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CASE REPORT

Simple Virilizing Congenital Adrenal Hyperplasia: A case Report of Sudanese 46, XY DSD male with G293D variant in *CYP21A2*

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Abstract: Congenital Adrenal Hyperplasia (CAH) is a family of inherited disorders that constitute the largest group of Disorders of Sexual Development (DSDs). The classical CAH has two types; the salt-wasting (SW-CAH) and the simple virilizing (SV-CAH). This study is a report of an SV-CAH regarding 46, XY DSD Sudanese male with early signs of puberty at the age of six years.

We designed a customized panel that included 48 genes associated with Disorders of Sexual Development (DSDs) and using Next Generation Sequencing (NGS) technology, detected the pathogenic G293D alteration in the *CYP21A2* gene. This variant has been reported in the salt-wasting (SW) form of 46, XX CAH.

Keywords: Congenital adrenal hyperplasia, CAH, Simple virilizing (SV) salt wasting (SW), Next Generation Sequencing (NGS), Early puberty, Disorders of sexual development (DSDs).

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1. BACKGROUND

Congenital Adrenal Hyperplasia (CAH) is the largest classified group in Disorders of Sexual Developments (DSDs). CAH has two forms; the severe classical /salt wasting (SW-CAH) and the milder non-classical/ simple virilizing (SV-CAH). Both forms are caused by deficient or decreased cortisol biosynthesis [1 - 4]. Inefficient cortisol biosynthesis leads to increased production of corticotrophin-releasing hormone, adrenocorticotropic hormone and hyperplasia of the adrenal glands. Thus this leads the adrenals to produce excessive amounts of androgens as early as 6–7 weeks of gestation [5 - 7]. Therefore, most of the CAH patients are unable to synthesize sufficient amounts of aldosterone and are prone to the life-threatening SW crises. So in SV-CAH, the aldosterone is normal and is usually ignored in boys because excess of androgen during childhood results in overgrowth and early signs of puberty [8 - 10].

Individuals with CAH have a deficiency in 21-hydroxylase enzyme (21-OHD) [11]. This enzyme, encoded by the *CYP21A2* gene [12 - 20] is mutated in 95% of CAH cases [21 - 23, 15] and is located within the HLA region of the short arm of chromosome 6 (6p21.3), next to its inactive pseudogene (*CYP21A1P*) [11, 24, 25]. The *CYP21A2* and *CYP21A1P* have 98% homology in exons and 96% in introns [21, 25, 26]. Both gene and pseudogene contain 10 exons with 3.1 kb in length [27].

The molecular genetics of CAH has identified different mutations which have been classified based on 21-hydroxylase activity [28]. Over 100 mutations in *CYP21A2* have been discovered to cause 21-hydroxylase deficiency. Approxi-mately, 65–70% of these occur due to micro-conversions derived from its pseudogene [25] such as the most common c.293-13A/C>G in intron 2 or p.I172L gene changes [29]. The rest are caused by copy number variants (CNV) (25-30%) or point mutations [25]. Compound heterozygous mutations have been also identified in the SW form, even though a single mutation could have a mild effect [30]. Moreover, previous studies have shown that p.Q318Y and p.R356W had 0% enzyme activity, while c. 293-13A/C>G and p.I172L presented minimal residual activity and were associated with the classic form of the syndrome [31].

2. CASE REPORT

Here we present a 12 years old boy, with 46, XY karyotype who developed early signs of puberty at age 6, including pubic

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hair, Tanner stage II without axillary hair, acne, and dark facial skin. His voice also changed to be more like an adult male voice. Penile length and testicular volume were 7.5 cm and 2 ml, respectively. His testes were not diagnosed with pathological condition. Testosterone level was 2.8 ng/ml and normal level of electrolytes. Accordingly; the boy was diagnosed with SV-CAH and administered hydrocortisone 17 mg (10 mg in the morning and 7 in the evening). At age 8 years old, his bone age was equal to that of 12-13 years old male. Testicular volume was 4 ml, FSH was 7.67mU/ml and LH 1.21 mU/ml. His weight was 37 Kg and was 137 cm tall. Furthermore, there wasn't a family history of this particular condition; however, the patient had a sister who presented with ambiguous genitalia at an early age but died right after that. Their biological parents were not relatives and there was no information of a similar conditions in their extended family.

Genomic DNA was isolated from peripheral blood leukocytes using the automated MagPurix 12S system from Zinexts (Zinexts Life Science Corp., Taiwan) and the MagPurix Blood DNA Extraction Kit 200 (Zinexts Life Science Corp). Genetic alteration causing Disorders of Sexual Development (DSD) was analyzed using a customized gene panel designed with the Ion AmpliSeq Designer software (https://www.ampliseq.com) that included all exons and exon-intron boundaries of the selected 48 genes (Table **1**). Libraries were prepared according to the manufacturer's instructions and samples were sequenced using the Ion Torrent PGM platform (Thermo Fisher Scientific). Amplicons (9.3%) not appropriately covered (<20x fold) were assessed by Sanger sequencing (Sequences of the primers are available under request).

