Analysis of partial azoospermia factor c deletion and DAZ copy number in azoospermia and severe oligozoospermia

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AZFc region—azoospermia factor—male infertility—Y chromosome microdeletion

Summary
Microdeletions of the azoospermia factor (AZF) regions in the Y chromosome are a well-known genetic cause of male infertility, resulting in impairment of spermatogenesis. However, the partial deletions of AZFc region related to spermatogenic impairment are controversial. We investigated partial deletion of AZFc region and DAZ copy number in a population of Iranian infertile men and normozoospermic controls. In total, 154 infertile men (113 patients with azoospermia, 41 with oligozoospermia) and 111 normozoospermic controls were analysed using PCR. Gene dosage analysis of the DAZ genes was performed by fragment analysis. Our results showed that the frequencies of gr/gr deletion in the azoospermic, severe oligozoospermic and normozoospermic men were 4.4% (5/113), 7.3% (3/41) and 1.8% (2/111) respectively. In the azoospermic patients, the frequency of b2/b3 was 1.8% (2/113). Partial AZFc deletions were not significantly different between the infertile and normozoospermic men. The frequencies of gr/gr deletions and b2/b3 were not significantly different between the azoospermic/severe oligozoospermic men and normozoospermic controls. Our data suggested that gr/gr deletion was not associated with azoospermia/oligozoospermia in an Iranian population.

Introduction
Infertility affects 10–15% of couples worldwide, and in approximately 40% of couples, the male partner has a problem in his reproductive system (Agarwal et al., 2015). After the Klinefelter syndrome, microdeletions of the long arm of the Y chromosome are the second most frequent genetic cause of male infertility (Krausz et al., 2013). To avoid vertical transmission of Yq microdeletions to the male offspring via assisted reproductive technology such as intracytoplasmic sperm injection (Krausz & Degl’Innocenti, 2006), screening for these microdeletions has become a part of the routine diagnostic workup for men with azoospermia or oligozoospermia (Krausz et al., 2013).

The male-specific region of the Y chromosome (MSY) consists of long, Y-specific repeats called amplicons. Homologous recombinations between amplicons generate deletions, commonly resulting in spermatogenic failure that is the most common form of male infertility (Lu et al., 2009). Four recurrent microdeletions of the Y chromosome are clinically relevant and are found in men with azoospermia and severe oligozoospermia including AZFa, AZFb (P5/proximal P1), AZFbc (P5/distal P1 or P4/distal P1) and AZFc (b2/b4). The most frequent deletion type is the AZFc region deletion (~80%) followed by AZFa (0.5–4%), AZFb (1–5%) and AZFbc (1–3%) deletion (Krausz et al., 2013).

In addition to the complete deletion of the AZFc region, three recurrent partial AZFc deletions have been described (The gr/gr, b1/b3 and b2/b3 deletions), resulting from nonallelic homologous recombination. The gr/gr deletion seems to be a significant risk factor for spermatogenic failure, while the b1/b3 and b2/b3 deletions do not seem to affect sperm production (Repping et al., 2003, 2004; Fernandes et al., 2004). The gr/gr deletion is divided into five rearrangement types including simple gr/gr deletion, gr/gr deletion-b2/b4 duplication, gr/gr deletion-b2/b4 multiple duplication, gr/gr deletion-CDY1 and DAZ amplification (Krausz et al., 2009; Shahid et al., 2011).

The results of gr/gr deletions in spermatogenetic impairment have been different among populations (Choi et al., 2012). To determine the role of the partial AZFc deletion in spermatogenesis and evaluate their clinical consequences, we conducted this study in idiopathic
infertile men with azoospermia/severe oligozoospermia and normozoospermic controls in an Iranian population.

Materials and methods

Subjects
In total, 156 infertile men including 115 patients with idiopathic nonobstructive azoospermia and 41 patients with severe oligozoospermia (Sperm concentration $< 5 \times 10^6$ ml$^{-1}$), who admitted to the Avicenna Infertility Clinic, Tehran, Iran, were included. The control group consisted of 111 normozoospermic fertile men with at least one child. Cytogenetic analysis for all the infertile men showed a normal 46, XY karyotype. Patients' information including age, serum level of FSH, LH, testosterone and semen analysis results was obtained from database of Avicenna Infertility Clinic laboratories. The ethics committee of the Avicenna Research Institute approved the study, and written informed consent was obtained from all participants.

Screening for partial AZFc deletions
Genomic DNA was extracted from peripheral blood leukocytes by the salting out method (Miller et al., 1988). In the case group, two azoospermic men with classical AZF deletions, using STS analysis recommended by the EAA/EMQN, were excluded, which were detected in our previous study (Saliminejad et al., 2012). Partial deletions of the AZFc region were detected by the STS markers sY1161, sY1197, sY1191, sY1291, sY1206 and sY1201.

The gr/gr partial deletion removes about half of AZFc region, including two copy of DAZ, one copy of CDY1 and BPY2 genes, and characterised by the absence of amplification of sY1291. The b2/b3 and b3/b4 deletions were detected by absences of sY1191 and sY1206 respectively. On the other hand, absences of the two STS markers sY1291 and sY1191 indicated a b1/b3 deletion (Choi et al., 2012).

