Gene deletions in an infertile man with sperm fibrous sheath dysplasia

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BACKGROUND: Asthenozoospermia may sometimes be related to genetic structural defects of the sperm tail detectable by transmission electron microscopy. Dysplasia of the fibrous sheath (DFS) is a genetic sperm defect, characterized by dysplastic development of the axonemal and periaxonemal cytoskeleton. We report the case of an infertile man with normal sperm count and total sperm immotility in which dysplasia of the fibrous sheath, Akap3, Akap4 gene deletions, meiotic segregation of chromosomes 18, X and Y and Y microdeletions were investigated. METHODS: A 32-year-old man with a 3-year history of primary infertility presented at our Regional Referral Center for Male Infertility. Family medical history, lymphocyte karyotype, PCR analysis, physical examination, hormone assays and semen analysis were performed. RESULTS: Ultrastructural sperm evaluation showed dysplasia of the fibrous sheath. Immunostaining of AKAP4 protein was negative in sperm tails. PCR analysis revealed intragenic deletions of the Akap3 and Akap4 genes. Fluorescence *in situ* hybridization on sperm showed a high frequency of XY disomy. CONCLUSION: In this infertile patient, our results suggest a possible relationship between dysplasia of the fibrous sheath, partial deletions in the Akap3 and Akap4 genes and absence of AKAP4 protein in the fibrous sheath. These findings, however, were not detected in another four patients with dysplasia of the fibrous sheath. Our results require future confirmatory molecular analyses.

Key words: Akap3/Akap4 deletions/DFS/FISH analysis/male infertility/TEM

Introduction

Infertility affects an estimated 15% of couples: a male factor has been identified in approximately 50% of infertility cases (Bhasin *et al*., 1994). Poor sperm morphology or motility may be responsible for male infertility. In rare cases, asthenozoospermia may be caused by genetic structural defects of the sperm tail detectable by transmission electron microscopy (TEM). The genetic sperm alteration dysplasia of the fibrous sheath (DFS) (Chemes *et al*., 1987, 1998) is characterized by dysplastic development of the axonemal and periaxonemal cytoskeleton. The genetic origin of this defect has been suggested by different authors (Chemes, 2000; Baccetti *et al*., 2001). In humans, two major proteins of the sperm fibrous sheath, AKAP82 and its precursor pro-AKAP82, were cloned and characterized (Carrera *et al*., 1996; Turner *et al*., 1998). Recently, Eddy *et al*. (2003) demonstrated that two A-kinase anchoring proteins (AKAP3, AKAP4) are the most abundant structural proteins of the fibrous sheath. AKAP3 is involved in organizing the basic structure of the fibrous sheath, whereas AKAP4 has a major role in completing fibrous sheath assembly (Brown *et al*., 2003). No evidence has yet been found to support the hypothesis that mutations in either gene for AKAP3 and AKAP4 proteins are responsible for DFS in humans (Turner *et al*., 2001b).

Genetic male infertility may also be associated with deletions of specific regions of the long arm of the Y chromosome. Y microdeletion screening is especially worthwhile in patients with a sperm count below 5×10^6/ml or even below 10×10^6/ml. PCR analysis for Yq microdeletions in 392 normospermic men did not reveal any deletions (Krausz *et al*., 2003). A new model of deletions in which AZFb and AZFc regions overlap has emerged. Repping *et al*. (2002) suggested at least three different deletion patterns as a consequence of AZFb and AZFc deletions.

Most studies using multicolour fluorescence *in situ* hybridization (FISH) demonstrate that infertile men may also have a higher frequency of sperm chromosomal abnormalities (Martin *et al*., 1996; Bernardini *et al*., 1997; Rives *et al*., 1998), in particular when DFS sperm defect is present (Baccetti *et al*., 2005).

In this paper we report the results of different molecular analyses performed in an infertile man with DFS. PCR was carried out on DNA extracted from peripheral blood lymphocytes to analyse specific Y microdeletions and partial sequences

of the AKAP4 and AKAP3 genes. Meiotic segregation pattern was also investigated by three-colour FISH sperm analysis.

