CASE REPORT

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Identification of a Novel *CYP11B2* Variant in a Family with Varying Degrees of Aldosterone Synthase Deficiency

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What is already known on this topic?

Isolated aldosterone synthase deficiency is a rare autosomal recessive disorder caused by pathogenic variants in *CYP11B2*, resulting in impaired aldosterone synthesis. To date, over forty different pathogenic *CYP11B2* variants have been reported, most of which are missense and nonsense variants that greatly compromise enzymatic activity.

What this study adds?

We report a novel variably pathogenic homozygous variant in the *CYP11B2* gene Chr8:NM_000498.3:c.400G>A p.(Gly134Arg). Interestingly, this homozygous variant led to different clinical phenotypes in two affected relatives. Our study shows that clinicians and geneticists should be alert to the potential pitfalls of *CYP11B2* sequencing due to its homology to *CYP11B1*.

Abstract

Isolated aldosterone synthase deficiency is a rare autosomal recessive disorder caused by pathogenic variants in *CYP11B2,* resulting in impaired aldosterone synthesis. We report on a neonate with isolated aldosterone synthase deficiency caused by a novel homozygous *CYP11B2* variant Chr8:NM_000498.3:c.400G>A p.(Gly134Arg). The patient presented shortly after birth with severe signs of aldosterone deficiency. Interestingly, segregation analysis revealed that the patient's asymptomatic father was also homozygous for the *CYP11B2* variant. Biochemical evaluation of the father indicated subclinical enzyme impairment, characterized by elevated aldosterone precursors. Apparently, this homozygous variant led to different clinical phenotypes in two affected relatives. In this manuscript we elaborate on the biochemical and genetic work-up performed and describe potential pitfalls in *CYP11B2* sequencing due to its homology to *CYP11B1*.

Keywords: Aldosterone synthase, mineralocorticoid, CYB11B2, hypoaldosteronism

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Introduction

Isolated aldosterone synthase deficiency is a rare autosomal recessive disorder caused by pathogenic variants in *CYP11B2* causing impaired aldosterone synthesis. The resulting hyperreninemic hypoaldosteronism leads to a potentially fatal condition, characterized by failure to thrive, dehydration, hyponatremia, hyperkalemia and mild metabolic acidosis (1,2).

We report on a neonate with isolated aldosterone synthase deficiency caused by a novel homozygous *CYP11B2* variant. The proband presented shortly after birth with severe signs of aldosterone deficiency. Segregation analysis revealed that the proband's father harbored the same homozygous *CYP11B2* variant. Although the proband's father appeared asymptomatic, biochemical evaluation showed low aldosterone levels with increased mineralocorticoid precursors suggesting subclinical enzyme impairment. This case complements other reports describing the lack of a clear genotype-phenotype correlation in *CYP11B2* mutations.

Furthermore, this report addresses the encountered pitfalls of *CYP11B2* variant analysis due to its extensive homology with *CYP11B1*, which may delay the genetic diagnosis.

Subjects

A family of Pakistani origin was studied. They were unrelated to previously reported families with pathogenic variants in *CYP11B2*. Written informed consent was obtained from all participants or their legal guardians. All procedures were performed in accordance with the Declaration of Helsinki.

Biochemical Analysis

Steroid hormones were measured in serum using isotope dilution liquid chromatography coupled to tandem mass spectrometry (ID-LC-MS/MS). The steroid profile consisted of cortisol, 17-OH-progesterone (17-OHP), 11-deoxycortisol (Compound S), 11-deoxycorticosterone (11-DOC) and corticosterone (3). Limit of quantitation (LOQ) for these steroids were 1 nmol/L for cortisol and 17-OHP), 0.4 nmol/L for Compound S, 0.1 nmol/L for 11-DOC and 0.5 nmol/L for corticosterone.

