Genetic Aetiology of Primary Adrenal Insufficiency in Chinese Children

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Primary research

Keywords: primary adrenal insufficiency, geneticdiagnosis, WES, congenital adrenal hyperplasia, triple A syndrome, X-linked adrenoleukodystrophy, congenital adrenal hypoplasia

Posted Date: July 22nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-45355/v1

Version of Record: A version of this preprint was published at BMC Medical Genomics on June 30th, 2021. See the published version at https://doi.org/10.1186/s12920-021-01021-x.

Abstract

Background

Primary adrenal insufficiency (PAI) is a life-threatening condition, and a definitive aetiological diagnosis is essential for management and prognostication. We conducted this study to investigate the genetic aetiologies of PAI in South China and explore their clinical features.

Results

Among the 70 children, 84.29% (59/70) were diagnosed with congenital adrenal hyperplasia (CAH), and a diagnosis of 21-hydroxylase deficiency (21-OHD) was subsequently genetically confirmed in 91.53% of the cases. Salt wasting (SW), simple virilization (SV), and non-classic (NC) CAH accounted for 66.10% (39/59), 30.51% (18/59), and 3.39% (2/59) of the cases, respectively. Interestingly, 17-hydroxyprogesterone (17-OHP) and testosterone levels in females were significantly higher than those in males among both SW and SV CAH patients. Additionally, 15.71% (11/70) of the patients were diagnosed with PAI, 72.73% (8/11) of whom had positive genetic findings. Among all the cases, two novel variants in *CYP21A2*, c.833dupT (p. 279GfsX17) and c.651 + 2T > G, were harboured by CAH patients. A microdeletion (Xp21.2-21.3) and five novel variants, including 2 in the *NR0B1* gene (p. 108S > X, p.L411Vfs*6, c.1231_1234delCTCA, p.L411Vfs*6), 2 in the *AAAS* gene (c.399 + 1G > A, p.V103Afs*8, c.250delT, p.W84Gfs*10) and 1 in the *NNT* gene (p.I758Mfs*10), were detected. The novel variant c.399 + 1G > A in the *AAAS* gene was further confirmed to lead to exon 4 deletion in mRNA transcription and produce a truncated ALADIN protein.

Conclusions

We found ethnic differences in the *CYP21A2* gene variant spectrum among different study populations. Female 21-OHD patients tended to have higher 17-OHP and testosterone levels, which warrants caution in relation to the virilization effect both physically and psychologically. Novel gene variants detected in the *CYP21A2*, *NR0B1*, *AAAS* and *NNT* genes expanded the genetic spectrum of paediatric PAI; however, further improvement of genetic testing tools beyond our protocol is still needed to uncover the complete aetiology of PAI in children.

1. Background

Primary adrenal insufficiency (PAI) is an infrequent but critical clinical condition due to inadequate steroid hormone secretion that mainly affects cortisol with or without aldosterone and adrenal sex steroid deficiency[1]. PAI in children is quite inconsistent with that in adults as congenital disorders caused by genetic aberrations are much more frequent[2]. Based on the affected biological process, the aetiology of inherited PAI can be categorized as impaired steroidogenesis, adrenal hypoplasia, adrenocorticotrophic hormone (ACTH) resistance, defects in complex lipid metabolism, autoimmune destruction, mitochondrial diseases and miscellaneous. Congenital adrenal hyperplasia (CAH) accounts for most paediatric PAI cases, ranging from approximately 70–80% of cases[2, 3]. Among the remaining patients, the aetiology is quite diverse. Based on the study population and a test platform, the disease spectrum contains adrenoleukodystrophy (ALD), X-linked adrenal hypoplasia congenita (X-linked AHC), autoimmune polyglandular syndrome, Wolman disease and so on.

In clinical practice, the differential diagnosis and management of paediatric PAI are quite challenging[4]. Ori Eyal and colleagues reviewed the epidemiology and risk factors for adrenal crises in children with adrenal insufficiency (AI) between 1990 and 2017 at four Israeli paediatric endocrinology units and found that diagnosis and long-term management of paediatric patients remained a challenge[5]. Adrenal crises are life-threatening emergencies, but studies on the rate and risk factors for adrenal crises in children with AI are scarce[5]. Currently, the exact cause of more than 5% of paediatric PAI cases is undetermined despite continual detection of novel genetic causes[6]. Considering the limited data for Chinese PAI children, we used multiple molecular test strategies to explore genetic causes of PAI in Chinese children and to determine the genotype and phenotype correlation.

