สัญญาเลขที่ RSA5780054

รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาลักษณะทางคลินิกและอณูพันธุศาสตร์ในผู้ป่วยโรคต่อมหมวกไตโตแต่กำเนิดชนิดวินิจฉัยยาก

(Clinical, hormonal, and molecular characterization of patients with atypical congenital adrenal hyperplasia)

หัวหน้าโครงการวิจัยผู้รับทุน รศ.พญ.ธนินี สหกิจรุ่งเรือง สถาบัน จุฬาลงกรณ์มหาวิทยาลัย

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Project Title: Clinical, hormonal, and molecular characterization of patients with atypical congenital adrenal hyperplasia

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Abstract

Objectives: To study the clinical, hormonal, and molecular characterization in patients with atypical congenital adrenal hyperplasia (CAH) and non-autoimmune Addison disease.

Methods: Clinical data and steroid profiles of patients with atypical CAH and Addison were collected. The g.DNA was extracted and the entire coding regions of the *StAR, CYP11A1, HSD3B2, CYP11B1, CYP17A1* and *POR* genes were assessed by polymerase chain reaction and sequenced. The identified novel missense mutations were recreated in expression vectors, and protein/enzymatic activities were measured as steroid production in COS-7 cells. For novel splice site mutations, the mutant minigene constructs were created and transfected into COS-7 cells; then total cellular RNA was extracted and used for RT-PCR. Those with no mutations in known genes undergo exome sequencing to identify new disease genes.

Results: A total of 34 patients with atypical CAH from seven university-based hospitals were enrolled. A genetic diagnosis was reached in 27 patients (79%). We found 11 patients had mutations in *StAR* gene with 5 novel mutations (p.P230L>WfsX, IVS6-1G>A, IVS3+(2-3)insT, p.W147R, p.Q264R). One patient had p.A359V mutation in *CYP11A1* gene, and two patients had *HSD3B2* mutations [p.Q334X, p.Y180X (novel)]. Two siblings had compound heterozygous *CYP11B1* mutations (R141X/IVS7+1G>A). One patient had homozygous p.R440H mutations in *CYP17A1* gene. Eight patients had DAX-1 mutations and 7 being novel. Novel *StAR* missense mutations were re-created in expression vectors and StAR activity was measured as

pregnenolone production in COS-7 cells. The respective activities of W147R, and Q264R were 3.9%, and 1.6% of wild-type activity. A minigene assay was used to determine the effects of the splicing mutation. The IVS7+1G>A mutation caused aberrant splicing of *CYP11B1* leading to exon skipping. The IVS6-1G>A mutation caused intron retention in the StAR gene. The functional studies of novel DAX-1 mutations are being under investigation.

Conclusion/Discussion: We report clinical, genetic, hormonal, and functional effects of steroidogenic gene mutations in the large cohort of patients with atypical CAH. *StAR* mutations may not be rare in Southeast Asian population.

Further plan: To establish the molecular diagnostic center of pediatric adrenal disorders in Asia-Pacific region which would have important translational impact for counseling families, early diagnosis, personalized therapy, predicting comorbidities and prognosis, and targeting clinical genetic testing in the future.

Keywords: Congenital adrenal hyperplasia, Mutation, Novel, Steroidogenesis, Genotypes, Phenotypes

ทุนพัฒนานักวิจัย สำนักงานกองทุนสนับสนุนการวิจัย
รหัสโครงการ RSA5780054

ชื่อโครงการ: การศึกษาลักษณะทางคลินิกและอณูพันธุศาสตร์ในผู้ป่วยโรคต่อมหมวกไตโตแต่กำเนิดชนิดวินิจฉัยยาก

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บทคัดย่อ

วัตถุประสงค์โครงการ: เพื่อศึกษาลักษณะทางคลินิกและอณูพันธุศาสตร์ในผู้ป่วยโรคต่อมหมวกไตโตแต่กำเนิดชนิดวินิจฉัยยาก และต่อมหมวกไตทำงานบกพร่องแต่กำเนิด และศึกษาผลที่เกิดจากการกลายพันธุ์ (mutation) ที่แตกต่างกันต่อการทำหน้าที่ของโปรตีน/เอ็นไซม์ นำไปสู่ความเข้าใจเกี่ยวกับกลไกการเกิดโรค

วิธีทดลอง: ผู้วิจัยได้รวบรวมผู้ป่วยที่มีลักษณะทางคลินิกเข้าได้กับโรคต่อมหมวกไตโตแต่กำเนิดชนิดวินิจฉัยยาก ได้จำนวน 34 ราย และทดสอบทางห้องปฏิบัติการทางชีวเคมีและ steroid hormone profiles และสกัด DNA จากผู้ป่วย เพื่อตรวจหาการกลายพันธุ์ของยีน *StAR, CYP11A1, CYP17A1, HSD3B2, CYP11B1* และ *POR* โดย PCR และ direct sequencing สำหรับ novel missense mutations จะถูกทดสอบการทำหน้าที่ โดยสร้าง cDNA expression vectors และ transfect ใน COS-7 cells และศึกษา in vitro activities ของโปรตีน โดยการวัด steroids ใน culture media สำหรับ novel splice site mutations จะถูกทดสอบด้วย minigene experiment สำหรับผู้ป่วยที่ไม่พบการกลายพันธุ์ใน known genes จะพิจารณาส่ง whole exome sequencing เพื่อหา novel candidate genes ต่อไป

ผลการศึกษา: พบการกลายพันธุ์ในผู้ป่วยทั้งหมด 27 รายจาก 34 ราย (ร้อยละ 79) โดยพบการกลายพันธุ์ในยีน *StAR* ในผู้ป่วย 11 ราย, พบ *CYP11A1* และ *CYP17A1* mutations อย่างละ 1 ราย, พบ *HSD3B2* mutations ในผู้ป่วย 2 ราย และพบ *CYP11B1* mutations ในผู้ป่วย 2 ราย ซึ่งพบทั้งส่วนที่เป็น novel missense mutation, novel splice site และ novel frameshift mutation สำหรับผู้ป่วยที่ตรวจ

พบ *StAR* mutations ผู้ป่วย 3 รายจาก 11 รายมีอาการแสดงของ nonclassic/atypical lipoid CAH ซึ่งพบภาวะ adrenal insufficiency ได้ช้ากว่า ผลการศึกษา StAR activities ใน transfected cells ของ W147R (novel), Q264R (novel), R188C, Q258X พบว่ามี activities ลดลงเหลือเพียง 3.9, 1.6, 6.7, 2.8% ของ wild-type activity ส่วน novel splice site ของ StAR (IVS6-1G>A) ได้สร้าง construct สำหรับ minigene experiment และพบว่า mutation ดังกล่าวทำให้เกิด intron retention สำหรับ splicing analysis ของ CYP11B1 ได้ทำการทดลอง minigene experiment เช่นกัน โดยพบว่า IVS7+1G>A mutation ทำให้เกิด exon skipping ซึ่งมีผลให้สูญเสียการทำงานของ CYP11B1 protein สำหรับผู้ป่วยรายอื่นๆ ซึ่งตรวจไม่พบการกลายพันธุ์ ได้ทำการศึกษา mutation analysis เพิ่มเติมของยีนอื่นๆที่เป็นไปได้ ซึ่งเป็นสาเหตุของ congenital adrenal insufficiency ที่พบได้น้อย และมีอาการแสดงคล้ายคลึงกับกลุ่มโรค CAH ได้ ขณะนี้สามารถ identify ผู้ป่วยที่พบ DAX1 mutations ได้เพิ่มเติมอีก 8 ราย (เป็น novel mutations จำนวน 7 ราย)

สรุปและวิจารณ์: ผู้วิจัยได้รายงานลักษณะทางคลินิกและความผิดปกติทางอณูพันธุศาสตร์ในผู้ป่วยโรคต่อมหมวกไตโตแต่กำเนิดชนิดวินิจฉัยยาก ใน cohort ขนาดใหญ่สำหรับประชากรเด็ก และพบว่า StAR mutations พบได้บ่อยได้กลุ่มประชากร Southeast Asian

ข้อเสนอแนะ/แผนงานในอนาคต: พัฒนาศูนย์การตรวจวินิจฉัยทางอณูพันธุศาสตร์ในโรคต่อมหมวกไตโตแต่กำเนิดชนิดวินิจฉัยยาก เนื่องเป็นกลุ่มโรคที่มีความซับซ้อนและมีอาการแสดงทางคลินิกได้หลากหลาย เป็นโรคที่จำเป็นต้องได้รับการวินิจฉัยและรักษาอย่างรีบด่วน ผู้ป่วยส่วนใหญ่ต้องได้รับการรักษาด้วยฮอร์โมนทดแทนตลอดชีวิตและต้องได้รับการตรวจติดตามภาวะแทรกซ้อนระยะยาวต่อไป การตรวจทางอณูพันธุศาสตร์ ทำให้การตรวจวินิจฉัยเป็นไปได้อย่างถูกต้อง รวดเร็ว ช่วยในการให้คำปรึกษาทางพันธุศาสตร์ ช่วยวางแผนการรักษาอย่างถูกต้องและบอกพยากรณ์โรคได้ดี

คำหลัก: โรคต่อมหมวกไตโตแต่กำเนิด, การกลายพันธุ์, การสร้างสเตียรอยด์, จีโนไทป์, ฟีโนไทป์

Introduction

Congenital adrenal hyperplasia (CAH) is one of the most common inherited metabolic disorders and is associated with significant morbidity and mortality in affected children. It comprises a group of autosomal recessive disorders caused by the mutations in the genes encoding for steroidogenic enzymes that involved cortisol synthesis. Impaired cortisol secretion results in hypersecretion of corticotropin-releasing hormone and adrenocorticotropic hormone (ACTH) and consequent hyperplasia of the adrenal glands. The clinical phenotypes and biochemical characteristics depend on the specific enzymatic defect. There is a broad clinical spectrum of this disorder. CAH is the most common cause of primary adrenal insufficiency in children. In most forms of CAH, it can be fatal if not diagnosed early in infancy (1).

The adrenal cortex is the production site for three classes of steroid hormones: mineralocorticoids, glucocorticoids, and sex hormones. The cortex is divided into three zones by different cellular arrangements, each one functionally distinct due to the enzymes required for different hormone production. The outer zona glomerulosa does not express P450c17 (2) and hence produces 17-deoxysteroids leading to aldosterone (the most potent mineralocorticoid), and is regulated primarily by the renin/angiotensin system. The middle zona fasciculata expresses the 17α-hydroxylase activity but very little of the 17,20-lyase activity of P450c17, and hence produces 21-carbon, 17-hydroxysteroids, leading to cortisol under the influence of ACTH. The inner zona reticularis expresses both the 17α- hydroxylase and 17,20-lyase activities of P450c17, and hence produce the 19-carbon 17-hydroxy steroid dehydroepiandrosterone (DHEA), the precursor of sex steroids (1, 3).

ACTH regulates steroidogenesis (chronic regulation) by inducing the transcription of genes encoding various steroidogenic enzymes, but acute regulation is at the level of cholesterol access to P450scc. The Steroidogenic Acute Regulatory protein (StAR) facilitates the movement of cholesterol into mitochondria, where it is converted to pregnenolone by P450scc (4).

Simplified diagram of adrenal steroidogenic pathways was shown in Fig.1.

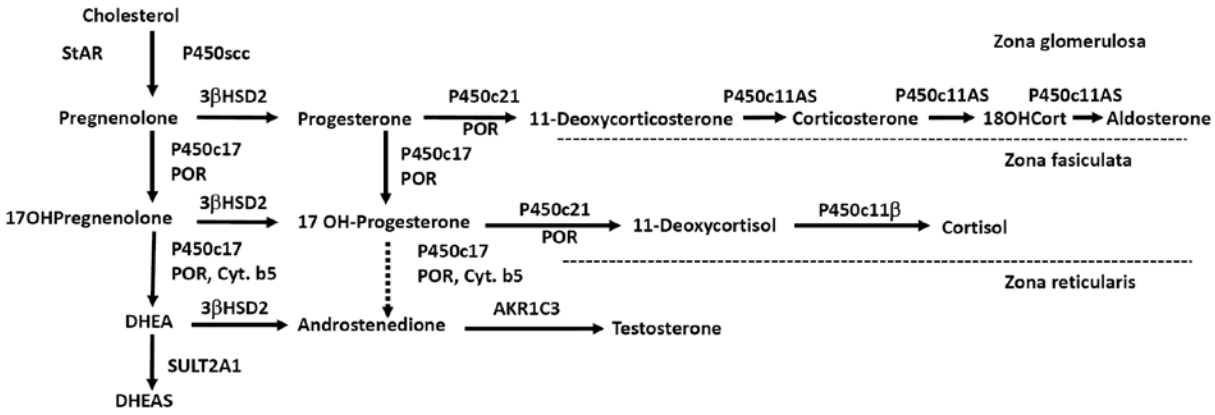

Fig. 1 Simplified scheme of adrenal steroidogenesis

AKR, aldo-keto reductase; Cyt.b5, cytochrome b5; P450scc, cholesterol side-chain cleavage enzyme; P450c17, 17α-hydroxylase/17,20-lyase; 3βHSD2, 3β-hydroxysteroid dehydrogenase type 2; P450c21, 21-hydroxylase; P450c11AS, aldosterone synthase; P450c11β, 11β-hydroxylase; POR, P450 oxidoreductase; StAR, Steroidogenic Acute Regulatory Protein; SULT2A1, sulfotransferase; 18OHCort, 18-hydroxycorticosterone

In Thailand, there has not been nationwide newborn screening program for CAH, although the recent pilot study suggested the high prevalence of CAH in our country (5, 6). More than 90% of cases are caused by a defect in the enzyme 21-hydroxylase (P450c21). Four other enzyme deficiencies in the steroid biosynthesis pathway (P450scc, P450c17, P450c11β, 3βHSD), along with one cholesterol transport protein defect (StAR), and one electron-transfer protein (P450 oxidoreductase; POR) account for the remaining cases. In these uncommon forms of CAH, the clinical and hormonal phenotypes can be complicated, and are not widely recognized by endocrinologists or pediatricians. In Thailand, there are very limited data regarding the types of CAH, and its genetic characterization in our population.

The clinical symptoms of the different forms of CAH result from the particular hormones that are deficient and those that are produced in excess. A characteristic feature of CAH is genital ambiguity or disordered sex development (DSD), and all variants are associated with glucocorticoid deficiency. Each variant of CAH is summarized in Table 1 (7).

Table 1: Characteristics of different forms of congenital adrenal hyperplasia (7)

	21-hydroxylase deficiency	11β-hydroxylase deficiency	17α-hydroxylase deficiency	3β-hydroxysteroid dehydrogenase deficiency	Congenital Lipoid adrenal hyperplasia	P450 oxidoreductase deficiency
Gene involved	*CYP21A2*	*CYP11B1*	*CYP17A1*	*HSD3B2*	*StAR/ CYP11A1*	*POR*
Chromosome location	6p21.3	8q24.3	10q24.3	1p13.1	8p11.2/ 15q24.1	7q11.2
Ambiguous genitalia	Yes in XX	Yes in XX	Yes in XY	Yes in XY, ± in XX	Yes in XY	Yes in both XX and XY
Adrenal crisis	Yes	Rare	No	Yes	Yes	Rare
Serum cortisol	↓	↓	↓	↓	↓	Normal/↓
Mineralocorticoid	↓	↑	↑	↓	↓	Normal
Androgens	↑	↑	↓	↓	↓	↓
Serum Na	↓	↑	↑	↓	↓	Normal/↓
Serum K	↑	↓	↓	↑	↑	Normal
Metabolite elevated	17OHP	DOC, 11-deoxycortisol	DOC, corticosterone	DHEA, 17OHPreg	None	17OHP

17OHP, 17-hydroxyprogesterone; DOC, deoxycorticosterone; DHEA, dehydroepiandrosterone; 17OHPreg, 17-hydroxypregnenolone

CAH is the most common cause of primary adrenal insufficiency in children. However, there is considerable overlap in the clinical and biochemical phenotypes of CAH and several genetic causes of adrenal hypoplasia (DAX-1, SF-1). For example, nonclassic lipoid CAH caused by StAR or CYP11A1 mutations that retain partial activities may have later onset of adrenal insufficiency resembling non-autoimmune Addison disease (8-13).

Establishing a specific genetic diagnosis of adrenal insufficiency is extremely valuable for early identifying children who could benefit from treatment before the onset of potentially life-threatening symptoms and for counseling family members appropriately. Knowing the genetic etiology can also help to personalized treatment, predicting comorbidities (such as impaired puberty or fertility) and prognosis, and targeting clinical genetic testing in the future.

In this study, we investigate the clinical, hormonal, and molecular characterization in patients with atypical CAH (excluding 21-hydroxylase deficiency) or non-autoimmune Addison disease in a cohort of 34 children with primary adrenal insufficiency of unknown etiology.

Patients and Methods

Patients

A pediatric cohort study was performed with patients with atypical CAH or unknown causes of non-autoimmune Addison disease recruited from 7 pediatric endocrinology clinics in Thailand and Singapore. Inclusion criteria were defined as the presence of signs and symptoms of adrenal insufficiency together with high plasma ACTH and low serum cortisol at initial presentation. Exclusion criteria were as follows: 1) congenital adrenal hyperplasia (21α-hydroxylase deficiency) diagnosed by a distinctive serum steroid hormone profile; 2) X-linked adrenoleukodystrophy in boys with neurological findings and elevated very long-chain fatty acids 3) clinical and biochemical evidence of autoimmune Addison disease.

All patients were assessed by a pediatric endocrinologist. All clinical, biochemical, and imaging data related to the diagnosis and treatment and family history were collected. Studies were performed with the approval of the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB No.505/53). Patients and/or parents provided written informed consent, and all studies were conducted in accordance with the principles of the Declaration of Helsinki.

A total of 34 patients (13 girls, 21 boys) were included. The most common presenting symptoms were salt-losing symptoms/electrolyte imbalance (53%), hyperpigmentation (25%), and ambiguous genitalia (18%). Detailed clinical findings are provided in Table 2.

Table 2. Clinical characteristics and genetic mutations identified in our pediatric cohort with atypical CAH/unknown causes of non-autoimmune Addison disease.

StAR (Chr. 8p11.23)			
Patient ID	**Clinical features**	**Mutations**	
		Allele 1	Allele 2
1.	A 46,XX non-virilized Thai female with primary adrenal insufficiency at 3 months of age	IVS3(ds,+2)insT*	
2.	A 46,XY sex reversal Singaporean female with primary adrenal insufficiency at 3 months of age	c.719delC	p.Q264R* (c.791A>G)
3.	A 46,XX Singaporean female with salt-losing crisis at 3 months of age	c.719delC	p.Q258X (c.722C>T)
4.	A 46,XY phenotypic female with salt-losing at 10 days of life with severe pigmentation	p.230L>WfsX* (c.687delG)	

5.	A 46,XY phenotypic female with salt-losing at 2 weeks of age	p.W147R* (c.439T>A)	
6.	A 46,XX female with salt-losing at age 1 weeks with severe pigmentation	c.719delC	p.R188C (c.562C>T)
7.	A 46,XX female with hypoglycemia, skin pigmentation and salt-losing at 3 days of life.	p.Q77X (c.229C>T)	IVS6-1G>A*
8.	A 46,XX female with salt-losing at 7 days of life.	Deletion	Deletion
9.	A 46,XX female with adrenal crisis at 11 months of age	p.R188C (c.562C>T)	
10.	A 46,XY male with hyperpigmentation (sibling of patient 9)	p.R188C (c.562C>T)	
11.	A 46,XX female with progressive pigmentation since 1 years old without salt-losing symptoms	p.R188C (c.562C>T)	
CYP11A1 (Chr. 15q24.1)			
12.	A 46,XY female with clitoromegaly and late-onset adrenal crisis	p.A359V (c.1076C>T)	
HSD3B2 (Chr. 1p12)			
13.	A 46,XY Indian boy with ambiguous genitalia and salt-losing crisis since 4 days of life with markedly elevated 17OHP.	p.Y180X* (c.540C>A)	
14.	A 46,XY Thai boy with ambiguous genitalia and salt-losing symptoms at 10 days of life.	p.T259M (c.776C>T)	
CYP11B1 (Chr. 8q24.3)			
15.	A 46,XX girl with ambiguous genitalia, hypertension	p.R141X (c.421C>T)	IVS7(ds,+1)G>A*
16.	A 46,XY boy with hyperpigmentation, and sexual precocity (sibling of patient 16)	p.R141X (c.421C>T)	IVS7(ds,+1)G>A*
17.	A 11-year-old boy (46,XY) with severe hypertension and pigmentation	p.I289N* (c.562C>T)	
CYP17A1 (Chr. 10q24.32)			
18.	A 46,XY phenotypic girl with delayed puberty and mineralocorticoid hypertension	p.R440H (c.1319G>A)	
WT1 (Chr. 11p13)			
19.	A 46,XY boy with ambiguous genitalia and kidney failure	p.R394W (c.1180C>T)	Normal
DAX-1 (Hemizygous mutation) (Chr. Xp21.2)			
20.	A 46,XY boy with salt-losing crisis since 1 months of age.	c.805_807delGTC* (p.Val269del)	
21.	A 46,XY boy with hyperpigmentation, and salt-losing crisis at 24	c.1148_1149delGG* (p.Gly383Aspfs*5)	

	days of life, later developed hypogonadotropic hypogonadism	
22.	A 46,XY boy with late-onset Addison disease and delayed puberty	c.1156C>T* (p.Leu386Phe)
23.	A 46,XY boy with adrenal crisis at 1 months of age and later developed sexual precocity	c.363delG* (p.Gly122Valfs*142)
24.	A 46,XY boy with adrenal crisis at 3 days of life, later developed growth hormone deficiency	c.501_502insG (p.Ala170Argfs*15)
25.	A 46,XY boy with early onset salt-losing crisis	c.1062delC* (p.Ala355Profs*17)
26.	A 46,XY with early onset salt-losing crisis at 1 months old, later developed sexual precocity	c.233G>A*, p.Ser78Asn
27.	A 46,XY boy with hypoglycemia at DOL3 and severe hyperpigmentation	c.805_807delGTC* (p.Val269del)

Molecular analyses

Mutation analyses

Leukocyte genomic DNA was extracted using the QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA). The entire coding regions of the *StAR, CYP11A1, HSD3B2, CYP11B1*, *CYP17A1*, and *POR* genes were amplified by polymerase chain reaction (PCR) and sequenced using previously described oligonucleotides and amplification conditions. Patients who had no mutations in these genes were assessed for mutations in *DAX-1* gene or undergo exome sequencing to identify new disease genes.

