American Journal of Medical Genetics 116A:152–158 (2003)

Lack of Association Between Y Chromosome Haplogroups and Male Infertility in Japanese Men

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The Y chromosome carries several genes involved in spermatogenesis, which are distributed in three regions in the euchromatic part of the long arm, called AZFa (azoospermia factor a), AZFb, and AZFc. Microdeletions in these regions have been seen in 10–15% of sterile males with azoospermia or severe oligozoospermia. The relatively high de novo occurrence of these microdeletion events might be due to particular chromosome arrangements associated with certain Y chromosome haplogroups. To test whether there is any association between Y chromosome types and male infertility, we studied a sample of 84 Japanese oligozoospermic or azoospermic males. The patients were analyzed for the presence of Yq microdeletions and also typed with a battery of unique event polymorphisms (UEPs) to define their Y haplogroups. Six of the infertile patients presented likely pathological microdeletions detectable with the sequence tagged sites (STS) markers used. There was no significant association between Y chromosome haplogroups and the microdeletions. We also compared the Y haplogroup frequencies in our subset sample of 51 idiopathic azoospermia patients with 57 fertile control Japanese males, and did not observe any significant differences. Contrary to previous reports, our data suggest that Y microdeletions and other molecular events causally associated with male infertility in Japan occur independently of the Y chromosome background.

KEY WORDS: Y chromosome; haplotypes; human male infertility

DATABASES: http://www.gdb.org/ (Genome Database [GDB]); http://www.ncbi.nlm.nih.gov/genome/seq/ (NCBI Database); http://www.ncbi.nlm.nih.gov/BLAST/ (BLAST program)

INTRODUCTION

The Y chromosome is small (2–3% of the haploid genome); most of the long arm is heterochromatic and contains only 30 known functional genes [Lahn and Page, 1997]. The non-recombinant region (NRY) occupies 95% of the chromosome and bears most of the Y-specific coding region, including the male determining gene *SRY* and many other genes involved in spermatogenesis, which are usually present in several copies and transcribed specifically in the testis. In 1976, Tiepolo and Zuffardi observed the involvement of Yq deletions in male infertility for the first time when they were analyzing cells from idiopathic infertile males. Since then, molecular studies have showed that microdeletions at Yq11 may represent the etiological factor in as many as 10–15% of cases with idiopathic azoospermia or severe oligozoospermia [Vogt et al., 1996; Foresta et al., 1997; Pryor et al., 1997]. Deletion mapping [Foote et al., 1992; Vollrath et al., 1992; Yen, 1998] directed the discovery of genes related to spermatogenesis, and defined three regions as the azoospermia factors (AZFa, AZFb and AZFc) mapped to Yq11.

Grant sponsor: CNPq of Brazil; Grant sponsor: Capes of Brazil.

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Received 1 February 2002; Accepted 7 June 2002
DOI 10.1002/ajmg.a.10827

AZFa contains two main candidate genes: *USP9Y* (ubiquitin-specific protease 9, also known as *DFFRY*) and *DBY* (*DEAD/H* box polypeptide). The main candidate in AZFb is the *RBMY* gene family [Ma et al., 1993] whose expression is restricted to the testis. *RBMY* consists of approximately 30 copies of genes and pseudogenes found on both arms of the Y chromosome, but Elliott et al. [1997] suggested that functional genes are clustered at the Yq in the distal interval 5 to proximal interval 6, i.e., the AZFb region.

AZFc represents the most frequently deleted region among infertile males [Ferlin et al., 1999; Kuroda-Kawaguchi et al., 2001]. The main candidate gene in AZFc is the *DAZ* (deleted in azoospermia) cluster, a set of genes transcribed in the adult testis and expressed exclusively in germ cells, apparently encoding a RNA binding protein [Reijo et al., 1995]. Saxena et al. [2000] reported the existence of at least four *DAZ* copies with different numbers of intragenic tandem repeats organized in two blocks, each one comprising an inverted pair of *DAZ* genes localized in the AZFc region.

Because the Y chromosome does not recombine in most of its length and it is passed from father to son, population geneticists have made use of its variability to study human male lineages, tracing male migrations and reconstructing human history [Jobling and Tyler-Smith, 1995; Santos and Tyler-Smith, 1996]. Most Y chromosome polymorphisms are biallelic markers, which can be combined in haplogroups that define Y lineages with specific geographic distributions around the world [Hammer et al., 1997; Santos et al., 1999], reflecting past demographic events shaped by population evolution.

