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Combined deletion of *DAZ2* and *DAZ4* copies of Y chromosome *DAZ* gene is associated with male infertility in Tunisian men

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ABSTRACT

The relationship between male infertility and AZFc micro-deletions that remove multiple genes of the Y chromosome varies among countries and populations. The purpose of this study was to analyze the prevalence and the characteristics of different *Deleted in azoospermia* (*DAZ*) gene copy deletions and their association with spermatogenic failure and male infertility in Tunisian men. 241 infertile men (30.7% azoospermic (n = 74), 31.5% oligozoospermic (n = 76) and 37.7% normozoospermic (n = 91)) and 115 fertile healthy males who fathered at least one child were included in the study. Three *DAZ*-specific single nucleotide variant loci and six bi-allelic *DAZ*-SNVs (I–VI) were analyzed using polymerase chain reaction (PCR)–restriction fragment length polymorphism and PCR. Our findings showed high frequencies of infertile men (73.85%) and controls (78.26%) having only three *DAZ* gene copies (*DAZ1*/*DAZ2*/*DAZ3* or *DAZ1*/*DAZ3*/*DAZ4* variants); so deletion of *DAZ2* or *DAZ4* were frequent both in infertile (36.5% and 37.3%, respectively) and fertile groups (33.9% and 44.3%, respectively) and removing *DAZ4* copy was significantly more frequent in oligospermic than in normospermic men ($p = 0.04$) in infertile group. We also report for the first time that simultaneous deletion of both *DAZ2* and *DAZ4* copies was significantly more common in infertile men (12.4%) than in fertile men (4.3%) ($p = 0.01$). However, deletions of *DAZ1*/*DAZ2* and *DAZ3*/*DAZ4* clusters were very rare. Analysis of *DAZ* gene copies in Tunisian population, suggested that the simultaneous deletion of *DAZ2 and DAZ4* gene copies is associated with male infertility, and that oligospermia seems to be promoted by removing *DAZ4* copy.

1. Introduction

Deletions on the human Y chromosome long arm (Yq) can often lead to spermatogenic impairment, one of the main causes of male infertility (Reijo et al., 1995; Tiepolo and Zuffardi, 1976). At least three spermatogenesis controlling loci, named azoospermia factors a, b and c (*AZFa*, *AZFb* and *AZFc*), have been mapped in Yq11 (Vogt et al., 1996). *AZFc* is the most frequent region involved in deletions in many populations (Zhang et al., 2006). Indeed, approximately 80% of AZF micro-deletions occur in AZFc region and most of them result in entire *DAZ* (deleted in azoospermia) gene deletion (Li et al., 2013; Simoni et al., 2004). *DAZ* gene has four copies, organized in two distinct clusters: *DAZ1*/*DAZ2* and *DAZ3*/*DAZ4* that play an important role in spermatogenesis (Fernandes et al., 2006; Reynolds and Cooke, 2005). This gene encodes RNA-binding proteins exclusively in testicular tissue (Kim et al., 2009; Writzl et al., 2005). Polymorphic expression among DAZ proteins has been reported, indicating unequal activities of different *DAZ* copies (Kim et al., 2009). In the literature, several investigators found an association between removing *DAZ* gene copies and spermatogenic failure (de Llanos et al., 2005; Giachini et al., 2005; Repping et al., 2003), whereas others did not (Carvalho et al., 2006; Hucklenbroich et al., 2005). Thus, research on *DAZ* gene or on other related genes is necessary to determine the relationship between AZFc polymorphisms or deletions and male infertility (Ye et al., 2013). So the aim of the current study was to evaluate frequency and different types of *DAZ* deletions in Tunisian population, in order to determine the contribution of these *DAZ* deletions to male infertility. A combined methodological approach including detection of different *DAZ* gene copies by sequence nucleotide variant (SNV) analysis and *DAZ*-specific sequence-tagged site (STS) markers was used.

2. Materials and methods

2.1. Patients

The studied populations consisted of 115 fertile healthy males who fathered at least one child (control group) and 241 male partners

Abbreviations: *DAZ* gene, *Deleted in AZoospermia* gene; AZF, AZoospermia factors; PCR-RFLP, Restriction Fragment Length Polymorphism-PCR; SD, standard deviation.

