

CASE REPORT

Molecular genetic study in two patients with congenital hypoaldosteronism (types I and II) in relation to previously published hormonal studies

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Abstract

We performed a molecular genetic study in two patients with congenital hypoaldosteronism. An original study of these patients was published in this Journal in 1982. Both index cases, a girl (patient 1) and a boy (patient 2), presented with salt-wasting and failure to thrive in the neonatal period. Parents of patient 1 were not related, whereas the parents of patient 2 were cousins. Endocrine studies had shown a defect in 18-oxidation of 18-OH-corticosterone in patient 1 and a defect in the 18-hydroxylation of corticosterone in patient 2. Plasma aldosterone was decreased in both patients, whereas 18-OH-corticosterone was elevated in patient 1 and decreased in patient 2. Plasma corticosterone and 11-deoxycorticosterone were elevated in both patients, whereas cortisol and its precursors were in the normal range. According to the nomenclature proposed by Ulick, the defects are termed corticosterone methyl oxidase (CMO) deficiency type II in patient 1, and type I in patient 2 respectively. Genetic defects in the gene CYP11B2 encoding aldosterone synthase have been described in a few cases. In patient 1, we identified only one heterozygous amino acid substitution (V386A) in exon 7, which has no deleterious effect on the enzyme activity. In patient 2 and his older brother, we identified a homozygous single base exchange (G to T) in codon 255 (GAG), causing a premature stop codon E255X (TAG). The mutant enzyme has lost the five terminal exons containing the haem binding site, and is thus a loss of function enzyme. This is only the second report of a patient with CMO deficiency type II without a mutation in the exons and exon-intron boundaries, whereas the biochemical phenotype of the two brothers with CMO deficiency type I can be explained by the patient's genotype.

European Journal of Endocrinology 139 96–100

Introduction

Aldosterone is the principal mineralocorticoid in humans. It is synthesised from 11-deoxycorticosterone in the mitochondria of zona glomerulosa cells of the adrenal cortex. The pathway of the terminal aldosterone biosynthesis involves three sequential reactions: hydroxylation of 11-deoxycorticosterone at position 11 β to form corticosterone, hydroxylation of corticosterone at position 18 to form 18-OH-corticosterone, and oxidation at position 18 to convert 18-OH-corticosterone to aldosterone. The terminal steps of aldosterone biosynthesis in the zona glomerulosa are catalysed by a single cytochrome P450 enzyme termed aldosterone synthase (P450c11Aldo) (1). The gene encoding for P450c11Aldo has been cloned and is termed CYP11B2 (2).

Congenital hypoaldosteronism caused by aldosterone synthase deficiency is an autosomal recessively inherited

disorder. Visser & Cost (3) were the first to describe a patient with deficient 18-hydroxylation of corticosterone (4). Subsequently, Ulick *et al.* (5) and Rappaport *et al.* (6) described two patients with deficient 18-OH-dehydrogenase. The two biochemically different forms of selective aldosterone deficiency are termed corticosterone methyl oxidase (CMO) deficiency type I and type II (7). In both CMO deficiency types, aldosterone biosynthesis is impaired, while corticosterone of zona glomerulosa origin is excessively produced. The two defects differ biochemically in that 18-OH-corticosterone is deficient in CMO deficiency type I, but overproduced in CMO deficiency type II. Both disorders are characterised clinically by salt-wasting, failure to thrive and growth retardation. Molecular genetic analysis of the genomic DNA from CMO deficiency type I and II patients led to the identification of mutations in the CYP11B2 gene (8–13). However, one study showed no CYP11B2 gene

mutations in a patient with CMO deficiency type II (14). In the present study, we have performed a molecular genetic analysis of the CYP11B2 gene in two patients with congenital hypoadosteronism. Endocrine data on the patients were originally published in this Journal (15).

Subjects and methods

Patients

Two infants with salt-wasting in the neonatal period have been reported by Drop *et al.* (15). We had the opportunity to perform a molecular genetic study in both patients and in the parents and an older brother of one of them.

Patient 1. This Caucasian girl (born 10 September 1964) was first admitted to hospital at age 1 month because of dehydration, salt-wasting and failure to thrive. On admission, serum sodium was 126 mmol/l and serum potassium 8.2 mmol/l. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency was excluded. Salt-wasting was reversed after administration of deoxycorticosterone acetate and salt supplementation. The parents were not related.