2. Results

2.1 Clinical characteristics of children with CAH

Among the 70 children, 84.29% (59/70) had CAH, and 15.71% (11/70) had uncharacterized PAI. The gender ratio was almost equal among CAH patients (male 29, female 30), but a male predominance (81.82%,9/11) was found for uncharacterized PAI. Among the 59 CAH patients, salt wasting (SW), simple virilization (SV) and non-classic (NC) patients accounted for 66.10% (39/59), 30.51% (18/59), and 3.39% (2/59) of the sample, respectively. A prader genital scale (PGS) score ≥ 3 was found in 93.75% (15/16) of SW girls and 38.46% (5/13) of SV girls (Supplement Table 1). Testosterone (TES) and 21-hydroxylase deficiency (21-OHD) levels were significantly higher in SW children than in SV children ($p = 0.018$ and $p = 0.034$, respectively). Among SW patients, 17-OHP and cortisol levels in females were significantly higher than those in males ($p = 0.003$ and $p = 0.033$, respectively). Among SV patients, 17-OHP, cortisol, dehydroepiandrosterone sulfate (DHEA-S) and TES levels in females were significantly higher than those in males ($p = 0.004$, $p = 0.005$, $p = 0.042$, $p = 0.008$, respectively) (Fig. 1).

Table 1
The spectrum of *CYP21A2* variants in Asian patients and in other ethnic groups

Country	Sample	c.293-13C > G	Del	I173N	G111_113VfsX4	R483PfsX58	R357W	P31L	Q319X	E6 cluster	V282L	Other
Croatia[21]	93	34.9%	18.8%	11.3%	2.2%	0.0%	16.7%	5.9%	4.8%	2.2%	0.0%	3.2%
Brazilia[15]	480	21.1%	9.0%	7.5%	1.8%	0.0%	5.4%	0.6%	6.1%	1.2%	26.6%	20.7%
Serbia[16]	61	18.5%	13.0%	2.8%	1.4%	0.0%	11.1%	13.0%	4.6%	0.9%	4.6%	30.1%
Argentine[13]	454	20.6%	11.2%	8.2%	0.8%	1.5%	4.2%	0.7%	6.7%	2.0%	26.2%	19.7%
UK[19]	153	30.3%	45.0%	7.0%	0.0%	0.0%	16.7%	0.0%	4.8%	0.0%	0.0%	3.8%
Tunisian[14]	50	6.0%	22.0%	8.0%	2.0%	0.0%	1.0%	1.0%	26.0%	1.0%	12.0%	9.0%
Germany + Austria[22]	538	29.2%	29.6%	13.1%	2.5%	0.0%	4.1%	2.6%	4.6%	1.5%	7.8%	5.0%
China[11]	43	20.9%	8.6%	36.0%	1.2%	0.0%	2.3%	7.0%	7.0%	2.3%	4.7%	10.0%
China[10]	230	35.0%	19.6%	14.3%	4.3%	0.0%	5.9%	0.2%	4.6%	1.3%	0.2%	14.6%
China[9]	30	38.3%	15.0%	11.7%	0.0%	0.0%	5.0%	1.7%	1.7%	3.3%	0.0%	23.3%
China[8]	35	27.0%	27.0%	17.6%	1.4%	0.0%	9.5%	0.0%	5.4%	1.4%	1.4%	9.3%
China[12]	166	42.5%	23.8%	12.7%	3.0%	2.1%	6.0%	0.0%	5.1%	0.9%	0.0%	3.9%
China (this study)	59	31.36%	18.64%	16.95%	5.08%	4.24%	3.39%	1.69%	0.85%	0.85%	0.85%	16.10%

2. 2 Mutation spectra in the *CYP21A2* gene and the genotype–phenotype correlation in 21-OHD patients

Combined Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) tests yielded 91.53% positive (54/59) variant findings. The most common variants were c.293-13C > G (31.36%), Del (18.64%), p.I173N (16.95%), E3 Δ8 (5.08%), p.R483PfsX58 (4.24%) and p.R357W (3.39%) (Table 1). Two novel variants, c.833dupT (p. 279GfsX17) and c.651 + 2T > G, were found in two patients (supplement Fig. 2). The variant c.833dupT (p. 279GfsX17) in exon 7 of the *CYP21A1* gene was found in an SV patient with the p.I173N variant on the other allele. The splicing variant c.651 + 2T > G at the end of exon 5 of the *CYP21A1* gene was found in an SW patient with the c.293-13C > G variant on the other allele (Table 2). Complete concordance was observed only in group 0, while the concordance values were 83.33% and 84.62% for groups A and B, respectively. Two SW patients in group D with only one pathogenic allele presented with elevated 17-OHP, growth retardation, hyperkalaemia, hyponatremia and adrenal hyperplasia. The other two SW patients in group D with the same presentations had no pathogenic variants in the *CYP21A2* gene. All the above patients refused to undergo further genetic examination. One NC patient in group D who developed increased 17-OHP and dehydroepiandrosterone with mild pigmentation and bilateral adrenal hyperplasia had a monoallelic variant (Supplement Table 2).

