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Genotype-phenotype correlation in 27 pediatric patients in congenital adrenal hyperplasia due to 21-hydroxylase deficiency in a single center

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Purpose: The purpose of the study was to evaluate endocrine patterns of patients with congenital adrenal hyperplasia and each gene mutation and to analyze the correlation between each phenotype and genotype.

Methods: This was a retrospective study of the patients with congenital adrenal hyperplasia in the pediatric outpatient clinic at the Samsung Medical Center from November 1994 to December 2012. We analyzed the medical records of 27 patients (male, 19; female, 8) with congenital adrenal hyperplasia who had been diagnosed by genetic testing to have 21-hydroxylase deficiency.

Results: In genetic analysis of 54 alleles from 27 patients, 13 types of mutations were identified. The distribution of 21-hydroxylase deficiency gene mutations revealed that intron 2 splice site (c.293-13A/C > G) mutations and large deletions were the most common, at 31.5% and 22.2% respectively, followed by p.I173N, p.R356W, and p.I172N mutations at 11.1%, 9.3%, and 9.3%, respectively. Other mutations were observed at 1.9–3.7%. No novel mutations were detected

Conclusion: The analysis of 54 alleles revealed 13 types of mutation. The salt wasting form showed a good correlation between genotype and phenotype, but the simple virilizing and nonclassic forms showed inconsistencies between genotype and phenotype. The distribution of *CYP21A2* mutations was evaluated for 21-hydroxylase deficiency patients from a single center. This study provides limited data on mutation spectrum and genotype-phenotype correlation of 21-hydroxylase deficiency in Korea.

Keywords: 21 hydroxylase deficiency, Human CYP21A2 protein, Genotype, Phenotype

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive genetic disease that affects the process of cortisol synthesis in the adrenal gland. The cause of CAH in 90–95% of affected individuals is a deficiency of 21–hydroxylase[1,2]. Deficiency of this enzyme causes a reduction in cortisol and aldosterone synthesis in the adrenal gland. This results in increased secretion of adrenal corticotropic hormone (ACTH), adrenal hyperplasia, and increased synthesis of testosterone[2].

Based on the severity of the clinical manifestations, the 21-hydroxylase deficiency is classified into classic form, known as salt-wasting (SW) and simple virilizing (SV) type, and a nonclassic (NC) form called late-onset type. The classic form generally occurs at incidence of 1:10,000–1:15,000, while the NC form occurs in 1:1000 persons[3]. A higher prevalence of both classic and NC forms is found in particular races; in particular, Yupik Eskimos (Alaska) have a prevalence of 1:282, and La Reunion (France) of 1:2141[4] for the classic form, while Ashkenazi Jews have a prevalence of 1:27, and Hispanics of 1:53 for the NC form[5].

Clinical symptoms may appear at diagnosis. Symptoms that appear during the neonatal

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period in the SW form are repetitive vomiting, diarrhea, tachypnea or shortness of breath, and weight loss. Cases with severe lack of cortisol and aldosterone can show hyponatremia, hyperkalemia, dehydration, and shock in the early postnatal period[6,7]. Girls with the SV form may show ambiguous external genitalia at birth, but newborn boys show no abnormality of the external genitalia. In later years, the excessive increase in testosterone in both men and women increases the frequency of precocious puberty because of the early activation of the hypothalamic pituitary gonadal axis. Bone age (BA) also advances and short stature occurs because of the early epiphyseal fusion[8,9]. The late-onset NC form is not characterized by ambiguous external genitalia at birth, but hirsutism, irregular menstruation, and other signs appear later due to the androgen excess at puberty[10].

The 21-hydroxylase genes, *CYP21A2* and *CYP21A1P*, are located in the human leukocyte antigen (HLA) class III region of the short arm of chromosome 6p21.3[11]. The two genes are composed of 10 exons, and structurally similar. The exons are approximately 98% identical, while the introns are 96% identical[1] and mutations arise due to this structural similarity[12]. Intergenic recombination is associated with 95% of the 21-hydroxylase deficiency, while 5% is associated with spontaneous mutations rather than gene conversion[13]. At present, over 100 *CYP21A2* mutations have been reported[14].

