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CYP21A2 Mutation Analysis in Korean Patients With Congenital Adrenal Hyperplasia Using Complementary Methods: Sequencing After Long-Range PCR and Restriction Fragment Length Polymorphism Analysis With Multiple Ligation-Dependent Probe Amplification Assay

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CYP21A2 mutation analysis of congenital adrenal hyperplasia (CAH) is challenging because of the genomic presence of a homologous *CYP21A2* pseudogene and the significant incidence of pseudogene conversion and large deletions. The objective of this study was to accurately analyze the *CYP21A2* genotype in Korean CAH patients using a combination of complementary methods. Long-range PCR and restriction fragment length polymorphism analyses were performed to confirm valid amplification of *CYP21A2* and to detect large gene conversions and deletions before direct sequencing. Multiple ligation-dependent probe amplification (MLPA) analysis was conducted concurrently in 14 CAH-suspected patients and six family members of three patients. We identified 27 *CYP21A2* mutant alleles in 14 CAH-suspected patients. The c.293-13A>G (or c.293-13C>G) was the most common mutation, and p.Ile173Asn was the second, identified in 25% and 17.9% of alleles, respectively. A novel frame-shift mutation of c.492delA (p.Glu 164Aspfs*24) was detected. Large deletions were detected by MLPA in 10.7% of the alleles. Mutation studies of the six familial members for three of the patients aided in the identification of haplotypes. In summary, we successfully identified *CYP21A2* mutations using both long-range PCR and sequencing and dosage analyses. Our data correspond relatively well with the previously reported mutation spectrum analysis.

Key Words: *CYP21A2*, Pseudogene, Restriction fragment length polymorphism, Multiple ligation-dependent probe amplification, Korea

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21-Hydroxylase deficiency is the most common cause of congenital adrenal hyperplasia (CAH, OMIM 201910). More than 90% of patients with CAH have *CYP21A2* mutations including conversions to the *CYP21A1P* pseudogene or large deletions [1]. 21-Hydroxylase-deficient CAH is an autosomal recessive disorder that is manifested in a variety of clinical severities comprised of three subtypes: (1) classic salt-wasting, (2) classic simple virilizing, and (3) non-classic forms [2]. The incidence of classic CAH ranges from 1 in 10,000 to 1 in 20,000 births worldwide [2].

Analysis of the *CYP21A2* mutation is challenging owing to the presence of a highly homologous *CYP21A1P* pseudogene (98% in exons and 96% in introns) [3], which is known to interfere with targeted *CYP21A2* amplification during sequencing. Furthermore, the RCCX module (the genes *RP*, *C4*, *CYP21*, and *TNX* arranged in tandem) in the 6p21.33 chromosome region shows high homology between the functional genes (*RP1*, *CYP21A2*, and *TNXB*) and the corresponding pseudogenes (*RP2*, *CYP21A1P*, and *TNXA*). This phenomenon leads to gene conversions and gene deletions due to homologous recombination, which results in inactivation of the functional gene [4]. Since a large number of *CYP21A2* mutations are composed of large pseudogene conversions and deletions (20-30%), additional modalities other than direct sequencing are essential to accurately identify these mutations. The objective of this study was to accurately analyze the *CYP21A2* genotype using a combination of complementary methods.

Genetic analyses were performed in retrospectively selected 14 patients (eight males and six females) with CAH who were referred for *CYP21A2* mutation analysis from 2008 to 2013 at Samsung Medical Center, Seoul, Korea. Six family members from three of these patients were also included in the study. Informed consent was obtained from the patients or the parents for pediatric patients. The Institutional Review Board of Samsung Medical Center approved this study.

All CAH-suspected patients showed elevated levels of 17-hydroxyprogesterone (17-OHP). Nine were classified as salt-wasting, two were non-classic, and one patient was simple virilizing according to clinical features. One female patient only presented with irregular menstruation (case 8), and clinical information was not available for one patient (case 4).