Table 2
Genotype-phenotype correlations in CAH patients

Group	Allele 1	Allele 2	Phenotype			Positive predictive value
			SW	SV	NC	For the predicted phenotype (%)
Group 0	Del [a]	Del	5	0	0	100%
	Del	R483PfsX58	1	0	0	
	Del	R357W	1	0	0	
	Del	L308FfsX5-Q319X-R357W	1	0	0	
	Del	P31L- c.293-13C > G- E3 Δ8	1	0	0	
	E3 Δ8-Q319X	c.293-13C > G- Q319X	1	0	0	
	Q319X	L308FfsX5- Q319X	1	0	0	
	R357W	E3 Δ8 [b]	1	0	0	
Group A	c.293-13C > G	c.293-13C > G	6	2	0	83.33%
	c.293-13C > G	Del	5	0	0	
	c.293-13C > G	E3 Δ8	2	1	0	
	c.293-13C > G	R357W	2	0	0	
	c.293-13C > G	R483PfsX58	2	0	0	
	c.293-13C > G	E6 cluster [c]	1	0	0	
	c.293-13C > G	c.293-13C > G- E3 Δ8	1	0	0	
	c.293-13C > G- E3 Δ8	G292S	0	1	0	
	c.293-13C > G	c.1223-1G > A	1	0	0	
Group B	I173N	I173N	0	5	0	84.62%
	I173N	Del	0	2	0	
	I173N	c.293-13C > G	1	1	0	
	I173N	E3 Δ8	0	2	0	
	I173N	R483PfsX58	0	1	0	
	I173N	L308FfsX6	1	0	0	
Group C	V282L	I173N	0	0	1	Not Applicable[d]
	P31L	Del	0	1	0	
	P31L	L308FfsX6	0	1	0	
Group D	p.279GfsX17	I173N	0	1	0	Not Applicable[e]
	c.651 + 2T > G	c.293-13C > G	1	0	0	
	V305M	No pathologic mutations detected	0	0	1	
	c.293-13C > G	No pathologic mutations detected	1	0	0	
	R483PfsX58	No pathologic mutations detected	1	0	0	
	No pathologic mutations detected	No pathologic mutations detected	2	0	0	
Total			39	18	2	

[a] Large fragment deletion

[b] c.331_339delGAGACTAC

[c] c.[7010T > A;713T > A;719T > A], p.[I237N;V238E;M240K]

[d] The positive predictive value for the expected phenotype was not calculated due to the small sample

[e] The positive predictive value for the expected phenotype was not calculated because enzyme activity was unable to predict novel mutations

2.3 Mutation spectra and the genotype-phenotype correlation in uncharacterized PAI children

Highly diverse genetic defects were detected in 72.73% (8/11) of probands from 7 families, and five novel variants were detected, including 2 in the *NR0B1* gene (p. 108S > X, p.L411Vfs*6; c.1231_1234delCTCA, p.L411Vfs*6), 2 in the *AAAS* gene (c.399 + 1G > A, p.V103Afs*8; c.250delT, p.W84Gfs*10) and 1 in the *NNT* gene (p.I758Mfs*10) (supplement Fig. 3). Case 1 (mutant *ABCD1* gene) showed elevated very long-chain fatty acid levels. Case 2 (*IL1RAP-NR0B1-GK* deletion, Xp21.2-21.3) presented with undetectable adrenal glands, increased triglycerides evels, hypothyroidism and cryptorchidism in addition to typical AI and mineralocorticoid deficiency. Cases 3 and 4 (mutant *NR0B1* gene) had mineralocorticoid deficiency and smaller adrenal glands. Cases 2, 3 and 4 with *NR0B1* gene variants showed reduced adrenal gland sizes, accounting for 75% (3/4) of the patients with similar adrenal glands sizes. Cases 5, 6 and 7 (mutant *AAAS* gene) had alacrima with or without achalasia and/or neurological symptoms and were all offspring of consanguineous parents. Case 8 (mutant *NNT* gene) presented no other features in addition to PAI (Table 3).

