Research Article

Variations in the 3′UTR of the CYP21A2 Gene in Heterozygous Females with Hyperandrogenemia

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Heterozygosity for CYP21A2 mutations in females is possibly related to increased risk of developing clinical hyperandrogenism. The present study was designed to seek evidence on the phenotype-genotype correlation in female children, adolescents, and women with CYP21A2 mutations and variants in the 3′UTR region of the gene. Sixty-six patients out of the 169 were identified as carriers of CYP21A2 mutations. Higher values of stimulated 17 hydroxyprogesterone (17-OHP) levels were found in the carriers of the p.Val281Leu mutation compared to the carriers of other mutations (mean: 24.7 nmol/l versus 15.6 nmol/l). The haplotype of the *52C>T, *440C>T, and *443T>C in the 3′UTR was identical in all heterozygous patients with p.Val281Leu and the haplotype of the *12C>T and *52C>T was identical in all heterozygous patients with the p.Gln318∗. In conclusion, hyperandrogenemic females are likely to bear heterozygous CYP21A2 mutations. Carriers of the mild p.Val281Leu mutation are at higher risk of developing hyperandrogenism than the carriers of more severe mutations. The identification of variants in the 3′UTR of CYP21A2 in combination with the heterozygous mutation may be associated with the mild form of nonclassic congenital adrenal hyperplasia and reveal the importance of analyzing the CYP21A2 untranslated regions for the appropriate management of this category of patients.

1. Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by mutations in one of the genes involved in steroidogenesis. More than 95% of all cases of CAH are due to 21-hydroxylase deficiency (21-OHD) resulting from defects in the steroid 21-hydroxylase (CYP21A2) gene. CAH is classified into the severe classical form with an overall estimated incidence of 1:10,000 to 1:15,000 and into the mild nonclassical form (NC-CAH) ranging from 1:500 to 1:100 live births [1–3].

Since the prevalence of CAH and NC-CAH has been reported to vary among populations, the expected heterozygote frequency for 21-OHD also varies considerably and ranges from 1:10 to 1:60 in specific populations [4–8]. Moreover, for certain ethnic groups such as the Ashkenazi Jews, the carrier frequency was reported to be 1:3, thus making it to be the highest ever reported for the disease [9].
To date, the occurrence of genetic mutants of 21-OHD has overwhelmingly been investigated and ethnic detailed distribution of mutations has been described [10–20]. Roughly 95% of the CYP21A2 alleles bearing mutations are due to recombination events among the homologous inactive CYP21A2P pseudogene and the CYP21A2 gene. The remaining 5% of the CYP21A2 mutations is the result of new casual mutagenic events [21, 22]. It should be noted though that an estimated 20% of the alleles in the NC-CAH form remain without identified causative mutations, thus signifying the necessity to investigate the CYP21A2 regulatory regions [23].

At this time, numerous studies have implicated a number of variations in the promoter and the 5′UTR of the CYP21A2 [24–26]. Recently, in vitro and bioinformatics analysis for the sequence variation *13G>A in the 3′UTR predicted a modification of the RNA expression and folding was found to correlate with a mild form of NC-CAH [27]. The 3′UTR plays a vital role in gene expression by regulating the localization, export, stability, and translational competence of an mRNA. Variations in the 3′UTR might change the mRNA secondary structure and are identified to be accountable for human diseases [28]. In population studies with a large number of nonclassical patients, the percentage of alleles with recognised mutations is variable, ranging from 80% to 100% [29–32], signifying the stipulation to evaluate the CYP21A2 regulatory regions.

Numerous studies in the Mediterranean Basin, including research from our group, have confirmed as the most predominant genetic defects, the mutations IVS2-13A/C>G, p.Gln318*, p.Val281Leu, and c.329_336del (8bpdelE3) [12, 13, 33, 34]. Compared to normal female individuals, female carriers for 21-OHD frequently demonstrate an increased secretion of the 21-OH precursors 17-hydroxyprogesterone (17-OHP) and progesterone (P4) [35–43] and lower levels of 11-deoxycorticosterone [37] as expected. Furthermore in obligate heterozygotes, patients for the simple virilizing form of CAH, aldosterone secretion was found to be reduced following ACTH stimulation, suggesting an impairment of the zona glomerulosa in addition to zona fasciculata [44].

