Idiopathic cases of male infertility from a region in India show low incidence of Y-chromosome microdeletion

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Chromosomal and Y-chromosomal microdeletion analysis has been done in cases of idiopathic infertility with the objective of evaluating the frequency of chromosomal and molecular anomaly as the causal factor of infertility. Barring a few cases of Klinefelter syndrome (XXY or XY/XXY mosaics), no chromosomal anomaly was encountered. Y-microdeletion was analysed by PCR-screening of STSs from different regions of the AZF (AZFa, AZFb, AZFc) on the long arm of the Y, as well as by using DNA probes of the genes RBM, DAZ (Yq), DAZLA (an autosomal homologue of DAZ) and SRY (Yp; sex determining gene). Out of 177 cases examined, 9 (azoospermia – 8 and oligoasthenospermia – 1) showed partial deletion of AZF. The size of deletion varied among patients but AZFc was either totally or partially removed in all of them. In contrast, no deletion was detected in AZFa. Testis biopsy done on a limited number of cases (50) showed diverse stages of spermatogenic arrest with no specific correlation with the genotype. The frequency of Y-chromosome microdeletion in our samples (~ 5%) is much lower than the frequency (~ 10%) reported globally and the two previous reports from India. We contend that the frequency may be affected by population structures in different geographical regions.

[Ambasudhan R, Singh K, Agarwal J K, Singh S K, Khanna A, Sah R K, Singh I and Raman R 2003 Idiopathic cases of male infertility from a region in India show low incidence of Y-chromosome microdeletion; *J. Biosci*. **28** 605–612]

1. Introduction

Nearly 10% of all married couples go childless despite unprotected intercourse. Nearly 50% of these are accountable to the male partner. A large proportion of male infertility cases are associated either with systemic defects such as diabetes, obesity, varicocele, cystic fibrosis or with infections for mumps, herpes or else with imbalance in levels of gonadal steroids and trophic hormones [e.g. testosterone, dihydrotestosterone, follicle stimulating hormone (FSH), leutinizing hormone (LH), androgen receptor]. However, in nearly 15% cases of male infertility no organic cause is identified (idiopathic infertility). Since infertility is largely due to impairment of gametogenesis, in which a number of genes participate, it is logical that mutations in spermatogenic genes would result in impaired spermatogenesis, leading to infertility. Mutations in autosomal cystic fibrosis transmembrane conductance regulator (CFTR), c-kit receptor (c-kitR) and X-linked androgen receptor (AR) genes all lead to sterility due to defects in germ cell proliferation or urogenital system. Matzuk and Lamb (2002) have reviewed the various genes

Keywords. AZF; azoospermia; DAZ; idiopathic infertility; Klinefelter syndrome; oligoasthenospermia; Y-chromosome

Abbreviations used: AZF, Azoospermia factor; DAZ, deleted in azoospermia; FSH, follicle stimulating hormone; KFS, Klinefelter syndrome; PCR, polymerase chain reaction; SCO, Sertoli cell only; STS, sequence tagged site.

and their influence on spermatogenesis and fertility. That the male-determining Y-chromosome also carries genes for spermatogenesis became evident when Tiepolo and Zuffardi (1976) showed association of deletions or rearrangements involving the euchromatic segment of the long arm of Y-chromosome (Yq) with azoospermia/oligospermia. Molecular studies on Y-chromosome microdeletion have led to the identification of an azoospermia factor (AZF) region in Yq11·2 (Vogt *et al* 1996). Most deletions occur *de novo* and fall in three nonoverlapping regions, designated AZFa, AZFb and AZFc, the last of these being the most frequently deleted (Kobayashi *et al* 1994). A number of genes have been located in these regions. Ubiquitously expressed *DFFRY*, *DBY* and *UTY* occur in AZFa (Brown *et al* 1998; Foresta *et al* 2000). *DAZ*, *BPY1*, *BPY2*, *CDY*, *PRY*, *TTY1*, *TTY2* and *XKRY*, all existing in multiple copies in AZFc, are expressed specifically in testis (Lahn and Page 1997). RNA-binding motif (RBM, formerly YRRM) family (Ma *et al* 1993), consists of about 30 copies on both arms of the Y-chromosome (Chai *et al* 1997). Deleted in azoospermia (DAZ) family, having at least 7 functional copies in interval 6D (Yen *et al* 1997; Yen 1998) represents the most frequently deleted region in infertile men. Either entire or partial loss of the DAZ family of genes is clearly associated with azoospermia, occasionally also with cases of oligospermia, independent of the testicular phenotypes (Reijo *et al* 1995). A global estimate places about 10% cases of idiopathic azoo/oligospermia due to deletion in AZF region. Therefore it is the most common molecularly diagnosable cause of spermatogenic failure in man (Reijo *et al* 1995), and is highly recommended in cases where intracytoplasmic sperm injection (ICSI) is to be adopted for assisted reproduction.

