Gene 503 (2012) 215–221

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

In silico structural, functional and pathogenicity evaluation of a novel mutation: An overview of *HSD3B2* gene mutations☆

Bahareh Rabbani [a,b,*,1], Nejat Mahdieh [b,d,e,1], Mohammad Taghi Haghi Ashtiani [b,c], Aria Setoodeh [b,c], Ali Rabbani [a,b,c,**]

[a] Growth and Development Research Center, Tehran University of Medical Sciences, Tehran, Iran
[b] Pediatrics Center of Excellence, Tehran University of Medical Sciences, Tehran, Iran
[c] Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
[d] Medical Genetic Group, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
[e] Division of Human Genetics, National Institute of Genetics, Mishima, Japan

ARTICLE INFO

Article history:
Accepted 23 April 2012
Available online 8 May 2012

Keywords:
HSD3B2 gene
Endocrine disorder
Mutation

ABSTRACT

Mutations of 3 beta hydroxysteroid dehydrogenase type II (HSD3B2) gene result in different clinical consequences. We explain a patient who demonstrated a salt wasting form of 3βHSD deficiency in infancy. Signs of hyponatremia and hyperkalemia were recognized in the infant with ambiguous genitalia and perineal hypospadias. The 46,XY male was genotyped by direct sequencing of *HSD3B2* gene. Steroid profiles showed elevated concentration of 17 hydroxyprogesterone, and decrease in concentration of cortisol, and testosterone. Dehydroepiandrotone (DHEA) to androstenedione ratio had 6 fold increases. Direct sequencing of the patient revealed homozygous missense A82P mutation in exon 3. This mutation was confirmed by segregation analysis of the parents. Bioinformatic tools were used for in silico structural and functional analyses. Also, the pathological effect of the mutation was validated by different software. Alanine is a conserved amino acid in the membrane binding domain of the enzyme and proline substitution was predicted to destabilize the protein. This report may highlight the importance of the screening programs of the disorder in Iran.

1. Introduction

3-beta hydroxysteroid dehydrogenase (3βHSD) deficiency is a rare autosomal recessive form of congenital adrenal hyperplasia (CAH) (Thilen and Larsson, 1990) due to mutations in type II (*HSD3B2*) gene. The gene encodes a nicotine adenine dinucleotide $(NAD)^+$ membrane associated enzyme (Headon et al., 1978), located in the endoplasmic reticulum and mitochondria. 3βHSD2 is a bifunctional dimeric enzyme required in steroid biosynthesis pathway which regulates the conversion of steroid precursors into respective final products including aldosterone, cortisol, and sex hormones in the adrenal gland by sequential oxidation and isomerization (Lachance et al., 1991; Penning, 1997). NAD+ is reduced to NADH by dehydrogenase activity of the enzyme which is then needed for the activation of isomerase residing on the same enzyme that adapts different conformation (Thomas et al., 2003).

The *HSD3B2* gene locating on chromosome 1p13.1 (Berube et al., 1989) is expressed in adrenal and gonads (Rheaume et al., 1992). It has four exons expressing into a 1.12 kb coding RNA and 372 amino acids. To date, nearly 40 mutations including point mutations, and small insertions or deletions have been found which are listed in the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk). Related genotypes may lead to a wide spectrum of clinical manifestations (Simard et al., 2005). Mutations have been distributed among all populations (Simard et al., 2005).

3βHSD deficiency is divided into classic and non-classic forms. Impaired steroidogenesis is related to both adrenals and gonads. The salt wasting form of classic *HSD3B2* may be life threatening if not treated early; however, non salt wasting would be diagnosed later in life. Males would present hypospadias, and ambiguous genitalia; females would show normal or mild virilized external genitalia because

Abbreviations: HSD3B2, 3 beta hydroxysteroid dehydrogenase type II; DHEA, dehydroepiandrosterone; CAH, congenital adrenal hyperplasia; NAD, nicotine adenine dinucleotide; CBC, complete blood cell count; 17OHP, 17 hydroxyprogesterone; ACTH, adrenocorticotropic hormone; I-TASSER, iterative threading assembly refinement; Phyre 2, protein homology/analogy recognition engine; SNP, single nucleotide polymorphism; SIFT, Sorting Intolerant From Tolerant; PolyPhen-2, Polymorphism Phenotyping; PON-P, Pathogenic or Not pipeline.

☆ This work is supported by grant no. 7383, 2008 of Growth and Development Research Center, and Endocrine and Metabolism Research Center of Tehran University of Medical Sciences.

* Correspondence to: B. Rabbani, Gharib St., Pediatrics Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran, P.O.Box: 14155-6386.

** Correspondence to: A. Rabbani, Tehran University of Medical Sciences, Tehran, Iran, Gharib St., Pediatrics Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran, P.O.Box:14155-6386. Tel.: +98 2166428998; fax: +98 2166923054.

E-mail addresses: baharehrabbani@yahoo.com (B. Rabbani), rabania@tums.ac.ir (A. Rabbani).

[1] Equally first authors.

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2012.04.080

this deficiency lowers the testosterone concentration for development of external genitalia (Pang, 1998).

Molecular analysis of Iranian CAH patients has been carried out for *CYP21A2* gene (Vakili et al., 2005; Ramazani et al., 2008; Rabbani et al., 2011a, 2011b) which accounts for more than 95% of the CAH defects in Iran. However, the remaining causal defects of CAH are unknown among this population.

Here, we report a novel variant in an Iranian family with signs of 3βHSD deficiency. The pathogenic effect of missense variant was evaluated using different bioinformatic tools. In silico structural and functional platforms were analyzed for this mutation. To our knowledge this is the first molecular analysis of HSDB2 gene in this population. We also indicated different reports on HSD deficiency and the related mutations.

2. Methods

2.1. Patient

A twenty-day-old infant boy, born as the first child of a consanguineous marriage in northern part of Iran, was referred due to ambiguous genitalia and dehydration presented to his general practitioner; he was born from a normal delivery and the mother had not used any medication. No other clinical finding or medication during pregnancy was written on his history document. Physical examination revealed tachycardia/pulse rate of 160 beats/min and respiratory rate of 60 breaths/min at rest. His blood pressure was 60/40 mm Hg, and his pulse rate was 100 beats/min. Examination on admission showed growth measurements of 51 cm of length (5th percentile), weight of 3000 g, and head circumference of 36 cm. The patient underwent fluid therapy. The newborn's length, weight and head circumference at birth were 49 cm, 4100 g, and 35 cm, respectively. Genitalia examination determined mild hyperpigmentation, microphallus with chordee, bifid scrotum, palpable testis in scrotum, perineal hypospadias (score IV) and inguinal hernia at the right side.

