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The IVS1-2A>G mutation in the *SRD5A2* gene predominates in Cypriot patients with 5α reductase deficiency

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ABSTRACT. ***Background:*** **5α steroid reductase deficiency (5αSRD) is an autosomal recessive enzymatic deficiency and mutations in the *5α steroid reductase type 2* gene (*SRD5A2*) result in male pseudohermaphrodism caused by decreased dihydrotestosterone (DHT) synthesis.** ***Aim:*** **To identify the specific mutations of the *SRD5A2* gene in Cypriot patients with 5αSRD.** ***Subjects and methods:*** **Five unrelated patients with 46,XY karyotype were examined. Four of them were born with ambiguous genitalia and 1 patient, who was raised as girl, presented with primary amenorrhea. The hCG test was informative (elevated testosterone/DHT) of 5αSRD in 3 out of 4 subjects. Sequencing of the *SRD5A2* gene was completed for all patients. Genomic DNA was also isolated from a total of 204 healthy unrelated Cypriot subjects. Screening for the IVS1-2A>G mutation was performed by using direct sequencing and restriction enzyme analysis.** ***Results:*** **The IVS1-2A>G was identified in homozygosity in 3 patients and in a compound heterozygote state in the other 2 patients, in combination with p.P181L and p.R171S in exon 3, respectively. The carrier frequency in the Cypriot population for the IVS1-2A>G mutation was estimated to be 0.98% or 2 in 204.** ***Conclusions:*** **The same IVS1-2A>G mutation in the *SRD5A2* gene seems to characterize all Cypriot patients with 5αSRD diagnosed so far. Furthermore this relatively rare genetic defect, which has only been reported previously in a single case in the Eastern Mediterranean region, is very likely to be the result of a founder effect.**

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INTRODUCTION

Sex determination is exclusively associated with the fate of the gonad whereas all other sexual development is the domain of the hormones. There is a growing body of knowledge about the genes that control sex determination and differentiation. Sex-determining genes influence the development of the gonads and testicular hormones are essential and sufficient causation of all secondary sexual development (1). Given the significant advances in understanding molecular causes of abnormal sexual development, it became necessary to integrate current knowledge on establishing the exact diagnosis of intersex disorders.

The development and virilization of the external genitalia in the human male depends on the production and action of dihydrotestosterone (DHT), which is synthesized from testosterone (T) in a reaction catalyzed by the membrane-bound steroid 5α-reductase enzyme. Impaired DHT synthesis as a result of 5α steroid reductase deficiency (5αSRD), leads to incomplete masculinization of the external genitalia of a 46,XY individual (2). The clinical spectrum of the 46,XY individual with 5αSRD ranges from complete female appearance at birth to nearly complete male phenotype. The patients show virilization at puberty without breast development, which is often accompanied by gender identity change, from female to male (3-6).

Steroid 5α reductase isoenzymes have different pattern of expression. The type 1 isoenzyme is encoded by the *SRD5A1* gene on chromosome 5 and is expressed at low levels in the prostate, whereas the type 2 isoenzyme is encoded by the *SRD5A2* gene that maps on chromosome 2 and contains 5 exons and 4 introns. The *SRD5A2* gene is expressed at high levels in the prostate and many other androgen-sensitive tissues (7).

5αSRD is caused by mutations in the *SRD5A2* gene, whereas the 5α reductase 1 gene retains its normal DNA sequence. At least 54 different disease-causing mutations scattered throughout the gene have been described (www.hgmd.cf.ac.uk/ac/gene.php?gene=*SRD5A2*). Forty-two of these are missense/nonsense mutations. Several mutations are recurrent and have been reported in different populations. Other mutations have been described in specific ethnic groups while some mutations reflect a founder gene effect in individuals with a common ancestry.

In this report, we present the identification of the same genetic defect in the *SRD5A2* gene in patients of Cypriot origin. Moreover, the relatively high frequency of this identical molecular finding, which was detected in all individuals diagnosed with 5αSRD so far, further supports its predominance in the Cyprus population.

