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No association found between gr/gr deletions and infertility in Brazilian males

Cláudia M.B.Carvalho[1], Luciana W.Zuccherato[1], Luciana Bastos-Rodrigues[1], Fabrício R.Santos[2] and Sérgio D.J.Pena[1,3]

[1]Departamento de Bioquímica e Imunologia and [2]Departamento de Biologia Geral Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[3]To whom correspondence should be addressed at: Departamento de Bioquímica e Imunologia, ICB, UFMG, Av. Antônio Carlos 6627 31270-010, Belo Horizonte, Minas Gerais, Brazil. E-mail: spena@dcc.ufmg.br

The Y chromosome carries several spermatogenesis genes distributed in three regions: AZFa, AZFb and AZFc. Microdeletions in these regions have been seen in 10% of sterile males with azoospermia or oligozoospermia, the most frequent of them being characterized by a complete deletion of AZFc region. A partial AZFc deletion named gr/gr has been singled out as a risk factor for spermatogenic failure. However, other authors have diagnosed it as a polymorphic deletion with no clinical relevance. We decided to investigate the association of gr/gr deletion and infertility in Brazilian males. We analysed 350 individuals (110 azoospermic, 122 fertile and 118 presumably fertile) and observed 12 g/gr deletions: five in infertile men (4.5%), three among fertile males (2.5%) and four in probably fertile individuals (3.4%). These differences were not statistically significant. Then, we decided to ascertain whether the clinical impact of the gr/gr deletion was associated with the type of Y chromosome. We have identified Y-chromosome haplogroups using 22 unique event polymorphisms (UEPs). Among the individuals with the gr/gr deletion, we found haplogroups R, K*, F*, E1, E3b2 and E3b*, all of which are common in white Brazilian males, and none revealed a particular association with infertility. Taken together, these results show no evidence of association between the occurrence of gr/gr deletion and male infertility.

Key words: AZFc/gr-gr deletion/male infertility/minisequencing/Y chromosome

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Introduction

Infertility, defined as 1 year of unprotected intercourse without conception, affects approximately 15% of human couples, with men being responsible in approximately 50% of the cases (reviewed by Wieacker and Jakubiczka, 1997). Although the delineation of the male factor in an infertile couple may be elusive, in approximately 10% of cases, a severe defect in sperm production can be unveiled. In roughly 10% of these cases of severe oligospermia or non-obstructive azoospermia, microdeletions of the Y chromosome can be found in one of three 'azoospermia factor' (AZF) regions: AZFa, AZFb and AZFc. The most common site for microdeletions is AZFc, where there are several copies of the DAZ (deleted in azoospermia) and *CDY1* genes (Kuroda-Kawaguchi *et al*., 2001; Vogt, 2005).

New complete deletions of AZFc are estimated to occur in approximately one in 4000 males and lead almost invariably to infertility (Kuroda-Kawaguchi *et al*., 2001). The complete sequencing of the AZFc in the human Y chromosome showed the reason for this high rate of *de novo* deletions: the region has a highly repetitive structure with many palindrome segments (Kuroda-Kawaguchi *et al*., 2001). Thus, homologous recombination between sister chromatids or within a chromatid will lead to loss of Y chromosome material (Vogt, 2005). Accordingly, the recombination between the direct repetitions b2/b4 produces the complete deletion of AZFc (3.5 Mb) (Kuroda-Kawaguchi *et al*., 2001), with removal of all genes in the region leading to spermatogenic failure.

More recently, Repping *et al*. (2003) studied another type of common AZFc deletion, called 'gr/gr deletion', also involving direct repeats, but encompassing only 1.6 Mb. Although this does not eliminate all the genes present in the AZFc region, Repping *et al*. (2003) presented evidence suggestive that the deletion might also lead to spermatogenic failure, although with low penetrance. Subsequent studies on the phenotypic impact of gr/gr deletions have led to conflicting reports: de Llanos *et al*. (2005), Ferlin *et al*. (2005) and Lynch *et al*. (2005) claimed to be able to reproduce Repping's finding, whereas Machev *et al*. (2004), Hucklenbroich *et al*. (2005) and Ravel *et al*. (2006) have not.

