Endocrine (2013) 44:212–219
DOI 10.1007/s12020-012-9861-2

ORIGINAL ARTICLE

Two novel mutations in CYP11B1 and modeling the consequent alterations of the translated protein in classic congenital adrenal hyperplasia patients

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Received: 4 April 2012 / Accepted: 12 December 2012 / Published online: 24 January 2013

Abstract Mutations in the 11β-hydroxylase (*CYP11B1*) gene are the second leading cause of congenital adrenal hyperplasia (CAH), an autosomal recessive disorder characterized by adrenal insufficiency, virilization of female external genitalia, and hypertension with or without hypokalemic alkalosis. Molecular analysis of *CYP11B1* gene in CAH patients with 11β-hydroxylase deficiency was performed in this study. Cycle sequencing of 9 exons in *CYP11B1* was performed in 5 unrelated families with 11β-hydroxylase deficient children. Three-dimensional models for the normal and mutant proteins and their affinity to their known substrates were examined. Analysis of the *CYP11B1* gene revealed two novel mutations, a small insertion in exon 7 (InsAG393) and a small deletion in exon 2 (DelG766), and three previously known missense mutations (T318M, Q356X, and R427H). According to docking results, the affinity of the protein to its substrates is highly reduced by these novel mutations. DelG766 has more negative impact on the protein in comparison to InsAG393. The novel mutations, InsAG393 and DelG766, change the folding of the protein and disrupt the enzyme's active site as it was measured in the protein modeling and substrate binding analysis. Molecular modeling and sequence conservation were predictive of clinical severity of the disease and correlated with the clinical diagnosis of the patients.

Keywords Congenital adrenal hyperplasia · *CYP11B1* · Mutation · Protein modeling · Prenatal diagnosis

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by defects in different enzymatic

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steps required to synthesize cortisol from cholesterol [1, 2]. Cortisol deficiency and the compensatory high adrenocorticotropic hormone (ACTH) levels result in a reactive hyperplasia of the adrenals and an increase in steroid precursors, which are shunted into androgen synthesis [1–3].

Deficiency of 11β-hydroxylase (11β-OH), detected in ~1 in 100,000 births, is the second most common form of CAH and accounts for 5–8 % of affected patients [4, 5]. Inherited defects of 11β-OH, required for conversion of 11-deoxycortisol and 11-deoxycorticosterone (DOC) to cortisol and corticosterone, respectively, lead to accumulation of steroid intermediates with mineralocorticoid activity (DOC) in combination with an excessive production of adrenal androgens. Consequently, the most common clinical features include hypertension due to sodium retention in the classic form, and prenatal virilization in females and precocious pseudopuberty in both sexes as a result of overproduction of androgens [1, 5, 6].

The 11β-OH gene (*CYP11B1*) is located on chromosome 8q24.3 and contains 9 exons. This gene is 5.4 kb in size and is functionally similar to the *CYP11B2* gene which synthesizes aldosterone. These two genes share a 95 % sequence homology and are located about 40 kb apart. The deficiency of 11β-OH activity is caused by mutations mainly clustered at exons 2, 6, 7, and 8 of the *CYP11B1* gene [6–8]. More than 60 different mutations are listed in the Human Gene Mutation Database, of which more than two-thirds are nucleotide substitutions (nonsense or missense mutations) [4, 8, 9].

In this study, we performed mutation analysis for 5 unrelated families with at least one 11β-OH deficient child. While in three patients, affected chromosomes were characterized with at least one previously known mutation, a novel 2 bp insertion in exon 7 (InsAG393) and a single nucleotide deletion (DelG766) in a heterozygous form in the maternal and paternal allele of an affected child was detected. To predict the molecular consequences, the mutants were analyzed individually using bioinformatic techniques and the latest three-dimensional (3D) structural model of the human CYP11B1 protein.