Aldosterone was measured in serum using ID-LC-MS/MS (4). The LOQ was 0.03 nmol/L. Plasma renin activity (PRA) was determined in EDTA plasma by measuring the production of angiotensin 1 from angiotensinogen over time using an in-house radio-immunoassay (5). The LOQ was 1 µg angiotensin 1/L/h. Further biochemical analyses of sodium, potassium, chloride, glucose and creatinine were measured on the ISE and C502 module of the Cobas 8000 (Roche). Blood gas parameters pH, pCO_2, bicarbonate and base excess were determined in capillary samples using a RapidPoint 500e (Siemens).

Genetic Analysis

Genomic DNA was extracted from EDTA blood of the patient, his sibling and their parents by standard methods. The coding region and exon–intron boundaries of the *CYP11B2* gene (GenBank: NM_000498.9) were amplified from genomic DNA. Sanger sequencing was performed using the BigDye Terminator Sequencing Kit (Thermo Scientific, Thermo Fisher Scientific, Waltham, USA) and ABI 3730 XL (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). Coding sequences and flanking intron sequences were analysed with SequencePilot (SeqPatient, JSI Medical Systems GmbH, Ettenheim, Germany). PCR and sequencing primers are available on request.

In silico Analysis

A number of pathogenicity prediction methods were used for *in silico* analysis of variant pathogenicity, including Align GVGD (6), CADD (7), PolyPhen-2 HumVar (8), SIFT (9) and MutationTaster (10). The DUET and DynaMut servers were used to evaluate protein stability upon mutation (11,12). The effects of residue substitution on *CYP11B2* structures, interatomic interactions and vibrational entropy energy were inspected in PyMOL using crystal structure model 4dvq of *CYP11B2* in complex with deoxycorticosterone (13).

Case Report

The proband was the second child of consanguineous, healthy parents of Pakistani origin. He was born at term after an uncomplicated pregnancy and was large for gestational age (LGA) with a birth weight of 3880 grams (91st percentile). There were no congenital anomalies. Because of LGA, glucose levels were measured, revealing hypoglycemia in the first hours after birth, which was treated with intravenous glucose. After two days, he was discharged from hospital on a normal newborn feeding formula, without any medication or supplements.

At the age of three weeks, the proband was seen at our outpatient clinic and was subsequently readmitted because of persistent vomiting and failure to thrive. At that time his weight was more than ten percent below birth weight (3415 grams). Physical examination revealed dehydration without apparent signs of infection.

Laboratory evaluation (Table 1A) showed hyponatremia, hyperkalemia and metabolic acidosis. Initially, primary adrenal insufficiency was suspected and after performing

Table 1. Laboratory results of the index patient (A) at presentation (age three weeks) and of the patient's asymptomatic father (B)

A. Results of the index patient		
Measurement		**Reference interval***
Sodium	124	133-142 mmol/L
Potassium	7.6	4.0-6.0 mmol/L
Chloride	89	98-113 mmol/L
pH (capillary)	7.3	7.32-7.43
pCO_2	5.6	3.5-5.5 kPa
Bicarbonate	20.1	23-29 mmol/L
Base excess	-6.1	-2-10 mmol/L
Glucose	4.4	2.8-4.5 mmol/L
Creatinine	42	35-80 µmol/L
Aldosterone	0.04**	< 0.54 nmol/L
Plasma renin activity	66	< 7.5 µg angiotensin 1/L/h
Cortisol	186	250-650 nmol/L
B. Results of the patient's father		
Measurement		Reference interval
Sodium	140	–135-145 mmol/L
Potassium	4.2	3.5-4.5 mmol/L
Aldosterone	< 0.03***	< 0.54 nmol/L
Plasma renin activity	6.1	< 7.5 µg angiotensin 1/L/U
Cortisol	284	250-650 nmol/L
17-OH progesterone	1.3	< 7.6 nmol/L
11-deoxycortisol	1.4	< 3.2 nmol/L
11-deoxycorticosterone	3.8	< 0.82 nmol/L
Corticosterone	27.7	1.8-56 nmol/L