The sequencing data were analyzed using the Ion Reporter software (Thermo Fisher Scientific). Variants were filtered to include those with a *p-value* <0.001 and a Minor Allele Frequency (MAF) ≤0.001 in the annotation settings of the Ion Reporter software (dbSNP, ClinVar and 5000 exomes databases). Moreover, the allele frequency was further checked for each ethnic group in 1000 genomes browser (http://browser.1000genomes.org/) and others. Variants were discounted if they were common. Accordingly, there were 12 variants in different genes (Table **2**). The impact of protein functionality of missense alterations was evaluated using *in silico* prediction programs (Table **2**), including SIFT (http://sift.jcvi.org/), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/index.php), Mutation Taster (http://www.mutationtaster.org/), SNPs & GO (https://snps-and-go.biocomp.unibo.it/snps-and-go/), and Panther (http://www.pantherdb.org). We identified the path-ogenic homozygous missense mutation c.878G>A (p.G293D) in exon 7 of the *CYP21A2* gene (NM_000500.7) (Table **2**). Sanger sequencing was then used for confirmation of the mutation.

3. DISCUSSION

Diagnosis of congenital adrenal hyperplasia (CAH) is challenging especially within clinical settings of the developing countries. Here, the patient was diagnosed with SV-CAH, based on his clinical presentation and available laboratory investigations. Genotyping analysis showed that the patient had the G293D variant in homozygosis in *CYP21A2*. The patient's sister might have been another carrier of this point mutation as she was born with ambiguous genitalia. To the best of our knowledge, a single study has detected the G293D protein change in a female with CAH. Tardy *et al*. described a newborn girl with Prader stage 4 and severe virilization of genitalia [32]. Although biochemical values were not determined, the patient was diagnosed with SW-CAH form.

Table 1. Included genes in the DSD panel.

Gene	Name	Locus	Gene	Name	Locus
AMH	Anti-Mullerian Hormone	19p13.3	*INHA*	Inhibin Alpha Subunit	2q35
AMHR2	Anti-Mullerian Hormone Receptor Type 2	12q13.13	*INSL3*	Insulin-Like 3	19p13.11
AR	Androgen Receptor	Xq12	*KISS1*	KiSS-1 Metastasis-Suppressor	1q32.1
ATRX	Mental Retardation, X-Linked	Xq21.1	*KISS1R*	KISS1 Receptor	19p13.3
BMP15	Bone Morphogenetic Protein 15	Xp11.22	*RXFP2*	Relaxin/Insulin Like Family Peptide Receptor 2	13q13.1
CBX2	Chromobox 2	17q25.3	*LHCGR*	Luteinizing Hormone/Choriogonadotropin Receptor	2p16.3
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1	15q24.1	*MAMLD1*	Mastermind Like Domain Containing 1	Xq28
CYP11B1	Cytochrome P450 Family 11 Subfamily B Member 1	8q24.3	*MAP3K1*	Mitogen-Activated Protein Kinase Kinase Kinase 1	5q11.2
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1	10q24.32	*NR0B1*	Nuclear Receptor Subfamily 0 Group B Member 1	Xp21.2
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1	15q21.2	*ESR1*	Estrogen Receptor 1	6q25.1
CYP21A2	Cytochrome P450 Family 21 Subfamily A Member 2	6p21.33	*ESR2*	Estrogen Receptor 2	14q23.3
DHH	Desert Hedgehog	12q13.12	*NR5A1*	Nuclear Receptor Subfamily 5 Group A Member 1	9q33.3
DMRT1	Doublesex And Mab-3 Related Transcription Factor 1	9p24.3	*POR*	Cytochrome P450 Oxidoreductase	7q11.23
DMRT2	Doublesex And Mab-3 Related Transcription Factor 2	9p24.3	*PSMC3IP*	Proteasome 26S ATPase Subunit 3-Interacting Protein	17q21.2

(Table 1) contd.....

Gene	Name	Locus	Gene	Name	Locus
FGF9	Fibroblast Growth Factor 9	13q12.11	*RSPO1*	R-Spondin 1	1p34.3
FOG2	Zinc Finger Protein, FOG Family Member 2	8q22.3	*SOX3*	SRY-Box 3	Xq27.1
FOXL2	Forkhead Box L2	3q22.3	*SOX9*	SRY-Box 9	17q24.3
FOXO3	Forkhead Box O3	6q21	*SRD5A2*	Steroid 5 Alpha-Reductase 2	2p23.1
FSHR	Follicle Stimulating Hormone Receptor	2p16.3	*SRY*	Sex Determining Region Y	Yp11.2
GATA4	GATA Binding Protein 4	8p23.1	*STAR*	Steroidogenic Acute Regulatory Protein	8p11.23
HARS2	Histidyl-TRNA Synthetase 2, Mitochondrial	5q31.3	*TSPYL1*	Testis-Specific Y-Encoded-Like Protein	6q22.1
HSD17B3	Hydroxysteroid 17-Beta Dehydrogenase 3	9q22.32	*WNT4*	Wingless-Type MMTV Integration Site Family, Member 4	1p36.12
HSD17B4	Hydroxysteroid 17-Beta Dehydrogenase 4	5q23.1	*WT1*	Wilms Tumor 1	11p13
HSD3B2	Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 2	1p12	*WWOX*	WW Domain Containing Oxidoreductase	16q23.1

