Recognition of 5α-reductase-2 deficiency in an adult female 46XY DSD clinic

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Abstract

Context: The late presentation of steroid 5α-reductase-2 (SRD5A2) deficiency in females is poorly characterised. The ratios of 5α/5β-reduced metabolites of adrenal steroids in a urine steroid profile (USP) can give an indication of SRD5A2 deficiency, although the diagnostic cut-off for 5α/5β ratios are not clearly defined in genetically confirmed cases.

Objective: The aim of this study was to establish the frequency of SRD5A2 deficiency in an adult clinic for disorders of sexual development (DSD) focussing on 46XY partially virilised adult female subjects. We investigated the relationship between USP results and SRD5A2 genetic sequence and determined the cut-off for USP 5α/5β-reduced steroid ratios compared with gene sequencing for the identification of SRD5A2 deficiency.

Methods: USP and SRD5A2 genetic analyses were performed in 23 adult females, aged 19–57 years, with 46XY DSD and in four males with confirmed SRD5A2 deficiency. 5α-Reductase activity was assessed using the USP ratio of androsterone to aetiocholanolone (A/Ae), 5α-tetrahydrocortisol (5α-THF)/tetrahydrocortisol (THF) and 5α-tetrahydrocorticosterone to tetrahydrocorticosterone (5α-THB/THB).

Results: The SRD5A2 gene mutations were found in 10/23 (43%) females and in all four males. Totally, four novel mutations were identified. All mutation-positive subjects had A/Ae and 5α-THB/THB ratios below the lower limit of normal (100% sensitivity) while the sensitivity of 5α-THF/THF ratio was 90%.

Conclusion: SRD5A2 deficiency is more prevalent than expected in the adult female 46XY DSD population. The clinical spectrum of this disorder may extend to a more female phenotype than previously considered to include individuals with little or no virilisation.

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Introduction

The diagnostic pathway for disorders of sexual development (DSD) is almost entirely based on paediatric experience (1). There is little guidance for making an accurate diagnosis in adults with a female phenotype who might present with amenorrhoea or who have had a previous diagnostic workup that cannot be verified. For instance, the phenotype of women with a 46XY genotype, absent uterus and a degree of virilisation may have several genetic aetiologies including mutations in genes encoding the androgen receptor (AR), 17β-hydroxysteroid dehydrogenase-3 (HSD17B3), 5α-reductase-2 (SRD5A2) and steroidalogenic factor (NR5A1). In practice, this group often comes under a poorly defined clinically based label of partial androgen insensitivity syndrome (PAIS) (2).

After gonadectomy, it is particularly difficult to make a biochemical diagnosis in adults when the original endocrine data are often not available (3), although those with SRD5A2 deficiency characteristically show decreased ratios of 5α- to 5β-reduced steroid metabolites. The ratios used are androsterone/aetiocholanolone (A/Ae), reflecting metabolism mainly of the C19 steroid dehydroepiandrosterone sulphate; 5α-tetrahydrocortisol (5α-THF)/tetrahydrocortisol (THF) and 5α-tetrahydrocorticosterone to tetrahydrocorticosterone (5α-THB/THB).
and virilisation at puberty (6–8). We used both biochemical and genetic methods to document the prevalence of SRD5A2 deficiency in partially virilised 46XY DSD living as women. We also aimed to define the most informative cut point for 5α/5β-reduced steroid ratios in adults that could identify subjects who potentially harbour SRD5A2 gene mutations.

**Subjects and methods**

**Subjects**

In total, 23 females attending DSD clinics at the University College London Hospitals were recruited with the clinical characteristics of 46XY DSD presenting as phenotypic female with an absent uterus and partial virilisation. The degree of virilisation varied from those with the clinical characteristics of 46XY DSD presenting with ambiguous genitalia at birth (n=11 including three considered to have micropenis), those with normal early appearances and virilisation at or after puberty (n=11) and one female with minimal virilisation whose sister clearly virilised at puberty. Women with complete androgen insensitivity syndrome (CAIS), defined clinically as no signs of virilisation and no family history of unusual virilisation, were not included. A comparison group of four males with the classical biochemical presentation of SRD5A2 deficiency was included. This group was characterised by ambiguous genitalia at birth with virilisation at puberty with subsequent male gender identity.

