Screening for Y-Chromosome Microdeletions in a Population of Infertile Males in the Gaza Strip

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Abstract

Infertility is an extraordinary public health problem in the Arab world, as it affects about 15% of couples seeking children. The male partner is responsible for infertility in approximately half of these cases. Classic microdeletions of the Y-chromosome involving the azoospermia factor (AZF) regions are known to be associated with spermatogenic impairment, and non-obstructive azoospermia must be differentiated on the basis of endocrine evaluation and testicular biopsy. Partial AZFc deletions remain controversial because there is no clear agreement regarding their role in spermatogenic failure. In the current study, 50 fertile males (controls) and 125 patients with primary idiopathic male infertility were studied in order to describe the frequency of Y-chromosome microdeletions among male infertility patients in the Gaza Strip-Palestine area. No Y chromosome classical microdeletions could be detected in any of the 125 infertile men, suggesting that ethnic factors, genetic background, and Y chromosome haplogroups are key factors in such deletions. On the other hand, six gr/gr and one b1/b3 AZFc partial deletions were detected in the infertile population. The gr/gr deletion was also noted in relatives of four of the six patients with this deletion, and in one of the fertile controls. In conclusion, our study shows that the incidence of Y-chromosome microdeletions in our population is rare; these data suggest that other genetic, epigenetic, nutritional and/or local factors are responsible for impairments in semen parameters observed in this Gazan population. We further hypothesise that the gr/gr deletion is not associated with male infertility, at least in this sub-group.

Keywords

Idiopathic male infertility; A/oligozoospermia; Y-chromosome microdeletions; AZFc partial deletion

Introduction

Male causes of infertility are found in about 50% of couples struggling with infertility [1,2]. Reduced male infertility can be a result of congenital and/or acquired abnormalities. Frequently, however, male infertility is difficult to diagnose and about 60–75% of cases remain idiopathic. These idiopathic cases present with no previous family history associated with fertility problems and have normal findings on physical examination [3]. Interstitial and terminal deletions in AZFa, or AZFb, or AZFc alone or in any combination of the Y-chromosome long arm (Yq) are all associated with dramatic nonobstructive spermatogenic failure. Therefore, there is a clear connection between deletion/s at particular AZF loci and male infertility [4–12]. These gross “microdeletions” are associated with divergent testicular histological profiles, ranging from Sertoli cell-only syndrome (SCOS), hypospermatogenesis (HS) to spermatogenic arrest (SGA) [13].

Apart from infertility, men presenting with AZF microdeletions appear otherwise healthy. These microdeletions are usually detected by performing sequence tagged site (STS) based PCR techniques on patient peripheral blood genomic DNA. In addition to standard GTG karyotyping, Y-chromosome microdeletion (by PCR) is a mandatory test in the evaluation of the azoo/oligozoospermic patient. Worldwide, the Y-chromosome microdeletion assay has
become a routine test, and current research indicates that about 10% cases of idiopathic azoo/oligozoospermia may be due to deletion in AZF. Disruption of AZF therefore can be viewed as the most common molecularly diagnos-
cable cause of spermatogenic failure in the setting of non-
obstructive azoospermia or severe oligozoospermia [7,
12,14–19]. Previous investigators have reported that com-
plete deletion of the entire AZFc region (spanning 3.5 Mb
of the Y-chromosome) is the most common known genetic
cause of human male infertility [11,15–18,20–23].

AZFc partial deletions probably occur through non-allelic
homologous recombination events between amplicons
within the AZFc region [24–26]. These recombinant
events can yield different AZFc deletion patterns (e.g., gr/
gr, b1/b3 and b2/b3) and are characterized by particular
region-specific STSs [17,20,25,27–29]. While there is no
consensus on whether partial AZFc deletions affect sper-
matogenesis, some authors have suggested that such dele-
tions represent a risk factor for male infertility [28–30].
Other authors, however, found no association between
certain AZFc partial deletions and infertility [31–33].

The association between Y-chromosome microdeletions
and male infertility has not been specifically studied in
patients from the Gaza Strip (Palestine) until now. To do
so we employed a PCR STS-based technique to detect Y-
chromosome microdeletions in a group of azoo/oligozo-
spermic Gazan infertile patients.

