Mutations of the Steroid 21-Hydroxylase gene among Filipino Patients with Congenital Adrenal Hyperplasia

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ABSTRACT

Congenital adrenal hyperplasia (CAH), an autosomal recessive disorder, is due to deficiency of the enzymes involved in adrenal steroidogenesis. Phenotypic manifestations vary as a result of the degree of glucocorticoid or mineralocorticoid deficiency and androgen excess present. Among Filipinos, the estimated crude incidence of CAH is approximately 1 in 7,000, which is higher than what is reported in most populations. More than 90% of all cases result from a 21-hydroxylase (21-OH) (cytochrome P450c21) enzyme deficiency involving two 21-OH genes, the active gene (CYP21) and a pseudogene (CYP21P). Studies have shown that mutations result from unequal crossover during meiosis which leads to complete deletion of the gene, gene conversion events or to point mutations. To date, there are no published data on the types of mutations present among Filipinos diagnosed with congenital adrenal hyperplasia. The objective of this study is to describe the profile of Filipino patients diagnosed with CAH and to determine the disease-causing alleles in the 21-OH gene of these patients. Using a method of combined differential polymerase chain reaction and amplification created restriction site approach, direct probing for the presence of known mutations in exons 1,3,4,6,7,8 and intron 2 of the CYP21 and CYP21P genes among Filipino patients with CAH was performed. A total of 12 unrelated CAH patients were examined. A majority of these cases had a premature splicing error mutation at nucleotide 656 of intron 2. The determination of the most frequent alleles in our population can facilitate rapid screening for mutations in the 21-OH gene and lead to a definitive diagnosis of CAH.

Key Word: congenital adrenal hyperplasia, 21-hydroxylase gene, mutations

Introduction

Congenital Adrenal Hyperplasia (CAH), an autosomal recessive group of disorders, is due to deficiency of enzymes involved in adrenal steroidogenesis.[1] Enzyme deficiencies resulting in CAH include 21-hydroxylase (21-OH) enzyme, 3 beta-hydroxysteroid dehydogenase enzyme, 17 alpha-hydroxylase enzyme, 11 beta-hydroxylase enzyme and steroidogenic acute regulatory protein (StAR) enzyme.[1,2] Deficiency of the 21-OH enzyme is the most common cause of CAH. Severe enzyme deficiency with prenatal onset characterizes the classic forms of the disorder, both salt-wasting (SW) and simple virilizing (SV). Androgen excess produces prenatal masculinization (ambiguous genitalia) in females and postnatal virilization in both sexes.[1] Classic SW-CAH is life-threatening in the newborn period. On the other hand, classic SV-CAH is a milder, non-salt-wasting form.[1,2,3] The non-classic (NC) form has moderate enzyme deficiency, no salt loss, postnatal onset and females are not virilized at birth. [1,2]

The worldwide incidence of the classic forms of CAH (both SW- and SV-) is approximately 1 in 15,000, with ethnic and racial variability.[1] The incidence in France, Italy, Scotland, New Zealand and Japan ranges from 1 in 10,000 to 1 in 23,000.[4] A higher population prevalence is seen in Saudi Arabia with a rate of 1 in 5,000. Among the Yupik Eskimos of Alaska, the incidence is much higher at 1 in 282.[4] In the Filipino population, the newborn screening program reports an estimated crude incidence of classic CAH to be approximately 1 in 7,842 (Philippine Newborn Screening Program, 2008) which is considered high compared to prevalences reported in most populations.

Clinical diagnosis for 21-OH deficiency should be suspected in females who are virilized at birth, who become virilized postnatally or who have contrasexual precocious puberty or premature adrenarche (Figure 1). Males most often present with a salt-losing crisis during the first four weeks or virilization in childhood. Affected untreated individuals will have elevated serum levels of 17-hyroxyprogesterone (17-OHP). Among those with SW-CAH, plasma renin activity is high, serum aldosterone is inappropriately low for the renin concentration and electrolyte levels show decreased sodium and chloride, and increased potassium.[1] Management of SV-CAH deficiency involves glucocorticoid replacement and SW-CAH involves both glucocorticoid and mineralocorticoid replacement therapy, and virilized females will also undergo reconstructive surgery.[1,2]

The molecular basis of 21-OH CAH has been well described. The 21-OH genes, CYP21 and CYP21P are located on the short arm of chromosome 6. [1] The genes, arranged tandemly, consist of 10 exons spanning 3 kilobases of

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DNA and display 98% sequence homology.[1,5] Only CYP21 is active and gives rise to the enzyme product; CYP21P has several deleterious mutations that has rendered it an inactive pseudogene. The existence of highly homologous, tandemly arranged genes provides an opportunity for meiotic mispairing and unequal crossing over between sister chromatids.[2,7,8] Studies have shown that these can lead to mutations such as complete deletion of the gene, gene conversion events or point mutations.[9] The loss of the CYP21 gene on both chromosomes results in an almost complete loss of 21-OH activity.

