Detection of Y-chromosome microdeletions in infertile Libyan men using multiplex PCR in misurata.

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Abstract

Background: Infertility is considered as one of the main health problems affecting about 10- 15% of couples seeking children. However, in 50% of these the male partner is responsible for infertility. Y chromosome classical microdeletions in the azoospermia factor (AZF) regions are known to be associated with spermatogenic failure. Spermatogenesis is an essential reproductive process that is regulated by many Y chromosome specific genes. The Y-chromosome is the smallest chromosome that consists of 2-3% of the haploid genome and may contain between 70 and 200 genes.

Objective: This study was carrying out to understand prevalence and patterns microdeletions in azoospermia factor (AZF) region of Y chromosome in infertile Libyan men using PCR.

Material and methods: In the current study, 50 patients from male infertility and 10 healthy controls. were studied in order to explore the Y chromosome disorders of male infertility in Misurata of Libya. AZF microdeletions and their associated phenotypes in infertile males have been extensively studied, and were identified by sequence-tagged site polymerase chain reaction (STS-PCR).

Results : The results of this study showed deletions in two cases (3.33%) as one of them had a deletion in AZFc and AZFd regions and the other had a deletion in AZFd region

Conclusion: our study showed that the prevalence of Y chromosome microdeletions is 3.33% in our population , also our study proves that molecular analysis is mandatory in any diagnostic workup of idiopathic infertile males. Moreover,. the importance of examining a molecular genetics approach including AZF deletions must be emphasized

for those men who are considering intra cytoplasmic sperm injection ICSI, because this genetic defect is transmitted to their sons, affecting their fertility.

Keywords: Male infertility, Y chromosome micro deletion, multiplex PCR.

Introduction:

Infertility is considered as one of the main health problems affecting about 10- 15% of couples and male factor however, can be considered as half of these cases (Foresta *et al.*, 2001). Spermatogenesis can be affected by different means, such as systemic disease, cryptorchidsm, endocrinological disorders, obstruction in seminal pathways, and infection or immunological factors. However, the cause of male infertility is unknown in up to 50 % of cases (Foresta *et al.,* 2001). In these cases, genetic aetiology may be associated with abnormal spermatogenesis. A genetic factor located at Yq11 has been established to be important for male germ cell development and the gene cluster is referred to as azoospermia factor (AZF) (Tiepolo & Zuffardi., 1976). In the AZF region, four loci termed as AZFa, AZFb, AZFc and AZFd have been identified (Vogt *et al*., 1996; Kent-First *et al*., 1999).

Genetic factors have been claimed to be responsible for about 10 % of cases. These genetic disorders affect semen parameters by causing alteration of chromosome materials such as Klinefelter syndrome and Y chromosome microdeletions (O'Brien *et al*., 2010). Human Y-chromosome is a sex determining chromosome and required for maintenance and development of male germ cells (Kuroda-Kawaguchi *et al.,* 2001).

A large number of sequence tagged sites (STS) have been generated and mapped for the above AZF regions on Y chromosome (Foresta *et al*., 2001). STS are known sequences of genomic DNA that can be amplified by PCR (van der Ven *et al*., 1997; Foresta *et al* 1998). Yq chromosome microdeletions are the second most important genetic cause for spermatogenic arrest in male with infertility after Klinefelter syndrome (Katherine *et al*., 2010), as it contain several genes which are involved in spermatogenesis (Vogt *et al*., 1996; Foresta *et al*., 2001).

A microdeletion is defined as a chromosomal deletion that spans several genes but not large enough to be detected using conventional cytogenetic methods. Studies have

revealed that microdeletions are more prevalent in men who are azoospermic and severely oligozoospermic (Katagiri *et al*., 2004).

AZF microdeletions result from intra chromosomal homologous recombination between repeated sequence blocks organized into palindromic structures in the long arm of the Y chromosome (Vogt., 2004).

These microdeletions are associated with different testicular histological profiles, ranging from Sertoli cell-only syndrome (SCOS), hypo-spermatogenesis (HS) to spermatogenic arrest (SGA) to SCO type II syndrome (Vogt *et al*., 1996; Foresta *et al.*, 2001).

In this study we are investigating the roles of several genes on Y chromosome by looking at AZF microdeletions and try to understand how this kind of abnormality may cause infertility in Libyan men.

Objectives

The overall objective for this project is to investigate the microdeletions in azoospermia factor (AZF) region of Y chromosome by PCR using primers corresponding to four genes of the AZF region (Table 1) in infertile Libyan men.