We decided to ascertain the frequency of gr/gr deletions in Brazilian males and to verify their association with infertility. We screened the AZFc region using single-tagged sequence (STS) markers in idiopathic infertile, fertile and presumably fertile males. All groups presented Y chromosomes with gr/gr deletions without statistically significant differences. All patients with gr/gr deletions (12) were typed with a battery of unique event polymorphisms (UEPs) to define their Y haplogroups. Among the 12 gr/gr deleted Y chromosomes that we have found, at least six arose as independent events, and we did not observe any Y haplogroup exclusively found in infertile males.

Materials and methods

DNA samples

This study used 124 DNA samples, from infertile Brazilian males, extracted from peripheral blood leukocytes by standard methods. After clinical and

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cytogenetic tests, 117 patients were classified as having idiopathic infertility due to non-obstructive azoospermia, with a normal 46, XY karyotype. These patients were submitted to molecular analyses of Y-chromosome microdeletions AZFa, b and c, and seven patients, who showed complete AZFc deletion, were excluded from further analyses. The 'fertile' group was composed of 122 Brazilian males with at least one proved offspring. We also used another group composed of 118 individuals from the Brazilian general population (described in Alves-Silva *et al*., 2000; Carvalho-Silva *et al*., 2001). Because we had no data about their fertility, this group, on statistical grounds, was called 'probably fertile'. All studies were anonymous and participants provided written consent.

Deletion screening

All patients and control males were prescreened for the presence or absence of the marker sY1291 in order to identify the gr/gr deletions. Negative results were confirmed by repetition and the deleted samples were tested further with the following STSs: sY1161, sY1191, sY1206, sY1201 and sY1258. In order to avoid false-negative results due to PCR failures, we made all PCR assays in a duplex format with the Yp marker *SRY*+465, which is positive in all males. The primer sequences for STSs are available from GenBank under the following accession numbers: sY1161: G66148; sY1191: G73809; sY1291: G72340; sY1206: G68331; sY1201: G67170; sY1258: G75479. The primer sequence for *SRY*+465 was described by Shinka *et al*. (1999). Primer concentrations used in PCR were 0.5 µM of STS markers plus 0.25 µM of *SRY*+465.

All PCR assays were performed in 12.5 µl with 50 ng genomic DNA, 2.0 mM $MgCl_2$, 1× reaction buffer from *Taq* DNA polymerase *Promega*, 200 µM dNTP's, 1 unit of *Taq* DNA polymerase *Phoneutria*, or specifically for sY1258, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.4) and 50 mM KCl. The cycling protocol was as follows: 3 min at 94°C, 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, 35 cycles, 5 min at 72°C. The PCR products were resolved in 6% polyacrylamide gels and silver stained according to Santos *et al*. (1993).

Y-haplogroup analysis

The Y-chromosome haplotype analyses were performed hierarchically as proposed by The Y Chromosome Consortium (2002) and updated by Jobling and Tyler-Smith (2003). The UEPs used were as follows: M145, PN2 (also known as P2), M35, M174, sY81 (also known as M2), M33, M75, M58, M191, M116, M10, M78, M281, M123, M81, M213, M214, M74, M9, M207, M4 and 12f2. All UEPs (except 12f2) were analysed using a minisequencing protocol, whose basic methodology has been described in detail elsewhere (Carvalho and Pena, 2005).