Genomic DNA was extracted using Wizard Genomic DNA Purification kits (Promega, Madison, WI, USA) according to the manufacturer's instructions. We performed long-range PCR using the AccuPower Hotstart PCR PreMix (Bioneer, Daejeon, Korea), containing a high-fidelity polymerase, buffers, and a deoxynucleotide triphosphate mix. The reaction mixture included 500 ng of DNA and 10 pmol each of CYP779f and Tena32F primers [5]. The PCR amplification conditions were: 94°C for 5 min followed by 32 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min. The resultant product of CYP779f and Tena32F primers and the subsequent TaqI restriction endonuclease-digested products were analyzed by electrophoresis in 1.2% agarose gels as previously described [6, 7]. The presence of an 8.5-kb PCR product and the appearance of TaqI-produced 3.7- and 2.5-kb fragments represent intact *CYP21A2* and partial fragments of the *TNXB* gene in normal individuals, whereas TaqI-produced fragments that are 3.2 and 2.4 kb in size represent *CYP21A1P* pseudogene recombinations in patients with CAH [7]. An aliquot of the same CYP779f and Tena32F PCR product was subsequently submitted for direct sequencing of all the coding exons and the flanking intronic sequences for *CYP21A2*, using primers designed in this study (available on request). Direct sequencing was performed by using an ABI Prism 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA), and sequence variants were designated according to Human Genome Variation Society recommendations (http://www.hgvs.org/mutnomen/) by using the reference sequences from GenBank (NG_007941.2, NM_000500.7, NP_000491.3).

The multiple ligation-dependent probe amplification (MLPA) assay was performed concurrently by using the SALSA MLPA kit P050-B2 (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's instructions. The kit is designed to detect large deletions and duplications in one or more exons of the *CYP21A* and *TNX* genes, and contains five probes for different *CYP21A2* mutations and three *CYP21A1P*-specific probes. The MLPA data were analyzed by using GeneMarker v.1.9 software (SoftGenetics, State College, PA, USA).

We identified 27 mutations in 28 alleles of the 14 CAH-suspected patients. The clinical features and molecular data of the patients and family members are listed in Table 1. Eight different point mutations [c.92C>T (p.Pro31Leu), c.293-13A>G or c.293-13C>G, c.518T>A (p.Ile173Asn), c.844G>T (p.Val 282Leu), c.874G>A (p.Gly292Ser), c.955C>T (p.Gln319*), c.1054G>A (p.Glu352Lys), c.1069C>T (p.Arg357Trp)], three small deletions and/or insertions [c.923dupT (p.Leu308 Phefs*6), c.1451_1452delGGinsC (p.Arg484Profs*58), c.492 delA (p.Glu164Aspfs*24)], and one mutation cluster were identified. The mutation cluster was on exon 6, and it was defined as c.[710T>A;713T>A;719T>A] (p.[Ile237Asn;Val238Glu;Met 240Lys]). The most common mutation was the intron 2 splice site mutation (c.293-13A>G or c.293-13C>G) at 25%, followed by the p.Ile173Asn mutation with an allele frequency of 17.9%. Three or more mutations were detected in three patients (patients 7, 9, and 14).

Approximately 95% of the defective *CYP21A2* mutations seen in CAH patients fall into three categories [4]: (i) approximately 61 to 70% [8-14] are deleterious mutations due to small gene conversions derived from the *CYP21A1P* pseudogene, including c.293-13A>G or c.293-13C>G (20.6-30.3%), p.Ile173Asn (8.2-19.8%), p.Val282Leu (2.2-26.2%), p.Arg357Trp (3.0-

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Table 1. Clinical and genetic characteristics of 14 CAH-suspected cases and six family members