Table 3
Clinical features of uncharacterized PAI patients

Probands	Sex	Age (years)	Gene variants	Stimulation tests (baseline or baseline/peak)				Electrolyte		Adrenal CT imaging	Main clinica presentatio
				ACTH (pg/ml)	Cortisol (baseline) (ug/dl)	Cortisol (peak) (ug/dl)	17-OHP (ng/ml)	K^+ (mmol/l)	Na^+ (mmol/l)		
Case 1	M	5.22	*ABCD1*: c.1552C > T p.R518W	> 1250	1.46	1.58	0.16	4.1	140	Normal	pigmentatic alacrima, ep consanguin parents
Case 2	M	0.13	*IL1RAP-NR0B1-GK* Microdeletion of Xp21.2-21.3 (2.6 Mb)	> 1250	4.83	ND	3.37	6.2	130	Undetectable	pigmentatic mineralocor deficiency, adrenal cris abnormal li function, ele triglyceride, hypothyroid cryptorchidi
Case 3	M	3.70	*NR0B1(DAX1)* c.338–339 CG > GA p.108S > X	> 1250	4.00	4.09	0.06	5.4	122	Undetectable	pigmentatic mineralocor deficiency, adrenal cris abdominal lymphadene
Case 4	M	9.81	*NR0B1(DAX1)* c.1231_1234delCTCA p.L411Vfs*6	> 1250	1.75	2.17	0.16	5.6	125	Tiny adrenal glands	pigmentatic mineralocor deficiency
Case 5	M	8.40	*AAAS* c.399 + 1G > A p.V103Afs*8	710.00	0.15	ND	0.01	4.2	144	Normal	pigmentatic alacrima, achalasia, consanguin parents
Case 6	F	5.52	*AAAS* c.250delT p.W84Gfs*10	> 1250	1.00	ND	0.03	4.4	142	Normal	pigmentatic alacrima, ep consanguin parents
Case 7	F	3.23	*AAAS* c.250delT p.W84Gfs*10	> 1250	1.00	ND	0.01	3.4	135	Normal	pigmentatic alacrima, consanguin parents
Case 8	M	1.91	*NNT* c.2274delT p.I758Mfs*10	482.1	0.27	ND	0.03	5.3	136.3	Normal	pigmentatic
Case 9	M	0.22	-	313	2.42	3.22	0.19	4.5	134	Normal	pigmentatic hypophysis
Case 10	M	5.02	-	241	9.21	11.10	0.11	4.0	141	Tiny adrenal glands	Pigmentatic microphallu short statur microcepha hepatomega splenomega
Case 11	Male	2.22	-	250	14.64	16.53	0.40	3.9	134	Normal	Pigmentatic

M: male; F:female; 17-OHP: 17-hydroxyprogesterone; ACTH: adrenocorticotrophic hormone; TES: testosterone; DHS: dehydroepiandrosterone. Normal ranges: < 46 pg/ml; Cortisol 5–25 ug/dl; 17-OHP, 1 month-1 year 1.06–40.41 ng/ml, 1 year-13 years 0.07–1.53 ng/ml; TES, female 0–31 ng/dl, male 0–6 years 3–32 7–12 years 3–68 ng/dl; DHS 35–430 ug/d

2.4 Pathogenicity analysis of the novel splicing variant c.399 + 1G > A

Case 5

(Table 3) harboured a novel variant (c.399 + 1G > A) at the splicing juncture site at the end of exon 4 in the *AAAS* gene, which was predicted to affect splicing as the wild-type motif “TCTgtaagt” was changed to “TCTataagt” with no acceptor site or potential branch point influenced or created. Agarose electrophoresis of the cDNA from the proband, the parents and a healthy control demonstrated that the parents had two bands: one is a higher molecular band of the same size as the normal control individual, and the other one is a lower molecular band of the same size as the patient. Sanger sequencing confirmed the exon 4 deletion in the lower molecular band, and the protein was predicted to be truncated (Fig. 2).

3. Discussion

In the present study, we recruited 70 infants and children with PAI and sequentially performed *CYP21A2* gene Sanger sequencing, MLPA testing and biochemical plus clinical detailed examinations and found an overall 84.29% (59/70) diagnostic rate for CAH, with 91.53% (54/59) of CAH patients showing positive genetic findings. For uncharacteristic PAI, we found that 72.73% (8/11) of the cases had positive genetic findings by whole-exome sequencing (WES) and array-based comparative genomic hybridization (Array-CGH). Thus, a total of 88.57% (62/70) of the children were determined to have a positive genetic test, which is higher than the rate in a previous review by Tulay Guran (80%)[7] but lower than the 94.2% positive rate detected by Rebecca Perry and colleagues[2].

Among our 21-OHD CAH patients, the most prevalent *CYP21A2* variants were c.293-13C > G (31.36%), Del (18.64%), p.I173N (16.95%), E3 Δ8 (5.08%), p.R483PfsX58 (4.24%) and p.R357W (3.39%). The first three variants, c.293-13C > G, Del and p.I173N, were consistent with the most frequent variants in the Asian population[8–12]; however, our data showed higher E3 Δ8 and p.R483PfsX58 variant prevalence rates and a lower p.R357W variant prevalence. Ethnic differences were evident among different studies; the frequencies of p.P31L and p.V282L were 1.69% and 0.85% in our study, respectively, while the p.V282L variant had a dramatically high frequency in Argentina and Brazil[13–15] and the p.P31L variant was frequent in Serbia[16] (Table 1). Thus, the genetic background of 21-OHD differs by ethnicity.

Novel variants were found in the *CYP21A2* gene in two children, c.651 + 2T > G and c.833dupT (p. 279GfsX17) (Table 2), which were predicted to be damaging. Variant c.651 + 2T > G located at the end of exon 5 was predicted to alter the wild-type donor site and mostly likely led to whole exon 5 skipping, which would result in the loss of 184–217 amino acid residues. With 9 highly conserved residues[17], these residues are essential to construct the important functional domains as steroid-binding sites (residues 203–207) and large hydrophobic areas (211–218). This splicing site variant should impair enzyme activity because the proband displayed the SW phenotype. The other novel variant, c.833dupT (p. 279GfsX17), most likely causes complete enzyme activity loss because another comparable frameshift variant c.923dupT (p.L308FfsX6), which retains more amino acid residues, causes complete enzyme activity loss[18]. However, more functional studies are required to confirm the exact impact of the novel variants on enzyme activity in the future.

