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Research Article

Mutations in *AR* or *SRD5A2* Genes: Clinical Findings, Endocrine Pitfalls, and Genetic Features of Children With 46,XY DSD

Short Running Title: Mutations in *AR* or *SRD5A2* Genes of Turkish Children

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What is already known on this topic?
Androgen insensivity syndrome (AIS) and 5α-reductase deficiency (5α-RD) are the most common reasons of 46,XY disorders of sexual development (DSD). They can present indistinguishable phenotypes that usually necessitate molecular analyses for the definitive diagnosis in prepubertal period.

What this study adds?
T/DHT ratio may lead to diagnostic confusion. Genetic analysis for actual diagnosis seems to be essential. Four novel AR variants were identified in our study.

Abstract
Objective: Androgen insensivity syndrome (AIS) and 5α-reductase deficiency (5α-RD) present indistinguishable phenotypes among the 46,XY disorders of sexual development (DSD) that usually necessitate molecular analyses for the definitive diagnosis in prepubertal period . The aim was to evaluate the clinical, hormonal and genetic findings of 46,XY DSD patients who were diagnosed as AIS or 5α-RD.
Methods: Patients diagnosed as AIS or 5α-RD according to clinical and hormonal evaluations were investigated. Sequence variants of steroid 5-α-reductase type 2 (SRD5A2) were analyzed in cases with testostetone/dihydrotestosterone (T/DHT) ratio ≥ 20, whereas androgen receptor (AR) gene was screened when the ratio was below 20. Stepwise analysis of other associated genes were screened in cases with no causative variant found in previous analysis. For statistical comparisons, the group was divided into three main groups and subgroups according to their genetic diagnosis and T/DHT ratios.
Results: A total of 128 DSD patients from non-related 125 families were enrolled. Birth weight SDS and gestational weeks were significantly higher in 5α-RD patients than AIS and undiagnosed groups. Completely female phenotype was higher in all subgroups of both AIS and 5α-RD patients than in undiagnosed subgroups. In those patients with stimulated T/DHT lower than 20 in the prepubertal period, stimulated T/DHT ratio was significantly lower in AIS than in the undiagnosed group, and higher in 5α-RD. Phenotype associated variants were detected in 24% (n=18 AIS, n=14 5α-RD) of the patients, revealing four novel AR variants (c.94G>T, p.Glu32*, c.330G>C, p.Leu110=; c.2084C>T, p.Pro695Leu, c.2585 2592delAGCTCCTG, p.(Lys862Argfs*16), of those c. c.330G>C with silent status remained undefined in terms of its causative effects.
Conclusion: T/DHT ratio is an important hormonal criterion, but in some cases, T/DHT ratio may lead to diagnostic confusion. Molecular diagnosis is important for the robust diagnosis of 46,XY DSD patients correctly. Four novel AR variants were identified in our study.
Keywords: 46,XY disorders of sex development, 5α-reductase deficiency , androgen insensitivity syndrome, Androgen Receptor gene mutations, SRD5A2 gene mutations

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Introduction
A completely virilized male phenotype requires a 46,XY chromosome, adequate testosterone (T) and dihydrotestosterone (DHT) which is formed from T by the enzyme steroid 5-α-reductase type 2 (SRD5A2) and, fully functionally active androgen receptors (AR) and post receptor pathways[1-3]. A disturbance at any of those last stages can lead to 46,XY disorders of sex development (DSD), characterized by a range of female phenotype to incompletely virilized external

genitalia[1-3]. Although a significant proportion of 46,XY DSD cases may remain with aetiologically unclarified, the most common identifiable cause in the reported series is androgen insensitivity syndrome (AIS) and 5α-reductase deficiency (5α-RD) is the second common cause[1-3]**.** The diagnosis of AIS requires the exclusion of other aetiologies of 46,XY DSD, which are gonadal differentiation defects, testosterone biosynthesis and metabolism. In prepubertal period, phenotypes of AIS, 5α-RD or unknown aetiologies of 46,XY DSD are indistinguishable because of the similarities in their clinical findings. Although adequate serum T concentrations rule out a defect in T biosynthesis, a low T value at baseline or following human chorionic gonadotrophin (hCG) stimulation does not always rule out AIS[2]. On the other hand, phenotype at birth varies widely in 5α-RD, according to the levels of residual enzymatic function so, a very similar clinical picture to AIS at prepubertal age with a normal testicular T production can be observed in those patients. Although serum T/DHT ratio is an important screening tool for identifying patients with possible 5α-RD, cut-off values for diagnosis remain uncertain. Cases with a molecular diagnosis of 5α-RD and T/DHT ratio below the suggested cut-off values have also been reported[4]**.** So, there are overlaps in the diagnosis of AIS or 5α-RD based on clinical or hormonal data and to distinguish them from each other or to differentiate these two main or most common diagnosis from unknown aetiologies, molecular analyses is necessitated. The *AR* gene is located on the X-chromosome in the Xq12 region, composed of eight exons and encodes a protein with 920 aa in length (NP_000035), that function as transcription factor activated via binding of steroid hormones. Peptide chain of AR consists of three domains; residues at the N-terminal region between 6-449 aa encodes androgen receptor domain (NTD; N terminal domain), 558-627 aa zinc finger C4 type domain (DBD, DNA binding domain) and 690-881 ligand binding domain (LBD) of nuclear hormone receptor (EMBL-EBI; P10275, Pfam) (PubMed:16381856)[5,6]. Loss-of-function mutation of *AR* is responsible from X-linked androgen insensitivity (MIM#300068) and hypospadias (MIM# 300633) in humans. The *SRD5A2* gene is located at 2p23, comprises of five exons encoding 254 aa peptide chain that encircles 3-oxo-5-alpha-steroid 4-dehydrogenase domain encoded by, residues between 105-254 (EMBL-EBI; P31213, Pfam) (Pub Med:16381856). Bi-allelik pathogenic alterations of *SRD5A2* are associated with pseudovaginal perineoscrotal hypospadias (MIM# 264600) caused by steroid 5-alpha-reductase deficiency.

Since previous studies have indicated AR and SRD5A2 gene mutations as culprits behind 46,XY DSD for the most part[1,-3], we focused on these two genes in patients with 46,XY DSDs with normal testicular development. Here, a relatively large cohort of Turkish children is reported to present the clinical, hormonal and genetic features of 46,XY DSD patients who were considered as AIS or 5α-RD and to analyze the accordance between the clinical and laboratory results with genetic analysis.

Material and Methods

Participants

A retrospective medical chart review of 46,XY DSD patients was performed to collect data from the pediatric endocrinology outpatient clinic of Istanbul University, Istanbul, Turkey. DSD patients who were diagnosed as AIS or 5α-RD according to the clinic, hormonal or molecular evalutions, were included in the study. Since the study was performed respectively, patient-informed consent forms were not needed. The study has been reviewed by the ethics committee of Istanbul Faculty of Medicine, Istanbul University, and has therefore been performed in accordance with the ethical standards laid down in an appropriate version of the Declaration of Helsinki. Initially, we sought all cases with AIS, 5α-RD or undiagnosed groups according to their molecular diagnosis. In addition, the participants were also subclassified according to their T/DHT for statistical analysis. Clinical diagnosis of AIS or 5α-RD was based on normal T secretion without Mullerian duct structures. Criteria suggesting DSD included overt genital ambiguity, apparent female genitalia with or without clitoromegaly, posterior labial fusion or inguinal/labial mass, and apparent male genitalia with non-palpable testes, micropenis, isolated perineal hypospadias or mild hypospadias with undescended testis[7]**.** Also, file records of older children and adolescents who had incomplete or delayed puberty, lack of breast development or primary amenorrhea or virilization at puberty were retrospectively evaluated with respect to DSD. A detailed history including age at presentation, main complaints, sex of rearing, consanguinity were recorded for each patient. A clinical examination consisting of anthropometry, assessment of pubertal stage, severity of ambigious genitalia, penile lenght and associated anomalies or dysmorphic features were evaluated for each patient. Quigley scale for grading AIS was used to determine the degree of external virilization in 46,XY DSD patients[8-10]. Grades 2 through 5 quantify four degrees of increasingly feminized genitalia that correspond to partial AIS (PAIS). Grades 1 and 6/7 correspond to mild AIS (MAIS) and complete AIS (CAIS), respectively[10]**.** The external masculinization score (EMS, range 0–12) was also assessed in patients[11,12]**.** The standard deviation score (SDS) of height and weight were calculated according to the reported data of Neyzi for Turkish children and adolescence[13], whereas birth weight SDS based on gestation week, were calculated according to reported national data of Kurtoglu for Turkish newborns[14]. Patients were subdivided into three groups according to birth weight SDS: small for gestational age [SGA (<-2 SDS)], large for gestational age [LGA (>+2 SDS)] and appropriate for gestational age [AGA (between -2 and +2 SDS)].

