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Research paper

Genetic screening of non-classic CAH females with hyperandrogenemia identifies a novel *CYP11B1* gene mutation

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ABSTRACT

OBJECTIVE: Congenital adrenal hyperplasia (CAH) is an endocrine autosomal recessive disorder with various symptoms of diverse severity. Mild hyperandrogenemia is the most common clinical feature in non-classic CAH patients and 95% of the cases are identified by mutations in the *CYP21A2* gene. In the present study, the second most common cause for non-classic CAH (NC-CAH), 11β-hydroxylase deficiency due to mutations in the *CYP11B1* gene, is investigated. DESIGN: Screening of the *CYP21A2* and *CYP11B1* genes by direct sequencing was carried out for the detection of possible genetic defects in patients with suspected CAH. RESULTS: It was observed that *CYP11B1* variants co-exist only in rare cases along with mutations in *CYP21A2* in patients clinically diagnosed with CAH. A total of 23 NC-CAH female patients out of 75 were identified with only one mutation in the *CYP21A2* gene. The novel *CYP11B1* gene mutation, p.Val484Asp, was identified in a patient with CAH in the heterozygous state. The structural characterization of the novel p.Val484Asp was found to likely cause distortion of the surrounding beta sheet and indirect destabilization of the cavity that occurs on the opposite face of the structural elements, leading to partial impairment of the enzymatic activity. CONCLUSIONS: *CYP21A2* gene mutations are the most frequent genetic defects in cases of NC-CAH even when these patients are in the heterozygous state. These mutations have a diverse phenotype giving rise to a variable extent of cortisol synthesis impairment; it is also clear that *CYP11B1* mutants are a rare type of defects causing CAH.

Key words: CYP11B1, Hyperandrogenemia, Non-classic CAH, PCOS, Premature adrenarche

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INTRODUCTION

Congenital adrenal hyperplasia (CAH) is an endocrine autosomal recessive disorder characterized by the inability of the adrenal cortex to synthesize

cortisol, which stimulates the secretion of ACTH. Elevated levels of ACTH cause adrenal hyperplasia due to the induction of adrenocortical growth.[1] Steroid precursors are overproduced and deflected into the androgen pathway.[2] Mutations in the *CYP21A2* gene that cause 21-hydroxylase deficiency are the most common cause of the disease and account for approximately 95% of CAH patients. The second most common cause of CAH is 11β-hydroxylase deficiency due to mutations in the *CYP11B1* gene. In some rare cases, CAH can be due to mutations in the following genes: *CYP11B2, CYP17, HSD3B2* and *STAR*.[3]

11β-hydroxylase isozymes encoded by the *CYP11B1* and *CYP11B2* genes[4] are members of the cytochrome 450 superfamily and are located on human chromosome 8q, at a distance of approximately 40 kb.[4] Both genes have nine exons and are 95% identical in coding regions and 90% in introns,[5] with a 440 bp insertion in intron 5 of *CYP11B2* being their biggest difference.[2] *CYP11B1* and *CYP11B2* encode for proteins that share approximately 93% homology.[2] The *CYP11B1* gene encodes a protein with only 11β-hydroxylase activity. It catalyzes the synthesis of cortisol and is regulated by ACTH.[6] *CYP11B2* is responsible for aldosterone synthesis. It encodes a protein with 18-hydroxylase and 18-oxydase activities and much lower activity of 11β-hydroxylase. Thus it is known as "aldosterone synthase" and is regulated by angiotensin II and K^+ serum levels.[5] *CYP11B1* mutations are responsible for 11β-hydroxylase deficiency[6] with over 90 reported disease-causing *CYP11B1* mutations.[7,8] Recombination between these two homologous genes is common, resulting in a chimeric gene which usually encodes for a protein with aldosterone synthase activity regulated by ACTH, the *CYP11B1* promoter.[5] Nevertheless, the opposite case has been reported by Hampf et al concerning a chimeric gene encoding cortisol from the *CYP11B2* promoter.[5] Mutations in the *CYP11B1* gene cause 11β-hydroxylase deficiency, which is similar to 21-hydroxylase deficiency in the accumulation of steroid precursors due to stimulation of ACTH, and shift towards the androgen synthesis pathway (Table 1).[9]

CYP11B1 and *CYP21A2* gene mutations give rise to hyperandrogenemia which is the most common clinical outcome for NC-CAH patients. Since *CYP21A2* mutations account for 95% of CAH cases, it is not common practice for patients with hyperandrogenemia symptoms to undergo *CYP11B1* screening.[10,11] In this report, 75 patients with mild hyperandrogenemia identified with a single or no *CYP21A2* gene mutations were evaluated further with screening for *CYP11B1*. The evaluation of *CYP11B1* involvement in hyperandrogenemia can contribute to improved treatment strategies but also potentially reassesses the diagnostic criteria. In this report we examine the novel *CYP11B1* gene mutation.