The purpose of this study was to evaluate endocrine patterns of patients with CAH according to their genetic mutations. We also analyzed the correlation between each phenotype and genotype.

Materials and methods

1. Subjects

Clinical data were collected retrospectively from a review of medical records of patients who visited the pediatric outpatient clinic at Samsung Medical Center, from November 1994 to December 2012. Twenty-seven patients (male, 19; female, 8) with CAH who had been diagnosed by genetic testing to have 21-hydroxylase deficiency. The Institutional Review Board of Samsung Medical Center approved this study (2013-04-065).

Data for physical examination, height, weight, electrolyte level, and hormonal level were collected from medical records. Pubertal status was assessed with the Tanner stage of breast development for girls and genital development for boys. BA was measured using the method described by Greulich and Pyle[15]. Body mass index (BMI) was calculated from height and weight. The 27 patients with 21-hydroxylase deficiency were classified into the classic (SW and SV) forms and the NC form[16]. The electrolytes were measured to confirm hyponatremia, hyperkalemia, and dehydration, while clinical findings confirmed ambiguous genitalia due to excessive androgen, precocious puberty, short stature, and hirsutism. Endocrine tests for 17-hydroxyprogesterone (17-OHP), ACTH, renin, and testosterone were performed at the time of diagnosis. The differences in BA and chronological age (CA) ratios and BMI were investigated through follow-up period. Patients were classified according to their type of *CYP21A2* mutation.

The Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used to extract genomic DNA from peripheral blood samples of all patients. All exons of the *CYP21A2* gene and exon-intron boundaries were amplified by polymerase chain reaction (PCR) using each primer set, and DNA was amplified using a Nucleic Acid Amplification Kit (Applied Biosystems, Foster City, CA, USA). Long PCR was performed to detect deletion or duplication by unequal meiotic crossing-over by pseudogene, and the PCR was carried by each exon. Sequence analysis was performed using an ABI Prism 3130 analyzer (Applied Biosystems). Mutations in the *CYP21A2* cDNA sequence were analyzed using the reference sequences from GenBank (NM_000500.7).

2. Statistical analysis

The patients were divided into two groups: SW form and SV form. Each phenotype was further divided by gender. The age at diagnosis, ratio of BA to CA, BMI and hydrocortisone dose of different phenotypic groups were evaluated. The Wilcoxon signed rank test was used for between-group comparison. Statistical analyses were performed using IBM SPSS ver. 20.0 (IBM Co., Armonk, NY, USA). *P*-value of < 0.05 was considered statistically significant.

Results

1. Patient characteristics

Among the 27 patients for whom follow-up observations were possible, the classic form of 21-hydroxylase deficiency was found in 26 patients. These included 15 patients (57.7%) with the SW form (male, 11; female, 4), 11 (40.7%) with the SV form (male, 7; female, 4), and 1 male patient (3.7%) with the NC form. The mean follow-up observation periods were 9.00±5.17 years for patients with the SW form, 8.38±3.68 years for patients with the SV form, and 16.4 years for the patient with the NC form. The mean age at diagnosis was 0.11±0.14 years (male, 0.13±0.16; female, 0.05±0.04) for SW form patients and 7.19±5.62 years (male, 7.57±4.07; female, 6.52±8.48) for SV form patients. The SW form was diagnosed at a significantly earlier age in comparison with the SV form (P=0.001). The male patient with the NC form was diagnosed at the age of six (Table 1).

