

A Molecular Study of Azoospermia & Sever Oligospermia in a Sample of Infertile Males Al-Anbar Governorate

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Received on: 10/10/2011& Accepted on: 5/4/2012

ABSTRACT

Infertility is one of the major public health problems in the Arab world, as it affects about 15% of couples seeking children. in 50% of these cases, the male partner is responsible for infertility . Y microdeletions in the azoospermia factor (AZF) region are known to be associated with spermatogenetic failure.

In the current study, the aim was to determine the molecular background of male infertility using tag sequence tagged site (STS) associated with Azoospermia cases . We evaluated the incidence of Y chromosome microdeletions in (100) Iraqi infertile male patients with primary idiopathic male infertility and 50 fertile males (controls) by polymerase chain reaction using 11 STSs in the azoospermia factor (AZF) region.

Of the 100 infertile men, Y-chromosome Microdeletion in the AZFa , AZFb and AZFc regions were detected in (64%)cases. The pathological significance of these deletions is not clear yet or most probably belongs to environmental pollution by depleted uranium used by collision forces in Al-Anbar governorate .

Keywords; Idiopathic male infertility; Azoospermia; Y-chromosome microdeletions

دراسة جزيئية لحالتي اللانطفية وقلّة النطف في عينة من الرجال العقيمين في محافظة الانبار

الخلاصة

يعد العقم احد المشاكل الصحية الرئيسية في المجتمع العربي ، إذ يؤثر في 15% من الأزواج الراغبون في إنجاب الأطفال ، وفي 50% من هذه الحالات يعد الرجل مسؤولاً عن العقم . وتشترك المنطقة الجينية المعروفة باسم منطقة عامل اللانطفية (AZF) region في فشل نشوء النطف في كثير من الحالات . ان هدف الدراسة هو تحديد الأسباب الوراثية الجزيئية لعقم الرجال باستخدام بادئات متخصصة (STS) sequence tagged site لحالة اللانطفية ، شملت عينة الدراسة (100) رجل يعاني من عقم غير معروف الأسباب (غير مفسر) و(50) رجل لديه ذرية كعينة مقارنة .

تشير الدراسة الى أن 64 % من الرجال يعانون من حدوث حذف دقيق لمنطقة عامل اللانطفية في مناطق AZFa , AZFb , وAZFc ولأسباب غير واضحة ربما تعود الى التلوث البيئي بمخلفات اليورانيوم المستنقذ المستخدم في أسلحة القوات متعددة الجنسيات في محافظة الانبار .

الكلمات المرشدة : عقم غير مفسر ؛ اللانطفية ؛ حذف كروموسوم Y

INTRODUCTION

Infertility is a major public health problem. It approximately occurs in 15–20% of couples and male factor infertility represent <50% of the cases. men with Azoospermia and Oligospermia represents ~40-50% of all infertile men. However, in ~30% of cases the underlying genetic basis of male infertility remains largely unknown. No causal factor is found in 60-75% of cases (idiopathic male infertility). These men present with no previous history associated with fertility problems and have normal findings on physical examination and endocrine laboratory testing. Semen analysis reveals a decreased number of spermatozoa (oligozoospermia), decreased motility (asthenozoospermia) , and many abnormal forms on morphological examination (teratozoospermia). These abnormalities usually occur together and are described as the oligoastheno-teratozoospermia (OAT) syndrome. Unexplained forms of male infertility may be caused by several factors. such as chronic stress, endocrine disruption due to environmental pollution, reactive oxygen species and genetic abnormalities. [1,2]

The regulatory processes of gene expression in spermatogenesis have unique requirements in terms of chromatin organization. The azoospermia factor locus (AZF) is assumed to contain the genes responsible for spermatogenesis. Three major deletion intervals have been defined and termed AZFa, AZFb and AZFc. Especially the AZFc genes have recently been identified in infertile and fertile populations, Azoospermia and Oligospermia are major causes of male infertility. Some genes located on the Y chromosome are suggested as candidates for this. Point and/or major deletions of the Y chromosome are significant causes of spermatogenic failure. Such mutations in these genes are thought to be pathologically involved in some cases of male infertility associated with Azoospermia or oligozoospermia. [3,4].