The worldwide distribution of Y lineages is thought to be a consequence of random evolutionary forces, such as genetic drift, population expansion, and migrations. The influence of natural selection in these processes is unknown, but usually regarded to be of little significance. However, one cannot dismiss natural selection so easily, because the Y chromosome carries important genes involved in spermatogenesis, which can be targets for adaptive processes. Two possible selection mechanisms could conceivably have strong effects in the populational genetic variation of Y chromosomes: hitch-hiking and background selection. The consequences of these two processes can thus be the spread and eventual fixation, or the decrease and eventual extinction, of a particular Y haplogroup.

Infertile men with Y microdeletions do not pass their genotype to next generations under natural reproductive conditions. However, a predisposition to deletion of a chromosome region could be passed from father to son [Meschede et al., 1998; Jobling et al., 1998a]. Studying Japanese males, Kuroki et al. [1999] claimed to have found an association between reduced sperm count, as well as azoospermia, and a specific Y haplogroup that is very frequent in Japan. More recently, Krausz et al. [2001] reported the identification of a European Y haplogroup associated with reduced sperm count in Danish males. Taken at face value, these reports would be demonstrations of selective processes involving Y genes.

In the present work, we have typed the Y chromosomes of infertile Japanese males with or without AZF deletions, and compared their haplogroup frequencies with those of fertile male Japanese controls. We have found no evidence that specific haplogroups are associated with idiopathic infertility or with a genetic predisposition to undergo chromosomal microdeletions.

MATERIALS AND METHODS

DNA Samples

We studied 84 DNA samples from infertile Japanese males from the Kinki region (Honshu island) that were extracted from peripheral blood leukocytes or sperm provided from the Department of Urology, Kobe University School of Medicine. After clinical and cytogenetic tests, 51 patients were classified as idiopathic azoospermic with a normal karyotype [Fujisawa et al., 2001]. All 84 samples were submitted to molecular analyses of Y chromosome microdeletions and haplogroup typing. The control group was composed of 57 fertile Japanese immigrants from Pará, Brazil. Their paternal origin was from 22 different Japanese cities (situated in the three main islands from Japan: Hokkaido, Honshu, and Kyushu). The DNA was extracted from peripheral blood leukocytes by standard methods.

Microdeletion Analysis

Silver-stained polyacrylamide gels were used for all patients to detect the presence or absence of some Y deletion markers covering the three AZF regions: *sY84*, *MSY2*, *YRRM1* (*RBMY1A1*, exon 12), M9, YAP, *sY254*, *sY258* (=*sY255*, *DAZ*), *sY465* (*DAZ*), *sY254* (*DAZ*), 50f2C, and Amel (Fig. 1). To confirm this analysis, samples from selected patients were subjected to polymerase chain reaction (PCR) with a multiplex set of Cy5-labeled primers including the Amel, *sY258*, *YRRM1*, *sY84*, and *sY465* loci, and analyzed in a fluorescent automatic DNA sequencer (ALF*Express*; Amersham-Pharmacia, Uppsala, Sweden).

After this initial screening, we used other Y chromosome markers to confirm and to delineate the observed deletions, using the following markers: *sY81*, *sY103* (*DYS199*), Y-GATA-A.7.2, *DYS392*, Y-GATA-A.10, *sY207*, and *sY272* (Fig. 1). All markers were used in the following multiplex reactions at the given primer concentrations: 1) *sY258*, *YRRM1*, *sY465*, and *sY254*, all 1 μM; 2) Amel, 0.09 μM and *sY84*, 0.05 μM; 3) *sY81*, 0.5 μM and *DYS199*, 1μM; 4) *MSY2* and *RPS4Y*, both 1 μM; 5) Y-GATA-A.10, 0.5 μM and *RPS4Y*, 1 μM; 6) Y-GATA-A7.2, 0.5 μM and *RPS4Y*, 1 μM; 7) *DYS392*, 0.5 μM and *SRY*+465, 1μM; 8) *sY207* and *RPS4Y*, both 1 μM; 9) *sY272* and *RPS4Y*, both 1 μM.