Case	Sex	Age at diagnosis	Clinical information	Mutation 1	Mutation 2	MLPA
1	M	<1 month	SW CAH	exon 6 mutation cluster	c.492delA (p.Glu164Aspfs*24)†	ND
	F	Prenatal	Sibling of case 1	ND	c.492delA (p.Glu164Aspfs*24)†	ND
2	M	6 yr	SW CAH	c.293-13C>G	c.518T>A (p.Ile173Asn)	ND
	M	37 yr	Father of case 2	ND	c.518T>A (p.Ile173Asn)	ND
	F	37 yr	Mother of case 2	c.293-13C>G	ND	ND
	F	Prenatal	Sibling of case 2	ND	c.518T>A (p.Ile173Asn)	ND
3	F	14 yr	NC CAH	c.92C>T (p.Pro31Leu)	c.293-13C>G	ND
4	M	<1 month	Unknown	c.293-13A>G	c.955C>T (p.Gln319*)	ND
	M	37 yr	Father of case 4	ND	c.955C>T (p.Gln319*)	ND
	F	34 yr	Mother of case 4	c.293-13A>G	ND	ND
5	F	30 yr	SW CAH	c.518T>A (p.Ile173Asn)‡	ND	Large deletion
6	F	<1 month	SW CAH	c.518T>A (p.Ile173Asn)‡	ND	Large deletion
7	M	4 months	SW CAH	c.1069C>T (p.Arg357Trp)‡	5 mutations§	Large deletion
8	F	16 yr	Irregular menstruation	c.293-13A>G	ND	ND
9	M	19 yr	SW CAH	c.518T>A (p.Ile173Asn)	c.955C>T (p.Gln319*), c.1069C>T (p.Arg357Trp)	ND
10	M	3 months	SW CAH	c.293-13C>G	c.1069C>T (p.Arg357Trp)	ND
11	F	38 yr	SV CAH	c.293-13C>G	c.874G>A (p.Gly292Ser)	ND
12	F	<1 month	SW CAH	c.293-13A>G	c.1054G>A (p.Glu352Lys)	ND
13	M	<1 month	SW CAH	c.518T>A (p.Ile173Asn)	c.1451_1452delGGinsC (p.Arg484Profs*58)	ND
14	M	49 yr	NC CAH	c.92C>T (p.Pro31Leu)	c.293-13C>G‡	ND

†Novel mutation; ‡Homozygous pattern in sequencing analysis chromatograms; §5 mutations: c.92C>T (p.Pro31Leu), exon 6 mutation cluster, c.844G>T (p.Val282Leu), c.923dupT (p.Leu308Phefs*6), c.955C>T (p.Gln319*).
Abbreviations: CAH, congenital adrenal hyperplasia; SW, salt-wasting type; NC, non-classic type; SV, simple virilizing; ND, not detected; exon 6 mutation cluster, c.[710T>A; 713T>A; 719T>A] (p.[Ile237Asn; Val238Glu; Met240Lys]); MLPA, multiple ligation-dependent probe amplification.

8.4%), p.Gln319* (2.4-6.7%), p.Gly111Valfs*21 (0.8-4.3%), exon 6 mutation cluster [p.Ile237Asn, p.Val238Glu, p.Met240Lys (1.1-3.0%)], and p.Pro31Leu (0.3-2.6%); (ii) approximately 5% of defective *CYP21A2* mutations are relatively infrequent spontaneous mutations; and (iii) approximately 7.5 [10] to 32.2% [11] are large gene rearrangements generated by unequal meiotic crossover. In our study, six different mutations derived from the *CYP21A1P* pseudogene due to small gene conversions (p.Pro31Leu, c.293-13A>G or c.293-13C>G, p.Ile 173Asn, exon 6 mutation cluster, p.Gln319*, and p.Arg357Trp) were detected in 20 alleles (71.4%). Three relatively infrequent mutations (p.Gly292Ser, p.Glu352Lys, and p.Arg484Profs*58) were detected in three alleles (10.7%), and three large deletions were found in three alleles (10.7%). These findings implicate that although we performed molecular genetic analysis of *CYP21A2* in a limited number of cases, our mutational spectrum corresponded with previous data generated from large cohorts [8-14].

Patient 1 had a novel frame-shift mutation, c.492delA (p.Glu-

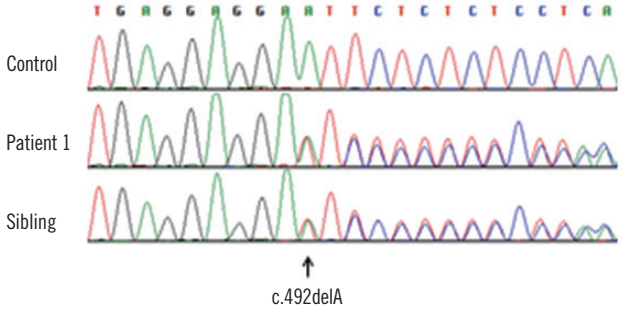

Fig. 1. Chromatogram of a novel mutation, c.492delA (p. Glu-164Aspfs*24), in patient 1 and the sibling of patient 1.