Laboratory Monitoring

As a part of routine evaluation of DSD, we performed hormonal measurements, karyotype analysis, abdominopelvic and scrotal ultrasound (if required, magnetic resonance imaging). Basal level of T, DHT, T/DHT ratio and gonadotropins were measured in pubetal age or in mini puberty. Short-term human chorionic gonadotropin (hCG) test was applied in the appropriate cases who were in prepubertal period. The hCG stimulation test was carried out by administering 1500 IU/m^2/dose of hCG daily IM for three consecutive days to determine the ability of the gonads to produce T and DHT. Blood samples were obtained before the first dose and 24 h after the last[15]. An increment in plasma T (Δ T) of more than 0.8 ng/mL or an absolute level greater than 0.9 ng/mL after hCG treatment was considered to be indicative of the presence of functioning testicular tissue and was defined as normal[16]**.** Laboratory diagnosis of AIS was identified as normal-sized testes, absent Mullerian structures, normal follicle-stimulating hormone (FSH), normal/mildly elevated luteinizing hormone (LH) and normal/elevated baseline or hCG-stimulated T level, and normal T/DHT ratio (T-to-DHT ratio less than 20). T/DHT ratio greater than 20 was accepted as suggestive of 5α-RD [17,18]. Also for exclution of 46,XY DSD causes related to congenital adrenal hyperplasia cortisol, 17-hydroxyprogesterone, dehydroepiandrosterone sulfate, androstenedione (A) were

also measured. T-to-A ratio (T/A) smaller than 0.8 was accepted as suggestive of 17β-hydroxysteroid dehydrogenase (17β-HSD) deficiency[11,12] and they were excluded. For statistical comparisons, the groups were divided into subgroups according to their genetic diagnosis and T/DHT ratios.

Hormones assays

ACTH, cortisol, dehydroepiandrosterone sulphate (DHEA-S), androsteneidone and 17-OHProgesteron (17-OHP) were measured using the IMMULITE 2000 system (immunochemiluminescence assay; ICMA; Siemens AG, Berlin and Munich, Germany) LH, FSH, T were analyzed by electrochemiluminiscense immunoassay (ECLIA) (Cobas, Roche Diagnostics, Mannheim, Germany). Enzyme-linked immunosorbent assay (Elisa) kits were also used for the direct quantitative determination of DHT although different laboratory results were included in the study as a result of retrospective design.

Molecular Analysis

Conventional karyotyping analysis were performed before molecular genetic investigations. Sequence variants of *SRD5A2* gene (NM_000348.3) screened in the cases who had T/DHT ratio equal or above 20 whereas *AR* gene (NM_000044.4) for patients who had the ratio under 20 for pathogenic alterations, by Sanger. If no pathogenic alteration detected in the first analysis than the second genetic analysis is applied for *SRD5A2* and/or *AR* whichever was not analysed in the first run. (Figure 1). Those who did not have a mutation in either *AR* or *SRD5A2* gene constituted the undiagnosed group. Each group were further subgroupped according the T/DHT value. Pathogenic variants were confirmed by database search (Human Genome Mutation Database, ClinVar) and literature search and classified according to ACMG guideline[19-21]. Human splice finder analysis is used for variants with silent status[22]. Segregation in family members are performed whenever available.

Statistical Analysis

The data were analyzed using SPSS software (Statistical Package for the Social Sciences, version 21; SPSS Inc., Chicago, IL, USA). The results are given as mean±SD or as percentages, where appropriate. Data were analyzed using descriptive statistical methods [mean, standard deviation, median, frequency, rate, ranges (minimum-maximum)]as well as some methods for comparing quantitative data. Mann-Whitney U test was used in the two-group comparisons of parameters without normal distribution. In comparison of three or more parameters without normal distribution Kruskal-Wallis test was used. A p value of less than 0.05 or $p < 0.01$ were considered statistically significant.

Results

Clinical Findings

A total of 128 DSD patients from unrelated 125 families were enrolled in the study. The flowchart of the distribution of the patients is given in Figure 1.

On admission, the mean age of AIS patients (n=18) was 3.7±5.5 years, of 5α-RD (n=14) it was 8.1±6.9 years and 1.3±2.2 in the undiagnosed group (n=96) ($p<0.05$). Review of patient charts indicated that Quigley scale grade and EMS on admission, reared gender, consanguinity, birth weight (BW) SDS and gestational age (GA, weeks) of patients were different between the 3 main groups. BW SDS and GA were significantly higher in 5α-RD patients than the other 2 groups. Median BW SDS in 5α-RD patients was 0.4 (min:-1.5, max:2.4) whereas median BW SDS were -1.0 (min:-4.2, max: 2.0) in AIS and -1.5 (min: -7.7, max:3.3) in undiagnosed group. Median values of GA in groups were also 40 weeks (min:38, max:40) in 5α-RD, 38 weeks (min:28, max:40) in AIS and 38 weeks (min:27, max:42) in undiagnosed group respectively. SGA rate was significantly different within the main groups. None of the 5α-RD patients had SGA whereas SGA rates of AIS and undiagnosed group were 27.8% and 40.2% respectively. Consanguinity rate was higher in 5α-RD patients than in both AIS and in the undiagnosed patients.

Comparisons of AIS and 5α-RD groups with the undiagnosed group according to their T/DHT ratios are detailed in Table 1, 2 and Table 3, 4, respectively. Although age at presentation did not differ between any subgroup of AIS and undiagnosed patients, 5α-RD patients presented later than undiagnosed patients (Table 1-4). BW SDS and GW of undiagnosed patients were significantly lower than 5α-RD patients whereas it did not differ between the subgroups of AIS and undiagnosed patients (Table 1-4). However, the percentages of SGA, AGA, LGA were same between the subgroups. Symptom on admission, additional findings, current anthropometry and pubertal status of all subgroups were also shown in Table 1,2,3 and 4. Additional findings or diagnosis including SGA or prematurity were significantly lower in 5α-RD subgroups than in the undiagnosed subgroups whereas it was not different between AIS and undiagnosed subgroups. However, other disease comorbidities (kidney disease, congenital heart disease, anal atresia and autism) were detected in only undiagnosed group (Table 1-4). According to Quigley scale, CAIS grade rate was higher in all subgroups of both AIS and 5α-RD patients than in the undiagnosed subgroups (Table 1-4). Comparison of EMS within subgroups differed significantly except between genetically proven AIS and undiagnosed patients with a normal T/DHT value (<20) (Table 1-4). Reared gender was also different between the subgroups (Tables 1-4).