A genetically functional Azoospermia factor (AZF) on the long arm of the human Y chromosome is an important part and we did not assume that there is a spermatogenic function of the highly polymorphic heterochromatin domain in Yq12, they postulated that this AZF locus should be located in the distal part of the euchromatic Y long arm, i.e., in Yq11.23 [5]. There was no idea about the genetic content of this AZF locus. Protein encoding AZF genes, functionally expressed in human testis tissue, were not expected due to the extremely variable length of the Y chromosome in fertile men [6].

There are increasing evidences for the presence of gene(s) located on the long arm of the Y-chromosome that are likely to be involved in the complex process of spermatogenesis [7]. Microdeletions of the long-arm of the human Y-chromosome which involves three regions termed Azoospermia factor (AZF), are associated with either reduced sperm count (oligozoospermia) or complete absence of spermatozoa [8].

Today we know that there are at least fourteen protein encoding Y genes which are part of the AZF locus [2,9] and that the premeiotic pairing process of the sex chromosomes along the AZF chromatin is essential for a proper meiosis of the male germ cells. Moreover, with knowledge of the euchromatic Y sequence it is now also evident that the two faces of this chromosome, namely, being polymorphic on one site, being functional for spermatogenesis at the other site, are structurally intermingled, i.e., functional AZF genes are structurally linked to the Y specific highly polymorphic DNA regions [10,11].

Y chromosomes have developed during human evolution and are distinguished now by a rooted pedigree of 153 Y chromosomal haplogroups (The Y Chromosome Consortium (YCC), [12]. Probably these Y structures were selected to drive the male reproductive fitness of the different human populations [13,14]. Molecular reasons of the dynamic Y sequence structure are mainly non-allelic homologous recombination (NAHR) of the locus-specific repetitive sequence block in distal Yp and Yq11 most of which are organised in large palindromes [9]. Here, we report the first comprehensive clinical, molecular studies of 100 Unexplained forms of infertile men with spermatogenetic failure, from Al-Anbar region.

MATERIALS AND METHODS

Study Population

A total of 100 infertile Iraqi males residing in Anbar governorate with non-obstructive sperm impairments were evaluated. These patients were confirmed to have non-obstructive Azoospermia or oligozoospermia by direct bed examination. These patients had Azoospermia (sperm count 0 M/ml), severe oligozoospermia (sperm count >0.1 and <10M/ml), and were recruited from private infertility clinics between January 2010 and November 2010. Patients presented with primary infertility and having sperm counts less than 10 M/ml on at least 2 consecutive occasions were included in the study. The control group consisted of 50 Anbar men with proven fertility, defined as conceiving at least one child without medical assistance.

Semen Analysis

In all 100 men, semen was assessed according to published guidelines in terms of the volume, sperm count, rapid progressive sperm motility and morphology by means of techniques described elsewhere in private lab side [15].

DNA Extraction and PCR

Approximately 3 ml venous peripheral blood samples were collected in K3-EDTA tubes. Genomic DNA from patient and control samples was extracted and purified by using new manual Genomic DNA Purification and extraction protocol mixing between two protocols [16,17] in Science Coll. Tekreet Uni. Microdeletion analysis of the Y-chromosome Yq region, aimed to detect AZFa, AZFb and AZFc complete microdeletions. 11 STSs (AZF loci), mapped at intervals 5 and 6 on the long arm of the Y chromosome were used: sY81, sY84, sY86, and sY87 for AZFa, sY117, and sY143 for AZFb, and sY157, sY277, sY254 and Variable sY157 for AZFc. In addition, SRY (sex determining region on Y) gene, X/Y homologous gene pair zinc-finger X (ZFX) and Zinc-Finger Y (ZFY) primers were used as positive

internal controls to detect amplification failures in case a microdeletion was detected.,Sequences of all primer pairs and primer size of their products are shown in Table1.