The median (range) age of the cohort was 33 (19–57) years, the median (range) age of first presentation was 13 (birth to 30) years and the median (range) age of gonadectomy was 14.5 (0.6–35) years. In particular, gonadectomy was performed after the age of 13 years in 13 subjects of whom ten described virilisation at puberty, two were unclear about the experience of puberty and one 17-year old who had no sign of virilisation either in childhood or at puberty and was included because she had an absent uterus and her older sister had virilised at puberty. Subjects from South Asia were over represented in this group compared with average UK data reflecting the population mix of central London. Parental consanguinity was recorded in 6/23 (26%) subjects.

At the first clinic appointment, 14 women had previously acquired a clinical label of PAIS of whom four had normal sequence of the AR previously established, two were considered to have SRD5A2 deficiency based on low blood concentrations of dihydrotestosterone and one had the vanishing testis syndrome. Exact diagnostic criteria were not always available in referral letters and historic documents. History was taken to record gender assignment at birth and sex of rearing. Of the 14 women, eight were primary referrals as adults with no previous diagnosis.

The project was approved by the ethics committee of UCL Hospitals and informed consent was obtained from all subjects.

**Urinary steroid profile analysis**

Female and male subjects provided either a 24 h urine collection (n=9) or a mid-morning random urine (n=18). Analysis of samples from 12 controls previously established that there was no significant diurnal variation in the ratios of 5α- to 5β-reduced metabolites comparing daytime to night-time urine samples (data available on request). A 5 ml aliquot of urine was used for analysis by gas chromatography and mass spectrometry (GC–MS) to provide a steroid profile as described previously (9). From the total ion current, chromatograms in the GC–MS data ratios of peak areas of A/Ae, 5α-THF/THF and 5α-THB/THB were used as indices of 5α-reductase activity. Reference ranges established in this laboratory for A/Ae and 5α-THF/THF were 0.67–2.20 and 0.42–1.1 respectively in 26 adult males whereas in 31 females ranges were 0.42–1.08 and 0.17–0.95 respectively. The data analyses for the ratio of 5α-THB/THB were performed retrospectively on archived MS data. Ratios were determined from the signals for specific ions at m/z 564 and 188. Insufficient data was available for 3/26 subjects for this parameter. Reference ranges for 5α-THB/THB were 0.9–4.0 for adult males. For the purposes of this study, it was considered that the male reference range was most appropriate to define abnormally low SRD5A2 activity. A fourth ratio, 11β-hydroxyandrosterone to 11β-hydroxyaetiocholanolone, proved unreliable as it was affected by co-elution of variable amounts of 17-hydroxyprogrenanolone with 11β-hydroxyandrosterone.

**SRD5A2 mutation screening**

DNA was extracted either from whole blood collected into EDTA or from saliva specimens. The SRD5A2 gene was analysed following amplification in three fragments containing exons 1, 2–4 and exon 5 respectively. Final reaction conditions for exons 1 and 5 contained 0.2 mM dNTP, 1.5 mM MgCl₂ and 500 nM of each primer, 1XGO taq buffer and 0.25 units Taq polymerase (GO Taq, Promega) in 25 μl reaction volume. For exons 2–4, the final concentration of reagents was 0.35 mM dNTP, 1× buffer (Expand system 2, Roche Diagnostics), 320 nM each primer and 0.1 unit Taq polymerase (Expand, Roche Diagnostics) in a final reaction volume of 25 μl. Following amplification, primers were removed using QIAquick PCR purification kit (Qiagen) and cycle sequencing performed using exon-specific primers. The products were sequenced on an ABI 310 genetic analyser.

Numbering of mutations was based on reference cDNA sequence NM_000348.3, with nucleotide +1 as the A of the ATG translation initiation codon. In silico analysis of missense changes was performed using SIFT
(16; http://sift.jcvi.org/) and Polyphen-2 (17; http://genetics.bwh.harvard.edu/pph2/) programmes. Potential splice site mutations were assessed using Human Splice Finder v 2.4.1 (15; http://www.umd.be/HSF/).

Results

Sequencing of the SRD5A2 gene revealed pathological mutations in 10/23 (43%) female subjects and in all four male patients (Table 1). Of these mutations, seven have been previously reported comprising six missense (p.Gln126Arg, p.Gly196Ser, p.Arg227X, p.His231Arg, p.Arg246Trp and p.Arg246Gln) (10–13) and one splice site mutation c.698+1G>T (14); four were novel mutations c.311G>A (p.Gly104Gln), c.358A>C (p.Thr120Pro), c.587G>A (p.Gly196Asp) and a mutation in the splice acceptor site of intron 4, c.699-1G>A. The mutations c.586G>A (p.Gly196Asp) and c.587G>A (p.Gly196Asp) were found in the same patient and were confirmed as being on separate alleles by cloning. The c.699-G>A occurs in the invariant region of the splice donor site and was predicted to affect splicing using Human Splicing Finder (15).

