Original Article

The low frequency of Y chromosome microdeletions in subfertile males in a Sinhalese population of Sri Lanka

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AIMS: This study was designed to determine the prevalence of azoospermia factor (AZF) microdeletions on the Y chromosome in Sri Lankan Sinhalese infertile men with azoospermia and severe oligozoospermia.
SETTINGS AND DESIGN: The patient group was 207 karyotypically normal infertile Sinhalese males.
MATERIALS AND METHODS: The presence of 13 sequence-tagged site (STS) markers in the AZF region was tested using multiplex polymerase chain reaction (M-PCR). One hundred and twenty unselected men were also studied as a control group.
RESULTS: Three (1.5%) had classic Y chromosome microdeletions in the AZFc sub-region.
CONCLUSIONS: These results suggest a much lower Y chromosome microdeletion frequency than previously thought, even among a strictly selected group of sub-fertile males in Sri Lanka.

Key words: Azoospermia factor a, azoospermia factor b, azoospermia factor c, male factor sub-fertility, Y-chromosome microdeletions

Introduction

Infertility is inability of a sexually active, non-contracepting couple to achieve pregnancy in one year.[1] In men, oligozoospermia, asthenozoospermia, teratozoospermia, and azoospermia are the main causes of infertility, which accounts for 20%-25% of cases worldwide. About one in ten couples are infertile for several possible reasons, with the male factor being responsible for approximately 50% of those cases. This group is typically split into 30% with strictly male factors, and 20% with both male and female factors.[2] However, male infertility is a multifactorial syndrome and in more than half of infertile men, the cause of their infertility is unknown (idiopathic) and could be congenital or acquired. Furthermore, up to 10% of male infertility cannot be explained medically.[3]

Genetic abnormalities have been identified in men with unexplained oligozoospermia and azoospermia, including numerical and structural chromosomal abnormalities.[4] *De novo* deletions of Yq are one of the most frequently-occurring chromosomal abnormalities in men and are believed to arise from recombination events between long stretches of highly repetitive DNA sequences during meiosis or early pre-implantation development.[5] The Y chromosome is a male-specific, 60 megabases (Mb) in size chromosome that consists of 60 million nucleotides and is one of the smallest human chromosomes with the least number of genes compared to any other chromosome. In 1976, Tiepolo and Zuffardi were the first to hypothesize a correlation between Y chromosome deletions and male infertility. It was postulated that at least one genetic Y factor essential for male germ cell development is located in the distal Yq11.[6] The spermatogenesis locus azoospermia factor (AZF) in Yq11 has been mapped to three microdeletion intervals designated as AZFa, AZFb, and AZFc[7] [Table 1]. Several genes located in the AZF regions are expressed in the testes and could, therefore, be viewed as "AZF candidate genes" [Figure 1]. The genetic complexity of the AZF locus located in the Yq could be revealed only

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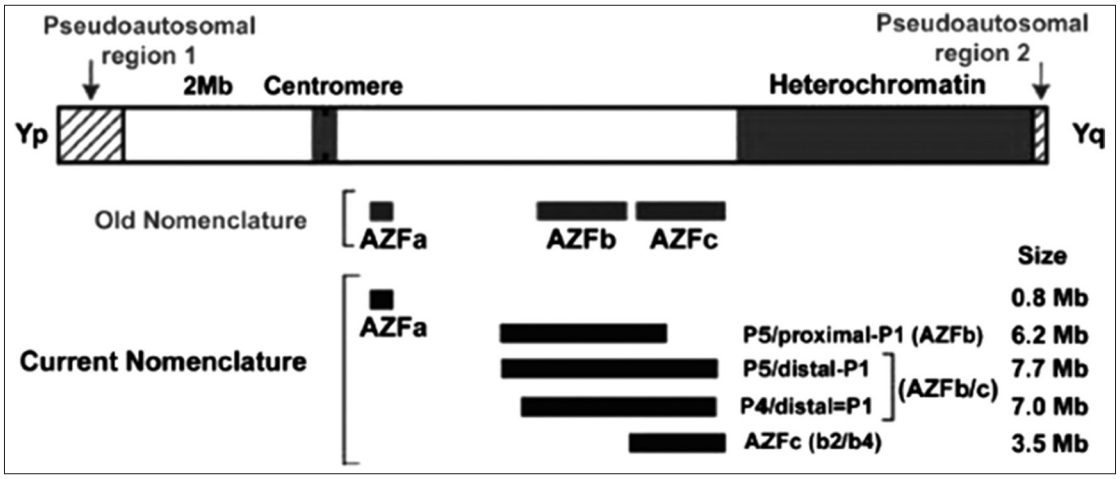

