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Absolute Polymorphic Teratozoospermia in Patients With Oligo-Asthenozoospermia Is Associated With an Elevated Sperm Aneuploidy Rate

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ABSTRACT: Infertile patients with abnormal sperm parameters have an increased sperm aneuploidy rate, despite a normal blood karyotype. The evaluation of sperm chromosome aberrations in patients with teratozoospermia only has shown a rate similar to that found in patients exhibiting oligo-astheno-teratozoospermia, which suggests that teratozoospermia is the critical parameter associated with aneuploidy. However, it is not known which alteration of the sperm morphology is associated with chromosome aberrations. The few cases reported so far have shown an association with the presence of abnormal head morphology and particularly with enlarged heads. We report the sperm aneuploidy rate of 3 patients with oligo-astheno-zoospermia who have absolute teratozoospermia (100% abnormal forms) and a different percentage of sperm head abnormalities. Fourteen healthy men with normozoospermia served as control subjects. Sperm aneuploidy and diploidy rates were calculated by using triple-color fluorescence in situ hybridization (FISH) for chromosomes 12, X, and Y, and double-color FISH was used for chromosomes 8 and 18. Patient K53, who had the highest number of spermatozoa with enlarged heads (54.3%), also had the highest aneuploidy and diploidy rates. The other 2 patients, K56 and K61, had sperm aneuploidy and diploidy rates lower than those of patient K53 but still well above the range found in normal men. Sperm chromosome abnormalities were intermediate in patient K61 and lower in patient K56, who had the lowest rate of spermatozoa with enlarged heads (18.9%). These data add further evidence that patients with teratozoospermia have an increased sperm aneuploidy rate and that this is particularly high in presence of an elevated percentage of spermatozoa with enlarged heads. For this reason, germ cells exhibiting this abnormality should not be used in in vitro fertilization programs.

Key words: Enlarged sperm head, fluorescence in situ hybridization, male infertility.

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The advent of intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of infertile patients with severe oligo-astheno-teratozoospermia (OAT), giving them an opportunity to procreate. However, these patients have an increased frequency of chromosomal abnormalities in their lymphocytes compared with fertile men (Meschede et al, 1998). This has raised concerns about the genetic safety of this technique, because these patients produce gametes with an abnormal karyotype. However, recent observations, using the fluorescence in situ hybridization (FISH) technique, have shown that patients with OAT and a normal blood karyotype have an increased sperm aneuploidy rate compared with normozoospermic men (Bernardini et al, 1998; Finkelstein et al, 1998; McInnes et al, 1998; Aran et al, 1999; Pang et al, 1999; Nishikawa et al, 2000; Ushijima et al, 2000; Vegetti et al, 2000; Calogero et al, 2001b; Härkönen et al, 2001). An inverse relationship between sperm aneuploidy and the percentage of normal forms has been reported (Ushijima et al, 2000; Vegetti et al, 2000; Calogero et al, 2001b; Härkönen et al, 2001). We have found that a group of patients with teratozoospermia only had a sperm aneuploidy rate similar to that found in a group of patients with OAT, which suggests that teratozoospermia was the critical parameter associated with aneuploidy (Calogero et al, 2001b). However, because the FISH technique alters sperm head morphology, thus preventing the differentiation between normal and abnormal spermatozoa, it is not possible to establish which type of sperm morphology alteration is associated with aneuploidy. The few cases reported in the literature have suggested that sperm aneuploidy seems to be restricted to spermatozoa with en-

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Table 1. *Clinical and seminal parameters of the 3 patients studied*