Endocrine Data

Laboratory results of AIS, 5α-RD and undiagnosed groups according to their T/DHT ratios were detailed in Table 1,2,3 and 4. 22.2% (n=4) of AIS patients and 36.5% (n=35) of undiagnosed patients had T/DHT ratio ≥ 20, whereas 28.6% (n=4) of 5α-RD patients had T/DHT ratio < 20 (Table 1-4). T/DHT ratios were obtained in 57% (n=73) during HCG test, whereas the remaining were obtained by basal hormone levels during puberty or minipuberty. Twenty four patients had more than one T/DHT ratio according to their ages. The mean values of T/DHT ratios used for hormonal diagnosis were significantly different between AIS, 5α-RD and undiagnosed groups ($p<0.05$). 5α-RD patients had significant higher T/DHT ratios than both AIS and undiagnosed groups. Although basal T/DHT ratio during the minipuberty or puberty did not differ, stimulated T/DHT ratio in prepubertal period was significantly lower in AIS subgroup with T/DHT<20 than in the undiagnosed subgroup with T/DHT<20 (Table 1). On the otherhand, stimulated T/DHT ratio in prepubertal period was significantly higher in 5α-RD subgroup with T/DHT<20 than in the undiagnosed subgroup with T/DHT<20 (Table 3). Clinical and laboratory findings of genetically diagnosed AIS and 5α-RD patients were presented in Supplemet 1 ve 2. Only pubertal LH was significantly higher in AIS patients than undiagnosed group when T/DHT≥20.

Molecular Genetics

Cytogeneticaly 32 patients had definitive diagnosis and 4 patients had non 46,XY karyotypes (3 with 47,XXY, and 1 with 47,XYY) . Fourteen patients were investigated for only AR, 10 for only *SRD5A2* and 104 patients were investigated for both of the genes. Variants attributed to disease in 32 (24%) patients, one prevailed uncertainty whereas the others (75%, n=96) remained undiagnosed for molecular genetic base. In the total cohort, 14% of patients (n=18) were molecularly diagnosed as AIS, and 10.9% (n=14) had 5α-RD genetically. Molecular genetic test results showed four novel *AR* variants (c.94G>T, p.Glu32*;, c.330G>C, p.Leu110=; c.2084C>T, p.Pro695Leu; c.2585_2592delAGCTCCTG, p.(Lys862Argfs*16). Among those, c.94G>T and c.2585_2592delAGCTCCTG were classified as pathogenic, c.2084C>T as likely pathogenic, while silent change (c.330G>C) as likely benign. *In silico* analysis for human splicing finder, it is not expected to have significant impact on splicing signals[22]**.** This silent change would cause the alteration of leucine encoded by CTG to CTC. In a study of translation-selection model of human genome, it has been revealed that, CTG is the major codon for leucine-tRNA, being more abundant in translation environment, an improtant factor determining translational efficiency[23]**.** Presently, there is no sufficient evidence to support causative status of c.330G>C and segregation analysis for this family was not achieved. The most frequent pathogenic *AR* variant in the study was c.1174C>T, (p.Pro392Ser) with the frequency of 33.3% (n=6) in all AIS patients (Table 5). All of the patients with this mutation presented with PAIS clinically. All of patients with CAIS (n=7, 38.9%) had different mutations (two of them novel mutation). However two siblings with c.2676T>A (p.Phe892Leu) mutation had different clinical presentations (one PAIS, one CAIS) and one of these siblings with PAIS had also 47,XXY karyotype (Supplement Table 1). The most frequent *SRD5A2* mutations were c.164T>A (p.Leu55Gln); c.453delC (p.(Leu152Tyrfs*8); and c.193G>C (p.Ala65Pro). Two patients with c.453delC (p.(Leu152Tyrfs*8)) had also different Quigley scores which caused PAIS in one and CAIS in another one. A patient who had heterozygous mutations with c.164T>A, p.Leu55Gln and c.269A>C (p.His90Pro) presented CAIS phenotype (Supplement Table 2).

Discussion

This study documented the clinical, hormonal and genetic features of 46,XY DSD who were considered as AIS or 5α-RD according to clinical and hormonal criteria. We summarized clinical, endocrine, and genetic data of 128 Turkish children with 46,XY DSD, collected in only one center with molecular analyses performed in a single laboratory.

In our cohort, 24% of the 46,XY DSD patients had any variants attributed to disease. This finding is consistent with other studies that report as around 20-40% of cases could get molecular diagnosis whereas the others remain without diagnosis[24]. On the otherhand, the lower rate of molecularly diagnosed cases such as 16.3% (11.6% 5α-RD2, 4.7% AIS)[25] and 12% (8% AIS, 4% 5α-RD2 and 88% without gene abnormality)[26] has also been reported in some studies. Different studies from Turkey also report different rates of *AR* or *SRD5A2* gene mutations in Turkish population[2,17,27,28]. One of this studies report their 51 patients with the mutation rates of *AR* gene 22% and *SRD5A2* gene 12% which are almost similar to our results[2]. Recently, in a large cohort of Turkish DSD patients, 143 patients of 46,XY DSD were evaluated and 45 (31.4%) of them were genetically proven. In this recent study, the distribution of the molecular diagnosis of 46,XY DSD patients were also presented as 26.6% *SRD5A2,* and 22.2% *AR*[28]. In concordance with most of the literature, our study results showed that the frequency of genetically diagnosed AIS patients were higher than 5α-RD patients in study sample [27,29-31]**.**

One of the remarkable point of the current study was the comparison of BW SDS and GA between the subgroups of study sample. The effect of androgens on fetal growth and BW difference between sexes has been reported in previous studies. Although some studies have shown that BW difference is dependent on fetal androgens, other studies reported that it is not generated by action of androgens[32]. Moreover, it is known that 46,XY DSDs due to nonspecific disorders of undermasculinization are more frequently associated with fetal growth restriction, SGA, and concomitant conditions[33-36]. In the current study, non of 5α-RD patients had SGA. Moreover, BW SDS and GA of 5α-RD patients were significantly higher than that of AIS and undiagnosed patients which may demonstrate that fetal androgens can also effect the fetal growth. The 19% of cases of nonspecific XY DSD without any clear diagnosis is reported to be SGA[16]. In this study, 40.4% of undiagnosed cases was shown to be SGA. The prematurity and IUGR rates were also higher in undiagnosed group in the current study. Additional conditions are frequent in DSD, with a rate of 27%, which is over 10 times the birth prevalence of congenital anomalies[16]**.** In our study, the other disease comorbidities was higher in undiagnosed group in concordance with literature. The presence of one congenital condition may be associated with the presence of further anomalies because disrupting factors, whether environmental or genetic, are likely to affect multiple developmental processes[16].

The different masculinization scores are standardized ways of recording and conveying the degree of virilization on physical examination. These scores are also used to distinguish between individuals with or without any mutation[37,38]. Some studies find a correlation between an identifiable genetic cause and masculinization scores whereas some of them do not[37,38]. In our study, CAIS phenotype was more frequent in all of mutation positive groups than genetically undiagnosed patients. Thus, having an identifiable genetic cause may be presented with a lower external masculinization score (EMS)[11,12] or higher Quigley score[10] compared to patients without an identifiable genetic cause and this trend appears consistent between historical and modern cohorts despite changes in genetic technology over time.

