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Evaluation of Possible Effects of Exon-4 Integrity and Distribution of Androgen Receptor Gene in Idiopathic Iraqi Male Infertility Groups

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Abstract : Infertility is a major health problem which affects approximately 22% of married couples in reproductive age. Androgens (testosterone and dihydrotestosterone) from another side are essential for male fertility and the maintenance of spermatogenesis, and to determine the expression of male phenotype, and their actions are mediated by single androgen receptor (AR).

So any mutation that disrupts (AR) functions completely or partially results in androgen insensitivity syndrome with impaired spermatogenesis and even XY genotype.

In the present study, male patients with infertility divided as (non-obstructive azoospermia, oligo and oligoasthenozoospermia) were studied in order to investigate the molecular genetics and molecular analysis for androgen receptor gene alteration, as a reason of male infertility in Iraq.

For molecular study 100 patients (39 azoospermia, 16 Oligo and 45 oligoasthenozoospermia) were examined, and 30 normal men were subjected for detection of androgen receptor gene alteration using molecular analysis by polymerase chain reaction (PCR) for exons (4) of androgen receptor gene.

The results show deleted exon 4 as detection by PCR in the groups of infertile men but control group. The Androgen receptor (AR) gene deletion was considered in all infertile groups as compared with control group, and in exons (4) the highest percentage of deletion was registered in oligoasthenozoospermic patients 40% from wild exon in a highly significant differences ($P<0.01$).

The patients with deleted exon 4, appeared decreased semen volume, progressive motility in a highly significant differences ($P<0.01$), and decreased grade B, and increased in liquefaction time in a significant differences ($P<0.05$) as compared with semen parameters of patients with wild exon (4).

The results demonstrated the necessity of the exons (4) presence and integrity for the AR function and spermatogenesis process.

Key words : Infertility, Androgen receptor, oligoasthenozoospermia, exons (4), spermatogenesis.

Introduction

Infertility is a disease (an interruption, cessation, or disorder of body functions, systems, or organs) of the reproductive tract which prevents the conception of a child or the ability to carry a pregnancy to delivery[1.] Infertility is a relatively common health condition, affecting nearly (7-15) % of all couples. Clinically, it is a highly heterogeneous pathology with a complex etiology that includes environmental and genetic factors. It has been estimated that nearly 50 % of infertility cases are due to genetic defects[2,3].

The end-organ resistance to androgens, called androgen insensitivity syndrome (AIS), is a rare disorder[4].

The end-organ resistance to androgens has been designated as Androgen Insensitivity Syndrome (AIS), an X-linked disorder caused bymutations in the Androgen Receptor (AR) gene. It is generally accepted that defects in the AR gene prevent the normal development of both internal and external genital structures in 46,XY individuals, causing a variety of phenotypes ranging from male infertility to completely normal female external genitalia[5].

Androgen insensitivity syndrome (AIS) or testicular feminization is a partial or complete inability of cell response to androgen. The cause is enzymatic defect in synthesis of testosterone, resulting sexually immature phenotypically female, with primary amenorrhea. There are three categories of AIS, complete, partial and mild, depending on the degree of external genital masculinization[6]. According to the molecular analysis for different exons within androgen receptor gene, the most common cause of AIS is mutations in the androgen receptor (AR) gene that the Complete AIS in this individual enrolled in the study was due to a G708E substitution in the AR protein result from a 2650G>A mutation (mRNA sequence reference) in exon (4) of the gene, resulting in replacement of glycine with glutamate at codon 708 in the ligand-binding domain of the AR protein[4].

These men have normal male external genitalia and a male gender orientation[7]. They usually present with gynecomastia at puberty. Spermatogenesis may or may not be impaired. In some instances the only observed abnormality appears to be male infertility; therefore,

MAIS could explain some idiopathic male infertility[8].Male infertility is a common cause of reproductive failure in humans[9].

Disturbances in the function of the androgen receptor can lead to several forms of male pseudohermaphroditism, such as androgen insensitivity syndrome, which can lead to infertility. Infertility affects around 20% of couples, and in half of the cases it is a male problem[10].

The gene has a length of about 90kb and includes 8 exons. The exons are separated by 7 introns. This gene codifies the AR receptor protein that is composed by 919 amino acids with a molecular weight of 110-112 Kd. The protein is characterized by four functional regions figure (1),11.

The hinge region encoded by(exon 4); as well as Ligand Binding Domain (LBD): region that represents the link region fo the steroid (exon 4-8),[11,12] as shown in figure (2).

