A possible association of a human tektin-t gene mutation (A229V) with isolated non-syndromic asthenozoospermia: Case Report

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Asthenozoospermia (AZS), characterized by grade A + B sperm motility (as in World Health Organization Guidelines) ≤50% or A <25% in fresh ejaculate, may exist as an isolated disorder, in combination with other sperm anomalies or as part of syndromic association. The majority of syndromic patients can be ascribed to mutations in dynein genes, while, to date, no genes have been described to be associated in humans with non-syndromic, isolated AZS. An interesting family of axonemal proteins, the tektins, has been recently identified in various mammals and they are thought to play a fundamental role in ciliary movement. Recently, the human tektin-t (or h-tekB1 or Tektin-2) gene has been cloned, showing specific expression in flagella of mature sperm. We report the screening of tektin-t gene in 90 isolated non-syndromic AZS patients. We found a heterozygous mutation (A229V) in one patient. Ultrastructural analysis showed anomalies in ≥80% of examined spermatozoa involving axoneme microtubules and mitochondria. Moreover, the viability and mitochondrial function of sperm were altered in the patient with the A229V mutation. This is the first description of human pathology linked to a tektin-family gene, since only murine models are available for these genes.

Keywords: asthenozoospermia; tektin-t; primary ciliary dyskinesia; sperm motility; axoneme

Introduction

Infertility affects ~15% of couples trying to conceive in western countries and genetic causes may be identified in a large proportion of them. Male infertility represents one of the clearest examples of a complex disease with a substantial genetic basis. In fact, a genetic abnormality may be recognized in about 15% of male infertile subjects (Ferlin et al., 2007). A multitude of irregularities, including sperm number, motility and morphology has been linked with male factor infertility. Azoospermia, oligozoospermia, asthenozoospermia (AZS) and teratospermia are the four major semen anomalies and are present in approximately half the cases of couple infertility and in almost 90% of infertile males (Huynh et al., 2002). In particular, AZS [A + B sperm motility ≤50%, or A <25% in fresh ejaculate, according to World Health Organization (1999) Guidelines] may exist as an isolated disorder, in combination with other sperm anomalies or as part of syndromic association.

AZS, as an isolated disorder, is found in as many as 24% of patients presenting for evaluation of male subfertility and may be a significant factor in another 55% of patients with combined defects in sperm density, motility and morphology (Lishultz, 1980). AZS might be due to different causes such as motility problems or increased level of sperm degradation (necrozoospermia). Decreased motility may be secondary to sperm dysfunction, prolonged periods of sexual abstinence, partial blockage, varicocele, infectious diseases or genetic factors. AZS is a phenotype often present in primary ciliary dyskinesia (PCD), a hereditary disorder characterized by immotile cilia, which leads to recurrent pulmonary and upper respiratory tract infections, which often result in bronchiectasis. When PCD is associated with situs inversus (dextrocardia and/or heterotaxia) the pathology is named Kartagener Syndrome (KS). A structural deficit in dynein arms (component of axoneme, internal structure of cilia and flagella) can be detected in ≥50% of PCD/KS patients (Afzelius, 1981). Approximately 90% of male KS patients are affected by AZS. The majority of KS patients can be ascribed to dynein genes mutations, whereas, to date, no genes have been described to be associated in human with non-syndromic, isolated AZS. From all these considerations, it is possible that defects in axonemal structure may be a cause of flagellar dysfunction in isolated AZS patients. An interesting family of
axonal proteins, the tektins, has recently been identified in various mammals, including mice (Iguchi et al., 1999) and humans (Xu et al., 2001; Roy et al., 2004, 2007), after 20 years of study in echinoderm and molluscan sperm flagella and blastula cilia (Setter et al., 2006). The tektins are a family of proteins, highly conserved from protozoa to human, forming coiled coils and having some structural properties similar to intermediate filament proteins. Tektins may function to provide stability and structural complexity to axonemal microtubules (MTs) and work as templates and guides for generating the three-dimensional organization of axoneme (Norlander et al., 2000). Thus, tektins are thought to play a fundamental role in ciliary movement (Setter et al., 2000). Recently, the human tektin-t (or h-tekB1 or Tektin-2) gene has been cloned (Iguchi et al., 2002; Wolkowicz et al., 2002), showing a specific expression in testis, precisely localized in flagella of mature sperm in mice and humans. The localization of this protein makes it a good candidate gene for screening in AZS.

