Molecular screening for Yq microdeletion in men with idiopathic oligozoospermia and azoospermia

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Infertility affects 15% couples attempting pregnancy and in 40–50% of these cases the male partner has qualitative or quantitative abnormalities of sperm production. Microdeletions in the azoospermia factor (AZF) region on the long arm of the Y chromosome are known to be associated with spermatogenic failure and have been used to define three regions on Yq (AZFa, AZFb and AZFc) which are critical for spermatogenesis and are recurrently deleted in infertile males. Semen analysis was carried out on one hundred and twenty five infertile males with oligozoospermia and azoospermia. Cytogenetic analysis was done for all the cases and in all cytogenetically normal cases (n = 83) microdeletion analysis was carried out on DNA extracted from peripheral blood using PCR. The sequence tagged sites (STS) primers sY84, sY86 (AZFa); sY127, sY134 (AZFb); sY254, sY255 (AZFc) were used for each case. Eight of the eighty three cases (9·63%) showed deletion of at least one of the STS markers. Correlation of phenotype with microdeletion was done in each case to determine any phenotype association with deletion of particular AZF locus. Based on the present study, the frequency of microdeletion in the Indian population is 9·63%. This study emphasizes the need for PCR analysis for determining genetic aetiology in cases with idiopathic severe testiculopathy.

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1. Introduction

Infertility is a major health problem today affecting 10% to 15% of couples. In approximately 30% of cases the pathology is found in man alone and in another 20% of cases both man and woman are affected. The male factor is therefore responsible in about 50% of infertile couples.

Forty to fifty percent of these men have qualitative or quantitative abnormality in sperm production. In more than 60% of cases the origin of reduced testicular function is unknown (Krausz *et al* 2000). Recent studies point to both environmental and genetic factors which have caused a decline in male reproductive health.

The Y chromosome has been shown for a long time to be involved in spermatogenesis. Since 1970 it was established that deletion of long arm of Y chromosome is associated with spermatogenic failure. It is only in the last few years that the loci involved in production and differentiation of sperms have been identified using molecular methods.

The entire length of the Y chromosome has been subdivided into 7 deletion intervals as shown in figure 1. Each of these intervals is further subdivided into subintervals (A, B, C, etc.) (Vergnaud *et al* 1986). In 1992, Vollrath and colleagues constructed a 43 interval deletion map of human Y chromosome which contained an ordered array of sequence tagged sites (STS) which span the long and

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Abbreviations used: AZF, azoospermia factor; FSH, follicular stimulating hormone; SCO, Sertoli cell only; STS, sequence tagged sites.

short arm of Y chromosome. The genes critical for spermatogenesis are located on long arm of Y chromosome in deletion interval 6 band 11·23 and deletion interval 5 also. This critical region for spermatogenesis is the azoospermia factor (AZF), the deletion of which is associated with azoospermia. The AZF region has 3 non-overlapping loci-AZFa, AZFb, AZFc which are required for normal spermatogenesis. The AZFa is located on proximal Yq from interval 5B to 5D. The AZFb region in centre of Yq from interval 5K to 6B and AZFc is immediately proximal to heterochromatin region from 6C to 6F. The microdeletion in the AZF are not only associated with azoospermia but with a varied testis histological profile ranging from sertoli cell only (SCO) to hypospermatogenesis (Vogt *et al* 1996). These AZF genes are putative RNA binding proteins and so may be involved in regulation of gene expression. In order to gain information about AZF deletions in the Indian patients and its relationship with infertile phenotype, we decided to analyse the DNA of cytogenetically normal infertile patients with idiopathic oligo and azoospermia.