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from consecutively enrolled couples who had their first infertility consultation in the Reproductive Biology Laboratory at the Faculty of Medicine of Sfax (Tunisia). All subjects and controls were of Tunisian ethnic origin. Written informed consent was obtained from each patient, and approval was given by the local ethics committee in Tunisia. The majority of patients had primary infertility (74.79%), with a mean duration of 4 years. They were aged between 23 and 57 years, the mean age was 34 years (standard deviation (SD) 5.69). For each patient, semen analysis was performed on a sample of semen collected by masturbation after 3–5 days of sexual abstinence. Patients were also subject to detailed clinical investigations and according to spermiogram analysis, endocrinology investigation, including FSH, LH and testosterone, and histology of testis biopsy were performed when possible. Patients with obstructive azoospermia, anatomical or syndromic abnormalities were excluded. Patients with chromosomal abnormalities were not excluded from the study. On the basis of spermiogram and according to the criteria of the World Health Organization (WHO, 2010), we subdivided patients into 3 groups: patients with normal sperm count (normozoospermia, $>15 \times 10^6$/ml; n = 91(37.7%)), patients with a complete absence of spermatozoa in the ejaculate (azoospermia, n = 74 (30.7%)) and patients with reduced sperm count (oligozoospermia, $<15 \times 10^6$/ml; n = 76 (31.5%)). The mean value of sperm concentration in oligospermic group was 4.49 million/ml ± 4.45.

2.2. DNA extraction

Total DNA was extracted from peripheral blood leucocytes using phenol/chloroform standard procedures (Lewin and Stewart-Haynes, 1992).

2.3. Standard screening of Y chromosome micro-deletions

We performed standard screening of Y chromosome AZFc microdeletions in all infertile men by PCR using pairs of oligonucleotide primers, to amplify two Y-specific STSs in AZFc locus (Sy254, Sy255). As positive control, we amplified one specific STS in *SRY* gene using one pair of specific primers. PCR was carried out in a total volume of 25 μl. Reaction mixture included 100 ng of each DNA sample, 5 μl of ammonium sulfate PCR buffer (15 mM), 200 mM of deoxynucleotidetriphosphates (dNTPs), 1 μM of each primers and 2 μl of Taq polymerase. After an initial denaturation step at 94 °C for 5 min, amplification was performed for 35 sequential cycles, each including 40 second denaturation at 94 °C, 50 second primer annealing at 55 °C and 50 second extension at 72 °C. A final extension step at 72 °C for 7 min ended the PCR program. PCR products were then analyzed by electrophoresis on 2% agarose gel containing ethidium bromide and visualized under ultraviolet light.

2.4. DAZ gene copy determination

To identify different *DAZ* gene copies in patients and fertile men, we analyzed six bi-allelic *DAZ*–SNVs (I–VI), two *DAZ1* + *DAZ4*-specific STS (*DAZ*-RRM3 and Sy152) and one *DAZ3*-specific STS (Y-*DAZ3*), whose sequences are deposited in GenBank. In *DAZ*–SNV analysis, Allele A and Allele B were named and analyzed by PCR-RFLP as described previously by Fernandes et al. (2002). For SNV and STS amplification, PCR reaction was carried out in a total volume of 25 μl and was composed as follows: 75 ng of each DNA sample, 2.5 μl of PCR buffer (BIOTAQ™ DNA Polymerase), 2 μl of $MgCl_2$ (BIOTAQ™ DNA Polymerase), 1 μl of deoxynucleotidetriphosphates (dNTPs) (10 mM), 1 μl of each primers (10 μM), 2.4 μl of Bétaïne (5 M) and 2 μl of Taq polymerase (BIOTAQ™ DNA polymerase). After an initial denaturation step at 94 °C for 5 min, amplification was performed for 35 sequential cycles, each including 30 second denaturation at 94 °C, 30 second primer annealing at 61 °C and 30 second extension at 72 °C. A final extension step at 72 °C for 7 min ended the PCR program.

