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Application of the DHPLC method for mutational detection of the *CYP21A2* gene in congenital adrenal hyperplasia

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ABSTRACT

Background: More than 90% of cases of congenital adrenal hyperplasia (CAH) are caused by a steroid 21-hydroxylase deficiency. Approximately 75% of the defective *CYP21A2* genes are generated through an intergenic recombination with the neighboring *CYP21A1P* pseudogene. These 2 duplicated genes share a 98% nucleotide sequence homology. Therefore, precisely identifying the *CYP21A2* gene in CAH patients is absolutely necessary.
Methods: We describe an established PCR-based amplification method, a denaturing high-performance liquid chromatography (DHPLC) analysis, to directly identify 11 different mutations commonly appearing in the *CYP21A1P* gene. Among these 11 mutations, 9 are found in CAH patients and 2 created mutations were from normal individuals.
Results: From the DHPLC analysis using 6 fragments of amplicons, the elution profiles of the 11 mutation sites were successfully used to distinguish these common disease-causing mutations of the *CYP21A2* gene. Based on this resolution, we were able to rapidly search existing sequences of mutations in the *CYP21A1P* gene for this malady.
Conclusion: DHPLC is an efficient and specific means to undertake such a program for screening patients with CAH caused by defects of the *CYP21A2* gene resulting from the neighboring *CYP21A1P* pseudogene.

1. Introduction

Congenital adrenal hyperplasia (CAH) is an inherited disorder mainly resulting from defects in the steroid 21-hydroxylase (*CYP21A2*) gene which causes about 90%–95% of all CAH cases. It is one of the most common inborn errors of metabolism in humans. The wide range of CAH phenotypes is associated with multiple mutations known to affect 21-hydroxylase enzymatic activity. Three forms of CAH are classified: the classic salt-wasting, classic simple virilizing, and nonclassical forms [1,2]. The incidence of disease caused by the 2 classic forms is reported to be 1:10,000–1:18,000, depending on race [3,4]. The non-classic one is milder with an estimated occurrence in the general population of 1:1700 [3,5].

Defective *CYP21A2* genes in CAH fall into 1 of 3 categories: (a) small-scale conversions of *CYP21A1P*, (b) spontaneous mutations, and (c) chimeric *CYP21A1P*/*CYP21A2* and *TNXA*/*TNXB* genes [6,7]. Most (up to 11 for the *CYP21A1P* gene) of *CYP21A2* mutations [8] identified so far are a result of small-scale conversions which account for about 70%–75% of all CAH cases. Among these mutations, the 3 most frequent ones are IVS2-12A/C>G (I2 splice), I172N, and R356W which show high similar incidences worldwide in different races [3,9–12]. The 707–714del mutation at exon 3 has a low frequency in most population [3,9–12]. However, its combination with the IVS2-12A/C>G (I2 splice) mutation (without the P30L mutation) presenting a 3.2 TaqI-produced fragment of the *CYP21A1P*-like gene is most prevalent in ethnic Chinese CAH patients [13]. In addition, a mutation of V281L, the most common nonclassical disease appearing with high frequencies in patients in France, Austria, Italy, Spain, Turkey, Argentine, and Portugal as well as Ashkenazi Jews [9,10,12,14], was not found in Japanese [15], Asian [12] or Tunisian patients [16]. The 2 mutations of P30L and V281L which reduce enzymatic activity to 20%–50% of normal are associated with this form [2]. In addition, cluster E6 of the I236N, V237E, and M239K mutations which are thought to be transferred together from the *CYP21A1P* gene [17] is not in a continuous stretch of DNA to the *CYP21A2* gene through recombination at meiosis [18]. The L307 frameshift mutation (F306ΛL307insT) has a low frequency or is not found in most populations [3,9–12,14,15]. The frequency of the non-sense Q318X mutation which results in the complete loss of enzyme activity has higher rates in East Indian populations [12] and Tunisian

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patients [16]. An increasing number of spontaneous mutations (5%–10% of cases) mostly consist of nucleotide changes in the coding sequence [19]. Chimeric *CYP21A1P*/*CYP21A2* and *TNXA*/*TNXB* genes, which result from unequal cross-overs (or deletions) during meiosis and occur in ~20% of CAH alleles in most populations [3], respectively resulted in the deletion of the _?_*CYP21A1P-XA-RP2-C4B*-_?_*CYP21A2* gene array which exists in 6 types of chimeric *CYP21A1P*/*CYP21A2* genes [20] and a deletion of the *RP2-C4B-CYP21A2*-_?_ *TXNB* gene array [7] which leads to CAH and is associated with the recessive disorder of Ehler–Danlos syndrome [21,22]. These are found in 5 types of chimeric *TNXA*/*TNXB* genes [23]. In fact, these 2 different types of large-gene deletions of the chimeric *CYP21A1P*/*CYP21A2* and *TNXA*/*TNXB* genes are generally considered to represent 1 event according to many studies [9,10,12,15,16,24,25].

Because of the high homology of ~98% of nucleotide sequences between the *CYP21A2* and *CYP21A1P* genes, separating the *CYP21A2* gene from the *CYP21A1P* pseudogene is absolutely essential during mutation analyses. Hence, direct molecular diagnosis of the *CYP21A2* gene has proven to be feasible and accurate. Several methods were established to separate the *CYP21A2* gene from the *CYP21A1P* pseudogene, including a 2-step *CYP21A2* amplification [24–26], 1-step differential polymerase chain reaction (PCR) amplification of the *CYP21A2* gene [8,27] and an allele-specific PCR for detecting 10 different mutations [28]. These PCR products of either 1 or 2 fragments as a template are subject to known or unknown mutational detection using more-practical methods, such as single-stranded conformation polymorphism (SSCP) [29], PCR/ligase detection [26], amplification-created restriction site (ACRS) [30], and direct DNA sequencing and multiplex minisequencing [31]. Recently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) was applied to identify both known and novel mutations and polymorphisms of the *CYP21A2* gene [32]. In the present study, we used a denaturing high-performance liquid chromatography (DHPLC) analysis to directly identify 11 nucleotide sequences commonly appearing in the *CYP21A1P* gene, including P30L, I2 splice, 707–714del, I172N, cluster E6, V281L, F306ΛL307inseT, Q318X, and R356W and to establish such a standard DHPLC heteroduplex profile for rapidly and precisely screening CAH patients which present 70%–80% in CAH cases.