Patient 2. This Turkish boy (born 23 July 1977) was first hospitalised at age 2 months because of dehydration, salt-wasting and failure to thrive. On admission serum sodium measured 128 mmol/l and potassium 6.2 mmol/l. Creatinine clearance and urine sediment analysis were normal. Plasma cortisol levels and 24 h 17-keto- and 17-hydroxycorticosteroid excretion in the urine were normal. Oral fludrocortisone medication was instituted because of low aldosterone levels accompanied by highly elevated plasma renin activity (PRA). The patient's clinical condition quickly improved, but whenever mineralocorticoid treatment was discontinued hyponatraemia and hyperkalaemia developed within 1–2 weeks. His father and mother, who are cousins, and three older brothers were in good health.

Methods

Treatment was suspended in both patients, at age 14½ years (patient 1) and 2½ years (patient 2), and they were placed on a salt-restricted diet (8.5 mmol sodium/day). Plasma steroids were measured using a method for the simultaneous determination of multiple adrenal steroids in a small plasma volume (1–2 ml), which was developed in our laboratory (16). The results are expressed in nmol/l; to convert to ng/ml divide by the following factors: aldosterone, 2.774; 18-OH-corticosterone, 2.759; corticosterone, 2.886; 18-OH-11-deoxycorticosterone, 2.886; 11-deoxycorticosterone, 3.026.

Nucleotide sequences of exons and exon–intron boundaries.

Genomic DNA was extracted from peripheral blood leucocytes, and the CYP11B2 gene was specifically amplified in two fragments containing the nine exons by PCR. Regions of the CYP11B2 gene with extensive mismatches with CYP11B1 were used for synthesis of primers (exon 1–4, 3510 bp, sense primer 5'-TCC TTC ATC TAC CTT TGG CTG GGG-3' and antisense primer 5'-CCG AGA CTG CCC CGA CAC CCA AAT-3'; exon 5–9, 2714 bp, sense primer 5'-ATT TGG GTG TCG GGG CAG TCT-3' and antisense primer 5'-TTG CTA TTT GAC AAG CCT GGC AAG-3'). PCR products were treated prior to sequencing, using exonuclease I and shrimp alkaline phosphatase. The nucleotide sequence of both strands of the PCR products was directly determined by thermocycle sequencing, using the Thermo Sequenase radiolabelled terminator cycle sequencing kit following the manufacturer's instructions (Amersham Life Science, Cleveland, OH, USA). The nucleotide sequences of the primers used for the sequencing reactions are shown in Table 1.

Results

Steroid determinations

Results of the plasma steroid determinations have already been published by Drop *et al.* (15). Plasma levels of aldosterone and its precursors are given in Table 2.

Table 1 Oligonucleotides used for sequencing of the CYP11B2 gene (the numbers in parentheses refer to the DNA sequence of CYP11B2 published in GenBank, accession number D13752).

Exon	Sense primer	Antisense primer
1	(544) 5'-GTTTCCAGAGCAGGTTTCTGGGTG-3' (567)	(933) 5'-TGGCAGTGCTGAGTGCC-3' (917)
2	(1116) 5'-CTGTGAAGCCGCTAAT-3' (1131)	(1441) 5'-CAGGCTGCCACCCTGC-3' (1425)
3	(3160) 5'-TTGCTGGGGCGGCCTCA-3' (3176)	(3445) 5'-CCACTCCAGGGTCTCTG-3' (3429)
4	(3507) 5'-CCTTGTGCTCAGCAGTG-3' (3523)	(3777) 5'-CCTTCCCACATAGCACTG-3' (3761)
5	(3965) 5'-GAGGACTGAAGGATG-3' (3981)	(4226) 5'-GCTTGGCATCACCCTCT-3' (4210)
6	(4952) 5'-TCCTCCTGTGCAAGGTC-3' (4968)	(5195) 5'-CCAGGGCCACAGGGAGG-3' (5179)
7	(5496) 5'-GGTGCTGAGAGCACAGG-3' (5512)	(5698) 5'-GGATCAGGGAATGACTG-3' (5682)
8	(5682) 5'-CAGTCATCCCTGATCC-3' (5698)	(6000) 5'-AATCACACCATGCAAGC-3' (5984)
9	(6264) 5'-TAATTGTTGCACCTGGG-3' (6280)	(6521) 5'-GGTCAGGCAGAGGGAAGCTGGTGG-3' (6498)

Table 2 Basal plasma levels of aldosterone and its precursors in two children with congenital hypoaldosteronism due to aldosterone synthase deficiency.