Different studies uses variable cut off levels of T/DHT to differentiate 5α-RD from AIS in laboratory[1,17,18,27,30-41]. Cut-off values ranging from 8.5 to 30 have been suggested for the T/DHT ratio[18]. The diagnostic interpretations of mean values of T/DHT ratio based on different age groups has also discussed until today. With different sensitivities from different studies, variable mean values of T/DHT in patients with 5α-RD during infancy (basal 19.1, peak 29.4), prepuberty (basal 8.0, peak 32.5), adolescence (basal 45.6, peak 71.8) and adulthood (basal 46.6) have been reported[18]. In the current study, same cutoff levels of T/DHT were used for all age groups as a result of missing standardized cutoff levels of ratio according to ages. Lack of precisely determined cutoffs stil compromises correct diagnosis, and improperly high or low ratios causes confusion for the reliability of T/DHT value in clinical practice. Although, AIS cases with T/DHT≥20 and 5α-RD cases with T/DHT<20 were detected in our study, the significant difference between the mean values of T/DHT ratios between the AIS, 5α-RD and undiagnosed groups may show that this ratio can still be a valuable determiner in laboratory diagnosis. Moreover, current study demostrated that when T/DHT was lower than 20, stimulated T/DHT ratio in prepubertal period was significantly lower in AIS than undiagnosed, and higher in 5α-RD. Thus, we can speculate that although the ratio is lower than 20, the lower values may be related to higher probability of AIS, whereas the higher values may indicate the probability

of being 5α-RD if it is close to 20. On the other hand, our study presented no significance between the basal T/DHT ratio between the subgroups during the minipuberty and puberty. Serum T and DHT shows fluctuations after birth before declining to normal prepubertal concentrations and also these hormones levels differ according to the Tanner Stage in puberty[1]. The ratio is reported to be typically higher in adolescents than infants and pre-pubertal children and a normal range of T/DHT may be 1.5– 17 in normal male infants[1]. These normal variations may influence the interpretation of basal T/DHT in mini puberty or puberty[1]. From this point of view, our study may also lead to new questions about the reliability of this ratio in minipuberty or puberty to differentiate AIS, 5α-RD or undiagnosed group when T/DHT ratio is in the same cutoff part. Also, it may suggest that HCG testing may be more useful in evaluating this ratio.

Although it is not common, 47,XXY, 47,XYY or different karyotypes with *AR or SRD5A2* mutations are reported[42,43]. In our study the rate of Klinefelter Syndrome was 2.3% (n=3, 1 patient with AIS, 1 patient with 5α-RD and 1 with uncertain diagnosis). Moreover, one patient with 47,XYY and AR gene mutation was described (0.8%) in the study.

Klinefelter patients classically have complete male sex differentiation, and genital anomalies are rarely recognized as associated features of the syndrome[44]. The evaluation of *AR* and *SRD5A2* genes in patients with karyotype anomalies and ambiguous genitalia is also essential to provide accurate genetic counseling for other members of the family.

Mutations in the *AR* are found in most subjects with CAIS, the rate has varied 28-73%, depending on the case selection[3,45]. In our study, *AR* mutations were found in 63.6% of patients with clinically completely female and this rate was almost consistent with that of some previous studies from Turkey[2]. On the other hand *SRD5A2* mutations and female external genitalia is reported as rare with nearly the rate of 3.9-7.3% in 5α-RD cases[41,46] . We described three patients with *SRD5A2* mutations who had clinical diagnosis as CAIS (21.4% of all *SRD5A2* patients and 2.3% of all patients) which was nearly fourth fold of the prior reported rate. Intersetingly, we also had one patient with clinically CAIS who could not be established genetic diagnosis and may require whole exome analysis to reveal the diagnosis.

In this research, 12 different variants (4 of them novel) in *AR* and 10 different variants in *SRD5A2* were detected. More than 1000 different mutations in *AR* leading to AIS have been reported[47]. Although exon 1 encodes more than half of the AR protein, the total of exon 1 mutations only represents 25% of all of the mutations in AIS patients[47]. More than 70% of AIS mutations in exon 1 appear to cause CAIS, and about 18% of exon 1 mutations are related with MAIS which is due to single-base substitution[47]. However, in the current study, most of the *AR* mutations (44.4%, n=8) were located in exon 1, and none of them presented CAIS phenotype. All of CAIS patients had *AR* mutation in ligand-binding domain. Also, the most common mutations of the *AR* gene in AIS are single point mutations that result in an amino acid substitution[45]. In parallel to this in our study, highest number of mutations were identified as missense type. The c.1174C>T (p.Pro392Ser) variant which was the most frequent pathogenic *AR* variant in this study, has been reported to be related with CAIS, MAIS, PAIS and testicular cancer phenotype before, although all of the patients with this mutation presented with PAIS clinically in this study. As compatible with the literature, c.2169G>T (p.Leu723Phe) variant caused CIAS rather than PAIS, c.1823G>A (p.Arg608Gln) caused PAIS rather than CAIS[47]. Two siblings of this research with c.2676T>A (p.Phe892Leu) variant had different clinical phenotypes (one PAIS, one CAIS). Identical AR mutations can lead to variable phenotypic expression because one mutation can produce different phenotypes and appear in different individuals within a family. Despite many studies, It is known that there is no specific correlations between genotypes and phenotypes identified in the AIS patients[6]. Forty-five allelic variants that may result in different phenotypes are currently recorded in the McGill AR mutation database. There are no available qualitative data on penetrance at present. The variable phenotypic expression of particular mutations may be due to differences in affected individuals such as somatic cell embedding[6]. On the otherhand, epigenetic repression of AR transcription in mutation-negative Androgen Insensitivity Syndrome (AIS Type II) has been studied recently[48]. Cofactors can influence AR activity at the transcriptional as well as posttranscriptional level. Methylation-dependent repression of AR mRNA expression can contribute to an incomplete male genital development in a subset of individuals with AIS type II. This epigenetic regulation of AR expression might be set up during embryonic development and maintained after differentiation to ensure proper cellular identity[48]. The identification of upstream factors responsible for this epigenetic AR mRNA repression will be the next planned step in undiagnosed cases for the future studies.

To date, more than 100 mutations have been described in *SRD5A2* gene (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php)[46]. It is found that approximately 60% are in homozygous and 40% are in compound heterozygous form[46]. However, in our study, we have only one compound heterozygous patient (7.1%) and high homozygous rate may be related to consanguinity rate in Turkish population. As a consequence, a wide phenotypic range has been described attributed to the residual enzymatic activity and probably to the individual genetic background without a strong genotype-phenotype correlation in literature[46]. One of our frequent mutation, p. Leu55Gln which causes decrease in enzymatic activity has only been described in Turkish patients until today. In parallel to this in the current study, we describe three patients with this mutation (one compound heterozygous, two homozygous). It is reported that this mutation causes less severe phenotypes, with EMS values ranging from 3.0 to 8.0, attributable to different residual enzymatic activities caused by different mutations and a genotype-phenotype correlation seems to be the most difficult in this group. In our study, two homozygous mutantion carrying patients presented PAIS whereas the compound heterozygous form of this mutation with a novel p.His90Pro mutation presented CAIS clinically which more case reports are needed to support our finding based on genotype–phenotype relationship. Pathogenic alterations that interfere with the NADPH binding domain that are within 3-oxo-5-alpha-steroid 4-dehydrogenase domain were also detected in our study (hom, p.Pro181Leu, hom. p.Gly196Ser, hom. p.Arg246Try], hom. p.Arg171Ser). The EMS scores of these mutations is reported to vary between 2.67 to 4.17 phenotype[46]. In the current study, homozygous p.Arg246Try alteration associated with CAIS phenotype. Although it has been reported that only the p.Gly196Ser variant seems to produce a less variable phenotype[46], allelic variants at exon 4 and indels consistently has been shown to cause more severe phenotypes recently[49]. Variant, p.Gly196Ser, identified in homozygous form is associated with PAIS phenotype with Quigley score 3 in this study. Another known mutations affecting NADPH domain, p.Arg171Ser, is frequently found in different populations (Mexican, Turkish, Spanish, Mediterranean), there is very few homozygous reported cases, being found more frequently in compound heterozygotes[49]. However we have one homozygous p.Arg171Ser mutant patient who presented with PAIS. Although female appereance genitala is infrequent in

5α-RD cases, we had three CAIS phenotype (one with p.[L55Q];[H90P], one hom. p.Arg246Try, and one with hom. p.(Leu152Tyrfs*8). Although we have two patients with the same hom. p.(Leu152Tyrfs*8) mutation and one heterozygous form of this mutation in the same family, they presented different undermasculization. The genotype–phenotype incongruence occurs even in individuals carrying the same variant and also in individuals from the same family, suggesting that other factors beyond the 5αreductase type 2 enzyme play a role in phenotype[49]. Thus, same as *AR* mutations, allelic variants in the SRD5A2 gene, lead to a broad spectrum of external genitalia phenotypes with no strong genotype–phenotype relationship[49] and some other factors that affect phenotype are still unclear for both AR and SRD5A2, and constitute a relevant field for future research.