*All reference intervals are age-specific reference intervals, except for aldosterone and plasma renin activity for which adult reference ranges are depicted. **Aldosterone LOQ: 0.03. ***The aldosterone concentration was below LOQ.
LOQ: limit of quantitation

a cosyntropin stimulation test, the patient was started on hydrocortisone 50 mg/m^2/day in four daily doses and intravenous isotonic (0.9%) saline. Basal cortisol and aldosterone levels were low-normal and very low (just above LOQ), respectively. PRA was increased and renal abnormalities were excluded by a renal ultrasound. The cosyntropin stimulation test (36 microgram/kg bodyweight) showed an appropriate rise of post-stimulation cortisol levels, ruling out glucocorticoid deficiency (Table 2). 17-OHP and 11-deoxycortisol levels were slightly increased at baseline, but only a marginal rise was seen following cosyntropin stimulation (Table 2). After ruling out glucocorticoid deficiency, isolated mineralocorticoid deficiency was suspected. Hydrocortisone was stopped after two days and treatment with fludrocortisone 62.5 µg twice daily was started. Precursors of the aldosterone synthesis pathway were measured in pre-treatment samples. Clearly elevated levels of 11-DOC and corticosterone (Table 2) were found, confirming the biochemical diagnosis of aldosterone synthase deficiency.

After initiating therapy, rapid clinical and biochemical recovery was noted. Intravenous saline treatment was changed to an oral hypertonic saline solution at the age of five weeks. Because of persistent subnormal sodium levels, the fludrocortisone dose was increased to 100 µg twice daily. With this treatment regimen, the patient was growing and developing well with normal sodium, potassium and PRA. At the age of one year, the patient was challenged by reducing the fludrocortisone dose to 62.5 µg twice daily. This resulted in recurrence of salt-loss necessitating reinstitution of the original fludrocortisone dose, confirming the persistence of clinically relevant hypoaldosteronism.

A heterozygous variant of unknown significance was detected in exon 3 of the *CYP11B2* gene after sequencing the coding region and the splice sites (NM_000498.3): Chr8:NM_000498.3:c.400G > A p.(Gly134Arg). As heterozygous variants are usually asymptomatic/subclinical, the heterozygous variant seemed an insufficient explanation for the observed phenotype of our patient.

To find an alternative explanation for the observed phenotype and because of the parental consanguinity, our first step was to search for interesting regions of homozygosity (ROHs). We performed genome wide array

Table 2. Results of a cosyntropin stimulation test in a three-week-old boy suspected of glucocorticoid and/or mineralocorticoid deficiency

Measurement	**T = 0 (unstimulated)**	**T = 30 min**	**T = 60 min**	**Reference interval (unstimulated)**
Cortisol	602	761	873	250-650 nmol/L
17-OH progesterone	5.6	9.7	11.3	< 3.2 nmol/L
11-deoxycortisol (Compound S)	10.9	12.2	13.8	< 3.2 nmol/L
11-deoxycorticosterone (11-DOC)	10.2	14.1	17.4	< 0.82 nmol/L
Corticosterone	353	547	639	1.8-56 nmol/L

All steroids were measured with LC-MS/MS. Reference intervals are intervals for the unstimulated state in adults, except for 17-OHP (age adjusted).
17-OHP: 17-OH progesterone

analysis using the CytoScan XON array platforms (Thermo Fisher Scientific, Inc., Waltham, MA, USA) on DNA from the index patient following the manufacturer's instructions. We did not identify a copy number loss or gain, but detected multiple, relatively large ROHs in the exome data (196 Mb of the autosomal genome (~6.5%), which is in agreement with the indicated parental consanguinity. The *CYP11B2* gene lies within a ROH of about 7 Mb. Due to the discrepancy between the results, another set of primers for exon 3 of the *CYP11B2* gene was designed, which led to the discovery that the c.400G>A variant was in fact present in a homozygous state. This is consistent with the genetic diagnosis of isolated aldosterone synthase deficiency due to a homozygous missense variant in *CYP11B2* (c.400G>A). The initially used primers had also recognized and amplified the highly homologous region of the *CYB11B1* gene, leading to false heterozygous detection of the variant.