Complete blood cell count (CBC) was normal; blood glucose level was 60 mg/100 mL. Electrolyte analysis showed that plasma Na was 120 mEq/L (normal range [NR]: 139–146 mEq/L) and K 7 mEq/L (NR: 3.5–5 mEq/L). Hormone concentrations were as follows: 17 hydroxyprogesterone (17OHP) 28 nmol/L (normal range [NR]: 0.2–2.3 nmol/L), cortisol 3 μg/dL (NR: 5–23 μg/dL), ACTH 500 pg/mL (25–100 pg/mL), dehydroepiandrosterone (DHEA) 3105 ng/mL (NR: 3–83 μg/L), testosterone 0.5 ng/mL (NR: 3–10 ng/dL), and androstenedione 510 ng/mL (NR: 0.28–1.75 ng/dL); plasma rennin activity was also high. Concentrations of hormones and electrolytes are shown in Table 1.To date the patient is undergone fluorohydrocortisone and hydrocortisone substitution.

2.2. Molecular analysis of HSD3B2 gene

After genetic counseling and signing the informed consent, blood sample was collected from the patient and his parents. DNA was extracted according to standard methods (Miller et al., 1988). PCR amplification of *HSD3B2* gene was performed as previously described (Rheaume et al., 1992, 1994; Sanchez et al., 1994; Simard et al., 1993). Six different fragments encompassed the four exons and 5′ and 3′ untranslated regions of the gene. PCR products were directly sequenced by BigDye termination method by sequencing analyzer ABI 3130XL (PE Applied BioSystems, US).

Table 1
Concentrations of electrolytes and hormones.

Electrolyte/hormone	Concentration	Normal range
Plasma Na	120 mEq/L	139–146 mEq/L
Plasma K	7 mEq/L	3.5–5 mEq/L
17 Hydroxyprogesterone (17OHP)	28 nmol/L	0.2–2.3 nmol/L
Cortisol	3 μg/dL	5–23 μg/dL
ACTH	500 pg/mL	25–100 pg/mL
Dehydroepiandrosterone (DHEA)	3105 ng/mL	3–83 μg/L
Testosterone	0.5 ng/mL	3–10 ng/dL
Androstenedione	510 ng/mL	0.28–1.75 ng/dL

2.3. Mutation data abstraction

A manual search of the literature on *HSD3B2* gene mutations was used to identify all the reported mutations in the database.

2.4. Multiple sequence alignment

A multiple sequence alignment in UniProt was carried out for 3βHSD protein family to demonstrate conserved domain among different paralogs and orthologs.

2.5. Structural and functional analysis

Human 3βHSD2 protein (UniProtKB/Swiss-Prot P26439) is a member of short chain dehydrogenase family of NAD or NADP-dependent oxidoreductase. There is no experimentally determined structure for 3βHSD2. Although some members of its family have a structured model, a structure based analysis is needed for 3βHSD2 protein. Based on template-based homology and folding recognition, protein homology/analogy recognition engine (Phyre 2) server (Kelley and Sternberg, 2009) and iterative threading assembly refinement (I-TASSER) server (Roy et al., 2010), the structure was predicted for 3βHSD2 protein. 3D structure of the protein was constructed with template structure 1r66A. Three-dimensional structures of the proteins were visualized by FirstGlance in Jmol.

Also, these servers were able to predict the function of protein sequence based on gene ontology and binding sites. SwissModel (Kiefer et al., 2009; Kopp and Schwede, 2004) tool was used for comparative molecular modeling of 3βHSD protein structure.

2.6. SNP annotation

Refseq (NG_013349.1) was searched from NCBI (www.ncbi.nlm.nih.gov/Nucleotide) and aligned to the obtained sequences for single nucleotide polymorphism (SNPs) search. SNPs that substitute residues in proteins may cause differences in protein function. The protein sequence was compared to the sequence on UniProt accession number P26439. The UniProtKB/Swiss-Prot contains all available protein sequences and protein functional annotation. Protein description, function and catalytic activity are available in this dataset.

Understanding the molecular cause of SNP for pathogenicity and/or disease causing amino acid substitution was determined in silico by available tools.

Sorting Intolerant From Tolerant (SIFT) (Ng and Henikoff, 2001) and Polymorphism Phenotyping (PolyPhen-2 v2.1) (Ramensky et al., 2002) servers were used to predict the effect of a non-synonymous SNP substitution on protein structure, function and phenotype, sequence conservation and/or protein structure. Pathogenic or Not Pipeline (PON-P) (Thusberg and Vihinen, 2009) service was used to describe the pathogenic effect of amino acid change in a simple and fast link. Various mutation analysis methods are provided by the pipeline to specify the structural changes due to side chain size, hydropathy, disorder, aggregation, stability, and tolerance. SNPs&Go was used to predict the relating mutation to the disease causing SNP (Calabrese et al., 2009). This bioinformatic tool predicts the mutation relating disease with the accuracy of 82%. Gene ontology, biological process, molecular function and cell components are compared to the investigated protein.

2.7. 3βHSD2 interactome

Interaction of 3βHSD2 protein was predicted by STRING database version 9 which is a database of known and predicted proteins. The interaction includes physical and functional associations derived from genomic context, high throughput experiments, co-expression and previous knowledge (Szklarczyk et al., 2011).

3. Results

3.1. Mutational verification

Direct sequencing of the six fragments revealed a homozygous transversion of G to C which modifies GCC to CCC codon at position 82 of exon 3 (chr1:119,962,142), which substitutes alanine to proline amino acid in the patient. This point mutation was confirmed by segregation studies in both parents as being heterozygous (Fig. 1).

Up to Jan 2012, the reported mutations of *HSD3B2* gene and the related phenotypes based on their ethnicity are listed in Table 2. The related SNP was searched for novelty in dbSNP and HGMD for the disease causing mutation. This mutation has not been reported so far.

3.2. Protein sequence alignment

The 3βHSD is a single pass membrane enzyme. The mutations found relating to different regions of the enzyme are depicted in Fig. 2.

Comparison of protein domain alignment with 3βHSD family delineates a conservation of amino acid in some domains of the enzyme. As indicated in literature, amino acid 82 of 3βHSD protein is part of membrane binding region (Thomas et al., 1999) (Fig. 2). A multiple protein alignment of the *HSD3B2* sequence with the related family members revealed that Ala82 is highly conserved among not only other mammalians, but also among other species (Fig. 3).

