Aetiological bases of 46,XY disorders of sex development in the Hong Kong Chinese population

Angel OK Chan *, WM But, CY Lee, YY Lam, KL Ng, PY Loung, Almen Lam, CW Cheng, CC Shek, WS Wong, KF Wong, MY Wong, WY Tse

ABSTRACT

Objective: Disorders of sex development are due to congenital defects in chromosomal, gonadal, or anatomical sex development. The objective of this study was to determine the aetiology of this group of disorders in the Hong Kong Chinese population.

Design: Case series.

Setting: Five public hospitals in Hong Kong.

Patients: Patients with 46,XY disorders of sex development under the care of paediatric endocrinologists between July 2009 and June 2011.

Main outcome measures: Measurement of serum gonadotropins, adrenal and testicular hormones, and urinary steroid profiling. Mutational analysis of genes involved in sexual differentiation by direct DNA sequencing and multiplex ligation-dependent probe amplification.

Results: Overall, 64 patients were recruited for the study. Their age at presentation ranged from birth to 17 years. The majority presented with ambiguous external genitalia including micropenis and severe hypospadias. A few presented with delayed puberty and primary amenorrhoea. Baseline and post–human chorionic gonadotropin–stimulated testosterone and dihydrotestosterone levels were not discriminatory in patients with or without *AR* gene mutations. Of the patients, 22 had a confirmed genetic disease, with 11 having 5α-reductase 2 deficiency, seven with androgen insensitivity syndrome, one each with cholesterol side-chain cleavage enzyme deficiency, Frasier syndrome, *NR5A1*-related sex reversal, and persistent Müllerian duct syndrome.

Conclusions: Our findings suggest that 5α-reductase 2 deficiency and androgen insensitivity syndrome are possibly the two most common causes of 46,XY disorders of sex development in the Hong Kong Chinese population. Since hormonal findings can be unreliable, mutational analysis of the *SRD5A2* and *AR* genes should be considered the first-line tests for these patients.

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New knowledge added by this study

- The most common likely causes of 46,XY disorders of sex development (DSD) in our local Chinese population are 5α-reductase 2 deficiency and androgen insensitivity syndrome.
- Blood hormone testing is unreliable in differentiating between androgen insensitivity syndrome and other causes of 46,XY DSD.
- Mutational analysis of the *SRD5A2* and *AR* genes should be considered the first-line investigation in patients with 46,XY DSD.

Implications for clinical practice or policy

- When encountering patients with 46,XY DSD, 5α-reductase 2 deficiency and androgen insensitivity syndrome should be considered early as their presence has implications for treatment and prognosis.

香港華籍人口中46-XY單純性腺發育不全綜合徵的病因

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目的：性腺發育不全主要由於先天性的染色體和性腺發育異常，或解剖性別非典型所致。本研究旨在探討香港華籍人口中性腺發育不全的病因。

設計：病例系列研究。

安排：香港5間公立醫院。

患者：2009年7月至2011年6月期間接受兒童內分泌科醫生診治的46-XY單純性腺發育不全綜合徵患者。

主要結果測量：量度患者的血清促性腺激素、腎上腺和睾丸激素水平，以及尿中類固醇激素水平測試。採用DNA直接測序方法和多重連接探針擴增術來分析基因突變。

結果：共64名患者被納入研究，病發年齡從出生至17歲不等。大多數患者的外生殖器分化模糊，如小陰莖和嚴重的尿道下裂。少數患者的青春期發育延遲，並有原發性閉經。不論患者是否有*AR*基因突變，其基線和後人類絨毛膜促性腺激素刺激睾酮和雙氫睾酮水平都沒有分別。22名患者確診有遺傳病，其中11例有5α還原酶2缺乏症，7例有雄激素不敏感綜合徵，另膽固醇側鏈裂解酶缺乏症、Frasier綜合徵、與*NR5A1*基因相關的性逆轉和Müllerian管發育不全綜合徵分別各1例。

結論：本研究結果顯示香港華籍人口中46-XY單純性腺發育不全綜合徵患者最普遍的兩種病因為5α還原酶2缺乏症和雄激素不敏感綜合徵。由於激素檢測的結果未完全可靠，應考慮為這些病人進行*SRD5A2*和*AR*基因突變分析作為第一線檢查。

Introduction

Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical.[1] Traditionally, diagnosis in these patients relies on extensive endocrine investigation. With advances in the understanding of the genes involved in sexual determination and differentiation,[2] molecular diagnosis is playing an increasingly important role and may even overtake the role of hormonal assessment as the first-line test, with the latter being reserved for assessment of disease severity rather than diagnosis.[3]

One of the most common causes of 46,XY DSD in the western population is androgen insensitivity syndrome (AIS).[4] Whether the same is true in our local population remains unknown. We performed a prospective multicentre study to explore the possible aetiological basis of 46,XY DSD in the Hong Kong Chinese population.

Methods

Patients

Patients who were referred to a paediatric endocrinologist for the first time or were followed up in their clinic at five public hospitals in Hong Kong between July 2009 and June 2011 were recruited for the study. Inclusion criteria were 46,XY ethnic Chinese patients who presented with incompletely virilised, ambiguous, or completely female external genitalia. Criteria that suggested DSD at birth were overt genital ambiguity, apparent female genitalia with an enlarged clitoris, posterior labial fusion, or an inguinal/labial mass, apparent male genitalia with bilateral undescended testes, micropenis, isolated perineal hypospadias, or mild hypospadias with undescended testes, and discordance between genital appearance and prenatal karyotype.[1] Micropenis is defined as stretched penile length of <2.5 cm based on the published norm for Chinese.[1] Written informed consent was obtained from the patients and/or parents and the study was approved by the local ethics committee. None of the patients/parents refused to participate in the study although seven refused genetic testing (Table 1).

Hormone analysis

Blood was taken from patients for electrolyte and baseline endocrine assessment and included measurement of cortisol, 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate, testosterone (T), androstenedione (A4), dihydrotestosterone (DHT), anti-Müllerian hormone (AMH), and gonadotropins. Human chorionic gonadotropin (hCG) stimulation test was performed to test for testicular Leydig cell function. The short synacthen test was also performed when indicated.