Parameter	K53	K56	K61	Controls (Range) (n = 14)
Age (year)	39	45	54	31 (25–35)
FSH (IU/L)*	5.6	8.3	12.3	3.2 (1.5–5.3)
Karyotype	46,XY	46,XY	46,XY	46,XY
Sperm density ($\times 10^6$ mL)	14.4	3.7	0.25	81 (20–180)
Total number of spermatozoa ($\times 10^6$)	43.2	13.3	1.25	201 (70–396)
Total motility (%)	14	13	8	54 (50–80)
Total abnormal forms (%)	100	100	100	62 (51–70)
Abnormal heads (%)	71.1	30.6	35.1	29.5 (20–42)
Pyriform (%)	0	0	0	0.5 (0–5)
Tapering (%)	8.4	0	0	8 (3–12)
Small oval (%)	0	0	0	0 (0–3)
Enlarged (%)	54.3	18.9	26.5	4.5 (1–7)
Duplicate (%)	0	0.9	0.6	0 (0–0)
Amorphous (%)	8.4	10.8	8	17 (6–24)
Abnormal midpieces (%)	12	12.6	16.7	24.5 (14–32)
Abnormal tails (%)	16.9	56.7	48.1	7.5 (4–11)

* FSH normal range, 1–8 IU/L.

larged heads or double tail (In't Veld et al, 1997; Bernardini et al, 1998; Viville et al, 2000). We report here the sperm aneuploidy rate of 3 oligo-asthenozoospermic patients with absolute (100% abnormal forms) polymorphic teratozoospermia and a different rate of sperm head abnormality. The results have shown that these patients had an elevated sperm aneuploidy rate for chromosomes 8, 12, 18, X, and Y that was higher in the patient with a greater number of spermatozoa with enlarged heads.

Materials and Methods

Patient Selection

The patients selected for the study were referred to us from the Private In Vitro Fertilization Center of Rabat, Morocco for the treatment of their infertile status, which lasted a duration of 2 to 4 years. They had OAT with absolute (100% abnormal forms) polymorphic teratozoospermia according to the World Health Organization (WHO) criteria in 2 consecutive semen samples (WHO, 1999). The rates of sperm head abnormalities are shown in Table 1. All 3 patients presented a normal 46,XY blood karyotype and testicular volume within the normal range. They had no history of abortions, and none of them was willing to undergo assisted reproductive techniques. The sperm aneuploidy rate was also evaluated in 14 healthy men with normal sperm parameters (WHO, 1999) and 46,XY karyotype, to establish the range of normality. The protocol was approved by the local ethics committee. An informed written consent was obtained from each patient and control subject.

Semen Preparation for FISH Analysis

An aliquot of spermatozoa was washed 3 times in phosphate-buffered saline (pH 7.2) and centrifuged at 650 × *g* for 10 minutes. The sediment was then fixed in methanol/acetic acid (3:1). The fixed specimens were stored at −20°C until further processing. The sperm heads were decondensed by incubating the spermatozoa with 25 mmol/L dithiothreitol, as reported elsewhere (Calogero et al, 2001b). The treatment did not disrupt the sperm structure, including the tail, which allowed an unequivocal differentiation between spermatozoa and the other cells present in the ejaculate.

DNA Probes

Double- and a triple-color FISH were done on each patient and control, using alpha-centromeric probes for chromosomes 8, 12, 18, X, and Y. The probe mixture for triple FISH consisted of a repetitive DNA sequence of centromeric probes: for chromosome X (pDMX1), labeled fluorescein isothiocyanate (FITC); for chromosome Y (pLAY5.5), labeled Cy3; and for chromosome 12 (pBR12), labeled FITC and Cy3. The probe mixture for the double-color FISH consisted also of a repetitive DNA sequence of centromeric probes for chromosome 8 (pZ8.4) and for chromosome 18 (2Xba), labeled FITC or Cy3, respectively. The probes were provided by Prof M. Rocchi, University of Bari (Bari, Italy).