Materials and Methods:

The target was a total of 60 male subjects were analyzed, of which, at least 50% - 70% of who are infertile patients (oligospermic and azoospermic) and the rest of the subjects were considered as fertile controls.

Routine semen analysis was carried as well and plasma follicle stimulating hormone (FSH) , LH and testosetron concentrations were determined for this subjects using by Vitros 3600 system (Johnson, American) at ,Alamal reproduction center / Misurata.

Analysis of Y-chromosome microdeletions was carried out by using polymerase chain reaction (PCR). The genomic DNA from the infertile as well as normal subjects was isolated by using phenol/chloroform extraction protocol. Subsequently, the isolated genomic DNA concentration was measured by using spectrophotometric analysis at 260 nm and the quality of the DNA were checked on 1.8 % agarose gel.

DNA was extracted from peripheral blood leucocytes according to standard procedure and the presence of sub-microscopic deletions of the *AZF* region on the Y chromosome

long arm was analysed using a multiplex PCR for Y-specific markers, including sY81(*AZFa*) and sY164 (*AZFb*), sY277 (*AZFc*) and sY153 (*AZFd*). All STSs PCR primers (indicated from 5`® 3`) adopted for Multiple regions on Y long arm in this study are shown in **Table 1.** Sense primers end with F (for forward), while antisense end with R (for reverse). All primer sequences were were chosen based on the laboratory guidelines described by Simoni *et al*. (Simoni *et al*., 1999).

Table 1 . Primers sequence and size for AZF microdeletions detection (using Multiplex PCR) (Simoni *et al*., 1999)

STS	Y- Region	Primer sequence	Size (bp)
sY81	AZFa	Forward 5′- AGG CAC TGG TCA GAA TGA AG-3′ Reverse 5′- AAT GGA AAA TAC AGC TCC CC-3	200 bp
sY164	AZFb	Forward 5′- AAT GTG CCC ACA CAG AGT TC-3 Reverse 5′- TGG AAG ACC AGG ATT TCA TG-3′	590 bp
sY277	AZFc	Forward5′-GGG TTT TGC CTG CAT ACG TAA TTA-3′ Reverse5′-CCT AAA AGC AAT TCT AAA CCT CCA G-3′	275 bp
sY153	AZFd	Forward 5′ -GCA TCC TCA TTT TAT GTC CA-3′ Reverse 5′-CAA CCC AAA AGC ACT GAG TA-3	135 bp

PCR was carried out in a 25-µL reaction volume containing: 200ng genomic DNA, 1.5mmol/L MgCl2, 200µmol/L dNTP, 0.1–0.5µmol/L of each primer, PCR buffer with mg++ (Qiagen, Germany) and 1 U HotStarTaq DNA polymerase (Qiagen, Germany). Thermocycling (Techne Cambridge Ltd, Duxford- Cambridge U.K.) were used for multiplex PCR and all samples were performed together under the same PCR conditions as follows: initial denaturation at 94°C for 5 min; followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 1 min, extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. PCR products were separated on 3% agarose gel electrophoresis (Invitrogen, USA), stained with ethidium bromide (Invitrogen, USA), and visualized using gel documentation system. The deletion of one PCR fragment was confirmed in single-primer pair PCR under the same experimental conditions. This analysis was performed at least three times on microdeleted sample. In each multiplex PCR assay, one sample from healthy female was used as negative control, and healthy fertile male was used as positive control.

Results

The study group included 50 subjects of infertile men from lamis clinic in addetion to 10 controls. A total of 9 out of 60 were found to be Azoo zoospermic (n=9)15 % , whereas 8 cases were sever oligozoospermic (n=3) 5 % , and with oligozoospermic (n=7)11.7% men with Teratozoospermics were counted (n=5)8.3%, and Terato together with astheno zoospermics were counted (n=26) 43.3% in contrast to the normal control (n=10)16.7 %. Hormonal profile (FSH, LH, total testosterone) data was available for all patients. Table summarizes the available hormonal data for the patient population.

Table 2 Summary of the available hormonal values in entire patient population.