All PCR reactions were performed in 12.5 μl with 50 ng genomic DNA, 1.5 mM or 1.0 mM (specifically for Y-GATA-A.10 and A7.2) $MgCl_2$, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μM dNTPs, and 1 or 0.5 unit (for *sY81*, M9, *MSY1*/50f2/C, *MSY2*) of *Taq* DNA polymerase. The PCR products were resolved in 6% polyacrylamide gels and silver-stained according to Santos et al. [1993].

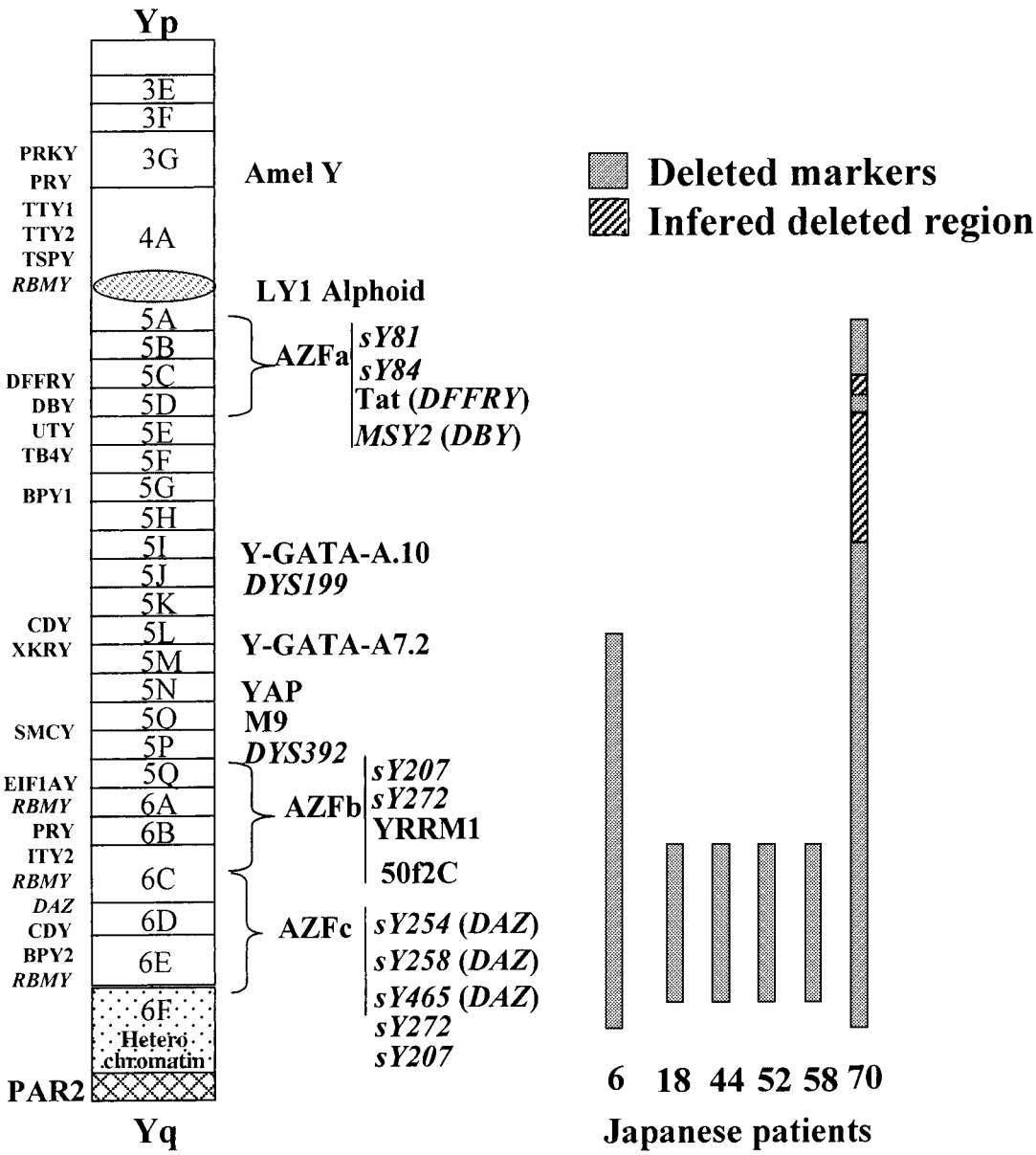

Fig. 1. Relative location of polymorphic and deletion markers, adapted from Yen [1999], and deletion mapping for the six infertile patients with microdeletions. The primer sequences for all markers are described elsewhere: *sY84* [Vollrath et al., 1992]; *YRRM1* [Stuppia et al., 1996]; *sY258*, *sY254*, and *sY465* [Mulhall et al., 1997]; Amel [Sullivan et al., 1993]; *sY81* [Vollrath et al., 1992]; *sY103* [Underhill et al., 1996]; Y-GATA-A.7.2 and Y-GATA-A.10.1 [White et al., 1999]; *DYS392* (Genome Database, www.gdb.org); *sY207* and *sY272* [Reijo et al., 1995]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Y Haplogroup Analysis