2. The ratio of BA and CA and the differences in BMI

The mean value of the BA to CA ratio at diagnosis was 1.39±0.40 (male, 1.23±0.31; female, 1.74±0.37) for the SW form patients and 1.57±0.72 (male, 1.52±0.46; female, 1.65±1.23) for the SV form patients; these differences were not statistically significant. The final BA to CA ratio was 1.25±0.14 (male,

1.26±0.16; female, 1.24±0.11) in the SW form patients and 1.23±0.19 (male, 1.31±0.17; female, 1.10±0.16) in the SV form patients; again, the differences were not statistically significant. The boy with the NC form had a BA to CA ratio of 1.0 at diagnosis and a final value of 1.03.

The mean BMI values at the last follow-up were 20.1±4.49 kg/m^2 (male, 21.0±4.85 kg/m^2; female, 17.7±2.20 kg/m^2) for patients with the SW form, and 21.2±3.26 kg/m^2 (male, 22.6±3.87 kg/m^2; female, 19.8±1.09 kg/m^2) for patients with the SV form. There were no significant differences between males and females and between the SW form and SV form groups (Table 1).

3. The changes in hormone levels

Endocrine tests performed on the 27 patients for whom follow-up observations were possible revealed that the levels of 17-OHP, ACTH, renin, and testosterone were normalized by hydrocortisone treatment (Table 1). The hydrocortisone dosage necessary to maintain normal endocrine function in patients with the SW form was 21.7±7.6 mg/m^2/day for boys and 16.5±3.6 mg/m^2/day for girls. The hydrocortisone dosage for patients with the SV form was 15.0±5.5 mg/m^2/day for boys and 15.5±3.6 mg/m^2/day for girls, and it was 17.3 mg/m^2/day for the male with the NC form. There were no significant differences between males and females and between the SW form and SV form groups.

4. The analysis of genes

The analysis of 54 alleles of genes in the 27 patients for whom follow-up observations were possible revealed 13 types of mutations. The distribution of 21-hydroxylase deficiency gene mutations revealed that intron 2 splice site (c.293-13A/C > G) mutations and large deletions were the most common, at 31.5% and 22.2% respectively, followed by p.I173N, p.R356W, and p.I172N mutations at 11.1%, 9.3%, and 9.3%, respectively. Other mutations were observed at 1.9–3.7%. No novel mutations were detected (Tables 2, 3). Most patients (70.3%) were compound heterozygotes. Homozygotes accounted for 22.2% (6 patients, all with the SW form), and complex allele accounted for 7.4% (2 patients only with the SV form) (Table 3).

The most common mutations in the patients with SW form were the c.293-13A/C > G and large deletion at 36.7% and 33.3% respectively, followed by p.R356W, p.I172N, and p.I173N mutations. Among these patients, a total of 6 patients were homozygotes, accounting for 40.0%: 2 patients had the c.293-13A/C > G mutation, 2 patients had large deletion, 1 patient had a p.I173N mutation, and 1 patient had a p.R356W mutation. The rest of the mutations were found in compound heterozygotes, but no complex alleles were detected (Tables 2, 3).

The most common mutations in the patients with SV form were c.293-13A/C > G , p.I173N, and p.I172N, at 27.3%, 18.2%, and 13.6% respectively, followed by large deletion, p.R356W, p.S171N, p.8bp-del, p.R484Pfs, p.L307FfsX6, and p.L306FfsX5. No homozygotes were found among these patients, but complex alleles were observed in 2 patients with large deletion + c.293-13A/C > G /c.293-13A/C > G+p.L306FfsX5 and p.I173N+p.Q318X+p.R357W (Tables 2, 3). The NC form patient showed large deletion and p.I172N mutations.

