Molecular Identification of Azoospermia in Iraqi Patients Based on (NR5A1) Gene Sequencing

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Abstract

Background:

Infertility is considered one of the main public health issues, as it affects about 15% of the couples of reproductive age. The male factor is involved in 40% - 50% of infertility cases. It is difficult to assess accurately the overall magnitude of the contribution of genetics to infertility as most, if not all, conditions are likely to have a genetic component. The genetic causes of infertility are varied and include chromosomal abnormalities, single gene disorders and phenotypes with multifactorial inheritance. Some genetic factors influence males specifically, whereas others affect both males and females. Frequently, however, in the clinic no clear cause for the observed infertility could be diagnosed which at least in part, it reflects our still poor understanding of the basic mechanisms that regulating and controlling the genetic networks of male infertility.

Objective:

To study the molecular identification of Azoospermic infertile patients by the gene sequencing of NR5A1.

Subjects, Materials and Methods:

A study carried out during the period from November 2014 to June 2015. Fifty specimens were collected from thirty azoospermic patients and twenty normal healthy subjects (normozoospermic subjects) as control their age ranged between 23 and 48 years old, the seminal fluid of cases indicated that liquefaction time, color and viscosity equal in normozoospermic subjects (control group) and patient group while there is significant difference $P \geq 0.05$ in sperm count reach to more than 20 million and motility reach to more than > 25% in control subjects while there is no sperm count found in azoospermic patients. Then blood was collected for hormonal assay, in azoospermic patients the results revealed a gradual decrease in serum testosterone levels with a concomitant increase in serum follicular stimulating hormone (FSH) level when compared with control subjects . NR5A1 gene were investigated in 30 samples of extracted DNA from azoospermic patients by using polymerase chain reaction (PCR) method it can directly detect the NR5A1 gene content after it had been molecularly identified then were sent

successfully for sequencing analysis ,this study examined the presence of two transversion and one transition mutation of NR5A1 gene in azoospermic patients compared with control subjects, there's no mutation in the same gene when compared with gene bank.

Key words: **Azoospermia; Molecular identification, PCR, Transversion and Transition**

Introduction:

About 10–15% of couples have problems conceiving and in half of cases a male co factor can be found upon clinical workup. Male infertility is mostly caused by spermatogenetic failure, clinically noted as oligo or azoospermia [1,2] .In recent years there has been increasing concern about a possible decline in reproductive health, and this trend is paralleled by an increasing demand for infertility treatments. In the majority of cases, the underlying cause of male infertility is unknown. Familial clustering of male subfertility as well as families with multiple infertile or subfertile men, in whom an autosomal-recessive or dominant mutation with sex-limited expression is likely to be present, indicates a genetic contribution to spermatogenic failure [3,4] .A chromosomal anomaly is carried by 5% of all infertile men (such as 47,XXY Klinefelter syndrome), and microdeletions of the long arm of the Y chromosome (MIM 415000) are present in 10% of azoospermic or severely oligozoospermic ($< 1 \times 10^6$ sperm/ml) men [5] Although rodent studies indicate that multiple genes have the potential to cause male infertility, only a few single-gene defects that cause male infertility have been identified in humans. These include *AURKC* (MIM 603495) mutations associated with large-headed, multiflagellar polyploid spermatozoa (MIM 243060), *SPATA16* (MIM 609856) mutations associated with globozoospermia (MIM 102530), *CATSPER1* (MIM 606389) mutations associated with recessive male infertility (MIM 612997), and mutations of the dynein genes that encode proteins of the axonemal dynein cluster (*DNAH1* [MIM 603332], *DNAH5* [MIM 603335], *DNAH11* [MIM 603339]) and are associated with asthenozoospermia [6] However, the collective prevalence of these mutations is extremely low. Steroidogenic factor-1 (SF-1, NR5A1, Ad4BP) Nuclear receptor subfamily 5, group A, member 1 was originally identified as a master-regulator of steroidogenic enzymes in the early 1990s following the seminal work of

[7,8]. It is located on chromosome 9q33 region between 127,243,514 base pair to 127,269,768 base pair. It is a 33182 bp long gene having 7 exons [9] It gives instruction for production of transcription factor, Steridogenic factor- 1 (SF1). It is a protein which binds to a particular region of DNA to control the activities of specific genes that are related to gonad development and adrenal glands (located in the upper portion of each kidney). The current study designed to investigate the molecular identification of azoospermia based on NR5A1 gene sequencing also biological and hormonal changes in the same patients.