Mutagenesis and transfection

Mutant full-length StAR cDNA expression vectors (14) were generated by PCR-based, site-directed mutagenesis and verified by direct sequencing. The PCR conditions were: 95 C for 30 sec, 16 cycles of 95 C for 30 sec, 55 C for 1 min, and 68 C for 18 min. Nonsteroidogenic monkey kidney COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics at 37 C in a humidified 5% CO2 incubator. Cells were divided into twelve-well plates (Falcon, BD Biosciences, Lincoln Park, NJ) and cotransfected using Effectene (Qiagen, Valencia, CA) at ~50% confluence. Co-transfections were done with a pCMV-StAR expression vector and the F2 plasmid expressing a fusion protein

of the cholesterol side-chain cleavage system (H2N-P450scc-adrenodoxin reductase-adrenodoxin-COOH) (15). To monitor transfection efficiency, cells were also cotransfected with 5 ng of Renilla luciferase reporter plasmid (pRL-CMV) (Promega, Madison, WI) per well. Culture media were collected 48 hours later, and pregnenolone production was measured by enzyme immunoassay (DIAsource ImmunoAssays, Belgium). The sensitivity of this assay is 5.4 ng/dL. Data are presented as the mean ± SEM for at least three independent experiments, each performed in triplicate.

Minigene construction and splicing analysis

We performed a minigene in vitro experiment of the novel *CYP11B1* and *StAR* splicing mutations. A segment of the wild-type (WT) and mutant genomic DNA (gDNA) of *CYP11B1* or *StAR* gene consisting of exons and their in-between introns was amplified by PCR. We used the gDNA of a normal control and the patients with splice site mutations as a template of minigene constructs. PCR reactions were carried out in a 20 µl volume containing 50 ng gDNA, 10xPCR buffer, 15 mM MgCl2, 10 µM dNTPs, 5 U/µl Taq polymerase and 10 µM of each primer, using the following parameters: 60s at 94°C, 90s at 68°C and 60s min at 72°C. The PCR product was cleaved with BamHI-HF and XbaI enzymes and cloned into the corresponding sites of pcDNA™3.1/myc-His B mammalian expression vector (Invitrogen, Carlsbad, CA) using T4 DNA ligase (New England BioLabs, UK). The wild-type and mutant vectors were confirmed by direct sequencing using NCBI Reference Sequences.

COS-7 cells were cultured in Dulbecco's Modified Eagles Medium, High Glucose (HyClone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Singapore) and 0.01% penicillin/streptomycin (HyClone Laboratories) at 37°C in a humidified 5% CO2 incubator. Cells were grown on 6-well plates and transiently transfected with the wild-type and mutant minigene constructs (1 µg) using Effectene® Transfection Reagent (Qiagen). Cells incubated for 48 h after transfection and then were washed 3 times with PBS and kept frozen at -20°C. Total cellular RNA was extracted using QIAamp® RNA Blood Mini Kit (Qiagen) and treated with DNaseI (Qiagen). The RNAs were then used as template for cDNA synthesis using ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, WI). Finally, both the WT and mutant cDNAs were amplified by PCR using the same primers and conditions as used for the minigene construction. The PCR products were analyzed by electrophoresis on a

1% agarose gel followed by staining with ethidium bromide. Each PCR product was confirmed by Sanger sequencing after subcloning into pGEM®-T Easy vectors (Promega).

Results

A molecular genetic diagnosis was obtained in 27 out of 34 children (79%) with atypical CAH using the targeted gene direct sequencing approach. Figure 2 showed mutations in various genes identified in this cohort. These included missense mutations (n=21), nonsense mutations (n=6), frameshift mutations (n=11), splice site mutations (n=5), and whole gene/exon deletions (n=2), summarized in Table 4.

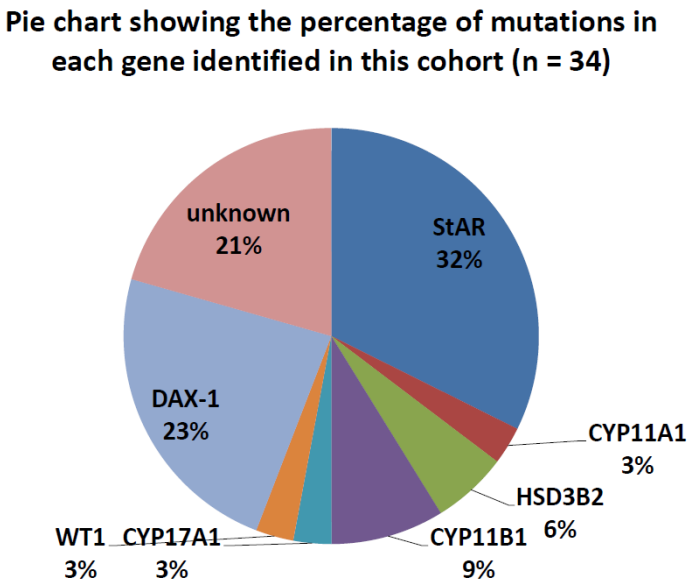

Fig 2. Pie chart showing the percentage of mutations in each gene identified in this cohort

Functional studies of novel mutations

Functional studies of the StAR mutants in transfected cells

We co-transfected nonsteroidogenic COS-7 cells with either wild-type or mutant StAR and a vector expressing the F2 fusion of the cholesterol side chain cleavage system and compared the amount of pregnenolone produced. An empty vector was used as a negative control, and 22R-hydroxycholesterol, which bypasses the action of StAR and thus indicates the maximal enzymatic capacity of the P450scc system was added as a positive control. Using endogenous cellular cholesterol and cholesterol in the serum in the culture media as substrate, COS-7 cells

expressing F2 and wild-type StAR made 2886 ± 600 ng/dl of pregnenolone, whereas cells transfected with the empty vector produced a low level of pregnenolone (24 ± 1 ng/dl) indicating the presence of StAR-independent steroidogenesis. The R188C mutant produced 217 ± 57 ng/dl of pregnenolone, whereas the W147R, Q264R and Q258X mutants generated 136 ± 17 ng/dl, 70 ± 15 ng/dl, and 104 ± 26 ng/dl, respectively. When the background of StAR-independent of steroidogenesis is subtracted, the R188C, W147R, Q264R, and Q258X mutants had 6.7%, 3.9%, 1.6%, and 2.8%, of wild-type activity, respectively (Fig. 3). In this assay, pregnenolone production in all five StAR mutations was significantly higher than vector control ($P < 0.05$).

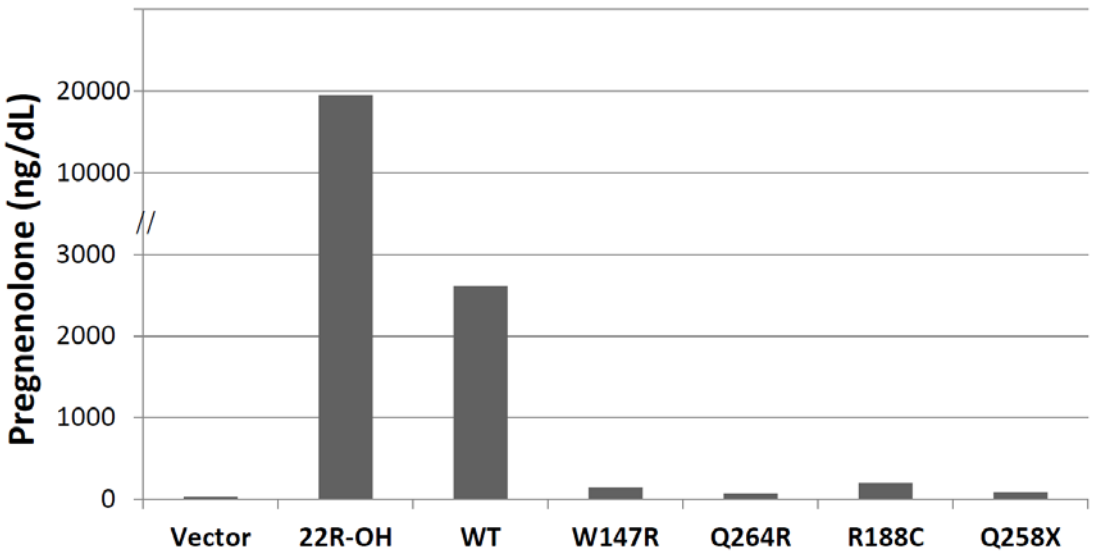

Figure 3. Activities of StAR mutants. A, Activity of full-length StAR in whole cells. COS-7 cells were cotransfected with expression vectors for the cholesterol side-chain cleavage system (F2) and either wild-type (WT) or mutant StAR, and pregnenolone was measured 48 h later by immunoassay. The StAR-independent substrate 22(R)-hydroxycholesterol (22R-OH) was added to the cell culture media to determine the maximum steroidogenic capacity of the cells.

<u>*StAR* minigene construction และ splicing analysis</u>

We performed an *in vitro* minigene experiment of the StAR mutation c.745-1G>A (IVS6-1G>A). A segment of the wild-type and patient human StAR gene which consists of exon 6, intron 6 and exon 7 was amplified by PCR using specific oligonucleotides; ggcggagcacggtcccacttgca (forward) and tcaacacctggcttcagaggcag (reverse). PCR reactions were carried out as described in the method section. The PCR products were cleaved with

BamHI and XbaI and cloned into pcDNA™3.1/myc-His B mammalian expression vector (Invitrogen, Carlsbad, CA) using T4 DNA ligase (New England BioLabs, UK).

Transient transfection was performed nonsteroidogenic monkey kidney COS-7 cells with wild-type and patient's minigene constructs using Effectene (QIAGEN, Valencia, CA) at approximately 50% confluence. Culture media were collected after transfected 48 hrs. Total RNA was extracted and treated with DNaseI. The RNA was used as template for cDNA synthesis. Then, the wild-type and patient cDNAs were amplified by PCR using the same primers and conditions as used for the minigene construction and were analyzed by electrophoresis on a 1% agarose gel followed by staining with ethidium bromide.

PCR amplification of the RNA produced from the transcription of the wild-type minigene showed that intron 6 was correctly spliced, yielding a product of the predicted size (208 bp) (Fig. 4). By contrast, PCR amplification of the RNA produced by the mutant minigene (IVS6-1G>A) produced a product of 1043 bp and no 208 bp product was seen, indicating that the sequences corresponding to intron 6 had been retained in the RNA. Thus the intronic mutation found in patient 7 prevents correct splicing of the *StAR* RNA, so that functional protein cannot be made.

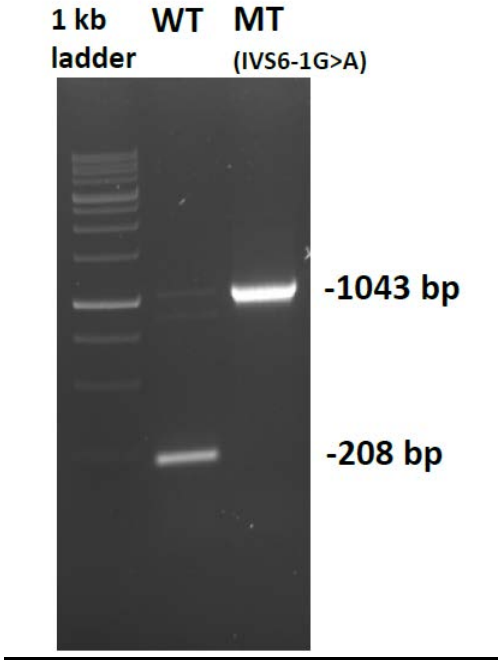

Fig 4. Minigene experiment. COS7 cells were transfected with vector expressing the wild-type or mutant StAR minigene construct. Total RNA from the transfected cells were used for RT-PCR, showing the expected 208-bp product from the wild-type construct and unspliced product from the mutant, sized 1043 bp.

Minigene construction and splicing analysis of CYP11B1 mutation

We hypothesized that the IVS+1G>A mutation located at the splicing donor site of intron 7 would create an abnormal splicing of the mRNA, likely skipping exon 7. To examine this possibility, we constructed expression vectors containing the WT and mutant c.1200+1G>A CYP11B1 minigene sequence from exons 6 to 9 (Fig. 5). The resultant minigenes were transfected into COS-7 cells. Then, total RNA was isolated and analyzed by the RT-PCR method. We found that the mutant c.1200+1G>A CYP11B1 minigene was processed to two major spliced products which were shorter than the WT minigene (Fig. 6), suggesting the exon-skipping or alternative splice site. Sequence analysis of the PCR products after subcloning into pGEM®-T Easy vector revealed that one of the mutant fragments contained the full sequence of exon 6, 8, 9, but exon 7 was entirely missing. The other mutant PCR product had the full sequence of exon 6 and 9, but totally skipped the exons 7 and 8 (Fig. 7).

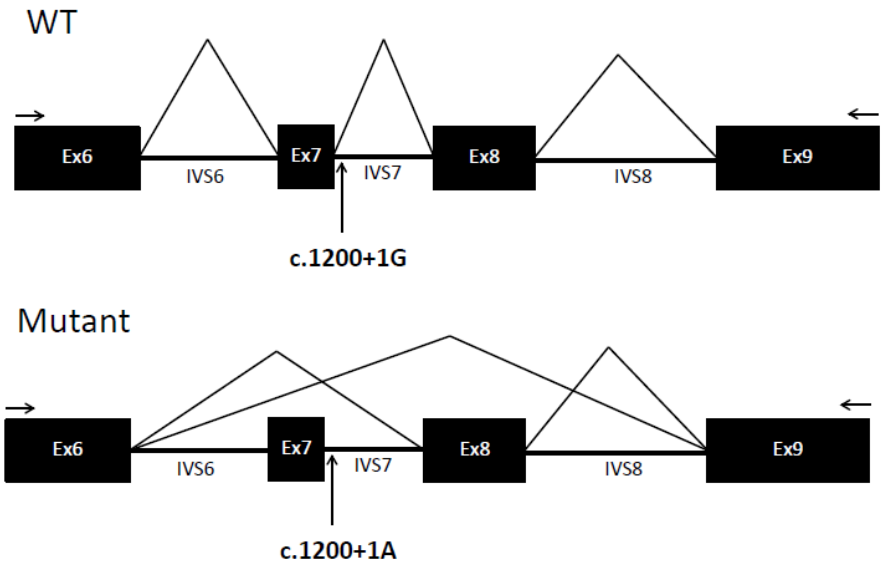

Fig. 5. The scheme shows the set-up of the minigene constructs for the splicing analysis in the WT and mutant expression vectors containing c.1200+1G>A mutation (arrows).

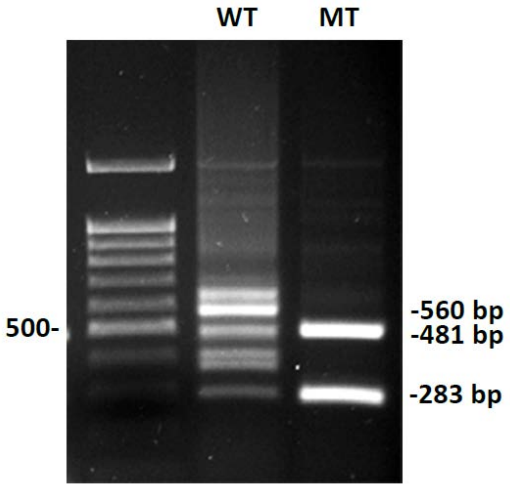

Fig. 6. Minigene experiment. COS-7 cells were transfected with the wild-type (WT) or mutant (MT) minigene constructs. Total RNA from the transfected cells were used for RT-PCR of *CYP11B1* cDNA. The figure shows the expected 560-bp PCR product from the WT construct and two shorter incorrectly spliced products from the mutant, sized 481 and 283 bp on an agarose gel.

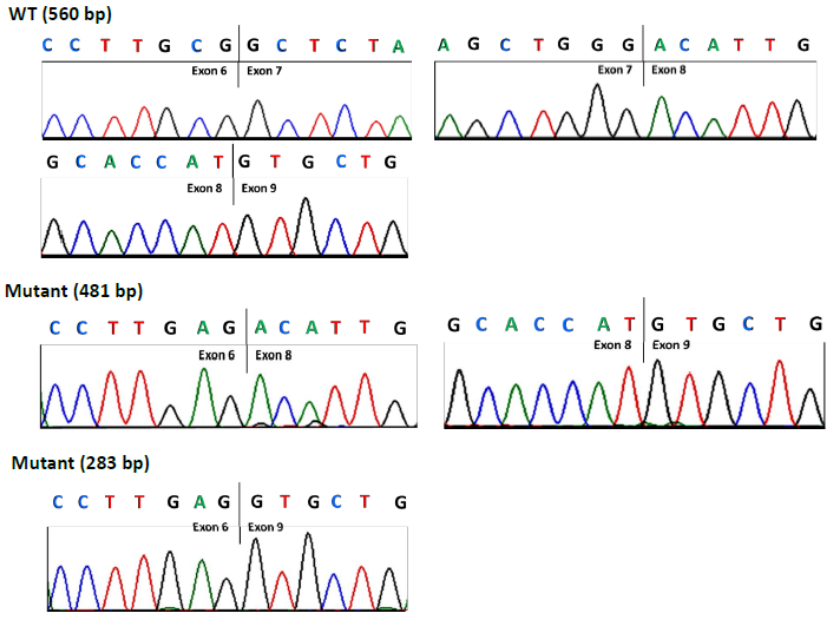

Fig. 7. Electropherograms of the minigene PCR products. The 481 bp mutant fragments skipped the entire exon 7, while containing the full-length sequences of exons 6, 8, 9. The 283 bp mutant PCR product skipped the exons 7 and 8, while retaining full-length sequences of exons 6 and 9. Black lines indicated exon–exon boundaries.

Functional studies of novel *DAX1* mutations (ongoing)

Gene cloning

We got human DAX-1 and SF-1 complementary DNA (cDNA) expression vectors from our collaborator, Prof. Han-Wook Yoo (University of Ulsan College of Medicine, South Korea). StAR promoter sequences from human genomic DNA will be cloned into luciferase reporter vector, encoding the luciferase reporter gene.

Site-directed mutagenesis

The mutations of DAX-1 will be created by QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies, United States) using full-length human DAX-1 cDNA as a template and overlapping PCR strategy with primers containing the appropriate nucleotide substitutions. Mutagenic primers will be designed to create the mutations corresponding to the mutation in the nucleotide sequence of DAX-1 from each patient by Primer3 Program (http://bioinfo.ut.ee/primer3-0.4.0/). Mutagenized plasmid, along with the wild-type plasmid, will be separately transformed into bacterial competent cells by heat shock transformation. The transformed colonies will be selected and extracted for plasmids by High-Speed Plasmid Mini Kit (Geneaid, Taiwan). Mutation sites and cloned plasmids will be confirmed via sequence analysis at Macrogen Inc. (Seoul, Korea) by using the Sequencher program (version 4.2; Gene Codes Corporation, Ann Arbor, MI) The verified plasmid will be transiently transfected into Human embryonic kidney 293T cells (HEK293T) by Lipofectamine 2000 reagent (Invitrogen, United States) according to the manufacturer's instruction.

Luciferase assay

A luciferase reporter construct containing the human StAR promoter region will be cotransfected with expression constructs containing human full-length cDNA of SF-1 and DAX-1 (wild type or mutants) using Lipofectamine 2000 reagent (Invitrogen, United States) according to the manufacturer's instruction. After 24 hours of transfection, the cell will be extracted and the luciferase activity of StAR will be determined. All transfections will be performed in triplicate. Results will be expressed as mean of each triplicate reaction that is a percentage of the DAX-1 empty vector control for that study to allow comparison of the statistical significance of repressive activity of mutants with wild type activity.

Discussion

Over the past 20 years, there has been a significant progress in our understanding of the genetic causes of childhood adrenal insufficiency. However, it is unclear how much these genes contribute to pediatric adrenal disease in Southeast Asian population. In this study, a nationwide cohort of 34 children with atypical CAH or unknown causes of Addison was recruited from 6 pediatric endocrinology centers across Thailand and one center in Singapore. A molecular diagnosis was reached in almost 80% of the children. The genetic etiologies found in this cohort were as follows: *StAR* (n=11), *DAX-1* (n=8), *CYP11B1* (n=3), *HSD3B2* (n=2), *CYP17A1* (n=1), *CYP11A1* (n=1), *WT1* (n=1), unknown (n=7). Several recurrent mutations were discovered, which likely represent founder effects. Some of these are localized to certain geographical areas i.e. p.R188C in StAR in the southern part of Thailand. This study has provided some useful clinical and novel molecular insight into several of these specific conditions.