Table 2. Identified variants in the patient and predicted function according to different software

Genes	Exon	Transcript	Variation	dbSNP	gnomAD[3]	PROVEAN	SIFT	POLYPHEN	Mutation Taster	SNPs & GO	PANTHER
KISS1	2	NM_002256.3	c.58G>A;p.E20K[2]	rs12998	0.03257	Del	Dam	Poss dam	DC	N	Poss dam
MAP3K1	3	NM_005921.1	c.743G>A;p.R248Q[2]	rs201579608	0.0001499	N	Dam	Poss dam	DC	N	Prob dam
MAP3K1	14	NM_005921.1	c.2845_2847delACA;p.T942del[2]	rs769777412	0.0004191	ND					
HSD17B4	7	NM_000414.3	c.420A>T; p.K140N[2]	rs28943589	0.007370	Del	Tol	B	P	D	Poss dam
HSD17B4	24	NM_000414.3	c.2182A>G;p.M728V[2]	rs28943594	0.01051	N	Tol	B	P	N	Prob dam
CYP21A2	7	NM_000500.7	c.878G>A;p.G293D[1]			Del	Dam	Prob dam	DC	D	Prob dam
TSPYL1	1	NM_003309.3	c.527_528insGGT;p.V176dup[2]	rs56100880	ND	ND					
GATA4	6	NM_002052.4	c.1129A>G;p.S377G[2]	rs3729856	0.09643	N	Tol	B	P	D	Prob B
GATA4	6	NM_002052.4	c.1138G>A;p.V380M[2]	rs114868912	0.005689	N	Tol	B	P	D	Prob B
DMRT2	4	NM_181872.4	c.815A>G;p.N272S[2]	rs138608089	0.001039	N	Tol	Poss dam	DC		Poss dam
CYP19A1	5	NM_000103.3	c.602C>T;p.T201M[2]	rs28757184	0.02471	N	Tol	B	P	N	Prob B
SOX3	1	NM_005634.2	c.307C>A;p.P103T[2]	rs201101913	0.006186	N	Dam	B	DC	N	Prob B

B, benign; D, disease; Dam, damaging; DC, disease causing; Del, deleterious; N, neutral; ND, not determined; P, polymorphism; Poss, possibly; Prob, probably; Tol, tolerated.

[1] Variant found in homozygous state. [2] Variant found in heterozygosis. [3] Allelic frequencies correspond to both Exome and Genome analyses.

It is well-known that the severity of the disease correlates well with the level of enzymatic deficiency and the location of the residue [33]. For example, point mutations that disrupt the binding of heme cofactor cause an SW phenotype, while alterations in hydrophobic residues lead to SV-CAH [34]. The highly conserved G293 residue, covering the proximal substrate-binding site is responsible for the flexibility of the I-helix. Therefore, the G293D mutation makes the bending and swiveling of the pocket difficult [30, 33]. Certainly, *in vitro* studies revealed that the mutation decreases the residual activity to <1% [32].

The main difference between the SW and SV-CAH forms is the insufficient aldosterone secretion that leads to a fatal drop of electrolytes in the first [35 - 39]. The normal electrolyte levels observed in SV-CAH might be explained by the role of testosterone as a down-regulator of aldosterone secretion [40]. Unfortunately, we were not able to measure the aldosterone level because the test is not available in Sudan. Functional studies by Toot *et al* in 2008, concluded that testosterone influences the excretion of Na (sodium) and K (potassium) through an androgen receptor dependent mechanism [41]. Similar findings were later published [42, 43]. However, CAH, either SW or SV, displays variable degrees of sodium depletion which is not always significant [44 - 49]. This might depend on the function of the testes. Cabrera *et al* found that patients with SW-CAH had a higher frequency of developing testicular nodules compared to SV-CAH [44]. Our patient was referred to early puberty signs but no nodules or testicular abnormalities affecting his function were observed. Thus, we can speculate that although the G293D mutation was found first in SW-CAH, his elevated testosterone levels could have played a role in electrolyte balance since gonads were normal.

CONCLUSION

In this study, the c.878G>A (p.G293D) variant in *CYP21A2*, previously related to CAH-SW, was identified in homozygous state in a patient diagnosed with SV-CAH. We believe that testosterone had balanced electrolytes through one of the androgen biological pathways. Thus, the level of electrolytes is not only affected by the severity of the enzymatic activity, but also by androgen levels in individuals diagnosed with CAH.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This patient is part of DSD-Sudan project approved by the

National Research Ethics Committee, National Ministry of Health, Sudan with Ethical Number (No. 93-5-09).

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

A written informed consent was obtained from the parents when they were enrolled.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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