Subjects, Materials and Methods:

Fifty specimens were collected from 30 azoospermic patients and 20 normal healthy subjects their age ranged between 23 and 48 years old .The study was conducted in the centre for infertility treatment and IVF in Kamal Al-Samarrai hospital ,the attending patients were suspected to have azoospermia as clinically identified by a physician; in seminal fluid analysis from the period from November 2014 to June 2015. All semen samples were collected to study different characteristics like liquefaction time, color, viscosity, sperm count and motility. The blood samples were drawn and serum was collected by centrifugation for the measurement of follicular stimulating hormone (FSH) levels and testosterone in serum by ELISA kits (Beckman-France), as presented in standard Assay [10]. The results were analyzed using one-way analysis of variance (ANOVA), the statistical level was set at $P<0.05$[11] .DNA was prepared and purified according to the genomic isolation kit provided by Geneaid Company/Taiwan (Cultured Cell Protocol Procedure Ver.11.21.13). The Nanodrop system (BioDrop/UK) was used for the measurement of the concentration and purity of the DNA according to Sambrook and Russel, 2001, using 2 µl of each DNA sample. The specific primers for NR5A1 gene and their sequences were chosen according to Kanbe *et al.*, [12], forward 5-GGCCCTGAAACAGCAGAAGA-3; reverse 5-CCAGAGAAGGGCTCTGGGTA-3. The polymerase chain reaction (PCR) was performed in 25 ml; each reaction mixture was heated to 95ºC for 10min. A total of 35 PCR cycles, each cycle at 0.3min at 94ºC for denaturation, 0.45 min at 55ºC for annealing, 1.15 min at 72ºC for

extension and a 10min final extension at 72ºC. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in (1x) TBE buffer (50 mM Tris acetate, 1 mM EDTA, pH 8.0) stained in 0.5 mg/ml Red stain, NR5A1cover the region from 240 to 1338 of regulatory gene with product size of 348 base pairs (bp) have been patented. The product PCR bands were visualized under UV light and photographed after staining the agarose gels with Red safe stain (10 mg/ml). Positive PCR product samples were sent for sequence analysis; 25 µl (10 pmol) from the forward primer. The samples were treated with AB13730XL Applied Biosystems machine in national instrumentation centre for environmental management NICM/USA company online at (http://nicem.snu.ac.kr/main/?en_skin=index.html). The result of the sequence analysis was analysed by blast in the National Centre Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and Bio Edit program [13].

Results and Discussion:

To study the cause of human male infertility "Azoospermia", disorder of sperm. The analysis of seminal fluid subjected and appeared that the significant differences between the normal healthy group and azoospermic patients during the period of study as appeared in table (1), the volume of seminal fluid in azoospermic patients was about 1.2 ml while in control group was more than 2ml, also the equal in liquefaction time, color and viscosity while the significant difference in sperm count which were about more than 20 million in normal healthy group while there is nil in

azoospermic patients, same findings were observed when study the motility parameters like grades A and B which were nil also in azoospermic patients , while its more than 25% in normal healthy subjects.

Table 1. Seminal fluid analysis during the period of the study.