Defects in StAR (encoding steroidogenic acute regulatory protein) disrupt the transport of cholesterol into mitochondria and classically lead to congenital lipoid adrenal hyperplasia. *StAR* mutations have been described in many ethnic groups, but are common in Japan, Korea, and some isolated population (10, 16, 17). The R188C mutation found in many Thai patients has been also reported in patients from Canada, Jordan, India and Pakistan (8, 9), suggesting a recurrent mutation. StAR mutations in intronic regions can also cause lipoid CAH (18). Most StAR missense mutations are found in the carboxy-terminal 40% of the 285 amino acid StAR protein, and totally eliminate StAR activity. Only five previously described mutations were associated with residual StAR activity in transfected COS cells; V187M (22%) (8), R188C (14%) (8), A218V (6%) (16), M225T (29%) (17), and L275P (10%) (16). The manifestations and severity of disease differed substantially in our patients. Patients 9, 10, 11 had milder phenotypes, were homozygous for R188C. In our assays of intracellular activity, R188C retained ~7% of wild-type activity. Previous studies of this mutant in different assays showed that R188C retained partial activities ~7-20% (8, 10). Hence, while there is some variability in these biochemical assays, it seems that 7-20% activity will dramatically alter the classic phenotype.

Until recently, CYP11A1 mutations in 46,XY individuals are associated with different degrees of disorder of sex development (DSD). In 2011, Parajes et al. first described two

brothers who presented with adrenal insufficiency at 2-4 years of age and normal male genitalia (13). These patients had homozygous CYP11A1 mutation, R451W. In vitro functional studies showed that R451W mutant retained ~30% of wild-type activity. Tee et al. also recently reported 7 children with adrenal insufficiency who lacked disordered sexual development and had CYP11A1 mutations (12). These data demonstrate a broad phenotypic spectrum in patients with CYP11A1 mutations, ranging from normal male, to normal female external genitalia, and from immediate postnatal adrenal failure, to delayed presentation in mid-childhood associated with intercurrent illness. Our patient presented A359V mutation, which was first reported in a 46,XY phenotypic female with life-threatening adrenal insufficiency at age 21 months (19). Our patient had a similar onset of first adrenal symptoms, but slightly different degree of DSD. The functional analysis revealed A359V mutant resulted in a severe functional defect in the P450scc enzyme (∼11%).

To date more than 40 mutations have been identified in the HSD3B2 gene in patients suffering from 3βHSD deficiency. Up to now, no mutations have been reported in the Thai or Indian population. In this study, we have found a previously-described missense mutation and a novel nonsense mutation in two unrelated boys (Thai and Indian) presented with ambiguous genitalia and salt-losing with elevated concentrations of 17OHP. Although underdeveloped genitalia in our 46,XY patients did not support the diagnosis of classic 21-OHD, they were initially misdiagnosed as having 21-OHD because of very high 17-OHP levels. This finding could be explained by the presence of 3βHSD1 isoenzyme in the peripheral tissues, which can extra-adrenally convert the accumulated $\triangle^5$-steroids (20). Basal and ACTH-stimulated 17-OHP levels in some previously reported cases with HSD3B2 mutations were higher than 10,000 ng/dL (300 nmol/L) (1, 21), which is the cut-off level for biochemical diagnosis of classic 21-OHD. Rare cases with 3βHSD deficiency came to medical attention due to a positive result of 17OHP newborn screening (21). Here, we describe the first HSD3B2 gene mutations in the Thai and Indian population; p.T259M and p.Y180X (novel) responsible for classic 3βHSD deficiency and emphasize that the clinical and hormonal phenotypes can be complex in this disorder.

Congenital adrenal hyperplasia due to steroid 11β-hydroxylase deficiency (11β-OHD) is a rare form of CAH associated with low renin hypertension, hypokalemia, hyperandrogenemia and ambiguous genitalia in affected females. In this present study, we describe two siblings

suffering from classic 11β-OHD who were compound heterozygous for a nonsense and a splice-site CYP11B1 mutations. The nonsense p. R141X is expected to lead to a premature stop in the exon 3 and yields a truncated enzyme lacking the essential residues for heme binding domain, consistent with our patients' clinical phenotypes and near-completely abolished in vitro CYP11B1 activity in a recent study (22). In addition, we identified a previously described IVS7+1G>A mutation in CYP11B1 affecting the consensus slice donor site of the exon 7 (23, 24). The minigene experiment confirmed that this splice site mutation caused exon skipping (either a complete loss of the exon 7 or both exons 7 and 8). To date, the therapies to modulate RNA mis-splicing using antisense oligonucleotide or small molecules are emerging (25). Our findings may help for better understanding of splice site mutation mechanism and facilitate the future new therapies targeted on splicing modulation to treat human disease.

CYP17A1 mutations cause 17α-hydroxylase deficiency, a rare form of CAH characterized by sexual infantilism, 46,XY sex reversal, hypertension and high ratios of C21 to C19 steroids (1). The lack of 17α-hydroxylase activity disrupts cortisol secretion, driving the compensatory overproduction of a glucocorticoid, corticosterone, and a mineralocorticoid, deoxycorticosterone, causing hypertension and hypokalemia (1, 26). Over 70 CYP17A1-inactivating mutations have been identified. There is no evidence of a hot spot in most large populations. Therefore, sequencing of the entire coding region is usually necessary. Our patient carried the previously-described mutation R440H and the in vitro studies showed this mutant had undetectable activity when expressed in COS-1 cells (27). Although rare, 17α-hydroxylase deficiency should be considered in any phenotypic female with delayed puberty and low renin hypertension.

DAX-1 mutations cause X-linked adrenal hypoplasia congenita (AHC) and hypogonadotropic hypogonadism in affected males. Affected individuals typically present with adrenal insufficiency in early infancy and later develop hypogonadotropic hypogonadism. Some rare cases can present with late-onset adrenal insufficiency (28). Hemizygous mutations in DAX-1 were found in 8 boys in this cohort. Most are frameshift or nonsense mutations that disrupt protein function, with a clustering of missense changes in three regions of the ligand-like binding domain (29). All patients were male and presented with salt-wasting crisis and hyperpigmentation within a few months of life. Two families had a history of unexplained death

in male relatives on their mother site. One patient had isolated micropenis and the remainder had normal genitalia at birth. Two patients showed signs of hypogonadotropic hypogonadism. Exceptionally, two boys developed macrophallia (penile enlargement) which is emerging as a rare feature of X-linked AHC (30-32). Their testosterone levels were elevated, but LHRH stimulation test suggested a GnRH-independent precocious puberty (GIPP). The association between AHC and GIPP had been rarely described. Although the exact mechanism of premature sexual development in this condition is still unclear, there are some postulated hypotheses include high ACTH stimulated testicular steroidogenesis via melanocortin receptor type 1 (MCR1) and autonomous transient Leydig cell hyperplasia in testes (30-32). Further studies are needed to confirm these hypotheses. However, both patients had spontaneous regression of puberty after increment of hydrocortisone dose.

Conclusions

There is a broad spectrum of the clinical and hormonal phenotypes of CAH depending on the specific enzymatic defect. The diagnosis remains a challenge in patients with atypical forms of CAH and requires thorough clinical and hormonal work-up. Lifelong treatment with steroids is required for most patients. This nationwide cohort study of comprehensive genetic testing of children with rare causes of Addison disease has provided novel clinical and molecular insights and has significant impact on the management of these patients and appropriate counseling for the families.

Acknowledgement

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Output จากโครงการวิจัย

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- Clinical and molecular characterization of patients with congenital adrenal lipoid adrenal hyperplasia in Southeast Asia. (manuscript in preparation, expected the Journal of Clinical Endocrinology and Metabolism)

2. **การนำผลงานวิจัยไปใช้ประโยชน์**

2.1 ความรู้ทางการแพทย์และสาธารณสุข

โรคต่อมหมวกไตทำงานบกพร่องแต่กำเนิด ในเด็กมักเกิดจากความผิดปกติจากการกลายพันธุ์ในยีนต่าง ๆที่เกี่ยวข้องกับการสังเคราะห์ฮอร์โมนจากต่อมหมวกไตหรือการเจริญพัฒนาของต่อมหมวกไต หากไม่ได้รับการวินิจฉัยที่ถูกต้อง อาจมีผลให้ผู้ป่วยเสียชีวิตได้ในช่วงวัยทารกตอนต้นเนื่องจาก salt-wasting crisis การวินิจฉัยโรคกลุ่มโรคดังกล่าวค่อนข้างยาก เนื่องจากลักษณะทางคลินิกและผลการตรวจทางฮอร์โมนมีความซับซ้อน ทำให้แพทย์จำนวนมากอาจมองข้ามการวินิจฉัยโรคดังกล่าว ยิ่งกว่านั้น ยังไม่เคยมีการรายงานหรือการศึกษา

อย่างเป็นระบบในผู้ป่วยเด็กไทยมาก่อน การศึกษานี้เป็นการรวบรวมผู้ป่วยที่มีอาการ อาการแสดง และการตรวจทางห้องปฏิบัติการซึ่งรวมถึง ACTH stimulation test ที่เข้าได้กับโรคต่อมหมวกไตทำงานบกพร่องแต่กำเนิดชนิดวินิจฉัยยาก มาทำการศึกษาหาการกลายพันธุ์ของยีนต่างๆใน adrenal steroidogenesis pathway นำไปสู่การยืนยันการวินิจฉัยโรค การรักษาที่ถูกต้องเหมาะสมและการให้คำปรึกษาทางพันธุกรรมแก่ครอบครัว เพื่อให้เกิดประโยชน์สูงสุดต่อผู้ป่วยและครอบครัว นอกจากนี้ การศึกษาเพิ่มเติมถึงการทำงานของยีนที่เกิดจากการการกลายพันธุ์ ช่วยให้เกิดความเข้าใจเพิ่มขึ้นเกี่ยวกับการทำงานของยีน นำไปสู่การสร้างองค์ความรู้ใหม่เกี่ยวกับกลไกการเกิดโรค ซึ่งความรู้เหล่านี้อาจจะถูกนำไปประยุกต์ใช้ในการพัฒนาวิธีการรักษาโรคต่อไปในอนาคต

2.2 เชิงวิชาการ (ผลิตบัณฑิตชั้นสูง สร้างนักวิจัยใหม่)

- โครงการวิจัยเรื่อง Splicing analysis of CYP11B1 mutation in a family affected with 11β-hydroxylase deficiency [BMC Endocr Disord. 2016;16:37] ได้ผลิตนิสิตในหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชาพันธุศาสตร์ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย 1 ราย (น.ส.ภัทรณัชชา ชาญวิชัย)
- โครงการวิจัยเรื่อง Mutation identification and functional analysis of mutant DAX-1 proteins found in Thai patients with X-linked Adrenal Hypoplasia Congenita (X-linked AHC) เป็นโครงการวิจัยเพื่อปริญญาวิทยาศาสตรดุษฎีบัณฑิต หลักสูตรวิทยาศาสตร์ชีวภาพ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย (น.ส.ชนิสรา สุทธิวรชัย)

2.3 เชิงสาธารณะ

- จากงานวิจัยเรื่องต่อมหมวกไตโตแต่กำเนิด ทำให้ผู้วิจัยได้รับเชิญเป็นที่ปรึกษาโครงการวิจัยของสถาบันชีววิทยาศาสตร์การแพทย์ กรมวิทยาศาสตร์การแพทย์ เพื่อทำโครงการวิจัยนำร่อง Pilot CAH newborn screening program ในเขตสุขภาพที่ 8 ซึ่งผู้วิจัยได้มีโอกาสไปบรรยาย และเข้าร่วมสัมมนาเชิงปฏิบัติการ ครั้งที่ 1 เมื่อวันที่ 29 มีนาคม 2560 สถานที่โรงแรม Centara Hotel & Convention center จังหวัดอุดรธานี ซึ่งขณะนี้กำลังอยู่ในระหว่างดำเนินการวิจัย และหาผลการศึกษาพบว่ามีความคุ้มค่า อาจทำให้มีการผลักดันนโยบายตรวจคัดกรองโรคต่อมหมวกไตโตแต่กำเนิด ในเด็กทารกแรกเกิดทุกรายในอนาคต (ใช้เลือดหยดกระดาษกรอง เช่นเดียวกับการตรวจคัดกรองโรคพร่องไทรอยด์แต่กำเนิด)

3. ผลงานอื่น ๆ

3.1 การเสนอผลงานในที่ประชุมวิชาการ

ผลงานวิจัยจากโครงการนี้ได้รับการตอบรับให้ไปเสนอผลงานวิชาการของการประชุมของ APPES & APEG joint meeting ซึ่งเป็นการประชุมวิชาการประจำปีที่ใหญ่ที่สุดของกุมารแพทย์ต่อมไร้ท่อในกลุ่มประเทศในภูมิภาค Asia Pacific และการประชุมวิชาการอื่นๆ ดังนี้

- เสนอผลงานทางวิชาการในรูปแบบ Oral เรื่อง “Clinical and mutational spectrum of patients with congenital lipoid adrenal hyperplasia in southeast Asia” ในงานประชุม APPES & APEG joint meeting วันที่ 30 ตุลาคม – 1 พฤศจิกายน พ.ศ. 2557 สถานที่ เมืองดาร์วิน ประเทศออสเตรเลีย
- เสนอผลงานทางวิชาการในรูปแบบ Poster เรื่อง “Clinical and molecular characterization of patients with classic 3β-hydroxysteroid dehydrogenase deficiency” ในงานประชุม APPES & APEG joint meeting วันที่ 30 ตุลาคม – 1 พฤศจิกายน พ.ศ. 2557 สถานที่ เมืองดาร์วิน ประเทศออสเตรเลีย
- เสนอผลงานทางวิชาการในรูปแบบ Poster เรื่อง “GnRH-independent Precocious Puberty in a Thai boy with NR0B1 novel mutation causing X-linked Adrenal Hypoplasia Congenita” ในงานประชุม the 9th Biennial Scientific Meeting of Asia Pacific Paediatric Endocrine Society (APPES) วันที่ 17-20 พฤศจิกายน พ.ศ.2559 สถานที่ กรุงโตเกียว ประเทศญี่ปุ่น
- เสนอผลงานทางวิชาการในรูปแบบ Poster เรื่อง “A WT1 mutation associated with severe form of Denys-Drash syndrome” ในงานการประชุมวิชาการพันธุศาสตร์แห่งชาติ ครั้งที่ 19 (National Genetics Conference 2015; NGC 2015) สถานที่ ณ โรงแรมเซ็นทารา แอนด์ คอนเวนชั่นเซนเตอร์ ขอนแก่น จ.ขอนแก่น
- เสนอผลงานทางวิชาการในรูปแบบ Oral เรื่อง “Mutation analysis of DAX-1 gene in Thai patients with X-linked Adrenal Hypoplasia Congenita (X-linked AHC)” ในงานประชุม The 21st Biological Sciences Graduate Congress (BSGC 2016) at University of Malaya, Malaysia วันที่ 15-17 ธันวาคม 2559

3.2 การได้รับเชิญเป็นวิทยากร

ความรู้จากงานวิจัยในโครงการนี้ ได้นำไปใช้ประกอบการบรรยายที่ได้รับเชิญไปในการประชุมต่างๆ ดังนี้

- วิทยากรบรรยาย (invited plenary speaker) เรื่อง “Diagnosis and Management of rare forms of CAH” ในการประชุม “APPES & APEG joint meeting 2014” (30 ตุลาคม 2557, Darwin convention center, เมืองดาร์วิน ประเทศออสเตรเลีย)
- วิทยากรบรรยายเรื่อง “Adrenal disorders: Management and long term follow-up” การประชุมอบรมระยะสั้น pediatric endocrinology: bridging theory to clinical practice (16 พฤษภาคม 2557, อาคารเฉลิมพระเกียรติฯ รพ.พระมงกุฎเกล้า, กรุงเทพฯ)
- วิทยากรบรรยายเรื่อง “Steroidogenesis & Adrenal disorders” ในการประชุม 3rd Pediatric Endocrine Fellow course 2014 ของชมรมต่อมไร้ท่อเด็กและวัยรุ่นแห่งประเทศไทย (13 ธันวาคม 2557, โรงแรมแรนโชชาญวีร์ จ.นครราชสีมา)
- วิทยากรบรรยายเรื่อง “CAH: pediatric to adult” ในการประชุมวิชาการต่อมไร้ท่อสัญจร (Endocrine weekend 2015) โดยสมาคมต่อมไร้ท่อแห่งประเทศไทย วันที่ 22 สิงหาคม พ.ศ. 2558 สถานที่ โรงแรมดุสิตธานี พัทยา จังหวัดชลบุรี
- วิทยากรบรรยายเรื่อง “Adrenal insufficiency” ในการประชุม Practical Endocrinology for Pediatricians 2016 โดยชมรมต่อมไร้ท่อเด็กและวัยรุ่นแห่งประเทศไทย วันที่ 27-29 กรกฎาคม พ.ศ.2559 ห้องประชุมชั้น 10 อาคารเฉลิมพระเกียรติ รพ.พระมงกุฎเกล้า

3.3 การเชื่อมโยงทางวิชาการกับนักวิชาการอื่น ๆทั้งในและต่างประเทศ

จากผลงานวิจัยชิ้นนี้ทำให้เกิดความร่วมมือกับแพทย์ต่อมไร้ท่อจากสถาบันอื่นๆในประเทศไทยและต่างประเทศ และได้มีการติดต่อร่วมมือต่อเนื่องในการศึกษาวิจัยผู้ป่วยโรค CAH รวมถึงความร่วมมือในการศึกษาวิจัยโรคอื่นๆ ใน steroidogenesis pathway อีกด้วย

ลงนาม.....

(รศ.พญ.ธนินี สหกิจรุ่งเรือง)

หัวหน้าโครงการวิจัยผู้รับทุน

ภาคผนวก

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Clinical and molecular review of atypical congenital adrenal hyperplasia

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Congenital adrenal hyperplasia (CAH) is one of the most common inherited metabolic disorders. It comprises a group of autosomal recessive disorders caused by the mutations in the genes encoding for steroidogenic enzymes that involved cortisol synthesis. More than 90% of cases are caused by a defect in the enzyme 21-hydroxylase. Four other enzyme deficiencies (cholesterol side-chain cleavage, 17α-hydroxylase [P450c17], 11β-hydroxylase [P450c11β], 3β-hydroxysteroid dehydrogenase) in the steroid biosynthesis pathway, along with one cholesterol transport protein defect (steroidogenic acute regulatory protein), and one electron-transfer protein (P450 oxidoreductase) account for the remaining cases. The clinical symptoms of the different forms of CAH result from the particular hormones that are deficient and those that are produced in excess. A characteristic feature of CAH is genital ambiguity or disordered sex development, and most variants are associated with glucocorticoid deficiency. However, in the rare forms of CAH other than 21-hydroxylase deficiency so-called "atypical CAH", the clinical and hormonal phenotypes can be more complicated, and are not well recognized. This review will focus on the atypical forms of CAH, including the genetic analyses, and phenotypic correlates.

Keywords: Congenital adrenal hyperplasia, Rare disease, Genotypes, Phenotypes

Introduction

Congenital adrenal hyperplasia (CAH) is one of the most common inherited metabolic disorders and is associated with significant morbidity and mortality in affected children. It comprises a group of autosomal recessive disorders caused by the mutations in the genes encoding for steroidogenic enzymes that involved cortisol synthesis. Impaired cortisol secretion results in hypersecretion of corticotropin-releasing hormone and adrenocorticotropic hormone (ACTH) and consequent hyperplasia of the adrenal glands. The clinical phenotypes and biochemical characteristics depend on the specific enzymatic defect. There is a broad clinical spectrum of this disorder. In most forms of CAH, it can be fatal if not diagnosed early in infancy[1].

The adrenal cortex is the production site for three classes of steroid hormones: mineralocorticoids, glucocorticoids, and sex hormones. The cortex is divided into three zones by different cellular arrangements, each one functionally distinct due to the enzymes required for different hormone production. The outer zona glomerulosa does not express 17α-hydroxylase (P450c17)[2] and hence produces 17-deoxysteroids leading to aldosterone (the most potent mineralocorticoid), and is regulated primarily by the renin/angiotensin system. The middle zona fasciculata expresses the 17α-hydroxylase activity but very little of the 17,20-lyase activity of P450c17, and hence produces 21-carbon, 17-hydroxysteroids, leading to cortisol under the influence of ACTH. The inner zona reticularis expresses both the 17α-hydroxylase and 17,20-lyase activities of P450c17, and hence produce the 19-carbon

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17-hydroxy steroid dehydroepiandrosterone (DHEA), the precursor of sex steroids[1,3].

ACTH regulates steroidogenesis (chronic regulation) by inducing the transcription of genes encoding various steroidogenic enzymes, but acute regulation is at the level of cholesterol access to cholesterol side-chain cleavage (P450scc). The steroidogenic acute regulatory protein (StAR) facilitates the movement of cholesterol into mitochondria, where it is converted to pregnenolone by P450scc[4]. Simplified diagram of adrenal steroidogenic pathways was shown in Fig. 1.

There have been nationwide newborn screening programs for CAH in only a few countries in Asia, although the recent pilot study suggested the high prevalence of CAH in Southeast Asian countries[5,6]. More than 90% of cases are caused by a defect in the enzyme 21-hydroxylase (P450c21). Four other enzyme deficiencies in the steroid biosynthesis pathway (P450scc, P450c17, 11β-hydroxylase [P450c11β], 3β-hydroxysteroid dehydrogenase [3βHSD]), along with one cholesterol transport protein defect (StAR), and one electron-transfer protein (P450 oxidoreductase; POR) account for the remaining cases. In these uncommon forms of CAH, the clinical and hormonal phenotypes can be complicated, and are not widely recognized by endocrinologists or pediatricians.

