

·Original Article·

Y-chromosomal microdeletions and partial deletions of the Azoospermia Factor c (AZFc) region in normozoospermic, severe oligozoospermic and azoospermic men in Sri Lanka

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Abstract

Aim: To assess for the first time the occurrence of Y chromosomal microdeletions and partial deletions of the Azoospermia Factor c (AZFc) region in Sri Lankan men and to correlate them with clinical parameters. **Methods:** In a retrospective study, we analyzed 96 infertile men (78 with non-obstructive azoospermia) and 87 controls with normal spermatogenesis. AZFa, AZFb, AZFc and partial deletions within the AZFc region were analyzed by multiplex polymerase chain reaction (PCR) according to established protocols. **Results:** No AZFa, AZFb or AZFc deletions were found in the control group. Seven patients in the group of infertile men were found to have deletions as following: one AZFa, two AZFc, two AZFbc and two AZFabc. The relative distribution of these patterns was significantly different compared with that found in the German population. Extension analysis confirmed that the deletions occurred according to the current pathogenic model. gr/gr deletions were found to be equally present both in the patients ($n = 4$) and in the control group ($n = 4$). One b2/b3 deletion was found in the patient group. **Conclusion:** These results suggest that the frequency and pattern of microdeletions of the Y chromosome in Sri Lankan men are similar to those found in other populations and confirm that gr/gr deletions are not sufficient to cause spermatogenetic failure. (*Asian J Androl* 2006 Jan; 8: 39-44)

Keywords: Y chromosome; male infertility; gr/gr; b2/b3; microdeletion; normozoospermia; azoospermia factor c

1 Introduction

Infertility affects approximately 15 % of married couples and, of these cases, approximately 50 % are due to male factors [1]. Although the causes of male infertility may vary with the geographical region and ethnic make-up of the population, a reasonable number of male

infertility cases are related to genetic factors. The most common chromosomal disorder in male infertility is Klinefelter syndrome, which is responsible for approximately 1.8 % of all cases [2]. The second most common genetic cause is microdeletions in the azoospermia factor (AZF) region of the Y chromosome [3].

The Y chromosome is the smallest chromosome in the human genome and is only transmitted from fathers to sons. In addition to the testis determining factor (SRY), located in the Yp region, the Y chromosome long arm (Yq region) harbors genes which regulate spermatogenesis [4]. Although the complete sequence of the Y chromosome is now known [4], the pathophysiological correlation between Y chromosomal microdeletions and the re-

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sulting impairment of spermatogenesis has not been fully established. Although it is difficult to establish precise genotype/phenotype correlations in patients with Y chromosomal microdeletions, deletions of *AZFa* or *AZFb* and deletions involving more than one region (*AZFbc* or *AZFabc*) have more severe effects on spermatogenesis than deletions of the *AZFc* region [3, 5]. Furthermore, different frequencies and different patterns of Yq deletions, both microdeletions and partial deletions of the *AZFc* region, have been reported in different geographical regions and ethnic groups. In Europe, the overall *AZF* deletion frequency is approximately 8 % of men with non-obstructive azoospermia or severe oligozoospermia, affecting the *AZFc* region in most cases [6]. Multi-region involvement (*AZFbc* or *AZFabc*) and deletions of *AZFa* were recorded at very low frequencies in the German population [7]. The published data for Asia indicated a certain variability in the deletion frequency depending on the selection criteria of the patients. When patients with azoospermia or severe oligozoospermia are considered together, the frequency of microdeletions varies from 5 % in Eastern Uttar Pradesh in India [8], to 7.6 % in Japan [9], 8.5 % in Calcutta, India [10], 9 % in China [11] and 10.6 % in Taiwan, China [12]. Interestingly, much higher frequencies of *AZFa* deletions (17.2 % in India) and *AZFbc* deletions (51.7 % in India and 36.6 % in China) have been recorded, compared with those in Europe [10, 11].

Recently, partial deletions of the *AZFc* region (e.g. gr/gr or b1/b3 resulting from homologous recombination between amplicons within the *AZFc* region, and removing smaller numbers of genes compared to complete *AZFc* deletions, have been described [13]. The exact effects of these partial *AZFc* deletions on spermatogenesis are still controversial. Some studies reported a significant association between partial *AZFc* deletions and spermatogenic failure [13, 14] but others did not [15, 16]. However, as the penetrance of these partial deletions is far lower than that of deletions involving the entire *AZFc*, it is to be expected that these partial deletions would not have severe effects on spermatogenesis. Data on the partial deletions of the *AZFc* region are still scant and indicate frequencies of between 4 % and 6 % of men with spermatogenetic failure [13–16]. In addition, some deletion patterns are more common in some populations, for example, in Eastern Siberian Yakuts [17], and are compatible with normal spermatogenesis and fertility [15, 16].