Several studies from various groups have conveyed that between 50 and 80% of carriers exhibit a 17-OHP level after ACTH stimulation and that is above the 95th percentile of the control value [37, 39, 42, 45]. The purpose of this study was to determine the frequency of identified defects of the CYP21A2 gene and also to weigh the regulatory 3′UTR region of the gene in a clinically symptomatic cohort of 169 females, characterized by hyperandrogenaemia. To test this hypothesis, the total of 169 patients was tested by Sanger DNA sequencing and MLPA analysis for defects in the CYP21A2 gene.

2. Subjects and Methods

2.1. Biochemical and Clinical Evaluation. A total of 169 unrelated Greek Cypriot females were diagnosed and 47 were children, 30 adolescents, and 92 women. The study was approved by the Cyprus National Bioethics Committee and written consent was obtained from all adult patients and the parents or guardians of the minors. The diagnosis of the patients was based on the clinical findings and elevated stimulated plasma 17-OHP [1, 10]. A standard dose of ACTH stimulation test was used. Measurements of the basal and after 60 minutes ACTH administration serum 17-OHP concentrations were obtained with the commercial RIA method (Beckman Coulter).

The patients of the study were clinically evaluated based on hyperandrogenaemia symptoms. These symptoms included early appearance of pubic or axillary hair (before the age of 8 years), severe acne or hirsutism (determined by a Ferriman–Gallway score of >8) in adolescents and adults, abnormal menstrual cycles with or without polycystic ovaries, and complete absence of virilisation.

2.2. Statistical Analysis. Comparisons of 17-OHP levels were accomplished using parametric methods (t-test procedures). Frequency distributions of the 3′UTR and 5′UTR/promoter variants of the CYP21A2 gene were tabulated and compared using chi-square methods. A two-tailed alpha level of 0.05 was used to establish statistical significance. All statistical analyses were done using SAS software Version 9.2, SAS Institute Inc., Cary, NC, USA. (i) The results of the 17-OHP levels are shown in the text and expressed as mean ± SD. (ii) The frequency distribution of the 3′UTR and the 5′UTR/promoter variants of the CYP21A2 gene was estimated using the FREQ procedure.

2.3. Amplification of the CYP21A2 Gene. The CYP21A2 gene of the total number of patients who participated in the study was analyzed by Sanger DNA sequencing. More specifically, the CYP21A2 promoter/regulatory regions from the patients participating in this study and 150 normal females were analyzed in this paper. The genetic investigation was done based on a cascade strategy as formerly described [12, 13, 33]. For the amplification of the 5′UTR region that is located in the first 167 nucleotides upstream the ATG codon of the CYP21A2 gene, the primers P1–P48 [46] were used to amplify a fragment of 370bp. The 3′UTR region that is 536 nucleotides downstream the TGA stop codon of the CYP21A2 gene was amplified using the primers: 5′AGATG CAGCCTTTGCCAAGTG3′ and 5′AGCACAGTGGAACCAT CAGGT3′ [27].

2.4. MLPA Analysis. The multiplex ligation-dependent probe amplification (MLPA) technique (MRC Holland, Amsterdam, Netherlands) was used to detect any possible large gene deletions, duplications, and large gene conversions in the CYP21A2 gene. DNA from the 169 female patients in this study analyzed by direct sequencing was also examined with MLPA [13].

2.5. Secondary Structure Analysis. The 2182 bp complete mRNA, including the 5′UTR, coding region, and 3′UTR, was submitted to the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) with default parameters to predict the potential secondary structure. Secondary structures were predicted for the wild-type, the 281I/ *52/*440/*443 mutant, and the 318Ter/*12/*52 mutant
Table 1: Phenotype-genotype correlation of the 66 females with CYP21A2 heterozygote mutations. The hormonal analyses for 17-OHP were performed in 52 patients. Two of the patients with p.Val281Leu/X had stimulated 17-OHP levels more than 60.5 nmol/l viewing the possibility of an additional unidentified mutation and were excluded from the statistical evaluation.