Materials and Methods

Study Population

A total of 125 infertile Palestinian males residing in Gaza
Strip with non-obstructive sperm impairments were eval-
uated. These patients were confirmed to have non-obstruc-
tive azoospermia or oligozoospermia by endocrine eval-
uation and testicular biopsy. These patients had crypto-
zoospermia (sperm count <0.1M/ml), severe oligozoos-
permia (sperm count >0.1 and <5M/ml), or oligozoosper-
mia (sperm count 5–10M/ml) and were recruited from
assisted reproduction centers and private infertility clinics
between June 2006 and August 2008. Patients presented
with primary infertility and having sperm counts less than
10 M/ml on at least 2 consecutive occasions were incudes
for study. The control group consisted of 50 Gazan men
with proven fertility, defined as conceiving at least one
child without medical assistance. All study subjects pro-
vided written informed consent in compliance with the
Helsinki Ethical Committee in Gaza.

DNA Extraction and PCR

Approximately 2 ml venous peripheral blood samples
were collected in K3-EDTA tubes. Genomic DNA from

patient and control samples was extracted and purified by
using Wizard® Genomic DNA Purification Kit (Promega)
following the manufacturer protocol. Microdeletion analy-
sis of the Y-chromosome Yq region involved two com-
ponents. The first step aimed to detect AZFa, AZFb and
AZFc complete microdeletions. 13 STSs (AZF loci) mapped
at intervals 5 and 6 on the long arm of the Y chromosome
were used: sY746, sY84, sY86, and DBY1 for
AZFa, sY117, sY125, sY127, sY131, and sY134 for
AZFb, and sY152, sY272, sY254 and sY255 for AZFc.
In addition, SRY (sex determining region on Y) gene and 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 micro-
deletion was detected. In the second step, when step one
does not show any AZF deletion for any patient, we looked
for AZFc partial deletions using sY1291, and sY1191 pri-
mer sets. Sequences of all primer pairs and expected size
of their products are shown in Table 1.

PCR was carried out in a monoplex fashion for each pri-
mer set. PCR was carried out in a 0.2ml PCR Microfuge
tube in a 20μl reaction volume containing: 2μl template
genomic DNA (100–200ng), 10μl PCR Master mix
(Promega, Madison, USA) 1.5 μl (2 μmol) each primer and
nuclease free sterile water to 20 μl. The amplification
reaction was performed in a programmable thermal cycler.
Amplification was started with initial denaturation step at
94°C × 15min, followed by 35 sequential cycles each
including 60sec denaturation at 94°C, 60sec primer
annealing at 57°C and 60sec extension at 72°C. The proto-
col was followed by a final extension step at 72°C ×
10min followed by cooling to 4°C until electrophoretic
detection.

For detecting AZFc partial deletions, the same reaction
mixture and volume were used as above, but instead
employed different primer sets. The following PCR pro-
tocol was employed: 5min initial denaturation (94°C),
followed by 35 sequential cycles of 30sec denaturation
(94°C), 45sec primer annealing (61°C) and 45sec exten-
sion (72°C). This was followed by an extension step of
7min at 72°C with subsequent cooling to 4°C until elec-
triphoretic detection. In case of detecting a partial dele-
tion, available first degree relatives were also tested for
the presence of that deletion. Note: Testing for origin of
AZFc partial deletions among family members of study
patients (i.e., inherited vs. de novo) was possible in only
four cases.

Positive and negative controls were run concurrently with
each patient sample. Female and fertile male DNA sam-
plexes were used as negative and positive controls, respec-
Water instead of genomic DNA was used as blank to check for any DNA contamination. The PCR product was added to the loading dye, mixed and run on a 2 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide in 1xTris Acetate EDTA (TAE). In addition, a 100bp DNA ladder was always run concurrently with each electrophoretic run to confirm product size. After electrophoresis at 70 volts × 45min, results were visualized and recorded using a documentation system (Vision, Scie-Plas Ltd, UK).

### Statistical Analysis

Partial deletion frequencies in the patient and control groups were compared using the Chi square; *p* < 0.05 was considered statistically significant.

### Results

#### Full AZF Microdeletions

No complete (classic) Y-chromosome microdeletions in AZFa, AZFb or AZFc were detected among the 125 infertile men included in this study. An example of PCR products confirming the lack of classical Y-chromosome microdeletions is shown in Figure 1.