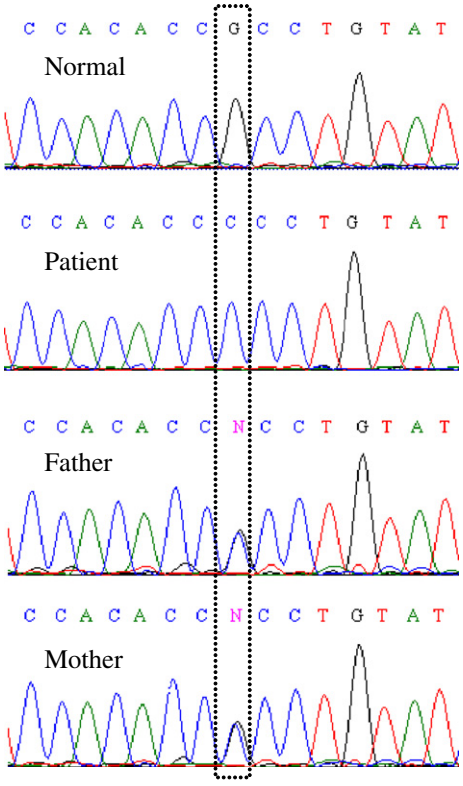

Fig. 1. Electropherograms of a normal control, the patient and his parents. GCC to CCC substitution cause Ala to Pro at protein level. Patient and normal control are homozygous for C and G respectively. The patient's parents are heterozygotes for this variant.

3.3. Structure prediction

Secondary structure prediction was achieved with online tools. I-TASSER prediction is basically based on threading model (Roy et al., 2010). Phyre 2 server predicted the structure based on template based homology modeling and fold recognition (Kelley and Sternberg, 2009). Secondary structure was determined as a coil structure by I-TASSER server (Fig. 4A) with mid confidence value. As illustrated, this position is buried in the protein with no solvent accessibility. The B-strand (E = extended) structure was predicted with mid confidence by Phyre 2 server (Fig. 4B). The position is structurally ordered that means this region is globular and relatively static. Disordered regions are flexible, dynamic and can be partially or completely extended in solution.

The highest similar structure 1sb8A (*Pseudomonas aeruginosa* UDP-N-acetylglucosamine 4-epimerase complexed with UDP-N-acetylgalactosamine, classified under *isomerase* family) was determined by I-TASSER server which constructed the 3D structure (Fig. 4C). The 3D model structure based on homology modeling of c1z45A (gal10 fusion protein galactose mutarotase/UDP-galactose 4-epimerase from *Saccharomyces cerevisiae* complexed with NAD, UDP-glucose, and galactose, family of isomerase) with coverage of 83% wherein 22% of the sequences had identity by Phyre 2 server (Fig. 4D). Also, the sequence was matched to the members of tyrosine-dependent oxidoreductase family, named d1r6da with 20% identity from superfamily of NAD (P)-binding Rossmann-fold domains based on fold recognition (Phyre server). The comparison of the template was designed based on template 3hsk (42% identity; aspartate semialdehyde dehydrogenase with NADP from *Candida albicans*, classified in oxidoreductases), 3l9w (22% identity; KefC C-terminal domain in complex with KefF and GSH), 2dfd (21% identity; human malate dehydrogenase type 2, classified in oxidoreductases) and 1r6d (20% identity; DesIV double mutant (dTDP-glucose 4,6-dehydratase) from *Streptomyces venezuelae* with NAD and DAU bound, classified in lyses) in SwissModel.

3.4. Functional analysis

The result of I-TASSER is based on enzyme commission, gene ontology, and ligand-binding site. The functional analysis elucidated isomerase activity with high homology to 1sb8A. Ligand binding site prediction identified 1EK6 (human UDP-galactose 4-epimerase complexed with NADH and UDP-glucose) with high score of binding site identity (data not shown). The Phyre 2 server looks for template binding site, cleft detection and consensus functional sites. This was done by 3DLigandSites (Wass et al., 2010) which is based on ligand binding sites on similar structures in protein library. Here, 1z45A (gal10 fusion protein galactose mutarotase/UDP-galactose 4-epimerase from *S. cerevisiae* complexed with NAD, UDP-glucose, and galactose) was used as model structure. Position 82 was also predicted as a binding site (data not shown).

3.5. In silico analysis of disease causing amino acid substitution

Based on different bioinformatic online software the probability of amino acid alteration and its effect were examined on the protein function and pathogenicity. SIFT 0 (≤0.05 pathogen) and PolyPhen 0.999 (0.5–1.5 possibly/probably damaging) were calculated for the amino acid substitution. PHD-SNP 4 (predictor of human deleterious single nucleotide polymorphism), and SNAP (screening for non-acceptable polymorphisms) values were exemplified as having pathogenic effect with POP tool. This suggested that the amino acid change could be deleterious. Also, SNPs&Go bioinformatic tool predicted the pathogenicity of the novel missense G to C mutation deleterious with the reliability index of 9 (unreliable: 0–10: reliable). Table 3

Table 2
Reported *HSD3B2* gene mutations and clinical features involved in congenital adrenal hyperplasia in the related populations.

Mutation type	Codon change	Phenotype	Population	References
Missense	L6F	HSD3B deficiency	Pakistani	Zhang et al. (2000)
	A10E	HSD3B deficiency	French	Alos et al. (2000)
	A10V	HSD3B deficiency	Egyptian	Moisan et al. (1999)
	G15D	HSD3B deficiency	Algerian	Rhéaume et al. (1995)
	A82T	Pseudohermaphroditism	Brazilian	Mendonça et al. (1994)
	A82P	HSD3B deficiency	Iranian	Present study
	N100S	Pseudohermaphroditism	French, English	Mébarki et al. (1995)
	L108W	Pseudohermaphroditism	Spanish/Portuguese	Sanchez et al. (1994)
	G129R	Adrenal hyperplasia	American, Brazilian	Rheaume et al. (1994)
	E142K	Adrenal hyperplasia	American, Caucasian	Simard et al. (1993)
	P155L	HSD3B deficiency	French	Moisan et al. (1999)
	L173R	Pseudohermaphroditism	Scottish	Russell et al. (1994)
	P186L	Pseudohermaphroditism	Spanish/Portuguese	Sanchez et al. (1994)
	L205P	Adrenal hyperplasia	Japanese	Katsumata et al. (1995)
	S213T	Idiopathic hypospadias	ND	Codner et al. (2004)
	S213G	Premature pubarche	ND	Moisan et al. (1999)
	P222Q	HSD3B deficiency	Algerian	Moisan et al. (1999)
	P222H	HSD3B deficiency	Brazilian	Moisan et al. (1999)
	P222T	HSD3B deficiency	Eastern European	Pang et al. (2002)
	L236S	HSD3B deficiency	French, American	Nayak et al. (1998)
	A245P	Adrenal hyperplasia	Turkish	Simard et al. (1993)
	Y253N	Adrenal hyperplasia	Dutch	Simard et al. (1993)
	Y254D	Adrenal hyperplasia	American	Sanchez et al. (1994)
	T259R	Adrenal hyperplasia	Japanese	Tajima et al. (1995)
	T259M	HSD3B deficiency	Taiwanese, Brazilian, French	Moisan et al. (1999)
	S284R	Idiopathic hypospadias	ND	Codner et al. (2004)
	G294V	HSD3B deficiency	French	Moisan et al. (1999)
	P341L	HSD3B deficiency	ND	Welzel et al. (2008)
	X373C	HSD3B deficiency	Caucasian	Pang et al. (2002)
Nonsense	E135X	Adrenal hyperplasia	Chilean	Marui et al. (1998)
	W171X	Adrenal hyperplasia	Swiss, American	Rheaume et al. (1992)
	R249X	Adrenal hyperplasia	Japanese	Tajima et al. (1995)
	Y308X	Adrenal hyperplasia	Japanese	Tajima et al. (1995)
	R335X	HSD3B deficiency	ND	Welzel et al. (2008)
	W355X	HSD3B deficiency	ND	Welzel et al. (2008)
Deletions	687del27	HSD3B deficiency	Sri-Lankan	Moisan et al. (1999)
	797delA	Pseudohermaphroditism	English	McCartin et al. (2000)
	818delAA	Adrenal hyperplasia	Afghan/Pakistani	Simard et al. (1993)
	867delG	HSD3B deficiency	French	Moisan et al. (1999)
	953delC	Adrenal hyperplasia	Pakistani	Zhang et al. (1996)
Insertions	558insC	HSD3B deficiency	American, Dutch	Rheaume et al. (1992)

