Sex Chromosomal Analysis of Spermatozoa from Infertile Men Using Fluorescence In Situ Hybridization

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Purpose: *To confirm an association between male infertility and chromosome aberrations of spermatozoa, we demonstrated the frequency of numerical abnormalities of spermatozoa from infertile men with abnormal semen parameters compared with fertile controls.*
Method: *Sperm cells from 10 infertile patients were investigated for disomy rates of sex chromosomes and chromosome 18 and diploidy by fluorescence in situ hybridization (FISH). All patients showed oligoasthenozoospermia with sperm counts 3–20 $\times$ 10^6/ml and motile rates 0–40%.*
Results: *Regarding XY disomy, a significantly higher frequency was found in 8 of 10 patients as compared to normal fertile men. The disomy rates of chromosome 18, XX, YY, and diploidy rate were not increased.*
Conclusions: *There is an association between male infertility and embryo with aneuploidy of sex chromosomes. Counseling about possible genetic risks should be provided to the infertile couples planning assisted reproduction treatment.*

KEY WORDS: FISH; infertile men; sex chromosome aneuploidy; spermatozoa; XY disomy.

INTRODUCTION

The introduction of intracytoplasmic sperm injection (ICSI) has recently revolutionized assisted reproduction and has made it possible for many male infertility cases to achieve fertilization. Only one sperm cell, even when motionless, immature, morphologically aberrant, and not having undergone acrosomal reaction, is able to fertilize the oocyte when injected directly into the cytoplasm, with pregnancy resulting in a large proportion of cases (1). Not only ejaculated spermatozoa from severely oligozoospermic men but also spermatozoa obtained from the epididymis (MESA, microsurgical epididymal sperm aspiration) and even from testicular tissue (TESE, testicular sperm extraction) can be used, making it feasible for azoospermic men to father children. The pregnancies arising from ICSI have been scrutinized for abnormalities because immature spermatozoa are utilized for fertilization and because artificially selected sperm is directly injected into the cytoplasm bypassing the zona. The Belgian group reported on prenatal diagnosis after ICSI resulting in 585 fetuses and detected five (0.85%) sex chromosomal aneuploidies: 47,XXY (2 cases); 47,XXX; 47,XYY; 46,XX/47,XXX (2). In't Veld *et al.* (3), in a study of 15 fetuses from 12 ICSI pregnancies, found five cases (33.3%) of sex chromosomal aneuploidy; two cases of 47,XXY; two 45,X; and one complex mosaic 45,X/46,X.dic (Y)(q11)/46,X.del (Y)(q11). The parents of the cytogenetically abnormal fetuses had normal peripheral lymphocyte karyotypes. Those frequencies of sex chromosomal aneuploidies after ICSI are higher than expected since the incidence in neonates is about one in 1000 births. On the other hand, Bonduelle *et al.* (4) reported 130 children after ICSI and 130 after in vitro fertilization (IVF) had all normal karyotypes by prenatal diagnosis.

Sperm chromosomes have been analyzed for structural and numerical abnormalities since Rudak first described the method of investigating human sperm chromosomes directly after sperm penetration of a golden hamster egg in 1978 (5,6). Recently, fluorescence in situ hybridization (FISH) has been developed. In contrast to the method of in vitro fertilization of hamster oocytes with human sperm cells, a much larger number of unselected spermatozoa in interphase per donor can be analyzed quickly by applying each specific DNA probe. Even spermatozoa of infertile men