Cortisol, dehydroepiandrosterone sulfate, and gonadotropins were measured by electro-chemiluminescence immunoassay (Modular Analytics E170; Roche, Mannheim, Germany); T was measured by a competitive immunoenzymatic assay (ACCESS 2; Beckman Coulter, Brea [CA], US); 17-OHP was measured by liquid chromatography–tandem mass spectrometry using an in-house method; AMH was measured by an enzyme-linked immunosorbent assay (AMH Gen II ELISA, A73818; Beckman Coulter, Brea [CA], US); DHT was measured by radioimmunoassay (DSL9600i; Beckman Coulter, Prague, Czech Republic); A4 was measured by solid-phase competitive chemiluminescent enzyme-labelled immunoassay (L2KAO2, Immulite 2000; Siemens, Tarrytown [NY], US). Male reference intervals were considered the most appropriate for data interpretation in this study.

Urinary steroid profiling

Spot urine from patients under 3 months of age and 24-hour urine from those at or older than 3 months of age were processed for steroid profiling as described previously.[5]

Molecular analysis

DNA was extracted from peripheral whole blood using a QIAamp DNA blood kit (Qiagen, Hilden, Germany). Polymerase chain reaction and direct DNA sequencing were performed on targeted genes when suggested by the clinical and hormonal findings. Otherwise all patients had their *AR* (androgen receptor) and *NR5A1* (steroidogenic factor 1) genes sequenced. Those patients with negative genetic findings were subjected to multiplex ligation-dependent probe amplification (MLPA) analysis (P185 Intersex probemix; P074 Androgen Receptor probemix and P334 Gonadal probemix; MRC-Holland) to test for gross deletion or gene duplication. The results were analysed by Coffalyser.Net. Family genetic studies were performed when mutation(s) were identified in the index patients.

In-silico analysis for novel missense mutations

The functional effect of novel missense mutations detected was tested by online in-silico analysis software SIFT, PolyPhen2, and Align GVGD.

Results

Overall, 64 patients (53 male, 11 phenotypic female), including 14 new patients, with 46,XY DSD were recruited into the study. The clinical and hormonal findings of individual patients are listed in Table 1. A genetic diagnosis was made in 10 patients prior to the study. Other major structural abnormalities were evident in eight (Table 2). Their age at presentation ranged from birth to 17 years. Five (8%) were born prematurely (24-35 weeks) and nine (14%) with low birth weight (0.59-2.32 kg). All had non-consanguineous parents. A family history of sexual ambiguity was present in six. Overall, 61 (95%) presented with ambiguous external genitalia including 15 with isolated micropenis, eight with isolated severe hypospadias, and one with discordance between the prenatal karyotype and the postnatal phenotype. Three presented after birth, one each with inguinal hernia, delayed puberty, and primary amenorrhoea.

Regarding the hormonal findings, Figure 1 shows the baseline and post-hCG–stimulated T and DHT levels in patients with mutations detected in the *AR* gene and those without, where the results overlapped between the two groups. Eight patients (patients 13, 19, 21, 30, 31, 40, 49, and 56) underwent short synacthen test and with the exception of patient 19, all had an adequate cortisol response (>550 nmol/L). Patient 27 had a relatively low T/A4 ratio before and after hCG stimulation but sequencing revealed no mutation in his *HSD17B3* (17β-hydroxysteroid dehydrogenase III) gene. All other patients had unremarkable T and A4 levels, as well as T/A4 ratio.

Eleven patients had characteristically low 5α- to 5β-reduced steroid metabolite ratios in their urine, compatible with the diagnosis of 5α-reductase 2 deficiency (5ARD). This was also confirmed by mutational analysis of the *SRD5A2* (steroid 5α-reductase 2) gene. All other patients had unremarkable urinary steroid metabolite pattern.

Overall, 22 (39%) patients had a confirmed genetic diagnosis (Table 3). The most common diagnoses in our cohort were 5ARD (n=11) and AIS (n=7). Other genetic diagnoses included cholesterol side-chain cleavage enzyme deficiency (n=1), Frasier syndrome (n=1), *NR5A1*-related sex reversal (n=1), and persistent Müllerian duct syndrome (PMDS; n=1). The clinical and laboratory findings of patients 19 and 20 have been reported previously.[6,7] Patients 12 and 15 had de-novo mutations in the *AR* gene and were in mosaic pattern. Patient 21 had a novel missense variant p.Ala260Val detected in his *NR5A1* gene. His AMH level was not low, contrary to some of the previously reported cases.[8] There was also a clinically significant rise in T level after hCG stimulation. Short synacthen test demonstrated an adequate cortisol response (baseline: 720 nmol/L; post–adrenocorticotropin hormone: 822 nmol/L). His father also carried the same heterozygous mutation although he denied any symptoms of DSD. This novel genetic variant was not detected in 100 normal Chinese subjects (control). Patient 22 had bilateral undescended testes. He underwent orchidopexy at the age of 1 year during which the presence of Müllerian duct structures was suspected. Further workup including pelvic ultrasound revealed Müllerian duct structures and extremely low AMH level. The diagnosis of PMDS was confirmed by the presence of three heterozygous novel missense variants in the *AMH* gene (Tables 3 and 4).

Six novel genetic variants were identified in the *AMH, AR*, and *NR5A1* genes (Fig 2). At least two of the three in-silico analysis programmes predicted the variants to be pathogenic (Table 4). Multiple sequence alignment showed that the amino acids of concern were highly conserved across different animal species. All these findings support the pathogenic nature of these variants accounting for the patients' phenotypes.

Eleven patients were reared as girls because of severe under-virilisation at birth, including three with 5ARD, three with AIS, and one with Frasier syndrome. The underlying genetic causes in the remaining four patients were undetermined. The longest follow-up period was 27 years. None of them has requested change of gender to date. Five patients (patients 2, 4, 7, 12, and 15) exhibited 'tom-boy-like' behaviour during childhood and required counselling by a clinical psychologist while two males (patients 17 and 47) requested exogenous T

to augment penile growth after puberty. Patient 20 developed germinoma in her dysgenetic gonad with no recurrence after surgery.