Materials and methods

CAH patients and family

A total of five children (all male) with 11β-hydroxylase deficiency from four unrelated Iranian families and an immigrant Afghan family were enrolled in this study for the mutation analysis. An informed consent was obtained from the patients' parents. The study was approved by the Ethics Committee of Mashhad University of Medical Sciences. The clinical and laboratory data of these patients are summarized in Table 1.

The first patient (P1: *T318M*) was admitted with general malaise accompanying with paresis at limbs and extremities and disability of gait. During the hospitalization, hypertension accompanied with a severe hypokalemia, precocious pseudo puberty and rapid somatic growth was detected which eventually led to the clinical diagnosis of CAH due to 11β-hydroxylase deficiency (11-OHD) and hormone replacement therapy with hydrocortisone was initiated and the symptoms suppressed afterwards.

The second patient (P2: *Q356X*) was presented with precocious pseudopuberty, accelerated growth rate, and hypokalemia at the age of three and severe hypertention later at the age of 10. The first hospitalization occurred 1 year later following two attacks of generalized tonic–colonic (GTC) seizures in addition to continued severe headaches, hemi paresis with ipsilateral involvement of facial nerve, severe hypokalemia, and hypertension along with a mild alkalosis (pH 7.46). The second admission to the hospital was at the age of 16 due to the presentation of elevated intracranial pressure. The imaging showed improved hyperplasic adrenal glands and brain sequels related to previous cerebrovascular accidents (CVA). Glucocorticoids and further unilateral adrenalectomy were not successful in controlling the hypertension. Bilateral shortness of the forth metatarsus was observed in the physical examination.

The third case (P3: *InsAG393*) was presented with accelerated growth rate and hyperpigmentation when he was 10-month old, following with severe acne, penis enlargement, Tanner pubertal stage G3, and pH 2, voice hoarsening and hypertension by the age of 3. Hormone therapy was initiated with prednisolone, later changed to hydrocortisone. His condition improved achieving real puberty at age 10. The treatment was continued by balancing the glucocorticoid dosage and prescribed growth hormone analogs for several years resulting in a normal stature at the age of 15. Unfortunately, the patient died in a car accident at the age of 16.

The fourth patient (P4: *R427H*) was a 9-year old boy of an Afghan immigrant family presented with accelerated growth and severe hyperpigmentation at the age of 6 months, later followed by signs of premature adrenarche. His clinical symptoms were not detected until the age of 38 months, when he presented with hypertension, hypokalemia, and a mild alkalosis in addition to a precocious pseudopuberty, a 10.5 cm full stretched penis and a bone age of about 8 years according to radiographic findings. The clinical and para-clinical findings led to the diagnosis of 11-OHD CAH. Hormone therapy was initiated by hydrocortisone and changed to prednisolone after 2 years. He had recurrent attacks of GTC seizures about once a year, accompanied by fever which stopped following treatment. Presenting with bilateral orchidomegaly, scrotal

Table 1 Clinical and laboratory information of the patients and their genotype

Pt	Mutation[a]	Age (years)	Age of Dx	Consanguinity	FH of CAH	*K*	BP (mmHg)	17 OHP (nmol/l)	11-Deoxycort-isosterone (ng/μl)	Metabolic alkalosis
P1	*T318 M*	10	2 years	+	Brother	2.1	160/90	–	207	–
P2	*Q356X*	17	3 years	+	–	2.1	170/100	18	68	+
P3	*InsAG393*	–	10 months	+	–	–	150/100	10.4	206	ND
P4	*R427H*	9	6 months	+	–	2.7	130/70	5.7	237	+
P5	*DelG123*	4.5	3 years	+	–	4.1	120/75	10.1	198	–

Pt patient, *ND* not determined, *BP* blood pressure, *17-OHP* 17-hydroxyprogesterone, *FH* family history, *Dx* diagnosis

[a] Presented genotypes are homozygote

pain, and palpable masses in scrotum, he was diagnosed with Leydig cell tumor with no metastasis at the age of 4. Bilateral fourth metatarsus shortness was noticeable in this patient, similar to patient 2.