Here, we report the screening of tektin-t gene in a cohort of isolated non-syndromic AZS patients, the finding of a heterozygous mutation in one patient among 90 showing this condition, and the results of relative functional and ultra-structural tests performed on this patient compared with controls.

Materials and Methods

Subjects
We evaluated 90 consecutive subjects (age range: 20–42 years old) affected by idiopathic isolated AZS (A + B sperm motility less than 50%, or A < 25% in fresh ejaculate) after three different evaluations of semen in 9 months. Exclusion criteria were: drug consumption, fever in the previous month, seminal infections, varicocele, systemic diseases, previous cryptorchidism or orchitis. Moreover, in all subjects, the presence of anti-sperm auto-antibodies was excluded by the SpermMAR-immunoglobulin (Ig)G and IgA test (FertiPro, Beernem, Belgium).

Controls (200 age matched men) had normal sperm parameters and were proven fertile, being recruited from men whose wives were in the first trimester of pregnancy. All patients and controls were of Caucasian origin and came from different Italian regions. The study was conducted in accordance with the guidelines in the Declaration of Helsinki and has been formally approved by the Hospital Ethics Committee. Each participant gave his written informed consent.

Semen analysis
Semen samples were obtained by masturbation after 2–5 days abstinence. Routine semen analysis was performed after liquefaction of the semen within 1 h according to WHO Guidelines (World Health Organization, 1999). For each sperm sample, motility was assessed manually by counting 200 spermatozoa from at least 10 individual fields using oil immersion with magnification of ×1000 under bright-field illumination. Viability of sperm was assessed by Eosin Y exclusion (normal value ≥ 50%). Morphology slides were stained with May-Grunwalds Giemsa. The percentage of normal sperm morphology was assessed according to WHO criteria.

Mutation detection
Mutation analysis of Tektin-t gene (RefSeq NM_014466, localized on 1p34.3) was performed by PCR amplification and direct sequencing, using a set of 14 oligonucleotide primers designed using available genomic sequence and covering the entire gene, from exon 1 to exon 10, plus flanking intronic sequence (Table I). Due to small introns dimensions, exons 2–3, exons 5–6 and exons 7–8–9 were amplified together.

Genomic DNA was extracted from peripheral blood leukocytes using a DNA isolation kit (Roche, Milano, Italy). PCR amplification was carried out in 30 μl reaction volume containing 200 ng of genomic DNA in standard PCR conditions, for 25 sequential cycles each including 1 min denaturation at 95°C, 1 min primer annealing at appropriate temperature (see Table I) and 1 min extension at 72°C. An initial denaturation step of 5 min at 95°C was added before the first cycle and a final extension step of 7 min at 72°C was added after the last cycle. PCR products were separated by electrophoresis on 2% agarose gel. For direct sequencing, PCR product were enzyme purified and both strands of the DNA fragment were sequenced using the same primers as for PCR. Additional internal primers Tekt-t8F (5'-GCCTGAGCTTCTCCTGCT-3') and Tekt-t9F (5'-CTAGCCAAAGTCTCCTC-3') were designed for sequencing the large PCR product of exon 7 to 9. Sequence analysis was performed by using the gap4 software of the Staden package available at the Staden Package Homepage. To verify the presence of mutation A229V and to screen the control subjects, we performed an enzymatic digestion by BsrBI (NEB, Ipswich, USA) restriction enzyme (cut site 5'-GAGCGG), mixing 500 ng of PCR-amplified exon 6 fragment, 1 U of BsrBI, NEBuffer 2 1× and water in a 30 μl total reaction volume. The reaction was then overnight incubated overnight at

Table I. Primer sequences and PCR annealing temperature for the 10 exons of the human Tektin-t gene (RefSeq NM_014466)

