviation or median (minimum-maximum), as appropriate.
SDS: Standard deviation score, BMI: Body mass index, 17-OHP: 17-hydroxyprogesterone, ACTH: Adrenocorticotropic hormone

Table III. Individual clinical characteristics including prader staging, pubertal development, and surgical outcomes

Patient ID	Phenotype	Prader stage at diagnosis	Pubertal stage at diagnosis	Surgical procedure	Age at surgery (years)
2	SW	3	1	Vaginal reconstruction + clitoroplasty	9
3	SW	3	1	None	-
6	SW	4	1	None	-
9	SV	3	1	Clitoroplasty	4
10	SV	4	1	Clitoroplasty	3
11	SV	4	1	Clitoroplasty	3
12	SV	4	1	Vaginal reconstruction + Clitoroplasty	12
13	SV	4	1	None	-
14	SV	3	1	Clitoroplasty	5
15	NC	2	2	None	-
16	NC	1	4	None	-
18	NC	1	5	None	-
20	NC	2	1	None	-
22	NC	1	5	None	-

SW: Salt-wasting, SV: Simple-virilizing, NC: Non-classical

When the variant distribution according to phenotype groups was examined, the most frequent variants were I2 splice, R356W, and Q318X in the SW phenotype; I2 splice and I172N in the SV phenotype; and P453S, V281L, and I172N in the NC phenotype (Table IV). A previously unreported P30L (c.92C>T)/p. E321Afs*60 (c.961_962delGA) variant was detected in one patient. According to ACMG variant classification criteria, this novel variant was considered *likely pathogenic* based on the presence of Pathogenic Very Strong 1 (PVS1) and Pathogenic Moderate 2 (PM2) evidence (15). The distribution of detected *CYP21A2* variants across the gene is summarized in a lollipop plot in Figure 1.

For genotype-phenotype correlation analysis, the patients were divided into four groups based on their residual enzyme activity. In Groups 1 and 2, the expected and observed phenotypes were completely concordant in all patients. In Group 3, one homozygous I172N patient exhibited the NC phenotype instead of the expected SV phenotype. In Group 4, the expected and observed NC phenotypes were completely concordant in all patients. The results of the genotype-phenotype concordance are presented in Table V. The genotype severity groups were significantly associated with the observed clinical phenotypes (p=0.001). Discordance was detected in only one case of homozygous I172N.

Table IV. Distribution of detected ***CYP21A2*** variant events and estimated residual enzyme activities

Variant	Protein change	Estimated residual enzyme activity	Salt-wasting (n=22)	Simple-virilizing (n=15)	Non-classical (n=19)	Total (n=56)
R356W (c.1066C>T)	p.Arg356Trp	0% (null)	6 (27.2%)	-	-	6 (10.7%)
Q318X (c.955C>T)	p.Gln319Ter	0% (null)	5 (22.7%)	-	1 (5.2%)	6 (10.7%)
E6 cluster	-	0% (null)	3 (13.6%)	-	-	3 (5.3%)
I2 splice (c.293-13C>G)	Splice-site variant	<1%	6 (27.2%)	8 (53.3%)	-	14 (25%)
Conversion	-	0% (null)	2 (9%)	-	-	2 (3.5%)
I172N (c.515T>A)	p.Ile172Asn	~1-2%	-	4 (26.6%)	4 (21%)	8 (14.2%)
P30L (c.92C>T)	p.Pro31Leu	~30-60%	-	2 (13.3%)	2 (10.5%)	4 (7.1%)
8-bp deletion (c.332_339del)	Frameshift variant	0% (null)	-	1 (6.6%)	-	1 (1.7%)
V281L (c.844G>T)	p.Val282Leu	~20-50%	-	-	4 (21%)	4 (7.1%)
P453S (c.1358C>T)	p.Pro453Ser	~20-50%	-	-	6 (31.5%)	6 (10.7 %)
p.R340H c.1019G>A	p.Arg340His	Unknown/likely mild-modifying	-	-	1 (5.2%)	1 (1.7%)
p.E321Afs*60 (c.961_962delGA)	p.Glu321Alafs*60	Unknown; frameshift component likely severe	-		1 (5.2%)	1 (1.7%)

Variant nomenclature follows Human Genome Variation Society (HGVS) recommendations using the ***CYP21A2*** reference transcript NM_000500.9.
Estimated residual enzyme activities and mutation severity classifications were derived from previously published functional and genotype-phenotype studies (1,2,12,15,20,25).
Null variants include non-sense, frameshift, splice-site, large conversion, and severe cluster variants associated with absent or markedly impaired 21-hydroxylase activity.