After amplification by PCR, digestion by adequate restriction enzymes was performed in a final volume of 10.3 μl, containing 8 μl of PCR product, 1 μl of reaction buffer, 0.3 μl of restriction enzyme, 1 μl of H_2O and incubated overnight at 37 °C for Afl III, Dra I, Alu I, Mbo I, and Fsp I and at 65 °C for Taq I. The products of each reaction (PCR and digestion) were verified by electrophoresis on 2% agarose gel containing ethidium bromide and visualized under ultraviolet light.

2.5. Statistical analysis

Comparison of the observed frequencies was made using the chi-square test (contingency tables $2 * 2$) with "contingent" program or "Epi InfoTM7" program. For contingency tables with a number of observed data less than five or zero, a Fisher's exact test and the Clump program were used. Probability values of $p < 0.05$ were regarded as statistically significant. For Power test calculation, IBM SPSS Statistics software version 20 (IBM Corporation, Somers, NY) was used.

3. Results

Using specific STS analysis, we verified the absence of classical Y micro-deletions in AZFc region in all infertile patients. Analysis of *DAZ*-specific SNVs allowed us to characterize the deleted *DAZ* gene copies in infertile and fertile men. We found high and similar frequencies of *DAZ2* and *DAZ4* gene copy deletions in both infertile (36.5% and 37.3%, respectively) and fertile groups (33.9% and 44.3%, respectively). The frequency of simultaneous deletion of *DAZ2* and *DAZ4* copies was significantly higher ($p = 0.01$) in infertile men (30/241) than in fertile men (5/115) (Table 1). However, low frequencies of *DAZ1*/*DAZ2* cluster deletion were found in infertile and fertile men (8.7% and 10.4% respectively) and a very rare *DAZ3*/*DAZ4* cluster deletion was noted in infertile men (0.8%); this last deletion was totally absent in fertile men. Moreover, the number of patients with *DAZ3*/*DAZ4* deletion was very low but it isn't significant ($p = 0.15$) with a power test = 0.29.

Comparison between the three subgroups of infertile men showed that *DAZ2* copy deletion was significantly more frequent in azoospermic and normospermic (41.8% and 42.8% respectively) than in oligospermic patients (23.6%) (Table 2). Removing *DAZ4* copy was frequent in oligospermic and azoospermic patients (Table 2) and was significantly more frequent in oligospermic than in normospermic men (44.7% vs 29.6%; $p = 0.04$).

4. Discussion

Micro-deletions in Y chromosome AZFc region can lead to different degrees of spermatogenic failure, with a significant variability between individuals, ranging from the absence of germ cells in testis to the presence of spermatozoa in the ejaculate (oligospermia) (Giachini et al., 2005; Shaqalaih et al., 2009). They involve more frequently *DAZ* gene that is present in multiple copies in AZFc. It is not clear if all the *DAZ* copies are expressed in testis but their deletion could have a deleterious impact on spermatogenesis. In this study we tried to elucidate the effect of *DAZ* gene copy variation on spermatogenesis and on fertility in Tunisian men. We found relatively high frequencies of *DAZ2* and *DAZ4* deletions in infertile and fertile subjects, tat means that individuals carrying four copies of *DAZ* gene seem to be rare in Tunisian population (4.14% of infertile and 6.95% of fertile men). Indeed, the majority (73.85% of infertile patients and 78.26% of fertile men) has only three copies of *DAZ* gene (*DAZ1*/*DAZ2* + *DAZ3* or *DAZ1* + *DAZ3*/*DAZ4*). This finding leads us to suggest that the presence of four copies of *DAZ* gene is not necessary for the achievement of spermatogenesis process, but losing the *DAZ4* copy (the presence of *DAZ1*/*DAZ2* + *DAZ3* variant) seems to favor oligospermia in Tunisian population, since the deletion of this *DAZ* copy was significantly more frequent in oligospermic (44.7%) than in normospermic patients (29.6%) ($p = 0.04$); this effect could be favored by additional genetic and environmental factors. However *DAZ2*