The detected variant has not been reported in the literature before and is present in a very low allele frequency (0.0012%) in the genome Aggregation Database (gnomAD, v.2.1.1) (http://gnomad.broadinstitute.org). Segregation analysis was performed in the asymptomatic parents and sibling. The proband's clinically unaffected mother and sister appeared to be heterozygous carriers of the novel *CYB11B2* variant but the patient's father harbored the variant in a homozygous state. This was an unexpected finding, as the father appeared clinically asymptomatic whilst on a regular Western diet, and had not experienced any symptoms during infancy or childhood. This result also argued against the pathogenicity of the detected *CYP11B2* variant, and for that reason an endocrine work-up of the father was performed. On physical examination, the father had normal height and his vital signs were unremarkable. Biochemical evaluation showed normal electrolytes and PRA, but low aldosterone and clearly increased 11-DOC (an aldosterone precursor) concentrations (Table 1B), suggesting a mild (subclinical) aldosterone synthase deficiency.

To investigate the variant's pathogenicity, *in silico* analyses were performed. Gly at position 134 is highly conserved across species (Figure 1) and appears to be an exposed residue in the Cytochrome P450 domain of *CYP11B2*. The variant is predicted to be deleterious by several pathogenicity prediction methods (Table 3). Results of protein structural analysis indicate that the surface of the mutated *CYP11B2* is changed (Figure 2). These changes may negatively influence protein-substrate dynamics. Protein stability analysis suggests decreased protein stability, which may lead to a misfolded protein (Table 3), perturbing substrate access to the active site or substrate binding and could also lead to increased degradation. These findings suggest that Gly at position 134 is a functional residue.

Discussion

Aldosterone is the principal mineralocorticoid in humans. Its main actions are to regulate intravascular volume

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P19099_C11B2_HUMAN      CRMILEPWVAYRQHRGHKCGVFLLNGPEWRFNRLRLNPDVLS 150
MUTANT_HUMAN            CRMILEPWVAYRQHRGHKCGVFLLNRPEWRFNRLRLNPDVLS 150
P15538_C11B1_HUMAN      HRMSLEPWVAYRQHRGHKCGVFLLNGPEWRFNRLRLNPEVLS 150
P15539_C11B2_MOUSE      RRMHLEPWVAHRELRGLRRGVFLLNGPEWRLNRLRLNRNVLS 150
P30099_C11B2_RAT        RRMHLEPWVAHRELRGLRRGVFLLNGAEWRFNRLKLNPNVLS 160
Q0P493_Q0P493_DANRE     RRMTLQPWATHRETRRHSKGVFLKNGTEWRADRLLLNREVMV 160
W0FJN1_W0FJN1_PIG       QRMFLEPWLAYRQLRGHKCGVFLLNGPTWRLDRLQLNPGVLS 150
F1PNC7_F1PNC7_CANLF     WRPPLDPWLAYRQHRGHKCGVFLLNGPEWRLNRLKLNPDVLS 150
                         *  *:** ::*: *    **** **  ** :** **  *:
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Figure 1. Sequence alignment. The Gly at position 134 of the *CYP11B2* gene is highly conserved across species suggesting structural and/or functional importance

Table 3. Pathogenicity score of the p.(Gly134Arg) variant in several pathogenicity prediction programs

Prediction method	Score
Align GVGD	C65 (very likely to interfere with function)
CADD	24.3 (top 1% deleterious variants)
MutationTaster	1 (disease causing)
PolyPhen-2 (HumVar)	1.000 (probably damaging)
SIFT	0 (deleterious)
DUET	ΔΔG: -1.26 kcal/mol (destabilizing)
DynaMut	ΔΔG: -0.59 kcal/mol (destabilizing)