We have carried out chromosomal, Y-microdeletion and testis analysis in patients of idiopathic infertility in cases from eastern Uttar Pradesh and western Bihar states of India with a view to assessing the frequency of deletions in idiopathic fertility and mapping the zone of deletion.

2. Materials and methods

2.1 *Patients*

From out-patient departments of the University hospital and an IVF and a Urology clinic in the Varanasi city 180 cases of idiopathic infertility – 142 azoospermy (sperm count nil), 33 oligospermy (sperm < 5 million/ml), and 5 severe oligo-astheno-teratospermy (sperm count < 5 million/ml with poor morphology and motility) – were examined. An institutional ethical committee had approved analysis of genetic disorders in the Department of Biotechnology (DBT) funded chromosomal and molecular genetic diagnostic unit. Informed consent for obtaining blood was obtained from each proband, who belonged to the age group of 22 to 40 years. A small part of the testis biopsy was obtained for histology in cases where the clinician considered it necessary to examine testis histology. In certain cases FNAC was also done but that was not sufficiently helpful in cytological determination of the stage of spermatogenic arrest. Patients consent was obtained in all these cases.

Patients were examined together by a clinician and a geneticist so that the genetic as well as clinical exclusion criteria could be implemented for patient selection. Three consecutive semenograms, done after 3–4 days of sexual abstinence, were considered to ascertain their infertility status. They were examined for the size, volume, consistency of testis, varicocele, hydrocele or physical injury and secondary sexual characters etc. It was ensured that the proband did not suffer from mumps, orchitis, or diabetes at any stage of life. A questionnaire recording history of habits like tobacco chewing, alcoholism, infertility or other illness in the family and drug insult to mother during pregnancy was maintained for each patient. Spouses of all patients were normal. Reports of *trans*-rectal sonography or vasography were obtained only when required. For chromosome preparation the PHA-stimulated whole blood was cultured for 72 h in RPMI 1640 medium supplemented with 10% fetal bovine serum. At least 20 metaphases were counted for verification of the diploid number. Chromosome preparations were GTG-banded and karyotyped.

2.2 *Deletion mapping by polymerase chain reaction*

Genomic DNA was extracted from peripheral blood and testis biopsy by standard methods. DNA was subjected to AZF-polymerase chain reaction (PCR) simplex on 31 sets of Y specific sequence tagged sites (STSs) spanning the euchromatic region of Y-chromosome from centromere to interval 7, with particular interest in interval 6 (AZF). A multiplex protocol with primers from all the three regions was also developed. All the STSs have been previously described (Vollrath *et al* 1992) and ordered into a sequence according to Vollrath *et al* (1992), Reijo *et al* (1995) and Sun *et al* (1999). All STSs were first tested in fertile men and women. In addition, specific primers for genes SRY (RG4– RG5), ZFY (ZFY1–ZFY2) (both in int. 2C), RBM1/RBM2 (F19/F20-E355), DAZ (DAZ20-TPX8) (Int.6d), were also used. A positive control (sample from a normal fertile male) and two negative controls [(i) normal female sample, (ii) every constituent except DNA], were included in every PCR assay. In the event of detecting deletion with a primer the PCR assay was repeated thrice for confirmation.

2.3 *Southern hybridization*

Genomic blot hybridization was carried out for patients with microdeletion in AZF region. DIG-dUTP (Boehringer

Mannheim, Germany) or a^{32}P-dCTP (sp. act. 4000Ci/mM BRIT, India)-labelled DNA probes from DAZ (pDP 1577, pDP 1593), RBM (MK5,MK29), SRY (pY53·3), pY6H52 (correspond to sY156) were hybridized with the *Eco*RI or *Pst*I-digested genomic DNA blotted to a nylon membrane, as per Sambrook *et al* (1982). The hybridized blots were finally washed with 0·5 × SSC, 0·1% SDS (65°C) and then exposed for autoradiography. Detection of signal in non-radioactive hybridization was done as per manufacturer's instructions (Boehringer Mannheim, Germany).