Table (1): Sequences of different primer sets employed to identify Y-chromosome microdeletions among Iraqi males.

Sr.	P length	P name	Locus	Primer sequence
1	495 bp	ZFY	Control	F 5'-ACCRCTGTA CTGACTGTGATTACAC -3'
				R 5'-GCACYTCTTTGGTATCYGAGAAAGT-3'
2	209 bp	Y81	AZFa start	F 5-AGGCACTGGTCAGAATGAAG-3
				R 5-AATGGAAAATACAGCTCCCC-3
3	320 bp	sY86	AZFa	F 5-GTGACACACAGACTATGCTTC-3'
				R 5'-ACACACAGAGGGACAACCCT-3
4	326 bp	sY84	AZFa	F 5'-AGAAGGGTCTGAAAGCAGGT-3'
				R 5'-GCCTACTACCTGGAGGCTTC-3'
5	Variable	Y87	AZFa	F 5'-TCTGTGCTTGA AAAAGAGGG-3'
				R 5'-ACTGCAGGAAGAATCAGCTG-3'
6	311 bp	Y143	AZFb	F 5'-GCAGGATGAGAAGCAGGTAG-3'
				R 5'-CCGTGTGCTGGAGACTAATC-3'
7	Variable	Y117	AZFb	F 5'-GTTGGTTCCATGCTCCATAC-3'
				R 5'-CAGGGAGAGAGCCTTTTACC-3'
8	285 bp	Y157	AZFc	F 5'-CTTAGGAAAAAGTGAAGCCG-3'
				R 5'-CCTGCTGTCAGCAAGATACA-3'
9	350 bp	sY254	AZFc	F 5'-GGGTGTTACCAGAAGGC AAA-3'
				R 5'-GAACCGTATCTACCAAAGCAGC-3'
10	Variable	Y157	AZFc	F 5'-CTTAGGAAAAAGTGAAGCCG-3'
				R 5'-CCTGCTGTCAGCAAGATACA-3'
11	Variable	Y277	AZFc	F 5'-GGGTTTTGCCTGCATACGTAATTA-3'
				R 5'-CCTAAAAGCAATTCTAAACCTCCAG-3'