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tekt-t1F</td>
<td>5'-CGCTACCTAAAGGCCCTC-3'</td>
<td>62</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>Tekt-t1R</td>
<td>5'-TTGGTTAACATCCTGCCCG-3'</td>
<td>62</td>
<td>244</td>
</tr>
<tr>
<td>2–3</td>
<td>Tekt-t2F</td>
<td>5'-CTGCTCCAAAGAGCTTTCC-3'</td>
<td>64</td>
<td>666</td>
</tr>
<tr>
<td></td>
<td>Tekt-t3R</td>
<td>5'-CGTGTCTCAGGAGTACATAGC-3'</td>
<td>62</td>
<td>488</td>
</tr>
<tr>
<td></td>
<td>Tekt-t4F</td>
<td>5'-GCTATCCTCCACAGCTAA-3'</td>
<td>62</td>
<td>488</td>
</tr>
<tr>
<td></td>
<td>Tekt-t4R</td>
<td>5'-GCTGAATTAGAGCAAGAGAGG-3'</td>
<td>64</td>
<td>488</td>
</tr>
<tr>
<td>5–6</td>
<td>Tekt-t5F</td>
<td>5'-ACTTGCGTGAGCTTCCATT-3'</td>
<td>62</td>
<td>594</td>
</tr>
<tr>
<td></td>
<td>Tekt-t6R</td>
<td>5'-GGCTGAGCTGAGATATAC-3'</td>
<td>62</td>
<td>594</td>
</tr>
<tr>
<td>7–9</td>
<td>Tekt-t7F</td>
<td>5'-ATGAGCTTCCACAGCTCCT-3'</td>
<td>62</td>
<td>822</td>
</tr>
<tr>
<td></td>
<td>Tekt-t9R</td>
<td>5'-GCTGCCAAAGTTTCTC-3'</td>
<td>62</td>
<td>822</td>
</tr>
<tr>
<td></td>
<td>Tekt-t10F</td>
<td>5'-GAGCACAGTGGAGCTGCC-3'</td>
<td>64</td>
<td>480</td>
</tr>
<tr>
<td></td>
<td>Tekt-t10R</td>
<td>5'-CCTGCTGAGCTCATACAGTG-3'</td>
<td>62</td>
<td>480</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.
37°C, followed by heat inactivation (80°C for 20 min). Normal C/C homozygote showed two cut bands (422 and 178 bp), whereas the mutated C/T heterozygote showed three cut bands (600, 422 and 178 bp).

**Transmission electron microscopy analysis**

For transmission electron microscopy (TEM) analysis ejaculates were immediately washed in Ham’s F-10 medium, pH 7.3 (GIBCO, Life Technologies Ltd., Paisley, UK), by centrifugation at 380 g for 20 min and pellets were resuspended in cold 100 mmol cacodylate buffer 1 \( \times \) pH 7.4, containing 3% (v/v) glutaraldehyde (AGAR Scientific Ltd, Essex, UK) for 2 h at 4°C. Sperm samples were subsequently washed in cacodylate buffer by centrifugation (380 g for 20 min), and pellets were post-fixed in 1% (w/v) osmium tetroxide in distilled water, dehydrated through graded ethanol and embedded in Epon 812 (AGAR Scientific Ltd). Ultra-thin sections were contrasted with uranyl acetate and lead hydroxide (AGAR Scientific Ltd) and evaluated in a Philips 100 transmission electron microscope (Philips Electronics, Eindhoven, Holland). Pictures were formatted with Photoshop 7.0 software (Adobe system, San Jose, CA, USA). TEM analysis was performed on 50 cross sections of the tails. This served to analyse both the axoneme and mitochondria and the latter were also analysed on longitudinal sections. For each axoneme, the number of outer doublet MTs, central singlet MTs, outer dynein arms, inner dynein arms, and radial spokes were assessed (Francavilla et al., 2006).

**Apoptosis analysis by annexin-V/propidium iodide assay**

The exposure of phosphatidylserine on sperm surface was detected by annexin-V-fluorescein isothiocyanate (FITC), Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). The staining procedure was conducted according to the manufacturer protocol with slight modifications. Briefly, for each sample, cells were resuspended in 100 μl of binding buffer at \( \sim 2 \times 10^8 \) cell/ml, and 10 μl of Annexin-V-FITC were added. Samples were then incubated for 10 min at room temperature under light-proof conditions. About 2 μl of propidium iodide solution (1:1000) were added to each sample for viability analysis. Finally, the spermatozoa were deposited onto slides and examined with an epifluorescence microscope.