The clinical symptoms of the different forms of CAH result from the particular hormones that are deficient and those that are produced in excess. A characteristic feature of CAH is genital ambiguity or disordered sex development (DSD), and all variants are associated with glucocorticoid deficiency. Each variant of CAH is summarized in Table 1. In this review, we focus on the molecular genetic basis of the variant forms of CAH other than 21-hydroxylase so-called "atypical CAH", including the genetic analysis, and phenotypic correlates.

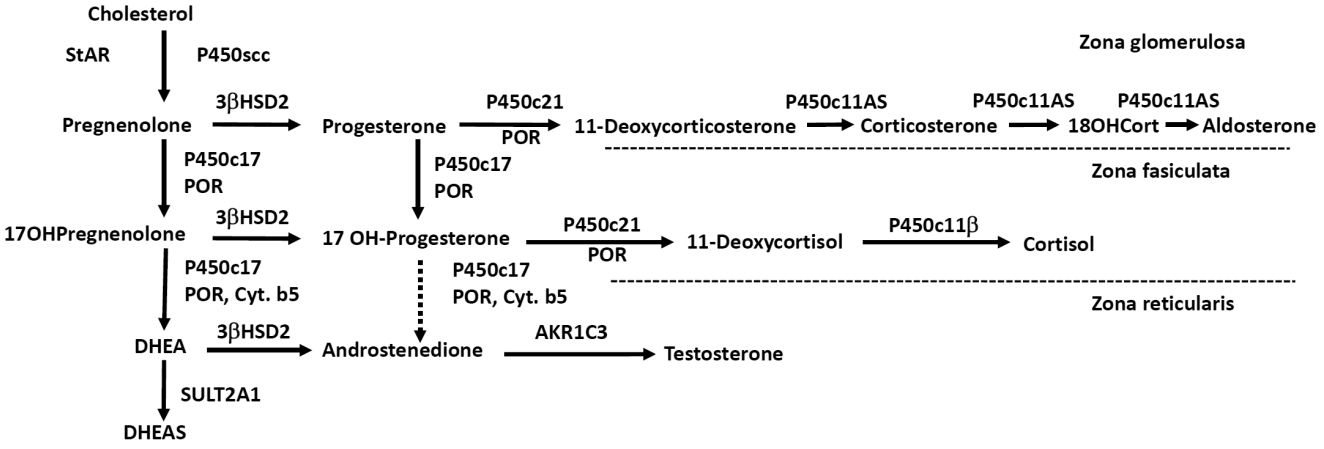

Fig. 1. Simplified scheme of adrenal steroidogenesis. AKR, aldo-keto reductase; Cyt.b5, cytochrome b5; P450scc, cholesterol side-chain cleavage enzyme; P450c17, 17α-hydroxylase/17,20-lyase; 3βHSD2, 3β-hydroxysteroid dehydrogenase type 2; P450c21, 21-hydroxylase; P450c11AS, aldosterone synthase; P450c11β, 11β-hydroxylase; POR, P450 oxidoreductase; StAR, steroidogenic acute regulatory protein; SULT2A1, sulfotransferase; 18OHCort, 18-hydroxycorticosterone.

Table 1. Characteristics of different forms of congenital adrenal hyperplasia (CAH)

Characteristic	21-Hydroxylase deficiency	11β-Hydroxylase deficiency	17α-Hydroxylase deficiency	3β-Hydroxysteroid dehydrogenase deficiency	Congenital lipoid adrenal hyperplasia	P450 Oxidoreductase deficiency
Gene involved	*CYP21A2*	*CYP11B1*	*CYP17A1*	*HSD3B2*	*StAR/CYP11A1*	*POR*
Chromosome location	6p21.3	8q24.3	10q24.3	1p13.1	8p11.2/15q24.1	7q11.2
Ambiguous genitalia	Yes in XX	Yes in XX	Yes in XY	Yes in XY, ± in XX	Yes in XY	Yes in both XX and XY
Adrenal crisis	Yes	Rare	No	Yes	Yes	Rare
Serum cortisol	↓	↓	↓	↓	↓	Normal/ ↓
Mineralocorticoid	↓	↑	↑	↓	↓	Normal
Androgens	↑	↑	↓	↓	↓	↓
Serum Na	↓	↑	↑	↓	↓	Normal/ ↓
Serum K	↑	↓	↓	↑	↑	Normal
Metabolite elevated	17OHP	DOC, 11-deoxycortisol	DOC, corticosterone	DHEA, 17OHPreg	None	17OHP

17OHP, 17-hydroxyprogesterone; DOC, deoxycorticosterone; DHEA, dehydroepiandrosterone; 17OHPreg, 17-hydroxypregnenolone.

Congenital lipoid adrenal hyperplasia (lipoid CAH)

Congenital lipoid adrenal hyperplasia is the most severe form of CAH in which the synthesis of all adrenal and gonadal steroid hormones is impaired and leads to the accumulation of cholesterol esters[7,8]. The true incidence of lipoid CAH is unknown, but it is clearly much higher in Japanese, Korean and Palestinian population. Patients with classic lipoid CAH usually present with adrenal failure and salt wasting, beginning within the first few months of life, and have female external genitalia irrespective of genetic sex[1].

Early clinical hormonal studies and incubations of affected tissue *in vitro* with various precursors identified a defect in the conversion of cholesterol to pregnenolone, so that the disorder was initially called "20,22 desmolase deficiency" as it was thought to result from a defect in the enzyme system converting cholesterol to pregnenolone; this enzyme was later identified as mitochondrial P450scc. However, in 1995 it was found that lipoid CAH results from mutations in the gene encoding the StAR[7]. StAR facilitates the movement of cholesterol into mitochondria, where it is converted to pregnenolone by P450scc[4]. StAR is expressed in the adrenals and gonads but not in the placenta[9]. Because placental production of progesterone is essential for the maintenance of human pregnancy, mutations in P450scc were thought to be incompatible with term gestation. Nevertheless, beginning in 2001 several patients with defects in *CYP11A1* gene causing P450scc deficiency have been reported[10-17]. Most of these cases were caused by severe loss-of function mutations and presented with severe, early onset adrenal failure and complete phenotypic 46,XY sex reversal in genetic males[14], although patients with late-onset nonclassic disease have been reported[15-18]. Thus, both of the defects of StAR and P450scc are now considered to be responsible for lipoid CAH, but most lipoid CAH may be caused by the mutations in the *StAR* gene.

More than 40 StAR mutations causing classic lipoid CAH have been described, but very few partial loss-of-function mutations have been reported[19-21]. The mutations are present in all exons. The mutations in intronic region are also found. The mutations causing premature translational termination or altering of the StAR reading frame are common, and they substantially alter the structure of the StAR protein. All missense mutations are found in the carboxy-terminal 40% of the amino acid StAR protein[22]. The Q258X mutation in exon 7 is very common in Japanese and Korean[23,24]. In Japanese this mutation is identified in 62% of the alleles and in over 80% of the patients. Other genetic clusters are found among Palestinian Arabs, most of whom carry the mutation R182L[8]; in eastern Saudi Arabia, carrying R182H[25]; and in parts of Switzerland, carrying the mutation L260P[26].

Nonclassic lipoid CAH is a recently recognized disorder caused by StAR mutations that retain partial activity[19]. Affected individuals can present with later onset of adrenal insufficiency resembling nonautoimmune Addison disease with only mildly disordered sexual development or normal development with hypergonadotropic hypogonadism[19-21]. We recently reported four patients with nonclassic/atypical lipoid CAH and demonstrated that there is a broad clinical spectrum of StAR mutations[21]. While there is some variability in these biochemical assays, it seems that 10%–20% activity will dramatically alter the classic phenotype[21]. The R188C mutation was found in patients from Thailand, Canada, Jordan, India and Pakistan[19-21], suggesting a recurrent mutation. To date, most patients with non-classic lipoid CAH carry R188C, although other mutations can cause this phenotype[19-21].

3βHSD deficiency

3βHSD or Δ5→Δ4-isomerase is a 42 kDa microsomal enzyme catalyzes steroidogenic reactions: the conversion of the hydroxyl group to a keto group on carbon 3 and the isomerization of Δ5 steroids precursors into Δ4 ketosteroids[27]. Therefore, 3βHSD is responsible for the conversion of pregnenolone to progesterone, 17α-hydroxypregnenolone (17OHPreg) to 17α-hydroxyprogesterone (17OHP), DHEA to androstenedione, and androstenediol to testosterone. Thus, 3βHSD is an essential enzyme for biosynthesis of all classes of active steroid hormones including aldosterone, and cortisol in adrenal cortex, and sex steroids in adrenals and gonads.

In humans, there are two closely linked genes *HSD3B1* and *HSD3B2* located on chromosome 1 encoded two isoforms of 3βHSD[28]. The type 1 enzyme (3βHSD1) encoded by *HSD3B1* is primarily expressed in placenta, mammary gland, liver, skin and some other tissues[29]. 3βHSD1 is required for placental progesterone synthesis during pregnancy. Mutations in *HSD3B1* gene have never been described, presumably because these would cause a spontaneous abortion due to lack of placental progesterone synthesis. In contrast, the type 2 enzyme (3βHSD2) encoded by *HSD3B2* gene is predominantly expressed in the adrenals and gonads[29]. Defects in *HSD3B2* gene causes 3βHSD deficiency, which is a rare form of CAH, and can be fatal if not diagnosed early in infancy[30].

The clinical spectrum of 3βHSD deficiency ranges from salt-wasting to non–salt-wasting forms. In its classic form, 3βHSD deficiency causes various degrees of salt-wasting in both sexes. In genetic males, 3βHSD deficiency in the testes impairs testosterone biosynthesis from early fetal life, so that these males have undervirilization of the external genitalia, and usually present at birth with severe hypospadias and micropenis. By contrast, genetic females have normal female genitalia or slightly virilized genitalia such as isolated clitoromegaly, because the fetal adrenal overproduces large amounts of DHEA, which can be converted to testosterone by extraadrenal 3βHSD1[1,31]. In this way, the presence of peripheral 3βHSD1 activity often complicates the hormonal diagnosis of this disorder in that very high 17OHPreg levels can be converted extra-adrenally to 17OHP confuses the diagnosis as 21-hydroxylase deficiency[31,32].

Mild forms of 3βHSD deficiency cause premature acne, premature pubarche, and growth acceleration in children[33] and a late onset variant manifesting with hirsutism, menstrual disorder, and polycystic ovaries in young women[34,35]. The newly proposed hormonal criteria for diagnosis for 3βHSD deficiency were elevated basal and ACTH-stimulated 17OHPreg and 17OHPreg to cortisol ratios, typically exceed 10 standard deviations above the mean[36]. These criteria were revised based on genotype-proven patients.

To date approximately 40 mutations have been identified in the *HSD3B2* gene in patients suffering from classical 3βHSD deficiency. In most cases, the functional consequences of *HSD3B2* mutations are in close agreement with the severity of the clinical manifestation. However, the *in vitro* 3βHSD activities alone cannot be used to predict the degree of male undervirilization[31].

P450c11β deficiency

11β-hydroxylase deficiency (11OHD) accounts for about 5%–8% of CAH in people of European ancestry but accounts for about 15% of cases in both Muslim and Jewish Middle Eastern populations[1]. P450c11β catalyses conversion of 11-deoxycortisol to cortisol, representing the final step in cortisol biosynthesis. The enzyme also catalyses the monooxygenase reaction converting 11-deoxycorticosterone (DOC) to corticosterone. Thus, deficient P450c11β activity results in decreased cortisol secretion and accumulation of 11-deoxycortisol and the mineralocorticoid precursor DOC. Thus, patients can subsequently suffer from significant hypertension, a hallmark feature of this CAH variant. Accumulated precursors are shunted into the androgen synthesis pathway, leading to hyperandrogenism. Classic 11OHD most commonly results in 46,XX DSD with severe virilization of the external genitalia, and precocious pseudopuberty in both sexes. Newborns may also have elevated concentrations of 17OHP, which accumulates two steps behind the enzymatic block, so that P450c11β deficiency may be detected in newborn screening for P450c21 deficiency[37]. The diagnosis is established by elevated basal concentrations of DOC and 11-deoxycortisol, which hyperrespond to cosyntropin. 11OHD is caused by mutations in the *CYP11B1* gene. At present, over 50 *CYP11B1*-inactivating mutations are described. Most are missense and nonsense mutations, but splice-site mutations, small deletions, small insertions, and complex rearrangements have also been detected[38-40]. The vast majority of mutations are associated with classic 11OHD, and only a few mutations causing nonclassic 11OHD have been described in otherwise asymptomatic women with hirsutism, and menstrual irregularities[41,42].

Generally, the *CYP11B1* gene is specifically amplified avoiding simultaneous amplification of homologous *CYP11B2* sequences. In the majority of cases, molecular genetic analysis is not difficult. However, special cases are reported, such as an unequal crossing-over between the *CYP11B2* and the *CYP11B1* genes as a cause of 11OHD[43].

P450c17 deficiency

P450c17 is the single microsomal cytochrome P450 enzyme that catalyzes both the 17α-hydroxylation required to produce the 17 hydroxy 21-carbon precursors of cortisol, 17OHPreg and 17OHP, and the 17,20-lyase activity needed to produce 19-carbon precursors of sex steroids[44]. P450c17 is encoded by *CYP17A1* gene, consisting of eight exons and located on chromosome 10q24.3[45]. *CYP17A1* mutations cause P450c17 deficiency, a rare form of CAH characterized by sexual infantilism, 46,XY sex reversal, hypertension and high ratios of C21 to C19 steroids. The lack of P450c17 activity disrupts cortisol secretion, driving the compensatory overproduction of a glucocorticoid, corticosterone, and a mineralocorticoid, deoxycorticosterone, causing hypertension and hypokalemia[1,46]. Rare patients may also have isolated 17,20 lyase deficiency, characterized by low C19 steroids with normal cortisol[47,48].

Over 70 *CYP17A1*-inactivating mutations have been identified. There is no evidence of a hot spot in most large populations. Therefore, sequencing of the entire coding region is usually necessary. Exceptions have been described in the some population, where mutations appear recurrently (1) a duplication of four nucleotides causing a frameshift is found among descendents of Dutch Frieslanders; (2) in-frame deletion of residues 487–489 is found throughout Southeast Asia; (3) a deletion of phenylalanine at position 53 or 54; and (4) the common W406R and R362C mutations, found among Brazilians of Spanish and Portuguese ancestry, respectively [1].

Mutations underlying isolated 17,20 lyase deficiency result in amino acid substitutions located within the redox-partner binding site of P450c17, thereby disrupting the electron transfer from POR to P450c17 specifically for the conversion of 17OHPreg to DHEA[47,48].

POR deficiency

POR deficiency is a unique and newly recognized form of CAH, biochemically manifesting with apparent combined P450c17 and P450c21 deficiency. POR transfers electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to all microsomal (type II) cytochrome P450 enzymes, including three steroidogenic enzymes: P450c17 (17α-hydroxylase/17,20 lyase), P450c21 (21-hydroxylase), and P450aro (aromatase)[1]. Although disruption of the *POR* gene in mice causes gross disorders of embryogenesis and embryonic lethality, in 2004 Fluck et al.[49] reported *POR* mutations in three children with ambiguous genitalia and skeletal malformations (Antley-Bixler syndrome, ABS) and in a phenotypically normal adult woman with primary amenorrhea and polycystic ovaries. The majority of patients with POR deficiency described to date have also had the ABS phenotype, characterized by craniosynostosis, radioulnar or radiohumeral synostosis,

bowed femora, and other variable skeletal disorders[1,50]. POR deficiency can cause ambiguous genitalia in both sexes. 46,XY males are typically undervirilized because decreased 17,20-lyase activity reduces androgen synthesis. 46,XX females are frequently virilized at birth, but this virilization is not progressive postnatally. There are two possible mechanisms for this virilization. First, because placental aromatase (P450aro) requires POR, a defect in this placental aromatase activity, either from mutation of POR or P450aro itself, will permit large amounts of fetal C19 steroids to enter and virilize the mother and the female fetus. Second, it appears to involve the "backdoor pathway" to fetal androgen production, in which 21-carbon steroid precursors are 5α-reduced and ultimately converted to dihydrotestosterone, bypassing the conventional precursors androstenedione and testosterone[1,50].

The human *POR* gene consists of 16 exons, spanning approximately 70 kb on chromosome 7q11.2. The overall incidence of POR deficiency in the general population remains unclear. However, over 50 *POR* mutations have now been described, suggesting that this disorder may be relatively common. There is the great variability in the clinical and hormonal findings in POR deficiency. Some patients with milder *POR* mutations do not have ABS, and the steroidogenic defect may present as hypogonadism and/or infertility[49-52]. Two mutations are especially common: A287P, the predominant mutation in patients of European ancestry, and R457H, the predominant mutation in patients of Japanese ancestry[53-55]. The genotype-phenotype correlation is not fully established yet[55,56], and future studies are needed.

Conclusions

There is a broad spectrum of the clinical and hormonal phenotypes of CAH depending on the specific enzymatic defect. The diagnosis remains a challenge in patients with atypical forms of CAH and requires thorough clinical and hormonal work-up. Lifelong treatment with steroids is required for most patients. Confirmation of the diagnosis by genetic analysis is of clinical importance.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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CASE REPORT Open Access

Splicing analysis of *CYP11B1* mutation in a family affected with 11β-hydroxylase deficiency: case report

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Abstract

Background: Congenital adrenal hyperplasia (CAH) due to steroid 11β-hydroxylase deficiency (11β-OHD) is a rare form of CAH associated with low renin hypertension, hypokalemia, hyperandrogenemia and ambiguous genitalia in affected females. Herein we describe the clinical, hormonal and molecular characteristics of two Uzbekistan siblings with 11β-OHD and analyze the effects of a splicing mutation.

Case presentation: A 46,XX girl presented with genital ambiguity and low renin hypertension; her 46,XY brother presented with precocious puberty. Hormonal studies suggested 11β-OHD. Mutation analysis was performed by PCR followed by Sanger sequencing of the entire coding regions and their flanking introns of the *CYP11B1* gene. Mutation analysis showed that both patients were compound heterozygous for IVS7 + 1G > A, and c.421C > T. Although the identified mutations have been previously described, this is, to our knowledge, the first report of these mutations in compound heterozygotes. A minigene assay was used to determine the effects of the splicing mutation. The constructs containing either the wild-type or the splice-site mutant *CYP11B1* genomic DNA of exons-introns 6–9 were transfected into COS-7 cells; subsequently, RNA splicing was assessed by reversed transcribed-PCR of *CYP11B1* complementary DNA. The minigene assay revealed that the IVS7 + 1G > A mutation resulted in two shorter incorrectly spliced products; one skipping the exon 7 and the other skipping the exons 7–8. The c.421C > T mutation leads to the introduction of a premature stop codon at residue 141 (p.R141X). These mutations are expected to code non-functional proteins.

Conclusion: Compound heterozygous mutations (IVS7 + 1G > A and p.R141X) in the *CYP11B1* gene were found to cause 11β-OHD. The IVS7 + 1G > A mutation causes aberrant splicing of *CYP11B1* leading to exon skipping. This finding could facilitate the future novel therapies targeted on splicing modulation to treat human disease.

Keywords: *CYP11B1*, Splicing, Mutation, 11β-hydroxylase deficiency, Congenital adrenal hyperplasia, Case report

Background

11β-hydroxylase deficiency (11β-OHD) caused by mutations in the *CYP11B1* gene accounts for approximately 5–8 % of congenital adrenal hyperplasia (CAH) in non-consanguineous populations, but accounts for ~15 % of cases in both Muslim and Jewish Middle Eastern populations [1]. Steroid 11β-hydroxylase (P450c11β, *CYP11B1*) converts 11-deoxycortisol to cortisol, representing the final step in cortisol biosynthesis, and 11-deoxycorticosterone (DOC) to corticosterone. Thus, deficient P450c11β activity results in impaired cortisol synthesis and accumulation of 11-deoxycortisol and the mineralocorticoid precursor DOC, which leads to significant hypertension, a hallmark feature of this CAH variant [1]. Accumulated steroid precursors are shunted into the androgen synthesis pathway, leading to androgen excess. Classic 11β-OHD results in virilization of the external genitalia in affected females (46,XX disorders of sex development) as well as precocious puberty, accelerated growth and bone maturation in both sexes. Patients with 11β-OHD can have elevated concentrations of 17-hydroxyprogesterone (17OHP), which accumulates two steps behind the enzymatic block, so that 11β-

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OHD may be detected by 17OHP newborn screening program [2]. The diagnosis is established by elevated basal concentrations of DOC and 11-deoxycortisol, which hyper respond to ACTH stimulation.

CYP11B1 is located on the long arm of chromosome 8 (8q21), consisting of 9 exons, and encodes 503 amino acids. To date, over 80 mutations in *CYP11B1* gene are described. Most are missense and nonsense mutations, but splice-site mutations, small or gross deletions/insertions, and complex rearrangements with *CYP11B2* have also been identified [3–5]. The majority of *CYP11B1* mutations are associated with classic 11β-OHD, and only a few mutations causing non-classic 11β-OHD which can manifest later in otherwise asymptomatic women with hirsutism, and menstrual irregularities [6, 7].

In this report, we describe two siblings with the clinical and hormonal phenotypes of 11β-OHD and identified compound heterozygous mutations in the *CYP11B1* gene. The splicing mutation was studied in vitro for its functional consequences with a minigene experiment and showed exon-skipping which confirmed the clinical diagnosis.

