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Low Incidence of Androgen Receptor Mutation Among Egyptian Children with Androgen Resistance

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ABSTRACT

Introduction: In Egypt, disorders of sex development (DSD) constitute a significant entity among the birth defect list. Previous studies have reported that end organ androgen unresponsiveness, i.e. Androgen resistance, was the most prevalent underlying mechanism among Egyptian 46,XY DSD cases. Based on cytogenetic and hormonal diagnostic criteria as well as few sporadic case reports, it was proposed that androgen receptor (AR) defects [i.e. Androgen insensitivity syndrome (AIS), OMIM#300068] might constitute a major etiology within this category. However, this has never been systematically ascertained through an AR molecular diagnostic approach.

Aim of the Work: The current study aimed to assess the role of AR mutations as an underlying etiology among a sample of Egyptian 46,XY DSD pediatric patients presenting with androgen end organ unresponsiveness.

Patients and Method: In the current study, 21 children [age<18years] with male undermasculinization due to androgen end organ unresponsiveness were selected from 46,XY DSD cases. The selection criteria included ambiguous genital phenotype or genitalia discordant to the genotypic sex; 46,XY Karyotype and normal testicular response to HCG stimulation in prepubertal patients or normal basal testosterone (T) levels in postpubertal subjects. Molecular studies of the AR entailed PCR amplification for screening of major deletions/insertions, single stranded conformational polymorphism (SSCP) screening for point mutations in the AR 2-8 exons followed by sequencing of these exons for all cases.

Results: The results showed that none had major deletions/insertions. Five exons out of 147 (3.4%) showed abnormal SSCP migrational patterns. Out of those 5, two mutations in two Egyptian patients were detected by sequencing. The first was R840G (Arginine 840 glycine), in exon 7 (The ligand binding domain). The other was A596T (Alanine 596 Threonine) in exon 3 (The DNA binding domain).

Conclusion:, This study shows that AR mutation is an uncommon underlying etiology among Egyptian paediatric 46,XY cases.

Key Words:

Androgen resistance, androgen receptor, mutation, disorders of sex development, birth defects.

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INTRODUCTION

In Egypt, disorders of sex development (DSD) constitute a significant entity among the birth defect list, where their incidence reaches 1 in 3000 live births.[1] Previous studies have reported that end organ androgen unresponsiveness, i.e. Androgen resistance, was the most prevalent underlying mechanism among Egyptian 46,XY cases.[2-4] Based on cytogenetic and hormonal diagnostic criteria, it has been proposed that androgen receptor (AR) defects [i.e. Androgen insensitivity syndrome (AIS), OMIM#300068] might constitute a major etiology in this category, in addition to the deficiency of 5 α-reductase 2 (5αR2) enzyme.[2-5]

The AR is a ligand-activated transcription factor required for male sex development and virilization. High affinity androgen binding triggers conformational changes required for AR transactivation.[6] The two major androgens, testosterone (T) and 5α-dihydrotestosterone (DHT), both act through the AR, which is encoded by a single-copy gene located at chromosome band Xq11.2-q12. The complete sequence of the human AR cDNA is 3.6 Kbp. It contains an open reading frame of 2757 bp encoding 919 amino acids. The human AR consists of the N-terminal transactivation domain (1–538), DNA-binding domain (DBD) (539–627), hinge region (628–644), and C-terminal ligand-binding domain (LBD) (645–919).[7]

Mutations in the AR gene cause a broad spectrum of abnormal phenotypes in humans, ranging from infertility in an otherwise normal male (Mild AIS), to ambiguous genitalia (Partial AIS), to a completely female external phenotype [Complete AIS (CAIS)].[8] The diagnosis of AIS is provisionally inferred from clinical and biochemical findings: a 46 XY karyotype, absence of Mullerian-derived structures, normal testicular histology, and age-appropriate androgen production by the testis.

More than 700 mutations in the AR gene have now been documented in AIS [http://www.mcgill.ca/androgendb/24/7/2007]. A number of reports have reported on single cases of AR mutations among Egyptian 46, XY DSD patients.[9-12] The current study employed a systematic molecular approach towards assessing the role of AR mutations as an underlying etiology among a sample of Egyptian 46,XY pediatric patients presenting with androgen end organ unresponsiveness.

PATIENTS AND METHODS

Patients:

Over a period of two years, 21 children [age<18years] with male undermasculinization due to androgen end organ unresponsiveness were selected from the DSD cases presenting to the division of Human Genetics and Genome Research, National Research Center (NRC).