Discussion

46,XY DSD is a heterogeneous condition caused by a wide spectrum of disorders. Making an accurate diagnosis is difficult but important for emergency medical treatment as some DSDs are associated with life-threatening Addisonian crisis. In addition, the diagnosis is essential so that relevant information and counselling can be provided to parents and clinical management can be formulated, bearing in mind the best interests of the child. Initial workup includes a detailed antenatal and postnatal history, physical examination, karyotyping, and hormonal assays. This will guide further workup such as imaging and genetic analysis. Nonetheless, there are often limitations to hormonal studies as illustrated in the present series. The non-distinct pattern of T and DHT at baseline and following hCG stimulation in *AR* mutation–positive and –negative patients suggest the need to reconsider our laboratory diagnostic algorithm for AIS.

TABLE 1. The clinical and hormonal findings of 64 patients with 46,XY disorders of sex development recruited in this study. Those baseline hormonal results below the age- and gender-specific reference limits are underlined, those above are in bold

Patient No.	Sex of rearing	Presentation	Age at presentation	Age at recruitment and hormonal evaluation	Other procedures	Baseline			
						LH (IU/L)	FSH (IU/L)	Cortisol (nmol/L)	DHEAS (μmol/L)
5ARD									
1	M	AEG	At birth	3 Years	O	<0.2	0.6	268	<u><0.4</u>
2	F	AEG	At birth	26 Years	BG+V	<0.2	1.7	-	-
3	M	Micropenis	At birth	8 Years	-	<0.2 (At 1 year)	0.8 (At 1 year)	163	-
4	F	AEG	At birth	20 Years	BG+V	-	-	-	-
5	M	Micropenis	At birth	7 Years	-	2.6 (On 18 days)	1.3	167	-
6	M	Hypospadia, micropenis	At birth	4 Years	-	2.7	2.4	68	-
7*	F	AEG	2 Months	26 Years	BG+V	-	-	-	-
8	M	Micropenis	1 Year	2 Years	-	-	-	-	-
9	M	Micropenis	2 Years	15 Years	-	4.3	4.0	207	2.9
10	M	Micropenis	2 Years	5 Years	-	0.5	1.0	-	-
11	M	Micropenis	17 Years	17 Years	-	6.6	7.1	-	-
Androgen insensitivity syndrome									
12*	F	AEG	At birth	27 Years	BG+V	-	-	152	<u>3.6</u>
13	M	AEG	At birth	5 Months	R	2.3	1.5	623	-
14	M	AEG	At birth	10 Years	R	<0.2	2.5	273	2.3
15*	F	AEG	At birth	14 Years	BG	-	-	73	5.8
16	M	AEG	At birth	15 Days	R	**22.7**	**11.5**	63	<0.4
17	M	AEG	1 Month	16 Years	R	**14.6**	5.9	347	8.0
18	F	Primary amenorrhoea	15 Years	16 Years	BG	**16.2**	11.6	-	-
Cholesterol side-chain cleavage enzyme deficiency									
19	M	AEG	At birth	2 Years	O	**7.3**	**27.3**	54	<u><0.4</u>
Frasier syndrome									
20*	F	Delayed puberty	15 Years	24 Years	BG	**54.7**	**181**	-	-
***NR5A1*-related sex reversal**									
21	M	AEG	At birth	5 Years	R	0.8	1.2	158	<u><0.4</u>
Persistent Müllerian duct syndrome									
22	M	Bilateral undescended testes	At birth	10 Years	Bilateral O	<0.2	1.1	173	2.4

Abbreviations: - = test not done; A4 = androstenedione; 5ARD = 5α-reductase 2 deficiency; AEG = anophthalmia-oesophageal-genital; AMH = anti-Müllerian hormone; BG = bilateral gonadectomy; DHEAS = dehydroepiandrosterone sulfate; DHT = dihydrotestosterone; FSH = follicle-stimulating hormone; hCG = human chorionic gonadotropin; LH = luteinising hormone; N = normal; O = orchidopexy; 17-OHP = 17-hydroxyprogesterone; R = repair; T = testosterone; USP = urinary steroid profiling; V = vaginoplasty

* Blood taken after gonadectomy

Androgen insensitivity syndrome is reported to be the most common cause of 46,XY DSD in a few ethnic groups,[9-11] while 5ARD, which is believed to be rare, was also a major aetiology in our cohort. It is important to differentiate between 5ARD and AIS as soon as possible so that patients with 5ARD can be raised as boys whenever practical.[12] The penile growth of patients with 5ARD can be promoted by topical DHT treatment and spontaneous virilisation may occur during puberty. Most of these patients who are reared as girls during childhood identify themselves as male and change their gender as an adult, although we have not received any such request from our cohort. Exposure to androgen during the antenatal, postnatal, and pubertal period may masculinise the brain and influence gender identity.[13] It was found that 5ARD is easy to diagnose by its characteristic urinary steroid excretion pattern and its high mutational detection rate in the *SRD5A2* gene.[14] Of the 11 patients with 5ARD, eight harboured the missense mutation p.Arg227Gln in their *SRD5A2* gene, a useful fact to enable screening for this mutation before proceeding to sequencing of the whole gene. Unfortunately, patients have

Baseline						Post-hCG stimulation					USP
17-OHP (nmol/L)	T (nmol/L)	AMH (pmol/L)	DHT (pmol/L)	A4 (nmol/L)	T/A4 ratio	T (nmol/L)	AMH (pmol/L)	DHT (pmol/L)	A4 (nmol/L)	T/A4 ratio	
0.5	<0.4	>100	<13.8	<1.0	-	8.1	>100	206	<1.0	-	5ARD
-	4.3 (At 2 months)	-	-	-	-	-	-	-	-	-	5ARD
-	<0.4 (At 1 year)	-	-	-	-	14.1 (At 1 year)	-	-	-	-	5ARD
-	-	-	-	-	-	-	-	-	-	-	5ARD
-	**6.8**	-	-	-	-	-	-	-	-	-	5ARD
-	**3.0**	-	-	-	-	10.0	-	-	-	-	5ARD
-	<0.4	-	-	-	-	7.4	-	-	-	-	5ARD
-	-	-	-	-	-	-	-	-	-	-	5ARD
2.4	15.7	51.8	236	6.2	2.53	-	-	-	-	-	5ARD
-	<0.4	-	-	-	-	5.5	-	-	-	-	5ARD
-	14.3	-	-	-	-	-	-	-	-	-	5ARD
<0.5	-	-	88.9	-	-	-	-	-	-	-	N
1.4	1.7	>100	299	2.6	0.66	28.3	>100	2601	4.6	6.13	N
1.3	<0.4	>100	19.0	2.6	-	<0.4	>100	116	2.7	-	N
<0.5	<0.4	<1.3	29.4	3.1	-	-	-	-	-	-	N
-	6.9	>100	**2392**	**7.4**	0.93	11.2	>100	4448	5.6	2.00	-
3.3	22.4	80.5	2115	4.2	5.33	-	-	-	-	-	N
-	**41.2**	>100	**4405**	**9.8**	4.20	-	-	-	-	-	N
0.9	<0.4	-	-	-	-	<0.4	-	-	-	-	N
-	<0.4	-	-	-	-	-	-	-	-	-	-
0.9	<0.4	>100	14.9	1.1	0.00	6.2	>100	1526	1.7	3.65	N
0.8	<0.4	<1.3	21.0	1.3	-	4.2	<1.3	1017	1.6	2.63	-