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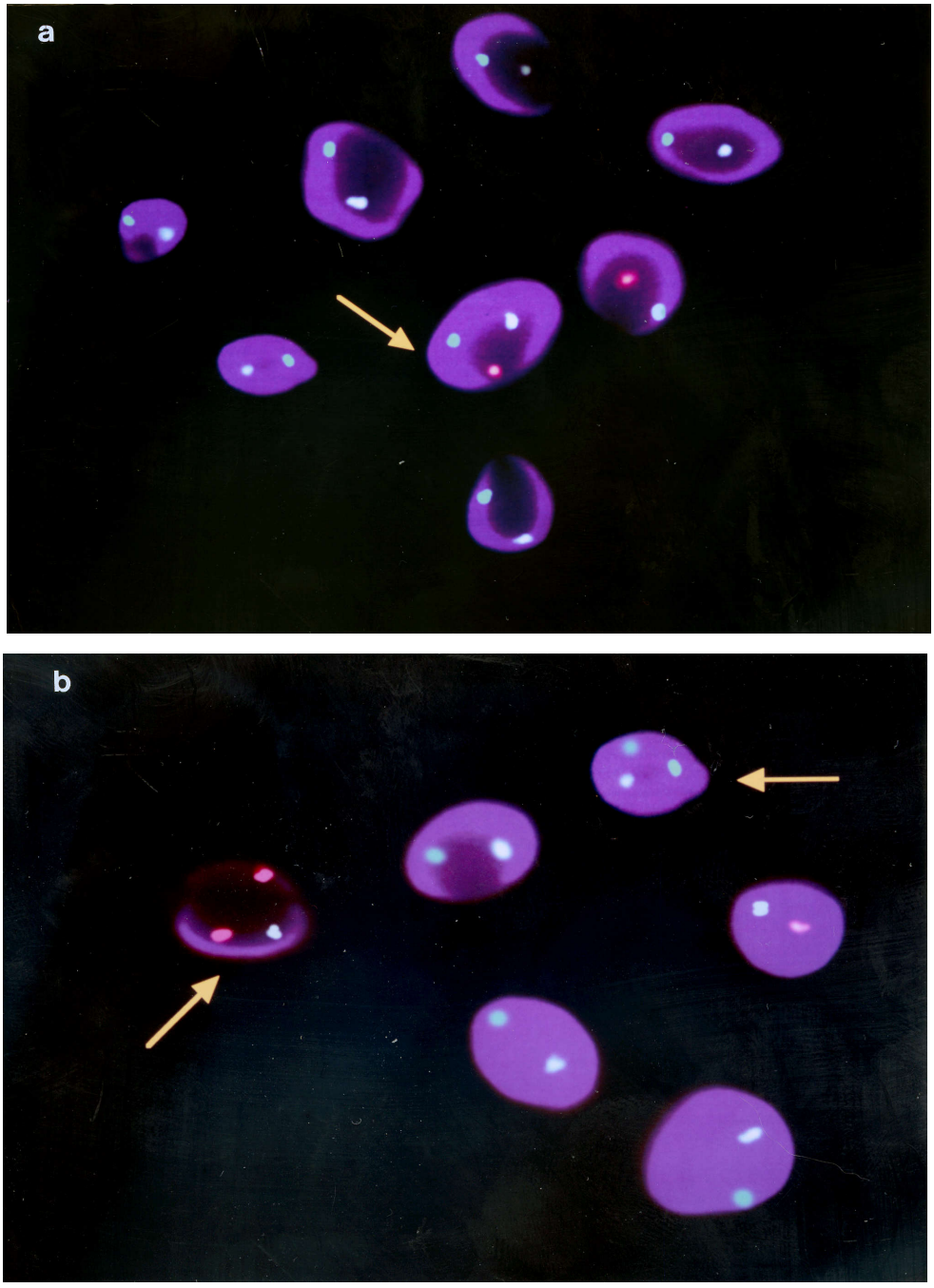

Fig. 1. Three-color FISH of spermatozoa. X, spectrum green; Y, spectrum orange; 18, spectrum aqua. (a) XY disomy sperm showing one green signal, one orange signal, and one aqua blue signal. (b) YY disomy sperm showing two orange signals and one aqua blue signal. XX disomy sperm showing two green signals, (left arrow) and one aqua blue signal (right arrow).

that are not capable of fertilizing an egg can be scored by this method. Multicolor FISH can be performed by the use of two or three probes. A large number of sperm have been scored for disomy rates for almost every chromosome except 19 and 22 (7–10). In this study, we investigate the rate of sex chromosome numerical abnormalities of spermatozoa obtained from infertile men by FISH.