In our study gonadectomy was applied to 16 patient (n=7 AIS, n=5 SRD5A2 and 4 patient without any mutation) and intratubuler germ cell neoplasia only was seen in one patient with no any detected mutation in *AR* or *SRD5A2*. Indeed, dysgenetic and undescended testes are the major risk factors for testicular cancer, the most common malignancy for men between the ages of 15 and 35 years[50]**.** Our patient with gonadoblastoma was 7.3 years old when the gonadectomy was applied. The other causes of gonadoblastoma in 46XY DSD patients except dysgenetic gonads are also reported as AIS, ovotesticular DSD, Klinefelter syndrome, 5α-RD, 17β-hydroxysteroid dehydrogenase deficiency respectively[50]**.** Unfortunately, we only studied *SRD5A2* and *AR* genes in this patient, and further genetic analysis will be essential to definitive diagnosis.

Limitations

Our study has somelimitations. First, the nature of the study required us to rely on data from medical records. Second, serum levels of inhibin B, anti-mullerian hormone were not examined due to missing data. Third, according to the wide age range of sample, T and DHT were measured in different years with a possibility of different methods that may have led to some confusions. These shortcomings can be overcome in future prospective studies by starting to use genetic analysis earlier with more specific methods like liquid chromatography linked with tanden mass spectrometry (LC-MS/MS) or immunoassays after organic solvent extraction to detect hormones. On the otherhand, this study used Sanger sequencing method for diagnosis of AIS and 5α-RD patients. However, next-generation sequencing-based targeted sequencing is a promising technique to improve the detection rate of DSD, and it will be more useful for future studies.

Conclusion

Four novel AR variants were identified in our study. T/DHT ratio in the diagnosis of AIS and 5α-reductase deficiency is an important hormonal criteria, but in some cases, T/DHT ratio may be lower or higher incorrectly that may lead to diagnostic confusion. So genetic analysis for actual diagnosis seems to be essential especially for determining the treatment pathway and the sex identity of patients.

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Table 1. Comparison of clinical and hormonal features of genetically proven AIS and undiagnosed patients with a normal T/DHT value (<20)

	Mutation (+)	**Mutation (-)**	***P***
T/DHT :Normal	**n=14**	**n=61**	
Age at presentation (years)	1.8±3.1	1.3±2.0	0.98
Consanguinity n(%)	25% (n=3)	23% (n=14)	0.87
Birth weight SDS	-0.8±1.9	-1.7±2.0	0.19
Gestational age (weeks)	37±4.1	36.6±3.7	0.46
Gestational age groups (weeks) n(%)			0.4
< 37	21.4% (n=3)	39.3% (n=24)	
≥ 37	78.5% (n=11)	60.6% (n=37)	
BW groups n(%)			0.6
AGA (-2 to +2 SDS) n(%)	64.3% (n=9)	54% (n=33)	
SGA (<-2 SDS) n(%)	28.6% (n=4)	42.6% (n=26)	
LGA (> +2 SDS) n(%)	7.1% (n=1)	3.3% (n=2)	
Symptom on admission			0.07
Ambiguous genitalia	78.5% (n=11)	93.4% (n=57)	
Same diagnosis siblings	-	1.6% (n=1)	
Mass in groin	14.2% (n=2)	1.6% (n=1)	
Undescended testes	-	1.6% (n=1)	
Micropenis	-	1.6% (n=1)	
Finding testis during inguinal hernia surgery	7.1% (n=1)	-	
At current			
Age (yrs)	6.7±0.9	6.5±5.4	0.40
Weight SDS	-0.4±1.6	-0.7±1.2	0.69
Height SDS	-0.3±1.4	-1.0±1.1	0.16
Puberty status			0.21
Tanner Stage 1	78.5% (n=11)	77% (n=47)	
Tanner Stage 2	7.1% (n=1)	8.2% (n=5)	
Tanner Stage 3	7.1% (n=1)	1.6 (n=1)	
Tanner Stage 4	7.1% (n=1)	-	

Tanner Stage 5	-	9.8% (n=6)	
Mini puberty	-	1.6% (n=1)	
Estrogen replacement after gonadectomy	-	1.6% (n=1)	
Reared gender n(%)			**0.00***
Male	71.4% (n=10)	96.7% (n=59)	
Female	28.5% (n=4)	1.6% (n=1)	
Female reared, changed identity after diagnosis	-	1.6 % (n=1)	
Ouigley scale			0.06
2	7.1% (n=1)	18% (n=11)	
3	35.7% (n=5)	57.4% (n=35)	
4	14.2%(n=2)	11.5% (n=7)	
5	7.1% (n=1)	4.9% (n=3)	
6/7	28.5% (n=4)	1.6% (n=1)	
applied after operation	7.1% (n=1)	6.5% (n=4)	
EMS			0.23
1	5.5% (n=1)	1.6% (n=1)	
2	16.7% (n=3)	0% (n=0)	
4	11.1% (n=2)	16.4% (n=10)	
5	0% (n=0)	23% (n=14)	
6	22.2% (n=4)	19.7% (n=12)	
7	11.1 % (n=2)	27.9% (n=17)	
8	5.5% (n=1)	4.9 %(n=3)	
applied after operation	5.5% (n=1)	6.5% (n=4)	
AIS groups n(%)			**0.02***
PAIS	71.4% (n=10)	98.4% (n=60)	
CAIS	28.5% (n=4)	1.6% (n=1)	
Additional findings			0.19
none	57.1% (n=8)	36%(n=22)	
Prematurity	21.4% (n=3)	34.4% (n=21)	
IUGR	-	9.8% (n=6)	
Multicystic dysplastic kidney	-	3.3% (n=2)	
Congenital heart disease	-	4.9% (n=3)	
Anal atresia	-	3.3% (n=2)	
Nephrolithiasis	-	1.6% (n=1)	
Autism	-	1.6% (n=1)	
Gonadoblastoma	-	1.6% (n=1)	
Infant of diabetic mother	-	1.6% (n=1)	
Klinefelter syndrome	7.1% (n=1)	1.6% (n=1)	
47,XYY	7.1% (n=1)	-	
Agenesis of the corpus callosum	7.1% (n=1)	-	
Target height SDS	-0.8±0.2	-0.9±0.09	0.97
Laboratory results according to the age			
0-6 months	n=7	n=26	
Age (months)	2.1±1.5	1.6±1.6	0.38
LH (mIU/ml)	4.9±4.3	4.1±3.7	0.62
FSH (mIU/ml)	1.7±1.2	2.2±1.6	0.42
Basal T (ng/ml)	1.5±1.4	1.6±0.8	0.76
Basal DHT (ng/ml)	0.3±0.2	0.4±0.1	0.59
Basal T/DHT ratio	10.5±7.3	6.9±6.3	0.22
Prepubertal	n=7	n= 38	
Age (years)	4.0±2.9	3.8±2.9	0.67
LH (mIU/ml)	0.7±0.9	0.5±1.6	0.23
FSH (mIU/ml)	1.2±1.0	1.5±2.5	0.86
Basal T (ng/ml)	0.3±0.4	0.2±0.4	0.82
Stimulated T (ng/ml)	5.1±2.9	4.3±2.4	0.38
Stimulated DHT (ng/ml)	1.5±1.6	0.6±0.5	0.12
Stimulated T/DHT	4.7±2.8	9.3±1.3	**0.04***
Pubertal	n=3	n=8	

Age (years)	10.8±0.3	12.7±3.0	0.13
LH (mIU/ml)	2.4±4.2	3.8±5.0	0.69
FSH (mIU/ml)	2±4.9	6.5±9.4	0.56
Basal T (ng/ml)	4.3±3.1	2.6±2.0	0.51
Basal DHT (ng/ml)	0.5±0.2	0.4±0.3	0.91
Basal T/DHT	9.5±4.5	5.8±3.0	0.16

