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Congenital Hyperreninemic Hypoaldosteronism in Israel: Sequence Analysis of CYP11B2 Gene

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Key Words
Aldosterone synthase · Hyperreninemic hypoaldosteronism · CYP11B2 gene

Abstract
Background/Aims: Isolated aldosterone biosynthesis defect causing congenital hyperreninemic hypoaldosteronism with otherwise normal adrenal function usually results from aldosterone synthase deficiency. Patients present with manifestations of mineralocorticoid deficiency during the first weeks of life. The largest numbers of cases have been described in Iranian Jews, who carried concomitantly two homozygous missense mutations (R181W and V386A). In a few cases with presumed aldosterone synthase deficiency no mutations in CYP11B2 gene have been identified. We describe a molecular and endocrine evaluation of seven cases of congenital hyperreninemic hypoaldosteronism in Israel. ***Patients/Methods:*** Two of the six Jewish patients are of Iranian origin. The parents of five other patients originated from Yemen, Syria and Morocco. One patient is a Muslim-Arab. CYP11B2's exons, exon–intron boundaries and promoter region were sequenced by multiple PCR amplifications. Gene size determination was performed either by long-range PCR or by Southern blot analysis. ***Results:*** Only two patients (Iranian Jews) carried a known homozygous R181W, V386A mutations, other two were compound heterozygotes for either the R181W or V386A and one additional novel amino acid substitution (A319V or D335G), and one patient was found to be a carrier of the two novel variations (A319V and D335G). We could not find a molecular defect in 2 patients: one was a carrier of the D335G mutation and the other had no detectable molecular change in the coding and promoter regions. ***Conclusion:*** The genetic and molecular basis of congenital hyperreninemic hypoaldosteronism is more heterogeneous than previously described. The significance of amino acid substitutions identified in this study remains to be determined.

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Introduction

Aldosterone regulates intravascular volume, blood pressure and serum electrolytes by controlling sodium absorption and potassium excretion in the distal nephron. Hereditary aldosterone biosynthesis defects may be caused by congenital adrenal hypoplasia, congenital adrenal hyperplasia due to 21-hydroxylase or 11-hydroxylase deficiencies resulting from mutations in CYP21 and CYP11B1 genes, respectively, that also affect cortisol and sex steroid biosynthesis or by congenital hyperreninemic hypoaldosteronism (CHH), and an isolated aldosterone biosynthesis defect. This defect usually results from deficiency of CYP11B2 gene product aldosterone synthase that converts 11-deoxycorticosterone to aldosterone in a three step reaction [1].

Patients present with signs and symptoms of mineralocorticoid deficiency during the first weeks of life with failure to thrive, hyponatremia, hyperkalemia, markedly elevated Plasma Renin Activity and low or inappropriately normal aldosterone levels [2].

In most of the subjects with CHH, CYP11B2 gene mutations were found to be responsible for the disease. The largest number of cases has been described in Iranian Jews originating from Isfahan [3–5], who carry two homozygous missense mutations (R181W in exon 3 and V386A in exon 7) [6]. The disease has also been documented in several European countries, North America, Australia and Japan [7–10]. In a small number of cases with presumed aldosterone synthase deficiency, no mutations in CYP11B2 gene have been identified [7, 11, 12]. In this collaborative study, we describe a molecular and endocrine evaluation of seven cases of CHH in Israel.

Patients and Methods

Patients

Study population included seven patients (five females and two males) who presented during early infancy with signs and symptoms of salt losing (table 1). All were born appropriate for gestational age to healthy non-related families from different ethnic backgrounds and no consanguineous parents. In five of the Jewish patients at least one of the parents was of Iranian origin. The origins of the other parents are: Yemen, Syria and Morocco. The seventh patient is a Muslim-Arab. The family history for documented salt wasting disease was negative in all the patients except the uncle of ML who was diagnosed in infancy and reported previously as a patient with corticosterone, methyloxidase II deficiency [5, 6].