Figure 1: Map of the Y chromosome (Yq) AZF region

Table 1: Azoospermia factor candidate genes

Gene	Length	Frequency (%)	Important candidate genes	Genotype/ phenotype
AZFa	792kb	1-2	DFFRY or UPS6Y, DBY	SCO syndrome
AZFb	1-3Mb	Intermediate. Between AZFa and AZFc	RBMY	Matuaration arrest of germ cells and azoospermia
AZFc	3.5Mb	2-10	DAZ, CDY	Mild/severe oloigozoospermia, azoospermia

AZF: Azoospermia factor; SCO: Sertoli cell only

with the development of sequence-tagged sites (STSs). Vollrath, *et al.*[8] reported the first polymerase chain reaction (PCR)-based sequence-tagged sites (STSs), interval map of the Y chromosome. These analyzes permit the detection of interstitial sub-microscopic deletions not visible at the cytogenetic level and detectable only by STS-PCR or southern hybridization. Such deletions are called microdeletions.[9] The AZFc region in the distal Y chromosome long arm, spanning 3.5 Mb between the b2/b4 amplicons, is critical for male fertility as it contains many gene families required for normal spermatogenesis.[10] Deletions in this region are the most frequent molecular genetic cause of severe infertility, observed with a prevalence of 5-10% in cases of azoospermia and severe oligozoospermia.[11] Deletions most frequently seen are the AZFc region including DAZ, less frequently the AZFb region including RBMY, and rarely the AZFa interval.[7] In addition to these deletions, several smaller sub-deletions exist within the AZFc region. The most prevalent of which is termed gr/gr[12] [Figure 1]. The clinical significance of gr/gr deletions remains controversial.[13]

The aim of this study was to detect the prevalence of azoospermia factor (AZF) microdeletions on the Y chromosome in Sri Lankan Sinhalese infertile men with azoospermia and severe oligozoospermia, as there are very few Sri Lankan studies carried out on this subject.

Materials and Methods

In the present study, 207 karyotypically normal infertile Sinhalese males were recruited from the fertility clinic at the Department of Obstetrics and Gynecology at the Faculty of Medicine, University of Colombo, assisted reproduction centers and private infertility clinics during the period from January 2008 to December 2010, according to a protocol approved by the Ethics Review Committee of the Faculty of Medicine, University of Colombo. Patients presented with primary infertility and having sperm counts less than 10 M/ml on at least two consecutive occasions were our candidates for the present study. Semen analysis results, family history, occupational and reproductive history, past medical history, including history of chemotherapy, radiotherapy, varicocele, and cryptorchidism, and habits concerning smoking, alcohol consumption were obtained from each patient. In addition, hormone profile (FSH, LH, total testosterone, and prolactin) and histological testicular biopsy reports were collected whenever available. In addition, 120 random DNA samples of males from the general population were also subjected to Y chromosome microdeletion testing to determine

the prevalence of Y chromosome microdeletions in the general population. These samples came from a population-based DNA collection maintained in our unit for studies of this nature with the approval of the Ethics Review Committee of the Faculty of Medicine, University of Colombo. Ethics clearance was obtained to use the samples for the current study.

Y chromosome microdeletion analysis

Peripheral blood (3 ml) was collected from each male in the study group. In addition to the blood sample, a semen sample was collected from each oligozoospermic male. Genomic DNA was extracted using the blood tissue DNA mini extraction kit (Qiagen, Germany) according to the manufacturer's protocol.