Table V. Genotype severity groups, hormonal characteristics, and genotype-phenotype concordance in patients with 21-hydroxylase deficiency

Group	Detailed genotype classification	n	SW	SV	NC	Median basal 17-OHP (ng/mL)	Median basal cortisol (μg/dL)	Expected phenotype	Genotype-phenotype concordance	Comments	p value
Group 1	**Null/severe variants**	5	5	-	-	20.0	15.0	SW	100%		
	R356W (c.1066C>T; p.Arg356Trp) / R356W (c.1066C>T; p.Arg356Trp)	1	1	-	-			SW	100%		
	Q318X (c.955C>T; p.Gln319Ter) / E6 cluster / R356W (c.1066C>T; p.Arg356Trp) / I2 splice (c.293-13C>G)	3	3	-	-			SW	100%		
	Large conversion variants	1	1	-	-			SW	100%		
Group 2	**Severe splice variants**	5	2	3	-	108.0	8.2	SW-SV	100%		
	I2 splice (c.293-13C>G) / I2 splice (c.293-13C>G)	4	2	2	-			SW-SV	100%		
	I2 splice (c.293-13C>G) / I2 splice (c.293-13C>G) / 8-bp deletion / P30L (c.92C>T; p.Pro31Leu) / P30L (c.92C>T; p.Pro31Leu)	1	-	1	-			SW-SV	100%		
Group 3	**Moderate residual activity variants**	4	-	3	1	14.0	14.0	SV	75% overall		
	I172N (c.515T>A; p.Ile172Asn) / I172N (c.515T>A; p.Ile172Asn)	2	-	1	1			SV	50%	One patient presented with an NC phenotype despite homozygous I172N genotype. Similar discordant cases were reported by Lao et al. (20) supporting phenotypic plasticity associated with moderate residual enzyme activity.	
	I172N (c.515T>A; p.Ile172Asn) / I2 splice (c.293-13C>G)	2	-	2	-			SV	100%		
Group 4	**Mild/non-classical variants**	6	-	-	6	24.0	20.95	NC	100%		
	I172N (c.515T>A; p.Ile172Asn) / V281L (c.844G>T; p.Val282Leu)	1	-	-	1			NC	100%		
	Q318X (c.955C>T; p.Gln319Ter) / I172N (c.515T>A; p.Ile172Asn) / V281L (c.844G>T; p.Val282Leu)	1	-	-	1			NC	100%		
	V281L (c.844G>T; p.Val282Leu) / P30L (c.92C>T; p.Pro31Leu)	1	-	-	1			NC	100%		
	P453S (c.1358C>T; p.Pro453Ser) / P453S (c.1358C>T; p.Pro453Ser)	2	-	-	2			NC	100%		
	P453S (c.1358C>T; p.Pro453Ser) / P453S (c.1358C>T; p.Pro453Ser) / p.R340H (c.1019G>A; p.Arg340His)	1	-	-	1			NC	100%		

Table V. Continued

Group	Detailed genotype classification	n	SW	SV	NC	Median basal 17-OHP (ng/mL)	Median basal cortisol (µg/dL)	Expected phenotype	Genotype-phenotype concordance	Comments	p value
Others	**Unclassified/novel variants or mutation-negative cases**	2	1	-	1	12.2	36.0				
	P30L (c.92C>T; p.Pro31Leu) / p.E321Afs*60 (c.961_962delGA); *p.Glu321Alafs*60)	1	-	-	1			SW/SV	-	Previously unreported compound heterozygous variant NC phenotype despite likely pathogenic frameshift variant; possible residual activity effect of P30L allele.	
	No mutation detected	1	1	-	-			Undefined	-	Clinically and hormonally confirmed SW phenotype despite negative molecular testing. Similar mutation-negative cases have been described in previous cohorts (6) and may reflect deep intronic variants or complex RCCX rearrangements undetectable by standard Sanger/MLPA methods.	
Total		22	8	6	8				90%	Strong overall genotype-phenotype correlation	*p=0.001

Variant nomenclature follows Human Genome Variation Society (HGVS) recommendations using the *CYP21A2* reference transcript NM_000500.9.
Median basal 17-OHP and cortisol values were calculated according to genotype severity groups using baseline hormonal measurements obtained at diagnosis.
*Genotype severity groups were significantly associated with observed clinical phenotypes (p=0.001). However, interpretation should be made cautiously because of the limited sample size and sparse subgroup distribution.
SW: Salt-Wasting, SV: Simple-Virilizing, NC: Non-classical, 17-OHP: 17-hydroxyprogesterone, MLPA: multiplex ligation-dependent probe amplification

