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Chromosomal studies in infertile men with oligozoospermia & non-obstructive azoospermia

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***Background & objectives*: Chromosomal anomalies have been postulated to be as one of the principal genetic factors in male infertility. Cytogenetic evaluation of men with severely compromised semen parameters reveals an increased incidence of chromosomal aberrations when compared with the normal population. The objective of this study was to determine the chromosomal constitution and sperm characteristics among Indian males with severe male factor infertility.**

***Methods*: In this prospective study we investigated 88 infertile men (42 men with azoospermia and 46 men with sperm count <5 x 10^6 million/ml) prior to intracytoplasmic sperm injection (ICSI) treatment. Karyoptying was performed on peripheral blood lymphocytes according to standard methods. Polymerase chain reaction (PCR) was performed to screen the microdeletions in the AZF region of the Y chromosome.**

***Results*: Constitutional chromosome abnormalities were identified in 14.3 per cent of azoospermic and 6.5 per cent of oligozoospermic men, with an overall rate of 10.2 per cent. Chromosomal abnormalities included gonosomal aberrations in 5 cases. Robertsonian translocation in one, trisomy 7 mosaicism in one case, deletion in chromosome 16 in one, and a marker chromosome in one case. Chromosome variants were observed in 33 (37.5%) subjects. Yqh- was the most frequent variant in sex chromosomes and increased length in heterochromatin and satellites were observed in autosomal chromosomes.**

***Interpretation & conclusion*: The high rate of chromosomal anomalies among infertile men strongly suggests the need for routine cytogenetic analysis prior to employment of assisted reproduction techniques. In addition, meticulous follow-up of babies born after ICSI, especially male offsprings, is necessary.**

Key words Chromosomal aberration - karyotyping - male infertility

Male factor infertility and recurrent miscarriages are distressing conditions that add to the psychological trauma of majority of the couples. Infertility affects about 15 per cent of all couples attempting pregnancy, with male-factor identified in approximately half the cases[1].

Male factor infertility has been linked with numerous irregularities including sperm number, motility and morphology. It is a known fact that in a range of causes of male infertility, chromosomal aberrations occur in about 2-3 per cent of unselected patients with proven subfertility[2]. In patients with

sperm counts below 10 x 10^6 spermatozoa/ml, the rate of chromosomal aberrations is estimated to be 5-7 per cent, with the percentage of cytogenetically abnormal cases increasing to 10-15 per cent in patients with azoospermia[3].

The application of the advanced techniques of assisted reproduction, such as intracytoplasmic sperm injection (ICSI) is a boon to infertile couples for circumventing male factor infertility. With the use of this technique, even spermatozoa from men with extremely low sperm count, and with poor sperm motility and morphology can be successfully used to fertilize the oocyte allowing pregnancy rates close to those of natural conception[4]. The use of ICSI has raised concern regarding the potential risks associated with this technique[5]. Some of the genetic anomalies observed in children born after ICSI arise *de novo* without a predisposing parental chromosomal aberration[6], others might be derived from a predisposing abnormality present in one of the parents[7]. It is worth mentioning that infertile men with azoospermia or severe oligozoospermia have a high occurrence of abnormal karyotypes compared to unselected male newborns[3,8]. Based on the increased incidence of chromosomal anomalies, cytogenetic analysis is widely recommended for male partners undergoing ICSI[8].

We undertook this study to ascertain the occurrence of various chromosomal aberrations in the Indian males with abnormal semen parameters prior to ICSI treatment.

Material & Methods

Infertile men (n=88) were prospectively recruited for chromosomal analysis from April 2001 to October 2003 at Inkus IVF Centre, Mumbai. The patients were selected prior to ICSI treatment because of severe male-factor infertility and past history of infertility for more than two years. The mean age patients was 35.4 yr (range 26-50 yr). All of them underwent an andrological work-up, which included medical history, physical examination, hormonal estimation (excluding those with primary testicular failure), human immunodeficiency virus (HIV) testing and semen analysis according to World Health Organization recommendations and standards[9]. There were 42 men with non-obstructive azoospermia and 46 with oligozoospermia with a sperm count of <5 x 10^6 /ml. Twenty five normozoospermic male donors with normal semen parameters (sperm count >20x10^6/ml, progressive motility >50% and normal morphology >30%) and proven fertility were included as controls. None of them had any hisitory of childhood disease, environmental exposure, radiation exposure or prescription drug usage that could account for their infertility.