ND = not determined.

shows the summary of the result of each tool on predicting the disease causality of amino acid change.

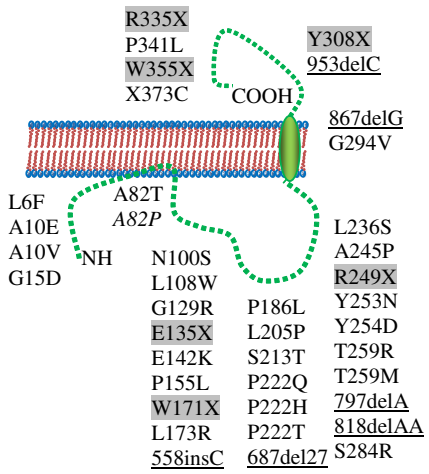

Fig. 2. Schematic inferred structure of 3βHSD2 domains and distribution of its mutations reported so far. As interpreted, 3βHSD2 enzyme is a single pass membrane structure (283–310) with a binding membrane domain at C-terminal (79–89 amino acids). Stop codon mutations are highlighted in gray. Deletion and insertion mutations are underlined. The new reported mutation is in italic.

3.6. Interactome analysis

The interactome analysis revealed that *HSD3B2* gene is related to CYP17A1 (17α-hydroxylase/17,20 lyase/17,20 desmolase), CYP11A1 (cholesterol side-chain cleavage enzyme), CYP11B1 (11 B hydroxylase), NR4A1 (nerve growth factor 1B), SRD5A1 (steroid 5 alpha reductase dehydrogenase alpha 1), SRD5A2 (steroid 5 alpha reductase dehydrogenase alpha 2), CYP19A1(aromatase), HSD17B2 (17 B hydroxysteroid dehydrogenase 2), HSD11B1 (11 B hydroxysteroid dehydrogenase 1), UGT2B15 (UDP glucuronosyl transferase 2) genes (Fig. 5).

4. Discussion

A few molecular studies have been published about CAH patients in Iran, and all of them have worked on *CYP21A2* gene mutations (Vakili et al., 2005; Ramazani et al., 2008; Rabbani et al., 2011a). In this study, a homozygous coding mutation is reported in a 20 day old pseudohermaphrodite male due to substitution of G to C of codon 82 (at position 244), encompassing the membrane binding domain of 3βHSD enzyme. Cosegregation analysis approved the existence of the mutation through parental gene.

Up to now, about 40 mutations have been reported to be deleterious in *HSD3B2* gene; of these mutations, 33 are base substitutions (27 missense and 6 nonsense mutations) and 6 are base deletions and insertions. That is, most of the mutations (84.6%) are substitutions (Table 2).

```
53   TKLTVLEGDILDEPFLKRACQDVSVVIHTACIIDVFGVTHRESIMNVNVKGTQLLLEACV  112  P26439   3BHS2_HUMAN
53   TKLTVLEGDILDEPFLKRACQDVSVVIHTACIIDVFGVTHRESIMNVNVKGTQLLLEACV  112  B2R8L0   B2R8L0_HUMAN
53   TKLTVLEGDILDEPFLKRACQDVSVVIHTACIIDVFGVTHRESIMNVNVKGTQLLLEACV  112  Q5QP01   Q5QP01_HUMAN
53   TKLTVLEGDILDEPFLKRACQDISVVIHTACIIDVFGVTHRQSIMNVNVKGTQLLLEACV  112  G1R000   G1R000_NOMLE
54   TKLTVLEGDILDEPFLKRACQDVSVVIHTACIIDVFGVTHRESIMNVNVKGTQLLLEACV  113  P27365   3BHS1_MACMU
54   TKLTVLEGDILDEPFLKRACQDVSVIIHTACIIDVFGVTHRESIMNVNVKGTQLLLEACV  113  P14060   3BHS1_HUMAN
54   IKLTLLEGDILDEQCLKGACQGTSVVIHTASVIDVRNAVPRETIMNVNVKGTQLLLEACV  113  P14893   3BHS_BOVIN
54   IKLTMLEGDILDEQCLKGACQGASVVIHTASIIDVVNAVGRETVMKVNVKGTQLLLEACV  113  Q9N119   3BHS_PIG
54   TKLTMVEGDILDEQCLKRACQGTSVVIHTASVIDVMNVIHRETIMNVNLKGTQLLLEACA  113  Q5IFP1   3BHS_CANFA
54   VKLTVLEGDILDEQFLKRACQGASAVIHTASIIDVTNLFNPQVTMNVNVEGTQLLLEACS  113  O46516   3BHS_HORSE
54   AKVTMLEGDILDAQYLRRACQGISVVIHTAAVIDVSHVLPRQTILDVNLKGTQNLLEAGI  113  F1LNS3   F1LNS3_RAT
54   AKVTMLEGDILDAQYLRRACQGISVVIHTASVMDFSRVLPRQTILDVNLKGTQNLLEAGI  113  P22072   3BHS2_RAT
54   AKVTMLEGDILDAQYLRRACQGISVVIHTAAVIDVSHVLPRQTILDVNLKGTQNILEACV  113  P22071   3BHS1_RAT
54   TKVTVLEGDILDAQCLRRACQGISVVIHTAAVIDVTGVIPRQTILDVNLKGTQNLLEACV  113  P24815   3BHS1_MOUSE
54   IKVTVLEGDILDTQYLRRACQGISVVIHTAAIIDVTGVIPRQTILDVNLKGTQNLLEACI  113  P26149   3BHS2_MOUSE
54   IKVTVLEGDILDTQYLRRACQGISVVIHTAAIIDVTGVIPRQTILDVNLKGTQNLLEACI  113  P26150   3BHS3_MOUSE
54   IKVTVLEGDILDTQYLRKACQGISVVIHTAAVIDVTGVIPRQTILDVNLKGTQNLLEACI  113  Q7TPU0   Q7TPU0_MOUSE
54   IKVTVLEGDILDTQYLRKACQGISVVIHTAAVIDVTGVIPRQTILDVNLKGTQNLLEACI  113  O35469   3BHS6_MOUSE
54   IKVTVLEGDILDTQCLRRACQGISVVIHTAALIDVTGVNPRQTILDVNLKGTQNLLEACV  113  Q62878   3BHS4_RAT
54   AKVRVLKGDILDAQCLKRACQGMSAVIHTAAAIDPLGAASRQTILDVNLKGTQLLLDACV  113  Q61694   3BHS5_MOUSE
54   TKVTVLKGDILDAQCLKRACQGMSAVIHTAAAIDPLGAASRQTILDVNLKGTQLLLDACV  113  Q61767   3BHS4_MOUSE
54   AKVTVLRGDIVDAQFLRRACQGMSVIIHTAAALDIAGFLPRQTILDVNVKGTQLLLDACV  113  P27364   3BHS5_RAT
54   TKVTVLEGDILDAQCLRRACQGISVVIHTAAAIDVWGIIPRQTIIDINVKGTLNLLEACV  113  Q64421   3BHS2_MESAU
54   TKVTVLEGDILDAQCLRRACQGISVVIHTAAAIDVFGAIPRQTVIDINLKGTQHLLDACI  113  O35296   3BHS3_MESAU
```

