Y chromosome azoospermia factors (AZF) microdeletions in azoospermic men.

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Abstract

Background: It becomes now evident that the abnormalities of chromosome Y espicially the microdeletions role the major causes of infertility and a number of studies linked the region Yq11 which contain the AZF factors to azoospermia.

Objectives: The current study was aimed to detect chromosomal abnormalities and Y microdeletions (AZFs deletions) among a number of azoospermic men.

Materials & methods: Five ml from peripheral blood was collected from 25 azoospermic men and four controls (one female and three fertile men) and used for DNA, PCR analysis and cytogenetic examinations in order to detect any kind of microdeletion in the AZF regions.

Results: Six individuals which accounts 24% of the total azoospermic men have a microdeletion in the AZF regions.The cytogenetic analysis revealed morphologically normal Y chromosome in all examined samples.

Conclusions: The microdeletions of the AZF regions cause quantitive loss in spermatogenesis.

Keywards: Infertility,AZF a,b,c , Y chromosome

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Introduction

During last few years, many Iraqi couples who are attempting pregnancy had a type of infertility. Although there is no official record about the true number of these couples. The number of men who attende the infertility clinics in Baghdad is increasing.

Men infertility can be classified into azoospermia, oligospermia, oligoastheno teratozoospermic and idiopathic and several factors behind each of them [1, 2]. Some of these factors are combined with some type of genetic abnormalities. Most of these abnormalities are associated with Y chromosome [3-5].

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It is now evident that the abnormalities of chromosome Y especially microdeletions role the major causes of infertility [6-8].

The argument of the association of Y chromosome abnormalities with infertility was strengthened by a number of studies which link the infertility to a number of microdeletions detected in the region Yq11, the region which contains the azoospermic factors AZFa,b,c and other genes such as RBM1,RBM2 and DAZ which are involved in the complex process of spermatogenesis [9-11].

AZFa,AZFb and AZFc have been identified as major cause of azoospermia leading to the disturbance of genes involved in spermatogenesis [8]. Several studies have demonstrated that microdeletion in AZF regions causes male infertility [12, 13]. Deletion of each AZF region has been found to have a different phenotypic effect [14, 15]. However, some of these deletions

are neutral which do not affect the fertility status and phenotype of the individual [16, 17].

The current study aimed to detect chromosomal abnormalities and Y microdeletions (AZF deletions) among a number of azoospermic men.

Materials and Methods

Semen and blood were collected from the azoospermic patients- aged between 20 to 51 years- who attended the infertility unit in the Institute of embryo research and infertility treatment/Al-Kadhimya from September 2006 to October 2007.

Semen sampling and analysis:

Using the 1999 WHO guidelines [18] a semen sample from each subject was collected into a clean, dry and sterile vial after abstinent of 3-4 days. After incubation at 37.5C for 30 minutes, the semen samples were centrifuged at 2500 rpm for 10 minutes and the pellets were examined under light microscope.

The azoospermia was defined as no sperm was present in the semen.

Blood Collection:

Five ml from peripheral blood was collected from 25 azoospermic men and four controls (one female +three fertile man).Each blood sample was divided into two aliquots, one aliquot was added to heparinized tube for cytological examination, the other aliquot was added to EDTA tube for DNA extraction.

The EDTA blood samples were centrifuged at 2000 rpm for 10 minutes. The serum of each blood sample was collected in a clean and sterile tube and used for further assays. The WBC layer from each sample was collected in a sterile tube and used in DNA extraction.

Blood Culture:

A half milliter from each heprinized blood sample was cultured in 5 ml of standard supplemented RPMI 1640 medium containing 20% fetal calf serum and 2% of phytohemagglutinin (PHA) (prepared by the molecular biology Department\Iraqi center for cancer and medical genetic research-ICCMGR-Baghdad-Iraq) in a sterile tubes. The tubes were cultured at 37°C for 72 hours. A hundred micro liter of cholchicine (0.45 mg\ml) was added to each culture. After 20 minutes, the cells from all culture tubes were harvested by centrifugation (2000rpm\10 mins).The supernatants were discarded and the cells redissolved with the remaining solution. The cells were exposed to mild hypotonic treatment with 3ml of 0.075 M KCL at 4°C.The cells was precipitated by another centrifugation. The supernatants were discarded, cells redissolved with remaining hypotonic solution and fixed with 5 ml fixative solution (3 methanol: 1 Glacial acetic acid).Centrifugation and fixation were repeated four times at intervals of 20 minutes. Slides were stained the following day for 10 minutes in 10 ml 5% buffered Giemsa solution, pH 6.8. Three slides were prepared for each sample and 50 metaphases were examined from each sample for chromosomal abnormalities.