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<td>2</td>
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<td>1</td>
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<tr>
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<td>15.6</td>
<td>8.3</td>
<td>2.2</td>
<td>2.4</td>
<td></td>
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<tr>
<td>Stimulated 17-OHP (nmol/l) N = 16</td>
<td>20–24.2</td>
<td>27</td>
<td>13.7</td>
<td>32</td>
<td>14.5</td>
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<tr>
<td>Adolescents</td>
<td>N = 17</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Basal 17-OHP (nmol/l) N = 13</td>
<td>2.6–12.4</td>
<td>4.2–9.2</td>
<td>3.5–6.7</td>
<td>2.4–5.1</td>
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<tr>
<td>Stimulated 17-OHP (nmol/l) N = 13</td>
<td>11–28</td>
<td>10.3–19.6</td>
<td>14.2–17.1</td>
<td>9.6–4.8</td>
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<tr>
<td>Adults</td>
<td>N = 30</td>
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<td>5</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Basal 17-OHP (nmol/l) N = 23</td>
<td>3.4–14.2</td>
<td>4.6–17.6</td>
<td>1.7–8.9</td>
<td>5.6</td>
<td>4.4</td>
<td>6.1</td>
<td>8.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulated 17-OHP (nmol/l) N = 23</td>
<td>11–30.2</td>
<td>1.4–18.1</td>
<td>8.9–14.7</td>
<td>12</td>
<td>21.1</td>
<td>15.8</td>
<td>12.3</td>
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CYP21A2 mRNA. Potential minimum free energy (MFE) structures, centroid structures, and positional entropies were obtained [47].

3. Results

3.1. Clinical and Laboratory Characteristics. In a total of 169 unrelated hyperandrogenaemic females, 66 were identified as carriers of CYP21A2 mutations (Table 1). Twenty-one of the CYP21A2 heterozygote female patients were presented in childhood with premature adrenarche (age 3–10 years). The remaining 45 CYP21A2 heterozygote female patients were diagnosed in adolescence (14–17 years) and adulthood (18–35 years) with clinical signs of hyperandrogenaemia. Among the 45 adolescent and adult CYP21A2 heterozygote female patients, the most common presenting symptom was irregular menses with or without multicystic ovaries on the ultrasound (32/45), followed by hirsutism in 21/45.

ACTH stimulation test was performed on 104 females (52 carriers and 52 noncarriers). Mean plasma basal 17-OHP (nmol/l) level in the noncarriers was 5.9 ± 1.9 (mean ± SD, range 10.2–2.2) and rose to 12.6 ± 3.8 (mean ± SD, range 19.4–7.0) after ACTH stimulation. Carriers demonstrated a higher mean plasma basal 17-OHP level (nmol/l) 6.5 ± 3.5 (mean ± SD, range 17.6–1.7) and after ACTH stimulation rose to 19.0 ± 12.6 (mean ± SD, range 30.2–1.4) (p < 0.0003). The hormonal analyses for 17-OHP were performed in 52 patients.

Two of these patients with p.Val281Leu/X which were hormonally evaluated had stimulated 17-OHP levels more than 60.5 nmol/l and were excluded from the statistical evaluation. Therefore, the implication of digenic inheritance in the above patient might be the case for the development of CAH.

3.2. CYP21A2 Genotypic Analysis. The most frequent mutation within the 66 unrelated alleles was p.Val281Leu (53.0%), followed by p.Gln318* (18.2%), p.Pro482Ser (10.6%), p.Val304Met (6.1%), p.Pro453Ser (6.1%), p.Ala391Thr (1.5%), large deletion/conversion exons 1–4 (1.5%), and microconversion of exons 6–8 and 8bpdelE3 (1.5%). MLPA results confirm that the carriers with the p.Gln318* are real and exclude the possibility of false positives due to influence of the pseudogene duplications. Phenotype-genotype correlation of the carrier hyperandrogenaemic females is presented in Table 1. Higher mean plasma values for 17-OHP after ACTH stimulation were exhibited in female carriers of the p.Val281Leu mutation when compared to the mean values observed in female carriers of other CYP21A2 mutations (24.7 nmol/l versus 15.6 nmol/l) (p < 0.0001).