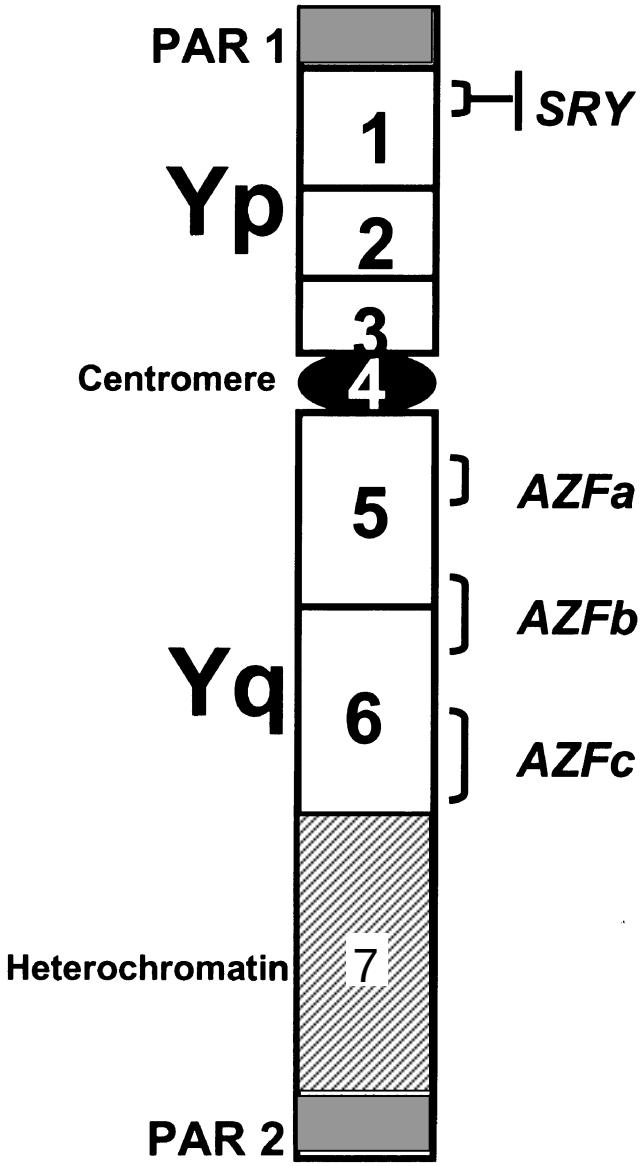

Figure 1. Schematic representation of the Y chromosome showing seven deletion intervals and pseudoautosomal region (PAR) 1 and 2.

2. Materials and methods

One-hundred and twenty-five men with idiopathic infertility and 25 age-matched fertile controls were included in this study. The diagnosis of azoospermia and oligozoospermia was made on the basis of semen analysis according to WHO guidelines (1992). Each patient was carefully examined to rule out other causes of infertility and a detailed history was taken on a predesigned proforma. Peripheral blood cultures were setup for chromosomal analysis in all the cases and 5 well spread G-banded metaphases were karyotyped using the standard protocols. The values of follicular stimulating hormone (FSH), luteinizing hormone and testosterone levels were recorded for all patients. Wherever possible, testicular cytopathological details following testicular fine needle aspiration cytology was collected as testicular biopsy was ethically not possible in these cases.

2.1 *PCR analysis*

In cytogenetically normal infertile men ($n = 83$), PCR screening was done for Yq microdeletions. Peripheral blood sample was collected from these cytogenetically normal infertile patients and DNA was isolated using phenol chloroform extraction method. Each of these patients were examined for 6 AZF loci which mapped to interval 5 and 6 of the Y chromosome. The STS primers used were – for AZFa: sY84, sY86; for AZFb: sY127, sY134; for AZFc: sY254, sY255. The internal control used was SRY (sex determining region of the Y) – sY14. Fertile male and female samples were used as positive and negative control and water was used as blank. This primer set was suggested by Simoni *et al* (1999) and prescribed by the European Academy of Andrology which enables the detection of over 90% deletions in the AZF loci and allows for minimal standardization and comparison of the data on AZF deletions from different laboratories in different countries.

Samples were subjected to PCR amplification using 35 cycles of 95°C for 1 min, 56°C for 30 s and 72°C for 1 min. Initial denaturation was done for 5 min at 95°C and final extension time of 7 min at 72°C was given.

The PCR products were analysed on a 1·8% agarose gel containing ethidium bromide (0·5 μg/ml). A STS was considered absent only after atleast 3 amplification failures in the presence of successful amplification of internal control (SRY).

3. Results

Of the 125 infertile men, 29 had numerical chromosomal aberrations and were not analysed further at the molecular level. These cases were Klinefelter syndrome 47, XXY (n = 11), mosaic Klinefelter (n = 9) and Klinefelter variants (n = 5), 46,XX male (n = 1), 46,XY(80%)/47, XYY(20%) (n = 1), 46,XX(90%)/46,XY(10%) (n = 1), 46,XY(Yq-). PCR analysis was done in 83 cytogentically normal infertile patients and 25 fertile controls.

PCR amplification produced a band of expected size for all the 7 loci investigated in 75 patients and 25 fertile controls. Eight patients (9·63%) showed a deletion of one or more STS namely sY84, sY86, sY127, sY134 (patients 1,2), sY84, sY86, sY127 (patient 3), sY127, sY134 (patient 4) and sY254, sY255 (patient 5, 6, 7, 8). All deletions were interstitial. PCR amplification performed on the genomic DNA of fathers of two of these eight patients (patient 4 and 8) did not show microdeletions and thus the deletions in patients 4 and 8 were *de novo* or the fathers were mosaic for these deletions. The fathers of the other six patients with microdeletion (patient 1, 2, 3, 5, 6 and 7) were not available. It can be summarized (table 1) that patients 1 and 2 had complete deletion of AZFa and AZFb loci, patient 3 had complete AZFa deletion and partial deletion of AZFb and patient 4 had AZFb deletion alone. In cases 5, 6, 7, 8 there were deletion of AZFc loci. Seven of these eight patients (patient 1, 2, 3, 4, 5, 7, 8) were azoospermic and patient 6 had oligo-asthenoteratozoospermia with a total mean sperm count of 1·5 million which had declined to 0·2 million after one year. Patient 4 and 8 with AZFc deletion were cryptorchid and testicular FNAC was not possible. Patients 1, 2 and 3 with AZFa and AZFb microdeletion had complete absence of germ cells in the semeniferous tubules (a picture characteristic of SCO syndrome). Patients 5 and 6 with AZFc deletion showed hypospermatogenesis and patient 7 with AZFc deletion had maturation arrest at the level of secondary spermatocyte (figure 2).