Seminal fluid analysis		
	Azoospermic	**Control**
Vol.	**1.2 ml**	**>2ml**
liquefaction time	**30 min.**	**30min.**
color	**white**	**white**
viscosity	**normal**	**normal**
sperm count	**Nil**	**>20 million**
motility		
Grade A(Linear Progressive)	**0%**	**> 25%**
Grade B(Linear non progressive or Non linear progressive)	**0%**	**> 25%**

Azoospermia means that the men who have no sperm found in their semen, it is a cause of human male infertility, also it's a disorder of sperm motility, and is implicated in 19% of infertile cases which found in 24% of the infertile men [5]. Hormonal changes can play important roles in the azoospermic patients . Table (2) Represented and summarized the hormonal profile of azoospermic patients compared with normal healthy subjects in the study ,the mean levels of serum FSH mIU/ml in normal healthy subjects was 6.52mIU/ml while in azoospermic patients was 16.0 mIU/ml which ranged between 6.9-21.4.The results showed a general trend for FSH levels to increase in azoospermic patients group, this increase reached statistical significance ($P<0.05$) where the mean values of serum testosterone level in control group was 9.2 while in azoospermic patients was 5.61 its ranged between 1.4 to 12.3 so the results appeared that testosterone were decreased in azoospermic patients in comparison with the normal healthy subjects(control).

Table (2): Average of Serum follicular stimulating hormone (FSH) and testosterone in patients with azoospermia compared with normal healthy subjects.

NO.	FSH, mIU/ml	Testosterone mIU/ml
Mean Values	Azoopsermic Patients *16.0±0.07	*5.61±0.05
	Control	
Mean Values	*6.5±0.02	*9.2±0.01

*P<0.05 Significant

The profile of FSH in current study may be caused by regulation of SF-1gene activity which is generally regulated by Adrenocorticotropin (ACTH) and gonadotropin (LH and FSH) and intracellular cAMP/PKA signal pathway which is a major signaling pathway that transmits the signal from extracellular stimuli to the nucleus [14]. Also the present result in hormonal changes may be related to the individuals might have external genitalia which cannot evaluate the difference in an individual's genitalia or abnormality in secondary reproductive organs and adrenal glands. Adrenal gland abnormality may rise due to deficiency of hormones which further might result in various health problems. The phenotype in genetic females would be expected to consist of adrenal failure, delayed puberty with absence of breast development, and primary amenorrhea, with raised gonadotropins [15]. The genomic DNA was extracted efficiently from blood patients using a genomic DNA extraction kit to yield intact DNA with a good quality and high purity for use in PCR techniques. The concentration of the extracted DNA ranged between 903-2202 ng/µl, with a purity of 1.6-2 was obtained. According to Saiki [16] *NR5A1*gene was successfully amplified using specific PCR primer amplification of *NR5A1gene* of different patients was performed in the present study to confirm the disorder in the patients included in the study compared with control. The DNA extracts was subjected to PCR analysis to confirm the possible presence of azoospermia gene. As expected DNA from all

patients produced clean bands upon amplification with *NR5A1* set of specific primer. **Figure (1) appeared that molecular weight of *NR5A1* gene was 348 bp in the patients and control groups.**

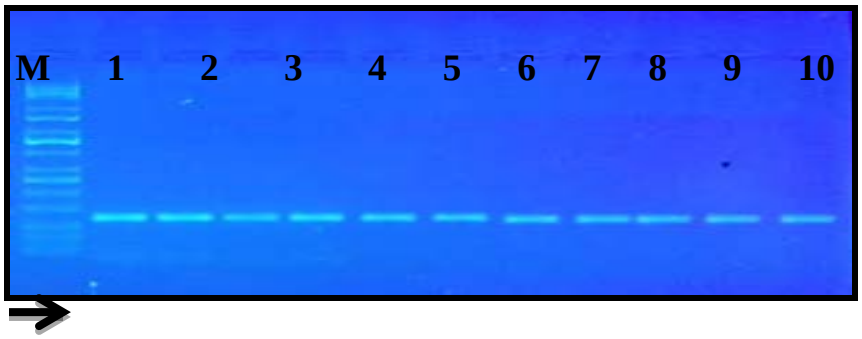

Figure 1. Electrophoresis of amplified PCR products for NR5A1 gene . M indicates the lane containing the 100 bp molecular size DNA marker. Lanes 1, 2, 3, 4, 5, 6 ,7,8,9,10 with molecular weight 348 bp.