Informed consent was taken from the patients and donors prior to collection of heparinised blood samples. Chromosome investigations were performed on cultures of peripheral blood lymphocytes using standard techniques[10]. From each patient, 50 well-spread metaphases were analysed by G-banding. To characterize the polymorphisms, specific techniques such as C-banding and NOR staining were additionally applied. All chromosomal abnormalities have been reported in accordance with the current international standard nomenclature[11]. The Y/F index was calculated as Y/F= total length of the Y chromosome/ average total length of the F group chromosomes (19 and 20). The Y chromosome belongs to the G group but its average total length is equivalent to the average total length of the F group chromosomes. Hence, the Y/F index was calculated to comparison to the F group. The average Y/F index for Y chromosome is defined as 0.95-1.09[12].

To screen for microdeletion in the AZF region of the Y chromosome by polymerase chain reaction (PCR), genomic DNA was prepared from the peripheral blood samples. Each man was analysed for the presence of sequence tagged sites (STS) in the AZFa, AZFb and AZFc regions. The STS probes used were sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc), sY160 (heterochromatin) and SRY and ZFX/ZFY (controls). Female genomic DNA and DNA samples from fertile male were included as negative and positive controls respectively. In addition, a water sample that contains all reaction components but water instead of DNA was used for reagent contamination. The PCR products were separated on 2 per cent agarose gel stained with ethidium bromide and visualized using ultraviolet illumination. A PCR of the expected size was scored positive for the given STS, and scroed negative if no product was detectable after 3 successive PCR reactions.