Fig. 3. Multiple amino acid alignment of 3βHSD family adapted from UniProt protein family members. Different orthologous and paralogous members are specified in the right side of the protein sequences. Ala82 as indicated in the box shows high conservation among different species.

Distribution of mutations of *HSD3B2* gene is illustrated in Fig. 2. Most mutations are located in the N-terminal region of the protein. It is noticeable that reported mutations of the C-terminal are mostly nonsense mutations. Three out of six mutations are nonsense mutations in the C-terminal hence most N-terminal mutations are missense mutations (Fig. 2). Up to now, two mutations were reported in transmembrane domain.

It seems that among all reported mutations, P222 is a hotspot in the enzyme due to various probabilities of mutation rate which has been reported deleteriously as P222Q, P222H and P222T (Table 2). Though, A10, A82, and T259 could also be considered as hotspot since different mutations were reported in these positions (Table 2). The position of Ala82 has been reported previously as a diseased variant in Swiss-Prot variant (VAR_010520) which was substituted to threonine (p.Ala82Thr) (Mendonça et al., 1994; McCartin et al., 2000). Despite common mutations found in *CYP21A2* gene, no common mutation has been reported for *HSD3B2* gene.

HSD3B2 gene encodes into 372 amino acids containing cofactor, putative substrate binding domain, substrate domain, NAD binding domain and membrane spanning domain. Two membrane associated

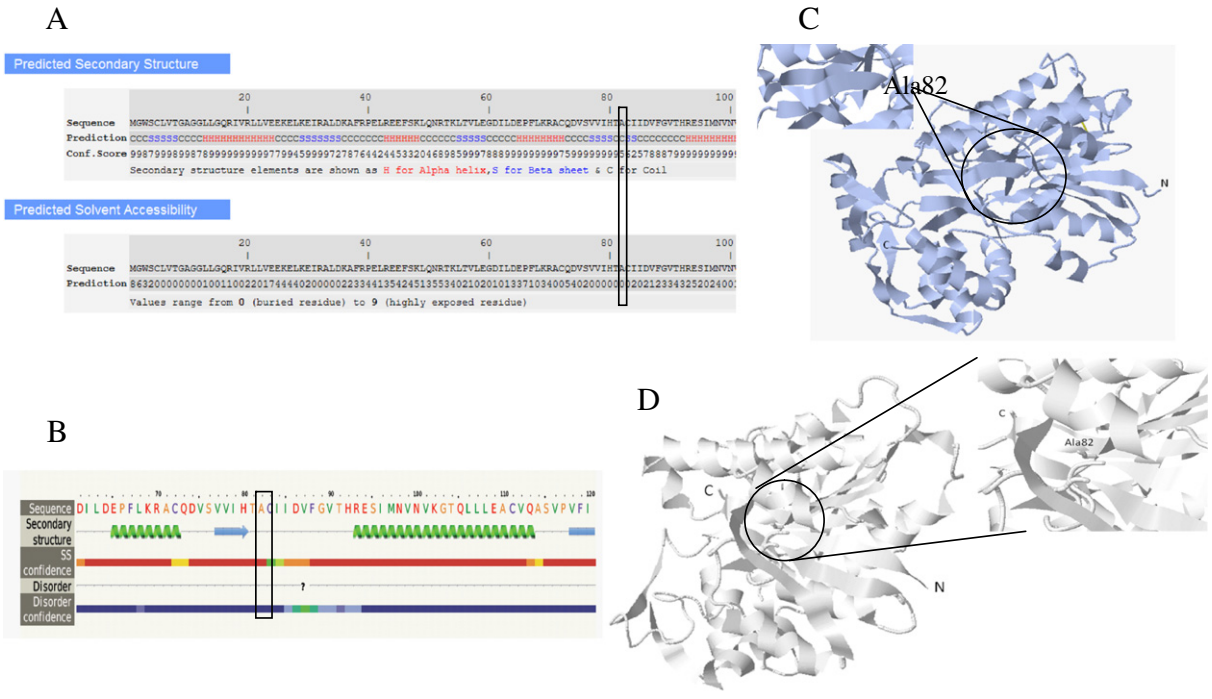

Fig. 4. Structure prediction of 3βHSD2 protein based on I-TASSER and Phyre servers. A) Predicted secondary structure based on threading model by I-TASSER, first line indicates the sequence, second line indicates the secondary structure which is determined to be coiled at position 82 with the confidence score of 5 (third line). The range of confidence is 0–9 wherein a higher score indicates a prediction with higher confidence. The solvent accessibility of the sequence is predicted as buried amino acid (range 0–9 wherein a higher value means higher accessibility). B) Prediction of the secondary structure based on template/homology modeling by Phyre 2 server. First line indicates the amino acid sequence and the second line is the secondary structure prediction which is determined as extended or B strand structure with the confidence value of high average (red, depicted in line three), also this position is ordered which means it is not flexible and dynamic with low value (blue); cartoon structural model of human 3βHSD protein, C) constructed by I-TASSER based on 1r66A (dTDP-glucose 4,6-dehydratase from *Streptomyces venezuelae*, NAD and TYD bound) with 22% folding aligned identity and 25% sequence identity. D) Constructed by Phyre 2 server based on c1z45A (gal10 fusion protein galactose mutarotase/UDP-galactose 4-epimerase from *Saccharomyces cerevisiae* complexed with NAD, UDP-glucose, and galactose, family of isomerase. The N-terminal and C-terminal are shown and the position of Ala82 is encircled in larger resolution in 3D structure.