2.4 *Testis biopsy*

Whenever available, testicular biopsy was obtained with the objective of assessing the stage of spermatogenic arrest and to check the possibility of mosaicism between somatic and germ cells. A part of the testis was fixed in Bouin's and sectioned (5 μ–6 μ) to stain with haematoxylin and eosin. Rest was used for air-dried chromosome preparation/surface spread synaptonemal complex preparations (in 4 cases) and/or DNA extraction. In 2 Yq microdeletion cases, bilateral FNAC was also performed, which matched with the histological findings.

3. Results

Chromosome preparations and GTG-banding was done on 180 azoospermic/oligospermic patients. Within the limited resolution of G-banding no case of chromosomal rearrangement or Y-chromosomal deletion was detected. However, 6 patients (pt #40, 63, 74, 101, 125, 128) were 47,XXY and two were mosaic (#11 and 147: 45,XO/46,XY/47,XXY). Except for hypogonadism and azoospermia, no other clinical or morphological feature typical of Klinefelter syndrome (KFS) was detected in these cases. That is, they were referred to us as cases of idiopathic infertility. Since chromosomal and DNA analyses were performed simultaneously DNA from the (KFSs) were also analysed for Y-deletion. However, they were excluded from the category of cases of idiopathic infertility.

3.1 *AZF deletion mapping*

Screening for the Y-chromosome microdeletions was done in a total of 177 patients with idiopathic oligozoospermia and azoospermia using multiple sets of primers for PCR. Using the criteria listed earlier, interstitial deletions of Yq11 in the AZF locus were recorded in eight azoospermia (#5, 17, 78, 88, 96, 117, 161, 175) and 1 oligospermia (#37) patients. FSH level was assayed in a few of these and was found to be elevated. With the exception of one (#175), in all the deletion cases, STSs covering the DAZ gene in AZFc (sY254, sY255) were deleted. The deletion always extended beyond the DAZ complex. In two cases (#17, #96) the deletions included the distal AZFb subregion (RBM1/RBM2 in #17 and up to sY117 in #96). In 4 cases, including the lone case of oligospermia, the deleted segment was between sY153–sY159. In this relatively low-resolution microdeletion analysis, distal break zone in all but one of the microdeletion cases was consistently sY159. In one of the cases (#161), the deletion was much smaller (SPGY), excluded DAZ and occurred ahead in the distal Yq in AZFc. For AZFa region, in addition to the standard primers (sY84 and sY83) other primers were also employed (see figure 5). However, none of these STSs was deleted in any of the patients. Figure 1 shows a representative picture of a PCR gel and a map of the deletion break zones in the patients. None of the Klinefelters showed a deletion.

Southern hybridization with probes for DAZ (pDP1577, pDP1593) coincided with the PCR results in the presence or absence of DAZ (figure 2). As expected, probe for RBM (MK29) gave multiple bands (*Eco*RI digested genomic DNA) in control normal male as well as patients but in the patient #17 the 9·5 kb band was missing and intensity of other bands was also low (figure 3a). The possibility that the faint band in patient #17 was due to less genomic DNA was ruled out by reprobing the blot with the Yp SRY (pY53·3) which exhibited an intense signal (figure 3b). Obviously at least some copies of RBM were deleted in this patient, most likely from AZFb.

3.2 *Analysis of testis biopsy*

In 50 patients (both azoospermia and oligospermia) testis biopsy (0·5–1 mm^2) was available. Chromosome preparations (24) and/or surface-spread testis preparations (only from 4) showed the presence of pachytenes in many of them but few post meiotic cells. DNA was extracted from 25 testis samples and PCR analysed for the same STSs, and in all of them the testis DNA gave the same pattern as that of the blood DNA from that patient. Thus, none of these 25 cases was a gonosomic mosaic.