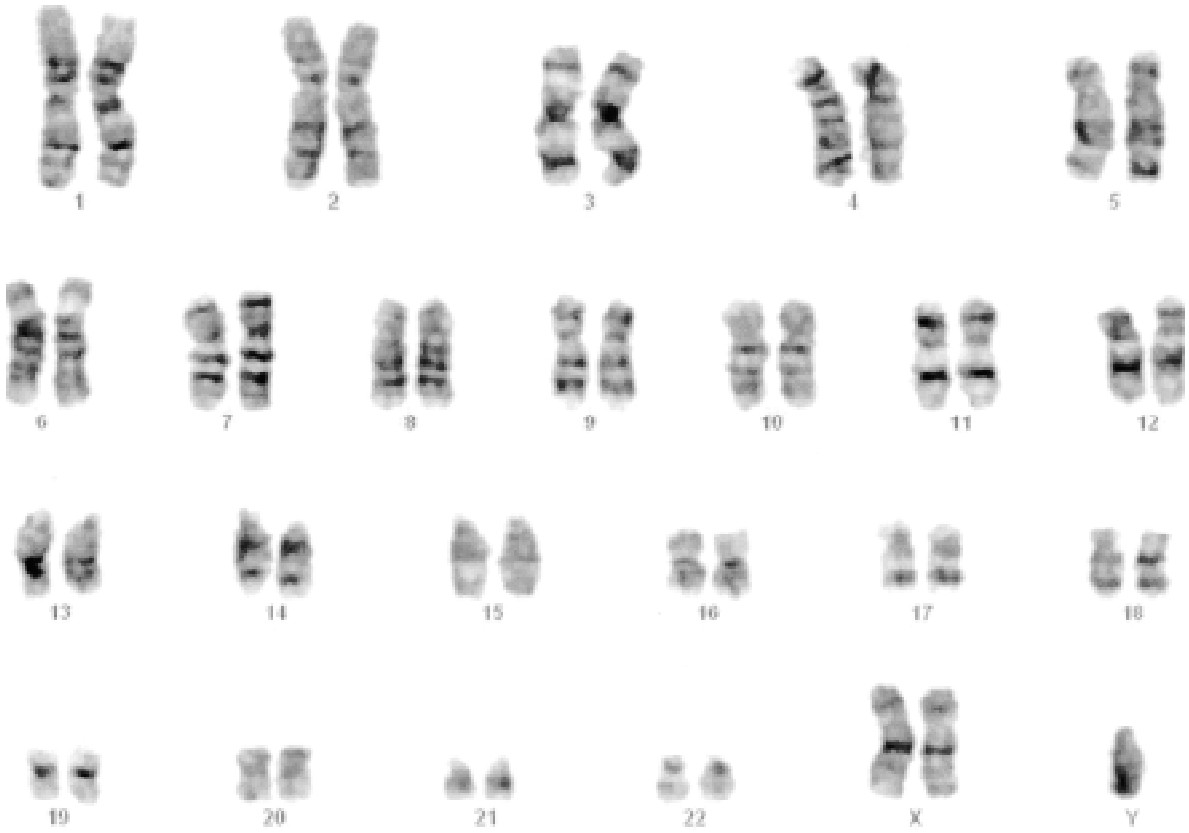

Fig. 1. Karyotype of Klinefelter's syndrome patient showing 47,XXY.

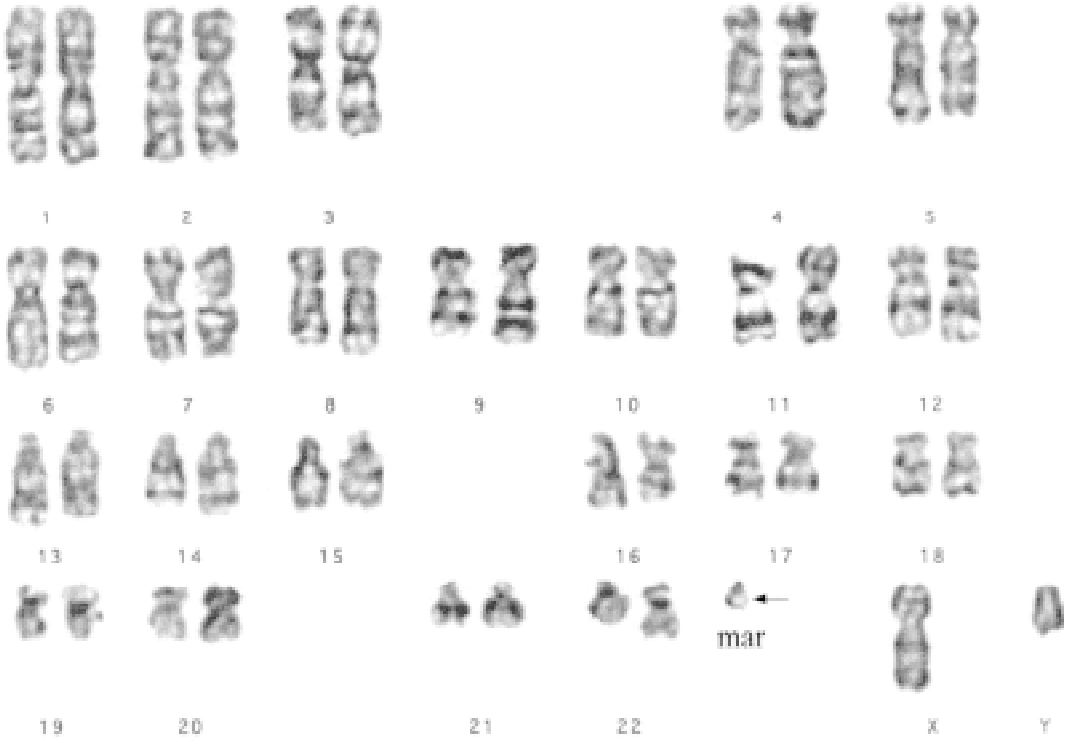

Fig. 2. Karyotype showing marker chromosome.