The last case (P5: *DelG766*) was a 4.5-year old boy referred with precocious pseudopuberty and accelerating growth indices and hypertension. The initial laboratory findings revealed a normal potassium level, lower limit sodium (135 mmol/l), and testosterone (2.37 ng/ml), and an elevated level of basic 17-OHP. As hormone therapy was initiated with hydrocortisone and spironolactone, the BP was controlled; however, the growth indices, especially the height, was only improved when an aromatase inhibitor was added.

Sequencing analysis

The genomic DNA was extracted from peripheral blood leukocytes using a standard salting-out method [10]. As previously described, the *CYP11B1* gene was amplified in a 6,200 bp fragment including all nine exons followed by five nested polymerase chain reactions (PCRs) for amplification of the exons and associated splice sites [10–12]. Forward and reverse PCR product strands were sequenced and analyzed with the DNA sequence assembly software (Sequencher 4.7, Gene Codes Corporation). Numbering of *CYP11B1* nucleotides and amino acids was based on the sequence published by Gene Bank accession nos NC_000008.

Structural analysis

To inspect the effects of the two novel mutations on the protein structure and function, 3D models for the normal and mutant proteins were developed and their affinity to 11-deoxycortisol, 11-deoxycorticosterone, and a previously found substrate, mitotane, was examined. The sequence of the wild-type CYP11B1 protein was retrieved from Uni-Prot (Gene Bank accession nos. P15538). Similarity search via Psi and Phi-BLAST was carried out as the first-line homology study in order to find highly identical proteins as templates. PDB ID: 3K9V [13], which is crystal structure of rat (*Rattus norvegicus*) mitochondrial P450 24A1 S57D with a resolution of 2.5 Å was selected as the template and the alignment was manipulated and refined in CLUSTALW, and different gap penalties and matrices were used to obtain better scores in the final models. The same template was used for the mutants; however, the alignments were manipulated to achieve better results. Homology modeling was performed by means of MODELLER 9v8 on a Dual Zeon® Windows® platform. Automodel, loopmodel, and refine classes of MODELLER were used to build models [14]. Heme was added to the protein structure from the start and structures were modeled as heteroatom containing molecules. MODELLER was set to build 20 models for each protein and compute DOPE score for every model. Best model in terms of DOPE was selected for refinement. Models were investigated in SPDBViewer and ViewerLite programs checking amino acids making clashes, Phi–Psi angles, and secondary structure matching the secondary structure prediction. At this step, structures were energy minimized using the MM + forcefield to an RMS gradient of 1 kcal mol^{-1} followed by backbone energy minimization and the procedure was finally completed by side chain energy minimization (backbone and sidechain minimizations were done using the MM + forcefield to an RMS gradient of 1 kcal $mol^{-1.}$). The models were then exposed to different tests: ERRAT, VERIFY3D, WHAT_CHECK, WHAT_IF, PROCHECK, and PROSA. Ramachandran plot was also contrived for the models. The models were refined and energy minimized according to evaluation scores. The refinement and modeling procedure and parameters were kept similar for all three proteins. Docking was performed on an Intel® Core2® Ubuntu 10.04 Linux platform using Autodock 4.2 [15]. Ligand structures were energy minimized using semi-empirical force fields with AM1 matrice. Docking grid box was created based on the X-ray structure of the template in conjugation with heme. Autodock was run once for each protein–ligand complex (a total of 9 times) and set to yield 50 docking complexes on each run. Best complex with the lowest energy of binding was selected as the final docking structure.

Results

Sequence analysis

Clinical characteristics of the patients at the time of presentation, are summarized in Table 1. The sequence analysis of *CYP11B1* gene in our patients resulted in the identification of three previously known missense mutations (T318M, Q356X, and R427H) [4, 8, 10–12, 16–19] and two novel mutations including a small insertion (InsAG393) and a single nucleotide deletion (DelG766) (Fig. 1).