Histological sections of testis revealed considerable variations in the nature and degree of spermatogenic failure. Four of these biopsies belonged to microdeletion cases (#17, #88, #96, #117) and 2 to the Klinefelter's (#11 and #40). In #17 majority of seminiferous tubules were poor in meiocytes and spermatid with no lumen and presence of a few darkly stained nuclei indicating apoptosis. Number 88 showed complete absence of germ cells characterized as Sertoli cell only syndrome type 1 (SCO I). In #96 while most tubules were distorted with thickened walls and were hyalinized with only fibrous tissue, occasional tubules had a few germ cells (SCO II). Number 117 showed maturation arrest. In the two Klinefelters the testis had immature tubules and gross fibrosis. Thus these

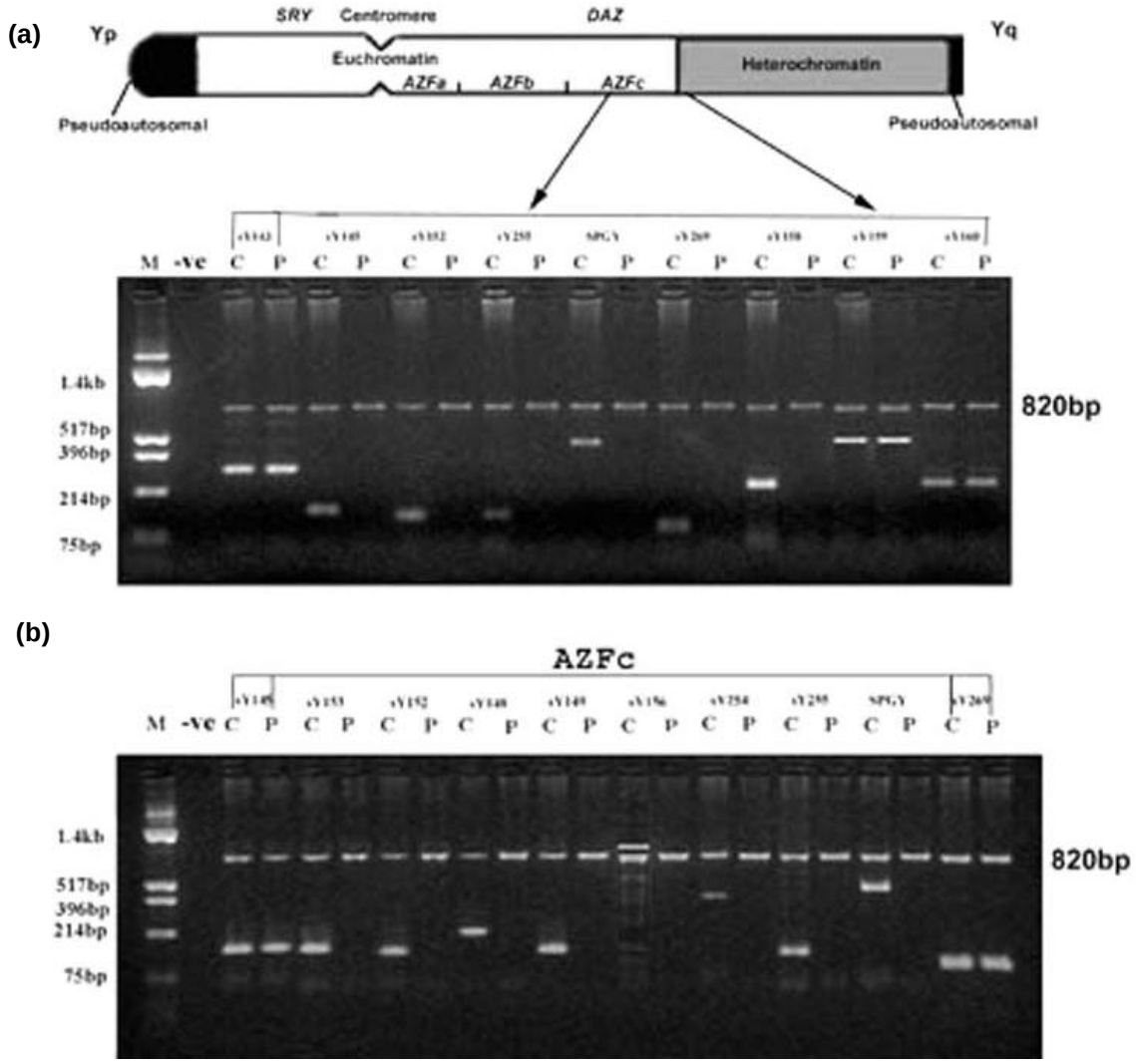

Figure 1. Mapping of the zone of deletion of the Y-chromosome in #117 and 161. Representative pictures of amplification of STS's from different regions of the Y-chromosome (a cartoon of the Y given at the top of the figure) for assessing the extent of deletion. Lane 1 shows the marker (pUC/Hinf1). In addition to the AZF primers, Yp SRY (820 bp) has been used as internal control for each reaction. For each STS a normal male DNA was amplified along with the proband DNA and run side-by-side in the gel. In **(a)** DNA from #117 shows deletion between sY153 and sY159 while in **(b)** #161 the deletion is much smaller (sY153 to SPGY).

50 azoospermia/oligospermia patients revealed a spectrum of testis phenotypes from SCO I to post-meiotic arrest. More importantly, identical Y-deletions showed different histology of testis, and though no deletion was recorded in AZFa region, SCO was encountered in two cases. Overall picture between the Y-deleted and undeleted cases was not very different, and even SCO's were seen in the non-Y-deleted cases (figure 4).

4. Discussion

More than SNPs and point mutations in any specific genes on the Y-chromosome, deletions of large regions within AZF have been found to be more frequent in cases of

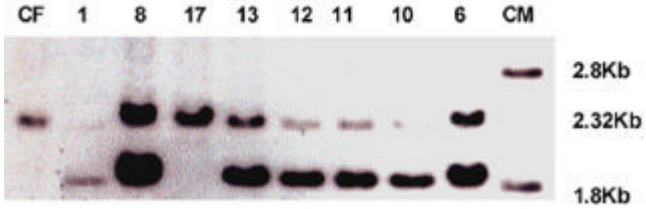

Figure 2. Deletion of DAZ in #17. Autoradiogram of the *Eco*R1-digested DNA from infertile males (numbers given at the top of each lane) hybridised with a probe of DAZ (325·7). The 2·3 kb band of the autosomal homologue of DAZ (DAZLA) is present in all the individuals, while the Y-specific band (1·8 kb) is missing in #17. The 2·8 kb band in the control male (CM; control female – CF) is a common polymorphism in normal males.