Sequencing of this gene was performed to detect mutation related to development of disease. Sequences alignment using BLAST and BioEdit showed that the 100% similarity of healthy sample, score 516 and expect 2e-142 with *NR5A*1 gene of *Homo sapiens* from the Gene Bank under Sequence ID: ref|NG_008176.1|, (Figure 2). The NR5A1 gene from 20 azoospermic patients showed 99% compatibility, score 422 and expect 4e-114 with NR5A1 gene from Gene Bank as shown in figure (3). There is transition mutations (missense) in the 20 cases that caused a change Alanine (A) to tryptophan (T) at 11889 and incidence two transversions, resulted in a change of amino acid (Arginine (R) to Methionine (M)) was location of gene bank in 11911, and in 11964 resulted in a change of amino acid Glutamic acid (Q) to histidin (H), there was a highly significant association with azoospermia as shown in table (3) and figure (4), the results were compared with data obtained from Gene Bank published ExPASY program which is available at the NCBI online at www.ncbi.nlm.nih.gov.

Homo sapiens nuclear receptor subfamily 5 group A member 1 (NR5A1), RefSeqGene on chromosome 9
Sequence ID: ref|NG_008176.1|

Score	Expect	Identities	Gaps	Strand
516 bits(279)	2e-142	279/279(100%)	0/279(0%)	Plus/Plus

```
Query  1      GGGGTGCCCCCGCCGCCCCCTCCCGCACCGGACTACGTGCTGCCTCCCAGCCTGCATGGG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11840  GGGGTGCCCCCGCCGCCCCCTCCCGCACCGGACTACGTGCTGCCTCCCAGCCTGCATGGG  11899

Query  61     CCTGAGCCCAAGGGCCTGGCCGCCGGTCCACCTGCTGGGCCACTGGGCGACTTTGGGGCC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11900  CCTGAGCCCAAGGGCCTGGCCGCCGGTCCACCTGCTGGGCCACTGGGCGACTTTGGGGCC  11959

Query  121    CCAGCACTGCCCATGGCCGTGCCCGGTGCCCACGGGCCACTGGCTGGCTACCTCTACCCT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11960  CCAGCACTGCCCATGGCCGTGCCCGGTGCCCACGGGCCACTGGCTGGCTACCTCTACCCT  12019
```

Figure (2): Sequencing of sense flanking the partial NR5A1 gene in healthy*: Homo sapiens* compared with standard NR5A1 obtained from Gene Bank. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

Homo sapiens nuclear receptor subfamily 5 group A member 1 (NR5A1), RefSeqGene on chromosome 9

Sequence ID: ref|NG_008176.1|

Score	Expect	Identities	Gaps	Strand
422 bits(228)	4e-114	234/237(99%)	0/237(0%)	Plus/Plus

```
Query  1      CCTCCCAACCTGCATGGGCCTGAGCCCAATGGCCTGGCCGCCGGTCCACCTGCTGGGCCA  60
              ||||||| ||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  11882  CCTCCCAGCCTGCATGGGCCTGAGCCCAAGGGCCTGGCCGCCGGTCCACCTGCTGGGCCA  11941

Query  61     CTGGGCGACTTTGGGGCCCCATCACTGCCCATGGCCGTGCCCGGTGCCCACGGGCCACTG  120
              ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  11942  CTGGGCGACTTTGGGGCCCCAGCACTGCCCATGGCCGTGCCCGGTGCCCACGGGCCACTG  12001

Query  121    GCTGGCTACCTCTACCCTGCCTTTCCTGGCCGTGCCATCAAGTCTGAGTACCCGGAGCCT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12002  GCTGGCTACCTCTACCCTGCCTTTCCTGGCCGTGCCATCAAGTCTGAGTACCCGGAGCCT  12061

Query  181    TATGCCAGCCCCCCACAGCCTGGGCTGCCGTACGGCTACCCAGAGCCCTTCTCTGGA  237
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12062  TATGCCAGCCCCCCACAGCCTGGGCTGCCGTACGGCTACCCAGAGCCCTTCTCTGGA  12118
```

Figure (3): Sequencing of sense flanking the partial NR5A1 gene in patient*: Homo sapiens* compared with standard NR5A1 obtained from Gene Bank.