Case presentation

The patients were siblings from a non-consanguineous Uzbekistan family. *Patient 1* was a 3-yr-old 46,XX female with ambiguous genitalia. She was previously evaluated for her abnormal genital development and underwent first genital surgery in Turkey. On an initial evaluation at age 2 y 8 m, her height was 100 cm (+2.1 SD), her weight was 15.8 kg, (+1.4 SD), blood pressure (BP) was 110/70 mmHg (94^{th}/96^{th} percentiles). The physical examination revealed that the phallus was 5 cm long and 2 cm wide (Prader grade IV); no gonads were palpable in the inguinal region. The areola and palmar creases were pigmented bilaterally. An ACTH stimulation test (250 μg) showed grossly elevated baseline ACTH (238 pg/mL) and basal cortisol of 4.7 μg/dL with non-response to ACTH and moderately elevated progesterone and 17OHP after 60 min; 11-deoxycortisol and androstenedione concentrations were markedly high (Table 1). The serum sodium was 136 mmol/L, potassium 3.1 mmol/L, plasma renin activity (PRA) was very low at 15 ng/dL/h (nl, 171–1115), and aldosterone 2 ng/dL (nl, 3–35). Her total testosterone levels were 132 ng/dL (nl, <3–10) and dehydroepiandrosterone sulfate (DHEAS) 165.2 μg/dL (nl, <5-57). She was followed up at the King Chulalongkorn Memorial Hospital (Bangkok, Thailand) due to the family relocation at age 3 yr for further management. After receiving the results of an ACTH stimulation test, she was started treatment with hydrocortisone, 5 mg thrice daily (10 mg/m^2/d) which improved BP into the normal range (90/60 mmHg), suppressed testosterone, and PRA became measurable (200–496 ng/dL/h).

Patient 2 is the younger brother of Patient 1. He presented at 2 years of age with acne and masculinization (isosexual precocious puberty). Physical examination revealed an advanced maturation of external genitalia as well as a low-pitched voice. His Tanner stages were G3 and PH1, and each of his testes was 3 mL in volume. His height was 97 cm (+3.2 SD) and weight was 17 kg (+2.9 SD). Height gain was accelerated from 12-month-old on the growth chart (from +2.6 SD to +3.2 SD). Skin pigmentation appeared consistent with his ethnicity, but no evident mucosal pigmentation. His BP was 110/65 mmHg (92^{th}/95^{th} percentiles). Labs revealed serum Na 136 mmol/L, K 4.3 mmol/L, bicarbonate 24 mmol/L, BUN 12 and Cr 0.3 mg/dL, respectively. An ACTH stimulation test showed elevated baseline ACTH, a low basal cortisol (2.3 μg/dL) with non-response to ACTH and moderately elevated 17OHP after 60 min (Table 1). 11-deoxycortisol concentrations were not measured. PRA was low at 106 ng/dL/h (nl, 171–1115), and low aldosterone 0.2 ng/dL (nl, 3–35). He was then treated with hydrocortisone 2.5 mg thrice daily (11 mg/m^2/d). His blood pressure was well controlled, and PRA was increased up to 738 ng/dL/h.

DNA sequencing

Genomic DNA from peripheral blood leucocytes of the patients and their parents was extracted by using the QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA) after taking informed consent. The coding sequence of

Table 1 Basal and 60 min post ACTH (250 μg) stimulated adrenal steroid profile

Steroids	Patient 1 (age 2 y 8 m)		Patient 2 (age 2 y)		Reference values	
	Basal	Stimulated	Basal	Stimulated	Basal	Stimulated
ACTH (pg/mL)	238	-	150	-	10–65	-
Cortisol (μg/dL)	4.72	4.65	2.3	2.3	3–22	27–50
17-OHP (ng/dL)	2880	2730	1310	2380	13–173	85–250
Progesterone (ng/dL)	464	499	-	-	[a]	[a]
Androstenedione (ng/dL)	2750	2710	-	-	<10-48	<10–87
11-deoxycortisol (ng/dL)	15100	-	-	-	7-210	95–323

[a]*Note*: Reference values are unavailable

CYP11B1 gene including exon-intron boundaries was amplified in eight fragments using specific primers (Table 2). PCR products were treated with *ExoSAP-IT* (USP Corporation, Cleveland, OH), and sent for direct sequencing at Macrogen Inc. (Seoul, Korea). Analyses were performed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI).

Minigene construction and splicing analysis

We performed a minigene in vitro experiment of the *CYP11B1* splicing mutation. A segment of the wild-type (WT) and mutant (IVS7 + 1G > A) genomic DNA (gDNA) of *CYP11B1* gene consisting of exons 6 to 9 and their in-between introns was amplified by PCR using the oligonucleotides listed in Table 2. We used the gDNA of a normal control and the patient with IVS7 + 1G > A *CYP11B1* mutation as a template of minigene constructs. PCR reactions were carried out in a 20 μl volume containing 50 ng gDNA, 10xPCR buffer, 25 mM $MgCl_2$, 10 μM dNTPs, 5 U/μl *Taq polymerase* and 10 μM of each primer, using the following parameters: 30s at 94 °C, 30s at 60 °C and 1.30 min at 72 °C. The PCR product was cleaved with *BamHI-HF* and *XbaI* enzymes and cloned into the corresponding sites of pcDNA™3.1/myc-His B mammalian expression vector (Invitrogen, Carlsbad, CA) using *T4 DNA ligase* (New England BioLabs, UK). The wild-type and mutant vectors were confirmed by direct sequencing using NCBI Reference Sequences (RefSeq) NG_007954.1 as the genomic reference and NM_000497.3 as the mRNA reference.

COS-7 cells were cultured in Dulbecco's Modified Eagles Medium, High Glucose (HyClone Laboratories, Logan, UT) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, Singapore) and 0.01 % penicillin/streptomycin (HyClone Laboratories) at 37 °C in a humidified 5 % CO_2 incubator. Cells were grown on 6-well plates and transiently transfected with the wild-type and mutant minigene constructs (1 μg) using Effectene® Transfection Reagent (Qiagen). Cells incubated for 48 h after transfection and then were washed 3 times with PBS and kept frozen at – 20 °C. Total cellular RNA was extracted using QIAamp® RNA Blood Mini Kit (Qiagen) and treated with *DNaseI* (Qiagen). The RNAs were then used as template for cDNA synthesis using ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, WI). Finally, both the WT and mutant cDNAs were amplified by PCR using the same primers and conditions as used for the minigene construction. The PCR products were analyzed by electrophoresis on a 1 % agarose gel followed by staining with ethidium bromide. Each PCR product was confirmed by Sanger sequencing after subcloning into pGEM®-T Easy vectors (Promega).

Results

Mutation analysis

DNA sequencing of the entire coding regions and their flanking introns of the *CYP11B1* gene showed that both siblings were compound heterozygous for a nonsense mutation c.421C > T in its exon 3 (NCBI RefSeq NG_007954.1), causing the introduction of a premature stop codon at residue 141 (p.R141X); and a splice site mutation, c.1200 + 1G > A which is at the 5' donor splice site of the intron 7 (IVS7 + 1G > A). These identified mutations were reported previously [8–10], but their pathogenic mechanisms have not clearly been elucidated. Sequence analysis of the parental gDNA demonstrated that the mother was heterozygous for the c.421C > T mutation and the father, heterozygous for the c.1200 + 1G > A mutation (Fig. 1).

Minigene analysis of the splice site mutation

We hypothesized that the c.1200 + 1G > A (IVS7 + 1G > A) mutation located at the splicing donor site of intron 7 would create an abnormal splicing of the mRNA. To examine this possibility, we constructed expression vectors containing the WT and the mutant c.1200 + 1G > A *CYP11B1* minigene sequence from exons 6 to 9 (Fig. 2a). The resultant minigenes were transfected into COS-7 cells. Then, total RNA was isolated and analyzed by the RT-PCR method. We found that the mutant c.1200 + 1G > A *CYP11B1* minigene was processed to two major incorrectly spliced products which were shorter than the WT minigene (Fig. 2b). Sequence analysis of the RT-PCR products after subcloning into pGEM®-T Easy vector revealed that one of the mutant

Table 2 Sequences of oligonucleotide primers used for PCR amplification and minigene construction

Primer	Sense Strand	Antisense Strand
CYP11B1_Exon 1	5'- GTTCTCCCATGACGTGATCCCTCT – 3'	5'- TCCAAAGGATGCAGAGTGCC – 3'
CYP11B1_Exon 2	5' – TGGACAGGAGACACTTTGGAT – 3'	5' – TCGCCGCTTACAGCAAGAAC – 3'
CYP11B1_Exon 3–4	5' – TGGGGACAAGGAGGATGGGATAC – 3'	5' – TGGTGGAGAGGGAGAAATTGGG – 3'
CYP11B1_Exon 4	5' – CGTGGGAAGATCCAGCCTCAG – 3'	5' – GGAAGGTGAGGAATCCCCGAC – 3'
CYP11B1_Exon 5	5' – AGGAGGAGGACACTGAAGGATG – 3'	5' – AGGCAGGCTTGGCATCACC – 3'
CYP11B1_Exon 6	5' – GGCTCTGTCGTTCTCAGGGTATGC – 3'	5' – GGCGTTGAAGAGGGATTCCAGAG – 3'
CYP11B1_Exon 7–8	5' – AGAGAGCACAGGAAGCCCCATC – 3'	5' – CAGTCCCACATTGCTCAAGC – 3'
CYP11B1_Exon 9	5' – GTTCCCCCTTCAGCATAATCTC – 3'	5' – GCCCTCGGGAGTTCCATTT – 3'
CYP11B1_IVS7_construct	5' - AGTCGGATCCCTTGCTGATGACGCTCTTTG - 3'	5'- GTACTCTAGAATGGCTCTGAAGGTGAGGAG - 3'

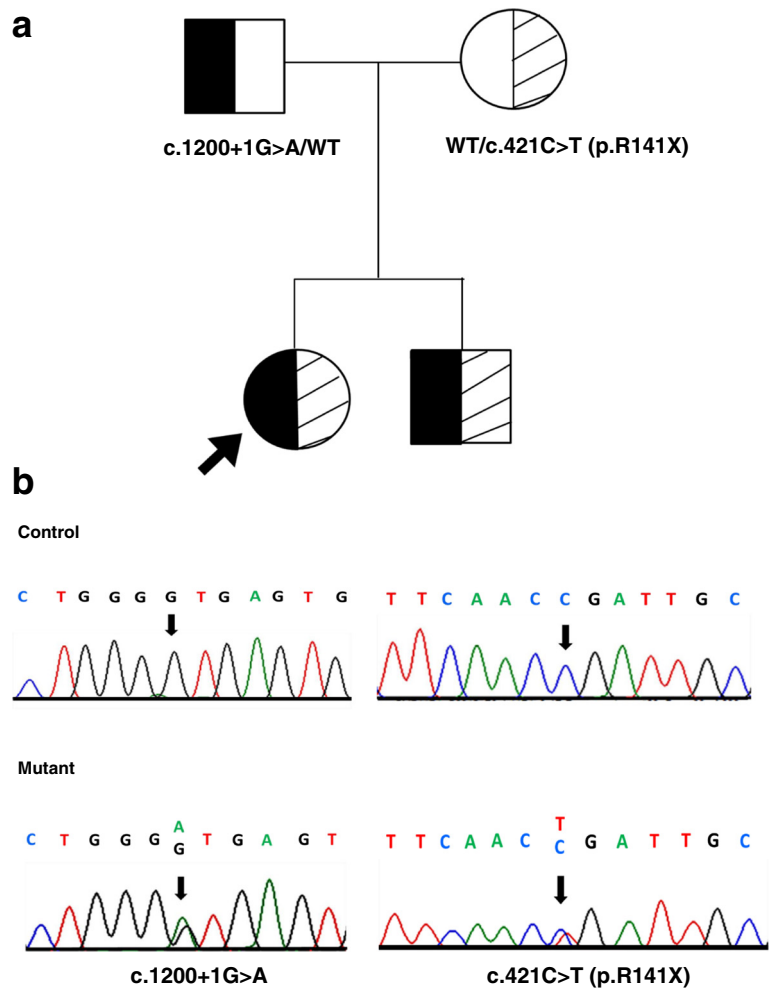

Fig. 1 Mutation analysis by direct DNA sequencing. **a** Family tree. The arrow indicates our proband. Both siblings carried compound heterozygous mutations; the point mutation at position bp 421 (c.421C > T) leads to the substitution of arginine to stop at amino acid position 141 (p.R141X), and the base change from G to A at the first position in intron 7 (c.1200 + 1G > A or IVS7 + 1G > A). The father is heterozygous for the c.1200 + 1G > A mutation and the mother is heterozygous for p.R141X. **b** Electropherograms of the patient and a healthy control

fragments skipped the entire exon 7, while containing the full-length sequences of exons 6, 8, 9. The other mutant PCR product skipped the exons 7 and 8, while retaining full-length sequences of exons 6 and 9 (Fig. 2c).

Discussion

In this study, we have described two severe *CYP11B1* mutations found in two siblings diagnosed with classic 11β-OHD in a family from Uzbekistan. Virilization and hypertension are the main clinical features of the classic 11β-OHD. Despite inability of aldosterone synthesis, overproduction of DOC, which is a less potent mineralocorticoid, causes salt retention and hypertension. However, affected newborns may have mild, transient salt loss presumably due to relatively high mineralocorticoids resistance in the newborn period [11]. Signs of mineralocorticoid excess generally correlate poorly with the degree of virilization in affected girls [3]. Blood pressure is usually normal during infancy and hypertension is often identified later in toddlerhood or in childhood, although its presence in infancy was demonstrated [12].

Most of the *CYP11B1* mutations described to date result in the classic form of 11β-OHD. Unlike 21-hydroxylase deficiency, molecular-genetic studies of 11β-OHD are relatively fewer, and a number of identified *CYP11B1* mutations have not been functionally characterized [5, 13]. Therefore, the exact genotype-phenotype prediction of 11β-OHD has not been well established. Previous studies showed that in vitro activities less than 5 % were considered severe and consistent with classic 11β-OHD [5, 13]. In this present study, we describe two siblings suffering from classic 11β-OHD who were compound heterozygous for a nonsense and a splice-site *CYP11B1* mutations. The nonsense p. R141X is expected

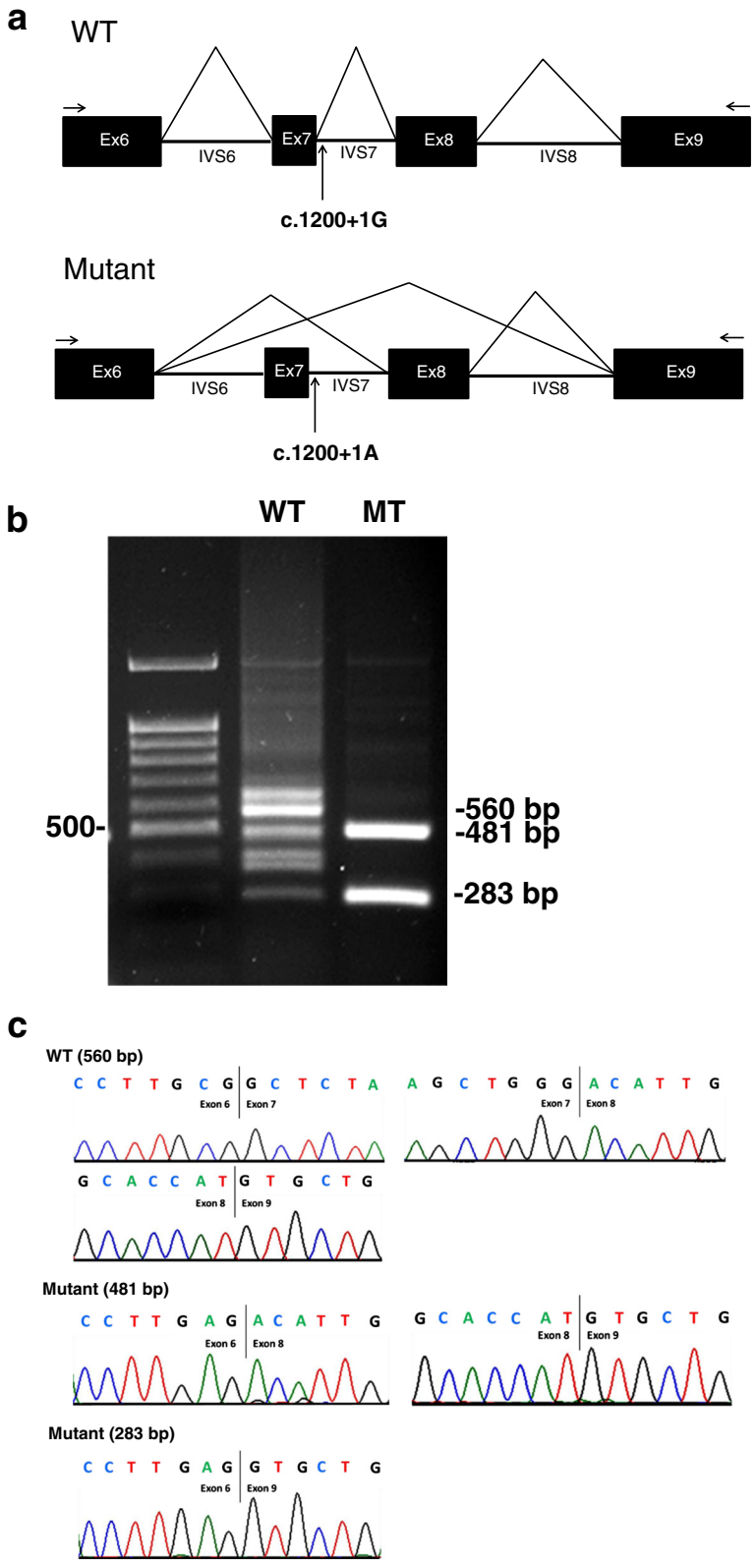

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Minigene experiment. **a** The scheme shows the set-up of the minigene constructs for the splicing analysis in the WT and mutant expression vectors containing c.1200 + 1G > A mutation (arrows). **b** COS-7 cells were transfected with the wild-type (WT) or mutant (MT) minigene constructs. Total RNA from the transfected cells were used for RT-PCR of *CYP11B1* cDNA. The figure shows the expected 560-bp PCR product from the WT construct and two shorter incorrectly spliced products from the mutant, sized 481 and 283 bp on an agarose gel. **c** Electropherograms of the minigene PCR products. The 481 bp mutant fragments skipped the entire exon 7, while containing the full-length sequences of exons 6, 8, 9. The 283 bp mutant PCR product skipped the exons 7 and 8, while retaining full-length sequences of exons 6 and 9. Black lines indicated exon–exon boundaries

to lead to a premature stop in the exon 3 and yields a truncated enzyme lacking the essential residues for heme binding domain, consistent with our patients' clinical phenotypes and near-completely abolished in vitro CYP11B1 activity in a recent study [14].

In addition, we identified a previously described IVS7 + 1G > A mutation in *CYP11B1* affecting the consensus slice donor site of the exon 7. The minigene experiment confirmed that this splice site mutation caused exon skipping (either a complete loss of the exon 7 or both exons 7 and 8). Most reported *CYP11B1* mutations are located in exons 6, 7, and 8 and 70 % of amino acid sequences in these exons are identical in human, ox, rat, and mouse, suggesting that exons 6–8 are essential for the enzymatic activity of CYP11B1 [15]. Recently, Nguyen et al. [9] studied a minigene experiment of this same mutation. Nonetheless, the authors designed a shorter minigene construct which had only exon 7, intron 7, and exon 8. They found that the IVS7 + 1G > A mutation caused an intron retention.

Splicing errors are well recognized causes of genetic diseases. Previous data point to an estimated frequency of sequence variations affect pre-mRNA splicing up to 50 % of the alleles causing human disease [16, 17]. Splice site nucleotide substitutions may result in skipping of the involved exon, intron retention, creation of a pseudo-exon within intron, usage of a cryptic splice site, or a combination of several of these [18, 19]. Hence, the design of minigene constructs is important to correctly identify the splicing effect of specific splice site mutations. Recent data have suggested that cassette exon skipping is the most common alternative splicing event in humans [19, 20]. To date, a +1G > A substitution at the 5'-splice donor site has been identified in a number of human diseases [21]. Functional studies of other +1G > A 5'-splice site mutations have shown either recognition of a 5'-cryptic splice site or exon skipping [22]. Therefore, we have designed the minigene constructs including exons 6–9 and introns 6–8 and our results confirmed that the IVS7 + 1G > A mutant construct results in exon skipping. To date, the therapies to modulate RNA mis-splicing using antisense oligonucleotide or small molecules are emerging [19]. The understanding of definite genetic mechanism could expand opportunities for gene therapy. Modulation of aberrant splicing transcripts can become a novel therapeutic approach for many diseases caused by splice site defects.

Conclusions

In summary, we describe two compound heterozygous *CYP11B1* mutations that severely affect normal protein structure explaining a severe phenotype of classic 11β-hydroxylase deficiency. Our findings suggest the mutation IVS7 + 1G > A causes aberrant splicing of *CYP11B1* leading to exon skipping. Our findings may help for better understanding of splice site mutation mechanism and facilitate the future new therapies targeted on splicing modulation to treat human disease.

