Characterisation of three novel *CYP11B1* mutations in classic and non-classic 11β-hydroxylase deficiency

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Abstract

Background: Congenital adrenal hyperplasia (CAH) is one of the most common autosomal recessive inherited endocrine diseases. Steroid 11β-hydroxylase (P450c11) deficiency (11OHD) is the second most common form of CAH.
Aim: The aim of the study was to study the functional consequences of three novel *CYP11B1* gene mutations (p.His125Thrfs*8, p.Leu463_Leu464dup and p.Ser150Leu) detected in patients suffering from 11OHD and to correlate this data with the clinical phenotype.
Methods: Functional analyses were done by using a HEK293 cell *in vitro* expression system comparing WT with mutant P450c11 activity. Mutant proteins were examined *in silico* to study their effect on the three-dimensional structure of the protein.
Results: Two mutations (p.His125Thrfs*8 and p.Leu463_Leu464dup) detected in patients with classic 11OHD showed a complete loss of P450c11 activity. The mutation (p.Ser150Leu) detected in a patient with non-classic 11OHD showed partial functional impairment with 19% of WT activity.
Conclusion: Functional mutation analysis enables the correlation of novel *CYP11B1* mutations to the classic and non-classic 11OHD phenotype respectively. Mutations causing a non-classic phenotype show typically partial impairment due to reduced maximum reaction velocity comparable with non-classic mutations in 21-hydroxylase deficiency. The increasing number of mutations associated with non-classic 11OHD illustrate that this disease should be considered as diagnosis in patients with otherwise unexplained hyperandrogenism.

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Introduction

Congenital adrenal hyperplasia (CAH), one of the most common autosomal recessive inherited endocrine diseases, is characterised by complete or partial impairment of adrenal steroidogenesis (1, 2). Although over 90% of cases of CAH are caused by 21-hydroxylase deficiency, steroid 11β-hydroxylase (P450c11, EC 1.14.15.4) deficiency (11OHD) accounts for 5–8% of cases, reflecting a frequency of ~1:100 000–1:200 000 live births in non-consanguineous populations (3, 4, 5).

The 11β-hydroxylase belongs to the cytochrome P450 system (P450c11) that facilitates the conversion of 11-deoxycortisol (S) to cortisol (F) and 11-deoxycorticosterone (DOC) to corticosterone (B) in the mitochondria of the adrenal cortex. 11OHD is characterised by deficient

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cortisol synthesis caused by mutations in the *CYP11B1* gene coding for 11β-hydroxylase (6, 7, 8, 9). An impairment of 11β-hydroxylase activity leads to an accumulation of the precursor steroids, which are shunted into the adrenal androgen synthesis pathway, resulting in prenatal virilisation of female external genitalia (46,XX disorder of sex development). Moreover, the excess of postnatal androgen causes androgenisation and rapid somatic growth as well as accelerated bone maturation in both sexes. The accumulation of DOC, which binds to and activates the mineralocorticoid receptor, leads to hypertension in about two-thirds of all patients (3, 10, 11, 12, 13). Hormonal diagnosis of 11OHD is verified by elevated baseline concentrations of DOC and S in classical forms as well as increased response to cosyntropin stimulation in non-classic forms (14).

The non-classic 11OHD form is caused by partial impairment of the P450c11 function (10, 15) with a phenotype resembling non-classic 21-hydroxylase deficiency (11). Non-classic 11OHD can manifest with mild virilisation and precocious pseudopuberty in children. Female patients with non-classic 11OHD are born with normal external genitalia and may have hirsutism and oligomenorrhea in adulthood, resembling signs and symptoms suggestive for polycystic ovary syndrome. However, only a minor percentage of women with hirsutism and oligomenorrhea suffer from non-classic 11OHD (10). Unlike in classic 11OHD, arterial hypertension is not commonly found.

In the current study, three novel *CYP11B1* mutations detected in three patients with classic and in one patient with non-classic 11OHD were studied in order to describe the effect of these variants.