Key-words: 46,XY disorders of sexual development (DSD), 5α-reductase, male pseudohermaphroditism, mutations, *SRD5A2* gene.

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Table 1 - *Clinical findings of patients with mutations in the* SRD5A2 *gene.*

Patients	Age at diagnosis	Clinical findings - History
#1	4 yr	Enlarged clitoris. Following incomplete hormonal investigation, the diagnosis of PAIS was suspected and she had gonadectomy.
#2	Birth	Almost female external genitalia with small (1 cm) phallic structure, urethral opening, vaginal orifice, and good size palpable gonads.
#3	1 month	Almost female external genitalia with enlarged clitoris and palpable gonads. Common urethral and vaginal opening with no fusion of the labioscrotal folds. The 3-cm blind vaginal pouch was connected to the urethra at the distal part.
#4	14 yr	Amenorrhea, absence of breast development, clitoromegaly. Blind vaginal pouch 4 cm long. Paplable gonads in the inguinal canals bilaterally and absence of Mullerian structures on pelvic ultrasound.
#5	2 months	Ambiguous genitalia: phallic structure 1.5 cm, single orifice at the base of the phallus, posterior fusion of the labioscrotal folds, palpable gonads bilaterally.

PAIS: partial androgen insensitivity syndrome.

MATERIALS AND METHODS

Patients

Five unrelated patients with normal male 46,XY karyotype and their parents of Cypriot origin, were studied. The clinical presentation of the patients, who were all raised as females, is shown on Table 1.

Molecular studies

Initially, exons 2 to 8 of the *AR* gene from patients #1 and #5 and their parents were amplified by PCR using sets of primers and reaction conditions previously described (8). PCR products were first verified for correct size on agarose gel, then purified using Qiaquick PCR columns (Qiagen, Courtaboeuf, France), sequenced using the ABI Prism Dye terminator sequencing kit and finally analyzed on an ABI 310 apparatus (Applied Biosystems, Courtaboeuf, France). No mutation was found in the *AR* gene for patients #1 and #5. As mutational analysis of the *AR* gene was not informative, DNA sequencing of all 5 exons of the *SRD5A2* gene were therefore carried out for the above-mentioned patients (9). Mutational analysis for the *SRD5A2* gene was also performed for the patients #2, #3 and, #4 including their parents.

Mutation detection of IVS1-2A>G mutation in healthy adults

Genomic DNA from 204 Cypriot healthy adults was isolated from peripheral blood leukocytes using the QIAamp Blood Midi Kit (QIAGEN, GmbH D-40724, Hilden, Germany). All DNA samples were screened for the IVS1-2A>G splice mutation by using restriction enzyme analysis and direct sequencing. Briefly, genomic DNA was subjected to PCR and was amplified with forward primer 5' gttaaggcgaaatggcagag 3' and reverse primer 5'acgaggtcattgcagtaggg 3' in a fragment of 382 bp, which selectively amplifies the intronic region of intron 1 and exon 2 of the *SRD5A2* gene. For the detection of the IVS1-2A>G mutation at the splice junction intron 1/exon 2, restriction enzyme digestion with *BstNI* was performed. For confirmation of the identified IVS1-2A>G mutation, direct sequencing was also employed on an automated Beckman Coulter CEQ 2000 sequencer.

Statistics

The carrier frequencies for the IVS1-2A>G mutation in the Cypriot population was carried out using the Poisson probability distribution and the Binomial model.

RESULTS

The diagnosis of 5αSRD in 3 out of 5 patients was suspected based on the biochemical findings, following hCG stimulation test, as shown on Table 2. The hCG test was not performed in patient #1, because she had already undergone gonadectomy. Therefore, stimulated testosterone (T) and DHT ratio (T/DHT) was calculated in 4 out of 5 patients, which showed elevated T/DHT ratio before (20, 16.6, and 13.5) and after stimulation (29, 29.5, 23.7, respectively) in 3 patients. The increased T/DHT ratio suggested 5αSRD and the diagnosis was confirmed by molecular analysis of the *SRD5A2* gene. Elevated T/DHT ratio was not found in patient 5, who was therefore diagnosed as having partial androgen insensitivity syndrome (PAIS). The patient was subsequently given a course of T treatment (T depot 25 mg im monthly × 3), which was not effective in increasing