#### AZFc Partial Deletions in Patients and Controls

In order to detect partial AZFc deletions our goal was to detect the unique fragments flanking the DAZ1/DAZ2 doublet at the u3 segment (proximal) and the P2/P1 palindrome junction (distal), corresponding, respectively, to sY1191 and sY1291 as described previously [17,18,39]. The patterns shown in Table 2 were used for assigning the different partial AZFc deletions.

In total, seven (5.6%) of 125 infertile men investigated had partial deletions within the AZFc region. In particular, we found two different patterns of partial AZFc deletions; the gr/gr (6/125, 4.8%) and the b1/b3 (1/125, 0.8%) deletions. One man with gr/gr deletion was oligozoospermic (sperm count 6.4 M/ml), one was severely oligozoospermic (sperm count 0.7 M/ml), but the others demonstrated total azoospermia. The patient with b1/b3 deletion was severely oligozoospermic (sperm count 2.6 M/ml). Testicular biopsy reports were available only for three of the seven patients with partial deletions of the AZFc region. One

| STS   | Primer Sequence                                      | Product Size (bp) | Reference |
|-------|-------------------------------------------------------|-------------------|-----------|
| ZFY-F | ACCRCTGCTACTGACATGAT-TACAC                           | 495               | [34]      |
| ZFY-R | GCACYTCATGGATCTACGAAGGCTCA                           | 495               | [34]      |
| SRY-F | GATATTCCCGTCTCTCCGGAA                                 | 472               | [34]      |
| SRY-R | GCTTGTCCTACTCTCATTCTGAG                               | 472               | [34]      |
| sY746-F | TTGACTGCATTACAACTACCA                                 | 216               | [35]      |
| sY746-R | CAGGGGAAAATGTTTGGTGGTTT                             | 326               | [34]      |
| sY84-F | AGAAAGGTCGAAAGCAAGAGGTATT                           | 320               | [34]      |
| sY84-R | GCCTACTCTGGAGGCTTC                                   | 277               | [36]      |
| sY86-F | GTGCACAGACTACTGCTTC                                   | 261               | [37]      |
| sY86-R | ACACACAGAGGCAACAACCT                                    | 261               | [37]      |
| DBY1-F | TATTGCAATCGTGAAGAC                                    | 200               | [38]      |
| DBY1-R | TGCCGGTTCCTCTACTGTC                                   | 274               | [34]      |
| sY117-F | GTTGGTCATGCATGCTCTCATC                                 | 143               | [38]      |
| sY117-R | CAGGGGAGCAAATTTTTTAAAC                                  | 143               | [38]      |
| sY125-F | GGGATAGGAAGGAAAGGTAAC                                 | 301               | [34]      |
| sY125-R | GGCACGACAGCTGTCCGCTG                                   | 301               | [34]      |
| sY127-F | CTGCAGGTCTGCAAGAACAAGGA                                | 125               | [38]      |
| sY127-R | ACAGAAGGCTGACTGACTGAG                                  | 125               | [38]      |
| sY131-F | ACATATGCCCTGGCCACTCTA                                  | 120               | [34]      |
| sY131-R | TCCAGGTCCTCTGCTGCTG                                   | 120               | [34]      |
| sY134-R | GTCTGCATGTCAGAACGAC                                   | 95                | [14]      |
| sY134-F | CACCAGATGGACACTGACTGAG                                  | 95                | [14]      |
| sY152-F | GAAGACTGTCGCAAGTCTTC                                   | 385               | [8]       |
| sY152-R | GACCAGGCTGCAAGTCTTC                                   | 385               | [8]       |
| sY254-F | GGGTGTTACGACAAGGCAAA                                   | 527               | [8]       |
| sY254-R | GAACGTATCTTACCAAGGAG                                      | 527               | [8]       |
| sY255-F | GTTACAGGATGGCCGTCGAT                                   | 123               | [34]      |
| sY255-R | CTCTGTCTGGCTGACACAC                                     | 123               | [34]      |
| sY272-F | GGTTGAGTCAAAATTGAT-CATG                                 | 385               | [8]       |
| sY272-R | CTTTACCAACAGAGAGAGG                                    | 385               | [8]       |
| sY1191-F | GAGCCCGAGTATCGTACCA                                    | 527               | [8]       |
| sY1191-R | TAAAAAGGCAAGACTGCCAG                                   | 527               | [8]       |
| sY1291-F | GGGAGGAGGAAAGTGCTGCAA                                  | 527               | [8]       |
| sY1291-R |
patient had Sertoli cell-only syndrome (SCOS), one had spermatogenic arrest and one patient had severe hypo-
spermatogenesis. One subject (1/50, 2.0%) in the control
group proved to have gr/gr deletion, which was the only
pattern of partial deletions of the AZF region observed in
the control group. The frequency of partial deletions in
experimental and control groups is given in Table 3. The