All the AZF regions, AZFa, AZFb, and AZFc, were tested for Y chromosome microdeletions in the Yq AZF region in two steps. In the first step, aimed at detecting AZFa, AZFb, and AZFc classic microdeletions, we used the multiplex PCR amplification system suggested in the state of the art recommendations made by the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/EMQN) guidelines.[14] Two multiplex reactions (A and B) were used for the analysis of the three AZF deletion regions on the Y chromosome. Both multiplexes contain five fragments, i.e., the three AZF loci and the two control fragments SRY and ZFY. The 25 μl PCR reaction mix contains: 12.5 μl 2x Quiagen Multiplex PCR MasterMix [containing HotStarTaq DNA Polymerase, Qiagen Multiplex PCR Buffer (containing 6 mM $MgCl_2$) and dNTP Mix], 2 μl ×10 Primer mix, approximately 1 μl template DNA and 25 μl of sterile distilled water.

Amplification conditions start with an initial activation step of 15 min at 95°C, followed by 35 cycles of 30 sec denaturation (94°C), 90 sec annealing (57°C) and 60 sec elongation (72°C), plus a final elongation step of 10 min and cooling to 4°C. The PCR products were separated by electrophoresis on a 3% agarose gel impregnated with ethidium bromide and visualized under UV light [Figure 1]. ZFY/ZFX (zinc finger protein) was used as an internal control. The SRY gene (sex-determining region of the Y) was examined to confirm the sex of the donor, while a negative control DNA was obtained from a woman.

Patients without the classical AZFa, AZFb, and AZFc deletions underwent assessment for AZFc partial deletions including all 120 control subjects. In the second step, we used a multiplex system to determine the presence or absence of the AZFc subdeletions by using sequence-tagged sites (STS) markers as described by Repping, *et al*.[12] Primer sequences were as described[12] except for sY1206, which used the primers sY1206* F (5´-ctgggctttctgtggcattt-3´) and sY1206 R (5´-gccaatttgaccagtgacttc-3´) from within the GenBank sY1206 sequence to allow better size separation of multiplex PCR products on agarose gels.[15] The sequences of all primer pairs and the expected size of the PCR products are shown in Table 2. A 2 μl aliquot of the genomic DNA was amplified by a multiplex PCR in a total volume of 25 μl containing 12.5 μl 2x Quiagen Multiplex PCR MasterMix, 2 μl 10x Primer mix and 25 μl sterile distilled water. The PCR result was visualized on a 2% agarose gel with ethidium bromide including a 100 bp DNA ladder as a marker. In all PCRs, a female DNA and a water sample (no template) were included as negative controls [Figure 1].

Results

The study included a total of 327 patients, among these, 153 (73.9%) were azoospermic (zero sperm count), 54 (26.1%) were severe oligozoospermic (>0.1 to <5 Million/ml), and 120 were healthy controls. All azoospermic patients were confirmed to have a non-obstructive etiology based on standard clinical evaluation. The mean age of the infertile men was of 34.8, ranging from 25 to 35 yrs. with a standard deviation of ± 5.34. A family history of infertility was found in 9.7% of the patients, 4.3% and 8.7% of the patients had a past medical history of vericocele and crytorchidism, respectively, while 42% were smokers or alcohol consumers [Table 1].

Three (1.5%) of the 207 infertile men had a deletion of AZFc loci [Figure 2]. The failed amplification of sY254 and sY255 markers indicates a complete deletion in the AZFc sub-region in one (0.5%) patient, while two (1%) patients had the absence of the sY254 marker. All three Y chromosome microdeletions were present in the azoospermic men, and none were observed in the

53 oligospermic cases and the 120 controls. No partial AZFc deletions were found. The mean age of patients with Y chromosome deletions was 33 yrs. Hormone profiles were available for 177 [Table 3]. Testicular pathology reports were available in 82 patients. The most frequent finding was spermatogenic arrest at the level of spermatogonia (SGA), which was seen in 42% of the patients. Hypo spermatogenesis (SGA) and sertoli cell only syndrome (SCOS) were 17% and 12%, respectively. The remaining 29% of the patients had normal testicular biopsies. Phenotypic data and clinical characteristics of these patients are shown in Table 3.