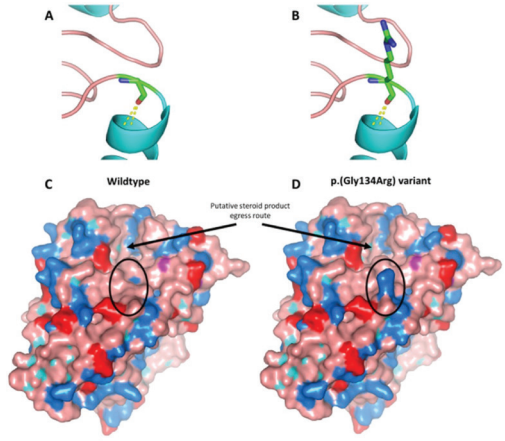

Figure 2. *In silico* protein modelling of *CYP11B2* p.(Gly134Arg)

Crystal structure model 4DVQ of human aldosterone synthase (*CYP11B2*) in complex with deoxycorticosterone (13) was used to study interatomic interactions upon mutation, employing PyMOL. Positively charged amino acids are blue, negatively charged amino acids are red. p.(Gly134Arg) substitution (panels A and B, the affected amino acid is given in green) did not result in steric clashes or changes to hydrogen bridges (yellow dashed lines). However, dihedral angles of p.(Gly134Arg) are considerably different to wild-type; the arginine sidechain (black circle, panel D) is pointed outwards, so that the otherwise smooth patch of protein surface has a positively charged appendicle (panels C and D). This may influence protein-substrate dynamics, since it is in close proximity to a putative steroid product egress route (13)

and electrolyte balance. Aldosterone is synthesized from cholesterol in the zona glomerulosa of the adrenal cortex. Aldosterone synthase is a mitochondrial cytochrome P450 enzyme, encoded by the *CYP11B2* gene located on chromosome 8q24.3 and containing nine exons (14). This enzyme is involved in the last three steps of the aldosterone biosynthesis pathway facilitating the 11-hydroxylation of deoxycorticosterone to corticosterone, the subsequent 18-hydroxylation to hydroxycorticosterone, and finally 18-oxidation to aldosterone (15). 18-hydroxycorticosterone can be measured to distinguish between 18-hydroxylase deficiency (type 1) and 18-oxidase deficiency (type 2). We chose not to measure 18-hydroxycorticosterone as this distinction would not have clinical implications.

Aldosterone synthase deficiency biochemically leads to hyperreninemic hypoaldosteronism, characterized by hyponatremia, hyperkalemia, metabolic acidosis, increased renin and low or low-normal aldosterone levels. As demonstrated in the present case, increased levels of mineralocorticoid precursors before the enzymatic block may be found. Clinically, the disease manifests in the neonatal period or early infancy with vomiting, dehydration, and failure to thrive (16,17,18). These symptoms are nonspecific and can easily be mistaken for more common conditions.

To date, more than forty different pathogenic variants in the *CYP11B2* gene causing isolated aldosterone synthase deficiency have been reported, most of which are missense and nonsense variants that greatly compromise enzymatic activity (17,18). In a recent study, the missense variant c.554C > T p.(Thr185Ile) was found, either in a homozygous or compound heterozygous state, in the vast majority of the studied patients (17). To the best of our knowledge, the missense variant c.400G > A p.(Gly134Arg) detected in the proband and his family members has not been described before.

Based on the location of the variant, in close proximity to two earlier described pathogenic variants (17), the known deleterious effect of the variant in the highly homologous *CYP11B1* gene (19), the low frequency in the gnomAD database, the results of prediction programs, the results of protein modeling and the appropriate clinical and biochemical findings in the proband, strongly suggest this variant to be pathogenic, in our opinion.

The proband's father with the same homozygous *CYP11B2* variant appeared asymptomatic, but biochemical analysis revealed low aldosterone levels and an elevated 11-DOC concentration, suggesting impaired enzyme activity. Apparently, this variant is not fully penetrant. Reduced penetrance has previously been reported in members of an extended Palestinian family, harboring a different variant in *CYB11B2* [c.1354G > T; p.(G452W)] (20). In this family, two clinically unaffected mothers were found to be homozygous for the same variant as their affected offspring. Why the severity of enzyme impairment can differ

between people with the same genotype is unclear. Faingelernt et al. (20) speculated that undetermined genetic or epigenetic factors, for example affecting sensitivity to mineralocorticoid action or impairing 11β-hydroxysteroid dehydrogenase type 2 activity, could play a role.