These studies suggest that geographical and ethnic

differences might influence the frequencies of *AZF* deletions and of partial deletions of the *AZFc* region, as well as the deletion patterns and, possibly, the phenotypic expression. In this study, the occurrence of Y chromosomal microdeletions and partial deletions of the *AZFc* region were assessed for the first time in Sri Lankan men.

2 Material and methods

2.1 Study group

The study group, consisting of infertile patients and semen donors attending the Reproductive Biology Laboratory, Faculty of Medicine, Galle, Sri Lanka, was selected retrospectively. All men gave written consent to voluntary participation in the study. Approval for the study was given by the Ethics Committee, Faculty of Medicine, Galle, Sri Lanka. Permission to use the available clinic data was obtained by the Reproductive Biology Laboratory administration.

Ninety-six patients were investigated, comprising two oligozoospermic men (sperm concentration $< 20 \times 10^6/\text{mL}$ but $> 1 \times 10^6/\text{mL}$), 15 severely oligozoospermic men (sperm concentration $< 1 \times 10^6/\text{mL}$) and 79 men with non-obstructive azoospermia. A questionnaire was distributed to each patient to collect demographic data, past medical and surgical history, including history of orchitis, mumps, testicular maldescent, testicular injuries, chemotherapy and radiotherapy, and habits concerning smoking and alcohol consumption. All patients were examined by a clinician for size, volume and consistency of the testis, hydrocele, varicocele and secondary sexual characteristics. Hormone profiles (serum follicle-stimulating hormone [FSH], luteinizing hormone [LH] and testosterone) of all patients were collected and, whenever available, historical testicular biopsy reports were collected.

Eighty-seven men of Sri Lankan origin, who were sperm donors for heterologous insemination with normal ejaculate parameters, according to the World Health Organization (WHO) criteria [18], were recruited as the control group.

2.2 Molecular analysis

Five mL of ethylene diamine tetraacetic acid (EDTA) blood was collected from each man in the study groups and genomic DNA was extracted using the Wizard DNA extraction kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Microdeletion analysis

of the Y chromosome Yq *AZF* region involved two steps. In the first step, aimed at detecting *AZF*a, *AZF*b and *AZF*c microdeletions, two multiplex polymerase chain reaction (PCR) systems (A and B) were carried out according to the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/EMQN) guidelines [3] using the recommended first choice primers and the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany). Each 50 μ L PCR reaction mix contained 25 μ L 2 \times Qiagen Multiplex PCR MasterMix (containing HotStarTaq DNA polymerase [Qiagen, Hilden, Germany], Qiagen Multiplex PCR Buffer [containing 6 mmol/L MgCl₂] and deoxyribonucleotide triphosphate mix), 5 μ L 10 \times Primer mix (2 μ mol/L each primer), ~ 1 μ g template DNA, and sterile distilled water to 50 μ L. Amplification started with an activation step of 15 min at 95 $^{\circ}$ C, followed by 35 cycles of 30 s denaturation (94 $^{\circ}$ C), 90 s annealing (57 $^{\circ}$ C) and 60 s elongation (72 $^{\circ}$ C), ended by an elongation step of 10 min and cooling to 4 $^{\circ}$ C. Reaction products (30 μ L) were separated on 2 % agarose (Peqbold Universal Agarose, Peqlab, Erlangen, Germany) plus 0.5 % DNA agar (Serva, Heidelberg, Germany) gels in 1 \times Tris borate EDTA (TBE) for 25 V overnight. Extension analysis was performed according to the EAA/EMQN guidelines [3]. In the second step, we used two multiplex systems, one including the sequence tagged site (STS) primers sY1291, sY1191 and sY1161 and the other including sY1201 and sY1206 to screen the partial deletions of the *AZF*c region, as described previously [15]. The samples, in which the deletions were detected by the above multiplex systems, were confirmed by repeating the single primer (simplex) reactions.

2.3 Statistical analysis

Fisher's exact test and the χ^2 test were used to compare the frequency of deletions by applying a commercially available software package (GraphPad Prism version 3; GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3 Results

Seven of the 96 infertile men (7.3 %) were found to have Y chromosome microdeletions in the *AZF* region (Table 1). One of these seven men (case 18) had severe oligozoospermia, and the rest had azoospermia. All men with microdeletions had normal serum testosterone levels. Five patients had high serum FSH levels. One patient (case 96) showed increased serum FSH and LH levels; the LH level was normal in the others.