MATERIALS AND METHODS

Semen Samples

Semen samples were obtained from 10 chromosomally normal patients experiencing idiopathic infertility who consulted Nagoya City University Medical School and showed oligoasthenozoospermia. For classification of the semenograms, the World Health Organization (11) criteria were used as a standard. Controls were five fertile men with normal semen parameters. All donors gave informed consent. Semen profiles for each patient are shown in Table I. For each patient, semen samples were collected after 3–5 days abstinence. The ejaculates from patients and control men were washed three times with phosphate-buffered saline (PBS), pH7.4, and centrifuged for 10 min, and then fixed in methanol-acetic acid (3:1). The specimens were stored at −20°C for 20 min. After two rinses with fresh fixative, the sperm cell suspension was dropped onto clean glass slides, air dried, and kept at −20°C until used.

Pretreatment of Sperm Nuclei

Sperm nuclei were decondensed by slide incubation at 37°C in TRIS buffer containing 5mM dithiothreitol (DTT) and Triton X-100 for 30–60 min. The slides were then washed in 2 × standard saline citrate (SSC: 0.15M NaCl/0.015M Na citrate, pH 7.0), dehydrated through an ethanol series and air dried (12,13).

DNA Probes

To distinguish disomic and diploid cells, both the autosome and sex chromosomes were investigated by three-color FISH. Probes included satellite probes for centromeric regions for chromosomes X (Spectrum Green: Vysis Inc. Downers Grove, IL), Y (Spectrum Orange, Vysis), and 18 (Spectrum Aqua, Vysis). All probes were directly labeled with fluorophores and obtained commercially.

Table I. Semen Parameters in Patients with Oligoasthenozoospermia

Patients	Count ($\times 10^6$/ml)	Motility (%)	Abnormal forms (%)
1	13	22	20
2	20	18	10
3	13	40	12
4	15	33	9
5	8	26	12
6	10	20	11
7	3	0	100
8	19	10	4
9	3	33	10
10	12	20	13

Fluorescence In Situ Hybridization

FISH incubation and detection were performed according to the manufacturer's instructions. Slides were denatured in 70% formamide/2 × SSC, pH7.0 at 75°C for 5 min and dehydrated in a 70%/85%/100% ethanol series. The probe predenatured was applied to the specimen target area on slides and hybridized overnight at 37°C. The post-hybridization wash was performed in 50% formamide/2 × SSC, pH7.0, three times for 10 min each and 2 × SSC/0.1% NP-40 at 47°C for 10 min. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI II).

Scoring of Sperm Nuclei and Analysis

Analysis was done using an OPTIPHOTO-2 (Nikon, Japan) epifluorescence microscope equipped with a triple-band pass filter for DAPI/Spectrum Green/Spectrum Orange and a single-band pass filter for Spectrum Aqua and a coupling CYTOVISION (chromosome and FISH analysis system; Applied Imaging Inc., USA). When scoring the sex chromosomes, normal sperm contained either a green (X) signal or an orange (Y) signal, disomic sperm contained two signals of the same color (XX or YY) or one signal of each color (XY). Scoring of disomic sperm nuclei was done according to the criteria described by Williams *et al.* (14): sperm nuclei were considered to be disomic for a specific chromosome if hybridization yielded two compact distinct signals of equal size that were separated from each other by a distance of at least one diameter of the signal size within that cell. Diploid sperm nuclei exhibited two signals of chromosome 18 and two sex chromosomes by three-color FISH. Fisher's exact test and χ^2 analysis were performed to compare results from oligoasthenozoospermic patients and controls using DA Stats of an Apple Macintosh Computer. A significance level of $P < 0.01$ was applied for all.

RESULTS

The sperm count of the oligoasthenozoospermic patients was scored to be ≥10,000 per case, a total of 101,532 sperm, and the rate of numerical chromosome abnormalities was compared with that of five normal

Table II. The Frequencies (%) of 18 and Sex Chromosome Disomy and Diploidy in Patients with Oligoasthenozoospermia and Controls

Patients	Disomy 18	Disomy XX	Disomy YY	Disomy XY	Diploidy	Total count
1	0.14	0.12	0.15	0.40[a]	0.18	10029
2	0.13	0.13	0.09	0.34[a]	0.12	11271
3	0.12	0.09	0,10	0.19	0.15	10000
4	0.20	0.10	0.17	0.45[a]	0.12	10082
5	0.16	0.08	0.12	0.37[a]	0.11	10120
6	0.18	0.15	0.11	0.40[a]	0.17	10030
7	0.11	0.06	0.20	0.46[a]	0.13	10000
8	0.16	0.13	0.13	0.26	0.09	10000
9	0.19	0.07	0.07	0.35[a]	0.19	10000
10	0.12	0.10	0.14	0.39[a]	0.10	10000
Controls average	0.14	0.15	0.14	0.14	0.10	50000