Steroid	Patient 1 (CMO II)	Patient 2 (CMO I)	Normal range
Aldosterone (nmol/l)	0.033	0.11	0.39–2.49
18-OH-Corticosterone (nmol/l)	22.9	0.17	0.55–1.38
Corticosterone (nmol/l)	34.3	21.4	0.14–14.4
18-OH-11-Deoxycorticosterone (nmol/l)	2.89	2.31	0.12–0.72
11-Deoxycorticosterone (nmol/l)	0.69	3.84	0.09–0.61
Corticosterone/18-OH corticosterone ratio	1.5	129	–
18-OH corticosterone/aldosterone ratio	688	1.5	–

Patient 1. High plasma renin activity, low plasma aldosterone levels, and elevated 18-OH-corticosterone values were fully compatible with CMO deficiency type II in this patient. The corticosterone/18-OH-corticosterone ratio was low (1.5) and the 18-OH-corticosterone/aldosterone ratio was high (688).

Patient 2. Patient 2 and his older brother, both studied in an untreated state, showed the typical steroid pattern of a defect in 18-hydroxylation (CMO deficiency type I). Aldosterone and 18-OH-corticosterone were decreased, whereas corticosterone and 11-deoxycorticosterone were increased. The corticosterone/18-OH-corticosterone ratio was high (129) and the 18-OH-corticosterone/aldosterone ratio was low (1.5).

Nucleotide sequences of exons and exon–intron boundaries

Patient 1. Direct sequencing of the patient's DNA showed only one heterozygous amino acid substitution V386A (GTG to GCG) in exon 7 of the CYP11B2 gene. There were no other mutations within the exons and the exon–intron boundaries of CYP11B2. Codon 181 of the CYP11B2 gene of this patient encoded arginine, which is the wildtype sequence of CYP11B2.

Patient 2. Direct sequencing of the patient's and his brother's DNA showed that both were homozygous for a single base exchange in their CYP11B2 genes. We identified a homozygous G to T transversion in codon 255 (Fig. 1). Codon 255 encodes glutamic acid (GAG) in the wildtype enzyme. The mutation causes a premature stop codon E255X (TAG). Direct sequencing of exon 4 showed that both parents were heterozygous carriers of the E255X mutation.

Discussion

Fifteen years after the report by Drop *et al.* (15) we had the opportunity to perform a molecular genetic study in the two patients with a biosynthetic defect of aldosterone production causing isolated hypoaldosteronism.

Both patients had low aldosterone plasma levels in the presence of elevated precursor steroid levels, suggesting a biosynthetic defect of aldosterone biosynthesis. Patient 1 had markedly elevated plasma levels of 18-hydroxycorticosterone which led to the diagnosis of CMO deficiency type II. Both patients had elevated levels of corticosterone, 11-deoxycorticosterone and 18-OH-11-deoxycorticosterone.

In patient 1 where the biochemical diagnosis has been checked repeatedly, we identified only one heterozygous amino acid substitution, V386A. Codon 181 of the CYP11B2 gene of this patient encoded arginine, which is the wildtype sequence of CYP11B2. Alanine is normally present in CYP11B1 at codon 386. It has been shown that V386A itself has a minimal effect on the enzymatic activity of CYP11B2 (7). In the CMO deficiency type II patients of Iranian–Jewish origin, all affected individuals were homozygous for two point

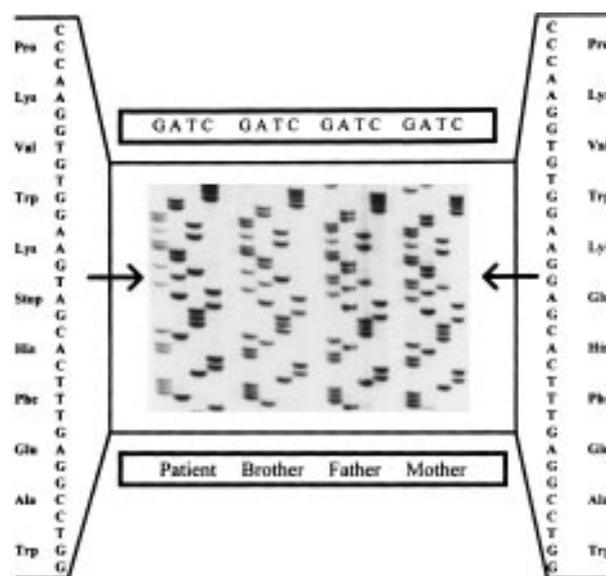


Figure 1 Direct sequencing of exon 4 of CYP11B2. The index case (patient 2) and one older brother were homozygous for the E255X mutation in exon 4, whereas the parents had the E255X mutation on one allele and the wildtype sequence on the other.

mutations, R181W and V386A. Individuals homozygous for the V386A mutation only were asymptomatic. Amino acid residues have been identified in a recent study using transfection experiments with cDNAs which encode hybrids between the highly homologous cytochrome P450 enzymes, CYP11B1 (11 β -hydroxylase) and CYP11B2 (aldosterone synthase), determining the different catalytic activities of both enzymes. Efficient 18-hydroxylation requires a glycine residue at position 288, and the subsequent 18-oxidation requires an alanine at position 320 (17). Thus, it can be stated that the heterozygous V386A mutation is not the cause of the patient's phenotype.