The microdeletion patterns observed were *AZF*c in two patients (cases 18 and 38), *AZF*b in two patients (cases 48 and 84), *AZF*a in one patient (case 87) and *AZF*abc in two patients (cases 20 and 96). The further characterization of the microdeletions by extension analysis showed a complete *AZF*a deletion in case 87 (sY82 and sY83 present, DBY, USP9Y and sY87 deleted, sY88 present) and deletion of the marker sY160 in cases 20 and 96, suggesting a large, terminal deletion in these two patients. Due to a shortage of DNA we could further analyze only one of the two patients with the *AZF*b deletion pattern. Using the STS markers, sY1264, sY1227, sY1228, sY1283, sY117, sY1291, sY639, sY1206 and sY1201, case 84 showed a pattern corresponding to the deletion model P4/distal P1 according to Repping *et al.* [13]. No microdeletions were found in the control group.

Table 1. Clinical findings in seven infertile men from Sri Lanka with microdeletions in the azoospermia factor (*AZF*) region. FSH, follicle-stimulating hormone; LH, luteinizing hormone; MA, maturation arrest at the spermatid stage; N/A, not assessed; SCOS, Sertoli cell-only syndrome.

Patient case No.	<i>AZF</i> deletion pattern	Spermconcentration ($\times 10^6$ /mL)	Testosterone (nmol/L)	FSH(IU/L)	LH(IU/L)	Testicular histology	Combined testicular volume(mL)
18	c	0.1	14.9	12.1	3.4	N/A	24.0
20	abc	0.0	12.6	23.5	2.7	SCOS	20.0
38	c	0.0	24.2	4.3	5.9	Normal	20.0
48	bc	0.0	33.4	16.6	5.5	SCOS	12.0
84	bc	0.0	23.3	27.8	1.8	MA	16.0
87	a	0.0	29.7	4.4	3.9	SCOS	20.0
96	abc	0.0	21.3	87.0	23.3	SCOS	12.0

Two different patterns of partial deletions within the AZFc region were observed in five men in the patient group. Four (4.17 %) had gr/gr deletions and one (1.04 %) had a b2/b3 deletion pattern (Table 2). One man with gr/gr deletion was oligozoospermic (sperm concentration $4.2 \times 10^6/\text{mL}$) but the others were azoospermic. Testicular biopsy reports were available for three of the five patients with partial deletions of the AZFc region. Two cases (cases 28 and 93) had Sertoli cell-only syndrome (SCOS) and one patient (case 24) had seminiferous tubule atrophy. Four subjects of the control group were detected to have gr/gr deletions, which was the only pattern of partial deletions of the AZFc region observed in the control group. All of them had normal ejaculate parameters (Table 3). The statistical analysis of gr/gr deletions in the control group and the patient group did not show any significant difference (Fisher's exact test, $P = 0.72$).

A comparison of the relative frequency of the type of microdeletions of the Y chromosome in Sri Lanka and Germany, according to the authors' previously published data [3], is shown in Table 4. A statistically significant difference in the distribution of the deletion pattern was observed. In particular, on the basis of the present results, AZFc microdeletions seem to be less frequent than the other patterns in Sri Lankan men compared to German men.

4 Discussion

Collecting demographic data is important in order to assess the appropriateness of the current analysis protocols [3], which might require adjustments of the STS panel according to the ethnic origin of the patients [19]. In this study we analyzed for the first time microdeletions of the Y chromosome in a small sample of Sri Lankan men. We found AZF microdeletions in 7.3 % of a selected group of infertile patients, a frequency similar to that of 8 % recorded as the overall frequency for AZF deletions among infertile patients [6]. Therefore, the frequency of Y chromosomal microdeletions in Sri Lankan men is similar to that observed in different regions of India [8, 10, 20]. The frequency distribution of the different types of AZF deletions, however, seems to be different from that observed in European men of German origin, suggesting that ethnic differences, possibly reflected by Y chromosome haplogroups, might result in a higher incidence of rare microdeletions in some populations. In a previous study conducted with Indian men, a similar overall frequency of Y chromosomal microdeletions was found, with a very high incidence (24.4 %) of AZFa microdeletions [10] and complex patterns within this region which, however, were not verified by Southern blotting. More data should be collected in order to verify whether Indian men might be more

Table 2. Clinical findings in five Sri Lankan infertile men with partial deletions in the azoospermia factor c (AZFc) region. FSH, follicle-stimulating hormone; LH, luteinizing hormone; N/A, not assessed; SCOS, Sertoli cell-only syndrome.