AGA: Appropriate for gestational age, AIS: Androgeninsensitivity syndrome, BW: birth weight, CAIS: Complet Androgen insensitivity syndrome, DHT: Dihydrotestosterone, EMS: External Masculinization Score , FSH: Follicle-stimulating hormone, IUGR: Intrauterine growth retardation, LGA: Large for gestational age LH: Luteinizing hormone, PAIS: Partial androgen insensitivity syndrome, SGA: Small for gestational age, T: Testosterone

Table 2. Comparison of clinical and hormonal features of genetically proven AIS and undiagnosed patients with a high T/DHT value (≥20)

	Mutation (+)	**Mutation (-)**	***P***
T/DHT : High	**n=4**	**n=35**	
Age at presentation (years)	4.0±4.4	1.3±2.8	0.11
Consanguinity n(%)	%0 (n=0)	25.8% (n=8)	0.38
Birth weight SDS	-0.7±1.9	-1.2±1.6	0.7
Gestational age (weeks)	35.0±7.0	35.9±4.1	0.93
Gestational age groups (weeks) n(%)			0.84
< 37	25% (n=1)	40% (n=14)	
≥ 37	75% (n=3)	60% (n=21)	
BW groups n(%)			0.88
AGA (-2 to +2 SDS) n(%)	75% (n=3)	60% (n=21)	
SGA (<-2 SDS) n(%)	25% (n=1)	34.3% (n=12)	
LGA (> +2 SDS) n(%)	0% (n=0)	5.7% (n=2)	
Symptom on admission			**0.00***
Ambiguous genitalia	75% (n=3)	94.3% (n=33)	
Mass in groin	-	-	
Undescended testes	-	2.8% (n=1)	
Micropenis	-	2.8% (n=1)	
Finding testis during inguinal hernia surgery	25% (n=1)	-	
At current			
Age (yrs)	7.7±8.6	3.9±4.7	0.34
Weight SDS	0.6±1.8	-0.7±1.2	0.20
Height SDS	-0.3±0.6	-0.9±1.3	0.38
Puberty status			0.07
Tanner Stage 1	25% (n=1)	82.9% (n=29)	
Tanner Stage 2	-	-	
Tanner Stage 3	-	-	
Tanner Stage 4	-	-	
Tanner Stage 5	-	11.4% (n=4)	
Mini puberty	-	5.7% (n=2)	
Estrogen replacement after gonadectomy	75% (n=3)	-	
Reared gender n(%)			**0.04***
Male	25% (n=1)	94.3% (n=33)	
Female	75% (n=3)	2.8% (n=1)	
Female reared, changed identity after diagnosis	-	2.8% (n=1)	
Ouigley scale			**0.00***
2	-	8.6% (n=3)	
3	25% (n=1)	85.7% (n=30)	
4	-	2.8% (n=1)	
5	-	2.8% (n=1)	
6/7	75% (n=3)	-	
EMS			**0.01***
1	50% (n=2)	0% (n=0)	
2	25% (n=1)	0% (n=0)	
3	0% (n=0)	2.9% (n=1)	
4	0% (n=0)	17.1% (n=6)	
5	0% (n=0)	8.6% (n=3)	

6	25% (n=1)	22.9% (n=8)	
7	0% (n=0)	40% (n=14)	
8	0% (n=0)	8.6% (n=3)	
AIS groups n(%)			
PAIS	25% (n=1)	100% (n=35)	**0.00***
CAIS	75% (n=3)	-	
Additional findings			0.95
None	75% (n=3)	51.4% (n=18)	
Prematurity	25% (n=1)	34.3% (n=12)	
IUGR	-	11.4% (n=4)	
Anal atresia	-	2.8% (n=1)	
Target height SDS	-0.8±0.3	-0.8±0.1	0.93
Laboratory results according to the age			
0-6 months	n=0	n=20	
Age (months)	-	2.5±2.1	-
LH (mIU/ml)	-	3.9±3.4	-
FSH (mIU/ml)	-	1.8±1.4	-
Basal T (ng/ml)	-	1.8±1.5	-
Basal DHT (ng/ml)	-	0.08±0.05	-
Basal T/DHT	-	40.0±18.5	-
Prepubertal	n=2	n=19	
Age (years)	5.2±5.0	2.2±2.2	0.23
LH (mIU/ml)	0.3±0.1	0.3±0.2	0.76
FSH (mIU/ml)	1.8±1.6	0.8±0.5	0.23
Basal T (ng/ml)	0.05±0.07	0.05±0.07	0.12
Stimulated T (ng/ml)	4.6±2.2	4.0±2.9	0.95
Stimulated DHT (ng/ml)	0.05±0.07	0.2±0.4	0.58
Stimulated T/DHT	94.8±28.7	81.6±15.9	0.49
Pubertal	n=3	n=3	
Age (years)	12.3±2.7	12.3±2.7	0.22
LH (mIU/ml)	23.9±3.0	6.1±2.9	**0.04***
FSH (mIU/ml)	20.6±10.4	6.6±5.7	0.12
Basal T (ng/ml)	6.5±1.9	4.4±2.2	0.28
Basal DHT (ng/ml)	0.2±0.08	0.09±0.1	0.64
Basal T/DHT ratio	55.8±29.7	57.3±21.2	0.31

AGA: Appropriate for gestational age, AIS: Androgeninsensitivity syndrome, BW: birth weight, CAIS: Complet Androgen insensitivity syndrome, DHT: Dihydrotestosterone, EMS: External Masculinization Score , FSH: Follicle-stimulating hormone, IUGR: Intrauterine growth retardation, LGA: Large for gestational age LH: Luteinizing hormone, PAIS: Partial androgen insensitivity syndrome, SGA: Small for gestational age, T: Testosterone

Table3. Comparison of clinical and hormonal features of genetically proven 5α-RD and undiagnosed patients with a normal T/DHT value (<20)

	Mutation (+)	**Mutation (-)**	***P***
T/DHT :Normal	**n=4**	**n=61**	
Age at presentation (years)	12.0±4.9	1.3±2.0	**0.00***
Consanguinity n(%)	75% (n=3)	23% (n=14)	**0.02***
Birth weight SDS	0.0±1.0	-1.7±2.0	**0,07**
Gestational age (weeks)	39.7±0.5	36.6±3.7	**0.04***
Gestational age groups (weeks) n(%)			0.12
< 37	0% (n=0)	39.3% (n=24)	
≥ 37	100% (n=4)	60.6% (n=37)	
BW groups n(%)			0.19
AGA (-2 to +2 SDS) n(%)	100% (n=4)	54% (n=33)	
SGA (<-2 SDS) n(%)	0% (n=0)	42.6% (n=26)	
LGA (> +2 SDS) n(%)	0% (n=0)	3.3% (n=2)	