[a] Frequency of XY disomy is significantly higher than that of controls at $P < 0.01$.

men (Fig. 1). In all cases ≥95% hybridization efficiencies could be obtained. Regarding XY disomy, a significantly higher frequency was found in 8 of the 10 oligoasthenozoospermic patients (ranging from 0.34 to 0.46%) as compared to the mean value of 0.14% in the normal group ($P < 0.01$). No differences were noted in the frequencies of 18 disomy, XX disomy or YY disomy, between the oligoasthenozoospermic and normal groups. The frequency of diploid did not differ between the two groups (Table II).

Next, by noting correlations with various sperm parameters, it was found that in all three cases with sperm counts of $\leq 10 \times 10^6$/ml the frequency of XY disomy was higher as compared to the normal group. However, in the oligoasthenozoospermic group, no significant correlations were found between sperm count and the frequencies of XY or other disomy.

With regard to motility and abnormal form rates, in Case 7 with a motility rate of 0% and abnormal form rate of 100%, the frequency of XY disomy was 0.46%, which was the highest value of any of the 10 cases. However, no significant correlations were noted between sperm motility, abnormal form rates and disomy frequencies.

DISCUSSION

During the past few years, ICSI has come to be widely applied, and assisted reproductive technology has undergone remarkable development. If fetuses resulting from ICSI therapy have numerous sex chromosomal aneuplodies in large population, then, of course, the injection of a single sperm artificially selected with this technique may also be associated with this risk. The infertile men, namely those with azoospermia or oligoasthenozoospermia must themselves have problem. This consideration prompted us to determine the frequency of sperm chromosomal abnormalities focusing on X and Y chromosomes in these patients.

Research on chromosomal abnormalities of spermatozoa in infertile men was first reported by Miharu *et al.* in 1994 (15). They studied 12 infertile males and donors comparing the frequency of 1,16,XY disomy using one-color FISH, and found no difference between the two groups. However, since one-color FISH was used, the frequency of XY disomy could not be detected, and some cases with normal semen parameters were included. Guttenbach *et al.* investigated the 1, 7, 10, 17, X, and Y chromosome disomy rates using one color and two color FISH in 45 infertile men with abnormal semen parameters (16). They found average disomy rates for all chromosomes of 0.10 to 0.14%, and a mean incidence of diploidy of 0.1%, with none of these values increased significantly as compared to those of healthy fertile males. Lähdetie *et al.* (17) compared the frequencies of sperm disomy for chromosome 1 and 7 and diploidy in 12 infertile men (eight with normal semen parameters, four with oligoasthenozoospermia) and 18 control fertile men using two-color FISH. They found no significant differences between group the infertile men with normal semen parameters and control group. In the oligoasthenozoospermic men, disomy of chromosomes 1 and 7 and diploidy were significantly more frequent as compared to control group. These results suggest that infertile men with abnormal semen parameters may be at high risk for chromosome abnormalities of spermatozoa. Pang *et al.* (18,19) compared nine oligoasthenoteratozoospermic patients and four fertile donors with regard to chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X, and Y of spermatozoa. The total frequency of abnormal spermatozoa, estimated as the sum of diploidy, and both autosomal and sex chromosomal aneuploidy, showed 33–74% for the patients and 4.1–7.7% for the fertile donors.

The frequency of XY disomy of infertile men was reported by Moosani *et al.* (20). They studied five infertile patients (two with teratozoospermia, two with asthenozoospermia, and one with oligozoospermia) by FISH using DNA probes specific for chromosomes 1, 12, X, and Y, and noted a significantly high frequency of 1 disomy in three cases and of XY disomy in four, but no increase in the frequency of 12 disomy or XX or YY disomy. Martin added the investigation for chromosomes 13 and 21 and found significant increase in the frequency of 13 and 21 disomy (21). Also, analyz-

ing sperm karyotype using the human sperm-hamster oocyte fusion, the numerical abnormalities were found to be more frequent in the infertile men as compared to the fertile donors (20).