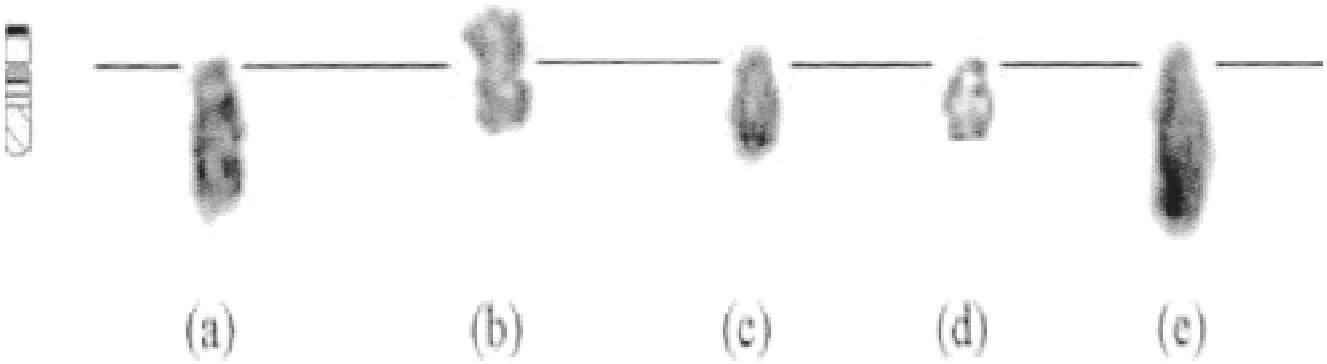

Fig. 3. Structural abnormalities of Y chromosome. (**a**) normal Y; (**b**) inv Y; (**c**) small Y; (**d**) del Y(q11.2); (**e**) long Y.