Symptom on admission			**0.00***
Ambiguous genitalia	0	93.4% (n=57)	
Same diagnosis siblings	50% (n=2)	1.6% (n=1)	
Mass in groin	50% (n=2)	1.6% (n=1)	
Undescended testes	0	1.6% (n=1)	
Micropenis	0	1.6% (n=1)	
At current evaluation			
Age (yrs)	15.9±2.8	6.5±5.4	**0.00***
Weight SDS	-0.8±0.7	-0.7±1.2	0.82
Height SDS	-0.6±0.6	-1.0±1.1	0.56
Puberty status			**0.00***
Tanner Stage 1	-	77% (n=47)	
Tanner Stage 2	25% (n=1)	8.2% (n=5)	
Tanner Stage 3	-	1.6 (n=1)	
Tanner Stage 4	-	-	
Tanner Stage 5	-	9.8% (n=6)	
Mini puberty	-	1.6% (n=1)	
Estrogen replacement after gonadectomy	75% (n=3)	1.6% (n=1)	
Reared gender n(%)			**0.00***
Male	-	96.7% (n=59)	
Female	75% (n=3)	1.6% (n=1)	
Female reared, changed identity after diagnosis	25% (n=1)	1.6 % (n=1)	
Ouigley scale on admission			**0.00***
2	-	18% (n=11)	
3	25% (n=1)	57.4% (n=35)	
4	-	11.5% (n=7)	
5	50% (n=2)	4.9% (n=3)	
6/7	25% (n=1)	1.6% (n=1)	
applied after operation	-	6.5% (n=4)	
EMS score			**0.00***
1	0% (n=0)	1.6% (n=1)	
2	25% (n=1)	0% (n=0)	
4	0% (n=0)	16.4% (n=10)	
5	0% (n=0)	23% (n=14)	
6	0% (n=0)	19.7% (n=12)	
7	0% (n=0)	27.9% (n=17)	
8	75% (n=3)	4.9 %(n=3)	
applied after operation		6.5% (n=4)	
AIS groups n(%)			**0.00***
PAIS	75% (n=3)	98.4% (n=60)	
CAIS	25% (n=1)	1.6% (n=1)	
Additional findings/diagnosis			**0.03***
none	100% (n=4)	36% (n=22)	
Prematurity	-	34.4% (n=21)	
IUGR	-	9.8% (n=6)	
Multicystic dysplastic kidney	-	3.3% (n=2)	
Congenital heart disease	-	4.9% (n=3)	
Anal atresia	-	3.3% (n=2)	
Nephrolithiasis	-	1.6% (n=1)	
Autism	-	1.6% (n=1)	
Gonadoblastoma	-	1.6% (n=1)	
Infant of diabetic mother	-	1.6% (n=1)	
Klinefelter syndrome	-	1.6% (n=1)	
Target height SDS	-1.0±0.2	-0.9±0.09	0.43
Laboratory results according to the age			

0-6 months	n=0	n=26	-
Age (months)	-	1.6±1.6	-
LH (mIU/ml)	-	4.1±3.7	-
FSH (mIU/ml)	-	2.2±1.6	-
Basal T (ng/ml)	-	1.6±0.8	-
Basal DHT (ng/ml)	-	0.4±0.1	-
Basal T/DHT	-	6.9±6.3	-
Prepubertal	n=1	n= 38	
Age (year)	4.8	3.8±2.9	0.23
LH (mIU/ml)	0.1	0.5±1.6	0.48
FSH (mIU/ml)	0.2	1.5±2.5	0.10
Basal T (ng/ml)	0.2	0.2±0.4	0.08
Stimulated T (ng/ml)	4.1	4.3±2.4	0.89
Stimulated DHT (ng/ml)	0.3	0.6±0.5	0.17
Stimulated T/DHT	16	9.3±1.3	**0.04***
Pubertal	n=4	n=8	
Age (years)	14.1±1.2	12.7±3.0	0.64
LH (mIU/ml)	6.6±7.2	3.8±5.0	0.23
FSH (mIU/ml)	12.1±14.6	6.5±9.4	0.23
Basal T (ng/ml)	2.6±2.1	2.6±2.0	0.85
DHT (ng/ml)	0.8±0.6	0.4±0.3	0.48
T/DHT	5±2.31	5.8±3.0	0.64

AGA: Appropriate for gestational age, AIS: Androgeninsensitivity syndrome, BW: birth weight, CAIS: Complet Androgen insensitivity syndrome, DHT: Dihydrotestosterone, EMS: External Masculinization Score , FSH: Follicle-stimulating hormone, IUGR: Intrauterine growth retardation, LGA: Large for gestational age LH: Luteinizing hormone, PAIS: Partial androgen insensitivity syndrome, SGA: Small for gestational age, T: Testosterone

Table 4. Comparison of clinical and hormonal features of genetically proven 5α-RD and undiagnosed patients with a high T/DHT value (≥20)

	Mutation (+)	**Mutation (-)**	***P***
T/DHT : High	**n=10**	**n=35**	
Age at presentation (years)	6.6±7.2	1.3±2.8	**0.04***
Birth weight SDS	0.6±1.4	-1.2±1.6	**0.007***
Gestational age (weeks)	39.2±0.8	35.9±4.1	**0.02***
Gestational age (weeks) n(%)			**0.03***
< 37	0% (n=0)	40% (n=14)	
≥ 37	100% (n=10)	60% (n=21)	
Consanguinity n(%)	50% (n=5)	25.8% (n=8)	0.14
BW groups n(%)			0.10
AGA (-2 to +2 SDS) n(%)	90% (n=9)	60% (n=21)	
SGA (<-2 SDS) n(%)	0% (n=0)	34.3% (n=12)	
LGA (> +2 SDS) n(%)	10% (n=1)	5.7% (n=2)	
Symptom on admission			0.07
Ambiguous genitalia	60% (n=6)	94.3% (n=33)	
Mass in groin	10% (n=1)	-	
Undescended testes	-	2.8% (n=1)	
Micropenis	-	2.8% (n=1)	
Primary amenorrhea	10% (n=1)	-	
Virilization in puberty	10% (n=1)	-	
Cliteromegali	10% (n=1)	-	
At current evaluation			
Age (yrs)	6.9±6.0	3.9±4.7	**0.03***
Weight SDS	0.6±1.0	-0.7±1.2	**0.01***
Height SDS	0.3±0.9	-0.9±1.3	**0.02***
Puberty status			0.95
Tanner Stage 1	60% (n=6)	82.9% (n=29)	
Tanner Stage 2	-	-	
Tanner Stage 3	10% (n=1)	-	
Tanner Stage 4	10% (n=1)	-	
Tanner Stage 5	10% (n=1)	11.4% (n=4)	
Mini puberty	-	5.7% (n=2)	
Estrogen replacement after gonadectomy	10% (n=1)	-	

Reared gender n(%)			**0.02***
Male	50% (n=5)	94.3% (n=33)	
Female	10% (n=1)	2.8% (n=1)	
Female reared, changed identity after diagnosis	4% (n=4)	2.8% (n=1)	
Ouigley scale			**0.03***
2	-	8.6% (n=3)	
3	50% (n=5)	85.7% (n=30)	
4	10% (n=1)	2.8% (n=1)	
5	20% (n=2)	2.8% (n=1)	
6/7	20% (n=2)	-	
EMS score			**0.00***
1	10% (n=1)	0% (n=0)	
2	10% (n=1)	0% (n=0)	
3	% (n=0)	2.9% (n=1)	
4	30% (n=3)	17.1% (n=6)	
5	10% (n=1)	8.6% (n=3)	
6	10% (n=1)	22.9% (n=8)	
7	% (n=0)	40% (n=14)	
8	30% (n=3)	8.6% (n=3)	
AIS groups n(%)			**0.03***
PAIS	80% (n=8)	100% (n=35)	
CAIS	20% (n=2)	-	
Additional findings/diagnosis			**0.04***
None	90% (n=9)	51.4% (n=18)	
Prematurity	-	34.3% (n=12)	
IUGR	-	11.4% (n=4)	
Klinefelter syndrome	10% (n=1)	2.8% (n=1)	
Anal atresia	-	51.4% (n=18)	
Target height SDS	-0.9±0.1	-0.8±0.1	0.35
Laboratory results according to the age			
0-6 months	n=2	n=20	
Age (months)	1.5±2.2	2.5±2.1	0.33
LH (mIU/ml)	4.7±1.9	3.9±3.4	0.76
FSH (mIU/ml)	2.3±1.0	1.8±1.4	0.31
Basal T (ng/ml)	2.1±2.0	1.8±1.5	0.73
Basal DHT (ng/ml)	0.5±0.02	0.08±0.05	0.13
Basal T/DHT	52.7±19.7	40.0±18.5	0.13
Prepubertal	n=6	n=19	
Age (years)	4.3±2.3	2.2±2.2	**0.01***
LH (mIU/ml)	0.4±0.4	0.3±0.2	0.89
FSH (mIU/ml)	0.9±0.5	0.8±0.5	0.47
Basal T (ng/ml)	0.07±0.07	0.05±0.07	0.43
Stimulated T (ng/ml)	3.4±2.4	4.0±2.9	0.52
Stimulated DHT (ng/ml)	0.08±0.07	0.2±0.4	0.64
Stimulated T/DHT	67.4±27.5	81.6±15.9	0.50
Pubertal	n=3	n=3	
Age (years)	15.6±2.3	12.3±2.7	0.25
LH (mIU/ml)	12.7±10.0	6.1±2.9	0.27
FSH (mIU/ml)	23.7±22.8	6.6±5.7	0.08
Basal T (ng/ml)	4.8±3.8	4.4±2.2	0.72
DHT (ng/ml)	0.1±0.2	0.09±0.1	0.22
T/DHT ratio	49.8±24.9	57.3±21.2	0.65