164Aspfs*24) (Fig. 1), and an exon 6 mutation cluster. Clinically, the patient showed markedly elevated 17-OHP in neonatal screening with salt-wasting signs and was treated with fludrocortisone and hydrocortisone. The sibling of patient 1 underwent prenatal mutation analysis with cultured chorionic villi samples and had only one mutant allele, c.492delA.

Patient 8, who had only one heterozygous mutation (c.293-

13A>G), was a 16-yr-old female who visited the obstetrics outpatient clinic for evaluation of irregular menstruation for three years without virilization. She showed an elevated basal 17-OHP level (620 ng/dL, reference range: <200 ng/dL) without abnormal results for testosterone or estradiol, which could imply non-classic type CAH according to the guidelines on CAH by Speiser *et al*. [2]. To rule out non-classic type CAH, she was evaluated after ACTH stimulation. The patient's 17-OHP level at 60 min after ACTH stimulation was 839 ng/dL, which was compatible with a genetically heterozygous carrier status. This patient is being followed up without any treatment.

Large deletions of *CYP21A2* were detected in three alleles (10.7%) by MLPA analysis. Of them, patients 5 and 7 showed compatible results for heterozygous deletion of entire *CYP21A2* (exons 1 to 8), and patient 6 showed results implying partial heterozygous deletion from exons 1 to 6. New *et al*. [8] reported the frequency of deletion and large gene conversions at 20.0% in the largest tested cohort to date including 1,507 families with heterogeneous ethnic backgrounds. Other relatively large studies that included different ethnicities showed variable deletion and large gene conversion frequencies from 11.2-32.2% [9, 11-13]. Ma *et al*. [14] showed rates of 15% for large deletions and 15% for conversions from 30 Chinese patients. However, Lee *et al*. [10] presented a lower occurrence of large gene deletions (or conversions) at 7.5% of the *CYP21A2* alleles in ethnic Chinese (Taiwanese) patients. Previous Korean studies [15-17] had used different methods from those of the current study to analyze *CYP21A2* mutations, and the largest study by Choi *et al*. [17] determined the frequency of large deletions to be 31.3%. The differences in the frequency of large deletions between previous reports [8-17] and our data may have resulted from differences in population size between the studies and/or differences in mutation analysis methods.

In terms of genotype–phenotype correlations, nine salt-wasting-type CAH patients had one or more mutations known to be associated with more severe CAH phenotypes (exon 6 mutation cluster, c.293-13A>G or c.293-13C>G, p.Ile173Asn, p.Gln319*, p.Arg357Trp, p.Arg484Profs*58, and large deletion) [8], while two patients with non-classic type CAH had the p.Pro31Leu mutation, which is frequently associated with non-classic CAH.

In a recent report by Xu *et al*. [18], a molecular analysis strategy for examining *CYP21A2* in CAH patients was discussed. They proposed a sequential approach according to primary testing using a PCR-restriction fragment length polymorphism (PCR-RFLP) method with two different sets of primers and MLPA. Then, patients with or without deletions proceeded to sequencing of the CYP779f/Tena32F amplicon, and patients with duplications proceeded to sequencing with an amplicon using an additional set of primers [18]. Although duplications are rare (1.53%) and were not detected by MLPA in our study, this possible limitation of the current method should not be overlooked when duplications are detected.

In the present study, we have employed complementary methods to analyze *CYP21A2*. The presence of pseudogene and a high incidence of gene conversion and large deletions of the *CYP21A2* could lead to analytic failure or post-analytic misinterpretation. Without complementary methods, when a homozygous mutant peak is detected, a hemizygous mutation due to a large deletion might be misinterpreted as a homozygous mutation in the absence of gene dosage analysis. The use of long range PCR and RFLP analysis confirmed valid *CYP21A2* amplification and also large scale deletion and/or conversion events before direct sequencing. In addition, MLPA assays were conducted for gene dosage analysis along with determination of approximate location and range of dosage mutations. In conclusion, we successfully identified *CYP21A2* mutations in CAH-suspected patients using both long-range PCR and RFLP analyses followed by sequencing and concurrent MLPA analysis.

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Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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