Table (3): Types of mutations detected in partial NR5A1 gene of azoospermic patients.

Type of mutation	Predicted effect	Amino acid change	Nucleotide change	Location of gene bank	Sequence ID
Transition	**Missense**	**Alanine (A) / tryptophan (T)**	**AGC>AAC**	**11889**	emb|CCQ43292.1|
Transversion	**Missense**	**Arginine (R) / methionine (M)**	**AAG>AAT**	**11911**	emb|CCQ43292.1|
Transversion	**Missense**	**Glutamic acid (Q) / histidin (H)**	**CAG>CAT**	**11964**	emb|CCQ43292.1|

Score Expect Method Identities Positives Gaps Frame

145 bits(365) 8e-42 Compositional matrix adjust. 75/79(95%) 76/79(96%) 0/79(0%) +2

Query 2 LPTCMGLSPMAWPPVHLLGHWATLGPHHCPWPCPVPTGHWLATSTLPFLAVPSSLSTRSL 181

Sbjct 26 LPACMGLSPRAWPPVHLLGHWATLGPQHCPWPCPVPTGHWLATSTLPFLAVPSSLSTRSL 85

Query 182 MPAPHSLGCRTATQSPSL 237

Sbjct 86 MPAPHSLGCRTATQSPSL 103

Figure (4): Amino acid sequence of the translated partial NR5A1gene of patients. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

The gene responsible for the expressed in the developing urogenital ridge, steroidogenic tissues was found in all studied case of patients *Homo sapiens.* Sequencing of amplified product for 20 samples of azoospermic patients were done to detect the presence of mutation within these sequences related to NR5A1 gene sequence [17]. NR5A1 regulates the müllerian inhibitory substance (MIS) by binding to a conserved upstream regulatory element and directly participates in the process of mammalian sex determination through müllerian duct regression [18]. Targeted disruption of NR5A1 (Ftzf1) in mice results in gonadal and adrenal agenesis, persistence of Meullerian structures and abnormalities of the hypothalamus and pituitary gonadotropes [19]. NR5A1 gene is expressed in the developing urogenital ridge, steroidogenic tissues

such as gonads, adrenals, and placenta, hypothalamus and anterior pituitary [20,21]. In general, it activates the expression of AMH in Sertoli cells leading to the regression of Müllerian structures in Leydig cells, it activates the expression of several enzymes involved in steroidogenesis, resulting in the virilization of external genitalia and testicular descent [18] and, in ovaries, NR5A1 is expressed in the granulosa and theca cells where it regulates genes required for ovarian steroidogenesis and follicle growth maturation [21], the nuclear receptor superfamily is involved in the functions associated with the steroidogenic tissues that are adrenal function and gonadal development, sex determination and differentiation and regulates the expression of steroidogenic P-450 enzymes [9]. The essential role of NR5A1 (SF-1) as a master steroidogenic gene was described by its forced expression in embryonic and mesenchymal stem cells experiments which gave the results that it was sufficient to activate steroidogenic genes and to initiate steroid expression [14]. Mutations associated with NR5A1gene are generally missense mutations, nonsense mutations caused by nucleotide deletions and duplications, one nucleotide polymorphism and a 3 Mb deletion spanning NR5A1. Studies on NR5A1 gene and ambiguous genitalia have reported that a heterozygous frame shift mutation results in ambiguous genitalia [3]. Heterozygote mutations of NR5A1 are associated with disorders of sex development, premature ovarian failure or male infertility [22]. Mutation may lead to a syndrome along with gonadal dysgenesis and lead to Sawyer syndrome. In this disorder, complete or pure gonadal dysgenesis patients are found. The formation of male sexual differentiation gets affected in such cases, which leads to the development of female appearance despite having the chromosome pattern typical of males. Other disorder which may be due to a mutation in this gene is partial gonadal dysgenesis [6,8,21,23].

Conclusion:

The study showed that there was significant difference between azoospermic patients and normal healthy subjects due to mutations at the *NR5A1 gene,* therefore the molecular method improved that it is more precise and less consuming time

in study of Azoospermia and this result may help in control and develop treatment for these cases.

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