Statistics	FSH MIU/ML	LH MIU/ML	TESTO Ng/ml
Mean ± Std. Deviation	6.57 ± 4.03	4.97 ± 2.33	11.15 ± 5.96
Minimum	2	1	3
Maximum	16	11	27
Total	60	60	60

All DNA samples were processed for Yq microdeletions analysis using PCR. Each of these samples was analyzed using 4 sets of primers the STS and used were for: AZFa: sY81(200bp),AZFb:sY164 (590 bp), AZFc: sY277(275bp), AZFd sY153(135bp) Fertile male and female samples were used as positive and negative control as Showed (fig 1) . From cases which analyzed for y chromosomal microdeletions using 4 STSs result this study showed deletions in two cases as Showed in figure 1 (3.33%) (one had AZFc + d and one had AZFd deletions)

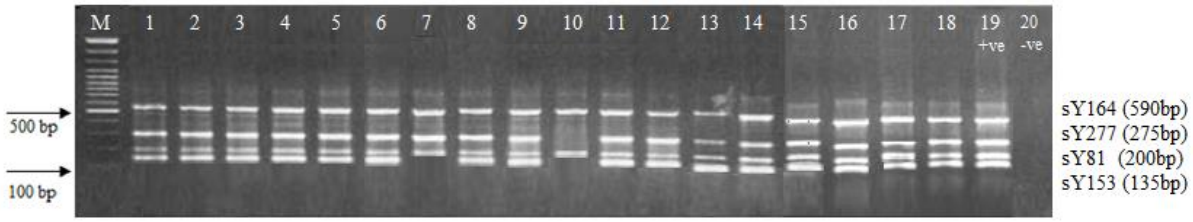

Figure 1 Results from multiplex polymerase chain reaction analysis; lanes M show 100-bp molecular weight markers. Lanes 1 – 18 are the randomly collected suspected samples. Lane 7 shows a deletion of sY153 whereas lane 10 shows deletions of both sY277 and aY153. The rest are undeleted samples, lane 19 is a positive control (normal male); lane 20 is a negative control sample of female DNA.

Confirmation of M-PCR results

A single-primer pair PCR under the same condition described above to confirm the multiplex PCR results.

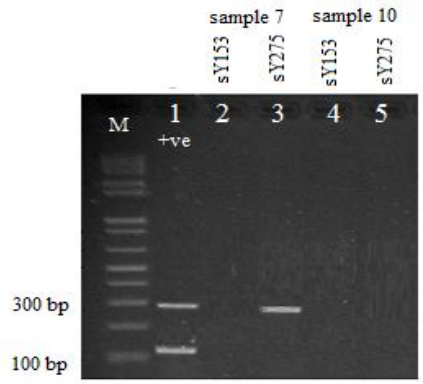

Figure 2 Lane M: molecular ladder. Lane 1 is a positive control from healthy male. Lane 2 and 3 represents sample 7 targeting sY153 and sY275 respectively. Lane 4 and 5 represents sample10 targeting sY153 and sY275 respectively. Confirmation of deleted sY153 region from sample 7, whereas, both sY153 and sY275 are deleted from sample 10.

Discussion

Molecular genetics techniques have unveiled a number of aetiopathogenetic factors, including microdeletions of the long arm of the Y chromosome (Yq) associated with male infertility.

Deletions of AZF regions are deletions of the euchromatine part of the Y chromosome long arm. Deletions of this part of Y chromosome can lead to or may damage genes in this region that is responsible for the proper course of spermatogenesis (Katagiri *et al*., 2004).

Furthermore, endocrine disorders are part of the parameters used as a diagnostic tool for the analysis of infertility (Sigman & Jarow, 1997). This study focused upon three major analysis including sperm parameters, biochemical analysis, and molecular investigations.

The sperm parameters results showed number of sperm defects which have been lately used in this study to divide the cases into 6 groups. These groups varies between (azoospermics, sever-oligozoospermic, oligozoospermic, astheno & teratozoospermic and teratozoospermic).

The hormonal analyses have showed no changes in the LH and testosterone values in all cases in contrast to controls. However, FSH values were inappropriate in many cases especially in azoospermics sever oligo zoospermics groups and claimed higher compare to the other groups and controls. This finding agrees with other studies in infertile men with or without deletions in AZF region. Thus, FSH concentration is a good parameter for evaluation of testicular function but still yet do not help differentiate cases with or without Yq microdeletions (Peterlin *et al* ., 2002; Oates *et al*., 2002; Mitra *et al* ., 2008 ; Wang *et al*.,2014).

The molecular work applied in this project showed that there were 2 cases with a microdeletion on their long arm of Y chromosome, these findings agree with other previous studies. We have found that patients with a microdeletion of AZFd alone or AZFc along with AZFd suffer from severe oligo zoospermia, meanwhile patients with such abnormalities have elevated FSH levels.