Subjects and methods

Patient 1

A male patient (karyotype 46,XY) of Turkish origin was referred to the Paediatric Endocrinology Department at Erciyes University, Kayseri, Turkey at the age of 2 years with the complaint of penile growth. His parents are first cousins and he has three siblings, one of them also suffering from 11OHD (patient 2). Results of the initial physical examination were as follows: height 97 cm (+3.1 SDS) (16), weight 17 kg, BMI 18 kg/m^2 (+1.07 SDS) (17), blood pressure 100/70 mmHg (90th percentile) (18), testicular volume 3 ml, penile length 9 cm (+5.3 SDS) (19), advanced bone age of 7 years and dark skin pigmentation especially at the genitalia. Abdominal ultrasound examination revealed hyperplasic adrenal glands. Steroid hormone analyses supported the diagnosis of 11OHD (Table 1). Hydrocortisone supplementation was initiated. During his last visit at 14 years of age, physical examination revealed the

Table 1 Laboratory data before treatment.

	Patient 1	Patient 2	Patient 3	Patient 4
Karyotype	46,XY	46,XX	46,XY	46,XX
Genotype	p.His125Thrfs*8/ p.His125Thrfs*8	p.His125Thrfs*8/ p.His125Thrfs*8	p.Leu463_Leu464dup/ p.Leu463_Leu464dup	p.His125Thrfs*8/ p.Ser150Leu
Age at analysis	2 years	2 days	26 years	5 years
11-Deoxycorticosterone (ng/ml)	ND	ND	ND	1.0→11.5 (bl. 0.06–0.52)
Corticosterone (ng/ml)	ND	ND	ND	1.4→22.5 (bl. 0.09–1.46)
Aldosterone (pg/ml)	ND	258 (70–540)	16.7 (35–300)	ND
17-OHP (ng/ml)	17.56 (0.6–3.4)	48.73 (0.6–3.4)	4.2 (0.6–3.4)	0.40→1.9 (bl. 0.06–0.56)
11-Deoxycortisol (ng/ml)	60.35 (0.1–14)	31.94 (0.1–14)	46.6 (0–8)	4.92→30.9 (bl. 0.09–1.11)
Cortisol (μg/dl)	38[a] (5–26)	73.7[a] (5–26)	2.2 (5–26)	90→158 (bl. 5.62–170.28 and stim. >150)
DHEA-S (ng/ml)	532 (50–480)	4692 (50–480)	7527 (300–3330)	ND
Androstenedione (ng/ml)	23.66 (0.08–0.5)	70.10 (0.2–2.9)	ND	109→97 (bl. 2.9–43)
Total testosterone (ng/dl)	657 (3–10)	4355 (20–64)	492 (11–80)	26→34 (bl. 2.9–20)
Renin (pg/ml)	ND	0.76 (1.9–6.0)	0.89 (1.9–6.0)	ND
ACTH (pg/ml)	2690 (5–60)	1540 (5–60)	4328 (5–60)	ND

Reference ranges in brackets (bl., baseline and stim., stimulated), ND, not done. Steroids in the patients 1, 2 and 3 have been detected by RIA.
[a]The cortisol RIA (ICN Biomedicals, Inc., Irvine, CA, USA, 1999) assay used in patients 1 and 2 has a 33% cross-reactivity with 11-deoxycortisol causing elevated cortisol concentrations in the case of 11OHD. Steroids in the patient 4 have been detected by LC–MSMS (56) before and 60 min after 250 μg ACTH i.v.

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following: height 169 cm (0.1 SDS) (20), weight 59 kg, BMI 20 kg/m^2 (+0.37 SDS) (17), blood pressure 110/70 mmHg (50th percentile) (17), bilateral testicular volume 25 ml, pubic hair Tanner stage 5, stretched penile length 13 cm (+0.0 SDS) (18) and a bone age of 16 years. Although he was advised to take up 20 mg/m^2 per day hydrocortisone by the general practitioner, the laboratory investigations revealed insufficient control of the disease due to non-adherence to the therapy (DOC 215.81 ng/ml (reference range (0.12–1.58), DHEAS 981 ng/ml (1200–3700), total testosterone 882 ng/dl (350–970) and androstenedione 9.42 ng/ml (0.33–1.9)).