Table 3
The bioinformatic software for predicting the pathogenicity of the amino acid change. The descriptions of the predicted tools are summarized with the range of value.

Software	Information	URL	Description	Value/range	Ref
SIFT	Pathogenic or not predictors	http://blocks.fhcrc.org/sift/SIFT.html	Deleterious	0 (≤0.05 pathogen; 0–1)	Ng and Henikoff (2001)
PolyPhen-2	Pathogenic or not predictors	http://www.bork.emblheidelberg.de/PolyPhen/ http://coot.embl.de/PolyPhen/	Deleterious	0.998 (0–1) damaging	Ramensky et al. (2002)
I-Mutant	Stability changes prediction	http://gpcr2.biocomp.unibo.it/cgi/predictors/IMutant2.0/I-Mutant2.0.cgi	Pathogenic	−1.47 (>0: decrease stability; <0: increase stability)	Capriotti et al. (2005)
SNAP	Pathogenic or not predictors	http://cubic.bioc.columbia.edu/services/SNAP/	Non-neutral	5 (>0)	Bromberg and Rost (2007)
PON	Pathogenic or not predictors	http://bioinf.uta.fi/PON-P	Pathogenic	9.95 (0–10)	Thusberg and Vihinen (2009)
PHD-SNP	Pathogenic or not predictors	http://snps.uib.es/phd-snp/phd-snp.html	Disease	4 (0.998)	Capriotti et al. (2006)
SNPs&Go	Gene ontology	http://snps-and-go.biocomp.unibo.it	Disease	9 (0–10:disease)	Calabrese et al. (2009)

regions are amino acid 79–89 and 283–310 regions. The latter is a helical transmembrane domain. Catalytic activity has been reported for amino acids 154–158 and 269–273. Comparing the two isoforms of HSD3B, His156 in 3βHSD1 and Tyr156 in 3βHSD2 are different in catalytic domain, Y154-X-H156/Y156-X-K158, of the isoenzymes (Thomas et al., 2002). However, His156/Tyr156 resides in the helical subunit interface of the dimeric enzyme. Also, a nucleotide-binding domain at the NH2 terminus of 3 beta HSD isozymes consists of beta strand-alpha helix-beta strand, providing a pocket for AMP part of the nucleotide factors (Scrutton et al., 1990). This site may function in both binding site for NAD and isomerase reaction of the enzyme. It is suggested that the function of 3βHSD2 is associated with conformational change of the enzyme (Pawlak et al., 2011). The interaction of 3βHSD2 with outer mitochondrial membrane and inner mitochondrial membrane translocases may promote the conformational changes. Therefore, it may lead to dual functionality of the protein. Also, different domains of the 3βHSD2 interact with Tom22 (translocase outer membrane protein), Tim23 (translocase inner membrane protein), and Tim50 complex proteins of the mitochondria.

Residues 72–89 in the NH2-terminal region form a membrane-binding domain. Deletion of this region causes the mutant protein to localize in the microsomes, mitochondria and cytosol (Thomas et al., 1999) and not attached as a membrane enzyme. Residues 72–89, therefore have an important role in membrane association, because the majority of the mutant protein is shifted into the cytosol. Deletion of these residues results in 8-fold loss of both 3 beta HSD and isomerase activity (Thomas et al., 1999). It may be acceptable that Ala82Pro located within the region can decrease the activities of the enzyme due to its impact on enzyme inclination to the membrane (Yohannan et al., 2004; Cordes et al., 2002). Previous studies have shown that increased polarity of the residues 75–91 in the rat 3 beta HSD type II leads to significant loss of its activity (Simard et al., 1991; Thomas et al., 1999). This highly conserved hydrophobic domain may play an essential role in the activity of not only 3 beta HSD type II but also the entire 3 beta HSD family. Thus, pathogenicity of Ala82Pro mutation is more suggested. Specifically, the residues 78–82 are very highly conserved among primates and this conservation highlights that it may be crucial to the activity of the enzyme.

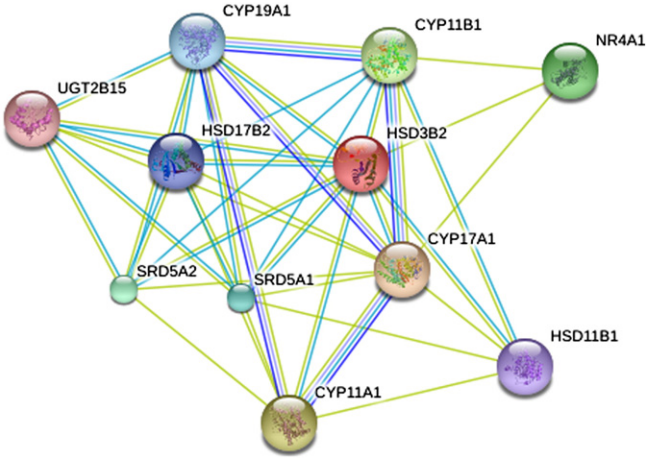

Fig. 5. Protein–protein interaction network of *HSD3B2*. This enzymatic system has a crucial role in the biosynthesis of all classes of hormonal steroids. Predicted functional partners are as follows: CYP17A1: cytochrome P450, family 17, subfamily A, polypeptide 1; CYP11A1: cytochrome P450, family 11, subfamily A, polypeptide 1; CYP11B1: cytochrome P450, family 11, subfamily B, polypeptide 1; NR4A1: nuclear receptor subfamily 4, group A, member 1; SRD5A2: steroid-5-alpha-reductase, alpha polypeptide 2; SRD5A1: steroid-5-alpha-reductase, alpha polypeptide 1; CYP19A1: cytochrome P450, family 19, subfamily A, polypeptide 1; HSD17B2: hydroxysteroid (17-beta) dehydrogenase 2; HSD11B1: hydroxysteroid (11-beta) dehydrogenase 1; UGT2B15: UDP glucuronosyltransferase 2 family, polypeptide B15. This figure was generated by STRING (V9.0) (Szklarczyk et al., 2011).