Materials and methods

Patient

A-32-year-old man was referred to our laboratory for semen analysis after 3 years of sexual intercourse without conception. His wife, age 30 years, did not have any fertility problems. Sexual development, medical history, physical examination and serum hormone levels (testosterone, cortisol, T3, T4, TSH, estradiol, FSH, LH, prolactin and inhibin-B) were normal. Semen and urethral fluid were tested for microbial infection. Consanguinity was excluded. Lymphocyte karyotype was 46,XY.

Semen analysis

Light and electron microscopy

A semen sample was collected by masturbation after 4 days of sexual abstinence and examined after liquefaction for 30 min at 37°C. Volume, pH, concentration and motility were evaluated according to WHO guidelines (World Health Organization, 1999).

For electron microscopy, semen was fixed in cold Karnovsky fixative and maintained at 4°C for 2 h. Fixed semen was washed in 0.1 mol/l cacodylate buffer (pH 7.2) for 12 h, postfixed in 1% buffered osmium tetroxide for 1 h at 4°C, dehydrated and embedded in Epon Araldite. Ultra-thin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate, and observed and photographed with a Philips CM10 transmission electron microscope (TEM; Philips Scientifics, Eindhoven, The Netherlands). Three hundred sperm in ultra-thin sections were analysed.

An aliquot from the same sperm sample was also processed for scanning electron microscopy, fixing the spermatozoa as described above and smearing them on polylysine (1%)-coated coverslides. After dehydration specimens were dried by the critical point technique, coated in gold and observed with a Philips CM 515 scanning electron microscope (Philips Scientifics, Eindhoven, The Netherlands).

PCR analysis

DNA was extracted from peripheral blood lymphocytes using the QIAamp DNA Blood Kit (Qiagen).

PCR analysis for Y microdeletion screening was performed according to European Academy of Andrology (EAA) guidelines (Simoni et al., 2004).

Control DNA was extracted from blood of 10 male donors, age 30–40 years, with a documented history of fertility. DNA extracted from blood of two fertile females was used as negative control.

PCR product corresponding to the region of pro-hAKAP82 that encodes the RII-binding domain was amplified according to Turner et al. (2001a). Oligonucleotide primers flanking the RII-binding site were used: sense primer, 5′-GCGATTATCACAGAGCATCC-3′; antisense primer. 5′-TGCTCTCTCTCTGGGACATC-3′.

PCR products corresponding to a region of hAKAP4 involved in binding to AKAP3 (site 1) and to a region of AKAP3 involved in binding to hAKAP4 (site 2) were amplified according to Turner et al. (2001b). Oligonucleotide primers flanking the respective binding sites, were used: site 1, sense primer, 5′-TCAGTGCCCTTATAGGT-GAG-3′; antisense primer, 5′-GCAGAGCTTCATCACAGATTC-3′; site 2, sense primer, 5′-TTGAGGAATCTCCACAGCG-3′; antisense primer, 5′-CCAACGGTCTTTCACACAACTTC-3′.

Control DNA was extracted from blood of a fertile man and four infertile men with DFS sperm defect.

Immunofluorescence

Semen samples of the patient and fertile men, used as controls, were washed twice in phosphate buffered saline (PBS), smeared on glass slides, air-dried, rinsed in PBS and fixed for 15 min in methanol at –20°C. Slides were then treated with blocking solution (PBS, 1% BSA, 5% normal goat serum [NGS]) for 20 min at room temperature and incubated overnight at 4°C with mouse monoclonal anti-β-tubulin (Sigma Chemical, St Louis, MO, USA) and mouse monoclonal anti-AKAP 82 (BD Biosciences, Erembodegem, Belgium) diluted 1:100 and 1:50 respectively in PBS, 0.1% BSA, 1%NGS. After three washes in PBS, the samples were treated with goat anti-mouse IgG-Texas Red-conjugated antibody (Southern Biotechnology Associates, Birmingham, AL, USA). Finally, the samples were washed three times in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Incubation in primary antibodies was omitted in procedure control samples. Observations and photographs were made with a Leitz Aristoplan light microscope equipped with fluorescence apparatus.