Patient 2

The female patient (karyotype 46,XX) was born after an uneventful pregnancy (height at birth 50 cm, weight 3400 g). She is the younger sister of patient 1. She was referred at the age of 2 days to the Paediatric Endocrinology Department at Erciyes University, Kayseri, Turkey because of ambiguous genitalia. She had virilised external genitalia reflecting Prader stage 3 without obvious hyperpigmentation of the skin. Pelvic ultrasonography revealed a small uterus, cervix and vagina. Steroid hormone analyses confirmed the diagnosis of 11OHD (Table 1). Hydrocortisone treatment was initiated. At her last visit to the Paediatric Endocrinology Department at the age of 12.5 years, the physical examination results were weight 42 kg (−0.1 SDS), height 145 cm (−1.04 SDS) (20), BMI 20 kg/m^2 (+0.17 SDS) (21), blood pressure 90/50 mmHg (10th percentile) (18), pubic hair and breast development Tanner stage 3 and bone age of 14 years. The patient was only irregularly followed up and adhered poorly to the treatment with hydrocortisone, which was increased up to 20 mg/m^2 per day by the general practitioner. Hormone analyses showed insufficient therapy (DOC 281 ng/ml (reference range 0.12–1.58), DHEAS 482 ng/ml (320–2260), total testosterone 269 ng/dl (15–35) and androstenedione 7 ng/ml (0.5–1.7)).

Patient 3

A 27-year-old male patient (karyotype 46,XY) of Turkish ancestry was referred to the Endocrinology Department at Erciyes University, Kayseri, Turkey with the complaint of infertility (Table 1). Premature pubarche led to clinical work up in infancy and revealed 11OHD. Hormonal data from that time are not available. Treatment with hydrocortisone was initiated at that time, but then he was lost to follow-up until the age of 27 years. At that time, his height and weight were 152 cm (−3.41 SDS) (20) and 65 kg, respectively, and his BMI was 28 kg/m^2 (+0.11 SDS) (17). Hormonal data at 27 years of age is shown in Table 1. Blood pressure was not taken on that occasion. Computed tomography scan of adrenal glands revealed nodular thickening. Semen analysis revealed severe oligospermia and mild-to-moderate asthenozoospermia. Follicle-stimulating hormone and luteinising hormone levels were elevated with 17 mIU/ml (reference range 1.5–12.4) and 10 mIU/ml (1.7–8.6) respectively. Scrotal ultrasonography revealed two heterogeneous hypoechogenic lesions (right testis, 38×20 mm and left testis, 24×155 mm) with lobulated contours and irregular boundaries reflecting testicular adrenal rest tumours (TART). The TART located on right testis was removed by testis sparing surgery. Histological examination was compatible with TART (Fig. 1). Prednisolone therapy was resumed and the patient's wife gave birth to a female child. The patient's fertility was not investigated in detail as he refused to perform a semen analysis.

Patient 4

The female patient (karyotype 46,XX) of Moroccan descent was diagnosed at the Paediatric Endocrinology unit at the Children's Hospital Altona, Hamburg, Germany, as non-classical 11OHD when she was 5.5 years old and came to attention with premature

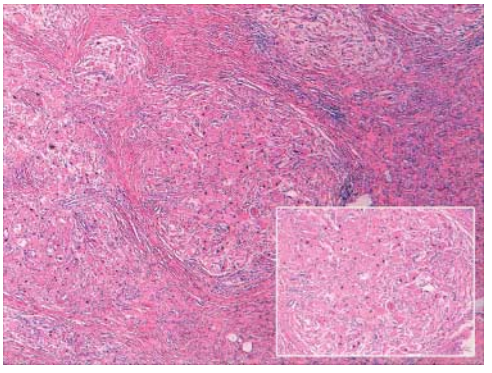


Figure 1
Histopathology from frozen tissue section of TART (magnification ×40). Multiple nodules are present having an appearance comparable with adrenal origin. The lesion was composed of sheets and nests separated by fibrous tissue. The individual cells were large, round with round nuclei with defined borders and abundant eosinophilic cytoplasm (cutout, magnification ×100).

