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Original Article

Phenotype and genotype characteristics of Indonesian 21-hydroxylase deficient patients

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Abstract

Background Congenital adrenal hyperplasia (CAH) is the most common cause of ambiguous genitalia in children and 90-95% cases show 21-hydroxylase deficiency. More than 100 mutations have been described and of these, four mutations have been frequently reported in Asia. Those mutations are deletion/large gene conversion (LGC), intron2 splice mutation (I2 splice), point mutations at codon 172 (I172N) and codon 356 (R356W). Genotyping is very valuable since close correlation observed between genotype and phenotype.

Objective To identify phenotype and genotype characteristics of CAH due to 21-hydroxylase deficiency (CAH-21OH) and correlation between them.

Methods From June to November 2006 we analyzed 37 confirmed CAH-21OH patients treated at the Department of Child Health, Cipto Mangunkusumo Hospital during the period of 1990-2006. Polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis or amplification-created restriction site (ACRS) were performed. We first identified deletion/LGC and I172N mutation that had been mostly reported in salt wasting (SW) and simple virilizing (SV) form patients respectively.

Results There were 37 patients, consisted of 6 males and 31 females with the ratio 1:5.2. Of those, 25, 10, 2 patients were SW, SV and non-classic (NC) form, respectively. PCR-RFLP or ACRS was performed to detect two mutations in 32 patients (64 alleles). Deletion/LGC was found in 6 alleles while I172N mutations in two. All deletion alleles showed SW phenotype but I172 mutated alleles showed SW and SV phenotype.

Conclusion There is a consistent close association between genotype and phenotype in our CAH-21OH patients. **[Paediatr Indones 2007;47:189-195]**.

Keywords: *congenital adrenal hyperplasia (CAH), phenotype, genotype, 21-hydroxylase deficiency*

Congenital adrenal hyperplasia (CAH) is a frequent autosomal recessive disorder which is caused by the loss or severely decreased activity of one of five steroidogenic enzymes necessary for cortisol biosynthesis.[1-8] This disease is known to be the most common cause of ambiguous genitalia in children (60-70%)[9,10] and 21-hydroxylase deficiency (CAH-21OH) is considered to be responsible for 90-95% cases.[1-3] Clinical manifestations of CAH-21OH vary from mild to severe, depend on the residual activity of 21-hydroxylase. It is classified into three forms: salt-wasting (SW) form, simple virilization (SV) form and non-classic (NC) form.[1-8] In severe impaired steroidogenesis, the affected female will experience intrauterine virilization therefore presenting ambiguous genitalia at birth. Aldosterone deficiency will result in postnatal salt losing and cortisol deficiency will cause inadequate response to stress. These problems will put the affected infants into life threatening condition.[11,12] In partial impaired

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cortisol biosynthesis, clinical manifestation will not appear soon after birth, but later in childhood or adolescence (hyperandrogenism).[3-5]

Previous studies have described certain mutations of CYP21 gene that cause 21-hydroxylase deficiency.[1-9] In general, there is a close correlation between phenotype and genotype of this disease. Certain mutation will establish certain phenotypic characterization.[9] More than 100 mutations have been reported in CAH-21OH.[5,8] Of these mutations, four have been frequently reported in Asia. Those mutations are deletion/large gene conversion (LGC), intron2 splice mutation (I2 splice), point mutation at codon 172 (I172N) and codon 356 (R356W).[13-17] Genotyping is very valuable in prenatal diagnosis as well as in neonatal screening because of high correlation between genotype and phenotype. Therefore, diagnosis can be established before clinical manifestations appear thus it will reduce morbidity even mortality of the disease. The first aim of this study was to elaborate phenotypic characterization of CAH due to 21-hydroxylase deficiency and allele frequencies of common mutations reported in Indonesia as well as in other Asian studies. The second one was to determine correlation between genotype and phenotype. In our study, we first identified deletion/LGC and I172N mutation that had been mostly reported in SW and SV form cases respectively.[13-17]

Methods

We conducted genotypic study during the period of June-November 2006 on 37 confirmed CAH-21OH patients treated in the Department of Child Health, Cipto Mangunkusumo Hospital during the period of 1990-2006. Phenotypic data were taken from medical records and parent information. Polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis or artificial-created restriction site (ACRS) were performed for genotyping all of the alleles.