FISH sperm analysis

FISH sperm analysis was carried out according to Baccetti et al. (2003) to evaluate aneuploidy frequency. A mix of α-satellite DNA probes (CEP, Vysis, IL) for chromosomes 18, X and Y, directly labelled with different fluorochromes, was used.

Results

Spermiogram evaluation showed that sperm count was 35×10^6/ml and sperm motility 0%. Extremely short tails were observed by light microscopy. The eosin Y test revealed 82% live sperm.

The genotypic sperm defect DFS was demonstrated by TEM (Figure 1a) in the whole sperm population. Sperm tails were extremely short, as confirmed also by scanning electron microscopy (Figure 1b). A disorganized fibrous sheath and altered axonemal structure, sometimes lacking dynein arms and microtubular doublets including the central pair, were observed. Although spermatozoa with these defects usually have well formed acrosomes and nuclei (Baccetti et al., 1993), in this case we found severe nuclear alterations, particularly in chromatin texture, in almost the entire sperm population.

Immunofluorescence staining using monoclonal anti-β-tubulin confirmed short, thick axonemal structure (Figure 2a, b). Immunofluorescence staining, using a monoclonal anti-AKAP82 antibody specific for human AKAP4 protein (previously named AKAP82), was completely negative (Figure 2c, d). In control samples (Figure 2e), the same antibody labelled the tail principal piece. Anti-tubulin antibody recognized the centriolar region and principal piece of the sperm tail in control samples (Figure 2f).

FISH was performed on sperm nuclei to evaluate aneuploidy frequency (Table I). We examined 4233 sperm. Only XY disomy was more frequent than in controls (Baccetti et al., 2003); the frequency of other disomies and diploidies was normal.

PCR screening of specific Y chromosome according to EAA guidelines (Simoni et al., 2004) did not evidence any deletions in the regions investigated.

When we performed PCR using two pairs of primers flanking sites 1 and 2, as described by Turner et al. (2001b), the expected PCR products corresponding to a region of hAKAP4 involved in binding to AKAP3 (site 1) and to a region of AKAP3 involved in binding to hAKAP4 (site 2) were both absent (Figure 3, lanes C2, 3). This was confirmed by three
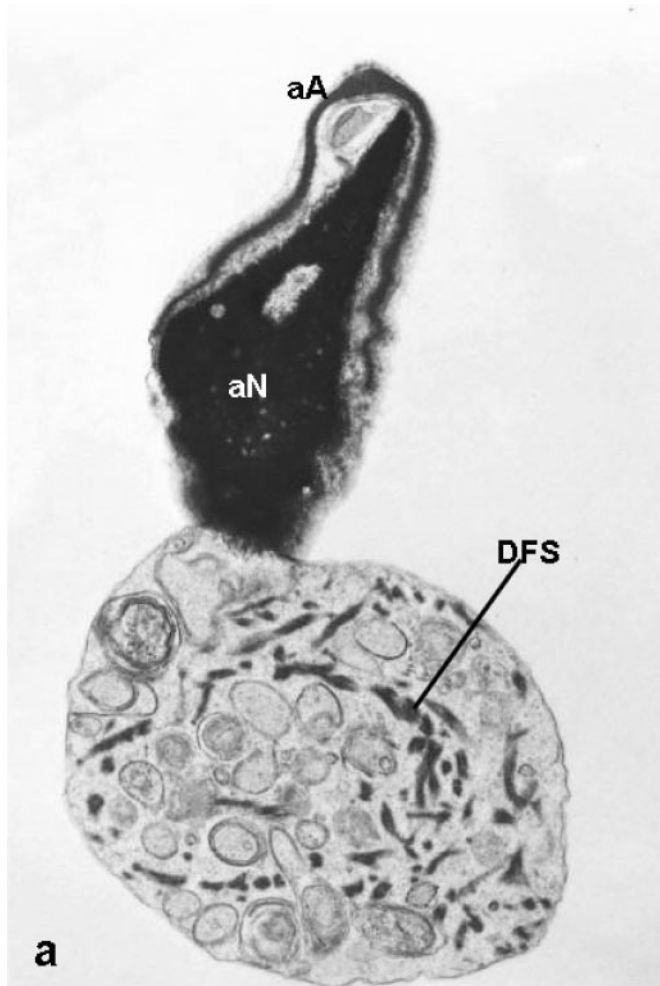
Figure 1. (a) TEM micrograph of a longitudinal section of sperm with altered acrosome (aA) and nucleus (aN), showing dysplasia of the fibrous sheath (DFS), which is completely disorganized in remnants embedded in a cytoplasmic residue. ×20 000. (b) SEM micrograph of a DFS sperm, with a very short tail. ×5400.