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pubarche without further signs of virilisation. Clinical characteristics at diagnosis were height 112, 2 cm (−0.7 SDS) (20), growth rate 10.7 cm/year (+5.2 SDS), weight 21.3 kg, BMI 16.9 kg/m^2 (0.86 SDS) (17) and bone age of 8.5 years. Ultrasonography of the ovaries and adrenals revealed no pathological changes. Baseline DOC and S were elevated and cortisol was within the reference range (Table 1). ACTH stimulation test showed significantly increased response of DOC and S with sufficient rise of cortisol compatible to non-classic 11OHD. Treatment with hydrocortisone (10 mg/m^2 per day) was initiated. At the age of 7.1 years, breast development started and therapy with triptorelin was started without performing further hormonal tests because of precocious puberty secondary to adrenal androgen excess.

The brother of patient 4 was first seen at the age of 8 months. Auxological data were height 68 cm (−1.1 SDS) (20), weight 9 kg and BMI 19.5 kg/m^2 (+1.1 SDS) (17). The androgen metabolites in urine were within the normal range for age. At the age of 16 months there were still no signs of virilisation, height 82.2 cm (+0.4 SDS) and growth rate accelerated with 20.3 cm/year within the last 8 months (+1.5 SDS). Bone age was not analysed yet.

Sequence analysis of the *CYP11B1* gene

The molecular analysis of the *CYP11B1* gene was carried out after receiving informed consent from patients and/or legal guardian for genetic studies. Genomic DNA was prepared from peripheral blood leukocytes and amplification of exons 1–9 of the *CYP11B1* gene was carried out as described previously (22, 23). Direct DNA sequencing and analysis of the coding region of the *CYP11B1* gene was done as described previously (23). Sequence variants were designated according to the recommendations of the Human Genome Variation Society (www.hgvs.org/rec.html) using the GenBank reference sequences NC_000008.10 (*CYP11B1* g.DNA), NM_000497 (*CYP11B1* c.DNA) and NP_000488.3 (*CYP11B1* p.protein).

Transient transfection assay

Each mutation was introduced into a *CYP11B1*-pcDNA3.1 expression vector construct (kindly provided by Prof. R Bernhardt, Institute of Biochemistry, University des Saarlandes, Saarbrücken, Germany) by site-directed mutagenesis as described previously (24, 25). *In vitro* transient transfection assay was performed in triplicates using the HEK293 cell line thrice. The cells were transfected with each pcDNA3.1-*CYP11B1* mutant constructs with additional transfection of adrenodoxin (pECE-*ADX*), Adx reductase (pECE-*ADR*) expression vectors (kindly provided by Prof. W L Miller, Department of Pediatrics, University of California, San Francisco, CA, USA) and pRK-TK (Promega) coding for Renilla luciferase as it was described in our previous paper (25). The kinetic constants of *CYP11B1* in intact HEK293 cells were determined 48 h after transfection. The cells were incubated in triplicates for 270 min at 37 °C with 1 ml DMEM medium containing 0.1, 0.25, 0.5, 1, 2, and 4 μmol/l DOC and S in parallel with 10 mM NADPH (Sigma–Aldrich). The steroid hormones DOC, B, S and F were simultaneously determined in the cell culture supernatant using an UPLC–MS/MS method as described previously (25, 26, 27). Western blot analysis was carried out to ensure the expression and translation of WT and mutant proteins as described previously (24, 25, 28).

All assays were performed in at least three independent triplicate experiments, and data are presented as mean±S.D. Kinetic parameters were established by nonlinear regression using the Michaelis–Menten equation to determine the Michaelis–Menten constant (K_m) and maximum velocity (V_{max}). Catalytic efficiency was defined as the ratio V_{max}/K_m expressed as percentage of WT. The 11-hydroxylase activity of the mutants was expressed as a percentage of substrate conversion in picomole per milligram of total protein per minute, defining *CYP11B1* WT activity as 100% after correction for total protein with Renilla luciferase activity. Enzyme kinetic parameters and enzymatic activity were calculated using the GraphPad Prism Software, version 5.0 (GraphPad, Inc., San Diego, CA, USA).

Molecular modelling

We used a fold recognition algorithm to show that the human CYP11B1 sequence is compatible with the architecture of the enzyme family (ProHit package, ProCeryon Biosciences GmbH, Salzburg, Austria). The template structure with the highest score of the pair potential was the X-ray structure of the cytochrome *CYP11A1* (PDB accession code: 3MZS) and served as the template for the three-dimensional model of *CYP11B1*. According to the alignment obtained by the fold recognition procedure, amino acid residues were exchanged in the template. Insertions and deletions in *CYP11B1* were modelled using a database search approach included in the software package WHATIF. The structural representation was generated with the Ribbons Software (Avatar Software AB, Stockholm, Sweden) as described previously (29).