Table 1. A representation of the clinical features observed in patients with 21-OHD, 11-OHD and Aldosterone Synthase Deficiency. As can be observed by the table below there are no similar clinical characteristics between 11-OHD and Aldosterone Synthase Deficiency

	21- hydroxylase deficiency *(CYP21A2)*	**11β- hydroxylase deficiency *(CYP11B1)***	**Aldosterone synthase deficiency *(CYP11B2)***
Normal Cortisol	✗	✗	✓
Hypertension	✗	✓	✗
Hypotension	✓	✗	✓
Hyperkalemia	✓	✗	✓
Hypokalemia	✗	✓	✗
Hyperandrogonemia	✓	✓	✗

SUBJECTS AND METHODS

Subjects

Seventy-five female patients of Greek-Cypriot origin with mild symptoms of hyperandrogenemia participated in this study and written informed consent was obtained from all patients. Group A included 37 females who displayed a premature adrenarche phenotype and group B consisted of 38 individuals with polycystic ovary syndrome who were selected for sequencing.

Hormonal assays

Blood for hormone analyses was drawn after an overnight fast. Serum/plasma was either assayed immediately or frozen for later use. Standard recommended biochemical methods for measured parameters were employed.

DNA amplification and sequence analysis.

Genomic DNA was isolated from peripheral whole blood and the desired regions were amplified by polymerase chain reaction in reaction volumes of 20μl using 100ng genomic DNA. The *CYP21A2* and *CYP11B1* genes were analyzed according to a cascade strategy as previously described.[12-14] The *CYP21A2* gene primers P1-P48[13] were used to amplify the fragment containing the -370 bp *CYP21A2* promoter, the 5' untranslated region of the *CYP21A2* gene that is mainly located in the first 167 nucleotides upstream the ATG codon and the 3' untranslated region that is 536 nucleotides downstream from the TGA stop codon of the *CYP21A2* gene.[14] Meanwhile, the *CYP11B1* region was amplified using 2 sets of primers, avoiding the homologous *CYP11B2* gene, which were then sequenced using sequencing primers that cover exons 1-2, 3–5 and 6-9, as previously described.[9,15]

MLPA analysis

All samples underwent DNA analysis using the multiplex ligation-dependent probe amplification (MLPA) technique (MRC Holland, Amsterdam, The Netherlands). MLPA was employed to investigate in the *CYP21A2* gene deleterious defects such as large gene deletions and large gene conversions which are harbored in the pseudogene *CYP21P* and can be transferred to the functional gene by homologous recombination.

Structural analysis

The characterization of the novel *CYP11B1* mutation protein structure was based upon the UniProt (Universal Protein Resource) P15538 structure of CYP11B2, that shares 93% sequence identity, using the PROMALS3D construct alignment database.[16,17] The CYP11B1 secondary structure elements were annotated with standard cytochrome P450 structural features [25a], as described in the alignment of CYP11A and CYP11B P450s.[18] The structure of CYP11B1 both wild type (V484) and mutant (V484D) was modelled on crystal structures of CYP11B2; pdb ID 4FDH (CYP11B2 crystallized in complex with heme and fadrozole) and pdb ID 4DVQ (CYP11B2 crystallized in complex with heme and 21-hydroxyprogesterone).[19] CYP11B1 was modelled using the protein-BLAST alignment [27a] with CYP11B2 using both 4FDH and 4DVQ structures as a template via modelling software Modeller version 9.11.[20,21] Of the twenty model structures generated the model with the lowest objective score was selected and used for structural analysis. The mutant V484D was modelled in the same manner. The structural analysis was carried out using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC, for structure visualization and residue conformation assessment. The residues in close proximity to the heme and steroid moiety were identified using PIC[22] LigPlot[23] and EBI pdb analysis suite PDBsum.[24]

Statistical analysis

A comparison was carried out between the female patients of group A with PA who were heterozygous for *CYP21A2* mutations and the patients of group B with polycystic ovary syndrome (PCOS) using a chi-square test. A significance level of 0.05 was used to establish statistical significance. The statistical analyses were performed using SAS software Version 9.2, SAS Institute Inc., Cary, NC, USA.