4. Discussion

Y chromosome deletions are emerging as an important cause of male infertility. The frequency of Y chromosome deletion increases with the severity of spermatogenic defect (Rejio *et al* 1996; Foresta *et al* 1997; Pryor *et al* 1997; Krausz *et al* 2001). About 15% azoospermic and 5% to 10% oligozoospermic men show Y chromosome deletions. However these Y chromosome microdeletions cannot be predicted cytogenetically or on the basis of clinical findings or on semen analysis. Thus PCR-based Y chromosome screening for microdeletions is necessary both for providing the correct diagnosis, and better management/counselling of these cases.

In this study, 8 infertile azoospermic and oligozoospermic men (9·63%) who were cytogenetically normal showed microdeletion of Y chromosome spanning the three AZF loci. In recent years several combined clinical and molecular studies have sought to define recurrently deleted region on long arm of Y chromosome in infertile males. Efforts have also been made to determine the inci-

Table 1. Clinical features in men with azoospermia factor microdeletions.

Patient No.	Age	Azoo/ oligo	Semen ($\times 10^6$ ml)	Testes Right	Testes Left	FSH mIU/ml	FNAC	STS deleted	AZF deletion
1	28	Azoo		Small	Small	22	SCO	SY84, sY86, sY127, sY134	AZFa + AZFb
2	25	Azoo		Mild Hydrocele		60	SCO	SY84, sY86, sY127, sY134	AZFa + AZFb
3	33	Azoo		Small	Varicocele	31·8	SCO	SY84, sY86, sY127	AZFa + partial AZFb
4	23	Azoo		Cryptorchidism		41·3	–	SY127, sY134	AZFb
5	30	Azoo		Small	Varico	4·6	Hypospermatogenesis	SY254, sY255	AZFc (figure 2)
6	40	Oligo	1·6–0·2	Small	Small	11·8	Hypospermatogenesis	SY254, sY255	AZFc (figure 2)
7	32	Azoo		Normal		6·2	Maturation arrest (sec. spermatocyte)	SY254, sY255	AZFc
8	20	Azoo		Cryptorchidism		6·8	–	SY254, sY255	AZFc

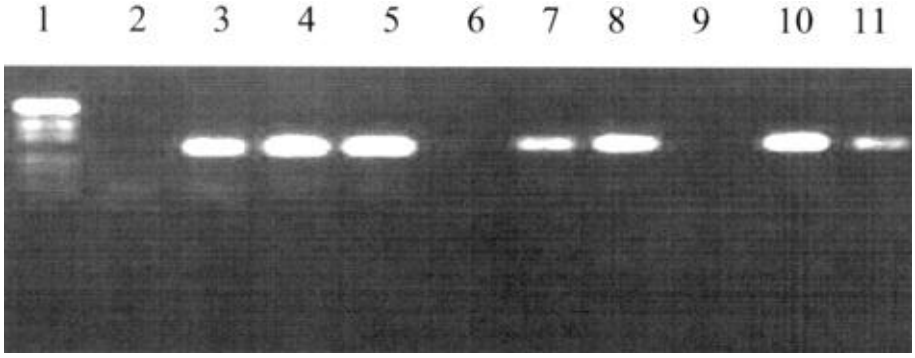

Figure 2. Gel photograph showing microdeletion of sY254. Lane 1, molecular weight marker Msp1 digest; lane 2, negative control (female sample), lane 6 and 9, AZFc (sY254) deletion in patients 5 and 6.