In the absence of expression studies, prediction of pathogenicity of the missense changes rely on the absence of the change in controls and in silico analysis of the effect of the changes on protein structure using SIFT (16) and Polyphen (17). Both SIFT and Polyphen predict that Gly196Asp is likely to be pathogenic with scores of 0.0 (<0.05 suggests pathogenicity) and 2.514 (>2.0 probably damaging) respectively; in addition, the variant was not found in 50 controls. A previously described mutation at the same position, Gly196Ser, is known to reduce the affinity of the enzyme for NADPH and reduces activity to 8.3% of normal (14). Thr120Pro was predicted to be possibly damaging by Polyphen (score 1.876), but not by SIFT (score 0.06). This change was not, however, found in the controls. Although this amino acid is not conserved throughout the species, an amino acid with some degree of hydrophobicity is usually expected with a benign variant. This change occurs at a residue that is fully conserved throughout species and the substitution with a larger, more polar amino acid could potentially interfere with conformation. As this mutation occurs with a documented mutation (Arg246Trp) on the other allele and in a biochemically confirmed subject with intact gonads, it is likely to be pathogenic, although final proof would require expression studies. The change did not affect a restriction enzyme site and could, therefore, not be easily checked in controls.

In comparison to the normal male reference range, reduced ratios of A/Ae metabolites (<0.67) were found in 12/23 (52%) subjects, 5α-THF/THF metabolites (<0.42) in 13/23 (57%) and 5α-THB/THB (<0.90) in 10/22 (45%) (Table 1 and Fig. 1). Figure 1 shows the mutation status according to urine steroid profile (USP) result, and it can be seen that the A/Ae and 5α-THB/THB ratios give better separation of mutation-positive subjects from the reference range compared with the 5α-THF/THF ratio. Using the established lower limits of normal A/Ae ratio was 100% sensitive for the identification of mutation-positive cases with 83% specificity; 5α-THF/THF ratio was 90% sensitive with 69% specificity: 5α-THB/THB ratio was 100% sensitive with 83% specificity. By combining results for all three ratios, the sensitivity was 90% and specificity increased to 92% with only one individual negative for SRD5A2 mutation who had low ratios for all three pairs of metabolites.

Discussion

Clarification of the precise genetic basis for 46XY DSD is important for genetic counselling for each family. In this study, we have shown that a significant proportion (41%) of 46XY DSD females have SRD5A2 deficiency and that this diagnosis should be considered for individuals labelled as PAIS, particularly, but not exclusively, those that virilise post-puberty. A similar prevalence of SRD5A2 mutations has been reported in a paediatric population of 46XY under-masculinised children (19), making this condition a more prominent component of the DSD population than previously considered.

Other disorders have been found in 46XY DSD cohorts of predominantly paediatric populations. From the Cambridge DSD database, AR mutations were found in 28% subjects with PAIS analysed by gene sequencing (20). The Dutch nationwide survey found 32/49 (65%) of possible AIS subjects to have AR gene mutations and 12 (24%) with mutations in the HSD17B3 gene (2). An earlier analysis from this same group reported that 13/18 cases previously thought to have AIS were ultimately found to have mutations in HSD17B3 (21). We now extend the understanding of 46XY DSD to estimate the proportion attributable to SRD5A2 deficiency. When considering the PAIS phenotype in all published cases, we estimate that a similar proportion of cases are accountable by AR, HSD17B3 and SRD5A2 gene mutations with ~25% unaccounted for.

In our study, the largest cohort of adult 46XY DSD females to be screened biochemically and genetically, six of the ten women with SRD5A2 deficiency had gonadectomy after puberty. In addition, this group included some subjects with unusually late diagnoses. There are several factors that can account for late presentation. First, some of the subjects originating...
Table 1 Clinical features, previous presentation, urinary steroid profile 5α to 5β ratio and SRD5A2 gene mutations in ten females (F1–F10) and four males (M1–M4) with SRD5A2 deficiency. Sex indicator is the gender currently chosen and Prader staging is estimated from history. Novel mutations are shown in bold.