Lastly, we would like to alert clinicians to the pitfalls we encountered trying to obtain a genetic diagnosis. The two 11β-hydroxylase iso-enzymes, 11β-hydroxylase enzyme (P450c11β) and aldosterone synthase enzyme (P450c11AS), are encoded by the 93%-identical *CYP11B1* and *CYP11B2* genes, respectively (21). Both genes are located in band q24.3 of chromosome 8 and are approximately 40 kb apart. Their sequence homology complicates genetic analysis as illustrated by the presented case and highlights the importance of using 100% specific primers for each gene.

There have been several reports of patients with hyperreninemic hypoaldosteronism in whom a genetic diagnosis could not be made (21,22). The present case raises the question whether similar false negative results, caused by the close homology between the two *CYP11B* genes, could have played a role in the inability to obtain a genetic diagnosis in these patients.

Conclusion

Herein, we report the case of a neonate with hyperreninemic hypoaldosteronism due to isolated aldosterone synthase deficiency, caused by a novel, homozygous *CYP11B2* variant. The patient's father was homozygous for the same variant and appeared asymptomatic. However, biochemical evaluation revealed suboptimal aldosterone synthase function, suggesting a phenotypical spectrum based on enzymatic residual activity that can vary within a family. The explanation for this observed lack of a clear genotype-phenotype correlation remains elusive. This case shows that establishing a genetic diagnosis of isolated aldosterone synthase deficiency may be complicated by the homology between *CYP11B2* and *CYP11B1*.

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Ethics

Informed Consent: Written informed consents were obtained from all participants and their legal guardians. All procedures were performed in accordance with the Declaration of Helsinki.

Authorship Contributions

Surgical and Medical Practices: Mark R. Garrelfs, Merijn W. Bijlsma, Martijn J.J. Finken, Joost Rotteveel, Nitash Zwaveling-Soonawala, Concept: Mark R. Garrelfs, Peter Lauffer, Max Nieuwdorp, A.S. Paul van Trotsenburg, Design: Mark R. Garrelfs, Peter Lauffer, Data Collection or Processing: Mark R. Garrelfs, Tuula Rinne, Jacquelien J. Hillebrand, Peter Lauffer, Merijn W. Bijlsma, Nicole de Leeuw, Martijn J.J. Finken, Joost Rotteveel, Nitash Zwaveling-Soonawala, Max Nieuwdorp, A.S. Paul van Trotsenburg, A.S. Paul van Trotsenburg, Christiaan F. Mooij, Analysis or Interpretation: Mark R. Garrelfs, Tuula Rinne, Jacquelien J. Hillebrand, Peter Lauffer, Hedi L. Claahsen-van der Grinten, Nicole de Leeuw, Martijn J.J. Finken, Joost Rotteveel, Nitash Zwaveling-Soonawala, Max Nieuwdorp, A.S. Paul van Trotsenburg, A.S. Paul van Trotsenburg, Christiaan F. Mooij, Literature Search: Mark R. Garrelfs, Tuula Rinne, Jacquelien J. Hillebrand, Peter Lauffer, Nicole de Leeuw, Writing: Mark R. Garrelfs, Tuula Rinne, Jacquelien J. Hillebrand, Peter Lauffer, Merijn W. Bijlsma, Hedi L. Claahsen-van der Grinten, Nicole de Leeuw, Martijn J.J. Finken, Joost Rotteveel, Joost Rotteveel, Nitash Zwaveling-Soonawala, Max Nieuwdorp, A.S. Paul van Trotsenburg, A.S. Paul van Trotsenburg, Christiaan F. Mooij.

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