Before performing the minisequencing protocol, it is necessary to amplify the specific region where the single-nucleotide polymorphisms (SNPs) are located. The primer sequences used and the PCR product size are provided in the Supplementary online material. We used the software Oligo version 4.0 to design most of the primers and they were synthesized commercially. Dr Peter A. Underhill from the Department of Genetics, Stanford University, kindly provided the Y-chromosome sequences of the region flanking each marker. Y-chromosome SNPs were resolved using either multiplex or simplex PCRs, whose conditions are provided in Table I (Supplementary data). Primers for the flanking regions of the following SNPs comprised the first multiplex, YD/Ea: M145, PN2, M174 and sY81. PCR assays were performed in a volume of 12.5 µl with 50 ng genomic DNA, 0.32 µM each primer, 2.0 mM $MgCl_2$, 1× *AmpliTaq* Gold buffer, 200 µM dNTPs and 1 unit of *Taq* DNA Polymerase (*AmpliTaq Gold*; Applied Biosystems, Foster City, CA, USA). The second multiplex, YD/Eb, was composed of M33, M35 and M75 primers. PCR assays were performed in a volume of 12.5 µl with 50 ng genomic DNA, 0.32 µM (M33 and M35) and 0. 4 µM (M75) of each primer. The third multiplex, YE3a, was composed of M10, M58, M191, M116 primers. PCR assays were performed in a volume of 12.5 µl with 50 ng genomic DNA, 0.4 µM of each primer. The fourth multiplex, YE3b, was composed by M78, M81, M281 and M123 primers. PCR assays were performed in a volume of 12.5 µl with 50 ng genomic DNA, 0.4 µM (M78 and M81) and 0.6 µM (M123, M281) of each primer. Amplification was achieved in a MJ Research Cycler (MJ Research, Watertown, MA, USA) and was done at 95°C for 5 min, followed by 29 cycles for 30 s at 95°C, 60 s at 50°C, 1 min at 72°C and a final extension for 10 min at 72°C. All samples were typed using multiplex YD/Ea. Samples typed as derived both to M145 and PN2 markers were tested to YD/Eb and YE3b. Samples typed as derived both to M145 and sY81 were tested to YE3a. Samples typed as M145 ancestral were tested to M213. M213 PCR was done in 12.5 µl with 50 ng genomic DNA, 0.4 µM of the primer pair, 1.5 mM $MgCl_2$, 1× buffer Phoneutria *Taq* DNA Polymerase, 200 µM dNTPs and 1 unit of *Taq* DNA Polymerase (Phoneutria, Belo Horizonte, Brazil). This protocol required a 'hot start' step: the enzyme was added 2 min after initial denaturation. The PCR touchdown program consists of 95°C for 5 min, followed by 15 cycles for 20 s at 94°C, 60 s at 63°C with an increment –0.5°C/cycle, 1 min at 72°C followed by 20 cycles for 20 s at 94°C, 45 s at 56°C and 60 s at 72°C. M213-derived samples were further tested with 12f2 and M9 markers. The 12f2 marker protocol is described in detail elsewhere (Rosser *et al*., 2000). M9-derived samples were further tested with M4, M214 and M74 markers. M74-derived samples were further tested with M207 markers. M9, M4, M214, M74 and M207 PCR protocols are similar to the M213, except for the primer concentrations, 0.4 µM (M9), 1.0 µM (M4, M74) and 1.2 µM (M207, M214). The PCR products were resolved in 6% polyacrylamide gels and silver stained according to Santos *et al*. (1993).

In order to eliminate the excess of primers and dNTPs, the PCR products were digested by an 'ExoSAP' mix consisting of 2 units/µl of *Escherichia coli* exonuclease I (Exo I) and 0.2 units/µl of shrimp alkaline phosphatase (SAP) and 1× SAP buffer. The enzymes were purchased from the USB Corporation (Cleveland, OH, USA). Five microlitres of PCR product were added to 5 µl of ExoSAP mix, incubated at 37°C for 30 min. The enzymes were inactivated afterwards by heating at 80°C for 15 min.