Endocrine Evaluation

Serum aldosterone level and Plasma Renin Activity were measured in all patients before initiation of therapy. To exclude other diseases associated with CHH such as congenital adrenal hyperplasia and congenital adrenal hypoplasia, basal and ACTH stimulated cortisol, 17-OH progesterone concentrations and basal DHEA-S, delta-4 androstenedione levels were measured in all patients. Since congenital adrenal hyperplasia due to mutations in CYP11B1 gene occurs in our population solely in Jews of Moroccan origin [3], compound-S levels were determined in two patients, one of them of Moroccan origin and the second a Muslim-Arab. 18-OH corticosterone and deoxycorticosterone concentrations were measured in five and three patients, respectively. 18-OH corticosterone/aldosterone ratios were determined in five patients.

DNA Isolation and Sequencing of CYP11B2

Genomic DNA was extracted from whole peripheral venous blood, using a commercially available kit (Gentra, Minn., USA). Informed consent form was obtained from all the parents.

Because of high homology between CYP11B1 and CYP11B2, we did not directly sequence genomic DNA. We first amplified exons 1–5 and 6–9, using the following primers: (1–5) Forward: CCAGTCCAGACCCCACGC Reverse: TAGCCTGGGGATGGGGAA, (6–9) Forward: AGGGCTCGTTTTTGCTCAG, Reverse: AAGCTGTGCACGTGGGAG. Polymerase chain reaction was performed in 50 μl containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2–2.5 m*M* $MgCl_2$, 250 μ*M* dNTPs, 1 μ*M* of each

Table 1. Patients with hyperreninemic hypoaldosteronism

	Patients						
	MD	FO	ML*	GN	ShT	IE*	OY
Sex	m	m	f	f	f	f	f
Age at presentation, week	3	7	5	1	17	2	3
Ethnic origin							
Mother	Iran	Iran	Iran	Muslim-Arab	Iran	Iran	Morocco
Father	Syria	Iran	Iraq	Muslim-Arab	Yemen	Iran	Yemen
Family history	no	no	uncle	no	no	no	no

* Patients that were found to be compound homozygous for Iranian mutations.

primer, 200 ng of genomic DNA and 1.25 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystem). Each exon was then amplified separately, using the above-amplified products as templates, according to table 2. Touchdown PCR conditions were used to amplify the exons 1–5 and 6–9 with the following conditions: exons 1–5: [94°C 30 s, 63°C 30s, 72°C 45 s] 9 cycles [1°C decrease in every cycle until 55°C] following [94°C 30 s, 55° C 30 s, 72°C 45 s] 35 cycles, exons 6–9: [94°C 30 s, 60°C 30 s, 72°C 45 s] 11 cycles [1°C decrease in every cycle until 50°C] following [94°C 30 s, 50°C 30 s, 72°C 45 s] 35 cycles. The promoter was sequenced by two 500 bp PCR products, using the following primers: a: F: CAGGGGGTACGTGGACATTTT and R: CTCTGCCTTTGCCCTCAGT. b: F: TGAAGGTGGAGTTGGAGAGA, R: AGGTACACCCTCCACCACAG. Long range PCR was performed using Elongase (Invitrogen, Carlsbad, Calif., USA) or blue-Taq DNA polymerase (EURx Ltd., Poland) according to the manufacturer's instructions. The primers were: Promoter bF and (6 + 9) R.

Sequencing was performed on an ABI sequencer and results compared to the wild type sequence (accession ENST00000323110) using the Ensemble database.

Southern blot analysis: Ten microgram samples of total DNA were analyzed by agarose gel electrophoresis following a 24 h digestion with BamHI (NEB, Beverly, Mass., USA). The digested DNA was separated by electrophoresis on 0.8% agarose gel and transferred to an N+ nylon membrane. The membrane was hybridized with 100 ng of 943 bp PCR product of exons 1 + 2 as described (table 2). The probe was radiolabelled with ^{32}P-dCTP labeled using Rediprime II random prime kit (Roche). Hybridization was performed at 72°C. The blot was washed once with 2× SSC, 0.1% SDS, once with 1× SSC, 0.1% SDS, and three times with 0.1× SSC, 0.1% SDS, exposed and read by a phosphoimager.