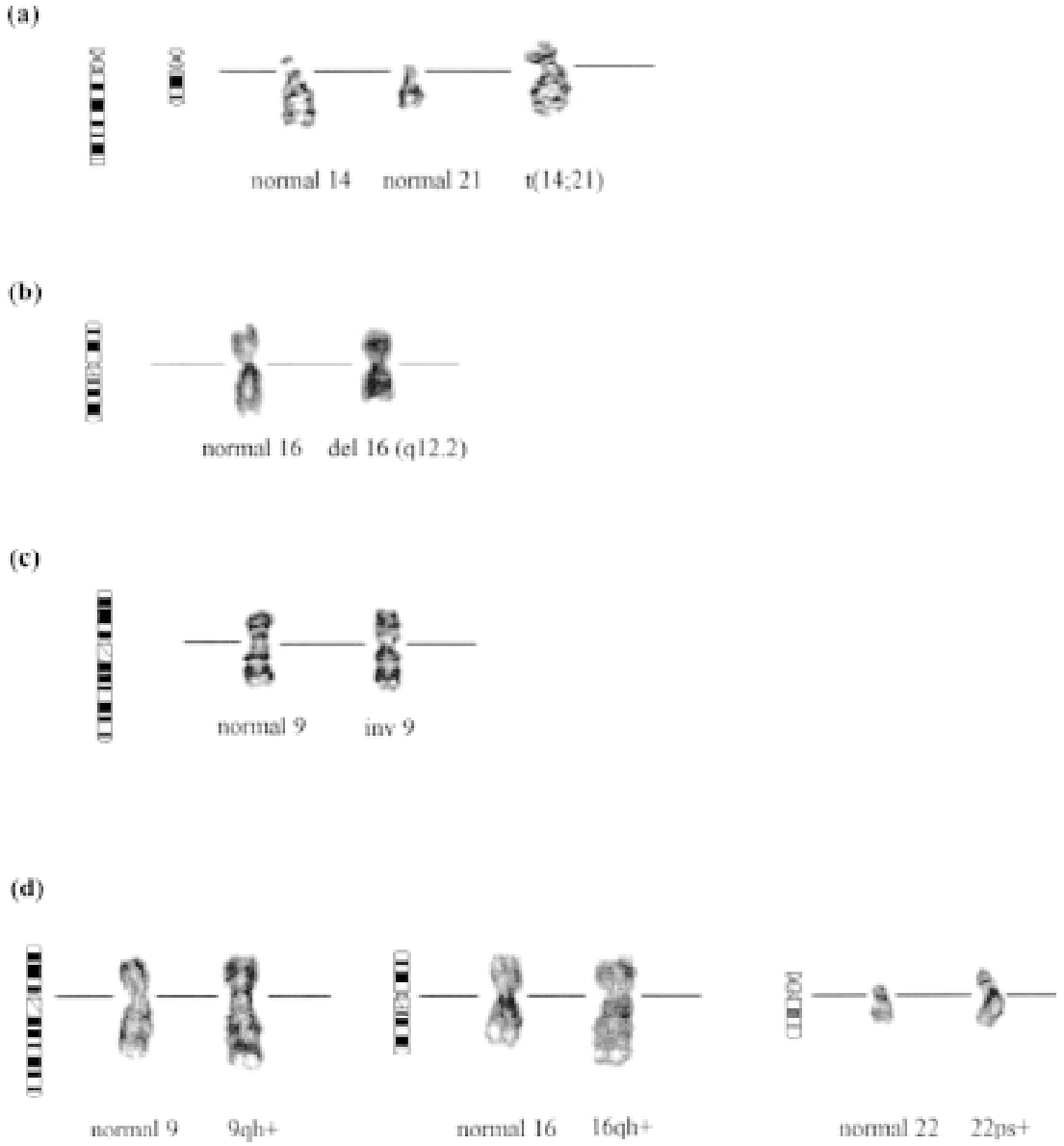

Fig. 4. (**a**) Robertsonian translocation between chromosomes 14 & 21 t(14;21); (**b**) deletion in chromosomes 16, del16(q12.2); (**c**) inversion in chromosome 9, inv 9; (**d**) autosomal polymorphism 9qh+, 16qh+, 22ps+.

Results

Among the 88 infertile men studied, 9 showed some kind of constitutional chromosomal abnormality corresponding to a frequency of 10.2 per cent. The occurrence of chromosomal abnormality in the azoospermics and oligozoospermics was 14.3 per cent (6/42) and 6.5 per cent (3/46) respectively. Chromosomal variants were seen in 14 men with azoospermia and 19 with oligozoospermia, resulting in an occurrence of 37.5 per cent. A normal karyotype of 46,XY was observed in all the controls.

The type of chromosomal aberrations detected in the azoospermic and oligozoospermic men are given in Table I. Men with azoospermia underwent percutaneous epididymal sperm aspiration (PESA) or testicular sperm extraction (TESE). An absence or decreased sperm count (range 0- 4 million/ml) with reduced motility (range 6-12%) and morphology (normal form 2-10 %) was seen in these patients (Table I). The abnormalities observed in the two groups comprised of gonosomal aberrations (n=5) (Fig. 1, 3b, 3d), Robertsonian translocation (n=1) (Fig. 4a), trisomy 7 mosaicism (n=1), supernumerary marker chromosome (n=1) (Fig. 2) and deletion in chromosome 16 (n=1) (Fig. 4b).

Aberration in the heterochromatin region of the Y chromosome was the most frequently identified polymorphism in 27 (30.7%) infertile men. Among 3 (3.4%) men the Y/F index ratio was >1 (Fig. 3e) and in 24 (27.3%) males the Y chromosome was smaller than the G group chromosomes (Table II) (Fig.3c). The Y/F index ratio in the fertile controls was 0.9-1.09 (mean 0.96 ± 0.043). Besides, autosomal chromosome variants were observed in 6 (6.8%) infertile men (Tables I, II) (Figs 4c, 4d). Inversion of chromosome 9 is a polymorphism but was included in Table I for the sake of completeness. The heterochromatin area was increased in chromosomes 9 and 16. NOR staining of chromosome 22 showed it to be only satellite (22ps+).