TABLE 1. (cont'd)

Patient No.	Sex of rearing	Presentation	Age at presentation	Age at recruitment and hormonal evaluation	Other procedures	Baseline			
						LH (IU/L)	FSH (IU/L)	Cortisol (nmol/L)	DHEAS (μmol/L)
Not confirmed genetically									
23	M	AEG	At birth	At birth	-	7.3	2.6	449	4.6
24	M	AEG	At birth	At birth	-	-	-	-	-
25	F	AEG, small gonads	At birth	At birth	-	0.4	0.9	162	0.8
26	M	Hypospadia	At birth	At birth	-	0.2	2.2	100	0.8
27	M	Micropenis	At birth	At birth	O	<0.2	0.3	311	0.9
28	M	Hypospadia, bifid scrotum	At birth	At birth	-	5.5	1.9	636	3.9
29	M	AEG	At birth	5 Days	O	1.5	1.9	364	1.6
30	M	Micropenis, bilateral undescended testes	At birth	2 Months	-	<0.2	0.4	236	1.8
31	M	Micropenis, epispadia	At birth	3 Years	R	1.0	0.6	287	-
32	M	Hypospadia	At birth	3 Years	R	<0.2	1.8	301	-
33	M	Hypospadia	At birth	4 Years	R	<0.2	**3.2**	307	<0.4
34	M	AEG	At birth	6 Years	R	<0.2	0.3	364	2.9
35	M	AEG	At birth	6 Years	R	<0.2	<0.2	217	-
36	M	Micropenis, left undescended testis	At birth	7 Years	Left O	0.6	0.9	788	-
37	M	AEG	At birth	8 Years	R	-	-	-	-
38	M	AEG	At birth	9 Years	R	1.2	**4.0**	348	4.1
39	M	AEG	At birth	9 Years	R	0.3	2.0	121	1.6
40	M	Micropenis, hypospadia	At birth	10 Years	Left O+R	0.5	2.9	78	2.8
41	M	AEG	At birth	10 Years	R	2.0	**4.4**	172	3.8
42	F	Amniocentesis 46,XY, phenotype at birth normal female external genitalia	At birth	10 Years	BG	0.7	**4.9**	157	1.7
43	M	Micropenis	At birth	11 Years	-	0.7	2.1	146	5.5
44	M	AEG	At birth	12 Years	R	2.3	4.7	197	-
45	M	AEG	At birth	15 Years	R	5.8	5.1	266	5.5
46	M	AEG	At birth	19 Years	R	5.0	8.4	352	-
47	M	AEG	At birth	23 Years	R	**27.3**	**13.3**	370	5.8
48	M	Micropenis	1 Month	6 Months	-	0.5	3.3	205	<0.4
49	M	Micropenis	1 Month	1 Year	-	<0.2	0.9	677	0.4
50*	F	Inguinal gonads with normal female external genitalia	1 Year	23 Years	BG	-	-	164	0.8
51	M	Hypospadia	2 Years	13 Years	R	1.8	2.4	151	2.8
52	M	Bilateral undescended small testis	5 Years	19 Years	Bilateral O	**32.6**	**70.7**	219	6.1
53	M	Hypospadia	5 Years	13 Years	R	3.7	**7.3**	193	4.7
54	M	Micropenis	6 Years	8 Years	-	<0.2	0.6	796	0.5
55	M	Hypospadia	7 Years	11 Years	R	0.4	2.9	-	-
56	M	Micropenis	9 Years	9 Years	-	0.7	3.5	159	-
57	M	Micropenis	17 Years	17 Years	-	1.4	2.2	166	-
Not consented for genetic testing									
58	M	Hypospadia	At birth	At birth	-	0.5	1.2	365	1.2
59	M	Hypospadia	At birth	At birth	R	1.0	1.7	464	2.6
60	F	AEG, inguinal gonads	At birth	2 Months	BG	<0.2	2.4	272	0.9
61	M	AEG	At birth	5 Years	R	<0.2	1.2	91	0.4
62	M	Micropenis	1 Year	1 Year	-	-	-	-	-
63	M	Micropenis, small gonads	11 Years	11 Years	-	<0.2	2.3	-	-
64	M	Micropenis	11 Years	12 Years	-	2.4	2.4	-	0.9