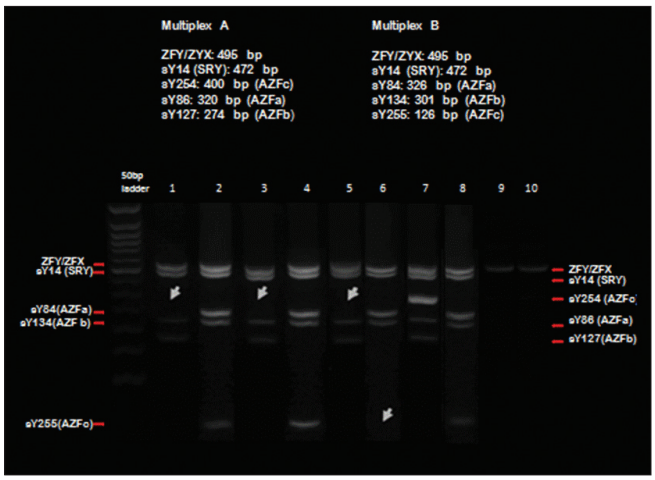

Figure 2: Gel electrophoresis profile of three azoospermic men with normal karyotype. Patients K17 and K25 in lanes 1, 2 and 3, 4, respectively, with the absence of the sY254 marker, patient (K6) with the absence of the sY254 and sY255 markers, indicating a complete deletion in the AZFc sub-region in the 5th and 6th lanes where the deleted bands are marked with an arrow. A normal male in the 7th and 8th lanes and a female sample as the negative control in the 9th and the 10th lanes.

Discussion

It is estimated that globally 60-80 million couples suffer from infertility every year.[2] In approximately 30%-50% of all cases of azoospermia or severe oligozoospermia, etiology is idiopathic.[16] The microdeletions in the AZF loci are associated with azoospermia and severe oligozoospermia as well as varied testis histology ranging from sertoli cell-only syndrome (SCOS) to hypospermatogenesis (HSG) and maturation arrest.[17-19]

In the present study, we tested 207 infertile males for Y chromosome microdeletions using the protocol recommended by the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/EMQN) (http://www.emqn.org)[14] and found microdeletions in the AZF c sub-region in three

Table 2: Sequences of the different primer sets adopted in the present study

STS	Primer sequence	PCR product size	Reference
ZFY-F	ACCRCTGTACTGACTGTG	495bp	Simoni, Bakker *et al.* 2004
ZFY-R	GCACYTCTTTGGTATCYGAGAAAGT		
SRY-F	GAATATTCCCGCTCTCCGGA	472bp	Simoni, Bakker *et al.* 2004
SRY-R	GCTGGTGCTCCATTCTTGAG		
SY84-F	AGAAGGGTCTGAAAGCAGGT	326bp	Simoni, Bakker *et al.* 2004
SY84-R	GCCTACTACCTGGAGGCTTC		
SY86-F	GTGACACAGACTATGCTTC	320bp	Simoni, Bakker *et al.* 2004
SY86-R	ACACACAGAGGGACAACCCT		
SY127-F	GGCTCACAAACGAAAAGAAA	274bp	Simoni, Bakker *et al.* 2004
SY127-R	CTGCAGGCAGTAATAAGGGA		
SY134-F	GTCTGCCTCACCATAAAACG	301bp	Simoni, Bakker *et al.* 2004
SY134-R	ACCACTGCCAAAACTTTCAA		
SY254-F	GGGTGTTACCAGAAGGCAAA	380bp	Simoni, Bakker *et al.* 2004
SY254-R	GAACCGTATCTACCAAAGCAGC		
SY255-F	GTTACAGGATTCGGCGTGAT	123bp	Simoni, Bakker *et al.* 2004
SY255-R	CTCGTCATGTGCAGCCAC		
SY1191-F	CCAGACGTTCTACCCTTTCG	385bp	Repping, Skaletsky *et al.* 2002
SY1191-R	GAACCGTATCTACCAAAGCAGC		
SY1291-F	TAAAAGGCAGAACTGCCAGG	527bp	Repping, Skaletsky *et al.* 2002
SY1291-R	GGGAGAAAAGTTCTGCAACG		
SY1201-F	CCGACTTCCACAATGGCT	677bp	Repping, Skaletsky *et al.* 2002
SY1201-R	GGGAGAAAAGTTCTGCAACG		
SY1206-F	CTGGGCTTCTGTGGCTTT	412bp	Lynch, Cran *et al.* 2005
SY1206-F	GCCAATTTGACCAGTGACTTC		
SY1161-F	CGACACTTTTGGGAAGTTTCA	330bp	Repping, Skaletsky *et al.* 2002
SY116-R	TTGTGTCCAGTGGTGGCTTA		