Abbreviations

11β-OHD, 11β-hydroxylase deficiency; 17OHP, 17-hydroxyprogesterone; ACTH, adrenocorticotropic hormone; CAH, congenital adrenal hyperplasia; DHEAS, dehydroepiandrosterone sulfate; DOC, 11-deoxycorticosterone; gDNA, genomic DNA; mRNA, messenger RNA; MT, mutant; nl, normal; PCR, polymerase chain reaction; PRA, plasma renin activity; RT-PCR, reverse transcription polymerase chain reaction; WT, wild-type

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Availability of data and materials

All data contained within the article.

Authors' contributions

PC carried out the molecular genetic studies and drafted the manuscript. The patients were under the care of VS[5]. TS conceived the idea of the report and drafted the manuscript. PY, KS, TS, and VS[2] participated in the writing, review of the literature, text editing and finalization of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Written informed consent was obtained from the patient's parent for publication of this Case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Ethics approval and consent to participate

All procedures were performed according to the Declaration of Helsinki and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from the patient's parent for participate in this study.

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ORIGINAL ARTICLE

Cardio-metabolic risk factors in youth with classical 21-hydroxylase deficiency

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Abstract Patients with congenital adrenal hyperplasia (CAH) appear to have adverse cardiovascular risk profile and other long-term health problems in adult life, but there are limited data in young CAH patients. We aim to evaluate the cardio-metabolic risk factors in adolescents and young adults with classical 21-hydroxylase deficiency (21-OHD). We performed a cross-sectional study of 21 patients (17 females) with classic CAH detected clinically and not through newborn screening, aged 15.2 ± 5.8 years, and 21 healthy matched controls. Anthropometric, biochemical, inflammatory markers, and body composition using dual-energy X-ray absorptiometry were measured. Obesity was observed in 33% of the CAH patients. The waist/hip ratio and waist/height ratio were significantly higher in CAH patients. Five out of 21 patients (24%) had elevated blood pressure. Silent diabetes was diagnosed in one patient (4.8%), but none in the control group. Serum leptin and interleukin-6 levels were not different between groups, but hs-CRP levels tended to be higher in CAH patients. Other metabolic profiles and body composition were similar in CAH and controls.

Conclusion: Adolescents and young adults with CAH appear to have an increased risk of obesity and cardio-metabolic risk factors. Close monitoring, early identification, and secondary prevention should be implemented during pediatric care to improve the long-term health outcomes in CAH patients.

What is Known:
- *Lifelong glucocorticoid (GC) replacement is the main treatment modality in patients with congenital adrenal hyperplasia which predispose to an adverse metabolic profile.*
- *Adult CAH patients have adverse cardiovascular risk profile and other long-term health problems.*

What is New:
- *Adolescents and young adults with CAH appear to have an increased risk of obesity and cardio-metabolic risk factors.*

Keywords Congenital adrenal hyperplasia · Metabolic syndrome · Adolescent · Body composition · Cardiovascular risk factors

Abbreviations

17-OHP	17-Hydroxyprogesterone
21-OHD	21-Hydroxylase deficiency
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMC	Bone mineral content
BMD	Bone mineral density
CAH	Congenital adrenal hyperplasia
DXA	Dual-energy X-ray absorptiometry
FBG	Fasting blood glucose
GC	Glucocorticoid
HbA1c	Hemoglobin A1c or glycosylated hemoglobin A protein
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
hs-CRP	High-sensitivity C-reactive protein

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IL-6	Interleukin-6
IQR	Interquartile range
LDL	Low-density lipoprotein
OGTT	Oral glucose tolerance test
SDS	Standard deviation scores
SV	Simple virilizing
SW	Salt-wasting

Introduction

Congenital adrenal hyperplasia (CAH OMIM #201910) is a group of autosomal recessive disorders of adrenal steroidogenesis defects. More than 90% of cases of CAH are caused by mutations in the *CYP21A2* gene responsible for 21-hydroxylase deficiency (21-OHD). Deficiency of 21-hydroxylase results in impaired adrenal synthesis of cortisol and aldosterone [37]. Accumulated steroid precursors are shunted into the androgen synthesis pathway resulting in excessive androgen production. 21-OHD is classified as classic (severe form) or non-classic (mild form) according to the severity of enzyme impairment. Classic 21-OHD is subdivided into the salt-wasting (SW) form and the simple virilizing (SV) form. Classic CAH patients typically present with potentially fatal salt wasting early in infancy and ambiguous genitalia in affected females [37].

Management of classic CAH consists of lifelong glucocorticoid (GC) replacement to prevent adrenal crisis and suppress androgen excess. Management of children with CAH remains a challenge because adequate androgen suppression usually requires GC supra-physiological doses and leads to iatrogenic Cushing's syndrome [36] which is characterized by central obesity, growth retardation, insulin resistance, dyslipidemia, hypertension, and low bone mass [44].

Previous studies reported a higher risk of obesity and increased body fat mass in pediatric and adult patients with CAH [9, 12, 18, 38]. Most previous studies, but not all demonstrated a high prevalence of hypertension and insulin resistance in CAH children and adults [8, 12, 14, 33, 42, 48]. A few recent studies indicated that CAH patients have adverse cardiovascular and metabolic risk profiles in later life [8, 30, 48]. However, there are relatively scarce data of cardio-metabolic risk factors in pediatric and young adult patients with CAH. In the current study, we aimed to evaluate comprehensive cardio-metabolic risk factors and metabolic syndrome in adolescents and young adults with classical 21-OHD compared with age-, sex-, and puberty-matched healthy controls.

Patients and methods

Patients

The study protocol was approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University. Informed consent was obtained from all subjects. Twenty-one patients with classical 21-OHD (17 females, F, and 4 males, M; aged 9–28 years) were enrolled in the study at King Chulalongkorn Memorial Hospital (KCMH) in Bangkok, Thailand, during 2013–2014. Diagnosis was made on the basis of clinical presentations and elevated serum 17-hydroxyprogesterone (17-OHP) levels [36]. All patients have been treated from the time of diagnosis. Four patients received hydrocortisone, nine received prednisolone, and the remaining cases received combination therapy (prednisolone and dexamethasone) due to poor hormonal control. GC dosage was converted to hydrocortisone equivalents according to the formula for growth-retarding cortisol equivalents (GRCE): 80 mg hydrocortisone = 16 mg prednisone = 1 mg dexamethasone [36]. At study entry, CAH patients received the mean hydrocortisone equivalent dose of 21.4 ± 5.8 mg/m^2/day. Twelve patients received 9-α-fludrocortisone treatment in a standard dose 0.05–0.15 mg/day.

Twenty-one healthy Thai adolescents and young adults with age-, sex-, and pubertal status-matched were recruited by the Clinical Research Center and included as controls. None of the controls received chronic medications or had known comorbidities.

Study protocol

All study subjects were examined as outpatients at the pediatric endocrine unit, KCMH, including measures of height (Ht), weight (Wt), waist and hip circumferences, blood pressure (BP), and heart rate. Body mass index (BMI) was calculated as Wt (kg)/Ht^2 (m^2). Ht and BMI values were expressed as the standard deviation scores (SDS) for chronological age from the age- and sex-specific references based on World Health Organization (WHO) standards [10]. Pubertal status was assessed by the method of Marshall and Tanner [24, 25]. Waist circumference was measured at the midpoint between the lower edge of the ribs in the mid-axillary line and the top of the iliac crest by the same physician. Hip circumference was measured around the widest portion of the buttocks [46]. Systolic BP (SBP) and diastolic BP (DBP) were measured in all subjects using a standardized automated Dinamap on the right arm. Blood samples were collected between 8 and 10 a.m. after an overnight fast for fasting blood glucose (FBG), insulin, HbA1c, lipid profile [total cholesterol, triglycerides, high-density lipoprotein (HDL) and directly measured low-density lipoprotein (LDL) cholesterol], liver enzymes (aspartate aminotransferase, AST; alanine aminotransferase,

ALT), leptin, interleukin-6 (IL-6), and high-sensitivity C-reactive protein (hs-CRP) levels in all patients and controls. In the CAH group, 17-OHP, testosterone, and plasma renin activity were also obtained before the administration of the morning medication. The family history was obtained by asking patients or parents whether any member in their first- or second-degree relatives experienced obesity, diabetes, hypertension, dyslipidemia, or cardiovascular disease.

A dual-energy X-ray absorptiometry (DXA) scan using a Hologic QDR-4500 densitometer (Hologic Inc., Waltham, MA) was performed in all patients and controls to assess total bone mineral content (BMC), fat mass, and lean mass. To adjust for height, lean mass and fat mass were divided by $(\text{height})^2$ (kg/m^2) [22]. Total body mass was calculated as follows: total body mass = total BMC + total fat mass + total lean mass. The percentage lean mass and percentage fat mass were calculated by dividing their absolute mass by the total body mass.

Hormonal assays

Commercial immunoassays were used to measure plasma insulin [electrochemiluminescence immunoassay (ECLIA); Diagnostic Products Corporation, Los Angeles, CA] and HbA1c (Immunoturbidity; Roche Diagnostics, Indianapolis, IN). Glucose was measured on a Cobas Integra 400 plus (Roche Diagnostics) using a hexokinase method. Liver enzymes and lipids were measured by standard enzymatic methods. Serum 17-OHP and plasma renin activity were measured using a radioimmunoassay kit (MP Biomedicals, OH, USA). Serum testosterone and IL-6 levels were measured by ECLIA (Cobas e411, Roche Diagnostics). Serum leptin concentrations were measured using an enzyme immunoassay method (Immunospec Corporation, Canoga Park, CA). C-reactive protein levels were measured with the use of particle-enhanced immunonephelometric assays on a BN II analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). All hormonal assays were performed according to the manufacturer's protocol.

Operational definitions

Overweight and obesity were defined as a BMI-SDS larger or equal to +1.0 and +2.0, respectively [10]. Hypertension was defined as an average SBP or DBP ≥95th centiles for gender, age, and height [15]. Patients with average SBP or DBP levels between 90th and 95th centiles or ≥120/80 mmHg were classified as pre-hypertension [15]. Insulin resistance was assessed by the homeostasis model assessment of insulin resistance (HOMA-IR) which was calculated as follows: fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5 [26]. Dyslipidemia was diagnosed if there were at least two abnormal values of the following: total cholesterol ≥200 mg/dl, serum LDL cholesterol ≥130 mg/dl, triglyceride ≥100 mg/dl (0–9 years) or ≥130 mg/dl (10–19 years), and HDL <40 mg/dl [4]. The International Diabetes Federation (IDF) consensus definition was used for the diagnosis of metabolic syndrome in children and adolescents [22]. Diabetes mellitus was diagnosed if FBG ≥126 mg/dl or the 2-h post oral glucose tolerance test (OGTT) glucose ≥200 mg/dl. Adequate hormonal controls were defined by 17-hydroxyprogesterone level <2000 ng/dl with normalization or near-normalization of testosterone according to pubertal stage.

Statistical analysis

Statistical analysis was performed using SPSS version 17 (SPSS, Chicago, IL). Normally distributed data are expressed as mean ± standard deviation (SD), whereas non-normally distributed data were expressed as median and interquartile range (IQR). Comparisons between two groups were analyzed by Fisher's exact test for categorical data and the unpaired *t* test or Mann-Whitney *U* test for continuous data. Pearson's correlation analysis was used to explore relationships. A *P* value <0.05 was considered significant.

Results

Baseline characteristics

Twenty-one patients with classical CAH and 21 healthy-matched controls were included in the analysis. In the CAH group, 10 patients (9 F and 1 M) had SW-CAH, and 11 (8 F and 3 M) had SV form. Up to now, there is no 17-OHP newborn screening established in Thailand. Thus, most patients with classic SW-CAH in our study presented with salt-losing crisis and ambiguous genitalia (in females). The median age of diagnosis in the SW-CAH group was 0.5 months (range 0.2–3 months). By contrast, SV-CAH patients presented later with sexual precocity in boys and ambiguous genitalia in girls. The median age of diagnosis in SV-CAH boys was 5 years (range 4–5.5 years) and in girls 1.5 years (range 0.3–48 months). Median serum levels of 17-OHP and testosterone (IQR1, IQR3) in 21 CAH patients were 12,740 (3260, 16,050) ng/dl and 155 (83, 204) ng/dl, respectively. Only four patients had excellent therapeutic control defined as no signs of virilization or hyperpigmentation, and 17-OHP levels were less than 2000 ng/dl. The baseline clinical characteristics of patients and controls are shown in Table 1. The mean age of patients was 15.2 ± 5.8 years, which was similar to controls (16.1 ± 5.3 years). All study subjects were in puberty (Tanner ≥2) at the study entry. The pubertal stage was not different between the two groups. Weight did not differ between the two groups, but CAH patients tended to have higher median BMI-SDS than in controls (0.89 vs. 0.15, $P = 0.07$).

Table 1 Baseline characteristics of patients and controls

	CAH (n = 21)	Controls (n = 21)	P value
Age (years), mean ± SD	15.2 ± 5.8	16.1 ± 5.3	0.593
Female (%)	17 (81%)	17 (81%)	1.000
Family history, n (%)			
Obesity	9 (42.9%)	3 (14.3%)	0.086
Diabetes	10 (47.6%)	10 (47.6%)	1.000
Dyslipidemia	7 (33.3%)	6 (28.6%)	1.000
Hypertension	9 (42.9%)	13 (61.9%)	0.354
Cardiovascular disease	4 (19%)	1 (4.8%)	0.343
Weight (kg), mean ± SD	50.6 ± 14.4	49.8 ± 9.7	0.833
Height (cm), mean ± SD	147.9 ± 8.6	155.4 ± 7.5	0.005*
Height SDS	−0.9 ± 1.5	−0.3 ± 0.9	0.096
Height SDS, median (IQR)	−0.89 (−1.99, −0.26)	−0.35 (−0.71, 0.18)	0.046*
BMI (kg/m^2), mean ± SD	23 ± 5.6	20.5 ± 2.8	0.077
BMI SDS, mean ± SD	1 ± 1.5	0.3 ± 1	0.069
BMI SDS, median (IQR)	0.89 (0.2, 2.24)	0.15 (−0.16, 0.96)	0.072
Pubertal staging			0.698
Early puberty (Tanner II–III)	5 (24%)	3 (14%)	
Late puberty (Tanner IV–V)	16 (76%)	18 (86%)	

*P < 0.05

Seven of the 21 (33%) CAH patients but none of controls were obese. Three patients with CAH and five subjects in the control group met the criteria of overweight. As expected, the CAH patients were shorter than controls (height 147.9 ± 8.6 cm vs. 155.4 ± 7.5 cm, P = 0.005, and median height SDS −0.89 vs. −0.35, P = 0.046).

Metabolic syndrome features

Comparisons of metabolic syndrome features between two groups are presented in Table 2. The waist to hip ratio and waist to height ratio were significantly higher in CAH patients than in controls (waist/hip 0.88 ± 0.05 vs. 0.82 ± 0.07, P = 0.007; waist/height 0.51 ± 0.08 vs. 0.46 ± 0.04, P = 0.007). Although there was no significant difference in mean SBP and DBP between CAH patients and controls, three of the 21 patients (14%) were noted to have pre-hypertension and two patients (9.5%) had hypertension. By contrast, almost all controls had normal blood pressure. Neither SBP nor DBP was significantly correlated with 9-α-fludrocortisone dose (r = 0.133, P = 0.566; r = 0.053, P = 0.819, respectively). In the CAH group, there was a positive correlation between BMI and BP (Fig. 1). However, there was no significant correlation between BMI and GC dose (r = 0.024, P = 0.918).

Interestingly, CAH patients had significantly lower mean FBG than normal subjects (77 ± 9.9 vs. 86 ± 6.5 mg/dl, P = 0.001), but fasting insulin, HbA1c, and the HOMA-IR index were similar for subject-control pairs. There was no significant correlation between HOMA-IR and BMI (r = 0.180, P = 0.260), GC dose (r = 0.023, P = 0.923), age (r = −0.237, P = 0.136), or 17-OHP levels (r = −0.081, P = 0.733). One out of 21 CAH patients (4.8%), but none of the control group was found to have type 2 diabetes and met the criteria for metabolic syndrome. Lipid profile was not different between patients and healthy controls except median HDL/total cholesterol ratio was significantly lower in CAH patients than in controls (0.61 vs. 0.98, P = 0.009). Four patients vs. five controls had dyslipidemia (P = 1.000). AST levels were significantly higher in subjects with CAH compared with controls (21.7 ± 6.1 vs. 18 ± 4.3 U/l, P = 0.029), and ALT levels also tended to be higher (17 ± 8.9 vs. 13 ± 5.7 U/l, P = 0.098).

Serum leptin and inflammatory markers

Serum leptin and IL-6 concentrations were not different between CAH patients and controls (Fig. 2). The median hs-CRP levels tended to be higher in CAH patients than in normal subjects (0.96 vs. 0.45 pg/ml, P = 0.078) (Fig. 2). There was a positive correlation between serum leptin levels and BMI (r = 0.632, P < 0.001) as well as leptin and HOMA-IR (r = 0.51, P = 0.002) (Fig. 3).

Body composition by DXA scan

Total BMD-SDS (z-score) was not different between patients and normal subjects (0.2 ± 1.4 vs. 0.4 ± 1.2, P = 0.585) (Table 3). There were no significant differences in total lean mass and total fat mass between groups even when adjusted for height (Table 3).

Table 2 Metabolic syndrome features in CAH patients and controls

	CAH (n = 21)	Controls (n = 21)	P value
Waist circumference (cm)	76.1 ± 12.8	70.8 ± 6.9	0.111
Waist/hip ratio	0.88 ± 0.05	0.82 ± 0.07	0.007*
Waist/height ratio	0.51 ± 0.08	0.46 ± 0.04	0.007*
SBP (mmHg)	104.4 ± 10.9	104.1 ± 7.6	0.922
DBP (mmHg)	66.5 ± 8.6	62.1 ± 7.1	0.076
Elevated BP	5/21 (23.8%)	1/21 (4.8%)	0.184
Fasting blood glucose (G_F)	76.9 ± 9.9	86 ± 6.5	0.001*
Fasting insulin (I_F)	6.45 (4.8, 13.65)	7.1 (5.7, 12.2)	0.990
HOMA-IR	1.23 (0.9, 2.5)	1.45 (1.22, 2.47)	0.481
HbA1c (%)	5.4 ± 0.7	5.3 ± 0.6	0.850
Triglyceride	100.7 ± 74.6	73.1 ± 39.8	0.143
≥130 mg/dl	4 (19%)	2 (9.5%)	0.663
Cholesterol	182.9 ± 49	184.2 ± 27.5	0.917
≥200 mg/dl	4 (19%)	5 (23.8%)	1.000
HDL-C	52.7 ± 15.4	58.6 ± 10.3	0.153
<40 mg/dl	5 (23.8%)	1 (4.8%)	0.184
LDL-C	107.2 ± 32.6	105.3 ± 25.6	0.834
≥130 mg/dl	4 (19%)	5 (23.8%)	1.000
HDL to cholesterol ratio, median (IQR)	0.61 (0.48, 0.84)	0.98 (0.88, 1.22)	0.009*
Aspartate aminotransferase (U/l)	21.7 ± 6.1	18 ± 4.3	0.029*
Alanine aminotransferase (U/l)	17 ± 8.9	13 ± 5.7	0.098
Met the criteria for metabolic syndrome	1/21 (4.8%)	0/21	1.000
IFG/T2DM	1/21 (4.8%)	0/21	1.000
Dyslipidemia	4/21 (19%)	5/21 (24%)	1.000

*P < 0.05

Obese vs. non-obese CAH patients

Seven of the 21 (33%) CAH subjects in this study were obese. Age, pubertal stage, GC type, hydrocortisone equivalent dose, and the family history of metabolic syndrome were not different between obese and non-obese patients. Three of the four male (75%) and four of the 17 female (24%) patients had BMI-SDS >2.0. There were no significant differences in serum concentrations of 17-OHP and PRA between groups except higher median testosterone levels in obese CAH patients [205 (187, 369) vs. 127 (81, 155) ng/dl, P = 0.007)].

Discussion

Our data show that our small cohort of adolescents with classical CAH appears to have an increased metabolic risk compared with matched healthy controls. We found a higher prevalence of obesity in CAH patients than in the reference population which was similar to previous studies [7, 39, 40]. In our study, BMI in CAH patients was neither associated with glucocorticoid dosage nor age. Similarly, Bachelot et al. showed no correlation between BMI and duration of treatment, glucocorticoid dosage, or 17-OHP levels [3], whereas Völkl et al.