Table I. Chromosomal anomalies in azoospermic and oligozoospermic men

Chromosomal findings	Karyotype	PESA/TESE	Sperm count (million/ml)	Motility (%)	Normal forms (%)
Numerical:					
Complete	47,XXY	ND	NA	NA	NA
Mosaicism	46,XY/47,XYY/47,XXY	TESE	0	0	0
	(84 : 12 : 4)				
	46,XY/47,XY+7	PESA	0	0	0
	(94 : 6)				
Structural:					
Inversion	46,X,inv Y	TESE	0.1	6	2
	46,X,inv Y	ND	2	9	8
	46,XY,inv 9	PESA	0.1	5	2
Deletion	46,X,delY(q11.2)	PESA&TESE	0	0	0
	46,XY,del 16(q12.2)	ND	4	12	10
Robertsonian translocation	45,XY,t(14;21)(q10;q10)	PESA	1	8	4
Marker chromosome	47,XY,+mar	ND	2	7	6

PESA, percutaneous epididymal sperm aspiration; TESE, testicular sperm extraction; ND, not done; NA, not applicable
No. of aberrations=1 in each case

Table II. Chromosomal polymorphisms in infertile men

Classification	Karyotype	No. of men with defects
Azoospermia	46,XYqh-	11
	46,XY, 9qh+	1
	46,XY,qh+,16, qh+,22ps+	1
Oligozoospermia	46,XYqh-	13
	46,XYqh+,	2
	46,XYqh+, 9qh+	1
	46,XY, 9qh+	2
	46,XY,22ps+	1

PCR analysis for the detection of AZF microdeletions showed deletion in the AZFc (sY254, sY255) region along with deletion of the heterochromatin (sY160) in only 1 male who presented with a karyotype of 46,X,delY(q11.2) on cytogenetic analysis. C banding in the same patient showed that the Y chromosome was not idic Yp. Y chromosome microdeletions were not observed in other men.

Among the 88 infertile men, the spouses of 64 undertooke the ICSI cycle. Fertilization rate and pregnancy rate were 57.8 and 15.6 per cent respectively.

Discussion

Among numerous aetiologic factors, chromosomal abnormalities play a prime role in male infertility with abnormal semen parameters. Our results demonstrated an inverse correlation between chromosomal anomalies and sperm count. The exact mechanism by which chromosomal anomalies induce infertility is not clear. It is likely that the presence of abnormally distributed chromatin interferes with meiotic division and thus reduces sperm production. Spermatozoa bearing abnormal chromosomes may cause abnormal embryonic development, which can in turn, cause early pregnancy loss[13].

The overall occurrence of 10.2 per cent constitutional abnormalities was observed in our study. The occurrence of karyotypic abnormalities among infertile men depends on a number of factors; the most important of these being the criterion for selection of patients based on the sperm counts. The frequency of chromosomal abnormalities ranged from 2.2 per cent in a non-selected group of males presenting with subfertility[2] to 10.3 per cent in infertile males with a sperm count below 10 million/ml[14]. The presence of 14.2 per cent chromosomal anomalies in males with azoospermia and 6.5 per cent in men with oligozoospermia is within the range of 10-15 and 5-7 per cent respectively, as reported in literature[3,15].

Sex chromosome abnormalities are the most frequent chromosome-related cause of infertility. In our study, we found one man with 47,XXY karyotype *i.e.*, Klinefelter's syndrome. This abnormality is associated with severe spermatogenic failure causing a marked reduction in testicular size and azoospermia resulting in infertility[13]. A mosaic 46,XY/47,XYY/47,XXY karyotype was found in one azoospermic male. Infertile men with gonosomal mosaicism have a range of spermatogenic profile ranging from severe impairment to apparent normality[16]. Gonosomal mosaicism may be a probable cause for the failure of assisted reproduction[17]. A deletion in the long arm of the Y chromosome was seen in one patient. Studies have indicated that deletions on the long arm of the Y chromosome involving a particular and consistent segment might lead to azoospermia[18] and sometimes to severe oligozoospermia[19]. There have been reports of vertical transmission of Yq deletions to the offspring via ICSI[20]. Two cases of inversion of Y chromosome were observed in our study. Pericentric inversion of Y chromosome is reported to be more prevalent among infertile men than in newborns[21]. A study on inversion of the Y chromosome in the Gujarati Muslim Indian population of South Africa failed to show any impairment in their reproductive fitness[22].