Hybridization Procedure

Hybridization was performed as reported elsewhere (Calogero et al, 2001b). In brief, each slide was denatured by means of a solution of 70% formamide/2× standard saline citrate (SSC; pH 7.5) at 80°C for 150 seconds. The slides were immersed in a 70%, 90%, and 100% ethanol series for 3 minutes each and dried by air. The probes, precipitated and denatured at 80°C for 8 minutes, were applied directly to the slides, which were then covered with a coverslip and sealed with rubber cement. Hybridization occurred overnight in a dark humidified container at 37°C, after which the coverslip was removed and the slides were immersed 3 times in a posthybridization wash of 50% formamide/2× SSC at 37°C for 5 minutes, 2× SSC 3 times at 42°C for 5 minutes, and 2× SSC/0.1% Tween 20 at room temperature

Table 2. *Chromosome 12, X and Y triple-color fluorescence in situ hybridization of 3 patients with oligo-asthenozoospermia and absolute (100% abnormal forms) teratozoospermia*

	K53	K56	K61	Normal Range*
X-bearing spermatozoa (%)	10.71	48.60	43.24	47.81–51.55
Y-bearing spermatozoa (%)	10.84	48.44	49.32	48.07–51.81
XY disomy (%)	15.94	0	0	0–0.17
XX disomy (%)	0.51	1.53	4.38	0–0.51
YY disomy (%)	2.55	1.11	2.70	0–0.39
XXY trisomy (%)	5.48	0	0	0–0
XYY trisomy (%)	0.26	0	0	0–0
XXXYYY (%)	0.38	0	0	0–0
Chromosome 12 disomy (%)	28.57	0	0	0–0.2
Chromosome 12 trisomy (%)	4.46	0	0	0–0
Chromosome 12 quadrisomy (%)	0.68	0	0	0–0
XY and chromosome 12 trisomy (%)	0.64	0	0	0–0
XX and chromosome 12 trisomy (%)	0.51	0	0	0–0
XXY and chromosome 12 disomy (%)	2.55	0	0	0–0
XYY and chromosome 12 trisomy (%)	0.13	0	0	0–0
Diploidy rate (%)	15.80	0.32	1.04	0–0.2
Number of cells scored	1784	1895	296	1475–2019

* Obtained in 14 normozoospermic men.

for 5 minutes. The slides were then mounted in 4′,6-diamidino-2-pheneylindole (DAPI) counterstain and antifade and stored in the dark at 4°C for the microscope observation. The slides were observed using an Axiophot fluorescence microscope (C. Zeiss, Oberkochen, Germany) with an appropriate set of single-band filters for DAPI, FITC, and Cy3. The hybridization efficiency was calculated dividing the total number of cells observed by the total number of cells with at least 1 hybridization signal and multiplying this ratio by 100. It was always higher than 99%. Spermatozoa were scored as reported elsewhere (Calogero et al, 2001b). Only intact spermatozoa with clear hybridization signals were scored. We excluded disrupted or overlapping spermatozoa. Spermatozoa were considered to be polysomic if they presented 2 or more distinct hybridization signals of equal intensity separated by at least 1 signal domain. Diploid spermatozoa displayed 2 signals for each tested chromosome with normal head and tail morphology.

Results

The clinical and seminal parameters of our 3 patients are reported in Table 1. All 3 patients had OAT with 100% morphologically abnormal spermatozoa. Patient K53 had a substantially higher number of spermatozoa with abnormal heads (71.1%). More than half of all the spermatozoa (54.3%) showed enlarged heads. Patients K56 and K61 had 30.6% and 35.1% of the spermatozoa with abnormal head morphology, respectively; 18.9% and 26.5% exhibited enlarged heads.

The results of triple- and double-color FISH for each patient are reported in Tables 2 and 3. Patient K53 had an extremely elevated sperm aneuploidy rate. Indeed, 25.13% of the spermatozoa had heterochromosome an-

Table 3. *Chromosome 8 and 18 double-color fluorescence in situ hybridization of 3 patients with oligo-asthenozoospermia and absolute (100% abnormal forms) teratozoospermia*