Baseline						Post-hCG stimulation					USP
17-OHP (nmol/L)	T (nmol/L)	AMH (pmol/L)	DHT (pmol/L)	A4 (nmol/L)	T/A4 ratio	T (nmol/L)	AMH (pmol/L)	DHT (pmol/L)	A4 (nmol/L)	T/A4 ratio	
6.6	**6.6**	>100	**2132**	**33.1**	0.20	-	-	-	-		-
-	1.5	>100	237	4.4	0.34	8.4	>100	2277	5.2	1.62	-
1.3	<0.4	>100	35.9	**15.9**		<0.4	>100	31.8	7.1	-	N
0.9	1.3	-	-	-	-	6.5	>100	36.7	13.9	0.47	N
0.8	1.1	>100	14.4	**15.0**	0.07	1.6	>100	115	19.3	0.08	N
4.5	**3.5**	>100	571	**32.4**	0.11	9.0	>100	2983	26.9	0.33	N
2.1	**7.2**	>100	1543	**14.0**	0.51	12.8	>100	3267	-	-	N
1.3	<0.4	<1.3	24.3	1.9	-	<0.4	<1.3	33.7	1.5	-	N
4.8	<0.4	>100	<13.8	<1.0	-	11.5	>100	1342	<1.0	-	N
<0.5	<0.4	>100	<13.8	<1.0	-	9.2	>100	362	<1.0	-	N
1.7	0.7	>100	<13.8	<1.0	-	7.4	>100	870	1.3	5.65	N
2.0	<0.4	>100	-	-	-	2.8	>100	397	3.0	0.93	N
0.5	<0.4	>100	30.5	1.7	0.00	2.6	>100	-	2.9	0.90	N
3.0	0.5	>100	24.1	**4.6**	0.11	2.7	>100	247	4.9	0.55	N
-	-	>100	16.6	1.1	-	2.8	>100	337	2.2	1.30	N
2.6	**2.7**	37.5	**226**	**5.1**	0.53	24.2	33.5	2542	9.4	2.57	N
0.7	0.4	>100	17.6	**2.4**	0.17	6.9	>100	264	1.8	3.83	N
1.1	<0.4	12.4	24.6	**2.6**	-	12.4	11.6	234	4.3	2.88	N
0.5	0.4	>100	87.8	1.3	0.31	5.5	>100	1198	2.0	2.75	N
0.6	2.0 (On 9 days)	-	-	1.1	-	-	-	-	-	-	N
<0.5	1.2	>100	88.4	3.6	0.33	18.3	>100	1446	3.9	4.67	N
1.6	4.1	>100	684	3.1	1.30	-	-	-	-	-	N
1.7	11.4	70.7	1220	6.6	1.73	-	-	-	-	-	N
3.7	11.1	16.3	1493	8.6	1.29	-	-	-	-	-	N
5.4	**32.7**	23	**4368**	9.0	3.63	-	-	-	-	-	N
3.9	0.2	>100	45.1	<1.0	-	17.3	>100	4899	3.6	4.79	N
5.7	<0.4	>100	<13.8	<1.0	-	8.5	>100	668	1.7	5.12	N
<0.5	<0.4	<1.3	139	<1.0	-	-	-	-	-	-	N
1.6	13.8	61.3	**1261**	2.8	4.98	-	-	-	-	-	-
0.5	3.7	<1.3	385	3.3	1.12	-	-	-	-	-	N
2.5	15.6	14.7	**2633**	6.0	2.62	-	-	-	-	-	-
5.1	<0.4	>100	14.8	**1.8**	-	6.0	>100	335	2.1	2.84	N
-	<0.4	>100	40.6	3.3	-	2.8	>100	213	1.9	1.47	N
<0.5	<0.4	>100	25.1	<1.0	-	6.4	>100	577	2.9	2.21	N
0.6	2.2	91.3	91.9	1.5	1.48	19.5	-	-	-	-	N
1.2	**2.5**	>100	79.3	**15.6**	0.16	9.7	-	-	-	-	N
3.9	**14.8**	-	624	-	-	38.2	-	-	-	-	N
1.3	<0.4	>100	66.2	**2.9**	-	25.1	>100	4413	5.5	4.60	N
<0.5	<0.4	-	-	-	-	4.9	-	-	-	-	N
-	<0.4	>100	<13.8	<1.0	-	6.7	>100	873	<1.0	-	N
-	<0.4	>100	36.4	3.5	-	1.6	>100	83.3	2.8	0.58	N
4.7	2.0	-	-	-	-	13.3	-	-	-	-	N

TABLE 2. Other structural abnormalities detected in eight of the patients in this study

Patient No.	Structural abnormalities
4	Neuroblastoma
13	Horseshoe kidney
27	Cleft palate
30	Right renal agenesis
41	Small patent ductus arteriosus with occlusion
47	Imperforated anus
59	Patent ductus arteriosus, ventricular septal defect
61	Right bronchogenic cyst

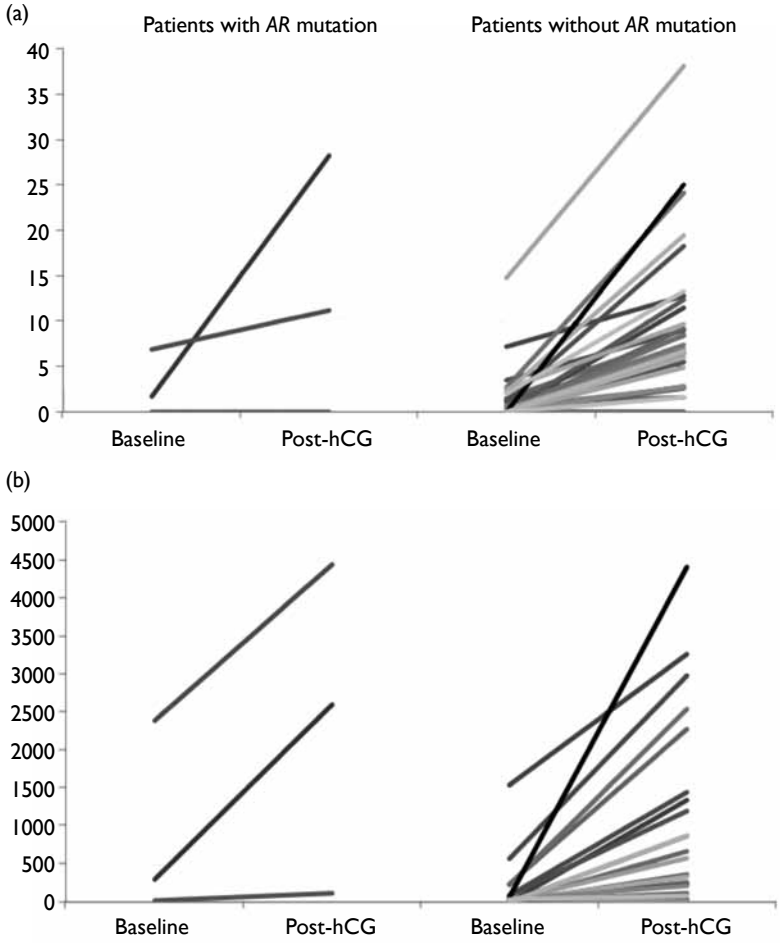

FIG 1. Responses in (a) testosterone and (b) dihydrotestosterone levels upon hCG stimulation in patients with *AR* mutation (left panel) compared with those without (right panel)
Abbreviation: hCG = human chorionic gonadotropin

previously been too easily labelled with AIS when laboratory diagnostic services were less advanced. This is illustrated by patient 7 who was labelled as AIS until her urine steroids were analysed and revealed classic features of 5ARD.[15] We recommend that 5ARD is excluded in all 46,XY DSD patients before other differential diagnoses are considered. Moreover, since the baseline and post-hCG–stimulated T and DHT results are unreliable when diagnosing AIS, genetic study of the *AR* gene should also be performed as a first-line investigation.