incidence of Yq microdeletions and to correlate the size and position of the Yq deletion with the infertile phenotype. Vogt *et al* (1996) correlated the position of the deletion with the phase in which spermatogenesis was blocked. Each AZF locus acts at a different phase of spermatogeneis. On the basis of testicular histology, the deletion of AZFa was associated with complete absence of germ cells and presence of sertoli cells in the semeniferous tubules – a picture characteristic of SCO syndrome. The deletion of AZFb is associated with developmental arrest of germ cells at pachytene stage, while deletion of AZFc with developmental arrest of germ cells at spermatid stage but can also be associated with hypospermatogenesis and maturation arrest. Thus deletion of a particular AZF loci results in a characteristic phenotype and genes at each loci act at a particular stage of germ cell differentiation. In the present study, patients 1 and 2 had AZFa + AZFb deletion and testicular cytopathology showed presence of Sertoli cells and absence of germ cells (SCO). Patient 3 had AZFa + partial AZFb deletion and FNAC showed total absence of germ cells (SCO). Patient 5, 6, 7 and 8 had AZFc deletion. Patients 5 and 6 showed hypospermatogenesis and in patient 7 there was maturation arrest. Thus in patients with AZFc deletion the testicular phenotype may vary as has been reported by Vogt *et al* (1996), Chang *et al* (1999) and Vogel *et al* (1999). Vogel and associates postulated that the heterogenous phenotype observed in AZF deletions may be due to variable penetrance and modifying effect of ones environment and expression of various modifying genes. Thus several factors, environmental, and genetic and epigenetic in nature influence the testicular phenotype in men with AZF deletions. Patient 6 showed a decline in sperm count from 1·5 million to 0·2 million over a period of one year. This quantitative decline in semen quality has also been reported in a patient with AZFc deletion (Simoni *et al* 1997). Thus the genotype–phenotype correlation in the present study is similar to that of previously published studies by Vogt *et al* (1996). No microdeletions were observed in 25 fertile controls, thus showing that Yq microdeletions are only observed in men with azoospermia and oligozoospermia. Two cryptorchid patients (patients 4 and 8) showed *de novo* AZFb and AZFc microdeletions respectively. It is possible that the fathers were mosaic for these Yq deletions and harboured the Yq deletion only in the germ cell line whereas the peripheral blood had normal Y chromosome and thus the deletions were transmitted to the sons but were not picked up by PCR microdeletion analysis. Similar finding was also reported by Krausz *et al* (2002). In an earlier study we have reported presence of Yq microdeletions in men with cryptorchidism (Dada *et al* 2002a,b,c). Thus AZF deletion not only leads to spermatogenic arrest but may also lead to impaired testicular descent and thus cryptorchidism. Yq microdeletions in cryptorchidism were also reported by Foresta *et al* (1999). They thus postulated that Yq microdeletions lead to oligozoospermia/azoospermia, and may also lead to cryptorchidism. This may be due to altered testicular response to mechanism regulating testicular descent. They detected Yq microdeletions in 27·5% men with cryptorchidism. Knowledge of these deletions is necessary to determine the prognosis in these cases after orchidopexy.

In the present study, 9·58% azoospermic men showed Yq microdeletions and 10% oligozoospermic men had Yq microdeletion. Several published data also report a higher frequency of Yq microdeletion in azoospermic (10–15%) vs oligozoospermic (5–10%) (Krausz *et al* 2001; Simoni *et al* 1999; Dada *et al* 2002b,c).

All the cases had a normal karyotype thus proving the importance of PCR to analyse infertility cases. These microdeletions may cause dysregulation of gene expression by position effect (Krausz *et al* 2001), but they postulated that it is equally likely that the deletion results in

absence of genes critical for spermatogenesis. The Y chromosome has the highest spontaneous loss of genetic material in the human genome. The Y chromosome genetic instability is due to the presence of highly repititive segments – the long and short interspersed repeats – and also because a large portion of the Y chromosome does not undergo recombination during meiosis. The incidence of microdeletions varies from 1 to more than 55% in different studies. These differences in deletion frequency and localization between different studies may reflect genuine population variances and may be related to a particular Y chromosome haplotypes, genetic background or environmental influences, and also on the selection criteria of the patients and different STS primers used. In the present study, only men with oligozoospermia and azoospermia were considered and the STS primers prescribed by the European Academy of Andrology were used. This allows for minimal standardization and determing the variation in deletion frequency in different populations in different parts of the world.

Though there is ethnic variation and diverse caste groups in the Indian population, the frequency of microdeletions in the Indian population was 9·63%, which is similar to that reported in 3 different European populations (Italian, French and Danish) suggesting that worldwide incidence of Yq microdeletions is likely to be similar, if a similar study group, and common clinical criteria and marker set are used.

Genetic causes has proven to be very important in context of new development of assisted reproduction. Intracytoplasmic sperm injection now allows men with sperm defects to procreate van Golde *et al* (2001). These defects can have a genetic origin and inconsequence can be transmitted to the offspring. Thus in order to establish a genetic aetiology and provide most adapted therapeutics to patients, such genetic aetiology of infertility should be established.

5. Conclusion

Therefore the search for microdeletions is compulsory preliminary step to define precisely the aetiology of spermatogenic failure and to determine the frequency and site of gene deletion. This will allow the validation of candidate genes and cloning of new ones.

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