The Y chromosome haplogroup analysis was performed using markers known to be polymorphic in Japanese or Asiatic populations. The markers used in our study are listed in Table I.

All PCR products were resolved in 6% polyacrylamide gels, except the alphoid heteroduplex system, which was run in mutation detection enhancement (MDE)FMC bioproducts) polyacrylamide [Santos et al., 1995] in order to resolve the heteroduplex bands. To visualize bands, the gels were silver-stained according to Santos et al. [1993].

Statistical Analysis

Tests were performed using the software ARLEQUIN ver. 1.1 [Schneider et al., 1997]. We used the Fisher's Exact Test of population differentiation and AMOVA (population genetic structure inferred by analysis of variance). The former tests the hypothesis of a random distribution of k different haplotypes among r populations, as described in Raymond and Rousset [1995]. The latter investigates the genetic structure of a population (fertile and infertile patients) using an analysis of variance that takes into account the number of mutations between haplogroups [Schneider et al., 1997].

TABLE I. Y-Chromosome Polymorphic Population Markers Used to Type Japanese Male

Marker	Mutation type	Reference
92R7	Transition C to T	Mathias et al. [1994]; Hurles et al. [1998]
Tat	Transition T to C	Zerjal et al. [1997]
MSY2 (*DYS440*)	Minisatellite consisted of three or four copies of a 99–110 bp repeat unit	Bao et al. [2000]
LLY22g	Transvertion C to A at a repetitive locus present at Yp and Yq	Zerjal et al. [2001]
αh	Alphoid heteroduplex system	Santos et al. [1995]
MSY1/50f2C	*MSY1*: minisatellite localized at Yp; used as a amplification control; 50f2C: a polymorphic deletion found in different haplogroups	Jobling et al. [1998b]
DYS199	Transition C to T	Underhill et al. [1996]; Santos et al. [1999]
LY1	Polymorphic L1 retroposon insertion in the centromeric alphoid array particularly frequent in Chinese	Santos et al. [2000]
47Z (*DXYS5Y*)	Point mutation at a locus present in the X and Y chromosome	Shin et al. [1998]
RPS4Y	Transition C-to-T at 711 position in the gene *RPS4Y*	Bergen et al. [1999]
M9	Transversion C to G	Underhill et al. [1997]
SRY – 1532	Recurrent G to A transition	Kwok et al. [1996]
SRY – 8299	Transition G to A	Whitfield et al. [1995]
SRY + 465	Transition C to T at the 155th codon	Shinka et al. [1999]
YAP	Polymorphic Alu insertion retroelement	Hammer and Horai [1995]

The PCR conditions and the primers used are described in detail at references cited.

RESULTS

Y Deletion Analysis

Among the 84 infertile patients analyzed, we found nine patients with Y chromosome microdeletions, although one of them, patient 70, presented a large deletion of Yq (Fig. 1). Three patients presented only a deletion of 50f2/C. This is a well-known polymorphic deletion [Jobling et al., 1996] that is not directly associated with infertility. Thus, we considered its presence in the infertile patients a coincidence of no clinical significance.

The observed deletions and the markers involved can be seen in Figure 1. It is interesting to note that all six patients also had 50f2/C deletions. Two previous reports [Nagafuchi et al., 1993; Nakahori et al., 1996] on Japanese infertile males had also found a high frequency (18/20 patients) of 50f2/C deletions. Perhaps these 50f2/C deletions should be studied and mapped carefully, because although this is believed to be a neutral polymorphic deletion, it can prove interesting as a screening tool for Japanese idiopathic infertile patients. The polymorphic deletions can be discriminated from likely pathological ones with the association to other markers, such as the LLY22g locus [Zerjal et al., 2001] that identifies haplogroup 12 and derivatives.