![Figure 1. Representative ethidium bromide-stained agarose gels for detection of classic (full) AZF microdeletions (case #83). The STSs and the PCR product sizes are indicated above each band. L: 100 bp DNA Ladder.](image)

Table 2:
Partial AZFc deletion classification scheme employing the sY1191 and sY1291 STSs.

| AZFc deletion pattern | sY1291 | sY1191 |
|-----------------------|--------|--------|
| No deletion           | +      | +      |
| gr/gr deletion        | −      | −      |
| b2/b3 deletion        | +      | −      |
| b1/b3 deletion        | −      | −      |

(+)= No deletion; (−)= deletion

Table 3:
Frequency of partial AZFc deletions in study and control groups.

| Group    | n  | gr/gr | b2/b3 | b1/b3 | Total |
|----------|----|-------|-------|-------|-------|
| Study    | 125| 6 (4.8%) | None  | 1 (0.8%) | 7 (5.6%) |
| Control  | 50 | 1 (2.0%) | None  | None  | 1 (2.0%) |

p = 0.409

The gr/gr deletion was present in both infertile (6/125, 4.8%) and control (1/50, 2%) groups. No statistically signif-
ificant difference in the frequency of this deletion was
found between the two groups (p=0.409). The deletion
frequency of b1/b3 in the infertile group was 0.8% (1/125),

sY1291 and sY1191 STS PCR results for selected patients with partial AZFc deletions are shown in Figures 2 and 3.
while it was not observed at all in the control group. The difference in deletion frequency between the two populations also was not statistically significant ($p = 0.528$). Considering both types (gr/gr and b1/b3) of partial deletions, no statistically significant difference ($p = 0.321$) could be found between the two groups.

First degree relatives of four patients with the gr/gr deletion were also determined to have this particular deletion, as shown in Figure 4.

No family history of infertility was noted for study subjects with a partial AZFc deletion, except one (case# 79, sperm count 0.7 M/ml) whose brother was found to have severe oligozoospermia (sperm count 0.4 M/ml). This brother was also found to have the gr/gr deletion (Figure 4, lane 11). Moreover, his maternal uncle suffered from infertility but this person was not available for testing.

Lane 5: father of case# 17, Lane 6: case# 49, Lane 7: brother of case# 49, Lane 8: case# 64, Lane 9: brother of case# 64, Lane 10: case# 79, Lane 11: brother of case# 79.

**Discussion**

**Y-Chromosome Classical Microdeletions**

The present study identified no classic AZF microdeletions in the long arm of the Y-chromosome in this population of Palestinian males. This is in general agreement with some previously published studies [40–42], although varied frequencies of Y-chromosome microdeletions (range=0.75 to 35%) have been reported by others [22, 23, 43–53]. The variance 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.

In support of the association between genetic background and Y-chromosome microdeletions, Kihale et al. (2005) studied the occurrence of Y chromosomal microdeletions in two different populations, Japanese and Africans [45]. They found a prevalence of 6.2% in the Japanese goup but no Y chromosome microdeletions in Africans. Similarly,
Y haplogroups seem to be a key factor in the occurrence of microdeletions in that certain haplogroups (e.g., haplogroup E) are more vulnerable to deletions than others. Indeed, certain haplogroups may confer protection (e.g., haplogroup J) against microdeletions [22,54,55]. This bizarre behavior of the different Y-chromosome haplogroups is related to the number, presence/absence and arrangement of certain DNA elements (e.g., LIPA4 element in HERV15q2) required for homologous intrachromosomal recombination leading to deletions.