Direct DNA sequencing of the complete *CYP11B1* gene in patient 1 revealed a C to T transition at bp 3,580 in exon 5 (g.3580C>T) in a homozygous form, leading to a threonine (T) to methionine (M) substitution at codon 318, T318M [8, 16, 17]. The same mutation was identified in a heterozygous state in his parents.

A C to T transition at bp 4,054 in exon 6 (g.4054C>T) was detected in patient 2 in a homozygous form. This point mutation changes a glutamine at codon 356 to a premature termination codon, Q356X [8]. His parents, as well as one of his healthy sisters, were heterozygote for this mutation. The two other children of the family had normal genotypes.

DNA sequencing of patient 3 provided the identification of a 2 bp insertion at nucleotide 4,565 of exon 7, insertion AG at codon 393 (4,565–4,566 InsAG) causing a frameshift mutation (Gene Bank accession no. EU236683) (Fig. 1a). Both parents had the same mutation at the same site in a heterozygous form.

Molecular analysis in patient 4 revealed a G to A transition at nucleotide 4,746 in exon 8 (g.4746G>A) in a homozygous form, resulting in an arginine (R) to histidine (H) substitution at codon 427, R427H [4]. His healthy brother had a heterozygote allele at the same site.

DNA sequencing in patient 5, revealed the identification of a single nucleotide deletion at the position 766 of exon 2, leading to a homozygote deletion at codon 123 which caused a frameshift and the introduction of a premature stop codon at amino acid 132 (Gene Bank accession no. GU201906). The parents also have this mutation as heterozygotes (Fig. 1b).

Structure analysis

Analysis of Ramachandran plot for the model of intact *CYP11B1* shows 84.3 % of amino acids in the most favored area, 14.2 % in allowed regions and 1.4 % in disallowed regions. These numbers for the new InsAG393 mutant protein model are 81.2, 17.8, and 1.0 %; and 87.4, 11.2, and 1.4 % for DelG766, respectively. The ERRAT score of the normal and mutant protein models is 86.681, 77.376, and 77.645, respectively. In VERIFY_3D test, the percentage of amino acids having a 1D to 3D score greater than 0.2 are 85.32, 84.68, and 67.43 for the 3 proteins, respectively. This assures the satisfactory quality of the original model and the decline in values in the mutant proteins may indicate that the mutations have destabilized the structures. Summary of docking results is presented in Table 2.

The normal and mutant proteins were modeled using the same template to eliminate differences in final models arising from the method. Docking complex of intact CYP11B1 and 11-deoxycortisol (Fig. 2a) shows that in normal attachment of the ligand to the protein, it is oriented so that it forms 3 hydrogens bonds in both ends. Three-ketone group is hydrogen bonded to Met111 oxygen rich substitutes on C17 of 11-deoxycortisol are fixed by formation of two hydrogen bonds to Val378 and Phe 487 and interacting with aromatic side chain of Phe487. The hydrophobic skeleton of the steroid is surrounded by