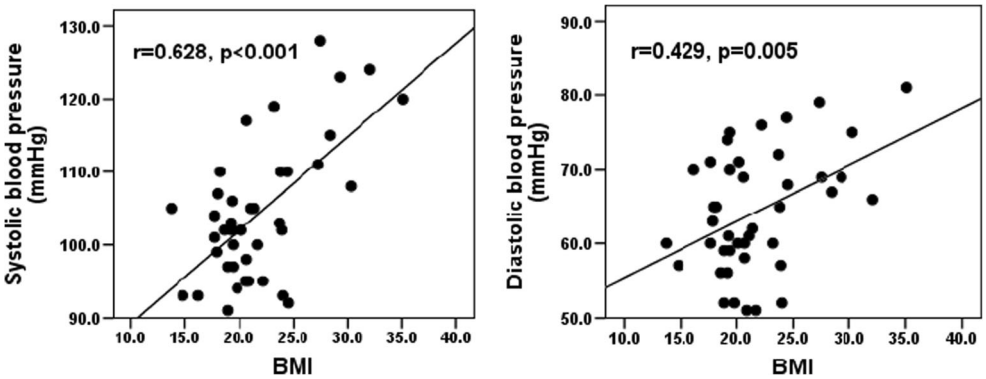

Fig. 1 Correlation between blood pressure and BMI of CAH patients

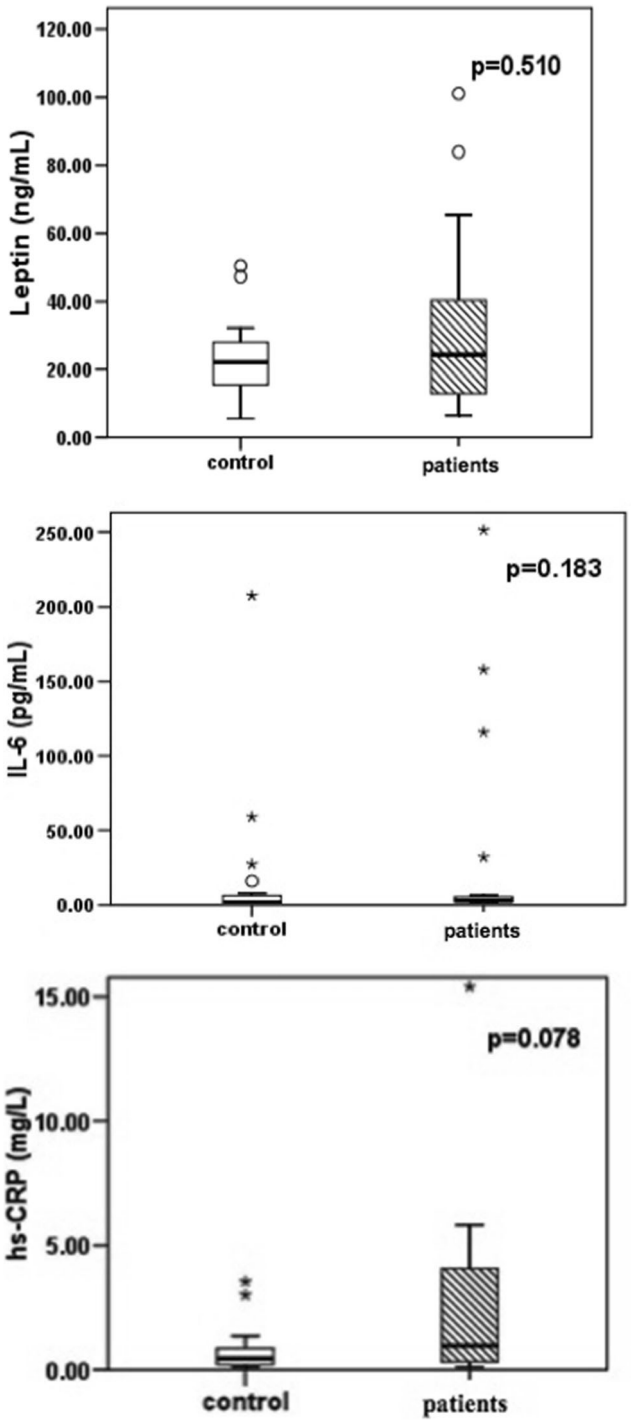

Fig. 2 Serum leptin, IL-6, and hs-CRP concentrations in patients and controls

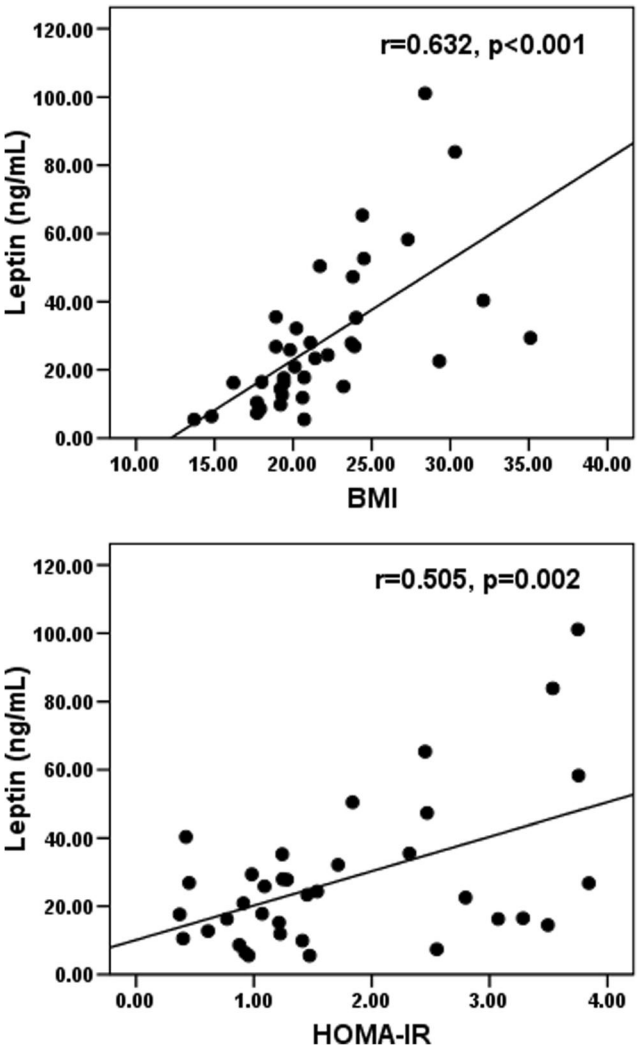

Fig. 3 Correlation between serum leptin levels and BMI or HOMA-IR in CAH patients

demonstrated a slightly positive correlation of BMI with the glucocorticoid dosage [40]. The cause of obesity among CAH patients remains unclear but several factors may contribute to it. Although it is speculated that obesity might be related to glucocorticoid dosage, Zhang et al. reported a higher BMI in untreated female adults with simple virilizing CAH [47]. Impaired adrenomedullary function with decreased adrenaline secretion may also play a role in the development of obesity [27]. Additionally, an altered leptin axis, the key regulator of energy balance, might contribute to the development of obesity in CAH patients. In this study, we found that serum leptin levels were not different in CAH patients compared to healthy controls, but leptin levels were positively correlated with BMI in CAH patients.

Several clinical studies have shown that increased androgen levels are associated with decreased leptin concentrations [21, 43]. An in vitro study also showed suppressive effects of testosterone on leptin production [43]. However, Charmandari et al. reported higher leptin levels in children with CAH than in normal subjects despite their high testosterone concentrations [8]. In contrast, Volkl et al. found leptin levels were not different between CAH children and matched controls, whereas serum levels of soluble leptin receptor (sOB-R) levels were significantly lower in CAH subjects [42]. Decreased sOB-R

Table 3 Body composition by DXA in CAH and control subjects

	CAH (n = 21)	Controls (n = 21)	P value
Total body bone density (z-score)	0.2 ± 1.4	0.4 ± 1.2	0.585
Total lean mass (kg)	30.5 ± 6.8	31.1 ± 5.5	0.748
% lean mass	61.6 ± 7.9	62.8 ± 5.1	0.542
Adjusted for height (kg/m^2)	13.8 ± 2.2	12.8 ± 1.5	0.094
Total fat mass (kg)	17.5 ± 8.2	16 ± 4.3	0.461
% fat mass	33.9 ± 8.3	32.4 ± 4.9	0.500
Adjusted for height (kg/m^2)	8 ± 3.6	6.6 ± 1.5	0.111

levels may lead to a "leptin resistance" state and contribute to an increased rate of obesity [42].

Consistent with Marra et al. [23], we observed that adolescents with CAH had significantly higher waist-to-hip ratio and waist-to-height ratio than in healthy controls indicating increased visceral obesity, which may contribute to an increased cardio-metabolic risk in adult life [16, 19, 23, 34].

Several studies have evaluated blood pressure in both adult and pediatric CAH patients and the results are conflicting. Some studies reported normal resting BP [12, 23] and 24-h BP profiles [11]. By contrast, Völkl et al. performed 24-h ambulatory BP monitoring and showed significant elevated systolic BP correlated with increased BMI in pediatric CAH patients, whereas normal-weight patients tended to have diastolic hypotension [41]. A few large cohort studies in adults showed SBP was slightly lower in men with CAH than population-based references [2, 6], but women with classic CAH had significantly higher diastolic BP than matched controls [2]. In our study, although there was no significant difference in SBP and DBP between CAH patients and controls, there was a positive correlation between BMI and BP but not glucocorticoid dosage, in agreement with previous studies [11, 41].

Glucose and insulin dynamics are infrequently studied in CAH children. Despite a higher proportion of obesity, we found lower fasting blood glucose levels in patients with CAH than in normal subjects, similar to previous recent study from the UK [39]. These findings may be explained by decreased epinephrine levels, the key regulatory hormone in fasting state, from adrenomedullary hypofunction in CAH patients [8, 27]. Although there was significant difference in fasting blood glucose, this finding may not be clinically important. In contrast to some previous studies [3, 8, 42], we could not demonstrate unfavorable changes in HOMA-IR in CAH adolescents compared to healthy controls. Insulin resistance appears to be associated with both over- and undertreatment in patients with CAH. Undertreatment leading to hyperandrogenism may induce insulin resistance, whereas overtreatment can also induce insulin resistance due to supraphysiological glucocorticoid dosage [31]. Nonetheless, our results revealed no significant correlation between HOMA-IR and GC dose, age, or 17-hydroxyprogesterone levels.

In this study, one out of 21 patients with CAH met the criteria to have diabetes (diagnosed by FBG and later confirmed by standard OGTT) and metabolic syndrome. To date, data on OGTT in pediatric and adult patients with CAH are scanty. Zimmermann et al. demonstrated higher fasting glucose and insulin levels in CAH children and young adults than in controls but the OGTT results were similar between groups [48]. Although the UK adult CAH cohort found insulin resistance defined by HOMA-IR in one third of the patients [2], but whether the prevalence of type 2 diabetes in CAH subjects would be higher than in normal population is currently unclear.

There are limited data on lipid profiles in pediatric CAH patients. Our data showed that lipid profiles were not different between patients and controls, except CAH patients had significantly lower HDL to cholesterol ratio. Most previous studies in children and adults with CAH suggested similar lipid profiles between CAH patients and healthy controls [3, 12, 39]. Zimmermann et al. found increased small dense low-density lipoproteins (sd-LDL) and decreased HDL in CAH children and young adults [48]. Hypercholesterolemia was found in 46% of the patients in a UK cohort study [2]. In contrast, a recent French study in 219 adult men with classic CAH found none of the patients was diabetic and lipid status was generally normal [6].

To the extent of our review, there have not been studies concerning the prevalence of non-alcoholic fatty liver disease (NAFLD) in pediatric CAH patients. Glucocorticoids are known to induce fatty liver [1] as well as obesity-related fatty liver disease [5]. Falhammar et al. reported elevated ALT and GGT levels in CAH adults [13]. By using the pediatric ALT thresholds [35], we found two CAH patients and one control had mildly elevated ALT which would need further periodic monitoring and evaluation [5]. Although AST levels were significantly higher in subjects with CAH compared with controls, the values were all in the normal ranges.

The hs-CRP and IL-6 concentrations are inflammatory markers associated with adverse cardiovascular outcomes. To date, there was only one study that evaluated these

inflammatory markers in adults with CAH and found no differences in IL-6 and hs-CRP concentrations between patients and controls [28]. Our results revealed serum IL-6 levels were not different between groups, but hs-CRP levels had trend towards higher in CAH patients than in healthy subjects. Increased C-reactive protein was associated with adverse cardiovascular in obese children and adolescents [45].

In this study, the total body BMD *z*-score, total lean mass, and total fat mass were not different between patients and controls. Most studies on BMD in CAH adolescents and young adults generally revealed normal bone density [9, 17, 29, 32]. However, previous studies in adults older than 30 years and in postmenopausal women demonstrated increased prevalence of impaired bone mineral density in CAH patients compared to healthy subjects [2, 12, 20]. Several previous studies showed increased fat mass in young adults with CAH particularly in male patients [9, 12, 18, 38]. Notably, most patients in our study were younger than 20 years. Falhammar et al. demonstrated increased fat mass only in patients older than 30 years while younger patients had similar lean and fat mass compared to controls [12]. These data suggested that low bone mass and abnormal body composition may be uncommon in pediatric CAH patients.

There are some limitations in our study. First, all patients in this study were detected clinically, not by newborn screening so a number of patients had delayed diagnosis and treatment as well as poor hormonal control. Second, the sample size is small so it would be difficult to reach statistically significant difference. In addition, there were only few males with CAH included, which did not permit us to perform subgroup analysis. Third, we used task force reference for office BP measurement instead of 24-h ambulatory monitoring which would have been more preferable. Finally, we did not measure intima media thickness which is the surrogate marker of early atherosclerosis.

In summary, adolescents and young adults with CAH appear to have an increased risk of obesity and cardio-metabolic risk factors. Close monitoring, early identification, and secondary prevention should be implemented during pediatric care to prevent metabolic complications and improve the long-term health outcomes in CAH patients.

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Authors' contributions KA and TS had the core idea for this study. All authors either analyzed the data or interpreted the results. KA wrote the draft of the article. All other authors participated in the review of the literature, text editing, and finalization of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards All procedures were performed according to the Declaration of Helsinki and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from the patient and/or the patient's parent for their participation in this study.

Conflict of interest The authors declare that they have no conflict of interest.

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A novel mutation in the *HSD3B2* gene causing classic 3β-hydroxysteroid dehydrogenase deficiency in two boys initially misdiagnosed as 21-hydroxylase deficiency

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Running title: Novel HSD3B2 mutation causing 3βHSD deficiency

Abstract

Background: Mutations of the *HSD3B2* gene encoding 3β-hydroxysteroid dehydrogenase (3βHSD) type 2 cause a rare form of congenital adrenal hyperplasia, "3βHSD deficiency", typically presenting with salt-wasting and ambiguous genitalia. Unusual cases may have elevated 17-hydroxyprogesterone (17OHP) due to peripheral conversion by 3βHSD type 1 isoenzyme which can complicate the diagnosis.

Methods: We report the clinical and molecular findings of two unrelated boys with 3βHSD deficiency.

Results: Patients 1 (Thai) and 2 (Indian) were 46,XY undervirilized newborns who developed salt-wasting since early infancy and initially misdiagnosed as 21-hydroxylase deficiency due to moderately elevated 17OHP. The ACTH tests revealed low cortisol response, elevated 17OHP and Δ^5/Δ^4 steroids. Both patients were identified to be homozygous for *HSD3B2* mutations including a novel p.Y180X.

Conclusion: We describe clinical and molecular findings of the classic 3βHSD deficiency for the first time in the Thai and Indian populations. A novel *HSD3B2* mutation is identified expanding its mutational spectrum.

Keywords: 3β-hydroxysteroid dehydrogenase deficiency, congenital adrenal hyperplasia, *HSD3B2*, mutation

List of Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase
11-OHD	11β-hydroxylase deficiency
17OHPreg	17-hydroxypregnenolone
17OHP	17-hydroxyprogesterone
21-OHD	21-hydroxylase deficiency
ACTH	Adrenocorticotropic hormone
ADD	Androstenedione
DHEA	Dehydroepiandrosterone
hCG	Human chorionic gonadotropin

Introduction

3β-Hydroxysteroid dehydrogenase (3βHSD) is a microsomal enzyme catalyzes the conversion of the hydroxyl-group to a keto-group on carbon 3 and the isomerization of Δ^5-steroids precursors [pregnenolone, 17-hydroxypregnenolone (17OHPreg), and dehydroepiandrosterone (DHEA)] into Δ^4-ketosteroids [progesterone, 17-hydroxyprogesterone (17OHP) and androstenedione (ADD)]. Thus, it is an essential enzyme for biosynthesis of all classes of active steroid hormones.[1] In human, there are 2 types of 3βHSD isoenzymes, type 1 and type 2, which share 94% homology[2] and are encoded by the adjoining genes (*HSD3B1* and *HSD3B2*) on chromosome 1p13.1. The 3βHSD type 1 (3βHSD1) is expressed in placenta, contributed in the placental progesterone synthesis, and peripheral tissues including mammary glands, prostate and skin. The 3βHSD type 2 (3βHSD2) is mainly expressed in adrenals and gonads.[2-4] Mutations in the *HSD3B1* gene have never been reported, probably due to placental progesterone synthesis defect would lead to spontaneous abortion in the first trimester.[1] In contrast, all reported cases with 3βHSD deficiency which is a rare form of congenital adrenal hyperplasia (CAH) are caused by defects in the *HSD3B2* gene. Its clinical spectrum ranges from salt-wasting to non-salt-wasting forms.[4, 5] In its classic form, affected individuals have salt-losing and ambiguous genitalia in both sexes. Biochemical characteristics include highly elevated pregnenolone, 17-OHPreg and DHEA with low levels of cortisol, aldosterone and sex steroids. Unusual cases with 3βHSD deficiency may have elevated 17-OHP levels due to extra-adrenal conversion of 17-OHPreg to 17-OHP by 3βHSD1 at peripheral tissues, which may mislead the diagnosis to 21-hydroxylase deficiency (21-OHD).[6]

Herein, we describe the clinical and hormonal phenotypes of two unrelated boys with classic 3βHSD2 deficiency presented with ambiguous genitalia, salt-wasting and high 17-OHP levels. Both patients were initially misdiagnosed as classic 21-OHD. Mutations of the *HSD3B2* gene were identified.

Methods

Genomic DNA from peripheral blood leucocytes of the patients and their parents was extracted after obtaining informed consent. The entire coding sequence of *HSD3B2* gene including exon-intron boundaries was amplified as described previously.[6] PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH), and sent for direct sequencing at Macrogen Inc. (Seoul, Korea). Analyses were performed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI).

Results

Patient 1

A 2-month-old Thai male infant born to non-consanguineous parents after an uneventful pregnancy, presented with vomiting and lethargy for 10 days. He was noted to have ambiguous genitalia since birth. His birth weight was 2.58 kg (-1.7 SD) and length 50 cm (+0.06 SD). He had frequent vomiting and poor weight gain since the age of one month. On initial evaluation, he had moderate dehydration, pulse rate was 150/min, and blood pressure 81/42 mmHg. His weight was 2.97 kg (-4.5 SD). The physical examination revealed no apparent skin hyperpigmentation, a 2.5-cm long and 1.2-cm wide phallus, penoscrotal hypospadias, separate labioscrotal fold with rugosity, and palpable gonads (Fig. 1). Labs showed serum Na 95, K 5.3, Cl 72, CO_2 11 mmol/L. His karyotype was 46,XY. He was treated with intravenous fluid and stress doses of hydrocortisone, with a provisional diagnosis of CAH. Oral hydrocortisone (15 mg/m^2/day) and fludrocortisone (0.15 mg) were initiated for maintenance treatment. An ACTH stimulation test (250 μg) after 24 h of hydrocortisone cessation showed very poor cortisol response, elevated baseline and stimulated 17OHP and ADD levels, but normal testosterone levels (Table 1). Considering moderately high 17OHP levels, the infant was initially thought to be affected by 21-hydroxylase deficiency. However, his undervirilized genitalia appeared to be inconsistent with 21-OHD. The patient was referred to our center for molecular genetic testing, and the diagnosis of 3βHSD deficiency was considered.

Patient 2

A 2-year-old Indian boy presented with genital ambiguity since birth. He was born to non-consanguineous parents at full term with a normal birth weight of 2.6 kg. He was noted to have genital ambiguity with a 2.5-cm long phallus, penoscrotal hypospadias with normal scrotal fold and palpable both gonads. Four days after birth, he had developed vomiting with significant weight loss (400 g in 4 days, over 10% reduction). The laboratory results showed Na 122, K 5.9 mmol/L and markedly elevated 17-OHP levels at 26,500 ng/dL (normal <200 ng/dL). His karyotype was 46,XY. He was initially diagnosed with classic 21-OHD due to evidence of salt-losing and markedly high 17-OHP levels. Stress dose of hydrocortisone and fludrocortisone therapy were given. He responded well with this treatment and his electrolytes became normal shortly after the hormonal treatment. At age 2 years, he was treated with a short course of testosterone (25 mg intramuscular once weekly for 3 weeks) to increase the size of the phallus before a 2-step hypospadias correction. Six months later, the combined ACTH and hCG stimulation tests were done (after stopped steroid replacement for 2 days) and the results showed high baseline ACTH levels, poor cortisol response to ACTH with elevated stimulated 17-OHP and DHEA levels (Table 1), and low stimulated free testosterone (1.01 pg/mL, normal range 3.3-8.0 pg/mL) after 72-hour hCG injection. Thus, 3βHSD deficiency was considered because of 46,XY undervirilization, evidence of primary adrenal insufficiency and the high levels of stimulated DHEA.

The results of direct *HSD3B2* gene sequencing showed a homozygous missense mutation c.776C>T (NCBI reference sequence NM_000198.3) in Patient 1, leading to an amino acid exchange of threonine with methionine at codon 259 (p.T259M). Parental DNA of Patient 1 was unavailable. Patient 2 carried a novel homozygous nonsense mutation (c.540C>A) that causes the introduction of a premature stop codon at residue 180 (p.Y180X). Both parents were heterozygous carriers for this mutation, and had normal phenotypes. The identified novel mutation was not found in 50 healthy individuals.