Results

All patients presented during infancy with hyponatremia, hyperkalemia, and failure to thrive. Increased Plasma Renin Activity, inappropriately normal or low aldosterone and normal basal and ACTH stimulated 17-OH-progesterone concentrations were found in all patients consistent with CHH (table 3). Androgens concentrations (DHEA-S, Androstenedione) were normal as well (data were not shown). In two patients (Moroccan-Jew and Muslim-Arab) compound S levels were normal (5.4, 6.3 mol/l, respectively, normal <23 nmol/l). 18-hydroxycorticosterone (18-OH-B) levels were high in 3 of 5 patients. 18-OH-B/aldosterone ratios were determined before therapy in five patients and were found ≤10 in four of them, suggesting aldosterone synthase deficiency type I. In one patient whose parents were of Iranian and Yemenite origin this ratio was markedly elevated suggesting aldosterone synthase deficiency type 2.

The patients were followed-up for 2–9 years. During follow-up, in repeated examinations, cortisol and androgen levels remained normal in all patients. None of them showed signs of adrenal insufficiency or hyperandrogenism and all had normal growth beyond infancy (table 3).

CYP11B2 Sequencing

Complete sequencing of all exons and introns, including all exon–intron boundaries, revealed 2 patients (ML and IE) homozygous for two mutations, R181W and V386A. Additional missense sequence variants identified in other patients are shown in table 4. We have found other polymorphisms with no amino acid alteration. Of the sequence variations, only I339T is considered a single nucleotide polymorphism. Patients FO and ShT are carriers of R181W, and GN and ShT are carriers of the V386A. The rest are carriers of new variants (A319V or D335G) (table 4). Sequencing of about 1,000 bp of the promoter region, revealed –344T/C variant in the SF-1 binding site in heterozygote state in three patients (MD, GN, ShT) and homozygote in one (OY). The sequences of the binding sites of transcription factors: CREB (TGACGTGA), Ad5 (CTCCAGCCTTGACCTT), and NBRE-1 (AAAGGCTA) were all normal. We found a novel homozygous polymorphic site (–664A/T) in all patients, except FO.

Long-range PCR amplification of the CYP11B2 gene, revealed a normal size fragment (6.4 kb) in all patients

Table 2. Primers used for Cyp11B2 sequencing

Exon	Primer forward	Primer reverse	Annealing temperature	Template	PCR product, bp
1+2	5′-GGG TGA GAT AAA AGG ATT TGG-3′	5′-CCAGGGCAGATGTGCTTTTGG-3′	60	1:500 of (1–5)	934
3	5′-AGG ATG GGA TAC GGG TCA GG-3′	5′-CCA CAC TCC CTT CAG TCC TC-3′	60	1:500 of (1–5)	349
4	5′-CTG CCT TGT GCT CAG CAG T-3′	5′-ATGGTGTCCCTTCCCCATAG -3′	55	1–5	282
5	5′-GAG-GAG-GAC-ACT-GAA-GGA-TG-3′	5′-AGT-GCC-TGG-GAG-GCA-GGC-TTG-3	60	1–5	300
6	5′-GAG TCC TCC TGT GCA AGG TC-3′	5′-AGC CCC AGA TTC TGT CTG C-3	60	1:500 of (6–9)	300
7+8	5′-GAA GGG TGC TGA GAG CAC AG-3′	5′-TGT GCA GGT CCC GCC TCT GC-3′	65	6–9	480
9	5′-CAG GTC CAT GGG CTA CTG AC-3′	5′-GTG CAC GTG GGA GAG AAG AC-3′	60	6–9	252

Table 3. Patients with hyperreninemic hypoaldosteronism: laboratory data at presentation