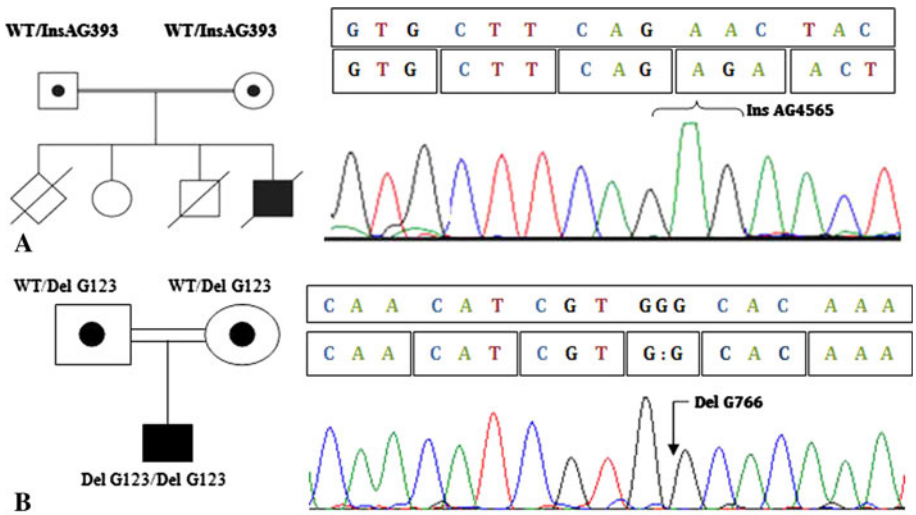

Fig. 1 Molecular genetic analysis of the *CYP11B1* gene by direct DNA sequencing in classical CAH parents of P3 and P5. **a** The pedigree and insertion of 2 nucleotides in exon 7 (g.4565Ins) in a heterozygous form results in a frameshift and the introduction of a premature stop codon at amino acid 431 (V431X). **b** The pedigree and deletion of single nucleotides G in exon 2 (g.766 Del) in a homozygous form results in a frame shift and the introduction of a premature stop codon at amino acid 132

Table 2 Energies and affinity of best docking complex for each set of protein ligand complex

	Binding Energy (kcal/mol)			Intermolecular energy (kcal/mol)			Ki		
	Intact protein	InsAG393	DelG123	Intact protein	InsAG393	DelG123	Intact protein	InsAG393 (μM)	DelG123
11-Deoxycortisol	−9.42	−8.97	−819	−10.61	−7.16	−9.39	124.85 nM	92.18	986.35 nM
11-Deoxycoricosterone	−9.53	−7.69	−8.06	−10.43	−8.59	−8.96	102 nM	2.31	1.23 μM
Mitotane	−6.98	−5.19	−6.06	−7.68	−6.09	−6.95	7.61 μM	156.55	36.17 μM