To date, there are no published data on the types of mutations present among Filipinos diagnosed with CAH. The objective of this study is to determine the disease-causing alleles in the 21-OH gene among Filipino patients diagnosed with CAH. Using a previously described combined differential polymerase chain reaction (PCR) and amplification created restriction site (ACRS) approach[9], direct probing for the presence of known mutations in exon 1,3,4,6,7,8 and intron 2 of the CYP21 and CYP21P genes among Filipino patients with CAH was performed. The determination of the most frequent disease-causing alleles in our population can facilitate rapid screening for mutations in the 21-OH gene and lead to a definitive diagnosis of CAH. [10]

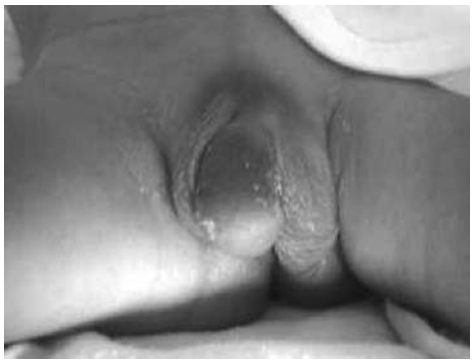

Figure 1. Ambiguous genitalia in a female with CAH

Materials and Methods

Subjects. Twelve unrelated individuals diagnosed with CAH were included in this study. Half of the cases were identified through the Philippine Newborn Screening Program and subsequently confirmed to have 21-OH CAH using a 17-OHP assay, and sodium and potassium determinations. Blood samples were collected and DNA was isolated using QIAmp Midi Blood extraction kit (Qiagen, Germany) and subsequently stored at -20°C prior to analysis.

Methods. The procedure used was a previously described PCR/ACRS method, directly probing for the presence of known mutations in exons 1,3,4,6,7,8 and intron 2 of the CYP21 and CYP21P genes.8 CYP21 was amplified using primers 21BF (TCGGTGGGAGGGTACCTGAAG)an d21BR(AATTAAGCCTCAATCCTCTGCAGCG). Primers 21AF (GGGTCGGTGGGAAGGCACCTGAG) and 21AR (GATTAAGCCTCAATCCTCTGCGGCA) were used for the amplification of CYP21P. The PCR reaction was carried out in a 50 μl solution of: 0.5 U of Taq polymerase (Invitrogen, USA), 5μl of 10X PCR buffer (200mM Tris-HCl, pH 8.4, 500mM KCl) (Invitrogen), 1.5μl of 10mM dNTP mix (Invitrogen), 2 μl of primers (10 pmol), 2.5 μl of gDNA (300 ng) and sterile distilled water. Primary PCR differential amplification of CYP21P and CYP21 was accomplished as follows: denaturation at 94°C for 4 min, followed by 10 cycles at 94°C for 30 sec, 59°C for 30 sec, and 68°C for 5 min, and another 15 cycles at 94°C for 30 sec and 68°C for 5 min. Primary PCR products were digested with EcoRI (Invitrogen) restriction enzyme, and separated on a 1% (LE analytical agarose, PROMEGA, USA). Secondary PCR was carried out using ACRS primers. The total volume of each reaction mixture was 50 ul which included: 0.5 μl of the primary PCR product, 5 μl of 10X PCR buffer (200mM Tris-HCl, pH 8.4, 500mM KCl) (Invitrogen,USA), 1.5μl of 10mM dNTP mix (Invitrogen,USA), 2 μl of primers (7.5 pmol), 1.5μl of MgCl (50mM), 0.5 U Taq polymerase (Invitrogen,USA) and distilled water. The samples were subjected to the following PCR conditions: 94°C for 1 minute, 25 cycles at 94oC for 30 seconds, 59°C 30 seconds and 72°C for 30 seconds. ACRS PCR product (5μl) was incubated for at east 3 hours with 5 to 10 U of a restriction enzyme. The reactions were then directly electrophoresed on 2% agarose (PROMEGA, USA) gel in the presence of ethidium bromide. Analysis was carried out on the enzyme digestion fragments of the PCR product. Gels were photographed using a UV transilluminator (Figure 2).

Results and Discussion

Twelve patients were included in the study, of which three were male (25%) and nine were female (75%). Ten of the patients had SW-CAH while two had SV-CAH. At the time of inclusion into the study, the ages of the subjects ranged from 3 weeks to 6 years of age. Six of the subjects had ages between 3-6 years of age, while the other six were ≤ 1 year of age. The average age at the time of diagnosis was around 3 weeks.

All female patients presented with dark skin and ambiguous genitalia at the time of diagnosis. Seven of nine female patients had a salt-losing crisis. Among the three male patients, two initially presented with adrenal crisis, while one presented with seizures. Only one patient had a positive family history of CAH.

Figure 3. ACRS amplification of the sequences containing the mutation sites in the CYP21 gene for the patients in this study with CAH.