subsequent PCR attempts. The same sequences were present in a fertile man (Figure 3, lanes A2, 3) and four infertile men with DFS (Figure 3, lanes B2,3, D2,3, E2,3, F2,3), who were analysed as controls (Figure 3). In all DFS patients, including our
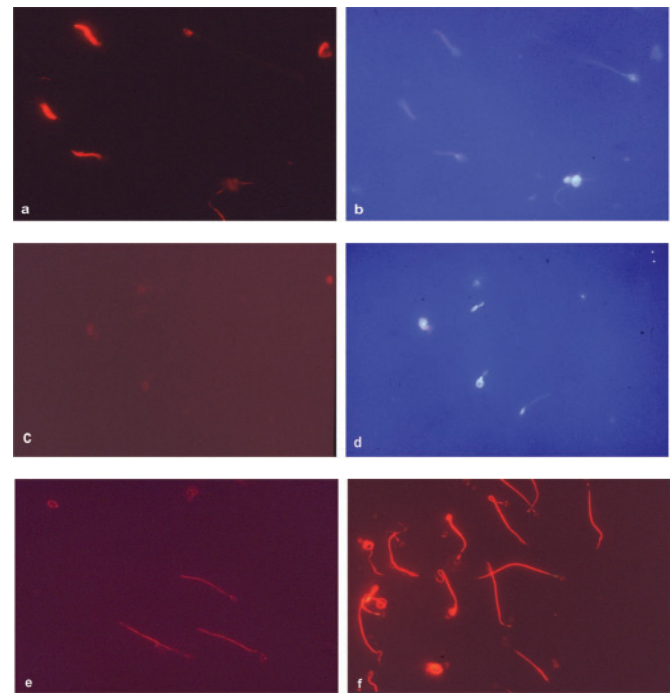
Figure 2. UV and blue light micrographs of ejaculated spermatozoa. (a, b) Staining with monoclonal anti-β-tubulin antibody highlighted very short thick axonemes. (c, d) Immunocytochemical labelling with anti-AKAP82 monoclonal antibody was completely negative. (e, f) Labelling with anti-β-tubulin and anti-AKAP82 monoclonal antibodies was normal in the principal piece of sperm of fertile men, used as control. ×4.

studied case, and in the control fertile man, PCR products corresponding to pro-hAkap82, which encodes the RII-binding domain, were present (Figure 3, lane 1, A–F)

Discussion

In the present study we report the case of an infertile patient who was a carrier of the genetic DFS sperm defect associated with deletion of partial sequences of potential AKAP4/AKAP3 binding regions.

TEM demonstrated the presence of a peculiar ultrastructural defect of the sperm tail, namely dysplasia of the fibrous sheath. This defect, like other genetic sperm defects, is more frequent in consanguineous patients and is related to the degree of consanguinity (Baccetti *et al.*, 2001). DFS includes a heterogeneous array of structural defects of the sperm tail (Chemes and Rawe, 2003) and causes almost complete immotility, but nuclear and acrosomal structure are generally unaffected. In our patient, however, we observed severe nuclear alterations, particularly in chromatin texture and condensation.