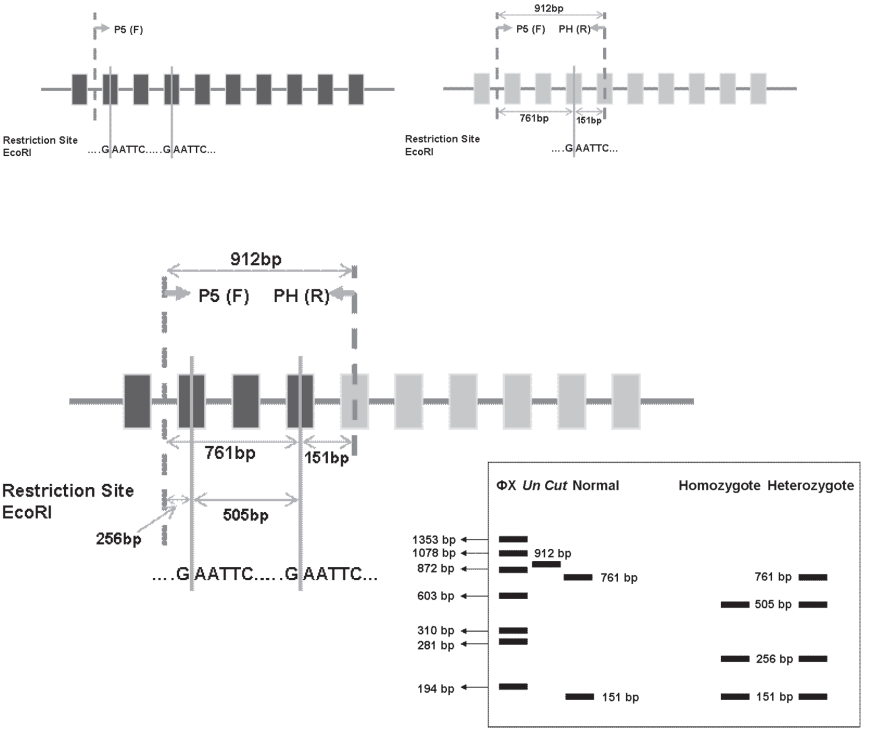
PCR was carried out on genomic DNA isolated from peripheral blood leukocytes by standard procedures using selective primers (Sigma-Proligo Ltd., Singapore.). These primer pairs were designed to selectively amplify the CYP21 gene only. For deletion/LGC detection we used forward primer P5: TCT GGC CCT CAC CAT AG and reverse primer PH: TTG TCG TCC TGC CAG AAA AGG AG. For I172N mutation, forward primer P3: TTC TCT CTC CTC ACC TGC AGC ATC G and reverse primer P4: CTG CAT CTC CAC GAT GTG ATC CCT C were used.[16] P3 contained G as amplification restriction site. Amplifications were performed in volume of 50 μL containing approximately 0.4 μg of genomic DNA, 40 ñmol of each nucleotide primer, 20 mM of each deoxynucleotide triphosphate, Q-solution (Qiagen, Research Biolabs), 10X PCR Buffer, 1.25 U of Hotstar Taq polymerase (Qiagen). For deletion detection, after initial denaturation at 95°C for 15 min, thirty cycles of amplifications were used at 95°C for 1 min, 54°C for 30 sec and 72° for 1 min, then 72°C for 3 min. For the detection of I172N mutation, PCR were carried out by initial denaturation at 94°C for 15 min, then 32 cycles at 94°C for 30 sec, 62°C for 30 sec for and 72° for 30 sec, then 72°C for 3 min. We then incubated 8 μL of each PCR product and 4-6 unit of the appropriate restriction enzymes to digest. Electrophoresis was performed in 2% agarose gel containing 0.5μg/mL ethidium bromide.

To detect any deletion/large gene conversion we used EcoRI as restriction enzyme. Naturally, CYP21 gene has one restriction site of EcoRI enzyme, so that EcoRI will cut DNA fragment into two pieces, 761 bp (base pair) and 151 bp fragments. In mutant allele, pseudo gene sequences are transferred into CYP21 therefore, it will have another restriction site for EcoRI. Thus, DNA mutant will be cut into three pieces, 505 bp, 256 bp, and 151 bp fragments. Since everyone has two alleles, we will find three bands in homozygote mutant (505 bp, 256 bp, and 151 bp fragments) but four bands in heterozygote one (761 bp, 505 bp, 256 bp, and 151 bp fragments). Sometimes, the smaller bands were unvisualized by agarose gel electrophoresis due to technical difficulty. (**Figure 1A**)