In our research, SW females had higher PGS scores than SV females, as reported by previous studies[19, 20], and the reason may be the higher 17-OHP and TES levels discovered in SW patients (Fig. 1). Interestingly, compared with males, females had higher serum 17-OHP and cortisol levels in both the SW and SV groups, which is consistent with another study[10]. Regarding the genotype-phenotype correlation, the PPV for group 0 was 100%, which is higher than those in most similar studies, while the PPVs in group A (82.61%) and group B (84.62%) are in accordance with those in previous studies[10, 13, 15, 16, 21]. Surprisingly, we found that 8.47% (5/59) of the patients clinically presented with the SW (n = 4) and NC (n = 1) phenotypes without biallelic variants (Supplement Table 2). Studies have reported that the monoallelic variant and absent variants accounted for 2.2–24% of enrolled children[11, 14–16, 21, 22]. The aetiology is unknown. We deduced that an amplification allele dropout effect may occur due to the high similarity between the *CYP21A1* and *CYP21A2* genes. Additionally, the *CYP21A2* gene promotor region and other intronic variants that have not been analysed in studies may also reduce transcriptional activity[4].

In our uncharacteristic PAI patients, we detected a total of 7 pathogenic variants, including one chromosome microdeletion and 5 novel variants in the *NR0B1*, *AAAS*, *NNT* and *ABCD1* genes. The *NR0B1* gene is the most frequent gene responsible for X-linked AHC in males (Table 4). The absence of *NR0B1* causes progenitor cells to prematurely differentiate into steroidogenic cells without adequate maturity[23, 24]. We found two patients each with one novel variant (c.338–339 CG > GA, p. 108S > X; c.1231_1234delCTCA, p.L411Vfs*6) and one infant with an *NR0B1* deletion that manifested as PAI, mineralocorticoid deficiency and diminished adrenal glands; however, no hypogonadotropic hypogonadism was noted due to the patient’s young age. We also found two novel homozygous pathologic variants in the *AAAS* gene (c.399 + 1G > A, p.V103Afs*8; c.250delT, p.W84Gfs*10) in 3 patients (Table 3, cases 6, 7, and 8) from 2 families, and both parents were consanguineous. Alacrima and AI were found in all three patients; however, achalasia was observed only in an 8-year-old boy, which manifested as swallowing difficulties. The other two sisters shared the same genotype with different phenotypes. These results suggest that careful estimation should be performed in every PAI child with alacrima or achalasia. The *ABCD1* gene is another causative gene that is often observed in male patients and results in X-linked ALD[24]. The clinical presentation of X-linked ALD is variable, and no phenotype-genotype correlation has been observed[25]. These phenotypes include cerebral, adrenal, spinal cord and peripheral nerve involvement[25]. Fifty percent of affected children will ultimately develop adrenomyeloneuropathy within 10 years[26]. Our patients showed clinical PAI, elevated serum long-chain fatty acid levels and normal neurological examination results but had white matter lesions detected by magnetic resonance imaging. We also found a novel pathogenic variant in the *NNT* gene (c.2274delT, p.I758Mfs*10). Researchers reported that 53% of patients with *NNT* gene variants presented with hyperpigmentation, while 17% had mineralocorticoid deficiency[27]. Cardiac and thyroid involvement may also exist[28]; therefore, a close long-term follow-up is still needed as our patient presented with only hyperpigmentation. Evident genetic defects were not detected in three PAI patients, and we deduced that our current test methods may fail to identify deep intronic variants and epigenetic changes[29–32].

Table 4
The gene spectrum of uncharacterized PAI in other ethnic groups

Country	Methods	*STAR*	*NR0B1*	*SMAD9*	*AAAS*	*NNT*	*MC2R*	*CDKN1C*	*AIRE*	*CYP11A1*	*MRAP*	*NR5A1*	*ABCD1*	*CYP11B1*
Japan[32]	targeted gene sequence	19	18	7	2	2	1	1	-	0	0	0	-	-
Canada[31]	targeted gene sequence	5	0	-	0	0	1	-	3	0	-	-	0	-
Turkey[30]	targeted gene panel + NGS	11	12	0	1	7	25	0	0	9	9	1	2	0
UK[29]	WES	0	4	0	2	2	1	0	2	6	0	0	0	1
China	WES+ Array-CGH	0	2	0	3	1	0	0	0	0	0	0	1	0

WES; whole-exome sequencing; NGS: next generation sequence; CGH; array-based comparative genomic hybridization

4. Conclusions

In conclusion, the genetic cause of PAI in children is very diverse, with a high prevalence of 21-OHD. A total of 7 novel gene variants were detected in the *CYP21A2*, *NR0B1, AAAS* and *NNT* genes in our cohort. SW 21-OHD females showed higher PGS scores than SV females, which was consistent with the higher levels of 17-OHP and TES. Ethnic differences existed in the genetic variant spectrum of the *CYP21A2* gene. Evident genetic variants were not detected in 11.43% of the PAI patients despite our combined genetic testing protocol, suggesting that testing tools must be improved in future studies.