Screening for each STS marker was performed by a multiplex PCR containing relevant STS and ZFY gene as internal control. Each multiplex PCR contains the following: 1 x PCR Buffer, 2 mM MgCl$_2$, 1U Taq DNA polymerase (BioFlux, Tokyo, Japan), 0.5 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 10 pmol of each primer, 50 ng of template DNA and sterile distilled water to 25 μl. Amplification conditions start with an initial denaturation step of 3 min at 94 °C, followed by 32 cycles of 30 s denaturation (94 °C), 30 s annealing (60 °C) and 30 s extension (72 °C), ended by a final extension for 10 min (72 °C). All PCR products were subjected to electrophoresis on 1.5% agarose gel prepared in 1 x TAE, stained with ethidium bromide and visualised by exposure to UV light.

Analysis of DAZ gene dosage
Dosage analysis of the DAZ gene was performed by fragment analysis on an Applied BioSystems 3130 Genetic Analyser. The PCR products were resolved on an ABI-3100 genetic analyser, and patient data were normalised to controls for copy-number differences using the GENE MARKER Software version 1.5 (SoftGenetics, State College, PA, USA). Because DAZ and autosomal DAZL are 90% identical, therefore, they were co-amplified using the forward 5'-TTAAGTACTACTGTAGAGC-3' and reverse 5'-GTTTCATTGATAATGAGAGTAGAGGC-3' primers. Different length of PCR products for DAZ (214 bp) and DAZL (217 bp) allowed the products to be distinguished. The forward primer was labelled at the 5' end with FAM fluorescent dye. Each diploid cell of a male contains two and four copies of the DAZL and DAZ genes respectively; accordingly, the peak height for DAZ is twice longer than DAZL (2 : 1 ratio). The gene dosage of DAZ was calculated by the comparison of peak area with DAZL, as an internal standard with known number of copies in the electrophoretograms. In the cases of gr/gr deletion, DAZ/DAZL patterns showed 1 : 1 ratio (Fig. 1).

The results were analysed by SPSS 11.5 (SPSS, Chicago, IL, USA). Fisher’s exact test was used to evaluate the gr/gr deletion between cases and controls. P values <0.05 were considered statistically significant.

Results
The mean ages of azoospermic and severe oligozoospermic patients were 39.8 (SD = 7.2) and 41.2 (SD = 7.3) years respectively. The mean plasma LH (IU/L), FSH (IU/L) and testosterone (ng/ml) in the azoospermic and severe oligozoospermic groups were 12.6 versus 6.7, 27.0 versus 11.7 and 9.8 versus 8.8 respectively.

Of 156 infertile men, two azoospermic cases, one with AZFc and the second with AZFbc deletions, were excluded from the partial AZFc deletion analysis. Finally, 154 azoospermic/oligozoospermic men and 111 normozoospermic controls (all with at least on DAZ copy) were further examined for AZFc gene copy numbers using the fragment analysis. We found two types of partial AZFc deletions, the gr/gr and b2/b3 deletions. In the azoospermic and severe oligozoospermic men, the frequencies of gr/gr deletion were 4.4% (5 of 113) and 7.3% (3 of 41) respectively. In the azoospermic patients, the frequency of b2/b3 was 1.8% (2 of 113). The distributions of DAZ copy-number alteration in the case and normozoospermic control groups in the nondeletion population
are shown in Table 1. Fisher’s exact test was used to evaluate the gr/gr deletion between cases and controls.

Partial AZFc deletions were not significantly different between the men with spermatogenic impairment and normozoospermic control group (P value = 0.20). The frequencies of gr/gr deletions (P value = 0.70) and b2/b3 (P value = 0.51) were not significantly different between the azoospermic/oligozoospermic men and normozoospermic control.

The results of fragment analysis showed that in two normozoospermic men with gr/gr deletion the DAZ/DAZL ratio was 2 : 1, which indicated the normal DAZ copy (Table 1). 87.5% (7 of 8) of infertile men with gr/gr deletion had two copy of DAZ gene (DAZ/DAZL ratio 1 : 1). One azoospermic patient with gr/gr deletion had eight copies of the DAZ gene (DAZ/DAZL ratio 4 : 1).

**Discussion**

The molecular diagnosis of Y chromosomal microdeletions has been a common routine diagnostic genetic test in azoospermic/severe oligozoospermic and severe oligozoospermic men. It has been demonstrated that the gr/gr deletion is a significant risk factor for impaired sperm production. However, the screening for gr/gr deletion as a routine diagnostic test is still a debated issue among experts (Krausz et al., 2013). Among a different number of partial AZFc deletion, only gr/gr is of potential interest in clinical application. As the carriers of the gr/gr deletion may show variable spermatogenic phenotypes ranging from azoospermia to normozoospermia, its clinical significance is still a matter of debate (Krausz et al., 2013).