For this reason, screening for Y microdeletion was carried out even though the patient had a normal sperm count. No Y microdeletions were detected. This coincides with the results of Krausz *et al.* (2003), who failed to detect Y microdeletions in normospermic individuals. On the other hand, we found an absence of the potential AKAP4/AKAP3 binding regions in the patient under study while the presence of these PCR products was detected in fertile men and other infertile men with

Table I. FISH analysis for chromosomes 18, X and Y

	Normal sperm	Frequencies of disomies				Frequencies of diploidies		
	X/Y-bearing	18, 18	X,X	Y,Y	X,Y	18,18,X,X	18,18,Y,Y	18,18,X,Y
Patient[a]	48%/50%	0.11%	0.05%	0.17%	0.45%	0.17%	0.05%	0.21%
Controls[b]		0.11 ± 0.003%	0.23 ± 0.003%			0.28 ± 0.004%		

[a]Number of cells scored by triple FISH analysis was 4233. The frequencies of nullisomies were: 18/0 = 0.02%; X/0 = 0.04%; Y/0 = 0.01%. Multiple fluorescent signals (>4) were detected in 0.21% of cells.
[b]Seven fertile men constituted the control group. Mean percentages (± SD) of disomies and diploidies are reported for the chromosomes examined. The number of sperm scored by triple-FISH analysis was 5600 ± 260.
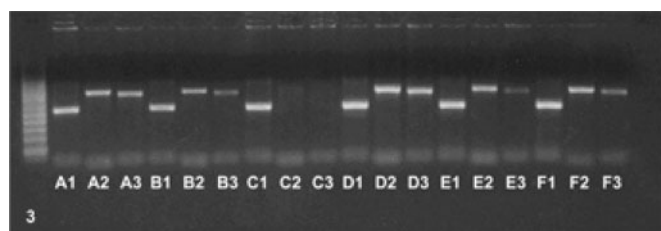

Figure 3. PCR analysis in five DFS patients (lanes B–F) and one fertile man (lane A) used as control. Lane 1: amplification products of pro-hAkap82 gene. Lane 2: amplification products of hAkap4 gene region involved in the binding to Akap3 (site1). Lane 3: amplification products of Akap3 region involved in the binding to hAkap4 (site 2). Lanes 2 and 3 were completely absent only in the patient (C).

DFS, analysed as controls. Turner *et al.* (2001b) did not find evidence to support the hypothesis that mutations in Akap4 or Akap3 are responsible for DFS in humans. However, in our case, deletion of the Akap4/Akap3 binding regions and failure to detect AKAP4 protein by immunofluorescence in the fibrous sheath of sperm tails suggests that, in this case, the lack of AKAP4 could be pathogenically responsible for the DFS phenotype.

Partial deletions of Akap3 and Akap4 gene sequences could therefore be related to defective assembly of fibrous sheath components and failure of compartmentalization of AKAP3 and AKAP4 proteins in the tail, causing sperm immotility. AKAP-3 is involved in organizing the basic structure of the fibrous sheath, while AKAP4 has a major role in completing fibrous sheath assembly. Their absence is linked to sperm immotility because the fibrous sheath lacks any signal transduction system (Miki *et al.*, 2002; Brown *et al.*, 2003).

Our findings suggest that, in some cases, the DFS sperm defect could be associated with alterations of Akap3 and Akap4 gene sequences.

Our results would require future confirmation. It would be useful to perform an immunoblot with the anti-AKAP4 antibody, in order to show whether there is any AKAP4 protein being formed.

Finally, FISH analysis highlighted an increased frequency of sex chromosome (XY) disomy in our patient, indicating a segregation anomaly at the first meiotic division. This rare combination of genetic and sperm defects is responsible for fertilization failure *in vivo*.

Intracytoplasmic sperm injection could be the only tool able to bypass the reproductive problem in these kinds of patients. Nevertheless, the possibility of transmission of different genetic anomalies to the offspring should be considered in genetic counselling before undergoing micromanipulative assisted reproduction.

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