DNA extraction:

The WBC layers collected from the EDTA blood samples were used in DNA extraction.

The DNA was extracted according to the Wizard genomic DNA purification kit (Progema/USA).One third milliliter from the WBC suspension was mixed with 900 ul of cell lysis buffer. Samples were incubated at 20°C for 10 minutes .The nuclei were pelleted by centrifuging at 3000 rpm for 10 minutes.The supernatant was discarded and the pellet redissolved with the remaining solution. Three hundred micro liter from nuclei lysis buffer was added to the nuclei suspension with gentle

mixing for one minute then 300 ul from protein lysis solution was added with another mixing. The samples were then centrifuged, the supernatants were collected in a clean tubes and the DNA precipitated with equal volume of isopropanol alcohol. DNA samples were pelleted by centrifugation, washed with 70% ethanol alcohol, air dried and re-suspended with 100 ul of distilled water.

The DNA concentration and purity were checked.The agarose gel electrophoresis was also adopted to confirm the presence and integrity of the extracted DNA.

PCR Assay:

Six primers supplied by alpha DNA company-Canada were used in PCR to determine the presence of Y chromosome microdeletions in AZFa, AZFb and AZFc locuses. The primers sequences were shown in Table 1.

Table 1: Primers sequences and products.

STS	Left primer	Right primer	AZF interval	Products in pb
SY84	5-GTGACACACAGACTATGCTTC-3	5-ACACACAGAGGGACAACCCT-3	AZFa	320
SY127	5-GGCTCACAAACGAAAAGAAA-3	5-CTGCAGGCAGTAATAAGGGA-3	AZFb	274
SY254	5-GGGTGTTACCAGAAGGCAAA-3	5-GAACCGTATCTACCAAAGCAGC-3	AZFc	400

PCR was performed according to (19) using a thermal DNA cycler machine (Tec gene-UK). Cinagene PCR Kit (Iran) was utilized. A hundred nano grams (ng) of denaturated DNA and 40 picomole from each primer were added to the PCR master mixture. The reaction was initiated in a volume of 50 ul. A total of 20 cycles of polymerization was carried out. Ten micro liter from each amplified DNA , 0.2 ug of lambda Hind III+EcoR1 fragments as a marker were mixed with 2 ul of loading buffer and electrophoresed through a 1% agarose gel for 30 minutes at 50 Hz volts. The gel was then stained, visualized under UV light and photographed.

Results

Screening of 25 azoospermic men with the sequence tagged sites- STS-markers specific to AZF regions showed deletion in 6 individuals (Figures 1, 2 and 3) which accounts for 24% of the total azoospermic men analyzed. Of 6 individuals with AZF deletions, deletion of the AZFc region alone was detected in 2 individuals which accounted for 33.3% of the total individuals (Table 2). One azoospermic man showed deletion in the AZFb region (16.7%) and 3 azoospermic men showed deletions in the AZFa +AZFc regions (50%).None of the control men showed deletion for STS markers.

The cytogenetic analysis revealed morphologically normal Y chromosome in all examined samples.