5. Methods

5.1 Subjects

Seventy children were diagnosed with PAI from July 2012 to August 2017 at Children's Hospital of Fudan University according to published criteria[33]: (i) Initial symptoms suggesting low cortisol levels (e.g., hyperpigmentation, fatigue, hypoevolutism, vomiting, electrolyte disturbances); (ii) Laboratory examinations indicating high plasma ACTH levels accompanied by low or normal cortisol levels and a less than 500 nmol/L peak during the ACTH stimulation test; and (iii) Availability for genetic testing. All the patients received detailed clinical evaluations, and all CAH female patients received PGS scoring for external genitalia. Patients with hyperkalaemia, hyponatremia, elevated serum 17-OHP, and external genitalia virilization manifestations were initially suspected of having 21-OHD, which was further verified through *CYP21A2* gene testing[34, 35]. The diagnostic algorithm for PAI patients is shown in Supplement Fig. 1. The study was approved by the Ethics Committee of the Children's Hospital of Fudan University. Written informed consent for the study was obtained from all patients' parents.

5.2 Biochemical measurements

Venous blood samples were drawn at approximately 8 am in the morning, and all hormones were tested within 2 hours, including serum ACTH (IMMULITE 2000 ACTH, Siemens, UK), cortisol (Access cortisol, Beckman Coulter, USA), DHEA-S (Access DHEA-S, Beckman Coulter), TES (Access Testosterone, Beckman Coulter) and 17-OHP (17 alpha-hydroxyprogesterone Radioimmunoassay Kit, Cisbio Bioassays, France).

5.3 Molecular analysis

Genomic DNA was extracted from peripheral lymphocytes, and four genetic tests were performed according to individual clinical presentations: (i) for 59 patients who presented with cortisol deficiency, androgen excess and/or elevated 17-OHP, the *CYP21A2* gene was amplified using PCR (Supplement Table 3) and double checked using MLPA (SALSA MLPA Probemix, MRC-Holland, Netherlands); (ii) for 2 patients with developmental delay/intellectual disability or congenital anomalies, array-CGH (Agilent Technologies, CA, USA) was performed, and the remaining patients underwent WES (SureSelectXT Human All Exon Kit V6, Agilent Technologies, CA, USA).

Variants found in all genetic tests except for array-CGH were verified by Sanger sequencing in the probands and some of their parents. Truncating variants such as nonsense, frameshift, and splice site variants, gene deletions and previously reported variants were regarded as pathogenic variants. For novel variants with frequencies lower than 0.01 in the ExAC (http://exac.broadinstitute.org/), dbSNP147 (http://www.ncbi.nlm.nih.gov/SNP/) and 1000 Genomes (http://www.internationalgenome.org) databases, pathogenicity was predicted with SIFT (http://sift.jcvi.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and Mutation Taster (http://www.mutationtaster.org).

5.4 Determination of the *AAAS* mRNA structure with the c.399 + 1G > A variant

One patient harboured a mutant *AAAS* gene with c.399 + 1G > A. Subsequent mRNA analysis was performed to test its pathogenicity. Human splicing finder (HSF) 3.0 software (http://www.umd.be/HSF3/index.html) was used to predict the potential donor or acceptor site alteration of the altered splice site. Total RNA was isolated from the peripheral blood of the proband, his parents and normal control individuals (Total RNA Kit, TIANGEN BIOTECH, Beijing, China). The

forward primer 5'-TGGATCAATCTTCCTGTCCTACAAC-3' covering the first 25 bp of exon 2 and the reverse primer 5'-TACAGTGAACAGCAGTCGG-3 46 bp upstream of the end of exon 11 were used to amplify the transcripts of both the wild-type and mutant *AAAS* gene. The amplification product of normal individuals was predicted to be 919 bp. The PCR products were separated by agarose electrophoresis and then extracted from the agarose gel (TIANgel Midi Purification Kit, TIANGEN BIOTECH, Beijing, China). The diversity of transcripts was verified by Sanger sequencing.

5.5 Genotype-phenotype correlation in CAH children

CAH patients' genotypes were categorized into 5 groups as previously described[21, 36]. Briefly, group 0 included null variants on both alleles; group A included homozygotes for the I2G variant or compound heterozygotes for the I2G variant and a group 0 variant; group B included patients who were either homozygous or compound heterozygous for the p.I173N variant with group 0 or A variants; group C included patients who were either homozygous or compound heterozygous for the p.P31L or p.V281L variants with group 0, A or B variants; and group D included unidentified variants and new variants. The genotype-phenotype correlation was assessed in the patients with both genetic and initial clinical information. Positive predictive value (PPV) for the predicted phenotype was calculated as the percentage of patients with the predicted phenotype. The predicted phenotypes were SW for groups 0 and A, SV for group B, and NC for group C.