idiopathic infertility. As such, barring a rare example in AZFa (Sun *et al* 1999), seldom a point mutation of any Y-chromosomal gene has been associated with azoospermia. Therefore, microdeletion analysis of the Y-chromosome has become a standard genetic marker for idiopathic infertility and much emphasis is laid on establishing uniform etiological parameters for non-obstructive idiopathic infertility, and authenticity of the molecular tests (Simoni *et al* 1999). While the vast majority of deletions are confined to AZFc, in a lower proportion they occur in AZFb and AZFa (Reijo *et al* 1995; Vogt *et al* 1996; Ferlin *et al* 2003). Sometimes the deletions are large enough to involve more than one region. The deletions in AZFc often include all the copies of DAZ gene, and they are frequently associated with azoospermia, rarely even with oligospermia (Reijo *et al* 1995). Deletions through AZFa have been seen to give more severe phenotypes such as SCO syndrome (Vogt *et al* 1996). This correlation, however, remains rather vague. In the present report (where cases of varicocele, cryptoorchidism and herpes or mumps have been excluded) only 5% (9/169) cases showed Y-microdeletion, and there was no case of AZFa deletion. On the other hand, deletion in AZFc was invariably involved in all of them. In two cases the deletions extended into AZFb region. Even in the 2 SCOS patients the deletion was in AZFc/AZFb region but not in AZFa.

The average frequency of Yq microdeletion in male infertility has been placed at around 8% (Simoni *et al* 1999). However, the prevalence differs in a wide range, from 1% (van der Ven *et al* 1997) to 55% (Foresta *et al* 1997). In north European populations (Scandinavian countries as well as France, Germany, The Netherlands etc.), for instance, the frequency of Y-chromosome deletion in infertility cases is rather low (1–4%) while in southern European population (e.g. Italy) the average frequency is greater than 15% (Foresta *et al* 1997). Among Asia-oceania populations, data are available from Australia, New Zealand and from southeast Asian countries (China, Japan, Korea, Phillipines etc.) and the frequency revolves around 10% (Kim *et al* 1999; Tse *et al* 2000). In the hitherto reported cases from Asian populations, vast majority of deletions is confined to AZFc region with only rare cases of AZFa/b deletions.