RESULTS

Genetic screening

Of the 75 female patients selected belonging to group A with PA and group B with PCOS, 23 (30.66%) were found heterozygous for *CYP21A2* gene mutations, whereas the remaining 52 (69.33%)

had no *CYP21A2* mutations (supplementary Table 1). Sixteen (43.2%) out of the 37 patients of group A with PA were found heterozygous for *CYP21A2* mutations *vs* 7/38 (18.4%) patients of group B with PCOS (p-value <0.020). Genetic screening for *CYP11B1* gene mutations revealed one heterozygous patient with the novel mutation, p.Val484Asp. One patient was identified with the known *CYP11B1* variant, p.Arg43Gln, and the severe *CYP21A2* mutation, p.Gln318Ter. The p.Gln318Ter mutation has a strong association with a trimodular haplotype which is non-disease causing.[25] Therefore, the p.Gln318Ter was verified, in addition to Sanger sequencing, using MLPA analysis.

Patient 1 is a female adolescent of Greek/Cypriot origin who was initially presented at the age of 7½ years with obesity (BMI: +2.23 SDS) and with premature adrenarche. She had pubic hair at Tanner stage 3 and axilary hair at Tanner stage 2 as well as body odor. Her bone age was advanced by 2 years according to the Greulich and Pyle method. She had normal blood pressure for age and height. Her fasting glucose, electrolytes, kidney function, lipid profile, liver and thyroid function tests were within the normal levels for age. Her baseline cortisol, insulin, 17-hydroxyprogesterone (17-OHP), androstenedione and DHEA-S were at normal levels, ACTH levels were 42 pg/ml and 11-deoxycortisol levels 105 ng/dl, i.e. both within the normal range for age. She started menstruating at the age of 11 years and, at this point, her BMI was dramatically increased. At the age of 12 she was obese with BMI +2.8 SDS and she presented with acanthosis nigricans, acne and oligomenorrhea. She had no hypertension. Her baseline 17-OHP was slightly elevated (1.8ng/ml, range: 0.2-1.2ng/ml) and she developed insulin resistance, evaluated by homeostatic model assessment (HOMA >2). The rest of her baseline hormonal and biochemical tests were within the normal ranges. She refused ACTH and OGTT tests. She was advised to start dieting and exercising and she was commenced on treatment with metformin. This female adolescent is also a carrier of the severe p.Gln318Ter mutation in the *CYP21A2* gene and, according to the bibliography, this mutation in heterozygosity could cause hyperandrogenemia. It seems that these mutations coexist and act synergistically in the premature adrenarche phenotype.

The patient with the novel *CYP11B1* mutation in heterozygosity, Patient 2, is a Greek-Cypriot female who initially presented at the age of 18 years due to oligomenorrhea and androgenic alopecia. She mentioned early adrenarche at the age of 8 years and menarche at the age of 12 years. She had had irregular menses since then. On the day of presentation her BMI was 27 kg/m^2 and she had no hypertension. She was diagnosed with PCOS according to the Androgen Excess Society criteria.[26] She had normal levels of electrolytes and normal kidney and liver function. Her fasting glucose, insulin, morning cortisol and 17-OHP levels were unremarkable (17OHP: 0.5ng/ml/l, range:0.2-1.2). She had elevated delta 4-androstenedione (Δ4) (5.4ng/ml, range:0.3-3.3) and slightly elevated DHEA-S (dehydroepiandrosterone -sulphate) (12.10μmol/L, range:0.94-11.67), measured on the 4th day of a spontaneous menstrual cycle. She was treated with a hormonal combination contraceptive pill. Genetic screening for both *CYP21A2* and *CYP11B1* mutations revealed a novel mutation on the *CYP11B1* gene. This mutation is located at codon 484 and replaces a non-polar hydrophobic amino acid (valine) for a positively charged polar amino acid (aspartic acid) in one of the two alleles.

Structural characterization

CYP11B proteins are steroid binding enzymes whose activity is dependent on colocalization of a heme molecule. Predominantly helical in composition, the helices arrange to form a deep cavity around the heme molecule and adjacently placed steroid substrate.[18]

As previously discussed, the *CYP11B1* and *CYP11B2* genes are arranged tandemly and encode proteins of 93% identity; nevertheless, the functions of these highly homologous proteins are distinct.[19] This distinction between CYP11B isoforms arises from the binding of different steroid substrates.