To determine allele's frequency of I172N mutation, we used a forward primer that was specifically designed to generate PCR products that contain restriction site of TaqI enzyme (artificial-created restriction site). In codon 172, CYP21 gene has ATC sequence for coding the amino acid isoleucine while pseudo gene has AAC sequences (aspargine). If point mutation occurs (T→A), the amplified fragment will have sequence 5'…ACC TGC ACC ATC GAC…3' with TCGA as restriction target for Taq1. Only mutant alleles have this restriction site, so that it will be cut into two pieces, 397 bp and 23 bp fragment. (**Figure 1B**)

Informed consent for mutation analysis was obtained from all subject/parents and this study was ap-

A. Deletion/LGC



B. I172N mutation

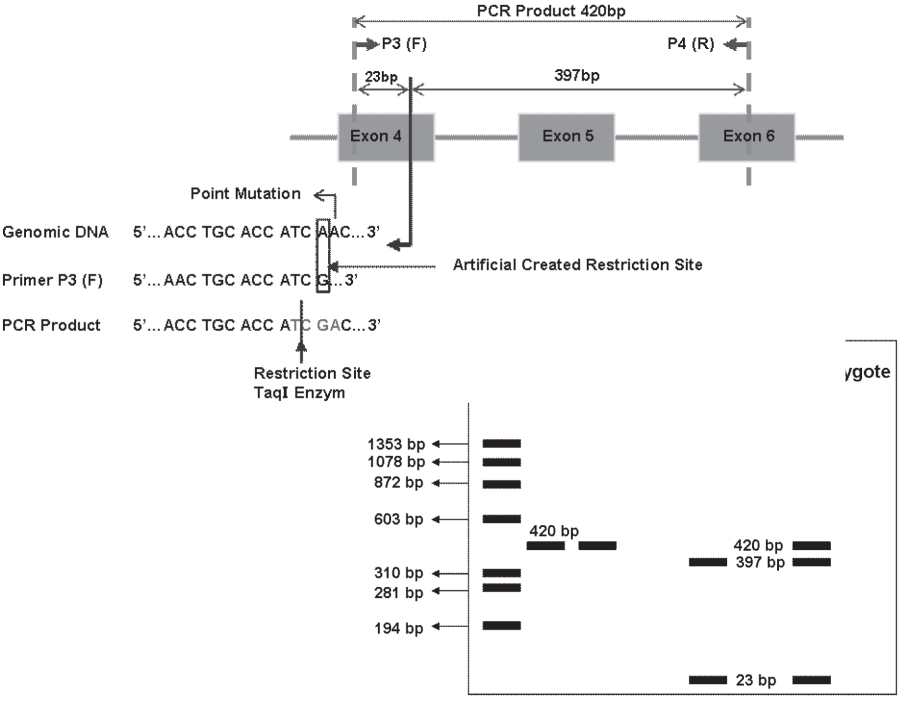


Figure 1. Strategy for mutation detection

proved by the Committee for Medical Research Ethics of the Faculty of Medicine, University of Indonesia.

Results

Phenotype of subjects

Thirty-seven CAH-21OH patients consisted of 6 males and 31 females (male: female ratio 1:5.2) were studied. All subjects born as term neonates with birth weight appropriate for gestational age. Mean gestational age was 38.8 (SD 0.8) weeks and mean birth weight were 3161.6 (SD 370.1) grams. The most frequent ethnics in this study were Java-Java. Subjects characteristics is shown in **Table 1**. Consanguinity was found in only one case, sibling death was found in five patients, but there was only one case whose sibling had the same disorder.

Twenty-five subjects were classified as SW form (21 females, 4 males), 10 subjects as SV form (8 females, 2 males) and 2 as NC form (both were females). Of 31 female subjects, 10 had been assigned as male gender before chromosome examination. Prader score III was observed in 17/31 female subjects, while Prader score II, IV, I were found in 7/31, 5/31 and 2/31 female subjects respectively.

Tabel 1. Characteristic of subjects

Characteristic	Total n (%)
Male /female (*ratio*)	6/31(1:5.2)
Gestational age, weeks (*mean*± SD)	38.84 ± 0.866
Birth weight, gram (*mean* ± SD)	3161.62 ± 370.15
Ethnic (father-mother)	
Java-Java	8
Betawi-Betawi, Java-Betawi (5 each)	5
Minang-Minang (3 each)	3
Sunda-Sunda, Chinese-Chinese (2 each) Batak-Batak, Bugis-Bugis, Java-Minang, Java-Sunda, Melayu-Melayu, Nias-Nias,Sunda-Minang (1 each)	2 1
Consanguinity	1
Infant sibling death	5
CAH Sibling	1