Ten of 12 (83%) patients had only one mutation that could be detected using the PCR-ACRS approach. Seven of these 10 patients had a premature splicing error involving

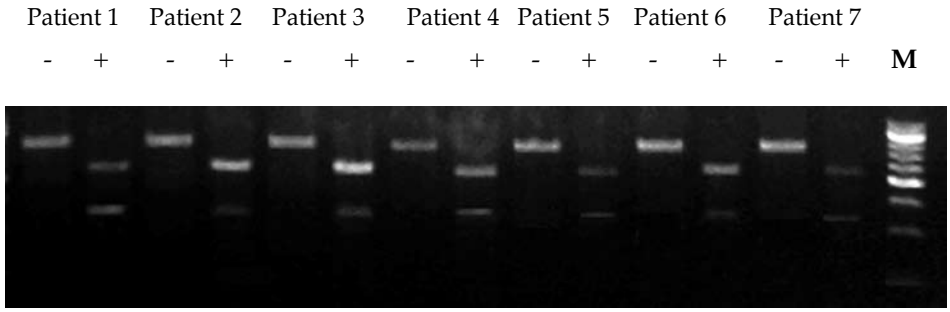

Figure 2. CYP21 primary PCR products used as templates for secondary PCR product. A 500 bp ladder (Promega, USA)was used as molecular marker (M).

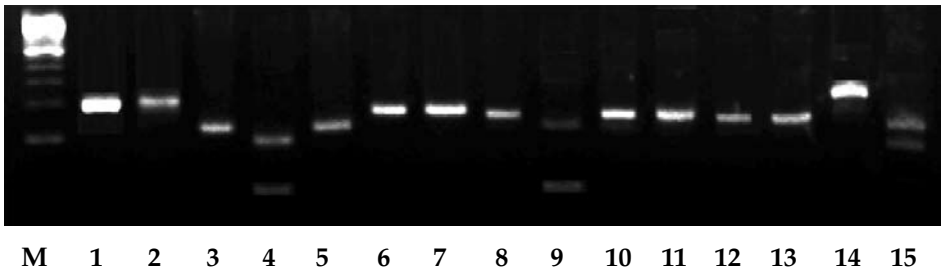

Figure 3. ACRS amplification of the sequences containing the mutation sites in the CYP21 gene for the patients in this study with CAH. Lanes 4 and 9 show the mutant (85 and 30 bp) fragments after digestion of *SacI* restriction enzyme. A 100 bp ladder (Invitrogen, USA) was used as molecular marker (M).

nucleotide 656 of intron 2 (Figure 3). Two of the 10 (20%) had a missense mutation in exon 4, codon 172, while one had a disease-causing allele in exon 8, codon 318. Finding only one mutation among these CAH patients suggests that they may be homozygous for this particular disease-causing allele. Demonstrating the same mutation in one of the alleles of each of their parents will help in establishing whether these patients are truly homozygous. Previous studies have shown that the majority of patients (65-75%) from heterogeneous population groups with 21-OH deficiency are compound heterozygotes for disease-causing mutations.[12,13] In our study, only 1 in 12 (8 %) demonstrated two different mutations in their CYP21 genes and is considered a compound heterozygote. This patient had the intron 2, nt 656 mutation on one allele and the exon 8, codon 318 mutation on the other allele.

One patient who presented with seizures during the first 25 hours, and subsequently developed an adrenal crisis, did not have any of the CYP21 mutational alleles examined. This patient may have a yet uncharacterized mutation which caused the clinical symptoms observed.

Cases of CAH which show multiple mutations in one chromosome have been reported. In such instances, a chimeric CYP21P/CYP21 gene may exist.[13] The chimera is caused by a deletion of a RCCX module including TNXA, RP2, and C4B genes.[11] The molecule of chimeric CYP21P/CYP21 results in unequal recombination between CYP21P and CYP21 genes. Single Strand Conformational Polymorphism (SSCP) analysis needs to be applied to find the second mutation in these cases where only one mutation was found. Other loci such as I172N, V281L, Q318X, and R356W require further analysis.

Molecular genetic tests for CYP21 gene are available in most laboratories abroad and can detect a panel of 9 common mutations and gene deletions.[6,10] These mutations account for 90-95% of disease-causing alleles in affected individuals and carriers. Functional mutations that have been characterized so far, which produce no enzyme activity, include deletions of exons 1 to 8, an 8 bp deletion in exon 3, several missense mutations on exon 6 (Ile 236 -> Asn, Val 237 ->Glu and Met 239 ->Lys), an insertion mutation on exon 7, a nonsense mutation in exon 8 and a premature splicing error on intron 2.[2,3,10] Those with severe impaired enzyme activity have shown a missense mutation in exon 4, while those with moderately impaired enzyme activity showed missense mutations in exons 1 and 7. In our study, the most common disease-causing allele identified was a premature splicing error in intron 2.

Conclusions and Recommendations

Among Filipino patients with CAH, the majority showed a premature splicing error mutation at nucleotide 656 of intron 2 of the 21-OH gene, one of the most common mutational alleles also reported in other populations. However, it is difficult to conclude whether the patients were homozygous for this disease-causing allele. It is likely that some of the patients carry an additional, uncharacterized mutation in other exons of the CYP21 gene, which could not be detected using the methods in this study. The small number of patients included also limits the significance of these results.

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