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Results

Sequence analysis of the *CYP11B1* gene

Patient 1 showed a novel homozygous guanine deletion at cDNA position 372 (c.372delG) in exon 2 of the *CYP11B1* gene, resulting in a frameshift and a premature stop codon (p.His125Thrfs*8). Patient 2 showed the identical homozygous deletion c.372delG. Parents were not available for analysis; therefore, a heterogeneous deletion of the *CYP11B1* gene cannot be ruled out. Genetic analysis in patient 3 depicted a novel homozygous duplication of six bases in exon 8, c.1387_1392dupCTGCTG, causing a duplication of two leucines in frame p.Leu463_Leu464dup. The *CYP11B1* gene analysis revealed that patient 4 was compound heterozygous for the novel mutation c.372delG (p.His125Thrfs*8) inherited from the mother and the novel mutation c.449C>T (p.Ser150Leu) located in exon 3 inherited from the father (Fig. 2).

In vitro functional 11-hydroxylase assays

The three novel *CYP11B1* mutations were functionally analysed using transiently transfected HEK293 cells measuring the conversion of DOC to B and S to F. The two mutations detected in patients with 11OHD (p.His125Thrfs*8 and p.Leu463_Leu464dup) had no residual enzymatic activity. The mutation p.Ser150Leu detected with non-classic 11OHD showed only partial 11-hydroxylase impairment with a reduced activity of 19.2±1.4% (mean±S.D.) and 14.7±0.5% (mean±S.D.) for the conversion to B and F compared with WT respectively. Determination of kinetic constants showed similar K_m values for both B and F production with significantly impaired V_{max} compared with the WT (Fig. 3).

Western blot analysis demonstrated that all mutations apart from the p.His125Thrfs*8 had translation efficiency similar to WT. The mutant p.His125Thrfs*8 was not detectable by western blotting either due to the loss of the antibody binding site or due to nonsense-mediated mRNA or protein decay (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Molecular modelling

The frameshift mutation p.His125Thrfs*8 terminates the translation at the B–C loop of the protein (30). By this, all relevant enzyme structures including the substrate recognitions sides and haeme-binding sites are not present, resulting in a complete loss of function (31, 32). The variation p.Leu463_Leu464dup is located in the amino terminal L-helix. Two leucines are inserted in a leucine-rich part of the L-helix carrying four leucines. The L-helix is involved in haeme binding as well as in interactions with redox partners (30). The distal L-helix forms parts of the protein's surface. The residue p.Ser150 is located in the C–D loop connecting the C-helix with the D-helix (Fig. 4).

Discussion

In the present study, we have functionally characterised three novel *CYP11B1* mutations detected in three families. The p.Leu463_Leu464dup and p.Ser150Leu mutations were unique for the studied families. On the contrary, the p.His125Thrfs*8 mutation was detected in both Moroccan and Turkish families where there is no known relationship. Reported *CYP11B1* mutations are p.Arg448Cys (33, 34), p.Trp116Cys, p.Leu299Pro (2), p.Leu489Ser (15), Glu338term (35) and p.Asn394 Argfs*74, p.Trp318Thr, p.Arg43Gln and Ala259Asp in the Turkish population IVS5+2T>G, p.Gly446Val (36) and p.Arg448Cys (22) in the Moroccan population. The elevated frequency of 11OHD in Morocco and Turkey is most likely due to the higher rate of consanguineous marriages producing an enrichment of recessively inherited diseases (37). The prevalence of consanguinity in Morocco reported to date varies between 20–28 and 59.1% in patients suffering from autosomal recessive diseases (38). In Turkey, around 20% of marriages are consanguineous (39).