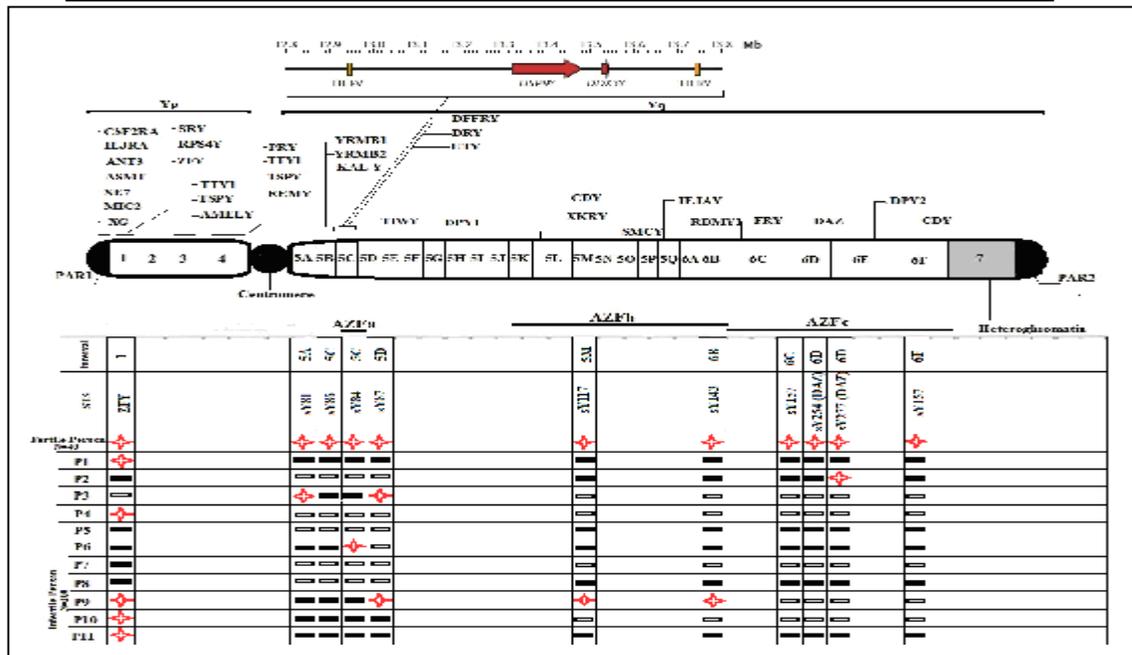


Figure (1)Gene map of the human Y chromosome. The Y chromosome is divided into seven intervals. The azoospermia factor (AZF) is divided into AZFa, AZFb, and AZFc regions .

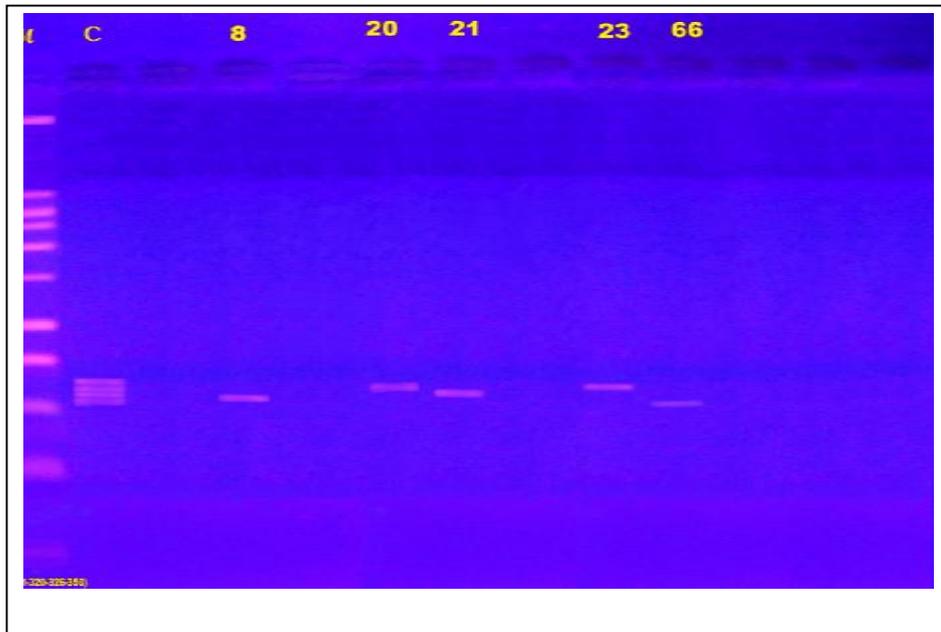
PCR was carried out in a monoplex fashion for each primer set. PCR was carried out in a 0.2ml PCR Microfuge tube in a 25µl reaction volume containing: 2µl template genomic DNA (100–200ng), 2 µl each primer, 2.5 µl PCR buffer, 1.5 µl MgCl₂, 0.5 µl dNTPs, 0.4 µl Tag and 14.1 µl free sterile water. The amplification reaction was performed in a played biosystem thermal cycler.

Amplification was started with initial denaturation step at 94°C × 5min, followed by 25 sequential cycles each including 60sec denaturation at 94°C, 45sec primer annealing at 58°C and 60sec extension at 72°C. The protocol was followed by a final extension step at 72°C × 7min followed by cooling to 4°C until electrophoresis detection. Fertile male DNA samples were used as positive controls, respectively.