The Table I in the Supplementary online material shows the minisequencing primers designed for each point mutation. Primers were designed with the 3′ base corresponding to the last base before the target point mutation. The primer sizes were adjusted by addition of a piece of a 'neutral' sequence at the 5′ ends. Multiplex and simplex PCR minisequencing was performed in a 12.5 µl volume with 1 µl purified PCR product. All minisequencing primers were used in 0.4 µM, except: sY81 (0.2 µM), M174 (0.04 µM) and M116 (0.08 µM). The PCR reaction contained 0.5 µM of ddATP or ddGTP labeled with fluorescein (NEL402 and NEL403-Perkin Elmer Life Sciences, Boston, MA, USA), 0.5 µM of unlabeled dCTP, dTTP and dGTP or dATP, 3.5 mM $MgCl_2$, 1× *Thermo Sequenase* buffer and 1 unit of *Thermo Sequenase* DNA Polymerase (Amersham Biosciences, Uppsala, Sweden). The thermal cycling was performed as 'hot start': the enzyme was added 2 min after an initial denaturation. The 'cycle sequencing' reaction consisted of 80°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 20 s.

The minisequencing products were applied in a 6% denaturing polyacrylamide gel and resolved in a fluorescent automatic DNA sequencer ALF (Amersham Biosciences, Uppsala, Sweden). The analyses were performed in the software package Allelinks (Amersham Biosciences, Uppsala, Sweden).

Statistical and population structure analyses

Tests were done using the software Epi Info™ for Windows Version 3.3.2, developed by Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/epiinfo) and available in Portuguese version at http://www.epiinfo.com.br. Specifically, we have used a Fisher Exact test to calculate a probability value for the relationship between the two variables as found in a two-by-two contingency table. The program calculates the difference between the observed and expected data, considering the given marginal and the assumptions of the model of independence of population differentiation.

Table I. Total samples screened for the AZFc partial deletion (gr/gr)

Samples	*n*	gr/gr deletion	%
Fertile males	122	3	2.5
Presumably fertile males	118	4	3.4
Infertile males	110	5	4.5
Total	350	12	3.4

Results

Y-deletion analysis

We initially analysed 125 azoospermic infertile men and found complete AZFc deletions in seven of them (5.6%). In the remaining 117 patients, we found five with a gr/gr microdeletion (4.3%). However,

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we also found four males with gr/gr deletions among 118 presumably fertile men and three individuals with gr/gr deletions among 122 men of proven fertility (Table I).

We performed pairwise Fisher Exact tests to compare the frequencies of gr/gr deletions in the three groups, and no significant difference was observed at the 5% level for any of the pairs.

Minisequencing development and Y-chromosome population analysis

All gr/gr-deleted patients were haplotyped using a hierarchical system according to The Y Chromosome Consortium (2002) and updated by Jobling and Tyler-Smith (2003). We used 22 biallelic DNA markers: one indel, 12f2, and 21 SNPs that included M145, PN2, M35, M174, sY81, M33, M75, M58, M191, M116, M10, M78, M281, M123, M81, M213, M214, M74, M9, M207, M4. In order to optimize the Y-chromosome typing, the multiplexes were developed by clustering nested monophyletic clades. This could be achieved using a minisequencing methodology (Carvalho and Pena, 2005). The exception was that the typing of markers of E clade was done in a simplex minisequencing format. In this fashion, all individuals could be reliably assigned to the haplogroups.

The haplogroups found in the gr/gr-deleted males were R, K* (×M, N, O, P), F*(×J, K), E1, E3b2, E3b*(xE3b1, E3b2, E3b3, E3b4). Thus, among the 12 gr/gr-deleted men there were six different Y-chromosome lineages, suggesting that the AZFc partial deletion occurred at least six times independently. Among the five infertile men with gr/gr deletions, the haplogroup F*(×J, K) was encountered twice and the haplogroups R, E1, E3b*(xE3b1, E3b2, E3b3, E3b4) were seen once each. Among the four presumably fertile males with gr/gr deletion, one presented haplogroup F*(×J, K), two K* (×M, N, O, P) and one E3b2. The three fertile individuals with gr/gr deletions presented two haplogroups: two were R and one was K* (×M, N, O, P) (Figure 1 and Table II).