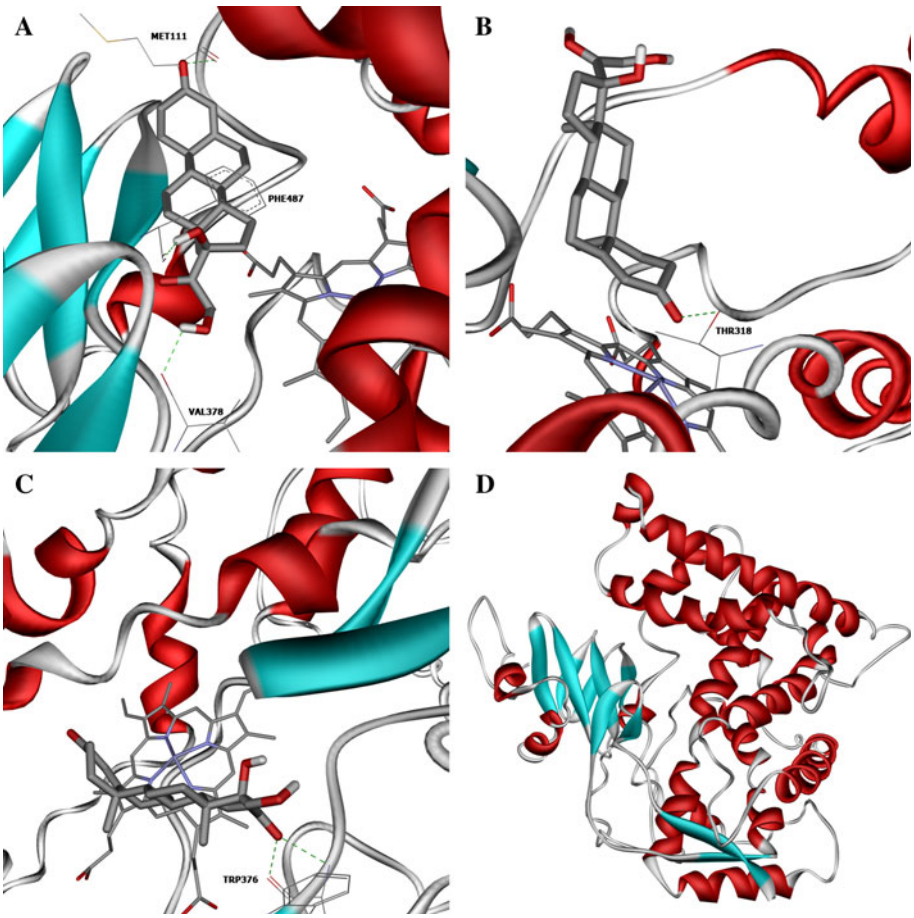

Fig. 2 **a–c** demonstrate 11β-hydroxylase in normal, InsAG393, and DelG766 mutants, respectively. Protein structures, heme, and 11β-hydroxylase are shown with *lines*, *sticks*, and *ball* and *stick* models, respectively. **d** shows overlay of normal and DelG766 mutant in active site area. Normal protein is displayed in *green* in contrast to *red* used for DelG766 mutant

hydrophobic amino acids and a good distance is maintained between them. Mutation InsAG393 (Fig. 2b) changes the formation of the active site making the pocket too big to form proper bonds with the ligand. Only one hydrogen bond is made between 3-ketone group of the ligand and Thr318. The orientation of the substrate is reversed in comparison to intact protein–ligand complex.

DelG766 mutant forms a very small binding pocket (Fig. 2c) which results in malorientation of the substrate and forces the substrate to get too close to heme group. The ligands make a double hydrogen bond to TRP376.

Figure 2d shows final model for CYP11B1. Structure of the protein and its mutants not only differ in terminal portions of the proteins where the sequences differ drastically, but the difference is also evident in amino acids which stand close to the terminal part in 3D structure and the effect of the mutation is observable everywhere in the protein structure.

Discussion

We have previously characterized patients with 21-hydroxylase (21-OH) deficiencies, as the most common cause of CAH, and reported novel mutations in the north-eastern Iran [12]. Pursuant to our characterization of CAH

in this region, we performed mutation analysis for five unrelated 11-OHD patients and considering their clinical manifestations, in this study. Mutations in *CYP11B1* gene are the most frequent cause of CAH due to 11-OH deficiency. Most of these mutations occur within coding regions resulting in an ineffective protein causing the 11-OHD phenotype [4, 20–24]. The analysis of *CYP11B1* gene in these patients revealed 3 previously reported substitutional mutations in exons 5, 6, and 8 and two novel mutations, a small insertion in exon 7 and a single nucleotide deletion in exon 2. Parental consanguinity in all of the studied patients reflects the concerning consequences of frequent consanguineous marriages in Iran and in turn, confirms the requisite of premarital genetic counseling, as well as newborn screening in the affected families.

In patient number three a 2 bp insertion of AG at codon 393 resulted in a frameshift mutation, making the pocket of active site too big to form proper bonds with the ligand. As a result, only one hydrogen bond is made between 3-ketone group of the ligand and Thr318, and the malorientation of the substrate is reversed in comparison to intact protein–ligand complex. All the changes may result in elimination of enzymatic activity. Disturbance in hydrophobic, hydrophilic properties of the binding pocket might also account for malfunction of InsAG393 mutant.

Despite InsAG393 mutation, DelG766 mutant (P5) forms a very small binding pocket which results in malorientation of the substrate. Consequently, it compels the substrate to get too close to heme group. The ligands make a double hydrogen bond to TRP376 which might account for a more favorable binding energy compared to the mutant InsAG393. However, wrong orientation of the ligand may result in decreased enzymatic activity far beyond the image binding energy gives.

Although the affinity of mitotane was less affected by the mutations, this can be explained by taking a substantially lower volume of mitotane in comparison to the examined steroids and the fact that one of the significant effects of these mutations is the change in the volume of the binding site.