HSD17B3 deficiency has been reported to be the most common cause of T biosynthetic defect leading to 46,XY DSD in some populations, with an estimated incidence of 1:147 000 in the Netherlands and as high as 1:200 to 1:300 in Arabians due to their high consanguinity rate.[16,17] Nonetheless, no patient in our cohort was diagnosed with this condition based on the hormonal pattern. Ethnic differences in disease spectrum may be one of the reasons for this observation. Another possible explanation is the lack of reliable diagnostic cutoff for the pre- and post-stimulated T/A4 ratios. George et al[18] have summarised the cutoffs used by various researchers, with the pre-stimulated cutoff range set at 0.006 to 1.64, and the post-stimulated level set at 0.09 to 3.4 for newborn to teenage groups. The difficulties in setting up reliable diagnostic cutoffs for the T/A4 ratio are similar to the T/DHT ratios and have been discussed in our previous study.[14] Furthermore, *HSD17B3* deficiency gives no characteristic findings on urinary steroid profiling.[5,19] Molecular analysis of the *HSD17B3* gene may have offered a means to diagnose this condition but unfortunately, due to budget constraints, we were unable to perform mutational analysis of this gene in all our patients, although a normal MLPA result in our patients made gross deletion in this gene unlikely.

The two novel mutations p.Asp266Asn and p.Thr576Pro in the *AR* gene lie within the N-terminal domain of the androgen receptor that is involved in transcription regulation and DNA binding, respectively. Missense mutations around these two codons have been reported in patients with AIS according to the Androgen Receptor Gene Mutations Database, April 2013.[20] Multiple sequence alignment shows that both amino acids are highly conserved among different species, suggesting that aspartic acid at codon 266 and threonine at codon 576 are critical for proper receptor function. Similarly, the alanine at codon 260 of the *NR5A1* gene is located in helix 3 of the ligand-binding domain of the nuclear receptor,[21] and is also a highly conserved region. Mutation in this region has been reported to result in 46,XY DSD.[8] Replacing alanine at this position by valine is therefore expected to be deleterious to the protein function. Phenotypic variability in *NR5A1* gene mutation within a kindred has been reported and this may explain why patient 21 had ambiguous external genitalia to such an extent that he required the attention of a paediatric specialist, even though his father was fertile, and denied any symptoms of DSD or need for medical

TABLE 3. Genetic findings of patients in this study

Patient No.	Gene	Genetic findings
1	*SRD5A2*	Compound heterozygous p.Gln6*/p.Arg227Gln
2*	*SRD5A2*	Homozygous p.Gly203Ser
3*	*SRD5A2*	Homozygous p.Arg227Gln
4*	*SRD5A2*	Compound heterozygous c.548-1G>A/p.Ala228Val
5	*SRD5A2*	Compound heterozygous p.Leu55Pro/p.Arg227Gln
6	*SRD5A2*	Compound heterozygous p.Val10Gly? p.Arg227Gln
7*	*SRD5A2*	Homozygous p.Arg246Gln
8*	*SRD5A2*	Homozygous p.Arg227Gln
9*	*SRD5A2*	Homozygous p.Arg227Gln
10	*SRD5A2*	Compound heterozygous p.Gln6*/p.Arg227Gln
11	*SRD5A2*	Homozygous p.Arg227Gln
12†	*AR*	Hemizygous p.Thr576Pro, mosaic
13	*AR*	Hemizygous p.Asp266Asn
14*	*AR*	Hemizygous p.Ala871Val
15†	*AR*	Hemizygous p.Met788Val, mosaic
16	*AR*	Hemizygous p.Ser176Arg
17	*AR*	Hemizygous p.Gln825Lys
18	*AR*	Hemizygous p.Arg841His
19	*CYP11A1*	Compound heterozygous p.Arg360Trp /p.Arg405*
20†	*WT1*	Heterozygous c.1228+4C>T
21	*NR5A1*	Heterozygous p.Ala260Val
22	*AMH*	Compound heterozygous p.Cys492Arg/p.Ala546Thr/p.His547Asp
30	*AMH, WT1*	Wild-type sequence
40	*AMH*	Wild-type sequence

* Genotyping was not performed on the parents
† De-novo mutation

TABLE 4. In-silico analysis of the novel variants detected in patients with 46,XY disorders of sex development

Gene	Novel variant	SIFT	PolyPhen2	Align GVGD
AMH	p.Cys492Arg	Not tolerated	Probably damaging	Class 65
AMH	p.Ala546Thr	Not tolerated	Probably damaging	Class 55
AMH	p.His547Asp	Tolerated	Probably damaging	Class 65
AR	p.Asp266Asn	Not tolerated	Probably damaging	Class 15
AR	p.Thr576Pro	Not tolerated	Probably damaging	Class 35
NR5A1	p.Ala260Val	Not tolerated	Benign	Class 65

Abbreviation: SIFT = sorting intolerant from tolerant

attention.[22] For the *AMH* gene, the 3' end of exon 5 is one of the mutational hotspots in patients with PMDS.[23] Exon 5 encodes the bioactive C-terminal domain. The three mutations detected in patient 22 are all located at highly conserved regions. Although in-vitro functional characterisation for the mutant proteins was not performed, the undetectable serum AMH level in this patient was compatible with the mutations being pathogenic, possibly due to abnormal protein folding and increased instability, as reported previously in mutations located in this region.[24]

Gonadal malignancy was rare in our series, probably because gonadectomy was performed early in life when the decision of female sex assignment was made. Although this helps to avoid further virilisation and to establish gender identity, the timing of corrective surgery and gonadectomy