In vitro 11-hydroxylase activity of <5% can be considered severe and is most often associated with classic 11OHD (7, 24, 40). The patients 1 and 2 carrying a completely inactivating mutation (p.His125Thrfs*8) of the *CYP11B1* gene show a typical classical 11OHD phenotype with highly elevated steroids hormones, prenatal virilisation of the external genitalia in females and macrogenitosomia in males (5). Neither patients showed obvious signs of hypertension. The latest clinical assessment revealed that hydrocortisone treatment with doses above the recommended has not been effective enough to normalise steroid hormone levels in either patient, most likely because of poor adherence to the treatment. This demonstrates the necessity for long-term follow-up of the patients by experienced physicians, which is obviously difficult in rural Turkey. The mutation p.His125Thrfs has a comparable effect on the protein structure and function with the mutant c.358-362dup

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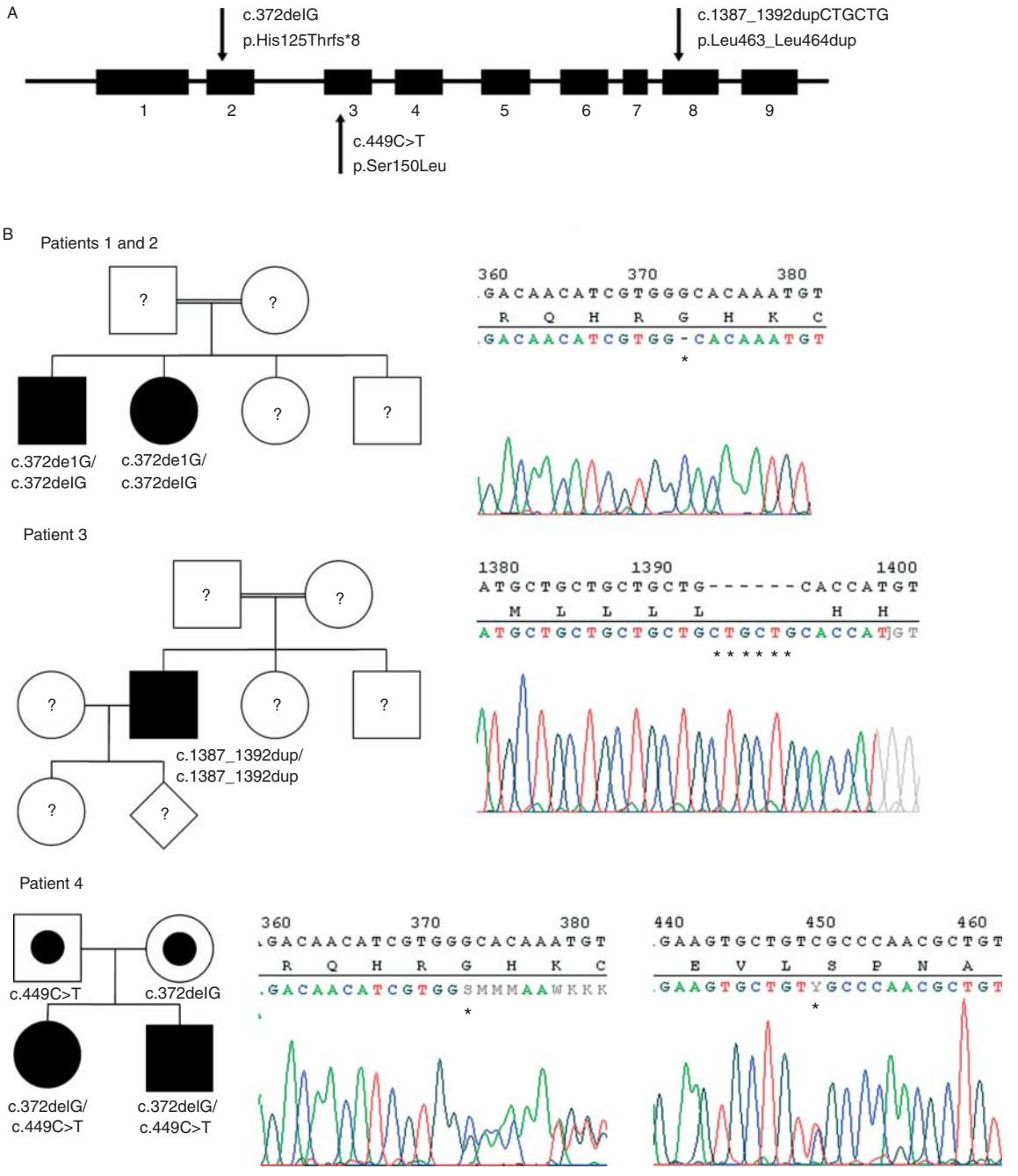