The selection criteria for the patients included ambiguous genital phenotype or genitalia discordant to the genotypic sex, 46,XY Karyotype and normal testicular response to HCG stimulation in prepubertal patients or normal basal T levels in postpubertal subjects.

Methods:

Molecular analysis of AR gene

1. ***Extraction:*** genomic DNA was extracted from peripheral blood lymphocytes of all patients by salting out technique[13] with some modifications.

2. ***Screening for major deletions or insertions:*** Exons 2-8 (Or B-H) of the AR gene were amplified by PCR using intron specific primers.[14] One hundred ng of DNA was used in amplification buffer containing 50mM KCl, 10mM tris HCl pH 8.3, 1.5mM Mg Cl2, 20µM dNTP, 0.2µM each primer and Taq polymerase1 (Unit/reaction) (Promega). Amplification conditions consisted of 35 cycles, each consists of denaturing at 94ºC for 75 seconds, annealing (At 52ºC for exon B, 53ºC for exon C, 50ºC for exon D, 64ºC for exon E, 60ºC for exon F, 64ºC for exon G and 55ºC for exon H) for 90 seconds, and extension at 72ºC for 2min. The amplified products were visualized by Ethidium bromide on 1-2% Agarose gel electrophoresis.

3. ***Screening for point mutations using SSCP technique:*** SSCP analysis is based on the principle that "A single base change between PCR products, which are amplified using the same primers, is sufficient to alter the mobility of single stranded products when electrophoresed through non-denaturing polyacrylamide gel".[15]

   In PCR-SSCP analysis, amplified double-stranded products were heat denatured and prevented from reannealing to its complement strand by cooling, and by the addition of formamide (As a denaturating agent). PCR products were prepared by adding 15 ul formamide loading buffer to 4 ul of the PCR products then denatured at 95ºC for 10 min, and immediately dipped in ice for 5 min before loading on 10% of non-denaturing polyacrylamide gel. The samples were loaded and run at 400 V for 3 hours at 7º C.

   Silver staining of the SSCP gel: To stain the SSCP gel, Silver Sequence TM DNA Sequencing System, (Promega, USA) was used. The gel was put in saran wrap then viewed on a light box to visualize bands migration.

4. ***Sequencing reaction:*** The amplified DNA products were purified using Centricon microconcentrators (Amicon, Beverly, MA 01915, USA). The purified PCR products were dried and resuspended in buffer (20mM MgCL2, 50mM NaCL, 40µM Tris, pH 7.5) and 2µM sequencing primer, in a total volume of 10µl After annealing at 65ºC for 2 minutes, dideoxy sequencing was performed using the sequence V.2 Kit (US Biochemical Corp. Cleveland, OH 44122,USA). The sequencing analysis was done using ABI Prism Dye terminator sequencing kit and the ABI 310 genetic analyzer apparatus (Applied Biosystems).

RESULTS

The age of the studied cases ranged from 0.5-17.9 years. Eleven cases were reared as males and 10 as females. Parental consanguinity was noticed in 17

out of the 21 cases, (81%). All patients had a 46,XY karyotype.

The T levels in the 4 post pubertal subjects ranged from 2.7-16 ng/ml, while in the prepubertal subjects, the post-HCG levels reached 2.2-16 ng/ml. The values of the postpubertal basal and prepubertal post-HCG T/DHT ratios varied from 6.2-98.5. Compared to our normal range[2], abnormally increased T/DHT values (i.e.>14) were noticed in 17 out of 21 cases (81%).

Molecular Data:

1. ***Screening for major deletions or insertions in the AR gene:*** Using PCR amplification of individual exons (2-8) of the AR gene, no major deletions or insertions were noted in any of the 21 studied cases.

2. ***Screening for point mutations in the AR gene:*** SSCP analysis for individual exons of AR gene was done as mentioned before. Four cases out of 21 (19%) showed changes in the migration patterns of five exons {Exon 7 (G), in case no. 18, exon 5 (E), in case no. 19, exon 3 (C), in case no. 20 Figure (1), and exons 5 (E) and 7 (G) in case no. 21}.

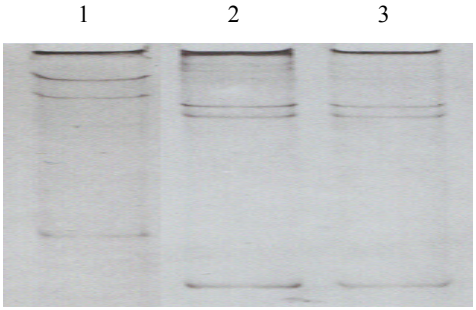

Fig. 1: SSCP analysis of exon C of AR gene: Lane 1 represents SSCP analysis of exon C of case no. 20 that displayed a change in electrophoretic migration pattern in respect to the normal controls (Lanes 1&2).

