Y-Chromosome Haplotypes in Azoospermic Israeli Men

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Abstract Among azoospermic and severely oligozoospermic men, 7–15% present microdeletions of a region on the long arm of the Y chromosome that has been called AZF (azoospermia factor). Because these deletions present varying relative frequencies in different populations, we decided to ascertain whether their presence was correlated with specific Y-chromosome haplotypes. For that, we evaluated 51 infertile Israeli men, 9 of whom had microdeletions in AZF. Haplotypes were identified using a hierarchical system with eight biallelic DNA markers. We also checked for the presence of the deletion marker 50f2/C, which was absent in all seven patients with isolated AZFc deletion and also in the one patient with isolated AZFb deletion, suggesting that these microdeletions overlap. As expected, haplogroup J was the most common (47%), followed by equal frequencies of haplogroups Y* (xDE, J, K), P* (xR1a, R1b8), K* (xP), and E. In six patients with AZFc deficiencies of comparable size, three belonged to haplogroup J, two belonged to haplogroup P* (xR1a, R1b8), and one belonged to haplogroup R1a. Also, there were no significant differences in the haplotype frequencies between the groups with and without microdeletions. Thus we did not identify any association of a specific haplogroup with predisposition to de novo deletion of the AZF region in the Israeli population.

The involvement of chromosome abnormalities in male infertility was first observed by Tiepolo and Zuffardi (1976), who found that 0.5% of infertile men had cytogenetically visible deletions on the long arm of the Y chromosome (Yq). Since then, intense investigations with molecular markers have demonstrated the presence of Yq microdeletions in 7–15% of males with idiopathic azoospermia or severe oligozoospermia (Reijo et al. 1995; Vogt et al. 1996; Pryor et al. 1997; Foresta et al. 1997; Kleiman et al. 1999; McElreavey and Krausz 1999). The

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Human Biology, June 2004, v. 76, no. 3, pp. 469–478.

KEY WORDS: Y-CHROMOSOME HAPLOTYPES, MICRODELETIONS, MALE INFERTILITY, AZOOSPERMIA FACTOR (AZF), AZOOSPERMIA, OLIGOZOOSPERMIA, ISRAEL, UNIQUE EVENT POLYMORPHISM (UEP), SEQUENCE-TAGGED SITE (STS)

microdeletions cluster in three critical regions, known collectively as azoospermia factor (AZF), of the long arm of the Y chromosome. These regions, AZFa, AZFb, and AZFc, are generally believed to be nonoverlapping (Vogt et al. 1996). Current research challenges include elucidating the genomic mechanisms that generate such recurrent deletions and identifying the genes that cause infertility when deleted.

The most common microdeletions involve the AZFc subregion, but deletions of AZFa and AZFb or deletions involving combinations or all three subregions can also be found (Kent-First et al. 1999; Kim et al. 1999). The usual technique to detect the presence and map the extent of the microdeletions is to use the polymerase chain reaction (PCR) to type specific monomorphic molecular markers called STSs (sequence-tagged sites) (Vollrath et al. 1992). The sequencing of the male-specific region of the Y chromosome (MSY) showed that some STSs previously thought to be unique are, in reality, multicopy, because the long arm of the Y chromosome has numerous duplications and repetitive elements, especially near the heterochromatic region (Smith et al. 1987; Skaletsky et al. 2003).

Homologous recombination between repetitive regions is believed to be the cause of the high incidence of de novo microdeletions in the Y-chromosome long arm. For instance, Kuroda-Kawaguchi et al. (2001) demonstrated that 47 of 48 men with AZFc deletions had the same proximal and distal breakpoints in 229-kb direct repeats flanking AZFc. Likewise, several research groups have shown that some AZFa deletions are caused by a recombinant event between repetitive endogenous retroviral elements (Blanco et al. 2000; Sun et al. 2000; Kamp et al. 2001). Repping et al. (2002) demonstrated that homologous recombination between repetitive palindromic regions in Yq can explain most isolated AZFb deletions and combined AZFb + AZFc deletions. More recently, Skaletsky et al. (2003) found evidence that gene conversion takes place, on average, once per generation in MSY, suggesting that homologous recombination events are recurrent in this region.