Among the 84 patients, we carried out further analysis with a subset of 51 patients identified as idiopathic azoospermic [Fujisawa et al., 2001]. Of the six patients with likely pathological microdeletions, three, all with isolated AZFc deletions, did not meet the criteria for the diagnosis of idiopathic azoospermia: patient 18 was oligozoospermic, and patients 44 and 58 did not undergo testicular biopsy. Thus, although unlikely, obstructive azoospermia could not be excluded in the latter two patients [Fujisawa et al., 2001].

Population Analysis

The haplogroup frequencies observed in infertile and normal Japanese males can be seen in Table II. The distribution of haplogroups obtained in the control population is in agreement with the literature [Nakahori et al., 1989; Hammer and Horai, 1995; Bergen et al., 1999; Karafet et al., 1999; Shinka et al., 1999; Kim et al., 2000; Underhill et al., 2000], and showed that we have a representative sample from Japan.

We analyzed specifically the subgroup of idiopathic azoospermia patients with and without microdeletions, considering their Y haplogroups. In order to verify if there is any association among the Y chromosome haplotypes and the infertile phenotype, we compared the data obtained for normal population and infertile population samples. Using Fisher's Exact Test of population differentiation and AMOVA, haplogroup frequencies were compared between deleted infertile population ($n = 6$) and the normal control group ($n = 57$), between infertile males without microdeletions ($n = 45$) and the normal control group ($n = 57$), and between infertile males without microdeletions ($n = 45$) and the deleted infertile population ($n = 6$). The test results showed that there were no statistically significant differences at the 5% level for all pairs of groups.

The diversity found in infertile Japanese males with Y chromosome deletions is great, because five of the 10 haplogroups observed in the normal population also were seen in the infertile males, including two rare haplogroups (13 and 16), and most of them were associated with distinct alphoid heteroduplex types (results not shown). Apparently, deletions can occur in any chromosome type and may be independent and sporadic events.

In our population analysis, we observed a close association between some alphoid types with haplogroups,

TABLE II. Frequency of Haplogroups Present in the Japanese Infertile Sample

	Control individuals			Idiopathic azoospermic patients		
Haplogroup	n = 57	Alphoid system typing αh	Minisatellite *MSY2*	n = 51	Alphoid system typing αh	Minisatellite *MSY2*
1	0	—	4 repeats	1 (2%)	II	4 repeats
4	20 (35%)	I: 2 XXXII: 18	4 repeats	13 (25%)	XXXII	4 repeats
5	10 (17,5%)	III	4 repeats	11 (21,5%)	III	4 repeats
10	11 (19,3%)	I: 2 III: 1 V: 1 XVIII: 6 XXXVII: 1	4 repeats	8 (15,6%)	III: 1 XVIII: 7	4 repeats
12	0	—	4 repeats	1 (2%)	XX	4 repeats
13	1 (1,8%)	III	4 repeats	1 (2%)	XLVIII	4 repeats
16	2 (3,5%)	XX	4 repeats	1 (2%)	XX	4 repeats
20	5 (8,7%)	II: 1 III: 4	4 repeats	4 (7,8%)	III	4 repeats
26	8 (14%)	II: 1 III: 5 XXX: 1 XLVIII: 1	4 repeats	11 (21,5%)	II: 1 III: 7 XXX: 2 XLVIII: 1	4 repeats 3 repeats: 1 3 repeats: 2 4 repeats

namely haplogroup 4 with αh XXXII, haplogroup 10 with αh XVIII, and haplogroups 12 and 16 with αh XX. These associations can be very informative, as shown in the case of patient 6, who had part of the long arm deleted, including the YAP locus; he was classified as haplogroup 4 based on the presence of αh XXXII.

DISCUSSION

Y chromosome microdeletions are seen at similar frequencies (10–15%) in idiopathic azoospermia patients of previously studied populations [Vogt et al., 1996; Foresta et al., 1997; Pryor et al., 1997]. The most frequently deleted region is AZFc, which is also true for Japanese patients, although most of studies in the literature did not screen for AZFa deletions [Nagafuchi et al., 1993; Iwamoto et al., 1995; Nakahori et al., 1996]. Our results are in agreement with these observations: we observed 7.1% (six of 84) of infertile patients with Y deletions, and AZFc was the main region involved. If we consider the idiopathic azoospermic group, we observed 5.8% (3/51) patients with Y microdeletions.