Discussion

The Y chromosome has evolved to carry out almost exclusively specialized male functions such as testes induction and spermatogenesis. The highly palindromic structure of the DNA sequences in the chromosome predisposes it to a high rate of *de novo* deletions that lead to infertility (Kuroda-Kawaguchi *et al*., 2001). In studies involving Japanese (Carvalho *et al*., 2003) and Israeli (Carvalho *et al*., 2004) azoospermic men, we did not find any association between the incidence of complete AZFc deletions and specific Y-chromosome haplogroups.

Although it is widely accepted that the complete deletion of the AZFc region leads to male sterility, the relationship between partial AZFc deletions and infertility has been controversial. In a case–control study, Repping *et al*. (2003) found that the frequency of gr/gr deletions was exclusively observed among men with spermatogenic failure or males whose spermatogenic status were unknown. They concluded that gr/gr deletions predisposed individuals to the development of infertility. Foresta *et al*. (2005), de Llanos *et al*. (2005) and Lynch *et al*. (2005) largely confirmed these findings. However, Machev *et al*. (2004) and Hucklenbroich *et al*. (2005) did a Y-chromosome microdeletion screening in oligozoospermic, azoospermic and normozoospermic men and found no association between semen analysis, or other clinical parameters, and partial AZFc deletions, and concluded that the gr/gr deletion was a neutral polymorphism.

Because of these controversies, we decided to carry out a study in 117 Brazilian azoospermic males, using as controls both fertile and presumably fertile males. We performed the gr/gr deletion screening using STSs proposed by Repping *et al*. (2003) and found this deletion in all the three groups studied, with no statistical difference between them. To eliminate the possibility of population stratification of our samples, we performed an analysis of the Y-chromosome haplogroups, similarly to the analyses that we did previously in males with complete AZFc deletions (Carvalho *et al*., 2003, 2004). The gr/gr deletions occurred in several haplogroups, independently of the spermatogenic phenotype, and there was no particular haplogroup associated with infertility, neither with gr/gr deletions (Figure 1 and Table II). Thus, in contrast with the conclusions of Repping *et al*. (2003), our results failed to show an association between the occurrence of gr/gr deletions and the infertile phenotype. Rather, on the basis on our data, we agree with Machev *et al*. (2004), Hucklenbroich *et al*. (2005) and Ravel *et al*. (2006) that the gr/gr deletion is a polymorphic trait with no phenotypic effect. The reason for the discrepant results in different studies is not apparent. It might be simply due to different methodologies and control conditions, or it might have biological causes, as reviewed by us elsewhere (Carvalho and Santos, 2005). Further studies should clarify this important question.