Two reports on Y-chromosome microdeletion on infertility patients from India have recently appeared (Dada *et al* 2003; Thangaraj *et al* 2003) and in both around 9%

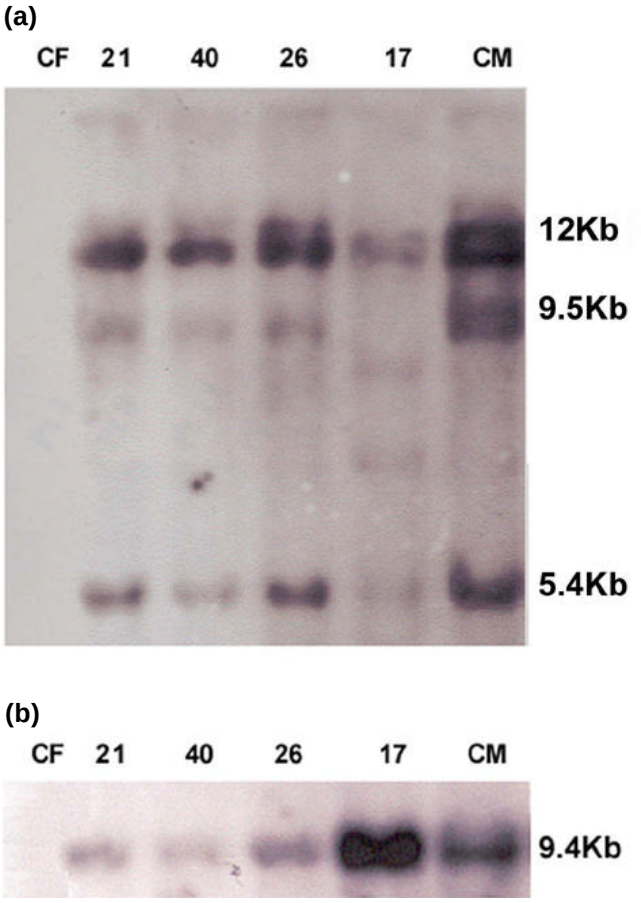

Figure 3. Partial deletion of RBM in #17. **(a)** Autoradiogram of the *Eco*R1-digested DNA from infertile males hybridised with an RBM probe. In #17 all the RBM bands are fainter than in other lanes and the 9·5 kb band is absent. **(b)** The same blot reprobed with SRY plasmid clone pY53·3. Strong signal in #17 confirms that the faint signal with RBM is not because of less amount of DNA in that lane.

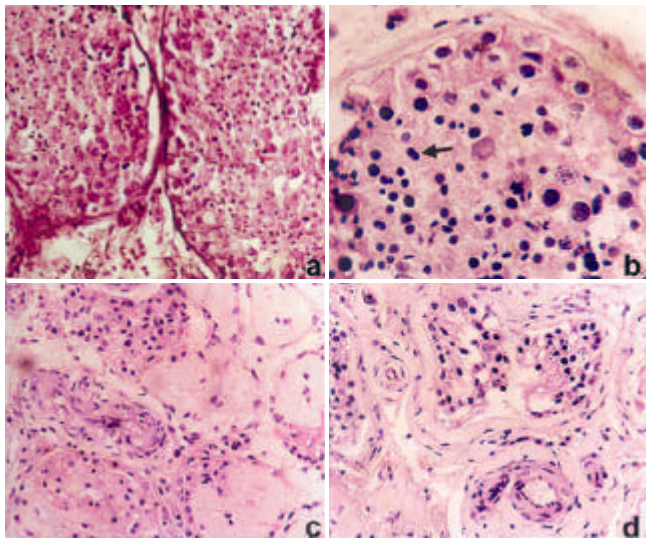

Figure 4. Histological picture of haematoxylene-eosin-stained sections of testis. (**a** and **b**) From #17, showing the Sertolis and a few meiotic cells but no sperm and lumen, arrow (**b**, enlarged view of **a**) showing an apoptotic cell. (**c** and **d** – enlarged view of **c**) From #96 showing hyalinised tubules amidst those with germ cells.