Figure (1): Structural Organization of Nuclear ReceptorsTop – Schematic 1D amino acid sequence of a nuclear receptor. Bottom – 3D structures of the DBD (bound to DNA) and LBD (bound to hormone) regions of the nuclear receptor. The structures shown are of the estrogen receptor. Experimental structures of N-terminal domain (A/B), hinge region (D), and C-terminal domain (F) have not been determined therefore are represented by red, purple, and orange dashed lines, respectively.

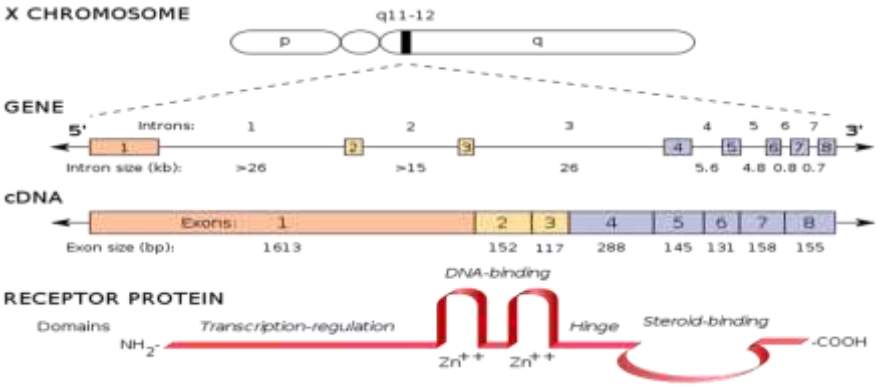

Figure (2): Location and structure of the human androgen receptor. Top, The AR gene is located on the proximal long arm of the X chromosome. Middle, The eight exons are separated by introns of various lengths. Bottom, Illustration of the AR protein, with primary functional domains labeled (not representative of actual 3-D structure).

The AR COOH-terminal domain (CTD), encoded by exon 4-8, harbors the AR ligand-binding domain (LBD) and transcriptional activation function 2 (AF2) co-regulator binding interface[13].

However, the somatic Sertoli, PTM, Leydig, vascular endothelial and vascular smooth muscle cells of the mature testis express androgen receptor[14], and it is widely accepted that the requirement of testosterone for spermatogenesis is mediated by these cell types.

2. Material and Method:

Genomic DNA was isolated from blood cells under aseptic condition according to the protocol described by Geneaid Biotechnology Companyfor wizard genomic DNA purification kit.

The sense primer of exon 4 was (5'ACA CTA CAC CTG GCT CAA TGG 3') and the antisense was (5' CGG AAG CTG AAG AAA CTT GG 3').

The components of PCR deionized water (9 µl), Green Master mix (12.5 µl), F-Primer (1µl), R-Primer (1µl) and DNA sample (1.5µl) at total volume 25µl. The optimization of amplification was performed under the following conditions **Tables (1).**

Table (1): Optimization of PCR conditions for exon (4) of Androgen receptor *gene*.

Name of cycle	Temperature °C	Time	Noof cycle
Initial danaturation	95	3 min	1 cycle
Denaturation	95	1 min	35 cycle
Annealing	55.3	1 min	35 cycle
Extension	72	1 min	35 cycle
Final extension	72	5 min	1 cycle
Soak	4	-	1 cycle

The solutions were prepared to transferred DNA by dissolved agarose 1 mg per 100 ml of T.B.E, while to transferred PCR products the solution performed by dissolved agarose 1.5 per 100 of T.B.E buffer[10]. Electrophoresis was then run for 10 min at 30v and 70v for 1hr, andwhen the electrophoresis was completed the gel was placed on a UV transilluminator[15].And the DNA ladder marker (50 - 1000 bp) used for correlation from Promega company USA.

3. Results and Discussion:

The results embedded in table (2) expressed the presence or absence (wild type or deleted) respectively of exon (4) in the PCR test figures (3), for different groups of infertility and control. The different groups of infertility showed variant percentage of wild type or deleted exon (4), and registered highly significant differences ($P<0.01$) between wild type exon and deleted exon (4) in all infertility groups, while all the men in control group indicate presence of exon (4).

On the other hand, as compared between infertility groups the highest percentage of exon (4) deletion was registered in the patients of Oligoasthenozoospermia group, and the lowest percentage of exon (4) deletion was in Oligozoospermia group.

Table (2): Distribution of Exon (4) in different groups of infertility and control group.