<table>
<thead>
<tr>
<th>Sex/ID/origin</th>
<th>Age</th>
<th>Clinical summary</th>
<th>Previous diagnosis/age of presentation</th>
<th>Prader stage</th>
<th>Age Gx</th>
<th>Virilise at puberty?</th>
<th>SRD5A2 mutation nucleotide/amino acid</th>
<th>Urinary steroid profile 5α to 5β ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 Pakistan</td>
<td>29</td>
<td>Primary amenorrhoea, clitoromegaly, short vagina, absent uterus</td>
<td>None 20</td>
<td>1/2</td>
<td>20</td>
<td>Yes</td>
<td>c.[698+1G&gt;T]+c.[698+1G&gt;T]</td>
<td>0.24 0.08 0.01</td>
</tr>
<tr>
<td>F2 Pakistan</td>
<td>23</td>
<td>Primary amenorrhoea, voice deepening, clitoromegaly, single perineal opening, no uterus</td>
<td>None 16</td>
<td>1/2</td>
<td>18</td>
<td>Yes</td>
<td>c.[737G&gt;A]+c.[737G&gt;A]</td>
<td>0.19 0.20 0.10</td>
</tr>
<tr>
<td>F3 Sri Lanka</td>
<td>29</td>
<td>Primary amenorrhoea, voice deepening, clitoromegaly, single perineal opening, no uterus</td>
<td>None 27</td>
<td>1/2</td>
<td>27</td>
<td>Yes</td>
<td>c.[689-1G&gt;A]+[689-1G&gt;A]</td>
<td>0.19 0.08 0.05</td>
</tr>
<tr>
<td>F4 Pakistan</td>
<td>33</td>
<td>Labial fusion, short vagina absent uterus</td>
<td>None birth</td>
<td>3</td>
<td>4</td>
<td>N/a</td>
<td>c.[699-1G&gt;A]</td>
<td>0.19 0.08 0.05</td>
</tr>
<tr>
<td>F5 UK</td>
<td>25</td>
<td>Female genitalia with clitoromegaly, short vagina absent uterus</td>
<td>SRD5A2 def birth</td>
<td>2</td>
<td>3</td>
<td>N/a</td>
<td>c.[358A&gt;C]+[358A&gt;C]</td>
<td>0.20 0.12 0.10</td>
</tr>
<tr>
<td>F6 UK</td>
<td>62</td>
<td>Female genitalia, virilisation at puberty, clitoromegaly, single perineal opening, no uterus</td>
<td>PAIS 13</td>
<td>0/1</td>
<td>17</td>
<td>Yes</td>
<td>c.[377A&gt;G]+c.[692A&gt;G]</td>
<td>0.19 0.06 –</td>
</tr>
<tr>
<td>F7 Ireland</td>
<td>22</td>
<td>Clitoromegaly at puberty, normal vagina with stenosis</td>
<td>PAIS 14</td>
<td>0/1</td>
<td>16</td>
<td>Slight</td>
<td>c.[692A&gt;G]+[692A&gt;G]</td>
<td>0.23 0.10 0.07</td>
</tr>
<tr>
<td>F8 Ireland</td>
<td>19</td>
<td>Female genitalia, short vagina, absent uterus</td>
<td>PAIS 12</td>
<td>0/1</td>
<td>14</td>
<td>No</td>
<td>c.[692A&gt;G]+[692A&gt;G]</td>
<td>0.54 0.04 0.31</td>
</tr>
<tr>
<td>F9 Pakistan</td>
<td>39</td>
<td>Labial testes at birth, short vagina</td>
<td>PAIS birth</td>
<td>3</td>
<td>6</td>
<td>N/a</td>
<td>c.[358A&gt;C]+[358A&gt;C]</td>
<td>0.35 0.11 0.15</td>
</tr>
<tr>
<td>F10 Pakistan</td>
<td>25</td>
<td>Primary amenorrhoea, clitoromegaly, 2 cm vaginal dimple</td>
<td>SRD5A2 def 18</td>
<td>1</td>
<td>20</td>
<td>Slight</td>
<td>c.[737G&gt;A]+c.[737G&gt;A]</td>
<td>0.20 0.02</td>
</tr>
<tr>
<td>M1 Pakistan</td>
<td>33</td>
<td>Ambiguous genitalia, raised as a female changed sex at 3</td>
<td>None 3</td>
<td>2</td>
<td>Never</td>
<td>Yes</td>
<td>c.[698+1G&gt;T]+c.[698+1G&gt;T]</td>
<td>0.31 0.02 0.06</td>
</tr>
<tr>
<td>M2 UK</td>
<td>69</td>
<td>Micropenis with perineal hypospadas, raised as female changed sex in adulthood</td>
<td>PAIS 20</td>
<td>4</td>
<td>Never</td>
<td>Yes</td>
<td>c.[311G&gt;A]+[736C&gt;T]</td>
<td>0.22 0.07 –</td>
</tr>
<tr>
<td>M3 Sri Lanka</td>
<td>21</td>
<td>Ambiguous female genitalia, changed sex at puberty</td>
<td>None 21</td>
<td>3/4</td>
<td>Never</td>
<td>Yes</td>
<td>c.[737G&gt;A]+[699-1G&gt;A]</td>
<td>0.20 0.09 0.05</td>
</tr>
<tr>
<td>M4 Pakistan</td>
<td>44</td>
<td>Ambiguous female genitalia, changed sex after puberty</td>
<td>SRD5A2 def 15</td>
<td>3/4</td>
<td>Never</td>
<td>Yes</td>
<td>c.[679C&gt;T]+[679C&gt;T]</td>
<td>0.25 0.03 0.04</td>
</tr>
</tbody>
</table>