5.6 Statistical analysis

SPSS Statistics (19.0) software was used for statistical analysis. Data are expressed as the median and minimum to maximum due to non-normal distributions. Comparisons between groups were performed using the Mann-Whitney U test, and differences were regarded as significant if $p < 0.05$.

Abbreviations

PAI
Primary adrenal insufficiency; ACTH:adrenocorticotrophic hormone; CAH:congenital adrenal hyperplasia; ALD:adrenoleukodystrophy; X-linked AHC:X-linked adrenal hypoplasia congenita; AI:adrenal insufficiency; PGS:prader genital scale; 17-OHP:17-hydroxyprogesterone; 21-OHD:21-hydroxylase deficiency; Array-CGH:array-based comparative genomic hybridization; WES:whole-exome sequencing; TES:testosterone; DHEA-S:dehydroepiandrosterone sulfate; HSF:human splicing finder; PPV:Positive predictive value; SW:salt wasting; SV:simple virilization; NC:non-classic:MLPA:multiplex ligation-dependent probe amplification.

Declarations

7.1 Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Children's Hospital of Fudan University. Written informed consent for the study was obtained from all patients' parents.

7.2 Consent for publication

Publication of this research was informed consent to all individuals involved in this study.

7.3 Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.They are also available from the corresponding author upon reasonable request.

7.4 Competing interests

The authors declare that they have no conflicts of interest in this work.

7.5 Funding

The study was supported by the Children's Hospital of Fudan University (no. EK112520180204).

7.6 Authors' contributions

Dr. Luo designed and supervised the study and critically reviewed and revised the manuscript.Dr. Chang performed the whole study, collected data and drafted the manuscript.Wei Lu, Zhu-Hui Zhao, Li Xi, Xiao-Jing Li, Rong Ye, Jin-Wen Ni, Zhou Pei, Miao-Ying Zhang, Ruo-Qian Cheng, Zhang-Qian Zheng, Cheng-Jun Sun, Jing Wu. Dr Lu, Zhao, Xi, Li, Ye, Ni, Pei, Zhang, Cheng, Zheng, Sun and Wu cared for the patients, helped collect data, and reviewed and revised the manuscript.

7.7 Acknowledgements

The authors thank Dr. Wu Bing-Bing and Dr. Yang Lin for their technical assistance with genetic testing. The authors are grateful to all the patients for their invaluable contribution to this study.