The remaining 103 females who manifested clinical signs of hyperandrogenaemia and were identified with no mutation in the CYP21A2 gene exhibited an unusual high allelic frequency of 56.3% for the p.Asn493Ser variant. On the contrary, the allelic frequency of the p.Asn493Ser variant was significantly different when compared to the one observed in the group of the 66 CYP21A2 heterozygous females (56.3% versus 19.7%, p < 0.0001).

3.3. Extended Sequencing of the Promoter/3’UTR and the 3’UTR of the CYP21A2. The aim of the study was to identify 3’UTR and 5’UTR variations in patients presented with clinical signs with hyperandrogenaemia who were found to be heterozygotes for the CYP21A2 gene. Therefore, these variations were not tested in patients with both forms of 21-OHD carrying two affected alleles.

Extended sequencing of the 3’UTR of the CYP21A2 in 66 female patients of the present study identified with one affected CYP21A2 allele demonstrated that 29/35 female patients who carried in heterozygosity the missense mutation p.Val281Leu also carry in the 3’UTR the variants *52T>C, *440C>T, and *443T>C in cis. In a similar fashion, 9/12 of the other heterozygote females with the severe p.Gln318* mutation were identified to also carry in the 3’UTR the variants *12T>C and *52C>T in cis (Table 2). For the children

### Table 2: Haplotypes of the alleles with the variants found in the 3’UTR of the CYP21A2 gene.

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Female controls (n = 150)</th>
<th>Female controls (%)</th>
<th>Hyperandrogenic females with no mutation (n = 90)</th>
<th>Hyperandrogenic females with no mutation (%)</th>
<th>CYP21A2 heterozygotes (n = 66)</th>
<th>CYP21A2 heterozygotes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>62</td>
<td>41.3</td>
<td>55</td>
<td>61.1</td>
<td>8</td>
<td>12.1</td>
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<td>1</td>
<td>12</td>
<td>8.0</td>
<td>1</td>
<td>1.1</td>
<td>29‡</td>
<td>43.9</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>18.7</td>
<td>16</td>
<td>17.8</td>
<td>11</td>
<td>16.7</td>
</tr>
<tr>
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<td>14.0</td>
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<td>8.8</td>
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<tr>
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<td>1</td>
<td>1.1</td>
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<td>13.7</td>
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<tr>
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<tr>
<td>6</td>
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<td>8</td>
<td>0</td>
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<td>2</td>
<td>2.2</td>
<td>3</td>
<td>4.5</td>
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‡All 29 females were heterozygote for p.Val281Leu, and all shared the same *12C>T and 52C>T combination of variants.

‡All 9 females were heterozygote for p.Gln318*, and all shared the same *12C>T and 52C>T combination of variants.
**Figure 1:** *CYP21A2* mRNA secondary structure prediction. (a) and (b) predicted *CYP21A2* mRNA secondary structures showing the MFE positional and centroid positional entropy, respectively, for the wild type, 281L/*52*/440/*443 mutant, and 318Ter/*12*/52 mutant. (c) Minimum free energy (MFE) and centroid secondary structure MFE values. mRNA secondary structures values with 1 kcal/mol above the minimum MFE observed in wild type are considered responsible for the destabilisation of the structure [48]. (d) Positional entropies for the wild type, 281L/*52*/440/*443 mutant, and 318Ter/*12*/52 mutant. Locations of the variations are indicated. 281L/*52*/440/*443 mutant variations are indicated with red colour. 318Ter/*12*/52 mutant variations are indicated with green colour. *52 variation which is common in the two mutants is indicated with black colour.
and adolescents that participated in the study, the parental samples were available and segregation analysis determined and proved that p.Val281Leu mutation and variations *52C>T, *440C>T, and *443T>C are in cis. For the rest of the women that participated in the study, no parental samples were available. Sequencing analysis of the CYP21A2 3′UTR of a cohort of 90 hyperandrogenaemic females with no detected mutations in the CYP21A2 gene identified both the combination of 3′UTR variants *52C>T, *440C>T, and *443T>C and *12C>T and *52C>T only once (1.1%) in two different patients (Table 2).

Concurrent screening of the CYP21A2 3′UTR in 150 control females with no hyperandrogenism and no detected mutations in the CYP21A2 gene identified the combination of 3′UTR variants *52C>T, *440C>T, and *443T>C in 12/130 (8%) and *12C>T and *52C>T in 8/150 (5.3%) (Table 2).