Y-chromosome-linked loci are haploid and paternally inherited, and with the exception of the pseudo-autosomal regions, there is no recombination with the X chromosome (Graves et al. 1998). Thus Y-chromosomal UEPs (unique event polymorphisms), microsatellites, and minisatellites are transmitted together as haplotypes (Y Chromosome Consortium 2002). Each male individual has the same Y-chromosome haplotype as his father, brothers, paternal grandfather, paternal uncles, etc., thus establishing patrilineages, which are storytellers of human evolution (Bertranpetit, 2000). Jobling, Bouzekri et al. (1998) showed that one class of infertile males, XX males with PRKX/PRKY translocation, arose predominantly on a particular Y-chromosome haplotype background that apparently was predisposed to the translocation. This observation led us to ask whether AZF microdeletions might also be associated with certain Y-chromosome haplotypes. We present here results of a molecular study of 51 infertile

men from Israel that does not support the hypothesis of an association between Y-chromosome haplotypes and infertility in males.

Materials and Methods

DNA Samples. We studied 51 DNA samples from infertile Israeli males from the Institute for the Study of Fertility, Lis Maternity Hospital, Tel Aviv, Israel. Forty-two of the patients were Jews from diverse geographic origins, eight patients were Palestinians, and one was European. The men underwent a series of tests, including semen analysis, hormone profile, cytology of the testis biopsy, and cytogenetic evaluation (Kleiman et al. 1999). After these tests the 51 patients could be classified as follows: 32 had nonobstructive azoospermia, 1 had obstructive azoospermia, 13 had severe oligozoospermia, and 5 were infertile with unknown semen analysis parameters. All 51 patients were analyzed for Y-chromosome microdeletions. However, four patients were removed from our sample before the Y-chromosome haplotype analysis because they had chromosomal defects involving the X chromosome or an autosome or because they did not have idiopathic infertility. DNA samples were extracted from peripheral blood leukocytes. Two patients with a 45,X/46,XisoY karyotype were also removed from further analyses. Written informed consent was obtained from all patients.

Detection of Microdeletions. All patients were tested both in Israel and in Brazil for the presence or absence of Y-chromosome STSs. The tests performed in Tel Aviv have been described in detail elsewhere (Kleiman et al. 1999). DNA molecular markers analyzed in Brazil covered the three AZF regions: sY84 (Vogt et al. 1992), YRRM1 (RBMY1A1, exon 12) (Stuppia et al. 1996), M9 (Underhill et al. 1997), YAP (Hammer and Horai, 1995), sY254, sY258 (= sY255), and sY465 (Mulhall et al. 1997). The indel marker 50f2/C was analyzed using the method of Jobling, Williams et al. (1998), which uses MSY1, a minisatellite localized at Yp, as an amplification control.

All PCR reactions were done in 12.5 μl with 50 ng genomic DNA, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 200 μ*M* dNTPs, and 1 unit or 0.5 unit (for MSY1/50f2/C) of *Taq* DNA polymerase (Phoneutria, Belo Horizonte, Brazil). The PCR products were resolved in 6% polyacrylamide gels and silver-stained according to the method of Santos et al. (1993). For confirmation of this analysis, selected samples were subjected to PCR with a multiplex set of Cy5-labeled primers that included amelogenin, sY258, YRRM1, sY84, and sY465 loci and analyzed in a fluorescent automatic DNA sequencer (ALFExpress, Amersham-Pharmacia, Uppsala, Sweden). Many of the polymorphic markers used for haplotyping were located at Yq (Figure 1) and were also helpful in establishing the extent of the microdeletions.