Discussion

CAH is a series of autosomal recessive diseases that produce a cortisol synthesis disorder due to enzyme deficiency imparted

Table 1. Clinical and laboratory finding of the patients with 21-hydroxylase deficiency

Variable	Salt wasting		Simple virilizing		Nonclassic	
	Male	Female	Male	Female	Male	Female
No. of patient	11	4	7	4	1	0
Age at diagnosis (yr)	0.13±0.16	0.05±0.04	7.57±4.07	6.52±8.48	6	
BA/CA						
Initial	1.23±0.31	1.74±0.37	1.52±0.46	1.65±1.23	1.00	
Final	1.26±0.16	1.24±0.11	1.31±0.17	1.10±0.16	1.03	
Body mass index (kg/m^2)						
Initial	13.50±2.17	12.20±2.89	17.42±3.70	17.80±3.65	16.64	
Final	21.01±4.85	17.72±2.20	22.63±3.87	19.81±1.09	28.00	
17-OHP (ng/mL)						
Initial	331 (30–1892)	114 (79–128)	151 (125–191)	342 (99–583)	38.43	
Final	13.64 (0.13–72)	1.52 (0.72–2.34)	3.02 (0.13–7.72)	4.84 (1.91–9.73)	64.00	
ACTH (pg/mL)						
Initial	168 (16–344)	601 (17–1780)	257 (119–473)	495.00 (7.13–1,493.00)	54.00	
Final	45 (7.24–158)	18 (18)	35 (18–48)	47 (25–71)	4.83	
Testosterone (ng/mL)						
Initial	0.83 (0.04–3.32)	11 (0.74–20)	3.33 (1.24–5.23)	1.03 (0.94–1.01)	1.67	
Final	0.62 (0.01–2.91)	0.13 (0.13)	2.54 (0.01–3.20)	0.07 (0.01–0.12)	4.28	
Renin (ng/mL), initial	32.00 (8.40–72.00)	34 (34)	19 (16–21)	8.34 (8.20–8.53)	6.75	

Values are presented as mean±standard deviation or median (range).
BA, bone age; CA, chronological age; 17-OHP, 17-hydroxyprogesterone; ACTH, adrenocorticotropic hormone.

by gene mutations that include cytochrome P450c21. The result is adrenal hyperplasia and an excessive accumulation of cortisol precursors and androgen. Several clinical findings, such as virilization of external genitalia and loss of salt, occur in response to intermediary metabolites[16]. To date, seven types of enzyme deficiency have been identified: 21-hydroxylase, 11β-hydroxylase, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase/17,20-lyase, and 18-hydroxylase. Among these, 21-hydroxylase deficiency accounts for 90–95% of this disorder[13,16].

Table 2. Phenotype and genotype of 27 patients with 21-hydroxylase deficiency

Patient	Sex	Phenotype	Genotype
1	Male	SW	c.293-13A/C>G/c.293-13A/C>G
2	Female	SW	c.293-13A/C>G/c.293-13A/C>G
3	Male	SW	c.293-13A/C>G/8-bp del
4	Male	SW	c.293-13A/C>G/large deletion
5	Male	SW	c.293-13A/C>G/large deletion
6	Male	SW	c.293-13A/C>G/p.R356W
7	Male	SW	Large deletion/large deletion
8	Male	SW	Large deletion/large deletion
9	Male	SW	Large deletion/c.293-13A/C>G
10	Male	SW	Large deletion/c.293-13A/C>G
11	Female	SW	Large deletion/conversion
12	Male	SW	p.I172N/large deletion
13	Female	SW	p.I173N/p.I173N
14	Male	SW	p.R356W/p.R356W
15	Male	SW	c.293-13A/C>G/p.R356W
16	Male	SV	Large deletion+c.293-13A/C > G/c.293-13A/C>G+p.L306FfsX5
17	Male	SV	Large deletion/p.R356W
18	Male	SV	c.293-13A/C>G/p.I173N
19	Male	SV	c.293-13A/C>G/p.R484Pfs
20	Male	SV	c.293-13A/C>G/p.S171N
21	Female	SV	c.293-13A/C>G/p.8-bp del
22	Male	SV	p.I172N/p.L307FfsX6
23	Female	SV	p.I172N/p.L306FfsX5
24	Female	SV	p.I172N/c.293-13A/C>G
25	Female	SV	p.I173N/p.I173N
26	Male	SV	p.I173N+p.Q318X+p.R357W
27	Male	NC	Large deletion/p.I172N

SW, salt wasting; SV, simple virilizing; NC, nonclassic; 8-bp del, 8-bp deletion.