AGA: Appropriate for gestational age, AIS: Androgeninsensitivity syndrome, BW: birth weight, CAIS: Complet Androgen insensitivity syndrome, DHT: Dihydrotestosterone, EMS: External Masculinization Score , FSH: Follicle-stimulating hormone, IUGR: Intrauterine growth retardation, LGA: Large for gestational age LH: Luteinizing hormone, PAIS: Partial androgen insensitivity syndrome, SGA: Small for gestational age, T: Testosterone

Table 5. The characteristics of *AR* and *SRD5A2* variants in patients with 46,XY DSD

GENE	Nucleotide	Peptide	Type	Variant ID	Classification*	Karyotype	Zygosity	Status in patients**	Allele/ Patients	References
AR (NM_000044.4, NP_000035.2), n=	c.94G>T	p.E32*	Nonsense	Novel	Pathogenic	46,XY	Hem.	c.[94G>T];[0]	1/1	This study
	c.330G>C	p.L110L	Silent	Novel	Likely benign	46,XY	Hem.	c.[330G>C];[0]	1/1	This study
	c.1174C>T	p.P392S	Missense	rs201934623	Pathogenic	46,XY	Hem.	c.[1174C>T];[0]	6/6	Bhangoo (2010)
	c.1823G>A	p.R608Q	Missense	rs137852573	Pathogenic	46,XY	Hem.	c.[1823G>A];[0]	1/1	Zhang (2019)
	c.2084C>T	p.P695L	Missense	Novel	Likely pathogenic	46,XY	Hem.	c.[2084C>T];[0]	1/1	This study
	c.2169G>T	p.L723F	Missense	-	Pathogenic	46,XY	Hem.	c.[2169G>T];[0]	1/1	Elfferich (2013)
	c.2482T>G	p.F828V	Missense	-	Likely pathogenic	47,XYY	Hem.	c.[2482T>G];[0]	1/1	Chavez (2001)
	c.2521C>A	p.R841S	Missense	-	Pathogenic	46,XY	Hem.	c.[2521C>A];[0]	1/1	Melo (1999)
	c.2585_2592delAGCTCCTG	p.(K862Rfs*16)	Frame shift deletion	Novel	Pathogenic	46,XY	Hem.	c.[2585_2592delAGCTCCTG];[0]	1/1	This study
	c.2668G>C	p.V890L	Missense	rs886041133	Likely pathogenic	46,XY	Hem.	c.[2668G>C];[0]	1/1	Audi (2010)
	c.2668G>A	p.V890M	Missense	rs886041133	Pathogenic	46,XY	Hem.	c.[2668G>A];[0]	1/1	Gottlieb (1999)
	c.2676T>A	p.F892L	Missense	-	Likely pathogenic	One with 47,XXY	Hem.	c.[2676T>A];[0]	1/1	Akcay (2014)
SRD5A2 ((NM_000348.3, NP_000339.2), n=	c.164T>A	p.L55Q	Missense	rs121434245	Pathogenic	46,XY	Hom. Com. het.	c.[164T>A];[164T>A] c.[164T>A];[269A>C]	2/3 1/3	Thigpen (1992)
	c.193G>C	p.A65P	Missense	-	VUS	46,XY	Hom.	c.[193G>C];[193G>C]	4/2	Savas Erdeve (2010)
	c.269A>C	p.H90P	Missense	-	Likely pathogenic	46,XY	Com. het.	c.[164T>A];[269A>C]	1/3	Gui (2019)
	c.453delC	p.(L152Yfs*8)	Frame shift deletion		Pathogenic	46,XY	Hom.	c.[453delC];[453delC]	2/1	Di Marco (2013)
	c.468-470delAAT	p.(Met157del)	In frame deletion	-	Likely pathogenic	46,XY	Hom.	c.[468-470delAAT];[468-470delAAT]	2/1	Boudon (1995)
	c.513G>C	p.R171S	Miss	rs75640	VUS	46,X	Hom.	c.[513G>C	2/1	Thigp

			ense	5261		Y		];[513G>C]		en (1992)
	c.542C>T	p.P181L	Miss ense	rs10575 17829	VUS	46,X Y	Hom.	c.[542C>T];[513C>T]	2/1	Nicole tti (2005)
	c.586G>A	p.G196S	Miss ense	rs12143 4250	VUS	47,X XY	Hom.	c.[586G>A];[586G>A]	2/1	Thigp en (1992)
	c.736C>T	p.R246W	Miss ense	rs12143 4244	Likely pathogen ic	46,X Y	Hom.	c.[736C>T];[736C>T]	2/1	Thigp en (1992)
	c.753delA	p.(Phe252S erfs*27)	Fram e shift deleti on	rs58777 6567	Pathoge nic	46,X Y	Hom.	c.[753delA];[753delA]	2/1	Can (1998)

Hom; Homozygous, Het: Heterozygous, Freq:Frequency of allele

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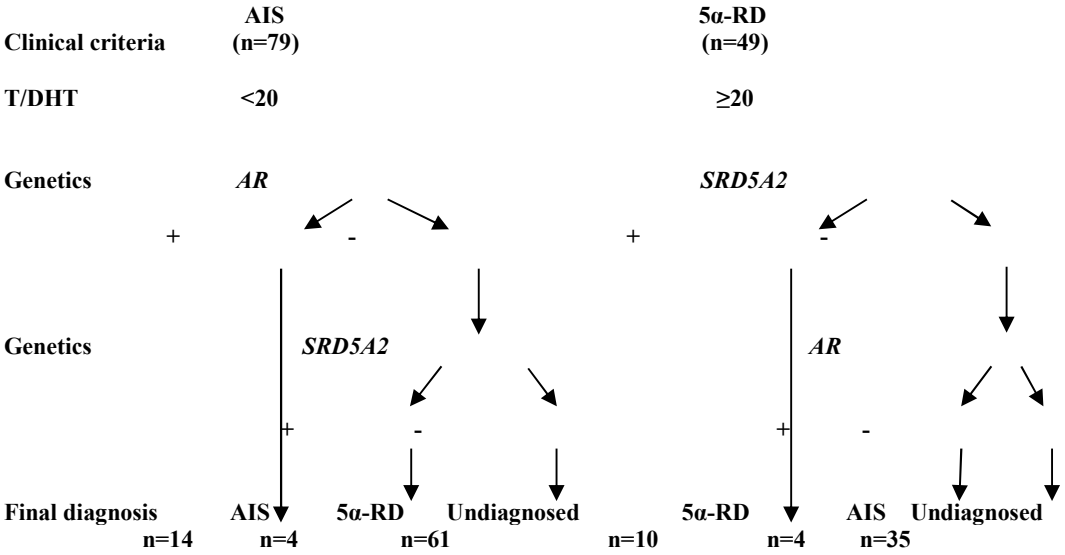

Figure 1. Analysis of the patients with 46, XY DSD