SD: standard deviation

Mutation analysis

PCR-RFLP and ACRS was performed and mutation analysis was done on 32 subjects (64 alleles). We found 8 alleles which have deletion/large gene conversion. These alleles belonged to 6 subjects and consisted of 4 heterozygote mutant and 2 homozygote mutants. Two alleles had I172N mutation. All of the alleles were heterozygote subjects. (**Table 2**)

Phenotype and genotype correlation

All subjects containing deletion/large gene conversion showed SW form while two subjects who had I172N mutation showed two different forms, SW and SV, respectively. These findings suggested that there is a general good correlation between genotype and phenotype in our CAH-21 hydroxylase patients. Details of phenotypic characteristics of deletion/LGC and I172N mutant is shown in **Table 2**.

Discussion

The male to female ratio in our CAH patients was 1:5.2; this is quite similar to that reported by Bajpai *et al*[18] and Kharrat *et al*[19], i.e., 1:5.8 and 1:4.1 respectively. Actually, these results were inconsistent

Tabel 2. Phenotypic charaterization in deletion/LGC and I172N subjects

Phenotype	Deletion/LGC							I172N
	CAH 12	CAH 16	CAH 22	CAH27	CAH11	CAH19	CAH 9	CAH 24
CAH Type	SW	SW	SW	SW	SW	SW	SW	SV
Sex	XX	XX	XY	XX	XX	XX	XX	XX
Gender	F	F	M	M	F	F	M	F
Parental Ethnicity	Minang Minang	Minang Minang	Melayu Melayu	Bugis Bugis	Sunda Java	Palembang Palembang	Java Java	Minang Sunda
Consanguinity	N	N	N	Y	N	N	N	N
Infant Sibling death	Y	N	N	Y	N	N	N	N
Sibling CAH	N	N	N	N	N	N	N	N
Infertility	N	N	N	N	N	N	N	N
Prader Score	IV	III	V	III	III	III	IV	III

SW:salt wasting; CAH 12,16,22,27 heterozygote sample; CAH 11,16: homozygote sample; F:female; M:male; Y:yes; N:No

with the characteristic of autosomal recessive inherited disease that is supposed to have male and female ratio of 1:1. In contrast, different results were reported by Kovacs *et al*[20] in Middle European Country and Loke *et al*[21] with male to female ratio of 1:1.5 and 1:1 respectively. These differences were presumably due to the presence or absence of neonatal screening, resulting in different detection of patients more commonly. In the absence of neonatal screening, most of the males will not be detected since there are no signs of ambiguous genitalia which usually raise the awareness of parents or medical attendants.[22] Thus, early diagnosis by prenatal diagnostics as well as neonatal screening have a very important role in reducing morbidity and mortality in CAH patients.[22]

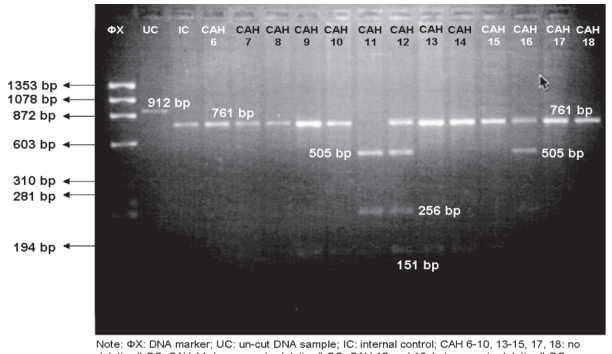
Consanguinity, sibling death and sibling CAH was present in 1, 5, and 1 patients respectively. Bajpai *et al*[18] reported these as much as 16.4%, 12.8%, and 25.5% respectively. The highest incidence of consanguinity that had ever been reported was in Tunisian population.[19] On the contrary, Koyama *et al*[16] reported that there were no consanguinity in CAH family in Japan. These facts emphasized that CYP21 gene and its pseudo gene are susceptible to genomic alteration due to adjacent structures and highly recombination events.[23,24]

Twenty five subjects showed SW form while the remaining had SV and NC form. Seventy percent of classic CAH patients showed SV form. This finding supported various reported epidemiological data, which showed that classic CAH-21 hydroxylase consisted of SW and SV form 70% and 30% respectively. For other comparison, study by Koyama *et al*[16] in Japan reported 67% SW form, 23% SV form and 10% NC form. Bajpai *et al*[18] reported that Indian CAH patients consisted of 48% SW form, 46.8% SV and 4.3% NC form.