This is the second report on a patient with CMO deficiency type II without a mutation in the coding region of the CYP11B2 gene. In the first report, the conversion of exons 3 and 4 from CYP11B1 to CYP11B2 did not cause the syndrome of CMO deficiency type II (14). The disease could be due to other undetected mutations in the CYP11B2 gene such as a mutation in the promoter or in an intron which disrupts splicing. We sequenced up to 40 nucleotides of the intronic sequence adjacent to each exon and detected no intron mutation. The hypothesis of a mutation in the promoter causing selective reduction of 18-oxidase activity of P450c11Aldo is unlikely because 18-hydroxylase as well as 18-oxidase activity would be expected to be reduced to the same extent in such a case. Alternatively, the disorder could be due to mutation of another gene. However, it has been shown that the human genome contains only two CYP11B genes (18). Thus, we conclude that the endocrine abnormalities of patient 1 are not likely to be caused by genetic variants of the CYP11B2 gene.

The plasma steroid pattern of patient 2 and his brother had originally shown a defect in 18-hydroxylation of corticosterone (15), which is termed CMO deficiency type I according to the nomenclature proposed by Ulick (7). Within the small number of patients with congenital hypoadosteronism due to aldosterone synthase deficiency, patients with CMO deficiency type I appear to have the more severely decreased enzyme activity of P450c11Aldo compared with those with CMO deficiency type II (8–13). The stop mutation in exon 4 which was identified in the two brothers might explain the biochemical phenotype, because the predicted enzyme has lost the five terminal exons encoding for several α -helices and β -strands which contain important residues for proton transfer, accessory protein binding, haem binding and substrate binding (19). Thus, it may be assumed that the P450c11Aldo expressed in the adrenals of the two brothers is a loss of function enzyme. We identified (20) the same homozygous stop mutation (E255X) in the patients reported in the original publication by Visser & Cost (3). Both families have a different genetic background. The family reported by Visser & Cost (3) has lived for generations in the Dutch area of Friesland, whereas the

parents of patient 2 came from Turkey. We did not find the E255X mutation in any of 60 other CYP11B2 alleles so far sequenced in our laboratory.

Why were corticosterone plasma levels elevated in patient 2? The predicted aldosterone synthase would be expected to have lost 11 β -hydroxylase activity also. Steroid 11 β -hydroxylase (P450c11) encoded by CYP11B1 remains intact in patients with aldosterone synthase deficiency. Because this enzyme is up-regulated by angiotensin II in cultured zona glomerulosa cells (21), it is likely that the increased PRA leads to increased transcription of CYP11B1 and a corresponding increase in 11 β -hydroxylase activity. The increased secretion of corticosterone, relative to cortisol, suggests that CYP11B1 is indeed expressed at high levels in the zona glomerulosa and that expression of this gene in the zona fasciculata is not markedly increased in patients with aldosterone synthase deficiency. If the elevated plasma levels of corticosterone were of zona fasciculata origin, cortisol and corticosterone would be expected to be increased to a similar extent. Elevated plasma levels of 18-OH-11-deoxycorticosterone should be explained accordingly, since 11 β -hydroxylase can convert 11-deoxycorticosterone to 18-OH-11-deoxycorticosterone (22).

Rösler (23) has shown that in each affected individual with aldosterone synthase deficiency the clinical severity of the disease decreases with age. Adolescents and adults may only show the abnormal steroid pattern which persists throughout life. Continued mineralocorticoid replacement therapy after childhood is not always necessary, as on clinical observation compensatory extra-adrenal salt-conserving mechanisms mature with age. The same natural course of the disease is observed in many patients with pseudohypoadosteronism (23).

Acknowledgements

The authors thank Mrs Jutta Biskupek-Siegiwart and Mrs Susanne Neumann-Olin for their technical assistance in the multiteroid analyses and Mrs Gisela Hohmann for her expert technical assistance in molecular biology techniques. We are grateful to Mrs Joanna Voerste for linguistic editing of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG Pe 589/1–1 and 1–2).

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Received 9 October 1997

Accepted 30 March 1998