The novel mutation identified in Patient 2 is not located within the binding cavity but is surface facing arranged on one of the few beta strands. The apo form of the amino acid is a valine which is conserved between CYP11B1 and CYP11B2, as are many of the amino acid networks in close proximity to this residue in the crystal structure(s) 4FDH and 4DVQ. These residues are located on the same beta strand β4-1 (K482, M483 and Y485), adjacent beta strand β4-2 (R490, P491 and S/G492), the beta hairpin loop

(S486) and one residue from a loop between helices αA' and αA at position E62. Of these compactly arranged residues only one is varied between C11B1 and C11B2, that is, G492 and S492, respectively (Figure 1).

Valine 484 is localized on the surface face of a beta strand, β4-1, along with K482, M483 and Y485 amino acids and adjacent to beta strand β4-2 and in close proximity to the αA' and αA loops. This region is not considered to have any steroid binding role but is within Van der Waals contact (approximately 5Å proximity[30]) to the hydrophobic residues F487 and I488 that is conserved between both CYP11B1 and CYP11B2 (Figure 1). It is apparent that the model we have produced of CYP11B1 based on the CYP11B2-Heme-Fadrozole complex contains the same hydrophobic-rich binding cavity. An electrostatic clash between the acidic aspartate at position 484 and glutamate at position 62 may indirectly cause destabilization of the binding cavity on the opposite face of the structural elements. It is postulated that the repulsion between electronegative oxygen moieties of D484 and E62 may cause a distortion on the surrounding beta sheet which includes residues F487 and I488 of the steroid binding pocket, thus indirectly destabilizing the binding of the steroid to CYP11B.

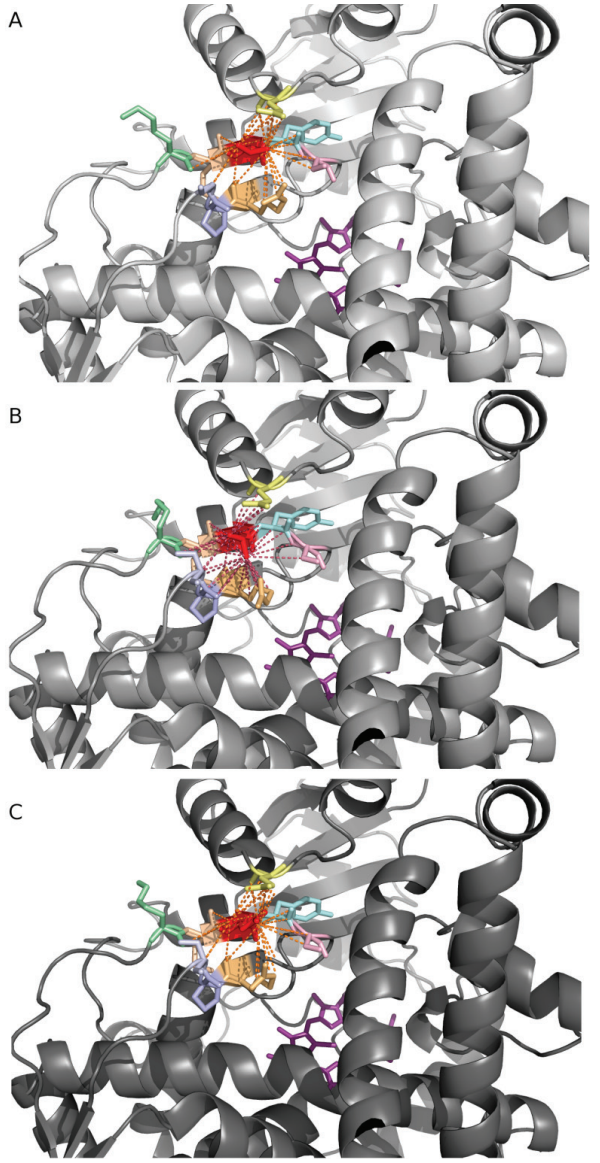

Figure 1. A) Wild Type crystal structure of CYP11B2. B) Wild type homology model of CYP11B1. C) Mutant V484D homology model of CYP11B1. Residue 484 valine in A) and B) and aspartate in C) shown in red with residues in close proximity (5Å or less) indicated by dashed lines: E62, yellow; K482, green; M483, salmon; Y485, cyan; S486, pink; R490, orange; P491, mauve; and S492 (CYP11B2) or G492 (CYP11B1), blue. The crystal structure is based on 4FDH. Based on the crystallized structure of CYP11B2 S486 is part of the beta hairpin loop and shares interatomic interactions with its neighboring residues. Residues K482, M483 and Y485 are located on the same beta strand β4-1 but adjacent to the beta strand β4-2 with residues R490, P491 and S/G492 and the beta hairpin loop that consist the S486 and one residue from a loop between helices αA' and αA at position E62. Within these compactly arranged residues of these beta hairpin loop structures, G492 of CYP11B1 is replaced with S492 of CYP11B2.