cases revealed AZF deletion. While AZFc deletion was expectedly higher in frequency, both reports recorded a number of cases with AZFa deletion. Actually, Dada *et al* (2003) found 3 deletions in AZFa as against 4 in AZFc (out of 83 cases). Thangaraj *et al* (2003) studying on a much larger sample (340), showed that about 17% of the deletion cases came from AZFa. However, they failed to see any deletion in the STSs (sY84, sY86) commonly recommonded for the analysis. On the other hand, the missing STSs were sY746, sY741, DFFRY, sY742, sY615, interspersed between or around sY86 and sY84. Thangaraj *et al* (2003) also found cases of multiple deletions in the Y-chromosome. It is remarkable that the AZFa deletions recorded by these authors are much smaller and different from those commonly observed. It is also noteworthy that in our study, no patient with AZFa deletion was recorded despite our examining all the STSs used by others and Thangaraj *et al* (2003). Dada *et al* (2003) report that out of 5 cases of AZFb (1) and AZFc deletions 2 each were cases of cryptoorchidism and varicocele. Although, it would be interesting to speculate on Y-chromosome affecting descent of testis into scrotum, at present this association appears a coincidental.

Obviously, there is much heterogeneity among the three reports from different regions in India, as also with other Asian reports in the overall frequency as well as the nature of deletions. In our case, almost all the patients came from eastern Uttar Pradesh and western Bihar. Not-