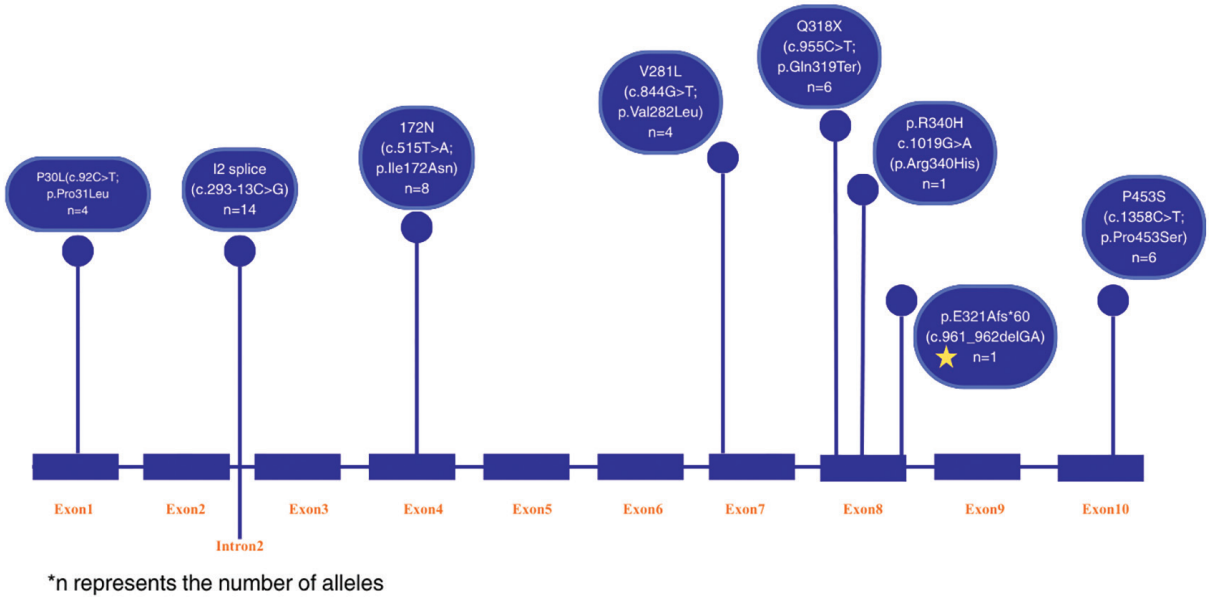

Figure 1. Lollipop plot demonstrating the distribution of *CYP21A2* variants identified in the cohort

Discussion

In this study, we evaluated genotype-phenotype correlation in pediatric patients with CAH due to 21-hydroxylase deficiency and identified a strong overall concordance between the *CYP21A2* genotype and the clinical phenotype. The I2 splice variant was the most frequently detected mutation, particularly among those patients with classical phenotypes, while I172N was mainly associated with the SV phenotype. A genotype-phenotype discordance was observed in one patient carrying homozygous I172N variants. In addition, we identified a previously unreported compound heterozygous variant, P30L (c.92C>T)/p.E321Afs*60 (c.961_962delGA), which may contribute to the expanding molecular spectrum of *CYP21A2*-related disease.

Complete phenotypic concordance was observed in Groups 1 and 2, which included null mutations and severe splice-site variants associated with an absence of enzyme activity, highlighting the importance of *CYP21A2* genotyping in predicting the severity of classical CAH. This finding is consistent with the strong correlation reported by New et al. (12) in their large cohort of 1,507 families. Furthermore, Speiser and White emphasized in their review that severe mutations were particularly strongly associated with the SW phenotype (16).

In our study, the most frequent variant was the I2 splice, which was consistent with previous reports (3,8,10,17). Baş et al. (6) identified the I2 splice as the most frequent variant (22%), primarily observed in patients with SW and SV forms. Similarly, in a large Turkish series of 124 patients reported by Kirac et al. (18), splice-site and deletion/conversion mutations represented the majority of the detected *CYP21A2* variants. The predominance of the I2 splice variant, particularly among those patients with classical phenotypes, further supports the phenotype-determining importance of this mutation in the Turkish population.

We detected the I172N variant as the second most frequent in our study. It was mostly associated with the SV phenotype, consistent with previous reports (10,16,19). In Turkish, Korean, and Chinese pediatric series, this variant has been shown to be frequently associated with the SV phenotype (3,6,10,18). One of the most striking findings in our cohort was the observation of a NC phenotype in a patient homozygous for the I172N variant (Case 15), rather than the expected SV clinical presentation. While the *CYP21A2* genotype is generally a robust predictor of clinical course, such discordances have been reported in 10% to 20% of cases in the literature (12,15,17). This phenotypic variability is particularly prevalent in variants with moderate residual enzyme activity, such as I172N. According to a comprehensive study by Lao et al. (20), which analyzed 457 individuals across the genotypic spectrum, the I172N variant represents a critical "threshold" in adrenal steroidogenesis. The I172N mutation typically retains approximately 1% to 2% of residual 21-hydroxylase activity, a level which is usually sufficient to prevent neonatal SW crises but resides on the borderline between the SV and NC phenotypes (20).