This finding also agreed with other studies carried by Kumar and his group in 2006 where they reported increment of serum FSH levels in all of his patients who have severe malfunction of their germ cell function, irrespective of the presence or absence of AZF deletions (Kumar *et al*., 2006). Other studies on the other hand, carried by Foresta and Ferlin and their group in 2011, 2007 respectively, reported a low FSH levels in patients with AZF microdeletion when compared to infertile men without deletion (Foresta *et al* .,2001; Ferlin *et al* ., 2007).

Therefore, and from the above discordant findings, we can conclude that hormonal data are not a useful tool to confirm any Y chromosomal deletion.

It was also reported that microdeletions in AZF region can lead to a variable phenotype with a significant reduction in sperm count and increased loss of germ cells and progressive decline in semen quality. According to European Andrology Association (EAA) guidelines for diagnostic (Krausz et al.,2014; Simoni et al .,2004) and most recent works(Kdous *et al*.,2015; Sathyanarayana *et al* .,2015)

AZF microdeletions are observed in 10 to 15% of infertile men with azoospermia or severe oligozoospermia, and are very rare in fertile men or men with a sperm density greater than 5 million/ml (Vineeth *et al*., 2011), frequency of AZF microdeletions was 31.71% in azoospermia and 21% in oligozoospermia patients (Nailwal *et al*., 2017).

This study showed the presence of microdeletions in AZFd, AZFc and AZFd regions produced a unique phenotypes in 2 cases out of 60 cases leading to sever oligozoospermia as explained above. The cause of this kind of chromosome abnormality is not clear yet, but still carrying further investigations such as family history or linkage analysis may help to uncover the confusion.

The incidence of Yq microdeletion presented in this study is (3.33%) out of the selected population. This result disagree with some previously published studies of which of Buch and colleagues (2004) in Spain and Shaqalaih (2009) in Palestine, Which their results did not report any type deletion on AZF regions.

While this result agrees with some previously published studies of which of Kihaile and colleagues (2005) studied the occurrence of Y chromosomal microdeletions within Japanese populations, and the prevalence was found to be 6.2% (Kihaile *et al.*, 2005) .

Carvalho and his group (2006) conducted study on Brazilian infertile males and found Y chromosome microdeletions with 10% frequency, and the most frequent of them being characterized by a complete deletion of AZFc region (Carvalho *et al*., 2006) . Hsu et al (2006) as well reported a total incidence of 24 (5.2%) in 460 infertile men in Taiwan the deletion was only region AZFc (Hsu *et al*., 2006).

Interestingly, the study that was presented by Ferlin and his group (2007) after carrying a prospective study that extended for 10 years (from January 1996 to December 2005) reported a total incidence of 3.2% in 3073 infertile men in Italy (Ferlin *et al*., 2007). This puts our results in scale with many studies particularly with countries in the rejoin such as Italy.

With regards to the Arabic region, this kind of studies was first carried out by El Awady (2004) in Egypt where he reported a frequency as high as 12% (4/33), two with AZFc deletion, one with AZFa deletion, and one with AZFa -b- c deletion. The two patients with AZFc deletion described in the study were found to be deleted only for a single STS (sY272) and not for the other AZFc STSs they used ,This will substantially reduce the frequency of microdeletions in the Egyptian study to 6% (El Awady *et al*., 2004).

The second study conducted by Hellani et al (2006) in Saudi Arabia they reported a frequency as low as 3.2% (8/247) of microdeletions (Hellani *et al*., 2006). Another study

was conducted among Moroccan infertile men by Imken and his group (2007) reported prevalence of 3.15% (4/127), with two cases with AZFc deletions and, two cases with AZFb + AZFc deletions. All the deletions were found only in azoospermic subjects (Imken *et al*., 2007).

Other studies in the Asian Arabic region was carried on Kuwaities by Mohammed et al (2007) where they reported 7 cases with AZF microdeletion out of 266 samples with prevalence of 2.4% (Mohammed *et al*., 2007).

This study is the first study run in Libya in the field, and from the above argument, it is very obvious that our finding aggress greatly with those studies with regards to Y chromosome microdeletion frequencies, despite neither ethnic nor the geographical differences.

Conclusion

The outcome of this study can be summarized in the coming points; first of all it showed the direct relationship between FSH levels and the high degree of spermatogenic failure, secondly; it is impossible use of the hormones data to distinguish Yq microdeletions. The third points in which we initially managed to predict the incidence of Y chromosome microdeletions at 3.33% in our population. Finally, this study confirmed the importance of using molecular analysis as a tool of diagnosis in the field of infertility. Moreover, introduce the molecular technologies to Libyan infertility clinics and importance of applying the molecular genetics approach including AZF deletions for those men who are considering ICSI, as these genetic defects could be transmitted to their future sons and may affect fertility.

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