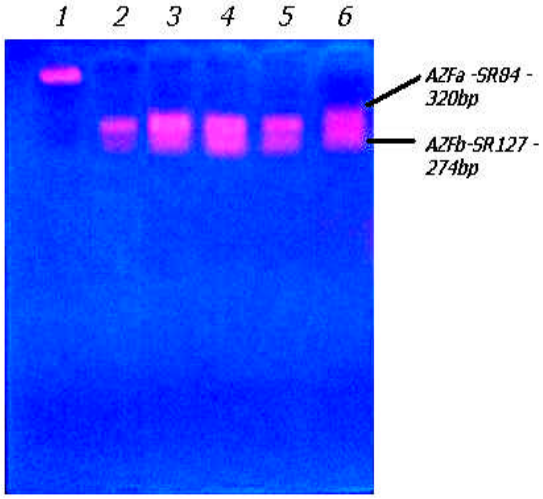

Figure-1 : Gel image showing PCR products of two markers represnting the AZFa and AZFb regions.
Line - 1: Female DNA sample without products.
Lines 2,3,4,5 and 6 azoospermic samples with normal AZFa and AZFb.

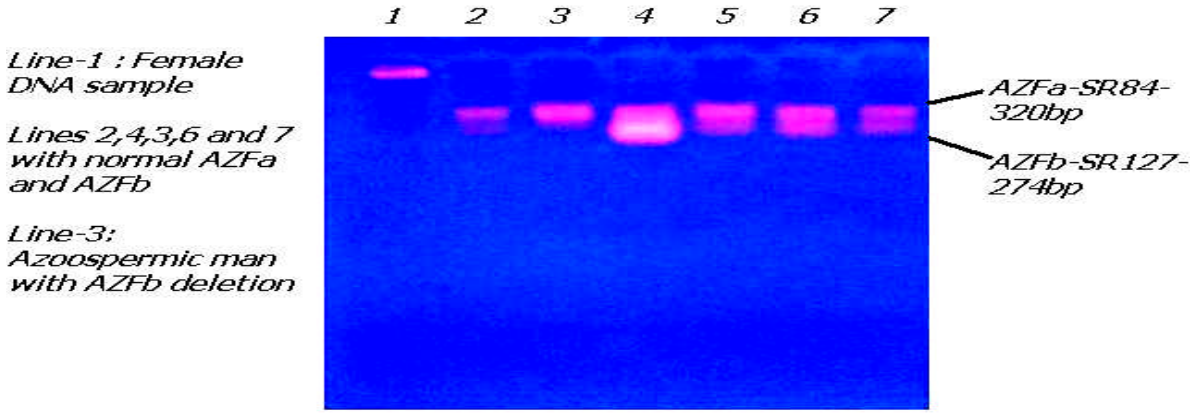

Figure-2 : PCR diplex of AZFa and AZFb products in azoospermic men.