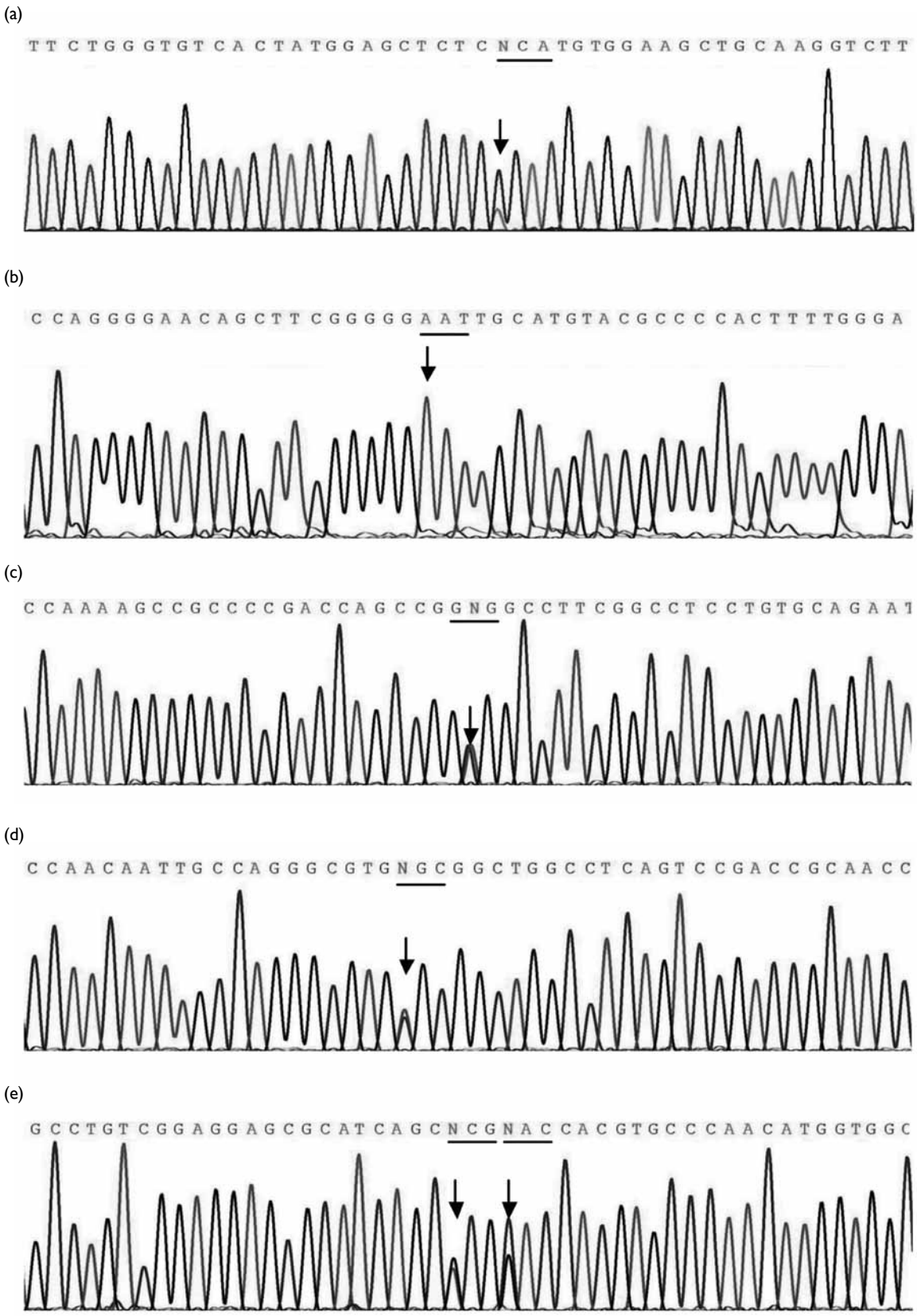

FIG 2. Segments of electropherograms showing the novel mutations

(a) Hemizygous c.1726A>C, p.Thr576Pro in mosaic pattern in the *AR* gene in patient 12; (b) hemizygous c.796G>A, p.Asp266Asn in the *AR* gene in patient 13; (c) heterozygous c.779C>T, p.Ala260Val in the *NR5A1* gene in patient 21; (d) heterozygous c.1474T>C, p.Cys492Arg; and (e) heterozygous c.1636G>A, p.Ala546Thr and c.1639C>G, p.His547Asp in the *AMH* gene in patient 22. The heterozygous sites are denoted by the letter N and the mutation site is indicated by arrows. The mutated codon is underlined

remain controversial. Patient advocacy groups have suggested delaying any surgery for cosmetic reasons until the patient is mature enough to give informed consent[25] but such practice has not been validated in our Chinese patients. Whether cultural factors have any impact on gender assignment remains uncertain in our community.

Prematurity or low birth weight was not uncommon in our series. This made diagnosis of DSD in our patients even more difficult because ethnic-specific and gestational age– or weight-adjusted anthropometric measurement of the external genitalia was not available. Assessment of the genital anatomy relies solely on the experience of the paediatric specialist and is obviously far from ideal. A conjoint effort by local paediatricians is needed to set up these normative data.

Less than half of our patients had a confirmed diagnosis in the present study. With the increasing availability of next-generation sequencing technology, and with its established role in molecular diagnostic services, including DSD,[3,26] it is hoped that sooner rather than later, most patients will have a confirmed genetic diagnosis. Nonetheless, we speculate that some patients have a non-genetic aetiology since environmental factors may alter the phenotypic expression. Several animal and human studies have shown that antenatal exposure to pesticides and plasticisers may lead to fetal genital malformation.[27]

Altogether there was an average of 11 250 male live births every year in the five public hospitals that participated in this study. Since 11 newborns with 46,XY DSD were born in these five hospitals and were recruited during our study period, this gives an estimated incidence of 46,XY DSD of 1:2045 male births requiring the input of paediatric endocrinologists. This figure may underestimate the true incidence of this group of diseases as some patients present late and others may have subtle defects that go unnoticed by our specialists. If the actual number of patients with chromosomal and 46,XX DSD in our population is considered, the actual incidence of DSD can be expected to be much higher.

There are a few limitations in this study. First, the number of patients was relatively small. This may have resulted in bias in our observation and the data do not represent the prevalence of disease in our population. Second, in-vitro study was not performed on the novel genetic variants for functional characterisation, although we believe that all the available evidence indicates the pathogenic nature of these variants. Third, due to budget constraints, we were unable to sequence all genes related to 46,XY DSD.