3. ***Sequencing of the AR gene:*** Sequencing of the 2-8 exons for all cases was carried out. A missense point mutation was detected in exon 7 of patient no. 18 (Figure 2) where the arginine residue (R) at codon 840 was replaced by glycine (G) (R840G).

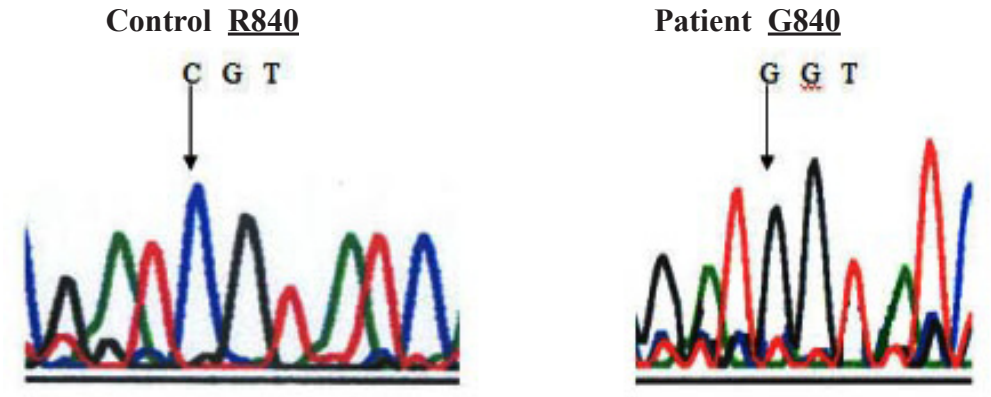

Fig. 2: Sequencing analysis of exon G in patient no. 18 with R840G mutation.

The patient (right panel) shows change in the first base of codon 840 leading to change of that codon from CGT (arginine) to GGT (Glycine).The left panel shows the normal sequence.

Another missense point mutation was detected in exon 3 in patient no. 19 (Figure 3) where alanine residue (A) at codon 569 was replaced by threonine (T) (A569T).

On the other hand, the other 3 exons that showed abnormal SSCP migrational patterns proved to have no mutations using sequencing of both the forward and reverse strands.

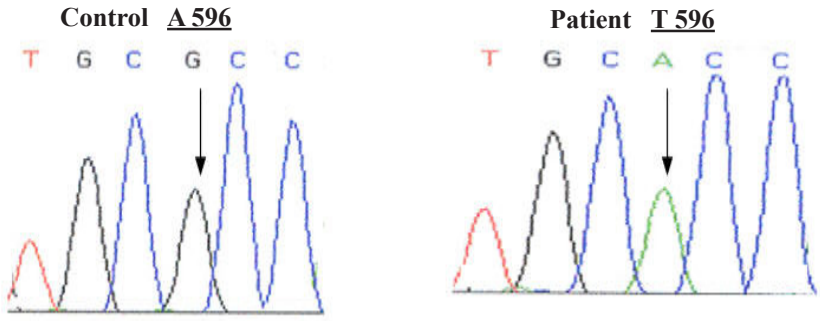

Fig. 3: Sequencing analysis of exon C in patient no. 20 with A596T mutation.

The patient (Right panel) shows change in the first base of codon 596 leading to change of that codon from GCC (Alanine) to ACC (Threonine).The left panel shows the normal sequence.

DISCUSSION

Abnormal sexual development is not uncommon in Egypt. A study reported an incidence of one newborn with ambiguous genitalia per 3000 live births.[1] Another study showed that 46, XY DSD owing to androgen resistance constituted ~25% of DSD presentations in Egyptian patients.[4] This makes it one of the two most common etiologies underlying 46,XY DSD states in Egyptian patients, in addition to 21-hydroxylase deficiency in 46,XX patients.

In the present study, a sample of patients (21 cases) presenting with 46,XY DSD due to androgen resistance was studied, aiming to evaluate the relative significance of AR mutations as an underlying etiology. The study comprised three main stages, the clinical phenotype assessment, the biochemical or hormonal evaluations and lastly the molecular detection of AR gene mutations.

The clinical presentation of all studied patients generally entailed ambiguous genitalia. However, the degree of ambiguity was variable from one case to another. The study showed that the external genital phenotypes varied from female to ambiguous to nearly male genitalia (Data not shown). This reflected the wide range of phenotypic heterogeneity in androgen resistance disorders.