DISCUSSION

CAH is a condition with diverse severity of symptoms that can be explained by various CAH causing genes. However, most importantly there are different types of mutations in the same gene that can cause from full to partial impairment of enzymatic activity. The combination of different types of CAH mutations affects the level of clinical expression of the disease.[27] Patient 1 did not have elevated 17-OHP levels as observed with other heterozygous NC-CAH cases with a single p.Gln318Ter mutation;[2,8-29] on the other hand, a higher than normal 17-OHP level might have been observed after ACTH stimulation. The p.Arg43Gln variant replaced a positively charged polar amino acid with an uncharged polar glutamine. This variation was initially characterized as non-causative,[5] but later on Barr et al and Parajes et al characterized this variant as causative with approximately 10-50% reduction of efficiency, leading to a mild phenotype.[30-32] Although

the different physical properties of the amino acids are a major reason for the changes in enzymatic activity, the short distance of a variation to an active site of the enzyme can also be causative. In 2001, Belkina et al studied the three-dimensional structures of the *CYP11B1* and *CYP11B2* genes and proposed the I helix of 11β-hydroxylase as the putative active site.[33] Since the variant p.Arg43Gln is located within the N-term[31] and not within the helix I, Barr et al assumed that this specific variant disturbs a substrate recognition site or influences the flexibility of the enzyme and disrupts the substrate-active site combination.[30] It should also be noted that p.Arg43Gln changes a basic arginine amino acid into a neutral polar glutamine amino acid, suggesting functional protein impairment.

Patient 2 was found to be a symptomatic carrier of the novel p.Val484Asp mutation and it can be assumed that this mutation contributes to the partial enzyme impairment. The specific mutation exchanges a non-polar valine hydrophobic amino acid for a positively charged polar aspartic acid amino acid at codon region 484, causing PCOS. Neighboring codons Tyr485 and Ile488 are parts of the active *CYP11B1* gene site.[33] It can therefore be predicted that mutation p.Val484Asp affects the local conformation and disturbs the efficiency of 11β-hydroxylase thereby contributing to the patient's phenotype. Interestingly, *in silico* mutation analyses that were performed initially using Provean software[34] predicted a deleterious effect of the p.Val484Asp alteration on *CYP11B1* function (Table 2).

Genetic screening for *CYP11B1* mutations in 75 Greek-Cypriot female patients with mild symptoms of hyperandrogenemia included: Group A with 37 females who displayed a premature adrenarche phenotype, and group B with 38 females with polycystic ovary syndrome all of whom had been previously examined for mutations in the *CYP21A2* gene. Of the 75 patients, 23 were found to be heterozygotes, whereas the remaining 52 had no *CYP21A2* mutations. The finding from our previous study that demonstrating the frequency of the underlying genetic *CYP21A2* defects in the Cypriot population to be one of highest ever reported and with an allelic frequency of 1:10 corroborated the observed heterozygotes of the present study.[35] Several studies have demonstrated *CYP11B1* gene defects to be rare, therefore, it is not surprising that of the patients tested in the present study only three NC-CAH patients were identified with *CYP11B1* gene mutations.[3,8,36]

Table 2. Provean function prediction of the p.Val484Asp variation in *CYP11B1*[39]

Variant	PROVEAN score	Prediction (cutoff= -2.5)
p.Val484Asp	-3.777	Deleterious

The detection of only two carriers of 11β-hydroxylase deficiency mutations among female patients with mild hyperandrogenemia is consistent with other studies and demonstrates that *CYP11B1* gene mutations account for a small minority of cases in females with hyperandrogenism.[37] The novel p.Val484Asp mutation was found to cause a distortion of the surrounding beta sheet and also to result indirectly in destabilization of the binding cavity that occurs on the opposite face of the structural elements, leading to partial impairment of the enzymatic activity. Reporting novel mutations and even combinations are of great importance as such information can enable a clearer diagnosis and contributes to a better treatment modality. Understanding the extent of cortisol synthesis impairment caused by specific mutations but also due to a combination of mutations is essential for correct diagnosis, prognosis and treatment. In this report we examine the deleterious effect of a novel *CYP11B1* mutation and, in addition, how heterozygosity in *CYP21A2* as well as cases with no identified mutations could lead to a NC-CAH phenotype similar to that observed in the most prevalent type of the disorder with two identified mutations.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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