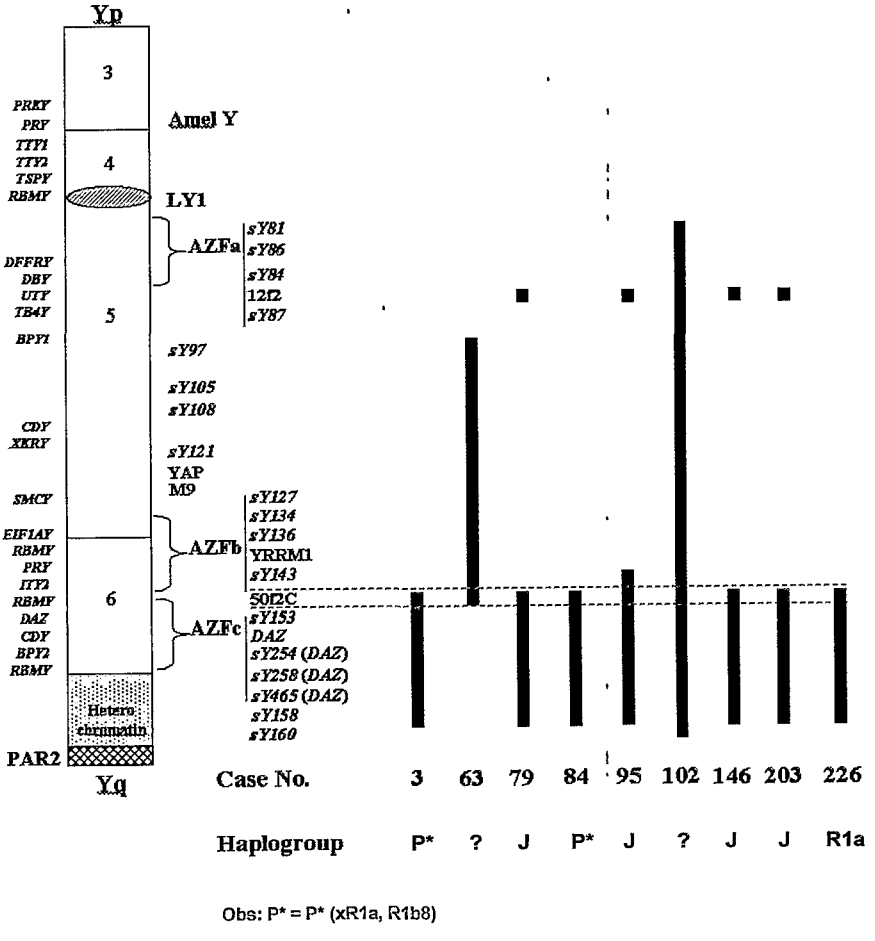

Figure 1. Relative location of polymorphic and deletion markers [adapted from Yen (1999)] and deletion mapping for the nine infertile patients with microdeletions. The primer sequences for all markers and the amplification protocols have been described elsewhere: YAP (Hammer and Horai, 1995); M9 (Underhill et al. 1997); 12f2 (Rosser et al. 2000); sY81 and sY84 (Vollrath et al. 1992); YRRM1 (Stuppia et al. 1996); sY258, sY254, and sY465 (Mulhall et al. 1997); amelogenin (Sullivan et al. 1993). The Y-chromosome haplogroup of each patient is shown.

Y-Chromosome Haplogroup Analysis. The Y-chromosome haplogroup analysis was performed using eight PCR unique event polymorphisms (UEPs) known to be polymorphic in the Israeli population: two indels (insertion–deletion polymorphisms) [YAP (Hammer and Horai, 1995) and 12f2 (Rosser et al. 2000)] and six single nucleotide polymorphisms (SNPs) [SRY −8299 (Whitfield et al. 1995), sY81 (Seielstad et al. 1994; Underhill et al. 1996), M9 (Underhill et al. 1997), SRY −1532 (Kwok et al. 1996), 92R7 (Mathias et al. 1994; Hurles et al.