Parental consanguinity was noted in 17 out of 21 patients (81%). This high frequency is much higher than the average parental consanguinity rate among the Egyptian general population which is nearly 1/3.[1] Androgen resistance entails several genetic defects (i.e.5αR2D, AR defects or post AR defects).[16] The presence of high inbreeding rate in the patient groups may provisionally infer the relevance of autosomal recessive DSD form(s) and undermine the possibility of AR defects as a prevalent underlying

etiology. Androgen resistance is commonly reported as a monogenic disease resulting from mutations in either SRD5A2 or AR genes.[17-21] Moreover, all patients presented with genital ambiguity as an isolated anomaly conforming with the notion that DSD in general are scarcely reported as a part of multiple congenital anomaly syndrome.[4,12]

General analysis of the patients, molecular data showed a number of findings:

First, there ware no major deletions or insertions in any of the patients. This conforms with the very low frequency of such genetic events worldwide (AR mutation database, http://www.mcgill.ca/androgendb/).

Second, the mutation rate in exons 2-8 of the AR gene was low among the studied groups (i.e. only 2/21, or 9.5%). Several explanations can be postulated in this regard. Firstly, Exon 1 was not analysed in the studied cases due to technical reasons but could harbor an underlying mutation. A second possibility entails a prevalence of 5αR2D among the patient sample. This disorder has been frequently reported among Egyptian DSD patients.[2,4,5,9,20,21] Thirdly, the criteria for selecting the cases focused on patients presented with ambiguous genitalia that manifested in infancy and childhood. That is in contrast with cases with complete testicular feminization who usually do not present in that age as they usually have a typical female phenotype (i.e. female external genitalia).[22] Moreover, at puberty subjects with complete AIS have excellent feminization resulting from high estrogen levels due to testicular estrogen secretion and peripheral aromatization of T. As a result, most of those cases are seeking clinical advice for causes of primary amenorrhea and delayed puberty at the time of puberty in gynecologic clinics.

Third, there was some discordance between the numbers of cases showing altered SSCP migrational patterns and those documented to have a mutation by sequencing analysis. Out of 147 analyses, 5 exons showed abnormal migration, two of them had a mutation. Thus, there were no false negatives (0/147, 0%), but 3/147, or 2% were false positive. This points out that SSCP may be a valid and sensitive screening tool for AR mutation detection but it still has some limitations regarding the assay specificity.

Among the patient sample, the 2 detected mutations comprised A596T and R840G. The A596T mutation was previously reported four times in cases with Reifenstein syndrome[23], somatic mosaicism[24], PAI[25] and ambiguous genitalia.[26] Although these patients were carrying the same mutation, they were presenting with different clinical phenotypes, suggesting variability in the expression of the same mutation in unrelated patients.

The mutation, A to T, on exon 3 replaced alanine to threonine at position 596 within the receptor DBD which makes up the D-loop of the receptor. The D-loop and sequences in the hormone binding domain together provide the interacting surfaces for receptor dimer formation and subsequent binding to DNA. It was demonstrated that the

destruction of dimerization of the AR is one of the causes of Reifenstein syndrome.[23]

The R840G mutation was characterized in another case in exon 7 of the AR-LBD. This mutation has been reported only once.[25] Three other mutations of the same residue (R840S; R840C, nonconservative; and R840H, conservative) have been reported in patients with PAIS. Each of these mutations was associated with a very diverse spectrum of phenotypes. These data highlight the role of the AR ligand-binding pocket (LBP) in the expression of transcriptional activity during prenatal sex differentiation. Four sites in the steroid-binding domain, arginine residues 774, 840, and 855, and valine 866, appear to have a particularly high frequency of mutation and together account for about one quarter of the missense mutations reported to date.[27]

The provisional diagnosis of AIS is usually supported by a normal T/DHT ratio. However, variable T/DHT values have also been observed among patients, depending on the patient's age and the HCG stimulation procedures.[28]

Moreover, 5αRD secondary to AR defects resulting in high T/DHT values have been reported and made diagnosis more difficult.[19] This has been illustrated in the current study where the R840G carrying patient had normal T/DHT ration [6.2], while the other having A596T showed a 50.7 T/DHT value. This reconfirms the notion that the molecular analysis remains the only definitive tool for diagnosis of AR defects, which were showed to be an uncommon finding among Egyptian Pediatric 46,XY DSD cases presenting with androgen endorgan unresponsiveness.

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