The groups	PCR/ exon 4	No.	Percentage (%)	Chi-square
Control	Wild type	30	100.00	14.75 **
	deleted	0	0.00	
Azoospermia	Wild type	36	92.31	14.272 **
	deleted	3	7.69	
OligoZoospermia	Wild type	15	93.75	14.108 **
	deleted	1	6.25	
OligoAstheno-zoospermia	Wild type	35	77.78	12.967 **
	deleted	10	22.22	
Total	Wild type	116	89.23	
	deleted	14	10.76	

** ($P<0.01$) means highly significant differences in each group.

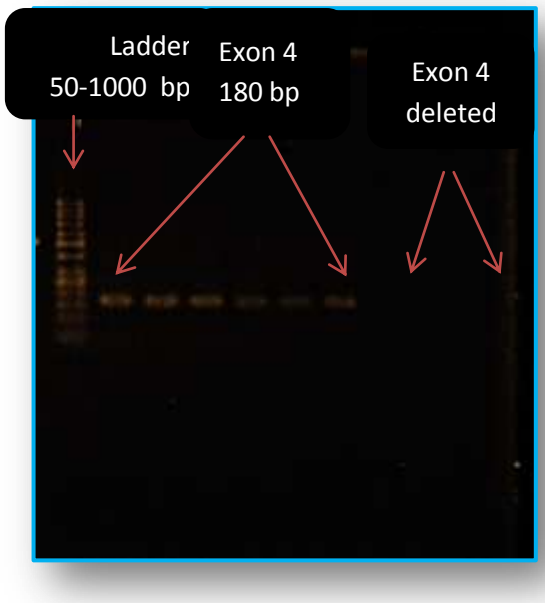

Figure (3):show the presence (wild type) of exon(4) bands on the left and right of the ladder and some bands lacked.

The results were embedded in table (3) showed the effect of the wild type or deletion of exon (4) on the hormonal values and semen parameters enrolled in the present study.

When compared between the patients group with presence (wild type) of exon (4) and those with absence (deletion) of exon (4), the results showed there were significant differences ($P<0.05$) in the semen volume which decreased and liquefaction time was increased in patients group with absence of exon (4) as compared with patients group have exon (4).

In addition the results were registered highly significant differences ($P<0.01$) in the progressive motility and grade (B) of sperm, they were decreased in the patients group with absence of exon (4) as compared with patients group have exon (4). In contrast, no significant differences registered in hormonal values and other semen parameters for both groups.

Table (3): shown the effect of the wild type or deletion of exon (4) on the hormonal values and semen parameters.

Parameters	Mean ± SE		T-test
	Wild type /exon 4	deleted/ exon 4	
Testosterone(ng/ml)	4.03 ± 0.15	4.96 ± 0.74	0.998 NS
FSH(mlU /ml)	5.87 ± 0.32	6.17 ± 1.04	1.881 NS
LH(mlU /ml)	3.91 ± 0.16	4.02 ± 0.45	0.926 NS
TSH(mlU /ml)	1.49 ± 0.06	1.528 ± 0.21	0.342 NS
T3(nmol /L)	115.35 ± 2.96	118.69 ± 7.79	16.845 BNS
T4(nmol /L)	84.60 ± 1.82	88.98 ± 5.06	10.431 NS
Semen volume (ml)	2.21 ± 0.08 **a**	1.71 ± 0.13 **b**	0.481 *
Liquefaction time	37.32 ± 0.98 **b**	43.21 ± 2.37 **a**	5.508 *
Sperm count	13.95 ± 2.75	5.71 ± 1.15	14.645 NS
Sperm morphology	30.11 ± 2.31	21.27 ± 3.56	11.346 NS
Sperm motility	35.75 ± 3.37	22.81 ± 3.46	16.035 NS
Progressive motility	16.65 ± 2.87 **a**	1.18 ± 1.02 **b**	13.271 **
SP. A %	2.69 ± 0.72	0.00 ± 0.00	3.471 NS
SP. B %	13.95 ± 2.30 **a**	1.18 ± 1.02 **b**	10.651 **

SP. C %	19.15 ± 1.64	21.63 ± 2.63	8.331 NS
SP. D %	63.86 ± 3.47	77.18 ± 3.46	16.663 NS
* (P<0.05), ** (P<0.01), NS: Non-significant.			

*(P<0.05) means significant differences with different letters in rows.
** (P<0.01) means highly significant differences with different letters in rows.