Patient case No.	Deletion pattern	Sperm concentration ($\times 10^6/\text{mL}$)	Testosterone (nmol/L)	FSH (IU/L)	LH (IU/L)	Testicular histology	Combined testicular volume (mL)
24	gr/gr	0.0	18.5	27.6	6.0	Atrophy	8.0
28	gr/gr	0.0	N/A	11.8	N/A	SCOS	16.0
51	gr/gr	0.0	9.9	63.9	34.0	N/A	4.0
78	gr/gr	4.2	23.3	4.3	2.1	N/A	20.0
93	b2/b3	0.0	8.8	25.0	5.4	SCOS	24.0

Table 3. Semen parameters in men in the control group with partial deletions in the azoospermia factor c (AZFc) region.

Subject case No.	Deletion pattern	Semen volume (mL)	Sperm concentration ($\times 10^6/\text{mL}$)	Total sperm count ($\times 10^6/\text{mL}$)	Motility (a+b, %)	Morphology (%)
3	gr/gr	2.3	71.5	164.45	63.0	30.0
6	gr/gr	3.5	21.5	75.25	60.0	30.0
21	gr/gr	2.0	22.5	45.00	60.0	90.0
28	gr/gr	3.5	27.5	96.25	65.0	47.0

Table 4. Relative distribution of microdeletion patterns in Sri Lanka (this study) and Germany [3] in patients with microdeletions of the Y chromosome. $P = 0.0155$ (*AZFc* vs. other deletions, Fisher's exact test).

Microdeletion pattern	Sri Lanka ($n = 7$ deletions)	Germany ($n = 34$ deletions)
<i>AZFa</i>	1 (14.2 %)	1 (3 %)
<i>AZFb</i>	0	3 (9 %)
<i>AZFc</i>	2 (28.6 %)	27 (79 %)
<i>AZFabc</i>	2 (28.6 %)	1 (3 %)
<i>AZFbc</i>	2 (28.6 %)	2 (6 %)

prone to *AZFa* deletions than European men.

Extension analysis of the two patients in our study with *AZFabc* deletions showed that the heterocromatic marker sY160 was absent in both cases, suggesting the occurrence of a terminal deletion. Cytogenetic investigation, however, was not performed in these men. The further molecular analysis of one case with *AZFa* and one case with *AZFbc* deletions confirmed the complete removal of the entire *AZFa* region, including the two genes present in it, and the deletion pattern P4/distal P1 in the latter case. This suggests that the mechanism of microdeletions, that is, homologous recombination between identical sequences flanking the deletion, should be valid independently of the ethnic origin of the Y chromosome.

The patients with *AZFa* or *AZFabc* deletion patterns showed SCOS in their biopsy reports. One of the two patients with an *AZFbc* deletion pattern showed maturation arrest. The second patient had SCOS. These findings are compatible with the recorded trend of genotype/phenotype correlation reported in other studies [5] and the concept that complete deletions of the *AZFa*, or *AZFb*, or *AZFbc* region result in SCOS or maturation arrest and the virtual impossibility of retrieving sperm by testicular sperm extraction attempts. Of the two patients with *AZFc* deletions, one was severely oligozoospermic and the other was azoospermic with, surprisingly, a normal testicular biopsy. The latter case suggests that an obstruction might be present in this patient, compatible with his normal serum FSH levels, and reinforces the concept that *AZFc* deletions might be compatible with spermatogenesis sufficient for fertility [21 and references therein], provided that no obstructions are concomitantly present.

In this study, we analyzed for the first time the occurrence of partial deletions of the *AZFc* region in a small

sample of Sri Lankan men. The commonest partial *AZFc* deletion pattern observed was the *gr/gr* pattern, which was detected in four (4.2 %) patients and four (4.6 %) control men, indicating no difference in the occurrence of *gr/gr* deletions between patients and controls. Our Sri Lankan data confirm previous findings in other populations that partial deletions of the *AZFc* region are not sufficient to cause spermatogenic failure. This finding is in contrast with some recent data reporting a significant correlation between the presence of *gr/gr* deletion and spermatogenic failure in Spanish men [14]. Similar to the present finding, however, Hucklenbroich *et al.* [15] and Machev *et al.* [16] found no significant differences between the control group and the patient group. *gr/gr* deletions have been associated with particular Y chromosomal haplogroups [13]. Although the correct distribution pattern of Y haplogroups in Asian populations is still not known, haplogroup N, which is susceptible to spontaneous *gr/gr* deletions, is reported to be common in this region [22]. In any case, our results show clearly that *gr/gr* deletions cannot be considered sufficient cause for spermatogenic failure. Haplogroup and *gr/gr* analysis in large groups of men of different ethnic origin with normal spermatogenesis need to be performed to characterize the role played by this type of chromosomal rearrangement in male fertility. Meanwhile, we see no basis to recommend screening for *gr/gr* deletions as a routine investigation in male infertility.

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