1998) and SRY −2627 (Bianchi et al. 1997)]. The nomenclature of Y-chromosome haplogroups is that of the Y Chromosome Consortium (2002). The primer sequences for all markers are described elsewhere (see Figure 1 caption). All PCR products were resolved in 6% polyacrylamide gels and silver-stained according to the method of Santos et al. (1993).

Statistical Analysis. All tests were done using the software Arlequin, version 1.1 (Schneider et al. 1997). Specifically, we used an exact test of population differentiation, which tests the hypothesis of a random distribution of k different haplotypes among r populations, as described by Raymond and Rousset (1995). This test is analogous to Fisher's exact test on a 2×2 contingency table extended to an $r \times k$ contingency table. Power analysis was performed with the software G*Power (Erdfelder et al. 1996).

Results

Y-Chromosome Deletion Analysis. We analyzed 51 infertile men and found AZF microdeletions in 9 of them (Figure 1). Isolated AZFc deletions were seen in seven patients, an isolated AZFb deletion was seen in one patient, and a large deletion involving all the markers tested in the long arm was seen in one patient. We did not see any case of isolated AZFa deficiency. Six of the patients with deletions had been previously described by Kleiman et al. (1999). Among our seven patients with an isolated AZFc deletion, six had the same STSs and thus the deletions apparently had the same or similar size, whereas the other patient had a deficiency that also included sY143 (Figure 1). All nine patients with AZF deletions were missing 50f2/C.

Population Analysis. All patients were haplotyped using a hierarchical system with nine biallelic DNA markers: two indels (YAP and 12f2) and seven single nucleotide polymorphisms (SNPs) [SRY −8299 (G → A), sY81 (A → G), PN2 (C → T), M9 (C → G), SRY −1532 (A → G), 92R7 (C → T), and SRY −2627 (C → T)]. In this fashion, all individuals could be reliably assigned to haplogroups according to the Y Chromosome Consortium (2002) (Figure 1; Table 1), except for the patient with an isolated AZFb deletion (patient 63) and the patient who had a very large deletion (patient 102), because key Yq UEP marker data, especially YAP, were missing for them. Two patients with the chromosomal mosaicism 45,X/46,XisoY were not included in the haplotype analysis.

The most common haplogroup in our infertile Israeli men was haplogroup J (47%), followed by haplogroups Y* (xDE, J, K), P* (xR1a, R1b8), K* (xP), and E in equal frequencies (Table 1). Among the patients with a microdeletion, several haplogroups were observed, even when the deletions were similar. For instance, of the six patients with comparable AZFc deficiencies, three belonged

Table 1. Frequency of Haplogroups Found in Infertile Israeli Men

Haplogroup	*Nondeletion Idiopathic Infertility Patients* ($n = 36$)	*Y-Chromosome Deletion Patients* ($n = 9$)	*Total* ($n = 45$)	*Jews (Hammer et al. 2000)* ($n = 336$)	*Palestinians (Hammer et al. 2000)* ($n = 73$)
P* (xR1a, R1b8)	3 (8.3%)	2 (28.6%)	5 (11.6%)	57 (17.0%)	7 (9.0%)
Y* (xDE, J, K)	5 (13.9%)	0	5 (11.6%)	47 (14.0%)	10 (13.0%)
R1a	2 (5.6%)	1 (14.3%)	3 (7.0%)	17 (5.0%)	0 (0.0%)
J	16 (44.4%)	4 (57.1%)	20 (46.6%)	121 (36.0%)	37 (51.0%)
E	5 (13.9%)	0	5 (11.6%)	67 (20.0%)	14 (19.0%)
K* (xP)	5 (13.9%)	0	5 (11.6%)	27 (8.0%)	5 (7.0%)
Not determined	0	2[a]	2[a]	0	0

a. In these patients the Y-chromosome haplogroup could not be established because key marker data were missing as a result of the large deletions of Yq.