Table 2 - *Results of the hCG stimulation test in patients with* SRD5A2 *mutations.*

Patient	T (nmol/l)	DHT (nmol/l)	T/DHT ratio	T (nmol/l)	DHT (nmol/l)	T/DHT ratio	Mutations	
	Baseline			Stimulated				
#1	-	-	-	-	-	-		
#3	26.5	1.3	20	52.3	1.8	29	Homozygous IVS2-2A>G	
#4	18.2	1.1	16.6	32.4	1.1	29.5		
#2	19.0	1.4	13.5	23.7	1.0	23.7	Heterozygous IVS2-2A>G	Heterozygous p.P.181L
#5	3.8	0.93	4.1	16.3	3.4	4.8	Heterozygous IVS2-2A>G	Heterozygous p.R.171S

T: testosterone; DHT: dihydrotestosterone.

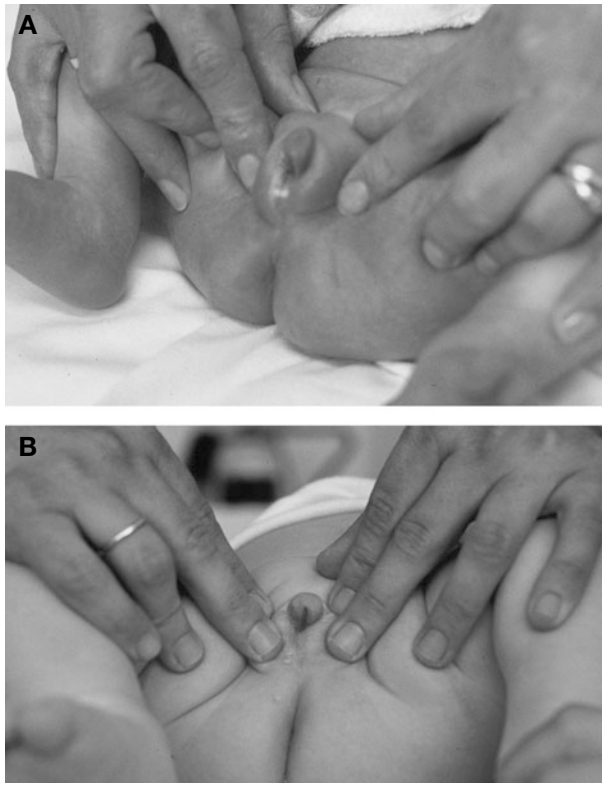

Fig. 1 - *Phenotypic appearance of patient 5 (A) before and (B) after testosterone treatment.*

the penile size (Fig. 1A and B). Removal of the testes was therefore performed and she was raised as a female. The underlying mutations detected in the patients are depicted on Table 2. The same homozygous mutation of *SRD5A2* gene was identified in 3 out of 5 patients in genomic DNA analysis. This mutation was A/G at splice junction intron 1/exon 2. Targeted genetic analysis for the IVS1-2A>G identified this mutation to be shared in heterozygosity in both parents of patients #1, #3, and #4. Patients #2 and #5 were compound heterozygote carriers of 2 mutations which were the same A/G substitution with p.P181L and p.R171S, both in exon 3, respectively (Fig. 2 and 3). The parents of patients #2 and #5 were also tested for the detection of mutations in the *SRD5A2* gene. Mutation IVS1-2A>G was found in the hetrozygote state in both mothers of patients #2 and #5. Mutations p.P181L and p.R171S, were respectively identified in the heterozygote state in the fathers of patients 2 and 5.
Because of the identification of this genetic defect in all patients, we further proceeded and studied the prevalence of this mutation in our population. The analysis of 204 healthy unrelated Cypriot adults detected 2 heterozygous for the IVS1-2A>G mutation, giving a carrier rate of 0.98%, or 2 in 204 [95% Poisson confidence interval (CI), 0.12-3.54% and/or using the binomial CI, 0.12-3.5%]. Both heterozygotes for the IVS1-2A>G mutation, who were detected with restriction enzyme analysis, were subsequently reconfirmed by direct sequencing.