In order to correlate the founded genotype to the disease causing phenotypes, bioinformatic tools evaluated the function of protein as pathogenic. The explanation for it could be that alanine side chain groups are non-reactive and particularly interact with non reactive atoms of the neighboring amino acids; this was homozygously substituted by proline. Although both Ala and Pro are from hydrophobic nonpolar amino acid's group, proline could not easily adopt different conformations in protein structure due to tighter link to the backbone of the protein; thus it affects the biological activity of the protein. Since in silico prediction of secondary structure of 3βHSD showed that A82 is part of coil region, proline substitution could decrease the stability of helices. Although the pathogenicity of the mutation was determined in silico but there is demand on the functional analysis of the mutation.

In silico analysis of the function of the enzyme based on template and fold recognition verified the analysis since it predicted the isomerase and NAD binding domains relating to other protein superfamilies in the databases. Also, binding site at position 82 was approved with the tools.

As known, the HSD enzyme has a crucial role in biosynthesis of all classes of steroid hormones. As the interactome analysis showed, the interaction is seen in sex differentiation hormones and estrogen and testosterone. Therefore, any changes in this enzyme would have an effect on many pathways.

Structural Biology Knowledgebase (http:sbkb.org) outlines the structures, theoretical models, structural genomic target information, protocols and clones from a sequence search. Four protein structures with 30% identity have been reported in this database for our protein (P26439) with 23 theoretical models which are not accurate. Despite the fact that the template alignment in the Phyre server was <30% identical to the query, the overall fold of the model would be correct and the central core of the model is accurate. Due to lack of good template regarding 3D model similarity in the protein structure library, I–TASSER could not define a very high value to construct a

3D model. Therefore, there is a need for experimental analysis for the modeling of this protein.

In conclusion, A82P may have deleterious effect on the enzyme structure and subsequently affect its function. However, mutations in *HSD3B2* gene are not a major cause of CAH in Iran; nevertheless, mutation analysis of this gene is required in subpopulations of Iran to get an accurate frequency of the gene mutation.

Acknowledgments

We appreciate the patient family for participating in this study. We would like to express our thanks to genetic laboratory members of Children's Center Hospital, Tehran. We would also like to thank Dr Susan Thomas for her comments.

References

Alos, N., et al., 2000. A novel A10E homozygous mutation in the HSD3B2 gene causing severe salt-wasting 3beta-hydroxysteroid dehydrogenase deficiency in 46,XX and 46,XY French-Canadians: evaluation of gonadal function after puberty. J. Clin. Endocrinol. Metab. 85 (5), 1968–1974.

Berube, D., et al., 1989. The gene encoding human 3-beta hydroxysteroid dehydrogenase/isomerase mapped to the p13 band of chromosome 1. Cytogenet. Cell Genet. 51, 962–963.

Bromberg, Y., Rost, B., 2007. SNAP: predict effect of non-synonymous polymorphisms on function. Nucleic Acids Res. 35 (11), 3823–3835.

Calabrese, R., et al., 2009. Functional annotations improve the predictive score of human disease-related mutations in proteins. Hum. Mutat. 30 (8), 1237–1244.

Capriotti, E., Fariselli, P., Casadio, R., 2005. I-Mutant2.0: predicting stability changes upon mutation from the protein sequence or structure. Nucleic Acids Res. 33 ((Web Server issue):W306-10).

Capriotti, E., Calabrese, R., Casadio, R., 2006. Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. Bioinformatics 22 (22), 2729–2734.

Codner, E., et al., 2004. Molecular study of the 3 beta-hydroxysteroid dehydrogenase gene type II in patients with hypospadias. J. Clin. Endocrinol. Metab. 89 (2), 957–964.

Cordes, F.S., et al., 2002. Proline-induced distortions of transmembrane helices. J. Mol. Biol. 323, 951–960.

Headon, D.R., Hsiao, J., Ungar, F., 1978. The intracellular localization of adrenal 3beta-hydroxysteroid dehydrogenase/delta5-isomerase by density gradient perturbation. Biochem. Biophys. Res. Commun. 82 (3), 1006–1012.

Katsumata, N., et al., 1995. A novel missense mutation in the type II 3 beta-hydroxysteroid dehydrogenase gene in a family with classical salt-wasting congenital adrenal hyperplasia due to 3 beta-hydroxysteroid dehydrogenase deficiency. Hum. Mol. Genet. 4 (4), 745–746.

Kelley, L.A., Sternberg, M.J., 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat. Protoc. 4 (3), 363–371.

Kiefer, F., et al., 2009. The SWISS-MODEL Repository and associated resources. Nucleic Acids Res. 37, D387–D392.

Kopp, J., Schwede, T., 2004. The SWISS-MODEL Repository of annotated three-dimensional protein structure homology models. Nucleic Acids Res. 32, D230–D234.

Lachance, Y., et al., 1991. Structure of the human type II 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD) gene: adrenal and gonadal specificity. DNA Cell Biol. 10 (10), 701–711.

Marui, S., et al., 1998. A novel homozygous nonsense mutations E135* in the type II 3beta-hydroxysteroid dehydrogenase gene in a girl with salt-losing congenital adrenal hyperplasia. Mutations in brief no. 168. Online. Hum. Mutat. 12 (2), 139.

McCartin, S., et al., 2000. Phenotypic variability and origins of mutations in the gene encoding 3beta-hydroxysteroid dehydrogenase type II. J. Mol. Endocrinol. 24 (1), 75–82.

Mébarki, F., et al., 1995. Nonsalt-losing male pseudohermaphroditism due to the novel homozygous N100S mutation in the type II 3 beta-hydroxysteroid dehydrogenase gene. J. Clin. Endocrinol. Metab. 80 (7), 2127–2134.

Mendonça, B.B., et al., 1994. Mutation in 3 beta-hydroxysteroid dehydrogenase type II associated with pseudohermaphroditism in males and premature pubarche or cryptic expression in females. J. Mol. Endocrinol. 12 (1), 119–122.

Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16 (3), 1215.

Moisan, A.M., et al., 1999. New insight into the molecular basis of 3beta-hydroxysteroid dehydrogenase deficiency: identification of eight mutations in the *HSD3B2* gene eleven patients from seven new families and comparison of the functional properties of twenty-five mutant enzymes. J. Clin. Endocrinol. Metab. 84 (12), 4410–4425.

Nayak, S., Lee, P.A., Witchel, S.F., 1998. Variants of the type II 3beta-hydroxysteroid dehydrogenase gene in children with premature pubic hair and hyperandrogenic adolescents. Mol. Genet. Metab. 64 (3), 184–192.

Ng, P.C., Henikoff, S., 2001. Predicting deleterious amino acid substitutions. Genome Res. 11 (5), 863–874.