PCR = Polymerase chain reaction

Table 3: Clinical details of the three azoospermic men with Y chromosome microdeletions

Testicular biopsy	Case no.	Age (yrs)	Karyotype	FSH (mIU/ml)	LH (mIU/ml)	STS deleted	AZF deleted	%
SCOS	K6	30	46, XY	19.8	8.0	sY254, sY255	AZFc	0.5
SCOS	K17	38	46, XY	15.3	8.0	sY255	AZFc	0.5
SGA	K25	34	46, XY	8.3	8.5	sY255	AZFc	0.5

SCOS = Sertoli cell only syndrome; SGA = Spermatogenic arrest

(1.5%) patients, out of which two patients showed SCOS (Sertoli cell-only syndrome) in their biopsy reports, while one patient showed SGA (spermatogenic arrest). These findings are compatible with the genotype/phenotype correlation observed in other studies.[19]

The proportion of infertile men with microdeletions in one or more sub-regions in the different countries is ranging from 0.7-34.5% with an average of 8.2%.[20] The changes in the prevalence could be also due to either different ethnicities, small sample size or endocrine disorders, or an unknown environmental factor, as has also been documented in different populations of male infertility.[21-25] In a study conducted on 3179 patients in Germany, 39 (2.4%) Y-chromosomal microdeletions were found,[26] indicating a much lower frequency of AZF deletions in Germany than in other countries. In a previous study conducted in Sri Lanka, in a group of infertile men coming from a diverse ethnic background, seven out of 96 men (7.3%) were found to have classic Y chromosome microdeletions in the AZF region.[27] It is possible that ethnic differences, selection criteria, and methodological aspects can contribute to the difference between the present and previous studies. However, a study conducted by Holly Cherian, in 2009, at the University of North Texas Health Science Center at Fort Worth, USA on Y chromosomal STR (short tandem repeat) concluded that the Sri Lankan population showed large amount of genetic diversity due to the high amount of various haplotypes observed (168 different haplotypes from a total of 171 samples analyzed). Additionally, when 10% of the Sri Lankan Y-chromosomal DNA samples were searched against the Applied Biosystems Yfiler® haplotype database, it was concluded that the Sri Lankan population haplotypes are unique to its own population, because they were not observed within this database.[28] Furthermore, it has been shown that the susceptibility to Y chromosomal microdeletions varies between different haplogroups.[29,30] Therefore, the lower frequency and disparity of Y chromosomal microdeletions in our study could be due to the fact that our study population was limited to the Sinhalese population, which is one of many ethnicities of Sri Lanka and that we used a selective panel of sequence tagged sites (STS) markers.

Although the percentage of Y chromosome microdeletions is lower in a selected population like ours, there are several considerations that support routine assessment of Yq deletions. Firstly, a positive test will provide a firm diagnosis of the man's problem, which, for some couples with longstanding infertility, can help resolve stress, blame, or feelings of guilt. Secondly, knowledge of the type of Yq deletion may assist the clinician in determining the best assisted reproductive technique (ART) treatment. Thirdly, couples should be offered this information, as they must understand that their male offspring will almost certainly be sub-fertile.[31] Fourthly, infertile men are known to be at increased risk of androgen deficiency and testicular neoplasia.[32] Lastly, the general heterogeneity and instability of the human Y chromosome suggests that AZF-microdeletions can also become "pre-mutations" for a subsequent complete loss of the Y chromosome in the AZF deleted patients' sperms, increasing the risk of embryonic X0 cells.[7]

Our study confirmed that detection of microdeletions of the AZF region in Sri Lankan Sinhalese sub-fertile patients is rare but important in the prognostic aspect. Therefore, the inherent heterogeneity and instability of the AZF loci on the human Y chromosome should be kept in mind when counseling men with AZF deletions for the risk of their inheritance after ICSI (Intra Cytoplasmic Sperm Injection) treatment. Furthermore, a routine Yq microdeletion testing before ART will inform affected couples of the cause of their infertility and the inevitability of vertical transmission (and likely infertility) in male offspring. Therefore, we recommend that Y chromosome microdeletion testing be a mandatory preliminary step to define accurately the etiology of spermatogenic failure, which has significant ethical consequences, particularly if the patient is a candidate for assisted reproductive techniques.

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