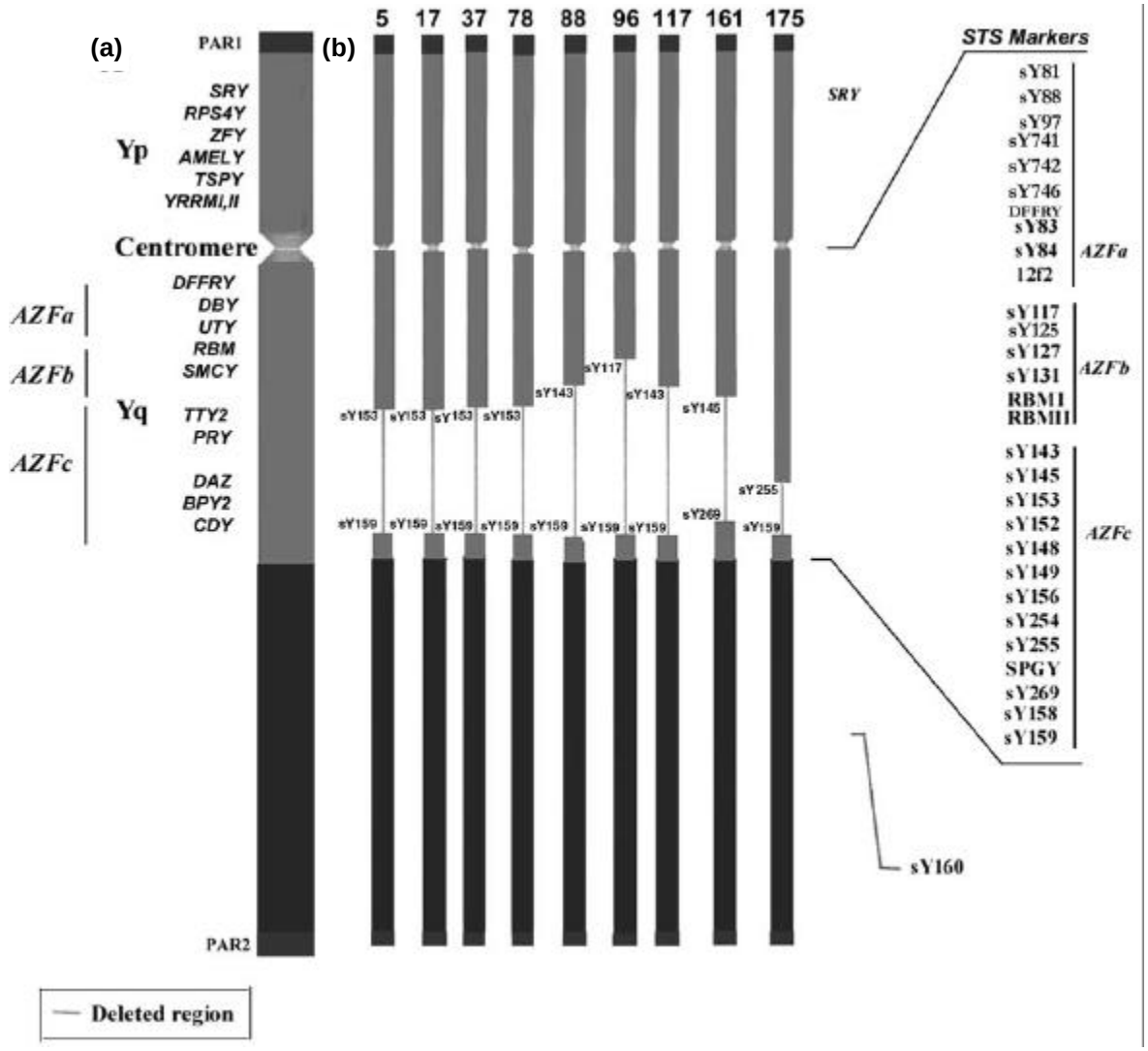

Figure 5. Y-Deletion map of each patient. **(a)** A cartoon showing AZF and other regions of the Y-chromosome. **(b)** Y-chromosomal ideogram from each deletion bearing patient; the STSs indicated in the ideograms are the extreme intact STS at either end of the deletion. The list of STSs used from different regions is indicated at extreme right.

withstanding the geographical proximity, there was no obvious ethnic homogeneity in the samples analysed. Thus in the diverse global reports minor but interesting differences in the frequency and genotypic profiles of patients are seen. Though the selection criteria of patients and the choice and number of STS markers may have a bearing on this diversity, the influence of different geographical regions on this difference is also indicated. Strikingly different deletion frequencies in different European regions and susceptibility of specific Y-haplogroups to male sterility in certain populations buttress this point (Krausz *et al* 2001; Kuroki *et al* 1999).

We have also carried out low resolution mapping of the deletion zones in these patients. It is noteworthy that the distal most intact STS is the same (sY159) in all but 1 of the cases, and the proximal intact STS was the same (sY153) in 4 of them (see figure 5). sY153 to sY159 is also the most frequent break zone in the earlier reported cases. One of the hallmarks of the Y-chromosome is the high frequency of amplified repeat sequences dispersed throughout the euchromatic and heterochromatic regions. That this becomes the precursor for intrachromosomal recombination is amply demonstrated in the HERV viral repeat-mediated deletion in AZFa (Sun *et al* 2000; Vogt *et al* 2000). Since massive palindromes and direct repeats also feature in AZFc region, they serve as substrates for homologous recombination, resulting in recurrent deletions (Kuroda-Kawaguchi *et al* 2001). sY153 is derived from a repetitive sequence (Yen *et al* 1998), whose isolated deletions in infertile men are also recorded (Kent-First *et al* 1999). Since sY159 is closer to heterochromatic region of Yq, it is possible that the zone of junction between eu- and heterochromatic region on the Y makes it more susceptible to breaks. Thus there is a strong case to screen the regions in the vicinity of sY153 and sY159 for the putative 'hot spots' on Yq.

We are also intrigued by the fact that in the group examined by us 20 had contracted chicken pox at some stage of their life, and that 5 of them showed the microdeletion (> 50%) and 1 was a Klinefelter. We are not aware of a comparable observation from elsewhere in India and other places, but it does appear to be a factor that could influence fertility. Therefore, it should be of critical interest to take geographical/environmental/ethnic axis into consideration in the genetic basis of infertility. In a multifactorial disorder, such as idiopathic infertility, where environment and the genetic components interact variously, data from more regions, and more data from each region, need to be generated to develop a more realistic picture.

Acknowledgements

We are grateful to Profs. David Page, Ann Chandley and Peter Vogt for the kind gifts of the probes respectively of DAZ, RBM and pY6H. We also thank Drs Anuradha Khanna and Madhukar Rai of IMS, BHU for referring some of their cases for the investigation. We record our appreciation for all the volunteers who contributed to this study by providing blood samples for getting DNA from normal persons. This work was supported by a grant from the Department of Biotechnology, New Delhi to RR.

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MS received 7 April 2003; accepted 11 August 2003

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