By three-color FISH including autosomal chromosomes, we were able to analyze the sex chromosome numerical abnormalities in sperm nuclei from oligoasthenozoospermic patients, with normal peripheral lymphocyte karyotyping, revealing a high frequency of XY disomy in 8 of 10 cases.

First, it is thought that chromosome pairing abnormalities are associated with abnormalities of sperm parameters. According to Egozcue *et al.*, infertile men have an increased frequency of pairing disruptions during meiosis, resulting in meiotic arrest (22). Meiotic arrest disturbs completion of spermatogenesis, and oligozoospermia or azoospermia may result. In particular, the sex chromosome bivalent is susceptible to pairing abnormalities since there is generally only one crossover in the pseudoautosomal region. Another reason for this high frequency of XY disomy is that some infertile men can be mosaic with an aneuploid cell line confined to the germ tissue. Guttenbach *et al.* used three-color FISH (1, X, Y) to analyze 2206 sperm nuclei of a Klinefelter male, and found that 43.4% were X chromosomes (23,X), 48.8% Y chromosomes (23,Y), with XX, XY, YY accounting for respectively, 1.22%, 1.36%, 0.098% (23). 47,XXY cells are able to complete meiosis and produce mature sperm nuclei. According to the reports of Cervret *et al.* and Cozzi *et al.*, 24,XY sperm were found to be significantly increased in a Klinefelter mosaic male in whom peripheral lymphocyte karyotyping revealed 46,XY/47,XXY (24,25). However, in these patients with a Klinefelter mosaic, the frequency of sex chromosome aberrations in sperm differed from the frequency in somatic cells. Roland *et al.* performed two-color FISH in an oligozoospermic male shown by peripheral lymphocyte karyotyping to be a Klinefelter mosaic (XXY/XXXY/XY) and investigated sex chromosome abnormalities in sperm (26). They noted such abnormalities in a total of 7.5%, namely 24,XY, 5.0%; 24,XX, 2.0%; and 25,XXY, 0.5%. In contrast with peripheral lymphocytes, 93.9% of which showed 47,XXY and 48,XXXY. They suggested that small amounts of germ cells with sex chromosome aberrations are capable of complete meiosis. To investigate whether the frequency of mosaics differs between peripheral lymphocytes and germ cells and whether some mosaics are limited to the latter, it will be necessary to analyze germ cell chromosomes by applying FISH and other methods to testicular biopsy material.

In this study, normal fertile men showed 0.14–0.15% for 18 disomy rate and XX, YY, and XY disomy and 0.10% diploidy. Disomy frequencies of sperm calculated with FISH have been reported to range from 0.03–0.36% for chromosome18, 0.02–0.70% for XX disomy, 0.01–0.60% for YY, 0.06–0.42% for XY, and 0.03–0.97% for diploidy (27–33). Guttenbath has attributed these variations to factors such as the decondensation method of sperm prior to in situ hybridization, types and numbers of DNA probes used, and scoring criteria of disomy (7). In the present study, sperm were decondensed with DTT, and using an α-satellite probe three-color FISH was performed. A minimum of 10,000 sperm per case were counted and spermatozoa were regarded to be disomic when two signals were separated by at least the distance of one diameter of the signal size (7,14).

Only one case showed teratozoospermia in this study, and so the association between morphological and chromosome numerical abnormalities could not be explored. In't Veld *et al.* reported that in a case of oligoasthenoteratozoospermia in which all sperm had large heads and multiple tails the frequencies of XY disomy (14%), diploidy (40%), and triploidy (24%) were all high (34).

In conclusion, our present results suggest that sperm from oligoasthenozoospermic infertile men have an increased frequency of sex numerical chromosome abnormalities, namely XY disomy. The transmission of this kind of gamete by intracytoplasmic sperm injection may lead to the birth of an infant with sex chromosome abnormalities. Accurate counseling about possible genetic risks must be provided to the patients based on knowledge of that chromosome abnormalities are more frequent in sperm from infertile men and this may influence numerical aberrations of embryo. And prenatal diagnosis should be carefully applied.

If spermatozoa with normal chromosome can be selected by any technique in future, it may be useful for patients with infertility or recurrent miscarriage to prevent failure of implantation or next miscarriage.

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