A relationship between balanced autosomal translocation and infertility has been reported[13]. A single case of azoospermic male with Robertsonian translocation t(14;21) is reported in our study. An increased number of carriers of Robertsonian translocations has been reported among severely oligozoospermic and azoospermic men[8]. The testicular histology of such carriers shows a variable picture ranging from severe impairment to near normality[2]. Balanced translocations interfere with normal chromosome pairing and segregation at meiosis I, thus

providing a potential for formation of unbalanced gametes and subsequent unbalanced abnormal offspring[23]. A unique finding observed in our study was the presence of deletion in chromosome 16q12.2 locus. Data published earlier point to the probable association of deletion in chromosome 16q with human neoplasias (including retinoblastoma, breast, ovarian, hepatic and prostatic cancers)[24,25]. However, we did not observe any of the above mentioned features in our case, indicative of a possible relation of such a deletion to subfertility. Further research in this direction is necessary.

Several reports on male infertility mention the presence of chromosomal variants or polymorphisms. The overall occurrence of chromosomal variants in the present study was 37.5 per cent. This was comparable with the one reported by Penna *et al*[26] but was more than the occurrence cited in another study[27].

Y chromosome polymorphisms have been preferentially seen in azoospermia and severe oligozoospermia[8]. 'Long Y chromosome'and 'short Y chromosome' are known to exist[21]. The variation in the length of Y chromosome is usually due to variation in the distal part of the long arm that is known to contain heterochromatin[21]. The occurrence of long Y (Yqh+) and short Y (Yqh-) was 3.4 and 27.3 per cent respectively, in our study. The presence of Yqh+ was quite similar to the frequency of 4.4 per cent reported in literature[28]. However, the occurrence of Yqh- was raised as compared to 1.6 per cent stated in a study[3]. Long Y chromosome has been seen to be associated with an increased risk of foetal loss[29]. However, another study[21] did not show any relationship between the size of the Y chromosome and the risk of abortion. Genest and Genest[30] reported that short Y chromosome does not seem to represent an increased risk of pregnancy loss. The contribution of Y chromosomal variants to alter the carrier's fertility is still a controversial topic and further studies are required to understand this.

In our study, autosomal polymorphisms were observed as increase in the centomeric heterochromatin in chromosomes 9 and 16 and as presence of satellites in acrocentric chromosome 22. Pericentric inversion of chromosome 9 was seen in one azoospermic patient. Although inversion 9 is included in the group of 'variant chromosomes', contradictory information is reported about its effect on reproductive fitness in males[31].

We found one phenotypically normal male having a trisomy 7 with a frequency of aneuploid mitoses of 6 per cent in the lymphocytes. Few cases of trisomy 7 mosaicism with skin pigmentary variation and dysmorphism have been described[32]. Marker chromosome was observed in one male. It has been postulated that presence of an extra structurally abnormal chromosome might lead to reduced fertility in males due to meiotic arrest and instability that results in maturation arrest at spermatocyte stage[23,33].

In conclusion, the occurrence (10.2%) of chromosomal anomalies among infertile men strongly suggests genetic testing and counselling prior to the ICSI treatment. Moreover, prenatal diagnosis in the case of abnormalities is of utmost importance. Such investigation is a pre-requisite to minimize the risk of propagation of chromosomal abnormalities into the next generation. Additionally, a thorough follow-up of babies conceived through ICSI, in particular the male progeny, is essential.

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