Figure 2

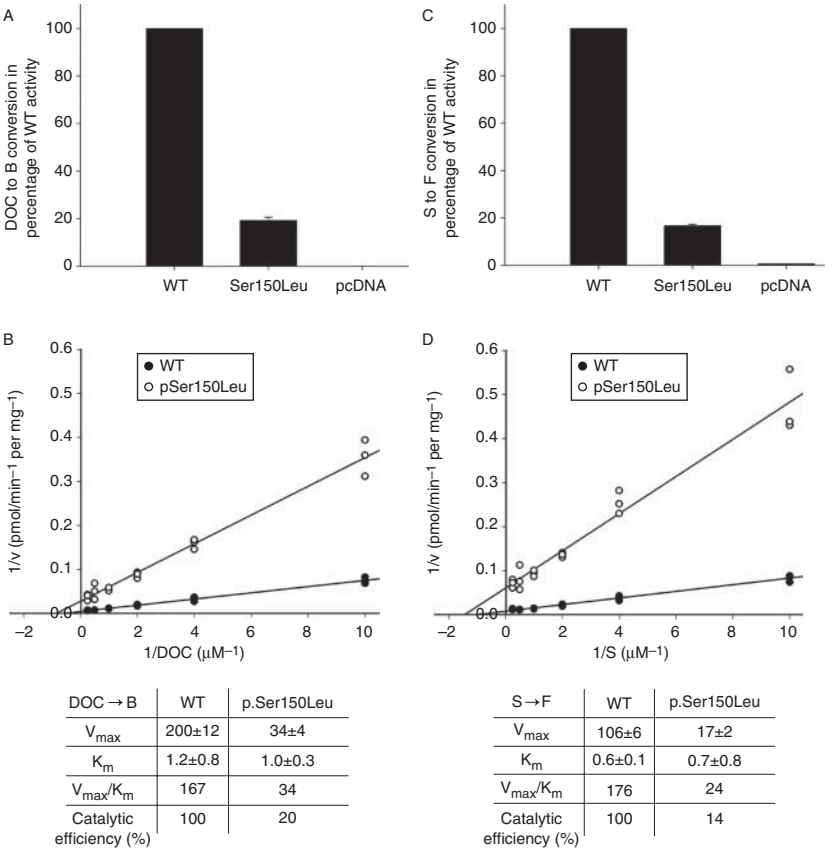
Molecular genetic analysis of the *CYP11B1* gene. (A) Schematic localisation of the mutations. (B) Pedigrees of the patients from three unrelated families with electropherograms of the mutations. Star indicates mutated nucleotides. Question marks indicate individuals not available for genetic analysis.

(p.H122Dfs*13) detected in a patient with a classic 11OHD phenotype described previously by Skinner *et al.* (41).

Patient 3, carrying a complete loss-of-function mutation (p.Leu463_Leu464dup) on the *CYP11B1* gene, came to clinical attention with premature pubarche in childhood. He was short statured due to insufficient treatment during childhood and adolescence. He developed bilateral TART which is the most important cause of infertility in men with CAH to 21-hydroxylase deficiency (42). Ectopically located adrenal tissues

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DOC → B	WT	p.Ser150Leu
V_{max}	200±12	34±4
K_m	1.2±0.8	1.0±0.3
V_{max}/K_m	167	34
Catalytic efficiency (%)	100	20

S → F	WT	p.Ser150Leu
V_{max}	106±6	17±2
K_m	0.6±0.1	0.7±0.8
V_{max}/K_m	176	24
Catalytic efficiency (%)	100	14

Figure 3

Residual 11-hydroxylase activity of the *CYP11B1* variants assessed in transiently transfected HEK293 cells co-expressing human WT or mutant *CYP11B1* with human Adx reductase and Adx respectively. (A) Corticosterone production of the mutant expressed as percentage of WT activity which is defined as 100%. Error bars represent the mean ± S.D. (B) Lineweaver–Burk plot of 11-hydroxylase activity converting DOC to corticosterone. One data point represents the mean of a triplicate detection. (C) Cortisol production of the mutant expressed as percentage of WT activity which is defined as 100%. Error bars represent the mean ± S.D. (D) Lineweaver–Burk plot of 11-hydroxylase activity converting 11-deoxycortisol to cortisol. One data point represents the mean of a triplicate detection.