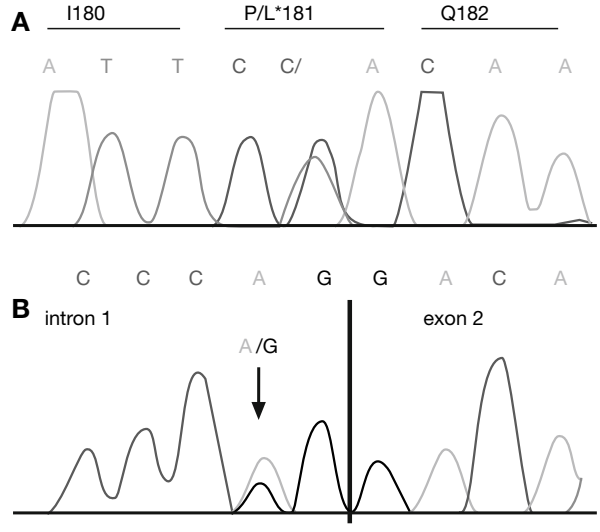

Fig. 2 - *A) Partial sequences exon 3 of SRD5A2 gene in patient 2 showing the C to T mutation leading to and heterozygous Proline to Leucine substitution at position 181. B) Partial sequence of the intron1/exon2 junction showing the A to G mutation at position -2 of the acceptor splice site. The mutation is heterozygous.*

DISCUSSION

The present report describes the molecular characteristics of 5 unrelated 46, XY patients of Cypriot origin with 5αS-RD, where the IVS1-2A>G mutation was identified either in homozygosity or in compound heterozygosity with p.P181L and p.R171S. Poisson model distribution which is commonly employed in case of rare and independent

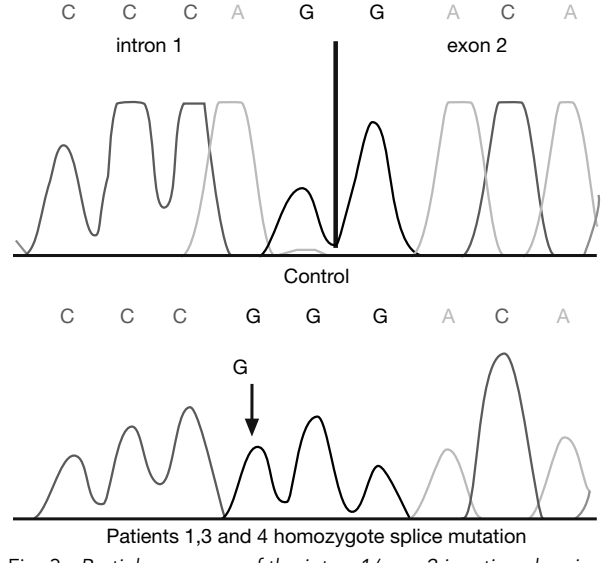

Fig. 3 - *Partial sequence of the intron1/exon2 junction showing the A to G mutation at position -2 of the acceptor splice site. The mutation is homozygous in patients 1, 3, and 4. A normal control is added.*

events was used (Lindsey JK, Modeling Frequency and Count Data, Clarendon Press, New York, 1995). Using this and the Binomial model, carrier frequencies of the IVS1-2A>G mutation in the Cypriot population were analysed. Both probability distributions are suggesting Cyprus and the Eastern Mediterranean region as the geographic center of this mutation. This is further supported by the fact that these mutations were reported in high frequency only in this geographic area so far (10).

Fifty-four different mutations of *SRD5A2* have currently been identified in patients with 5αSRD (6, 10-25). Approximately 2/3 of the patients with 5αSRD have been shown to have homozygous mutations, whereas some are compound heterozygotes. Deletions and disruptive mutations are rare; mostly missense mutations have been described.