Consistent with our findings, Lao et al. (20) provided evidence that the clinical expression of homozygous I172N is not monolithic; they identified several instances where individuals shared this genotype yet presented with a milder NC phenotype rather than the classic SV form. This "phenotypic plasticity" suggests that the final clinical outcome is modulated by factors beyond the primary *CYP21A2* sequence. Phenotypic variability in those patients carrying the I172N variant may be influenced by alternative splicing efficiency, androgen receptor sensitivity, extra-adrenal steroid metabolism, and/or modifier genes affecting residual enzyme activity (2,12,16,21-23). Our study identified a patient with a SW phenotype in whom no mutation was detected by standard Sanger sequencing and MLPA (Case 8). While seemingly contradictory, this phenomenon has been reported in other cohorts, including those by Lao et al. (20) and Baş et al. (6). These cases likely represent the technical limitations of the current diagnostic panels in identifying promoter region variants, deep intronic mutations, or complex RCCX module rearrangements (2,5,12,24). In conclusion, our data, supported by the recent large-scale findings of Lao et al. (20), underscore that while genotyping is essential for prognostic prediction, it must be interpreted with caution. The existence of "grey zones", especially for moderate variants such as I172N, highlights the necessity of a multidisciplinary approach which integrates molecular data with longitudinal clinical observation and biochemical monitoring in order to optimize patient management.

The novel P30L (c.92C>T)/ p.E321Afs*60 (c.961_962delGA) variant identified in our cohort appears to be rare and, to the best of our knowledge, has not been previously reported in the literature. Numerous novel variants have been identified in different series in the Turkish population previously (1,18). The novel frameshift variant c.961_962delGA (p.Glu321Alafs*60) was classified as *likely pathogenic* according to ACMG criteria based on the presence of PVS1 and PM2 evidence. Since frameshift variants are generally expected to result in a severe loss of enzyme activity through the disruption of the normal protein sequence and premature termination, a more severe clinical phenotype might be anticipated (5,20). However, the patient carrying this variant presented with a NC phenotype in compound heterozygosity with the mild P30L (c.92C>T) variant. This observation suggests that residual enzymatic activity associated with the P30L allele may partially mitigate the clinical severity of this novel variant (20). In addition, the discrepancy between predicted molecular severity and observed phenotype further supports the concept that clinical expression in *CYP21A2* deficiency may be influenced by additional genetic and biological modifiers beyond the primary genotype alone.

Beyond genotype-phenotype concordance, our cohort also demonstrated distinct clinical and hormonal differences among the phenotype groups. Those patients with classical phenotypes were diagnosed at earlier ages and showed more severe virilization, greater skeletal maturation advancement, and increased rates of genital corrective surgery compared to those with the NC phenotype. In addition, the increase in BMI SDS observed during follow-up, particularly among those patients with classical phenotypes, may reflect the long-term metabolic consequences of chronic glucocorticoid exposure, which has been reported previously in patients with CAH (25,26). Together, these findings suggest that detailed longitudinal clinical characterization may provide important complementary information beyond molecular analysis alone.

Study Limitations

The main limitations of this study include its retrospective single-center design and its relatively small sample size. Functional analyses could not be performed for the novel variant. However, this study provides detailed longitudinal characterization, comprehensive genotype-phenotype analysis, and the identification of a novel likely pathogenic *CYP21A2* variant.

Conclusion

Our study demonstrates a strong genotype-phenotype correlation in those patients with CAH due to 21-hydroxylase deficiency, while also highlighting that moderate-severity and novel variants may present with unexpectedly mild or variable clinical phenotypes. The individualized evaluation of clinically discordant cases provides additional insight into the complexity of phenotypic expression in *CYP21A2* deficiency. Therefore, integrating molecular findings with clinical, hormonal, and longitudinal follow-up data remains essential for accurate phenotypic prediction and long-term patient management.

Ethics

Ethics Committee Approval: Ethical approval for this study was obtained from the Non-Interventional Research Ethics Committee of İzmir Katip Çelebi University Faculty of Medicine on September 29, 2016, with decision number 220.

Informed Consent: Retrospective cohort study.

Footnotes

Authorship Contributions

Concept: G.Ç., B.N.D., Design: N.M.B., G.Ç., B.N.D., Data Collection or Processing: N.M.B., C.K., Analysis or Interpretation: N.M.B., B.N.D., Literature Search: G.Ç., C.K., B.N.D., Writing: N.M.B., G.Ç., C.K.

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