usually regress with advancing age except in CAH patients in whom low levels of cortisol induce ACTH secretion and hyperplasia of dispersed adrenal tissue (43). The presence of adrenal rests within the testes of adult males with classic 21-hydroxylase CAH is more frequent in the salt-wasting form and is associated with a higher risk for infertility (44). 11OHD patients with TART are rare. Up to our knowledge, only nine cases are presented in the literature so far (43, 45, 46, 47, 48). This is most likely due to the smaller incidence of CAH due to 11-hydroxylase deficiency. All reported cases were poorly compliant to treatment. However, the idea that poor control may lead to a higher risk of TART was just recently refuted in 21-hydroxylase deficiency (49, 50). Therefore, the findings in 11OHD may be a collection bias. In the patient, the TART located in the right testis was removed because of its size and the difficulty to distinguish it from a Leydig cell tumour during follow-up (51). TART cells showed eosinophilic cytoplasm with neither lipochrome pigments nor Reinke crystalloids (Fig. 1) (47, 52). The tumour on the left is under control with prednisolone treatment. Whether surgery has had a positive effect on the testicular function or whether resuming prednisolone treatment led to fertility remains unclear. TART surgery in 21-hydroxylase deficiency has no proven effect on fertility (53).

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Figure 4
Ribbon representation of the three-dimensional molecular model of *CYP11B1*. Helix C, the CD-loop and helix D are coloured light blue, red and light green respectively. The side chain of residue Ser150 located in the C–D loop is depicted.

The variation p.Leu463_Leu464dup detected in patient 3 is located in the amino terminal L-helix. Two leucines are inserted in a leucine-rich part of the L-helix carrying four leucines. The L-helix is involved in haeme binding as well as in interactions with redox partners (30). The distal L-helix forms parts of the protein's surface. As four residues are needed for a full helical turn, the addition of two residues will produce an additional half turn of the helix, leading to a displacement of the proximal part of the helix and disturbance of substrate binding and electron flux, or interference with the binding of redox partners due to changes in the protein surface. Both possibilities will result in a complete loss of function. Interestingly, Geley *et al*. described a patient with classic 11OHD, caused by a duplication of a single leucine at the identical position. This variant also caused a complete loss of 11-hydroxylase activity (7).

Patient 4 showed clinical signs compatible with non-classic CAH (25). The novel mutation p.Ser150Leu detected in this patient had a residual activity of 19.2% of the WT. By this, it is comparable with the previously described mutations associated with non-classic 11OHD. Interestingly, only 12 mutations causing non-classic 11OHD have been described so far (8, 10, 25, 54). This is most likely due to the overall rarity of 11OHD. However, the increasing number of reports on affected patients should increase the awareness for non-classic 11OHD. The residual activity of mutations associated with non-classic 11OHD ranged from 9 to 40% of WT activity, which is well comparable with the residual activity of mutations in the *CYP21A2* gene causing non-classic 21-hydroxylase deficiency (55). The residue p.Ser150 is located in the C–D loop connecting the C-helix with the D-helix of the CYP11B1 protein (Fig. 4). This loop is located at the surface of the molecule. The change to p.Leu150 does not lead to obvious steric problems with neighbouring residues *in silico*. However, the exchange of the polar serine by the hydrophobic leucine leads to a change in the surface properties of the molecule. Since the adjacent D-helix is part of the redox partner interaction side, the mutation may induce a decrease in electron flux which is consistent with the observed reduction of V_{max} (30, 32).

In conclusion, we have shown the inactivating nature of two novel *CYP11B1* mutations in classic 11OHD in addition to one variant with residual activity associated with non-classic 11OHD. The analyses of *in vitro* enzyme function broaden our understanding of *CYP11B1* function relationships and help to counsel families as the severity of the clinical disease expression can be estimated. The condition of non-classic 11OHD is a rare disease but should not be missed in the differential diagnosis of hyperandrogenism.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-13-0737.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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