It should be noted that Nebel et al. (2001) reported that Palestinians differ in their Y-chromosome pool from Europeans and other Middle Eastern populations [52]. They found a high proportion of Palestinians (55.2%) residing in Israel and the Palestinian Authority (West Bank) area belong to J haplogroup (which is assumed to be protective against microdeletions). This feature might explain the inability to observe classical 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 [23,44,47,51,56,57]. Our patient population included only one SCOS patient who proved to have gr/gr AZFc partial deletion. Patients with chromosomal abnormalities evident by GTG banding, however, 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 no Y-chromosome microdeletions were found in this study population.

Figure 3. Ethidium bromide-stained agarose gel photo for case# 71, where b1/b3 partial AZFc deletion was identified. L: 100 bp DNA Ladder, Lane1: sY1191 negative control (female DNA), Lane2: sY1191 positive control (fertile male DNA), Lane3: sY1291 negative control (normal female DNA), Lane 4: sY1291 positive control (fertile male DNA), Lane 5: sY1191 for case# 71, Lane 6: sY1291 for case# 71. Note that both sY1191 and sY1291 are absent in case# 71, consistent with b1/b3 partial AZFc deletion.

Figure 4. Ethidium bromide-stained agarose gel for determination of gr/gr deletion source (inherited vs. de novo). L: 100 bp DNA Ladder, Lane 2: negative control (female DNA), Lane 3: positive control (fertile male DNA), Lane 4: case #17.
AZFc Partial Deletions

The gr/gr deletion is associated with loss of about half the AZFc gene content, including two of the four copies of the major AZFc candidate gene, known as DAZ. Our analysis revealed this defect in 6 (4.8%) cases. Another deletion, b1/b3, is associated with a loss of nearly 1.8 Mb of the AZFc region and also eliminates two DAZ copies. This was present in 1 (0.8%) case. In terms of these deletions, there was no statistically significant difference between the experimental and control groups. The gr/gr frequency (4.8%) observed in our study is comparable to that previously reported [11,17,30].

The pathological significance of these partial deletions is not yet clear. The gr/gr 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 [17,28–30]. In our patient population gr/gr deletion was encountered in oligozoospermic, severely oligozoospermic, and azoospermic males, indicating that this deletion cannot be linked to a particular type of spermatogenic impairment. Other investigators found no association between AZFc partial deletions (gr/gr or b1/b3) and male infertility [11,18,31–33,49,58–60]. Whether such partial deletions are associated with certain male lineage haplogroup(s) remains unresolved. Indeed, the b2/b3 deletion (which was not detected in our patients) has been shown to consistently occur in Y haplogroup N [39], and the gr/gr deletion has been found in association with haplogroups D2b and Q1 [17,61,62]. Therefore, the effect of partial deletion on male infertility may vary according to the Y haplogroup of the study subjects.

Although the Y haplogroup(s) of our study patients was not specifically assessed, these data (especially regarding the gr/gr deletion) suggest that this deletion could be a heritable polymorphism rather than a de novo arrangement.

This is because the incidence of this deletion was not significantly different between patients and controls. Furthermore, in three cases with gr/gr deletion, the origin of the deletion appeared to be inherited and not a de novo rearrangement, as their fertile first-degree relatives had the same deletion. The fourth available relative was the brother of case# 79, who showed the same deletion pattern as his brother, i.e., another gr/gr deletion. Considering that this individual was severely oligozoospermic, it suggests that the Y-chromosome is not a major factor in this particular case, as their maternal uncle was also infertile.

The b1/b3 partial deletion was observed in only one 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 particular deletion has been observed in both control and patient groups previously however, leading some investigators to conclude that b1/b3 is probably irrelevant to spermatogenesis [60].

In conclusion, classic Y-chromosome microdeletions were not detected in this unselected population of idiopathic oligo- and azoospermic fertile patients. Results concerning gr/gr partial deletion suggest that this pattern does not represent a risk factor for male infertility and might be considered a heritable variant in this population. Further studies are needed in order to elucidate the structure of Y haplogroup(s) prevalent here, and to explore other genetic, epigenetic and/or nutritional factors that contribute to idiopathic oligo- and azoospermia in the Gaza population.

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