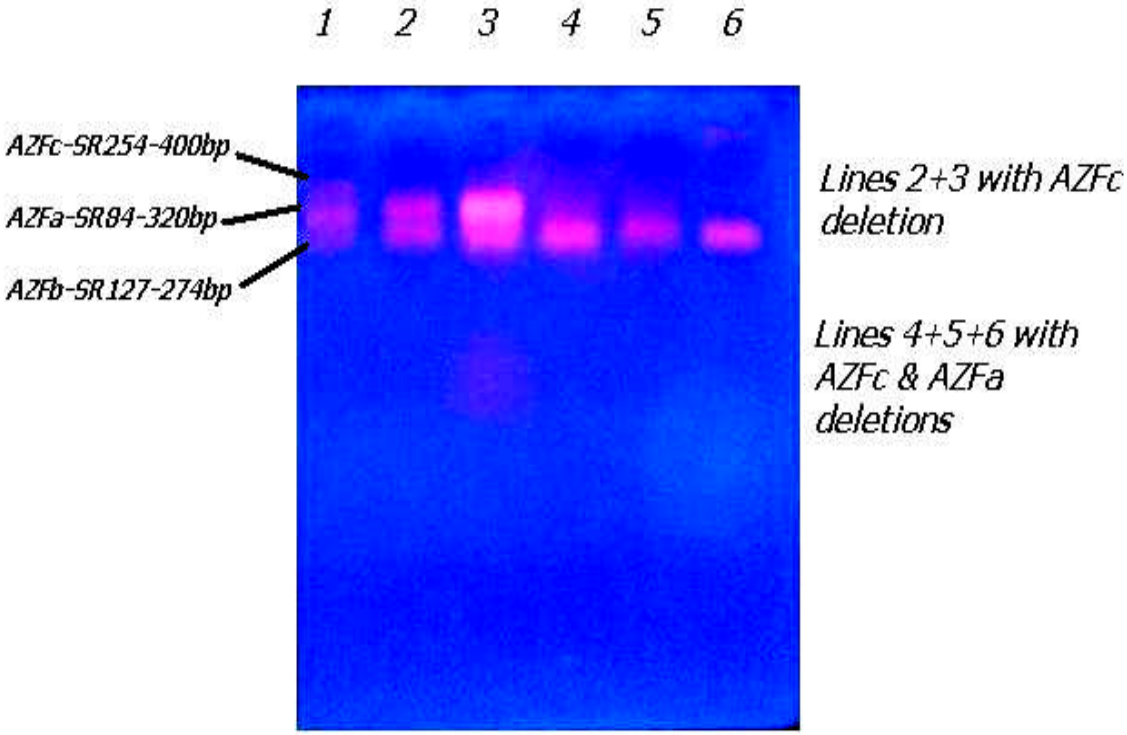

Figure- 3 : Gel image showing PCR products of three sequence-tagged sites (STS) markers representing the AZFa,AZFb, and AZFc regions.
Line-1: Fertlie man, Lines 2,3,4,5 and 6 : Azoospermic men .

Table 2: The Y chromosome deletions detected in the AZF region of azoospermic men.

Sample NO.	Y deletions		
	AZFa	AZFb	AZFc
1 (Female)	—	—	—
2	—	—	—
3	—	+	—
4	—	—	—
5	—	—	—
6	—	—	—
7	—	—	—
8 (Fertile man)	—	—	—
9	—	—	+
10	—	—	+
11	+	—	+
12	+	—	+
13	+	—	+

Discussion

PCR –based STS analysis of 25 azoospermic men revealed microdeletions on the Y chromosome in 6 individuals (Figure 1,2,3 and Table 2) accounting for 24% of the total azoospermic men analyzed . Other studies revealed that the Y chromosome microdeletions were responsible for 7% to 13% of the infertile men ([11, 20]).

Fifty five and half percentage(55.5%) of the Y chromosome deletions detected in this study were in the AZFc region, 22.2% of them with only AZFc deletion and 33.3% associated with AZFa deletion (Table-2). This indicates that gene making the AZFc region is extremely fragile comparing with other AZF regions and among the three AZF regions, deletion of AZFc has been found to be the most frequent abnormality followed by AZFa and then with AZFb. This is in agreement with the other studies showing that the incidence of deletion in the AZFc region was high compared with the AZFa and AZFb regions [8, 21, 22].

Whether the AZF deletion detected in this work associated with specific factors caused azoospermia or other types of infertility is not clear yet. However, many other studies have been found that each AZF deletion has a different phenotypic effect. Kamp et al, 2001 [23] found that AZFa is associated with sertoli cell-only syndrome type 1 (SCOS) phenotype. Also deletions in the AZFb region have been found to be associated with azoospermia, oligospermia and normozoospermia. While deletion of the AZFc region has been found to be associated with azoospermia and sever to mild oligospermia [24].

It has been found in many cases that similar deletion of AZFc region causes quantitative loss in spermatogenesis [25]. However, genotype-phenotype correlation has not been fully understood.

This high percentage of the AZF deletions accounted in our study for (24.4%) of cases suggesting that it is possible that AZFc is predominant in Iraq azoospermia. However, we believe that the etiology of male infertility may differ between ethnic populations. The deletions of AZF regions in azoospermic are not always detected. Martinez et al, 2000 [21] have analyzed 128 infertile men with SY84, SY85 and SY86 (AZFa) and found none of them had shown deletion. Dohle et al, 2002 [26], also did not see any deletion in the AZFa region during their screening of 37 azoospermic individuals with 2 STS markers for each AZF regions.

In the light of the above, further studies using other AZFc markers and more azoospermic subjects need to be done.

Most of the STS-based studies on male infertility have been carried out with a few markers for each AZF region [4]. Hence they failed to detect the Y chromosome deletion in many cases. Therefore, there is no collective opinion about the marker to be used for Y chromosome micro deletion analysis.

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