Serum/plasma values	Patients							Normal values
	MD	FO	ML	GN	ShT	IE	OY	
Na, mmol/l	131	127	129	120	114	121	125	136–146
K, mmol/l	6	6.3	5.7	7.8	6.7	8	6.4	3.5–5
Aldosterone, pmol/l*	2,320	321	137	301	332	588	198	140–3,051
PRA, ng/ml/h*	>180	>180	>180	>180	56.2	>180	>180	<36
18-OH-B, pmol/l	<140	ND	ND	1,044	21,479	3,559	2,011	560–1,460
DOC, pmol/l	ND	ND	ND	8,529	3,730	4,827	ND	<600
18-OH-B/aldosterone	<0.06	ND	ND	3.5	64.7	6.0	10	<10 type 1
ACTH test								
Cortisol 0′, nmol/l	328	215	297	474	173	273	136	116–634
Cortisol 60′, nmol/l	954	604	899	692	629	590	510	>500
17-OHP 0′, nmol/l	0.7	1.1	0.3	0.15	1.8	4.2	0.3	0.3–3.21
17-OHP 60′, nmol/l	2.0	ND	4.8	ND	5.1	8.9	2.3	2.57–6.26

ND: not done.
* Normal values below 3 months of age.

Table 4. Sequence variations in CYP11B2 coding regions and 1,000 bp promoter

Patient	R181W	A319V	D335G	I339T	V386A	SF-1(–344C/T)
MD			+/–	+/–		+/–
FO	+/–	+/–		+/–		–/–
ML	+/+				+/+	–/–
GN				+/–	+/–	+/–
ShT	+/–		+/–	+/–	+/–	+/–
IE	+/+				+/+	–/–
OY		+/–	+/–	+/–		+/+

(fig. 1). Southern blot analysis after BamH1 digestion revealed the expected 4.5 kb fragment of CYP11B2, in addition to a higher band which is probably the CYP11B1 (data not shown).

Discussion

Isolated aldosterone deficiency results from loss of activity of aldosterone synthase encoded by the CYP11B2 gene. This disease was previously termed CMO deficiency by Ulick [13]. He later suggested that the term aldosterone synthase deficiency types I and II reflects more appropriately the molecular basis of this disease [14]. Different mutations in the CYP11B2 gene do not explain the two hormonal phenotypes in patients with CHH type I and type II [15–17]. Moreover some patients with CHH do not have mutations in their CYP11B2 gene [15]. Thus we prefer to use the term CHH for our patients.

Rosler et al. [4, 5] were the first to describe a group of Israeli patients with aldosterone synthase deficiency. All patients were Jews originating from Isfahan, Iran, and were found to be homozygous for two missense mutations in the CYP11B2 gene [6]. These mutations were shown to reduce aldosterone synthase activity to less than 0.2% of normal activity [6, 16]. Homozygosity for either mutation alone and heterozygosity for both mutations were insufficient to cause the disease [6]. Hormonal data of these patients were consistent with aldosterone synthase deficiency type II.

In our collaborative study, we examined 7 patients with CHH. The clinical and biochemical characteristics of these patients were consistent with the diagnosis of congenital hypoaldosteronism due to aldosterone synthase