Gx, gonadectomy; PAIS, partial androgen insensitivity syndrome; def, deficiency; N/a, not applicable.
from South Asia as adults did not have access to specialist medical care. Secondly, if the original diagnostic work up was carried out in non-specialist units, the rate of missed or wrong diagnosis can be particularly high. In our sample, 28% of subjects referred to our DSD clinic with a diagnosis of PAIS (based on the phenotype and not genetically confirmed) turned out to have SRD5A2 deficiency. Finally, severe deficiency of activity of the SRD5A2 enzyme could result in late recognition if favouring a particularly female phenotype. In this regard, two females in our population with SRD5A2 mutations were sisters who grew up in a favourable social and medical context and were first investigated at puberty for primary amenorrhoea and mild virilisation (F7 and F8). The mutation in these individuals, p.His231Arg, alters testosterone binding (22) and has been described previously, in one case as a heterozygote (14) and in another homozygous for the change (23). The latter individuals presented and remained female but experienced virilisation at puberty, suggesting that this might be a severe mutation with a predominantly female phenotype. One of these sisters is important to note because on her own, she might have been classified as having CAIS and was not for her virilised sibling. This case raises the possibility that the female end of the SRD5A2 deficiency spectrum extends into the CAIS phenotype and this area is worthy of further exploration.

Of the four novel mutations we, one, Thr120Pro, was found in two unrelated subjects (F4 and F9 in Table 1) from Pakistan. The novel c.699-1G>A splice site mutation occurred in two unrelated subjects (F3 and M3) originating from Sri Lanka within the Tamil ethnic group.

USP has been used in the paediatric setting showing good agreement with SRD5A2 genetic testing (24). This report was interesting because of the inclusion of a heterozygote mother with a reduced A/Ae ratio of 0.39, that is within our diagnostic range for SRD5A2 deficiency. Other studies also show overlap of USP results for carriers (25–28). Our series extends this experience in a more heterogeneous adult group in an attempt to map the area where USP and genetic results may be discrepant. The USP appears to be a rapid and reliable method to screen subjects with 46XY DSD for SRD5A2 deficiency rationalising genetic screening in a targeted, cost-efficient manner. Allowing for the fact that mutation-positive cases may exist with a higher A/Ae ratio than the highest value we have found (0.54), we would recommend applying mutation screening to all 46XY subjects with an A/Ae ratio of <0.67 – the lower limit of the normal male range. There are some differences in reference ranges between laboratories for normal subjects, patients and carriers, probably reflecting differences in rates of hydrolysis of conjugates during the analytical procedure. An international EQA scheme operated from our centre is seeking to find the cause of assay variations.

**Limitations and bias in this study**

The main outcomes of this study are affected by the ascertainment bias of the clinical service at our centre. The majority of subjects with 46XY DSD in our clinics are referred through a gynaecology pathway and the population served by our centre has a high degree of ethnic diversity. It was not possible to include samples matched for ethnicity in the control group to be certain that the novel gene alterations exist in each ethnic subgroup.

**Conclusions**

SRD5A2 deficiency is more prevalent than that expected in an adult 46XY female DSD population and this suggests that this diagnosis is often missed. USP is
an effective preliminary diagnostic test for this disease, the A/Ae, 5z-THB/THB ratios and 5z-THF/THF ratio are effective in discriminating the SRD5A2 mutation-positive subjects from normal and identifies those patients in whom sequencing of SRD5A2 gene will be beneficial for definitive diagnosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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