The presence of this A/G splice junction intron 1/exon 2 mutation, which is considered to abolish enzymatic activity in all patients, indicates that this is a common mutation in the Cyprus population. Since 5αSRD is inherited in an autosomal recessive mode, the presence of one mutant allele with the mutation IVS1-2A>G does not cause the disease. In 2 out of 3 patients, who were homozygous for the IVS1-2A>G mutation, the phenotype indicates that there is not a complete deficiency of the enzyme, because of ambiguous genitalia. The 3rd patient, who was described by Ocal et al. (10) presented with a phenotypically female appearance, was raised as a girl and she underwent gonadectomy at the age of 7 yr when the diagnosis was made. Similarly, patient #4 was also raised as a girl and the diagnosis was made in adolescence when she presented with primary amenorrhea.

The presence of a compound mutation in 2 of the 5 patients examined supports previous findings that such mutations are frequent in the *SRD5A2* gene and are found in various ethnic groups. Other mutations presumably reflect a founder gene effect as they have been retrieved in individuals with a common ancestry. A study in 8 patients from unrelated Turkish families with 5αSRD revealed the presence of p.L55E mutation in 6 patients out of 8, which indicates the increased prevalence of this hot spot mutation in the Turkish population (10). On the contrary, a recent study in 6 unrelated Italian patients with 5αSRD identified 6 different causative mutations in the *SRD5A2* gene. Five of these mutations were missence, 1 was a nonsense, and a rare polymorphism was also identified (20).

The p.P181L mutation reported in this study most probably does not reflect a founder effect as it has been previously identified in Italo-American patients in a compound heterozygote state (p.P181L/p.H230P) (3). The p.P181L substitution was also detected in both homozygote and heterozygote states in patients of Italian origin (20). The clinical picture of the patient with the p.P181L mutation was similar to that of patient #2, because of clitoromegaly, urogenital sinus, short vaginal pouch, and palpable inguinal gonads.

The p.R171S mutation was initially reported by Thigpen et al. in Sicilian and Maltese patients (11). The same genetic defect was subsequently identified in patients of Turkish origin (22). Taken together, both p.R181L and p.R171S mutations seem to be originated in the Mediterranean area.

It is often not easy to identify the exact etiology of undervirilized males with 46,XY DSD based only on clinical phenotype and hormonal findings, as seen in patient #5. This patient did not respond to T therapy and was considered as suffering from PAIS obviously because he had 5αSRD and T was therefore not converted to DHT. The necessity of applying molecular techniques to establish the diagnosis, in such cases, is supported by a recent study in which 20 patients showing clinical features of 46,XY DSD were included. By applying molecular analysis of the *SRD5A2* gene, the diagnosis of 5αSRD was made in 4 of the 20 patients, who were distinguished and differently treated from patients with 46,XY androgen insensitivity syndrome (26).

In addition to the possible founder effect presented here, similar phenomena and impressive geographic clustering have been documented before for several other mutations or diseases among the population of Cyprus. Best examples are the high carrier frequency of 1:14 for *CFTR* gene mutation p.F508del (27), in a village nearby the capital Nicosia, and the frequency of 1:7 to 1:10 for Friedreich Ataxia gene *FRDA* in two neighboring villages and in the surrounding population, in Pafos in the west of the island (28). Moreover, various studies have established a high frequency of c.35delG of the *GJB2* gene, causing autosomal recessive hearing deficit, as the result of a founder effect in various populations of the Mediterranean region (29). This mutation was detected with a carrier frequency of 1:40 in the Cypriot population of the island (30).

In a previous report, we postulated that the IVS1-2A>G mutation is characteristic of the Eastern Mediterranean region and its presence in our patients may correspond to a founder effect (31). Even though the number of samples in this study is small, because of the rarity of the disease, we suspect that the IVS1-2A>G mutation in the *SRD5A2* gene is characteristic in the Cypriot population and is most likely due to an ancestor effect, further supported by the 0.98% carrier frequency.

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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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