The 21-hydroxylase gene is composed of *CYP21A2*, a functional gene, and *CYP21A1P*, a nonfunctional gene. The gene is arranged beside genes that encode complement factors called C4A and C4B and it consists of a tandem repeat composed of *C4/CYP21A2* and *C4/CYP21A1P*[11]. Since *CYP21A2* and *CYP21A1P* are structurally similar, 98% of exons are identical, and 96% of introns are identical[1]. Therefore, due to the structural similarity of these duplicated regions, misalignment occurs during meiosis, resulting in mutations[12]. Gene conversion accounts for 75% of intergenic recombination, which occurs via transfer to *CYP21A2* during mitosis[17,18]. Mutations such as deletions, *CYP21A2*/*CYP21A1P* chimeric genes, and duplication during meiotic crossover of *CYP21A2* account for the remaining 20–25%[19].

The correlation between 21-hydroxylase genotype and phenotype has been studied in a variety of ethnic groups[7,12,20]. The 21-hydroxylase gene mutations are classified into 3 groups according to genotype and phenotype based on enzymatic activity[7,12,20]. Group A is the SW form, which lacks enzyme activity and includes deletion, p.Q318X, and p.R356W, and c.293-13A/C > G mutations. Group B is the SV form, with enzymatic activity of 1–5%, and is mainly a p.I172N mutation[21]. Group C is the NC form, with enzyme activity of 20–50%, and includes p.V281L[22], p.P301L[23], and p.P453S[24] mutations.

Early diagnosis of this disease can occur for the SW form, which shows acute symptoms at a relatively early age, and for girls with SV, who show external genital abnormality at birth. However, no striking symptoms are observed in boys with the SV form, so diagnosis is often delayed, as seen in previous reports[25]. Likewise, this study also revealed a significant

Table 3. Allelic frequency of *CYP21A2* mutation in patients with 21-hydroxylase deficiency

Mutation	Salt wasting	Simple virilizing	Nonclassic	Total
c.293-13A/C>G	11 (36.7)	6 (27.3)	0 (0)	17 (31.5)
Large deletion	10 (33.3)	1 (4.5)	1 (50.0)	12 (22.2)
p.R356W (c.1066C>T)	4 (13.3)	1 (4.5)	0 (0)	5 (9.3)
p.S171N (c.512T>A)	0 (0)	1 (4.5)	0 (0)	1 (1.9)
p.I172N (c.515T>A)	1 (3.3)	3 (13.6)	1 (50.0)	5 (9.3)
p.I173N (c.518T>A)	2 (6.6)	4 (18.2)	0 (0)	6 (11.1)
Conversion	1 (3.3)	0 (0)	0 (0)	1 (1.9)
8-bp deletion (c.329_336delGAGACTAC)	1 (3.3)	1 (4.5)	0 (0)	2 (3.7)
p.R484Pfs (c.1451dupC)	0 (0)	1 (4.5)	0 (0)	1 (1.9)
p.L307FfsX6 (c.920_921insT)	0 (0)	1 (4.5)	0 (0)	1 (1.9)
p.L306FfsX5 (c.920dupT)	0 (0)	1 (4.5)	0 (0)	1 (1.9)
Large deletion+c.293-13A/C>G+p.L306FfsX5 (c.920dupT)	0 (0)	1 (4.5)	0 (0)	1 (1.9)
p.Q318X+p.R357W (c.955C>T+c.1069C>T)	0 (0)	1 (4.5)	0 (0)	1 (1.9)
Total	30 (100)	22 (100)	2 (100)	54 (100)

Values are presented as number (%).

difference in mean age at diagnosis, at 0.11±0.14 years for the SW form and 7.19±5.62 years for the SV form.