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Supplementary data

Supplementary data are available at: http://publicacoes.gene.com.br/gr-gr.htm

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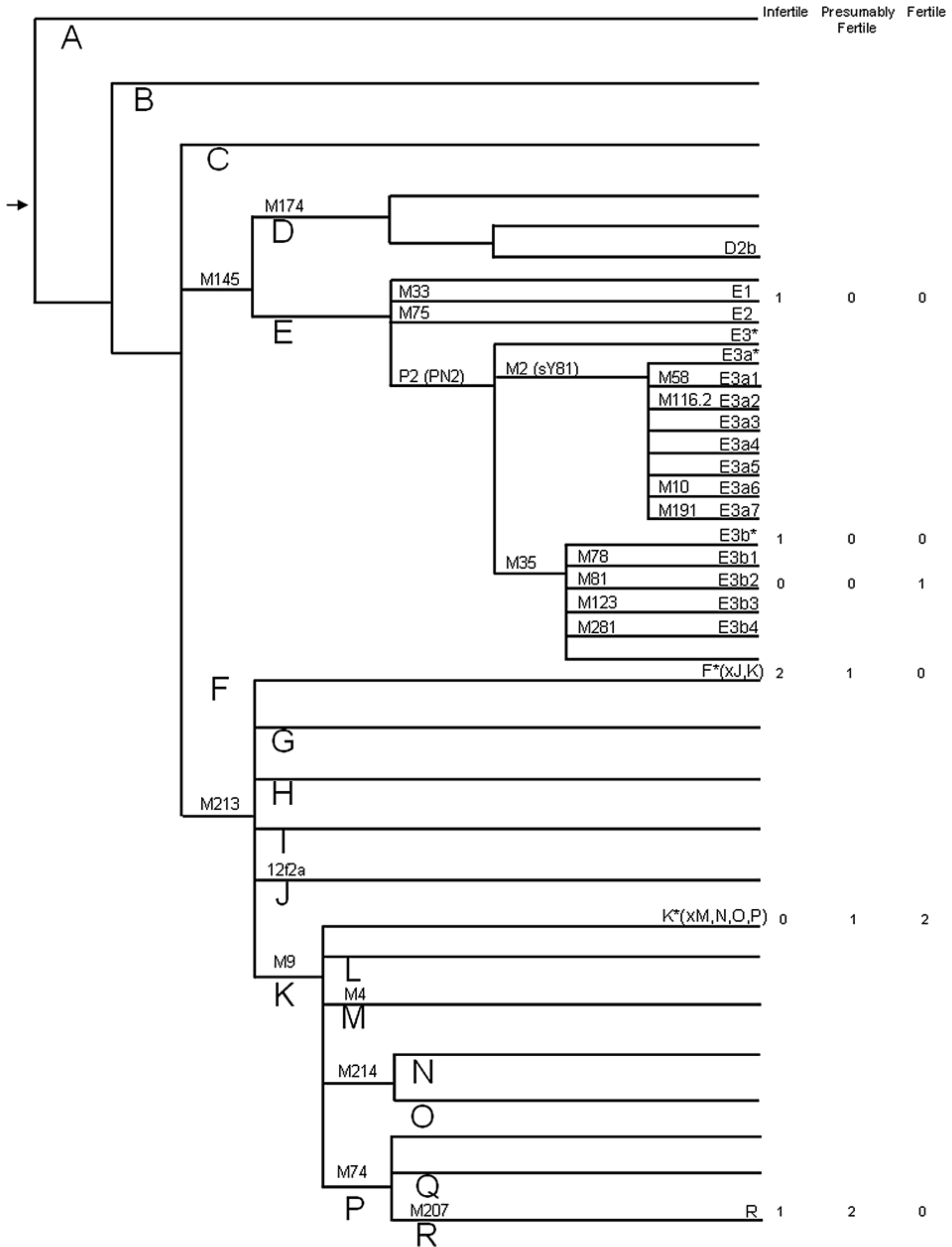

Figure 1. The genealogical tree of human Y-chromosomes and branches in which the gr/gr deletion was observed. Fertile, presumably fertile and infertile individuals were assigned to the biallelic polymorphisms indicated. The designations for the branches are based on The Y Chromosome Consortium (2002) and updated by Jobling and Tyler-Smith (2003).

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Table II. Proportion of each Y-chromosome haplogroups within gr/gr-deleted sample group

Haplotype	Infertile males (%)	Presumably fertile males (%)	Fertile males (%)
DE*(×D2b)	1.8	0	1.0
F*(×J, K)	1.8	1.0	0
K*(×M, N, O, P)	0	1.0	1.6
R	1.0	1.7	0

not gene copy loss; is strongly associated with male infertility. J Med Genet 41,814–825.

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Electronic database information

Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/epiinfo).

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