Discussion

3βHSD deficiency is a rare cause of CAH. The clinical spectrum ranges from classic salt-wasting to mild or non-classic form. Classic 3βHSD deficiency results in salt wasting early in infancy and

ambiguous genitalia in both sexes. Affected females exhibit normal genitalia or only mild degree of virilization due to the overproduction of DHEA from adrenals, which can be converted to testosterone by 3βHSD1 in extra-adrenal tissues.[1, 4] In contrast, affected genetic males have undervirilized external genitalia (typically severe hypospadias and micropenis) due to defects of testosterone synthesis.[5]

To date more than 40 mutations have been identified in the *HSD3B2* gene in patients suffering from 3βHSD deficiency. Up to now, no mutations have been reported in the Thai or Indian populations. In this study, we have found a previously-described missense mutation and a novel nonsense mutation in two unrelated boys (Thai and Indian) presented with ambiguous genitalia and salt-losing with elevated concentrations of 17OHP. Although underdeveloped genitalia in our 46,XY patients did not support the diagnosis of classic 21-OHD, they were initially misdiagnosed as having 21-OHD because of their very high 17-OHP levels. This finding could be explained by the presence of 3βHSD1 isoenzyme in the peripheral tissues, which can extra-adrenally convert the accumulated $\triangle^5$-steroids, including pregnenolone, 17-OHPreg, and DHEA to progesterone, 17-OHP and ADD, respectively.[5, 7] Basal and ACTH-stimulated 17-OHP levels in some previously reported cases with *HSD3B2* mutations were higher than 10,000 ng/dL (300 nmol/L)[6], which is the cut-off level for biochemical diagnosis of classic 21-OHD.[8] Rare cases with 3βHSD deficiency came to medical attention due to a positive result of 17OHP newborn screening.[6, 9] The differential diagnosis of an elevated 17OHP includes 21-OHD; 11β-hydroxylase deficiency (11-OHD); 3βHSD deficiency; and P450 oxidoreductase (POR) deficiency. Affected genetic male newborns with 21-OHD or 11-OHD should have normal genitalia. In 46,XY infants with underdeveloped external genitalia and elevated 17-OHP; 3βHSD deficiency or POR deficiency have been considered. However, patients with POR mutations have never been reported with salt-losing crisis. In addition, patients with severe POR mutations should have Antley-Bixler syndrome skeletal phenotype.[1] Thus, the clinical phenotypes and the complete adrenal steroid profile from the ACTH stimulation test are essentially leading to the correct definite diagnosis.

Hormonal criteria for the diagnosis of 3βHSD deficiency were proposed including baseline and ACTH-stimulated 17-OHPreg and 17-OHPreg to cortisol ratios, which typically over 10 SD above the mean.[10, 11] However, the assay of 17-OHPreg is generally unavailable in most laboratories especially in less-resource countries. Thus, the mutation analysis of the *HSD3B2* gene plays an important role for definite diagnosis and appropriate genetic counseling.

The exact genotype-phenotype prediction of *HSD3B2* mutations has not been well established. Previous studies suggested that *in vitro* 3βHSD activities correlate well with salt-wasting phenotype but cannot be used to predict the degree of male undervirilization.[5] The p.T256M mutation found in Patient 1 has been functionally studied by *Moisan et al.*[12], and caused the complete loss of *in vitro* 3βHSD activity. The p.Y180X mutation identified in Patient 2 has not been previously reported. The p.Y180X would lead to a predicted large truncation (192 amino acids) of 3βHSD2 protein and the loss of substrate binding and membrane-spanning domains[5] which are crucial parts for enzyme activity.

In summary, we described the novel mutation associated with classic 3βHSD deficiency. This study may provide further insight into the complex clinical and hormonal phenotypes as well as expands the genotypic spectrum of *HSD3B2* mutations.

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Ethical approval: All procedures were performed according to the Declaration of Helsinki and approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Competing interest: All authors declare no completing interests.

Contributors: KW carried out the molecular genetic studies and drafted the manuscript. The patients were under the care of KU and VS[3]. TS conceived the idea of the report, study design, and data analysis. TS and VS[4] revised the manuscript critically for important intellectual content. All authors approved the final version of the manuscript.

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Table 1. Basal and 60 min post cosyntropin (250 μg) adrenal steroid profile.

Steroids	Patient 1 (age 2 m)		Patient 2 (age 2 y)		Reference values	
	Basal	Stimulated	Basal	Stimulated	Basal	Stimulated
ACTH (pg/mL)	-	-	1250	-	10-65	-
Cortisol (μg/dL)	0.75	1.05	0.8	0.6	3-22	27-50
17-OHP (ng/dL)	605	1032	-	957	13-173	85-250
Progesterone (ng/dL)	111	275	-	-	*	*
DHEA (ng/dL)	-	-	-	2160	*	*
ADD (ng/dL)	89	105	44	-	<10-48	<10-87
Testosterone (ng/dL)	214	-	<10	-	#	-

*Note: Reference values are unavailable.

#Reference ranges for total testosterone in males (20-60 days) were 60-400 ng/dL, and in males (1-10 years) were <10 ng/dL

Figure legend

Fig. 1. Genital appearance in Patient 1. Micropenis, penoscrotal hypospadias, and separate labioscrotal fold with rugosity were noted.

Mutation in the *CYP11A1* Gene Resulting in Partial Deficiency of P450 Side-chain Cleavage Enzyme in a Patient with 46,XY Disorder of Sex Development (DSD) and Late-onset Adrenal Insufficiency

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Running title: *CYP11A1* mutation and 46,XY DSD

The capsule of abstract

There is a broad clinical spectrum of P450scc deficiency. *CYP11A1* mutation should be considered as a differential diagnosis of 46,XY DSD, particularly in context of any clinical features of adrenal insufficiency.

Abstract

Objective: To determine the cause of 46,XY DSD and late-onset adrenal insufficiency (AI) in a 2-y-old child.

Design: Case report

Setting: A university hospital

Patient: A female Venezuelan child presented at birth with discordant prenatal karyotype (46,XY) and postnatal genital appearance. The baby had palpable left inguinal gonad, minimal clitoromegaly, and no hyperpigmentation. At age 1 month (m), she had normal gonadotropins, but low testosterone (30.3 ng/dL) and high DHEAS (131 mcg/dL; reference range [RR]: 0.5-19.4). Left orchidectomy at 3m revealed an immature but otherwise normal testis. At age 1.5y, she presented with acute gastroenteritis, lethargy, mild hyponatremia; serum cortisol was 19.1 ug/dL. At age 2.3y, she presented with vomiting, fever, mild hypotension and tachycardia: Na+ 122 mMol/L, CO2 16 mMol/L, glucose 49 mg/dL. Critical sample prior to IV hydrocortisone showed high ACTH (250 pg/mL), cortisol 12 ug/dL, plasma renin activity 11.15 ng/mL/hr (RR: 0.25-5.82), and low (but measurable) steroids of the other pathways. She was started on hydrocortisone and fludrocortisone with a provisional diagnosis of high-level steroidogenic defect.

Interventions: The entire coding regions of the *StAR, HSD3B2*, and *CYP11A1* gene were assessed by polymerase chain reaction and sequenced.

Main outcome measures: Molecular characterization of the *StAR*, *HSD3B2*, and *CYP11A1* gene.

Results: *StAR* and *HSD3B2* gene sequences were normal. The patient harbored homozygous previously-described p.A359V mutation in the *CYP11A1* gene; parents were heterozygous carriers. Prior *in vitro* studies showed ~11-36% of native enzyme activity, consistent with the clinical diagnosis of partial P450scc deficiency.

Conclusions: Partial loss-of-function *CYP11A1* mutation should be considered as a differential diagnosis of 46,XY DSD, particularly in context of any clinical features of AI, such as hyponatremia or hypotension during illness, which may be life-threatening.

Key words: P450scc, *CYP11A1*, adrenal insufficiency, mutation, ambiguous genitalia, 46,XY DSD, congenital adrenal hyperplasia

Introduction

Formerly considered incompatible with fetal survival, P450scc (cholesterol side-chain cleavage enzyme) deficiency is a very rare form of congenital adrenal hyperplasia that disrupts the first step of steroidogenesis, resulting in deficiencies of all adrenal and gonadal steroids. Early reported cases had 46,XY DSD and early-onset adrenal insufficiency (AI)[1,2]. However, recently a handful of partial P450scc deficiency cases due to mutations of *CYP11A1* have been reported in patients with late-onset (often life-threatening) AI, with or without DSD[3,4].

P450scc is a 521-amino acid protein localized on the inner mitochondrial membrane that catalyzes three consecutive reactions: 20α-hydroxylation, 22-hydroxylation, and scission of the C20,22 carbon bond. These reactions result in the conversion of cholesterol to pregnenolone in the first step of steroidogenesis. P450scc is encoded by the *CYP11A1* gene, located on chromosome 15q23-24. P450scc deficiency was formerly thought to be incompatible with fetal survival because the high level of the steroidogenic defect disrupts placental production of progesterone, which is required for the maintenance of human pregnancy. However, several cases with mutations in *CYP11A1* cause defects of P450scc activity have been reported. Initial reported cases of P450scc deficiency had clinical features of complete 46,XY sex reversal and early-onset, life-threatening adrenal insufficiency[3,4]. Recently, a small number of cases with milder form of this disease have been reported in patients with later-onset adrenal insufficiency, with or without disorder of sex development[5-10]. Here, we describe the case of P450scc deficiency presenting with 46,XY DSD and late-onset AI caused by partial loss-of-function *CYP11A1* mutation.

Materials and methods

Patient

The patient was a 2-year-old Venezuelan phenotypic female girl. She was born at term with a birth weight of 3.5 kg. She was the first child to healthy parents who deny consanguinity, but her maternal and paternal grandparents came from the same small town in Venezuela. She was worked up on the basis of discordant prenatal 46,XY karyotype and postnatal female genital appearance. Physical examination in the neonatal period noted a palpable left inguinal gonad, mild clitoromegaly (length 1.4 cm) and no skin pigmentation. Her provisional diagnosis at that time was testosterone biosynthetic defect, based on findings of low testosterone levels at 1 month of age (Table 1). She had normal gonadotropins at age 1 month, but an elevated FSH level (45 IU/L) at age 3 months. She underwent left orchidectomy at 3 months of age. The gonad was reported as being an immature testicle, accompanied by epididymis, and otherwise normal testis, including some germ cells (Figure 1). Repeat hormonal evaluation at 1.3 years of age revealed measurable anti-Müllerian hormone (AMH) and inhibin B levels, suggested normal Sertoli cell function. At 1.5 years of age, she presented to an emergency room (ER) with acute gastroenteritis, lethargy, and mild hyponatremia. Her serum cortisol was 19.1 μg/dL. She received intravenous fluid to correct dehydration and electrolyte imbalance and was discharged without medication. A year later (age 2.3 years), she again presented to ER with fever, vomiting, mild hypotension and tachycardia. Physical examination revealed clinical signs of dehydration, mild clitoromegaly with posterior labial fusion, underdeveloped labia minora, and single perineal opening. She had no skin hyperpigmentation. Laboratory results showed serum Na 122 mmol/L, K-hemolyzed, CO_2 16 mmol/L, and plasma glucose 49 mg/dL. Her critical samples prior to IV hydrocortisone showed elevated ACTH at 251 pg/mL, cortisol 12 μg/dL, plasma renin activity 11.15 ng/mL/hr; low (but measurable) steroids of the other pathways (Table 1). Due to inappropriately low plasma cortisol level and other steroids given her stressed state, she was

treated with IV fluid and stress dose of hydrocortisone with a provisional diagnosis of a high-level steroidogenic defect. She was initiated with oral hydrocortisone and fludrocortisone for maintenance treatment. At age 3.1 years, she was admitted for additional diagnostic workup and elective right inguinal hernia repair and orchidectomy. Pre-operative investigations while receiving hydrocortisone (11 mg/m^2/day) and fludrocortisone 0.15 mg/day revealed elevated ACTH levels at 104 pg/mL. An ACTH stimulation test (cosyntropin 250 μg), showed no significant increases in any adrenal steroids (Table 2). Right inguinal hernia repair and orchidectomy was done and the pathology showed atrophic but otherwise normal testis, mild fibrosis of testicular parenchyma, and tubules contained Sertoli cells only, no identifiable germ cells or Leydig cells (Figure 1).

Hormonal studies (Table 1, 2)

PCR and DNA sequencing

With Institutional Review Board approval and informed consent, leukocyte genomic DNA was extracted and all protein-coding exons and at least 100 bp of flanking intronic DNA of the *StAR, HSD3B2* and *CYP11A1* genes were amplified by PCR using previously described primers and conditions[8,11,12], in 20 μL reactions containing 100 ng of genomic DNA, PCR buffer (Promega, Madison, WI), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 μM of each primer, and 0.5 U Taq DNA polymerase (Promega). The PCR products were verified for correct size on ethidium bromide-stained 1% agarose gel, and treated with exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT, USP Corporation, Cleveland, OH). DNA sequence analysis was performed by Macrogen Inc., Seoul, South Korea. The sequencing data was analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI, USA).

Results

DNA sequencing of coding regions and splice sites of the *StAR* and *HSD3B2* genes showed no mutations. The patient was homozygous for the previously-described *CYP11A1* missense mutation c.1076C>T substitution in exon 6 (Figure 2), changing alanine to valine at codon 359 (p.A359V). Both parents were heterozygous for the mutation.

Discussion

There are only nineteen patients with P450scc deficiency reported so far[1-10]. The clinical manifestations and phenotypic spectrum differed substantially in this disorder. Affected individuals with severe P450scc deficiency are phenotypically female and have severe salt loss in early infancy, whereas milder forms present with later-onset adrenal insufficiency, with or without disorder of sex development. Until recently, *CYP11A1* mutations in 46,XY individuals are associated with different degrees of DSD. In 2011, Parajes *et al.* first described two brothers who presented with adrenal insufficiency at 2-4 years of age and normal male genitalia[7]. These patients had homozygous *CYP11A1* mutation, R451W. *In vitro* functional studies showed that R451W mutant retained ~30% of wild-type activity. Tee *et al.* also recently reported 7 children with adrenal insufficiency who lacked disordered sexual development and had CYP11A1 mutations[10]. These data demonstrate a broad phenotypic spectrum in patients with *CYP11A1* mutations, ranging from normal male, to normal female external genitalia, and from immediate postnatal adrenal failure, to delayed presentation in mid-childhood associated with intercurrent illness. Our patient presented A359V mutation, which was first reported in a 46,XY phenotypic female with life-threatening adrenal insufficiency at age 21 months[4]. Our patient had a similar onset of first adrenal symptoms, but slightly different degree of DSD. The functional analysis revealed A359V mutant resulted in a severe functional defect in the P450scc enzyme (~11%).

When *in vitro* activities were reassessed using highly specific liquid chromatography tandem mass spectrometry to measure pregnenolone production, A359V retained ~36% of wild-type activity[7]. Therefore, while there is some variability in these biochemical assays, it seems that 10-40% activity will considerably alter the classic phenotype especially the onset of adrenal symptoms. Because of its location on the protein surface, one functional consequence of the A359V mutation may be to interfere with redox partner interactions[7]. The defect resulted in negligible prenatal and postnatal androgen production (manifest by failure of external genital masculinization) and apparent progressive decline in glucocorticoid and mineralocorticoid production, with adrenal failure in the toddler years. In retrospect, the child had evidence of adrenal insufficiency by 1.5 years of age, although she had survived significant illnesses without steroid replacement, likely due to the residual enzyme activity associated with this missense change. It is possible that her decompensation at age 2.3 years reflected progressive loss of adrenal can be the result of the "two-hit mechanism" that accounts for the pathophysiology of congenital lipoid CAH caused by StAR mutations[13], in which the disease progresses from mutation-induced impairment of steroidogenesis (the first hit) to the loss of steroidogenesis from accumulation of intracytoplasmic and intramitochondrial cholesterol esters leading to apoptosis of steroidogenic cells (the second hit). In addition, there is evidence that non-steroidogenic testicular function was retained such as absent Müllerian structures as well as postnatal detection of AMH and inhibin B in serum.

In summary, *CYP11A1* mutations in 46,XY individuals are associated with a broad phenotypic spectrum, from normal male, to normal female external genitalia, and from immediate postnatal adrenal failure, to delayed presentation in mid-childhood associated with intercurrent illness. The variations in clinical presentation are likely due, at least in part, to mutation-specific

abolition *vs.* partial retention of steroidogenic activity of P450scc. P450scc deficiency due to mutations in *CYP11A1* should be considered in the differential diagnosis of all patients presenting with adrenal insufficiency, irrespective of genital phenotype and age of presentation.

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Table 1. Summary of Patient's hormonal investigation

Test	Reference Range[1]	Age						
		1 m	5 m	1 yr	1 yr 3 m	1 yr 5 m	2 yr 2 m[3]	2 yr 6 m
17-OH pregnenolone (ng/dL)[1]	10-103 (2-5 y)						28	
17-OH progesterone (ng/dL)[1]	40-200 (1-2 m) 3-90 (1-10 y)	566					11	31
Androstenedione (ng/dL)[1]	6-68 (1-11 m) 8-50 (1-10 y)	330					<10	<0.3
DHEA (ng/dL)[1]	20-130 (1-5 y)						113	
DHEA sulfate (µg/dL)[1]	5-111 (1-12 m) <5-57 (1-5 y)	131.1				198.8	53	
Total testosterone (ng/dL)[1]	75-400 (1-7 m)	30.3		7	4.8		<3	<4.9
Free testosterone (pg/ml)[1]	0.15-0.6 (1-10 y)				0.45			
Dihydrotestosterone (ng/dL)[1]	<3 (prepubertal)				<6[2]			
Cortisol (µg/dL)[1]	3-21 (at 08:00)					19.1	12	4.3
Deoxycorticosterone (ng/dL)[1]	2-34 (2-10 y)						3.8	
Aldosterone (ng/dL)[1]	3-35 (2-9 y)						3.8	
Plasma renin activity (ng/mL/hr)	<10						11.15	
ACTH (pg/mL)[1]	6-48						251	191.7
LH ICMA (mIU/mL)[1]	0.02-7.0 (2w-11m) 0.02-0.3 (1-8y)	0.0	5.2	2.4			QNS	
FSH ICMA (mIU/mL)[1]	0.16-4.1 (1-11m) 0.26-3.0 (1-8y)	2.5	45.0	51.1			2.88	
Anti-Mullerian Hormone enzyme immunoassay (ng/mL)[1]	48-83 (1-6 y)				8.4[2]			
Inhibin B immunoassay (pg/mL)[2]	21-166 (5-9 y)				36[2]			
SHBG immunoassay (nMol/L)[2]	18-114 (1m–2y)				80.8[2]			
Notes						During GI illness with mild hyponatremia	Hydrocortisone (HC) and fludrocortisone (FC) started	HC 5 mg; FC 0.1 mg

Laboratory analyses in the first 2 years of life were performed in Caracas, Venezuela using unknown methods.

[1]In most cases, pediatric reference ranges were not provided for the lab in which the assay was run, so reference ranges shown here are based on pediatric normative data for males, from Esoterix Endocrinology. [2]Performed by Nichols/Quest, Valencia, CA; [3]Labs at 2y 2 mo were collected at Cornell during acute illness with salt wasting, and run at Esoterix.

Table 2. The results of 250 µg ACTH stimulation test

Steroid	Baseline	60 minutes
Pregnenolone (ng/dL)	<10	10
Progesterone (ng/dL)	<10	<10
17-hydroxypregnenolone (ng/dL)	17	11
17-hydroxyprogesterone (ng/dL)	<10	<10
Dehydroepiandrosterone (ng/dL)	<20	<20
Androstenedione (ng/dL)	<10	<10
Cortisol (µg/dL)	2.4	2.5

The patient was receiving 11 mg/m^2/day hydrocortisone, but her ACTH level was 104 pg/mL at 2 days before this test. Blood was drawn at baseline and 60 minutes after administration of 250 µg ACTH. All steroids were measured at Esoterix Endocrinology, California

Figure 1. Right inguinal hernia repair and orchidectomy was done at age 3 years and the pathology showed atrophic but otherwise normal testis, mild fibrosis of testicular parenchyma, and tubules contained Sertoli cells only, no identifiable germ cells or Leydig cells

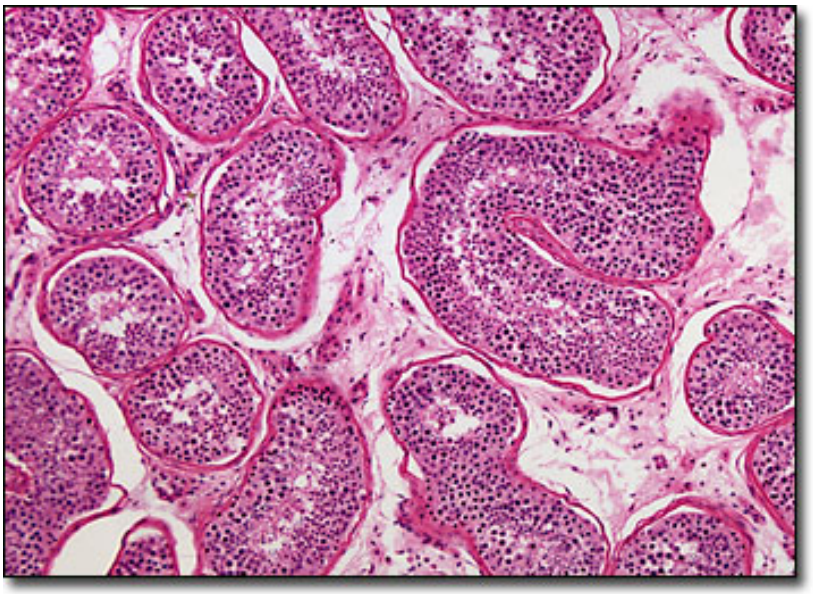

Figure 2. Mutation analysis by direct DNA sequencing. The base change from C to T at position bp 1076 results in alanine to valine change at amino acid 359 (p.A359V). The parents are heterozygous, and the patient is homozygous for the p.A359V mutation.