The PCR product was added to the loading buffer, mixed and run on a 2 % (w/v) agarose gel and stained in 0.5 µg/ml ethidium bromide. In addition, a 100bp DNA ladder was always run concurrently with each electrophoresis run to confirm product size. After electrophoresis at 70 volts × 65min, results were visualized and recorded using UV trans-illuminator.

RESULTS

Y-chromosome microdeletions in AZFa, AZFb or AZFc were detected among the infertile men included in this study. An example of PCR products confirming the lack of Y-chromosome microdeletions is shown in Figure 2.



**Figure (2) Multiplex Pcr Analyses of Y chromosome Microdeletions
(M: 100 Bp Ladder)(C: Control)**

In total, 64(64%) of 100 infertile men investigated had deletions within the AZF region. In particular, we found different patterns of partial AZFc deletions; 16 severely oligozoospermia, but others demonstrated total Azoospermia while it was not observed at all in the control group. No family history of infertility was noted in the study subjects with AZF deletion except three cases whose brother was found to have severe oligozoospermia

DISCUSSION

The present study identified *AZF* microdeletions in the long arm of the Y-chromosome in population studies. This is in general agreement with some previously published studies [16,17], in spite of the varied frequencies of Y-chromosome microdeletions have been reported by others [18,19,20,21]. The variation in Microdeletion frequency noted by different investigators could be attributed to several factors influencing *AZF* Microdeletion status, including genetic background and Y-chromosome haplogroups, patient selection criteria, and size of study sample.

The different Y-chromosome Microdeletions are related to arrangement of certain DNA elements required for homologous intrachromosomal recombination leading to deletions. This feature might explain the microdeletions in our study population. Another important factor influencing Microdeletion frequency is patient selection criteria. Significantly higher frequencies of microdeletions have been reported in the setting of histologically-confirmed Sertoli cell only syndrome (SCOS), Klinefelter syndrome, and among patients with chromosomal abnormalities, varicocele and

cryptorchidism, and idiopathic Azoospermia accompanied by elevated serum follicle-stimulating hormone (FSH) levels [19,22 ,23 ,24 ,25 ,26]. However, patients with chromosomal abnormalities evident by G banding, were excluded from our study population. Conversely, all idiopathic infertility cases with sperm counts <10M/ml were included for evaluation. These factors may offer additional insights as to why Y-chromosome microdeletions were found in the population studies.

The pathological significance of these deletions is not yet clear. The deletion, described in infertile men with varying degrees of spermatogenic failure, has been proposed by some authorities as a risk factor for spermatogenic failure or oligozoospermia [27,28,29]. In our patient population deletion was encountered in severely oligozoospermic, and azoospermic males, indicating that this deletion cannot be linked to a particular type of spermatogenic impairment. Other investigators found association between *AZFc* partial deletions and male infertility [30,31, 32,33,34,]. Whether such deletions are associated with certain male lineage haplogroup(s) remains unresolved. Therefore, the effect of deletion on male infertility may vary according to the Y haplogroups of the study subjects. Although the Y haplogroup(s) of our study patients was not specifically assessed, these data suggest that this deletion could be a heritable polymorphism rather than a *de novo* arrangement. This is because the incidence of this deletion was significantly different between patients and controls. The partial deletion was observed oligozoospermic patient (sperm count 2.6 M/ml). While interesting, this isolated observation is insufficient to make any conclusions regarding its effect on spermatogenesis, this lead some investigators to conclude that Y-chromosome microdeletions is probably irrelevant to spermatogenesis [16]. In conclusion, Y-chromosome microdeletions were detected in this population of idiopathic oligo- and azoospermic infertile patients. Results concerning deletion suggest that this pattern does represent a risk factor for male infertility and might be considered a heritable variant in this population in order to explore other genetic, epigenetic and/or nutritional factors that contribute to idiopathic oligo- and azoospermia in the Anbar population.

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