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Figures

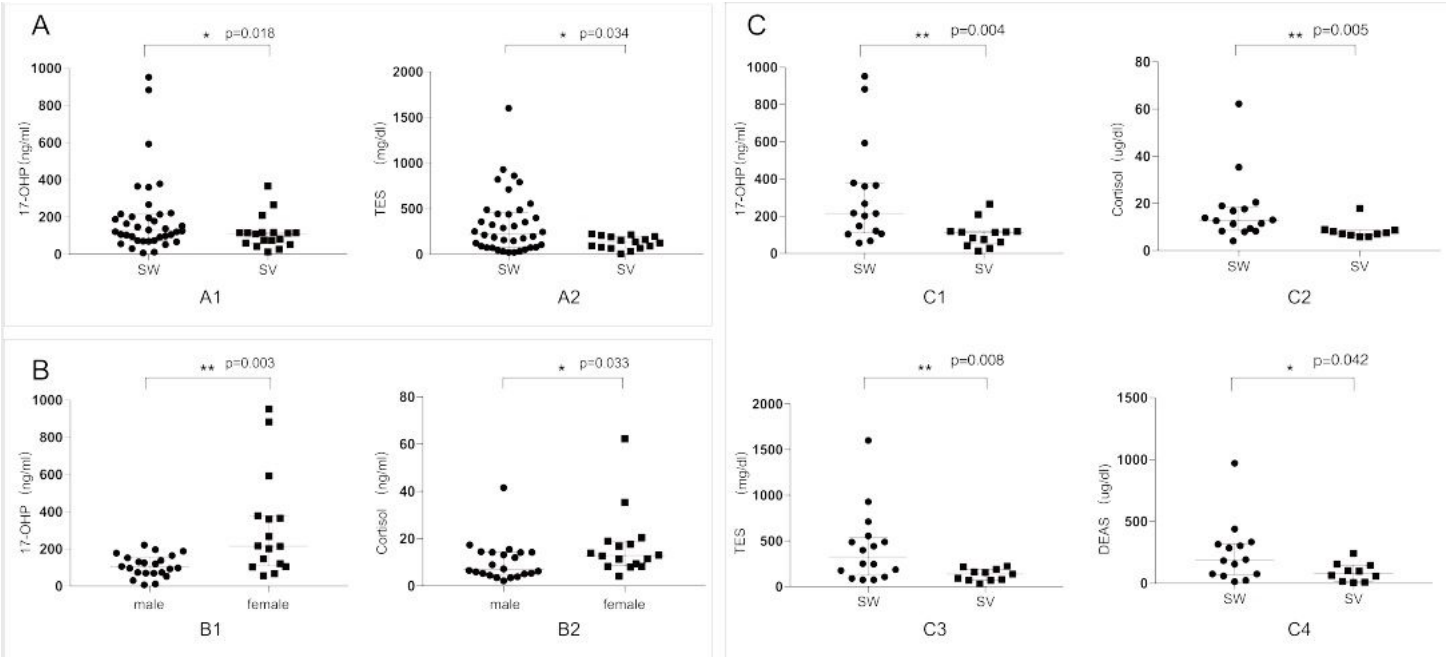

Figure 1

Differences in relevant serum hormones in CAH patients A: The 17-OHP level and the serum TES level were significantly higher in SW patients than in SV patients (A1, p=0.018 and A2, p=0.034, respectively). B: In SW patients, females had significantly higher serum 17-OHP levels (B1, p=0.003) and cortisol levels (B2, p=0.033). C: In female children, the SW group had increased 17-OHP, cortisol, TES and DEAS levels (C1-C4, p=0.004, 0.005, 0.008, and 0.042, respectively).

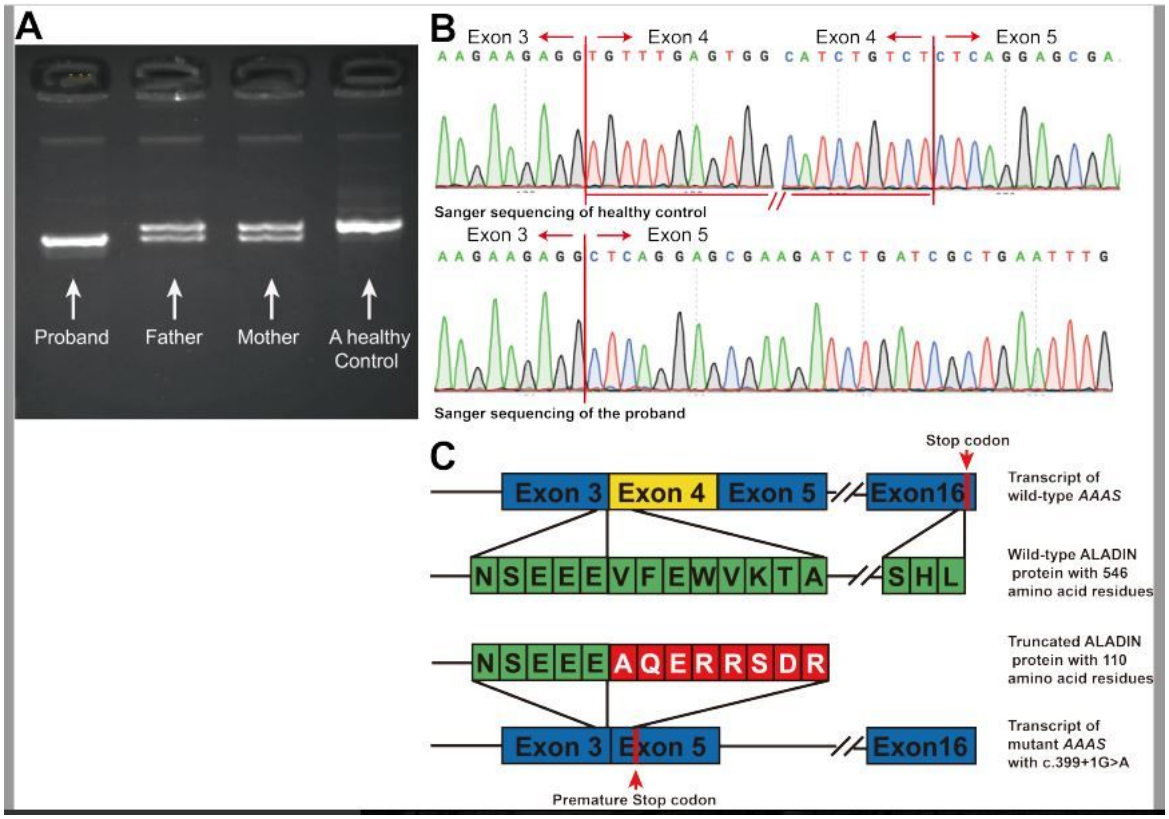

Figure 2

Transcripts of wild-type and mutant (c.399+1G>A) AAAS A: Agarose electrophoresis of the proband, his parents and healthy controls. B: Sequencing of the purified band. The front band lacks exon 4. C: Transcripts and translation of wild-type AAAS and mutant AAAS. The exon 4 deletion resulted in a premature stop codon and truncated ALADIN protein.

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