The *CYP11B1* A356V amino acid substitution, probably due to conversion from *CYP11B2,* was found in patient 1 and his father with T318M mutation. A similar pattern has been reported in a Chinese patient with T318P mutation [17]. A386V is a polymorphism which occur in a normal control subjects [17]. This mutation revealed a previously known mutation of Thr ACG → Met ATG at codon 318 in exon 5. A remarkable finding in the clinical manifestations in the patient with T318M mutation was the presence of hypertension despite the common presentation of association with a normal blood pressure [4]. On the other hand, this observation was in line with the report of Chabre et al. [25] for the same mutation in a case of CAH with intractable symptoms.

Another remarkable finding was the recurrence of GTC seizures in patients 2 and 4 with an unclear correlation to the underlying disease that requires further examination. We speculate that the seizure in patient 4 was associated with fever and it was considered as febrile seizure. Kawawaki et al. [26] reported 6 cases of CAH patients with febrile seizure and proposed unknown factors in the pathogenesis such as excess secretion of corticotropin releasing factor (CRF) under stress conditions, prolonged elevation of CRF during fetal life and linkage between CAH and febrile seizures on the chromosome 6. However, seizure in patient 4, the seizure was expected to be a consequence of sever hypokalemia ($K = 2.1$). As mentioned above, the seizures were accompanied with 2 CVA attacks in patient 2 which may be a consequence of severe hypertension, in spite of both glucocorticoid and anti-hypertensive regimen and an unsuccessful unilateral adrenalectomy procedure. Although not an established treatment, laparoscopic bilateral adrenalectomy may be beneficial for this patient as previously reported for rare cases [25, 27]. Genetics analysis of CAH could be considered to decide for adrenalectomy [25]. Along with resistant symptoms, Q356X genotype could be an indicator of aggressive therapy in the treatment of 11β-OHD CAH patient.

Another rare finding was the Leydig cell tumor in patient 4. Few reports of Leydig cell tumor have been published in both cases [28–30]. Bilateral testicular enlargement is a diagnostic concern with two important differential diagnoses in patients with CAH: testicular adrenal rest tumors (TART) which is more frequent and reported in 86 % of CAH patients [31], usually regress with corticosteroid therapy with a benign nature, and the Leydig cell tumors. For an accurate diagnosis, a thorough histopathological evaluation in addition to the patient's response to suppressive therapy should be considered [29, 32–34]. TARTs will eventually be found in the majority of patient with CAH if ultrasound is performed and that the most Leydig cells tumors in CAH probably are benign TARTs not needing surgery. These tumors usually respond to ACTH suppression therapy [35, 36]. As reported by Entezari et al. [34], a combination of the following findings led us to the diagnosis of Leydig cell tumor. ACTH suppression therapy was not successful in tumor regression, however, plasma testosterone and urinary 17-ketosteroids weaned down following the surgical treatment.

An interesting, but not necessarily associated, finding in the physical examination was bilateral shortness of the fourth metatarsus in both patients P2 and P4. Although it has not be reported as a relevant finding, looking for a same finding may be noteworthy in other CAH patients.

In conclusion, we detected two novel mutations, InsAG393 and DelG766, which change the folding of the protein and disrupt the enzyme's active site. Molecular

modeling and sequence conservation relatively predicted clinical severity of the disease and correlated with the clinical diagnosis of the patients. The *CYP11B1* gene analysis is clearly a useful method for the genetic diagnosis of CAH, and not only is applicable to the prenatal diagnosis of CAH due to 11β-OH deficiency using amniotic cells or chorionic villi, but also offers a potential, effective prenatal treatment of future affected pregnancies. Evaluating these novel mutations, in addition to the previously known mutations, which cause enzymatic defects, may be a valuable tool for predicting the clinical outcome in CAH and have therefore relevance for diagnosis, prognosis, and also counseling for affected families and consanguineous marriages.

Acknowledgments This study was supported by a Grant from Mashhad University of Medical Sciences (#86426).

Conflict of interest The authors have no conflict of interest.

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