Conclusions

Our findings indicate that 5ARD and AIS are possibly the major causes of 46,XY DSD in the Hong Kong Chinese population. Molecular analyses of the *SRD5A2* and *AR* genes were demonstrated to be more reliable than hormonal testing. Since the missense mutation p.Arg227Gln was a recurrent hotspot mutation in 5ARD in our local patients, all patients should be screened for this mutation.

Acknowledgements

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References

1. Lee PA, Houk CP, Ahmed SF, Hughes IA; International Consensus Conference on Intersex organized by the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. Pediatrics 2006;118.e488-500.
2. Ono M, Harley VR. Disorders of sex development: new genes, new concepts. Nat Rev Endocrinol 2013;9:79-91.
3. Arboleda VA, Lee H, Sánchez FJ, et al. Targeted massively parallel sequencing provides comprehensive genetic diagnosis for patients with disorders of sex development. Clin Genet 2013;83:35-43.
4. Jääskeläinen J. Molecular biology of androgen insensitivity. Mol Cell Endocrinol 2012;35:4-12.
5. Chan AO, Shek CC. Urinary steroid profiling in the diagnosis of congenital adrenal hyperplasia and disorders of sex development: experience of a urinary steroid referral centre in Hong Kong. Clin Biochem 2013;46:327-34.
6. Parajes S, Chan AO, But WM, et al. Delayed diagnosis of adrenal insufficiency in a patient with severe penoscrotal hypospadias due to two novel P450 side-chain cleavage enzyme (CYP11A1) mutations (p.R360W; p.R405X). Eur J Endocrinol 2012;167:881-5.
7. Chan WK, To KF, But WM, Lee KW. Frasier syndrome: a rare cause of delayed puberty. Hong Kong Med J 2006;12:225-7.
8. Allali S, Muller JB, Brauner R, et al. Mutation analysis of *NR5A1* encoding steroidogenic factor 1 in 77 patients with 46,XY disorders of sex development (DSD) including hypospadias. PLoS One 2011;6:e24117.
9. Bangsbøll S, Qvist I, Lebech PE, Lewinsky M. Testicular feminization syndrome and associated gonadal tumors in Denmark. Acta Obstet Gynecol Scand 1992;71:63-6.
10. Boehmer AL, Brinkmann O, Brüggenwirth H, et al. Genotype versus phenotype in families with androgen insensitivity syndrome. J Clin Endocrinol Metab 2001;86:4151-60.
11. Abdullah MA, Saeed U, Abass A, et al. Disorders of sex development among Sudanese children: 5-year experience of a pediatric endocrinology clinic. J Pediatr Endocrinol Metab 2012;25:1065-72.
12. Mieszczak J, Houk CP, Lee PA. Assignment of the sex of rearing in the neonate with a disorder of sex development.

Curr Opin Pediatr 2009;21:541-7.
13. Imperato-McGinley J, Peterson RE, Gautier T, Sturla E. Androgens and the evolution of male-gender identity among male pseudohermaphrodites with 5alpha-reductase deficiency. N Engl J Med 1979;300:1233-7.
14. Chan AO, But BW, Lee CY, et al. Diagnosis of 5α-reductase 2 deficiency: is measurement of dihydrotestosterone essential? Clin Chem 2013;59:798-806.
15. Chan AO, But BW, Lau GT, et al. Diagnosis of 5α-reductase 2 deficiency: a local experience. Hong Kong Med J 2009;15:130-5.
16. Boehmer AL, Brinkmann AO, Sandkuijl LA, et al. 17β-hydroxysteroid dehydrogenase-3 deficiency: diagnosis, phenotypic variability, population genetics, and worldwide distribution of ancient and de novo mutations. J Clin Endocrinol Metab 1999;84:4713-21.
17. Rosler A. 17 Beta-hydroxysteroid dehydrogenase 3 deficiency in the Mediterranean population. Pediatr Endocrinol Rev 2006;3 Suppl 3:455-61.
18. George MM, New MI, Ten S, Sultan C, Bhangoo A. The clinical and molecular heterogeneity of 17βHSD-3 enzyme deficiency. Horm Res Paediatr 2010;74:229-40.
19. Lee YS, Kirk JM, Stanhope RG, et al. Phenotypic variability in 17β-hydroxysteroid dehydrogenase-3 deficiency and diagnostic pitfalls. Clin Endocrinol 2007;67:20-8.
20. Androgen Receptor Gene Mutations Database. Available from: http://androgendb.mcgill.ca/. Accessed Aug 2013.
21. El-Khairi R, Martinez-Aguayo A, Ferraz-de-Souza B, Lin L, Achermann JC. Role of DAX-1 (*NR0B1*) and steroidogenic factor-1 (*NR5A1*) in human adrenal function. Endocr Dev 2011;20:38-46.
22. Ciaccio M, Costanzo M, Guercio G, et al. Preserved fertility in a patient with a 46,XY disorder of sex development due to a new heterozygous mutation in the *NR5A1*/*SF-1* gene: evidence of 46,XY and 46,XX gonadal dysgenesis phenotype variability in multiple members of an affected kindred. Horm Res Paediatr 2012;78:119-26.
23. Josso N, Belville C, di Clemente N, Picard JY. AMH and AMH receptor defects in persistent Müllerian duct syndrome. Hum Reprod Update 2005;11:351-6.
24. Belville C, Van Vlijmen H, Ehrenfels C, et al. Mutations of the anti-Müllerian hormone gene in patients with persistent Müllerian duct syndrome: biosynthesis, secretion, and processing of the abnormal proteins and analysis using a three-dimensional model. Mol Endocrinol 2004;18:708-21.
25. Consortium on the Management of Disorders of Sex Development: Clinical Guidelines for the Management of Disorders of Sex Development in Childhood. California, US: Intersex Society of North America; 2006. Available from: http://www.dsdguidelines.org/files/clinical.pdf. Accessed Aug 2015.
26. Hersmus R, Stoop H, Turbitt E, et al. *SRY* mutation analysis by next generation (deep) sequencing in a cohort of chromosomal Disorders of Sex Development (DSD) patients with a mosaic karyotype. BMC Med Genet 2012;13:108.
27. Kalfa N, Philibert PH, Baskin LS, Sultan C. Hypospadias: interactions between environment and genetics. Mol Cell Endocrinol 2011;335:89-95.