We investigated partial deletions of AZFc region and their clinical implications in an Iranian population. Briefly, we initially screened AZFa, AZFb and AZFc regions for classical Yq microdeletions and then partial AZFc deletion, and finally, the DAZ gene copy number was determined. The total frequency of partial AZFc deletions, regardless of deletion type, was not significantly different (P value = 0.20) between azoospermic/oligozoospermic (eight subdeletions of 154 infertile men).

**Table 1** Results of AZFc subdeletion and DAZ copy number in the azoospermic and severe oligozoospermic men and controls. Only gr/gr and b2/b3 subdeletions were seen in this study.

<table>
<thead>
<tr>
<th>Subjects no.</th>
<th>DAZL/DAZ ratio</th>
<th>Subdeletion</th>
<th>Deleted STS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoo 28</td>
<td>1 : 4</td>
<td>b2/b3</td>
<td>SY1191</td>
</tr>
<tr>
<td>Azoo 108</td>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azoo 8</td>
<td>1 : 1</td>
<td>gr/gr</td>
<td>SY1291</td>
</tr>
<tr>
<td>Azoo 29</td>
<td>1 : 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azoo 67</td>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azoo 92</td>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azoo 101</td>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligozo 6</td>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligozo 28</td>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligozo 31</td>
<td>1 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 33</td>
<td>1 : 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 35</td>
<td>1 : 2</td>
<td></td>
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</tbody>
</table>

**Fig. 1** Different gene dosages of DAZ/DAZL genes. The x-axis shows length of PCR products in base pairs and y-axis shows fluorescent intensity. (a) DAZ/DAZL 1 : 1 ratio in cases of gr/gr deletion and two DAZ copies deletion; (b) DAZ/DAZL 2 : 1 ratio in cases with no DAZ deletion; (c) DAZ/DAZL 1 : 4 ratio which indicate duplication of DAZ copy number; (d) DAZ/DAZL 0 : 1 ratio in the case of complete deletion of DAZ genes.
men and normozoospermic men (two subdeletions of 111 healthy men). This result suggested that such mutations could not be a risk factor for impaired spermatogenesis in Iranian population.

Two types of partial AZFc deletions including b2/b3 and gr/gr were identified in our population, none of which was statistically associated with impaired spermatogenesis. The frequencies of gr/gr deletions (P value = 0.70) and b2/b3 (P value = 0.51) were not significantly different between the azoospermic/oligozoospermic men and normozoospermic control. Our result is consistent with several studies, which showed no association between gr/gr deletions and azoospermia/oligozoospermia in Caucasian (Hucklenbroich et al., 2005; Stouffs et al., 2008), Asian (de Carvalho et al., 2006; Zhang et al., 2006; Lin et al., 2007; Wu et al., 2007; Lu et al., 2009). However, our result was not consistent with two other meta-analyses (Visser et al., 2009; Stouffs et al., 2010) and with the largest study on Caucasians (Giachini et al., 2008) and the Korean population (Choi et al., 2012).

We found the b2/b3 deletion only in azoospermic men, indicating that b2/b3 deletions might affect on spermatogenesis with mechanism yet to be revealed. This results in contrary to the previous study in a Korean population (Choi et al., 2012). Data from very large study populations in China and a North African population suggest that b2/b3 deletion is a risk factor for impaired sperm production (Wu et al., 2007; Lu et al., 2009; Eloualid et al., 2012).

Our results showed that the carriers of gr/gr deletion were mainly oligozoospermic 7.3% (3/41) than the azoospermic 4.4% (5/113), and in fact, this deletion is more likely to be associated with oligozoospermia than with azoospermia (Giachini et al., 2008; Stouffs et al., 2010). The overall frequency of gr/gr deletions in our study in Iranian patient with spermatogenetic failure (~3.2% or 7 of 154) was close to Europeans (~4.5%) and lower than Han Chinese (10.0% and 10.6%) and Korean (8.5%) populations (Navarro-Costa et al., 2010; Stouffs et al., 2010). This might be resulting from different origins of the study populations. In a recent study by Sen et al. (2015) in an Indian population, AZFc deletions were a major risk factor for male infertility. The authors found that the frequency of gr/gr was higher in oligozoospermic (10.5%) and azoospermic (11.6%) men as compared to normozoospermic controls (5.1%).

The results of fragment analysis showed that in two normozoospermic men with gr/gr deletion, DAZ copy number was normal. These data suggesting that partial duplications followed gr/gr deletion and compensated for the reduction in DAZ copy number caused by the gr/gr deletion (Lin et al., 2007).

Finally, despite the heterogeneity of the study populations, results of meta-analyses showed a significant association between gr/gr deletion and increased risks of reduced sperm output/infertility. A gr/gr deletion will be obligatorily transmitted to the male offspring and the partial deletion may expand to a complete AZFc deletion in the next generations; however, currently the data are sufficient to draw final conclusions on this specific risk. At present, no general agreement to advise routine testing of gr/gr deletion has been reached (Krausz et al., 2013).

Conclusion

Our data suggested that gr/gr deletion was not associated with azoospermia and oligozoospermia in Iranian population.

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References


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