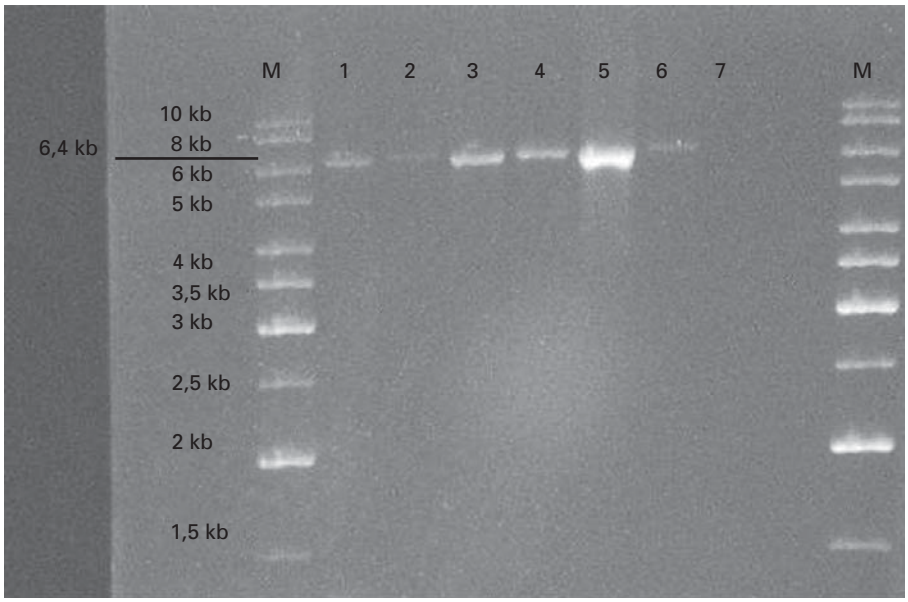

Fig. 1. Long-range PCR amplification of CYP11B2 gene in CHH patients. M, 1: kb ladder marker; 1–5: patients MD, FO, GN, ShT and OY, 6: normal individual; 7: blank.

deficiency. In general the clinical presentation in our patients did not differ from those of patients described by Rosler et al. [18]. However, special genetic and molecular features of our patients are noteworthy. The parents of our patients were not consanguineous and all have different ethnic origins. In our study, four patients had 18-OH-B/aldosterone ratios ≤ 10 consistent with aldosterone synthase deficiency type I including a patient whose parents are Iranian Jews. In contrast, the Iranian Jews described by Rosler had 18-OH-B/aldosterone ratios consistent with aldosterone synthase deficiency type II. The mutations described in Jews from Isfahan were found in only two of our patients who were also of Iranian origin (in one patient the father was an Iraqi Jew). Sequencing of the coding region, exon–intron junctions and a 1,000 bp of the promoter region of CYP11B2 gene, failed to detect any known disease-causing mutations in five other patients from different ethnic origins. Major alterations in the gene structure (e.g. introns) were ruled out by long-range PCR as well as Southern blot analysis for all patients.

According to the Genecard single nucleotide polymorphism database, only I339T is a polymorphic change, while the other variations found in this study have not been reported previously. One of our patients is a carrier of R181W and V386A mutations and in addition carries a third, new variant D335G. We do not yet know the functional significance of this sequence change.

One patient found to be heterozygous for R181W mutation carries an additional A319V mutation. Another patient is heterozygous for two variants: A319V and D335G. Expression studies are needed to determine whether these compound heterozygote alleles are disease-causing.

In two patients whose parents are Muslim-Arabs and Jews of Syrian and Iranian origin, respectively, we did not find a molecular defect. A previous study reported a missense mutation in a Muslim-Arab with CHH [19].

The expression of CYP11B2 gene is regulated by several transcription factors [20]. We have not found any change in a 1,000 bp region downstream from the transcription initiation site in any of our patients. It remains to be determined whether these patients have a mutation in a different region, in the function of a transcription factor, or possibly in the function of other genes that are involved in regulating aldosterone synthesis (Angiotensinogen, Angiotensin converting enzyme, AT1 type angiotensin II receptor, etc.).

There are few reports which failed to detect mutations in CYP11B2 gene in patients with CHH [7, 11, 12]. Most probably there is a bias toward publishing studies with positive findings. Our study reflects the need to report negative results as well, to obtain an estimate of the proportion of CHH infants without CYP11B2 mutations for the elucidation of other molecular causes for this phenotype. These findings also reflect the changes in the Israeli Iranian Jewish population from a homogeneous group (as found in Rosler's studies) to a heterogeneous one. A high index of suspicion is warranted for the diagnosis of CHH in children with salt wasting originating from the Middle East and Asia (Sephardic-Jews) and Muslim-Arabs.

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