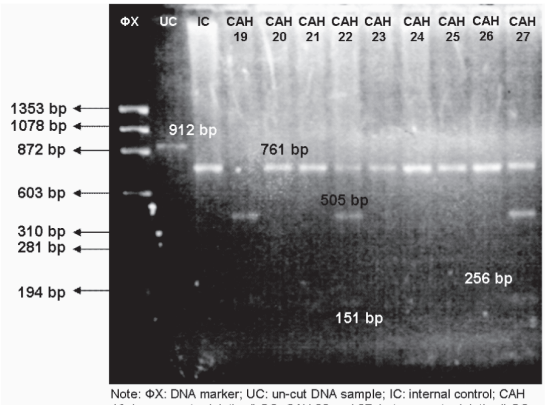
Almost one-third of females were incorrectly assigned as male gender before the real sex became chromosomally established. This gender mistake are prone to have psychosocial problems later. Hochbergt *et al*[25] reported psychosocial problem in 7 genotypic female (XX) who were treated as male gender. Of these children, three had severe personal disorder due to the relative "small penis" but the children who were treated according to their genetics, eventhough expressing tomboyish girl and poor body image, could adjust with their female gender identity. Severe virilized females are more likely to be raised as males in cultures that boys value more or in developing country where the diagnosis are likely to be delayed.[8]

More than half of our female subjects showed Prader score III virilization. Similar to our study, Bajpai *et al*[18] reported Prader score III virilization in 52.7% of subjects, Kharrat *et al*[19] reported Prader score III and Prada score IV in 22 and 45% of the subjects respec-

A. Deletion/LGC

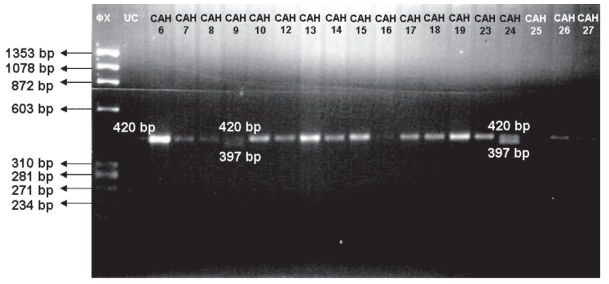


Note: ΦX: DNA marker; UC: un-cut DNA sample; IC: internal control; CAH 6-10, 13-15, 17, 18: no deletion/LGC; CAH 11: homozygote deletion/LGC; CAH 12 and 16: heterozygote deletion/LGC



Note: ΦX: DNA marker; UC: un-cut DNA sample; IC: internal control; CAH 19: homozygote deletion/LGC; CAH 22 and 27: heterozygote deletion/LGC; CAH 20, 21, 23-26: no deletion/LGC

A. I172N mutation



Note: ΦX: DNA marker; UC: un-cut DNA sample; CAH 6-8, 10-23, 25-27: no I172N mutation; CAH 9 and 24: heterozygote I172N mutation

Figure 2. PCR analysis

tively. All these studies indicated how important prenatal diagnosis is, because early treatment can be started immediately to prevent prenatal virilization.[1-8]

In our study, allele's frequency of deletion/LGC was 12.5%. The global predicted frequency were 25-30% with phenotype prediction was SW. Asian countries, such as India,[15] Lebanon,[26] and Turkey,[27] reported this mutation as much as 15.9%, 14%, and 32.1% respectively. The frequency of this mutation is remarkable high. Point mutation in codon 172 (isoleusin α aspargine), was found in 3.1% cases. Globally, it was predicted that this mutation has a frequency of 5-10% with phenotype prediction of the SV form. In Japan[16] Singapore,[13] China,[17] India[15] and Turkey,[27] alleles frequency of I172N was reported to be 2.9%, 23.1%, 23%, 71.4% and 11.4%, respectively.

SW phenotype in deletion/LGC subjects occurs because of CYP inability in coding the formation of active enzymes. In homozygous mutant, where there was completely no activity of residual enzyme, eventually will result in SW form[28]. All of the subjects with deletion/LGC in our study showed SW form, indicating the consistent correlation between genotype and phenotype. Subjects who had I172N mutation showed SW and SV form. This different phenotype was attributable to heterozygote mutation in these two subjects. Jaaskelainen *et al*[29] reported clinical variation in subjects with I172N mutation with genotype I172N/Deletion showing SW form. Wilson *et al*[30] suggested that genotype is not constantly able to predict phenotype in heterozygote mutant. Phenotype usually reflects the most unaffected alleles.

In conclusion, our series indicates that there are generally good correlation between genotype and phenotype in our CAH-21 hydroxylase patients that is similar to those found in Asian studies.

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