	K53	K56	K61	Normal range*
Normal pattern (%)	2.64	96.85	91.85	98.86–99.85
Chromosome 8 disomy (%)	2.03	1.12	1.51	0.05–0.38
Trisomy 8 (%)	4.06	0	0	0–0
Chromosome 18 disomy (%)	6.09	1.4	1.81	0.05–0.45
Chromosome 8 disomy and chromosome 18 trisomy (%)	8.11	0	0	0–0
Chromosome 8 disomy and chromosome 18 quadrisomy (%)	2.03	0	0	0–0
Chromosome 8 trisomy and chromosome 18 disomy (%)	6.09	0	0.91	0–0
Chromosome 8 trisomy and chromosome 18 trisomy (%)	28.40	0	1.81	0–0
Chromosome 8 trisomy and chromosome 18 quadrisomy (%)	8.11	0	0	0–0
Chromosome 8 quadrisomy and chromosome 18 disomy (%)	4.07	0	0	0–0
Chromosome 8 quadrisomy and chromosome 18 quadrisomy (%)	6.09	0	0	0–0
Diploidy rate (%)	22.31	0.63	1.41	0–0.2
Number of cells scored	1986	2065	263	1864–2037

* Obtained in 14 normozoospermic men.

euploidy (normal range, 0%–0.62%), 33.68% had chromosome 12 aneuploidy, and 3.83% had the simultaneous presence of chromosome 12 and heterochromosome aneuploidy. The double-color FISH showed an aneuploidy rate of 6.09% for chromosomes 8 and 18 (normal range, 0.15–0.70). A large percentage of spermatozoa (62.87%) had aneuploidies regarding both autosomes simultaneously. The total aneuploidy rate was 69.55% (normal range, 0.3%–1.55%), whereas the mean diploidy rate was 19.06% (normal range, 0%–0.20%). Patients K56 and K61 had sperm aneuploidy rates substantially lower than that observed in patient K53 but higher than those found in normal control subjects. K56 had an aneuploidy rate of 2.64% for the sex chromosomes and 2.52% for chromosomes 8 and 18. The total aneuploidy rate was 5.16%, whereas the mean diploidy rate was 0.47%. K61 had a decreased number of spermatozoa bearing the chromosome X. The aneuploidy rate was 7.08% for the sex chromosomes and 6.04% for chromosomes 8 and 18. The total aneuploidy rate was 13.12%, whereas the mean diploidy rate was 1.23%.

Discussion

These 3 oligo-asthenozoospermic, infertile men with absolute teratozoospermia (100% abnormal forms) had quite an elevated sperm aneuploidy rate compared with normal control subjects. There was a relationship between the rate of chromosome abnormalities and the percentage of spermatozoa with enlarged heads. Indeed, the aneuploidy rate was extremely elevated in patient K53, who had the greatest proportion of spermatozoa with this abnormality. The rate was intermediate in patient K61 and lower, although still greater than in normal control subjects, in patient K56, who had the lowest rate of spermatozoa with enlarged heads.

These results are in close agreement with other observations that have shown an increased sperm aneuploidy rate in patients with teratozoospermia. Aribarg et al (2000) examined the sperm heterochromosome aneuploidy (by double FISH) in 20 patients with an almost absolute teratozoospermia (abnormal forms ranging from 98% to 100%, WHO criteria) and found increased XY, XX, and YY disomy rates compared with 20 normal men. We reported that the sperm aneuploidy rate for chromosomes 8, 12, 18, X, and Y in 9 patients with isolated teratozoospermia (normal forms ranging from 1% to 9%, Kruger et al's [1988] strict criteria) was similar to that of 19 patients with oligo-astheno-teratozoospermia, which suggests that teratozoospermia is the hallmark for the abnormal chromosomal constitution (Calogero et al, 2001b). However, Gole et al (2001) found that 8 patients with isolated teratozoospermia (normal forms <10%, WHO criteria) had a sperm aneuploidy rate lower than that of patients with oligo-astheno-teratozoospermia but significantly higher than that found in normal controls. Härkönen et al (2001) reported that patients with less than 10% abnormally shaped spermatozoa (WHO criteria) had a significantly higher chromosome 7, chromosome 18, XY, and YY disomy rates than patients with a less severe degree of teratozoospermia or normal controls. An increased sperm chromosome 13, XX, and YY disomy rate has also been reported in 9 patients with teratozoospermia (normal forms <13%, WHO criteria) (Templado et al, 2002). However, those patients had also asthenozoospermia, and it has been shown that patients with asthenozoospermia (total motility ranging 3%–45%, WHO criteria) have increased chromosome 12 and XX disomy rates (Hristova et al, 2002).