to haplogroup J, two belonged to haplogroup P* (xR1a, R1b8), and one belonged to haplogroup R1a. This suggests that de novo deletions are not associated with a specific haplogroup. Moreover, when we used an exact population differentiation test to compare haplogroup frequencies between the Y deletion ($n = 9$) and nondeletion ($n = 36$) male infertility patients, no significant difference was observed ($P = 0.33$) at the 5% level for all pairs of groups. Obviously, the relatively small sample size limits the power of this statistical procedure. For a large d effect size (0.8) and $\alpha = 0.05$, we used the program G*Power to calculate the power ($1 - \beta$) as 0.68.

Discussion

Hammer et al. (2000) used basically the same marker set as we did to study Jewish and Arab populations of the Middle East, including Palestinians and several Jewish groups living in different world regions (Ashkenazi, Roman, North African, Near Eastern, Kurdish, Yemenite, Ethiopians), and establish their haplogroup frequencies (Table 1). They did not observe any significant differences in haplotype frequencies among these several groups. When the haplogroup frequencies in our infertile Israeli men were compared with those of Jews or Palestinians using an exact test of population differentiation (Arlequin, version 1.1), no significant differences were found ($P = 0.50$ and $P = 0.24$, respectively) at the 5% level for all pairs of groups.

All the patients with microdeletions analyzed in this study had deletion of the 50f2/C marker. This deletion has not been directly associated with infertility and indeed was described as a polymorphic trait in healthy Scandinavians, Asians, and, in smaller frequencies, Greeks (Jobling et al. 1996). We have interpreted the occurrence of this deletion in our patients as having no pathological significance, because 50f2/C appears to be contained within the AZFc region and

is thus deleted fortuitously. We searched for the 50f2/C sequence in the physical map of AZFc made by Kuroda-Kawaguchi et al. (2001) and found it in the BAC 010080, corresponding to a unique sequence stretch that they called u3. The 50f2/C marker was also absent in the patient with an isolated AZFb deletion. This suggests that 50f2/C was deleted with the AZFb interval and, consequently, that the deletion in this patient overlaps with AZFc. This is in good agreement with the recent findings of Repping et al. (2002).

The main objective of the present study was to ascertain whether there was an association between the occurrence of Y-chromosome microdeletions and specific Y-chromosome haplogroups. The fact that we observed similar isolated AZFc deletions in patients with different Y-chromosome haplogroups strongly suggests that no such association exists. This confirms our similar conclusions in Japanese infertile men (Carvalho et al. 2003).

Previous work with haplotyping infertile males has generated conflicting results. Some groups claim to find evidence of an association between sterility and certain Y-chromosome haplogroups (Kuroki et al. 1999; Krausz et al. 2001) and others do not (Carvalho et al. 2003; Previderé et al. 1999; Paracchini et al. 2000; Quintana-Murci et al. 2001). Population substructuring could explain this disagreement, as noted by Previderé et al. (1999). Initially, Previderé and coworkers found an association between a specific Y-chromosome haplogroup and idiopathic infertile patients, but when they separated the samples according to geographic origins, the association disappeared. The confounding element was that some Y-chromosome haplogroups show different frequency distributions in the same country, depending on the region. We propose that the deletions happen as independent and sporadic events caused by rearrangements in the long arm of the Y chromosome, reflecting the highly repetitive structure presented by all Y chromosomes (Skaletsky et al. 2003) independent of haplogroup.

Acknowledgments This research was supported by the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Technológico (CNPq), Coordenaçã de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). We wish to thank Rodrigo Redondo for the inestimable collaboration in statistical analyses.

Received 12 November 2002; final revision received 23 December 2003.

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Appendix: Electronic Database Information

BLAST program: http://www.ncbi.nlm.nih.gov/BLAST
Genome Data Base (GDB): http://www.gdb.org
NCBI Data Base: http://www.ncbi.nlm.nih.gov/genome/seq