Pang, S., 1998. The molecular and clinical spectrum of 3beta-hydroxysteroid dehydrogenase deficiency disorder. Trends Endocrinol. Metab. 9, 82–86.

Pang, S., et al., 2002. A novel nonstop mutation in the stop codon and a novel missense mutation in the type II 3beta-hydroxysteroid dehydrogenase (3beta-HSD) gene causing, respectively, nonclassic and classic 3beta-HSD deficiency congenital adrenal hyperplasia. J. Clin. Endocrinol. Metab. 87 (6), 2556–2563.

Pawlak, K.J., et al., 2011. Inner mitochondrial translocase Tim50 interacts with 3β-hydroxysteroid dehydrogenase type 2 to regulate adrenal and gonadal steroidogenesis. J. Biol. Chem. 286 (45), 39130–39140.

Penning, T.M., 1997. Molecular endocrinology of hydroxysteroid dehydrogenases. Endocr. Rev. 18, 281–305.

Rabbani, B., et al., 2011a. Mutation analysis of CYP21A2 gene in the Iranian population. Genet. Test. Mol. Biomarkers 16 (2), 82–90.

Rabbani, B., et al., 2011b. Molecular diagnosis of congenital adrenal hyperplasia in Iran: focusing on CYP21A2 gene. Iran. J. Pediatr. 21 (2), 139–149.

Ramazani, A., et al., 2008. The frequency of eight common point mutations in CYP21 gene in Iranian patients with congenital adrenal hyperplasia. Iran. Biomed. J. 12 (1), 49–53.

Ramensky, V., Bork, P., Sunyaev, S., 2002. Human non-synonymous SNPs: server and survey. Nucleic Acids Res. 30 (17), 3894–3900.

Rheaume, E., et al., 1992. Congenital adrenal hyperplasia due to point mutations in the type II 3 beta-hydroxysteroid dehydrogenase gene. Nat. Genet. 1 (4), 239–245.

Rheaume, E., et al., 1994. Molecular basis of congenital adrenal hyperplasia in two siblings with classical nonsalt-losing 3-B hydroxysteroid dehydrogenase deficiency. J. Clin. Endocrinol. Metab. 79, 1012–1018.

Rhéaume, E., et al., 1995. Identification and characterization of the G15D mutation found in a male patient with 3 beta-hydroxysteroid dehydrogenase (3 beta-HSD) deficiency: alteration of the putative NAD-binding domain of type II 3 beta-HSD. Biochemistry 34 (9), 2893–2900.

Roy, A., Kucukural, A., Zhang, Y., 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nat. Protoc. 5 (4), 725–738.

Russell, A.J., et al., 1994. Mutation in the human gene for 3 beta-hydroxysteroid dehydrogenase type II leading to male pseudohermaphroditism without salt loss. J. Mol. Endocrinol. 12 (2), 225–237.

Sanchez, R., et al., 1994. Functional characterization of the novel L108W and P186L mutations detected in the type II 3 beta-hydroxysteroid dehydrogenase gene of a male pseudohermaphrodite with congenital adrenal hyperplasia. Hum. Mol. Genet. 3 (9), 1639–1645.

Scrutton, N.S., Berry, A., Perham, R.N., 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature 343 (6253), 38–43.

Simard, J., de Launoit, Y., Labrie, F., 1991. Characterization of the structure–activity relationships of rat types I and II 3 beta-hydroxysteroid dehydrogenase/delta 5 -delta 4 isomerase by site-directed mutagenesis and expression in HeLa cells. J. Biol. Chem. 266 (23), 14842–14845.

Simard, J., et al., 1993. Molecular basis of congenital adrenal hyperplasia due to 3 beta-hydroxysteroid dehydrogenase deficiency. Mol. Endocrinol. 7 (5), 716–728.

Simard, J., et al., 2005. Molecular biology of the 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family. Endocr. Rev. 26 (4), 525–582.

Szklarczyk, D., et al., 2011. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res. 39 ((Database issue):D561-8).

Tajima, T., et al., 1995. Molecular analysis of type II 3 beta-hydroxysteroid dehydrogenase gene in Japanese patients with classical 3 beta-hydroxysteroid dehydrogenase deficiency. Hum. Mol. Genet. 4 (5), 969–971.

Thilen, A., Larsson, A., 1990. Congenital adrenal hyperplasia in Sweden 1969–1986. Prevalence, symptoms and age at diagnosis. Acta Paediatr. Scand. 79 (2), 168–175.

Thomas, J.L., et al., 1999. Creation of a fully active, cytosolic form of human type I 3beta-hydroxysteroid dehydrogenase/isomerase by the deletion of a membrane-spanning domain. J. Mol. Endocrinol. 23 (2), 231–239.

Thomas, J.L., et al., 2002. Structure/function relationships responsible for the kinetic differences between human type 1 and type 2 3beta-hydroxysteroid dehydrogenase and for the catalysis of the type 1 activity. J. Biol. Chem. 277, 42795–42801.

Thomas, J.L., et al., 2003. Structure/function relationships responsible for coenzyme specificity and the isomerase activity of human type 1 3 beta-hydroxysteroid dehydrogenase/isomerase. J. Biol. Chem. 278 (37), 35483–35490.

Thusberg, J., Vihinen, M., 2009. Pathogenic or not? And if so, then how? Studying the effects of missense mutations using bioinformatics methods. Hum. Mutat. 30 (5), 703–714.

Vakili, R., et al., 2005. Molecular analysis of the CYP21 gene and prenatal diagnosis in families with 21-hydroxylase deficiency in northeastern Iran. Horm. Res. 63 (3), 119–124.

Wass, M.N., Kelley, L.A., Sternberg, M.J., 2010. 3DLigandSite: predicting ligand-binding sites using similar structures. Nucleic Acids Res. 38 ((Web Server issue):W469-73).

Welzel, M., et al., 2008. Carboxyl-terminal mutations in 3beta-hydroxysteroid dehydrogenase type II cause severe salt-wasting congenital adrenal hyperplasia. J. Clin. Endocrinol. Metab. 93 (4), 1418–1425.

Yohannan, S., et al., 2004. Proline substitutions are not easily accommodated in a membrane protein. J. Mol. Biol. 341, 1–6.

Zhang, Y., et al., 1996. Human ovarian expression of 17 beta-hydroxysteroid dehydrogenase types 1, 2, and 3. J. Clin. Endocrinol. Metab. 81 (10), 3594–3598.

Zhang, L., et al., 2000. Characterization of two novel homozygous missense mutations involving codon 6 and 259 of type II 3beta-hydroxysteroid dehydrogenase (3betaHSD) gene causing, respectively, nonsalt-wasting and salt-wasting 3betaHSD deficiency disorder. J. Clin. Endocrinol. Metab. 85 (4), 1678–1685.