Letter **(a)** refers to increased value, and letter **(b)** refers to decreased value.

The androgen receptor (AR), encoded by AR gene, is a steroid receptor transcription and regulator factor that mediates the cellular activities of testosterone (T) and dihydrotestosterone (DHT)[16]. The AR-mediated androgen function plays important roles in the development and maintenance of male and female phenotype and reproduction activity[17,18].

Given the complicated structure and the necessary function of each AR domain represented by (8) exons, it is not surprised that AR alternative splicing would impairs the AR cellular signal and result in pathologic conditions. Several alternative spliced AR isoforms have been considered in different pathologic conditions such as prostate cancer, Kennedy disease, androgen insensitivity syndrome (AIS) and so on[19,20].The AIS is represented by a variety of phenotypes ranging from male infertility to completely normal female external genitalia with 46XY for both condition[21].

The alternative splicing of androgen receptor may be a critical pathogenic mechanism in human infertility[22].

The exon (4) responsible for coding the short flexible hinge region, which regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor[23].

In addition to the AR COOH-terminal domain (CTD), encoded by exon 4-8, contains the AR ligand-binding domain (LBD) and transcriptional activation function 2 (AF2) as co-regulator binding interface[24,13].The first zinc finger in the AR DBD determines the specificity of DNA recognition, which makes contact with major groove residues in an androgen-response element (ARE) half-site[25,26]. The second zinc finger is a dimerization interface that mediates binding with a neighboring AR molecule engaged with an adjacent ARE half-site. The short flexible hinge region, encoded by exon 4, regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor[24]. However, this region was shown to be involved in DNA binding as well as AR dimerization. It was suggested that the hinge region also acts to attenuate transcriptional activity of the AR gene.

The AR is not able to migrate into the nucleus as well as to link to the target genes DNA, and the interaction with the androgen leads to the dissociation between AR and heat shock protein (hsp), the further phosphorylation and the migration into the cellular nucleus, where the receptor undergoes a process of homodimerization. This process is characterized by the each other linkage of the complex hormone-receptors, allowing the recognition and the link to the responsive element present on the target gene and generating the synthesis of new specific proteins[27].

The androgen insensitivity syndrome (AIS), because of an inactivating mutation of the AR, the testes may exhibit some degree of dysgenesis, and are at elevated risk of developing cancer in testicular germ cell[28]. The ablated AR by Endocrine disruption pathways in male sexual differentiation, and transgenesis, both show 30–50% decreasing in Sertoli cell number at around the time of birth and a more severe decreasing (60–75%) in adulthood[29.]

Another study in newborn rats has shown that interference with androgen action neonatally, via administration of the AR antagonist flutamide, also decrease Sertoli cell number[30]. These results appear that androgens exert a proliferative action on Sertoli cells during the perinatal period, and the Sertoli cells do not express AR for all or most of this period[31].

Regardless of the mechanism of such influence, recognition the deficiency in androgen production or action in foetal or early postnatal life is the important point, likely to result in a testis with fewer Sertoli cells. As Sertoli cell count per testis determines how many germ cells can be supported through maturation into

spermatozoa[32], it can be appreciated idea that effects on sperm counts and/or fertility in an impaired individual are then a possibility.

This is particularly the case in humans, as men do not store sperm (as do many domestic animals), in the same context that Sertoli cell number and abstinence period (frequency of ejaculation) reflects the sperm counts are produced. Another important reason in this context is that spermatogenesis in the human is less efficient when compared with that in most mammals[33].

The leakage of these free radicals makes mitochondria a major intracellular source of reactive oxygen species (ROS). These unique features are probably the cause of faster accumulation of sequence variations in mitochondrial DNA than in nuclear DNA[34].The PCR amplification of mtDNA has shown a higher incidence of mtDNA deletion in asthenozoospermic patients as compared with unaffected individuals[35,36].

This excessive ROS production may induce the opening of membrane permeability transition pores and release of free radicals, cytochrome C and other apoptogenic factors that ultimately lead to apoptosis. Although mtDNA mutations have been identified in many studies, their role as a diagnostic marker in male infertility is still under debate. Nonetheless, male infertility due to mtDNA mutation can be successfully treated by ICSI, as mtDNA mutations are not transmitted to the offspring[37,38].

The results demonstrated the necessity of the exons (4) presence and integrity of the AR function and spermatogenesis process. So it is necessary to put the detection presence and integrity of exon (4), as well as chromosomes aberrations in the infertility prediction and diagnosis for infertility in men.

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