In our analysis, we paid special attention to haplogroup 4, defined by the Alu insertion (YAP), which is probably a very old mutation event, and is found in high frequencies in Africa, Tibet, and Japan [Hammer et al., 1997]. Hammer and Horai [1995] proposed that Japanese males with this insertion are descendent from the Jomon, a group that colonized Japan 10,000 years ago. Because only Japanese males characterized in haplogroup 4 have the alphoid type XXXII, this association enables us to distinguish YAP males that are descendent from the Japanese paternal lineage from others present worldwide, such as the African ones [Santos et al., 1995, 1996]. Haplogroup 4 still has a very high frequency today in Japan (28%, Kim et al. [2000]; 56%, Hammer and Horai [1995]; and our data), with some variation depending on the region studied.

Kuroki et al. [1999] suggested that the presence of haplogroup 4 was associated with low sperm count and a higher incidence of azoospermia. They used Y polymorphic markers in common with our study (*SRY* + 465 [haplogroup 20], 47z [haplogroup 5], YAP [haplogroup 4]), to type azoospermic patients and fertile controls from two Japanese cities. Their conclusions showed that there were significantly more individuals from haplogroup 4 among the azoospermic men than would be expected by chance only. Moreover, they observed that among 198 fertile Japanese men, those belonging to haplogroup 4 had an average sperm count lower than those from haplogroups 5 and 20. We did not address the question of sperm counts, but our data do not support the proposed association of haplogroup 4 and sterility or microdeletions. Haplogroup 4 was seen in 35% of our control fertile patients and in a lower proportion (25%) of the patients with idiopathic azoospermia (Table II). Moreover, only two of six (33%) patients with likely pathological deletions belonged to haplogroup 4.

We have reanalyzed Kuroki et al.'s [1999] data with the same tests that we have used here. There were no significant differences ($P < 0.05$) in AMOVA and Fisher's Exact Test when our control and azoospermic samples were compared to Kuroki et al.'s populations. AMOVA produced a single significant value obtained from the comparison between Kuroki et al.'s azoospermic and control populations without haplogroup subdivisions; however, the same comparison was not significant with the exact test. Besides using Kuroki et al.'s statistical approach, we used an odds ratio two-by-two table test to evaluate our data for comparison. We did not observe any statistically significant results for all comparisons in our sample. Indeed, a re-analysis of Kuroki et al.'s data has shown that while they found a significant odds ratio between azoospermic and control samples, subdivided in haplogroups 4 and 20 + 5 [Kuroki et al., 1999], non-significant values ($P = 0.0978$) were obtained when the comparison was between haplogroup 4 and other Y chromosomes (haplogroups 20 + 5 + 2), which is the relative chance of getting azoospermia between haplogroup 4 and non-haplogroup 4

individuals. Thus, in our point of view, Kuroki et al.'s data also shows that their conclusions about association of haplogroup 4 and sterility may be misleading.

Additionally, Previderé et al. [1999] found an association between a specific Y haplogroup and idiopathic infertile Italian males, but when they subdivided the samples according to their origins, they lost the association. The Italian population presents a heterogeneity similar to that observed in Japanese people: some haplogroups show different frequency distributions depending on the region of the country. Thus, we believe that the results obtained by Kuroki et al. [1999] associating azoospermia patients with haplogroup 4 could also reflect population substructuring due to distinct geographic, ethnic, or even social class origins of patients.

Furthermore, if haplogroup 4 predisposed men to microdeletions, we would expect the presence of a specific chromosome structure involving repetitive sequences prone to illegitimate recombination, like was seen for some AZFa deletions [Blanco et al., 2000]. Consequently, we would expect the deletions to have similar breakpoints, i.e., the same size. However, the deletions seen in our two haplogroup 4 sterile patients had different extensions (patients 6 and 18, Fig. 1).

Our analysis of the present study and previously published data lead us to conclude that there is no significant association between particular Y haplogroups and likely pathological deletions or infertility in Japanese males.

ACKNOWLEDGMENTS

We thank the anonymous sample donors, who allowed this work to be carried out. We thank also Núcleo de Genética Médica de Minas Gerais for the analysis of some Y deletion markers. We are grateful to Dr. Chris Tyler-Smith from the Department of Biochemistry of Oxford University, UK, for the *MSY2* primers and LLY22g information before publication. C.M.B.C. was supported by Capes of Brazil, and F.R.S. and S.D.J.P. were supported by CNPq.

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