Patients with the SW or SV form usually show a shorter final adult height than the average adult height, of approximately 1–2 SD (standard deviation)[16,25]. The reasons are considered to be the increased rate of bone maturation and early epiphyseal maturation due to excessive secretion of androgens, the promotion of bone maturity and epiphyseal maturation caused by precocious puberty, and the reduction in the pubertal growth time[26]. In this study, the BA to CA ratio increased in both the SW form and SV form, with no significant difference between the forms of the disease. Numerous reports[27] indicate that patients who received appropriate treatment showed a higher risk of obesity and greater increases in BMI. The mean BMI in each group was lower than 25 kg/m^2 which means there is no tendency toward obesity. Other recent studies have also reported no significant differences in BMI among patients with different phenotypes. Therefore, BMI cannot be considered a measure of the complications of disease[28].

Wilkins et al.[29] and Batter et al.[30] demonstrated in 1950 that cortisone is an effective treatment for CAH, and the combined use of cortisone and mineralocorticoid has greatly lowered the mortality and morbidity rates and increased the life expectancy of these patiens. The treatment of this disease is aimed at reducing the excessive secretion of ACTH and replenishing mineralocorticoid levels. The preferred drug in substitution therapy for glucocortocoid is hydrocortisone, which is administered at 10–20 mg/m^2/day three times. In this study, hydrocortisone was administered at 17.2 mg/m^2/day on average, and the administered doses did not differ significantly between phenotypes or genders.

Recently, Choi et al.[31] reported a genotype-phenotype correlation of 21-hydroxylase deficient patients in Korea. They concluded that the overall concordance between genotype and phenotype was 96.8% that is consistent with previous reports[19,32,33]. In this study, each mutation were divided into three groups to correlate their genotypes, positive predictive value in 83.3%, 40.9% with SW, SV, respectively. No gene mutation was associated with the patient with the NC form. As a result, there were the difference between the previous reports and a genotype-phenotype correlation. The mutations of gene causing 21-hydroxylase deficiency in Koreans reported in domestic research were observed in the sequence of c.293-13A/C > G (23%), deletion/large conversion (18%), p.I172N (11%), p.Q318X (9.3%), p.R356W (8.0%)[34,35]. Overseas reports indicate the main mutations to be deletion, c.293-13A/C > G, p.I172N, p.R356W, and p.Q318X[2]. In a study of Korean, the frequency of the deletion of the *CYP21A2* gene was 18.0%, Japan and China were 12.0%, 4.7%, respectively[36,37]. That is lower than in Western countries at 20–30%[7,12].

The frequencies of gene mutations causing 21-hydroxylase deficiency in this study showed discrepancy from the numerical values in other domestic and overseas reports, but the common mutation types were consistent, with c.293-13A/C > G , large deletion, p.R356W, p.I172N, and p.I173N accounting for most of the mutations. The *CYP21A2* mutation genotypes and phenotypes vary according to the severity of disease, and this also showed a good correlation[2]. The c.293-13A/C > G, deletion, and p.R356W mutations are as associated with the SW form[38], while c.293-13A/C > G is known to cause both the SW and SV forms[39]. p.V281L is mainly found in the NC form[21].

In conclusion, the mutations of the *CYP21A2* gene in the SW, SV, and NC forms of CAH were confirmed in 27 patients in a single center for whom follow-up observations were possible. The analysis of 54 alleles revealed 13 types of mutation. The SW form showed a good correlation between genotype and phenotype, but the SV and NC forms showed inconsistencies between genotype and phenotype. The SW and SV forms showed large deletion, p.I172N, and p.I173N mutations, while the NC form showed large deletion and p.I172N mutations.

The distribution of *CYP21A2* mutations was evaluated for 21-hydroxylase deficiency patients from a single center. This study provides limited data on mutation spectrum and genotype-phenotype correlation of 21-hydroxylase deficiency in Korea.

Conflict of interest

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

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