The extended sequencing of the CYP21A2 promoter/5′ UTR region in all groups of patients and controls mentioned above did not reveal any unusual variants or frequencies.

3.4. CYP21A2 mRNA Secondary Structure Prediction. Secondary structure prediction analysis of the wild-type CYP21A2 mRNA was compared to the 281L/*52/*440/*443 and the 318Ter/*12/*52 variant mRNAs that are displayed in Figures 1(a) and 1(b). In those figures, the MFE and the centroid positional entropy MFE values are, respectively, displayed and more structural changes caused by the CYP21A2 variants were observed between the two forms. Moreover, the MFE and the centroid secondary structure MFE values of the predicted mRNA secondary structures were also found to be higher in the 281L/*52/*440/*443 and 318Ter/*12/*52 variants when compared to the wild-type form. This finding could be attributed to a possible reduced stability of the secondary mRNA structure (Figure 1(c)). Furthermore, position entropy for the variation located at the *52 position in the 3′UTR is higher compared to wild-type CYP21A2 mRNA which might be responsible for destabilizing the mRNA secondary structure (Figure 1(d)).

4. Discussion

Up to date, a large spectrum of mutations in the CYP21A2 gene has been reported. Most of these reported mutations affect the coding region of the gene and to a lesser extent the introns and the promoter [27]. Interestingly, variants located in the 3′ UTR of CYP21A2 which among other regulatory elements contain several microRNA-binding sites have not yet been reported to be associated with CAH. As 3′UTR mutations can influence the disease susceptibility by altering protein and microRNA- (miRNA-) binding regions, we screened the CYP21A2 3′UTR for mutations in the CYP21A2 heterozygote hyperandrogenaemic females and the hyperandrogenaemic females with no mutations. Among the variations lying at the promoter and the 3′UTR noncoding regions, the combination of 3′UTR variants *52C>T, *440C>T, *443T>C, *12C>T, and *52C>T were present in a statistically significant number of CYP21A2 heterozygous females with the mild NC-CAH phenotype. Interestingly, the variants *52C>T, *440C>T, and *443T>C were present in all heterozygous females identified with the p.Val281Leu missense mutation. In a similar fashion, the variants *12C>T and *52C>T were present also in all females identified with the p.Gln318* mutation.

On the contrary, the distribution of both *52C>T, *440C>T, *443T>C and *12C>T, *52C>T variants was not observed in any of the other CYP21A2 heterozygote females of the present study. Twelve known CYP21A2 3′UTR variants were identified, the majority of which were unique to the three different examined cohorts. Additionally, the distribution of both *52C>T, *440C>T, *443T>C and *12C>T, *52C>T in the cohort of the sex-matched female controls and the cohort of hyperandrogenaemic females identified with no mutation in the CYP21A2 gene were very low and almost negligible.

Secondary structure for CYP21A2 mRNA molecule was predicted using the RNAfold WebServer, which provides the thermodynamically favoured structure. The mRNA secondary structure is a critical characteristic in the function of cis-regulatory elements located in the 3′UTR. Comparison of the CYP21A2 wild-type, 281L/*52/*440/*443 mutant, and 318Ter/*12/*52 mutant mRNA secondary structures showed distinct differences. The higher MFE of the mutant mRNA secondary structures and the distinct higher position entropy of the *52 variation at the 3′UTR indicate less stable mRNA secondary structure which might contribute to the clinical manifestation of the disease. However, mRNA structure differences cannot be proposed as a single factor responsible for a disease.

In conclusion, females with hyperandrogenism are likely to bear heterozygous CYP21A2 mutations. The identification of variants in the 3′UTR of the CYP21A2 gene in combination with the heterozygous mutation may be associated with the mild form of the disease and reveal the importance of analyzing the CYP21A2 untranslated regions to better characterize and treat this category of patients. Insights obtained from studies that identify the genetic basis of detrimental disorders such as CAH are particularly useful since they can provide a better understanding of disease pathogenesis, used for more effective diagnostic confirmation, assist in genetic counselling, and used in the development of newer therapeutic methodologies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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