An increased sperm aneuploidy rate in patients with teratozoospermia has further been supported by studies that have evaluated the chromosome constitution of spermatozoa with normal and abnormal morphology in the same patients. Bernardini et al (1998) showed an almost 30-fold increase of chromosomes 1 and 17 aneuploidy rates in spermatozoa with an abnormal morphology, compared with germ cells with normal morphology, in controls and infertile patients. That study was conducted using a colorimetric in situ hybridization method that preserves sperm head morphology and cytoplasmic structures (sperm tail). A greater sex chromosome aneuploidy rate has been reported in spermatozoa with abnormal morphology in a patient with Klinefelter syndrome. Spermatozoon images, captured before FISH analysis, showed an abnormal morphology in 18 of 24 identified spermatozoa. In particular, 7 spermatozoa had head vacuoles, 4 had a slightly amorphous head, 3 had a severely amorphous head, 2 were microcephalic, 1 was bicephalic, and 1 had neck abnormality. Eleven (61%) of these spermatozoa showed to carry an abnormal karyotype, whereas only 1 (16.6%) of 6 spermatozoa with a normal morphology was aneuploid (sex chromosome nullisomy) (Estop et al, 1998).

The greater sperm aneuploidy rate found in patient K53, whose teratozoospermia was mainly characterized by an increased rate of spermatozoa with enlarged heads, agrees with other observations. Viville et al (2000) reported an extremely elevated sperm aneuploidy rate (66.9%) only in a patient whose absolute teratozoospermia was characterized by an elevated number of spermatozoa with enlarged heads (64%). In contrast, the other 3 patients reported in that study, who presented an absolute teratozoospermia mainly related to sperm tail or acrosome (absence or irregularly shaped) defects, had a normal sperm aneuploidy rate. In't Veld et al (1977) also reported an elevated sperm aneuploidy rate in a patient with 100% macrocephalic spermatozoa. The presence of

multiple tails in a high proportion of these spermatozoa suggested, however, that they originated from a lack of cell division during meiosis. Accordingly, 40% of these mature germ cells were diploid. Another case of a patient with absolute teratozoospermia, characterized by 30% headless spermatozoa and 70% abnormal head structure (large, round, and with or without acrosome), showed aneuploidy in virtually all spermatozoa (99.2%) and diploidy in about 20% of them (Weissenberg et al, 1998). Therefore, altogether, these data suggest that patients with teratozoospermia characterized by enlarged heads have a particularly elevated aneuploidy rate.

Previous studies have shown that poor sperm morphology results in poor fertilization rates (Oehninger et al, 1988), pregnancy rates (Kahraman et al, 1999), and embryo quality (Cohen et al, 1991; Parinaud et al, 1993) in in vitro fertilization programs. In addition, a higher spontaneous abortion rate has been reported in conventional in vitro fertilization performed using semen with an abnormal morphology (Oehninger et al, 1988). Given that about 50% of spontaneous abortions are chromosomally abnormal (see Hassold, 1986 for review), it can be hypothesized that one reason for this is the elevated sperm aneuploidy rate found in spermatozoa with an abnormal morphology. Indeed, Yakin and Kahraman (2001) reported a 60% chromosomal abnormality rate in embryos developed from semen samples with enlarged heads. For this reason, they suggested that ''couples should be counseled about low rates of fertilization and pregnancy with ICSI when only macrocephalic spermatozoa are present in the ejaculate.'' The results of the present study add further evidence that these patients definitely need thorough genetic evaluation and counseling.

In conclusion, the present study adds further evidence that teratozoospermia is associated with an increased sperm aneuploidy rate. Abnormal chromosome constitution reaches a very elevated degree in spermatozoa with enlarged heads. The presence of these sperm abnormalities at an elevated rate may be viewed as a bad omen for pregnancy outcome (Calogero et al, 2001a).

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