2. Materials and methods

2.1. Subjects

From a concerted effort among hospitals across Taiwan from 1994 to 2006, we characterized 200 CAH patients [23]. All families requested an extensive molecular diagnosis and provided informed consent. Among these CAH families, we used 9 mutations in unrelated patients converted from the *CYP21A1P* gene (small-scale conversions) including I2 splice (designated B1), 707–714del (designated B2), I172N (designated C), cluster E6 (designated D), F306ΛL307inseT (designated H2), Q318X (designated J1), and R356W (designated J2) (Fig. 1) which accounted for ~79% cases [23]. Detection of the *CYP21A2* mutation in these patients was formerly determined by the ACRS method as previously described [30]. In order to produce the heteroduplex DNA fragment for the DHPLC analysis, patients with the haplotype of compound heterozygous mutations in the *CYP21A2* allele were selected. Since no patient with both the P30L (designated A) and V281L (designated H1) mutations (Fig. 1) was found in our population (1 patient with the V281L mutation was misdiagnosed in a report [23]), we created these 2 mutations from a normal individual.

2.2. A primary 3.5-kb differential PCR product of the CYP21A2 gene for identifying 9 mutations converted from the CYP21A1P gene

Because of the high homology between the *CYP21A2* and *CYP21A1P* genes, a 3.5-kb PCR product covering 10 exons of the *CYP21A2* gene was amplified with a differential paired primer, BF1/21BR, as described previously [8] (Fig. 1). To identify the *CYP21A2* mutations converted from the *CYP21A1P* gene, the 3.5-kb primary PCR product obtained from these unrelated CAH patients was then used as a template for detecting 9 mutation sites.

2.3. A primary 3.0-kb PCR product containing a mixture of the CYP21A2 and CYP21A1P genes for creating P30L and V281L heterozygous mutations in a normal individual

Because of no mutation of P30L or V281L found in our population (1 patient with V281L mutation was misdiagnosed in a report [23]), a 3.0-kb PCR product amplified with a universal paired primer, CYP-270f/Ex10R (Fig. 1), was derived from a normal individual. The 3.0-kb PCR product contained a mixture of the *CYP21A1P* and *CYP21A2* genes which present the haplotype of compound heterozygous mutations with 11 defective alleles as the *CYP21A1P* gene [8]. Primers CYP-270f (nt −270 to −250 [33], 5′-CCAGAAAGCTGACTCTGGATG-3′) and Ex10R (nt 2720 to 2739 [33], 5′-CTGAGGTACCCGGCTGGCAT-3′) are respectively located at the 5′and 3′ends of the *CYP21A1P* and *CYP21A2* genes. The PCR conditions used for 3.5-kb differential PCR amplification of the *CYP21A2* gene were as previously described [8]. The 3.0-kb PCR products as a template were then used to detect the P30L (designated A) and V281L (designated H1) mutations (Fig. 1).

2.4. Secondary PCR amplification of both the 3.5- and 3.0-kb PCR products for the DHPLC analysis

Both the 3.5-kb PCR product amplified with the differential paired primer, BF1/21BR, from CAH patients and the 3.0-kb PCR products amplified with the universal paired primer, CYP-270f/Ex10R, creating P30L and V281L mutations from the normal individual were used as a template for secondary PCR amplification using 6 DHPLC paired primers (Table 1) including 11 mutational loci (designated A to J2) (Fig. 1). The sequence and location of the DHPLC primers are listed in Table 2. The reaction mixture of the secondary PCR amplification

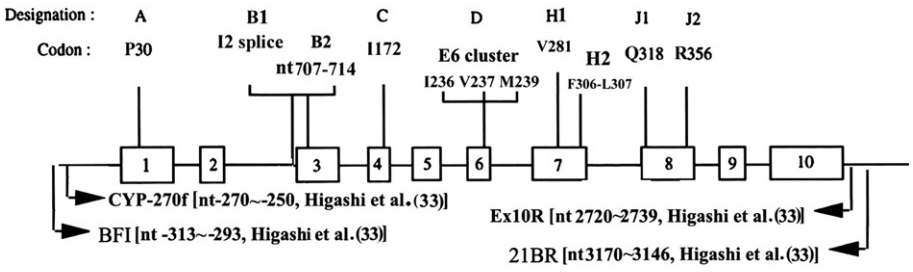

Fig. 1. Diagram of 11 *CYP21A2* mutations converted from the neighboring *CYP21A1P* pseudogene and the strategy for amplification of the *CYP21A2* gene. The structure of the *CYP21A2* gene is indicated by a *white box*. Two strategies for differential PCR amplification of the *CYP21A2* gene using the paired primers, BF1/21BR, for amplifying a 3.5-kb PCR product and for amplifying the mixture of the *CYP21A2* and *CYP21A1P* genes using the universal paired primer, CYP-270/Ex10R, for amplifying a 3.0-kb PCR product are shown. Designations of A to J2 indicate the 11 mutation sites converted from the *CYP21A1P* pseudogene.