**Assessment of sperm mitochondrial status**

Sperm mitochondrial activity was evaluated by staining with JC-1 (5,5’,6,6’-tetraclorofluorescein-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide) according with Molecular Probes, Eugene, OR, USA. JC-1 changes reversibly its fluorescence from green (monomeric status) to orange (multimeric status) when mitochondrial membrane potential is high. About 10 μl of 200 μM JC-1 in dimethylsulphoxide were added to 1 ml of 2 \( \times 10^8 / \) ml cell suspension in phosphate-buffered saline (PBS) and incubated for 20 min at 37°C. About 1 μl of 50 mM carbonyl cyanide m-chlorophenylhydrazone was added to control tube, and the cells were incubated at 37°C, 5% CO₂, for 20 min. After centrifugation and resuspension of pellet in 500 μl of PBS, samples were analysed on a flow cytometer (Becton Dickinson, Facs Scan, BD Biosciences, San Jose, CA, USA) with 488 nm excitation using emission filters appropriate for Alexa Fluor 488 dye and R-phycocerythrin.

**Results**

After screening 90 AZS patients, we found four sequence changes in tektins-t: c.-12G>A (in 5’ untranslated region), R46C in exon 3 (c.136C>T), T393T in exon 10 (c.1179A>G) and A229V (c.685C>T) in exon 6, each one in a different patient and always in the heterozygous condition. After screening the controls and an online database analysis, we established that the first three sequence changes were common single nucleotide polymorphisms, found also in controls, whereas we never found the missense mutation A229V in controls, confirmed twice in separate DNA samples by direct sequencing (Fig. 1A) and restriction enzyme digestion (data not shown). Amino acid (a.a.) 229 is a highly conserved residue from invertebrate to higher species (Fig. 1B). Moreover, we further analyzed the A229V mutation using Polyphen software (genetics.bwh.harvard.edu) for predicting the possible consequences of this mutation on the structure and the function of protein. The prediction showed a possibly damaging mutation with position-specific independent counts score difference of 1.827, which corresponds to a structural, buried site and cavity creation.

The patient showing this mutation was a 20-year-old man at the time of observation, son of healthy parents. Five different semen evaluations over 2 years showed variable AZS (A + B ranging from 45 to 5%), with 15–40% of angled-neck spermatozoa, with normal total and per/ml sperm number (70–110 million/ml, total volume ranging from 2.2 to 3.5 ml), as well as pH (ranging from 7.8–8.2) and viability (78–83%, normal values >65%). The testicular ultrasound showed...
normally descended testes, with normal morphology and size (right 23.4 cc; left 21 cc). His general condition was excellent, without varicocele, even if he reported frequent upper airways infections and catarrhal bronchitis since childhood, always treated by prolonged antibiotic therapies.

He was sexually active but, until now, had never tried to conceive, so we ignore the real outcome of his infertility. After analysis extension to the family, we discovered that the mother was carrier of the same mutation (data not shown), but she never reported problems with conceiving or respiratory involvement.

TEM analysis showed anomalies in the tail in more than 80% of examined spermatozoa (Fig. 2). Mitochondria were not regularly arranged in the mid piece (Fig. 2A and B) and most spermatozoa showed disorganization and rarefaction of the cristae (Fig. 2A and C), while dilatation of intracisternal space (Fig. 2B) was also observed. Axoneme showed a severe disorganization of MTs, consisting of central MTs absence and a reduced number of peripheral MTs (Fig. 2B). Supernumerary outer dense fibers were also frequently observed (Fig. 2C). Occasional spermatozoa showed a normal organization of the axoneme and of the mitochondria in the mid piece. No specific dynein arm defects were observed. Occasional heads showed an abnormal alignment of the head with the mid piece, resulting in sperm with bent heads.

Finally, we performed the functional tests on fresh ejaculate from the patient with the mutation and after 4 h (Table II), which showed a clear reduction of mitochondrial function and of viability (JC-1 and annexin test). In particular, after 4 h the active mitochondria were reduced from 80 to 47% and the live sperm from 73 to 34%, whereas the dead sperm were increased from 7 to 46%. The results of these tests confirmed the previous observations of the reduced motility of the patient’s sperm, highlighting a possible early mitochondrial energy depletion linked to scarce productivity of sperm movement.

**Discussion**

Tektins are the constitutive proteins of Mts in cilia, flagella (also sperm tail), basal bodies and centrioles (Norrander et al., 1998; Larsson et al., 2000) and were originally isolated from sea urchin as a set of proteins, named tektin A, B and C (Norrander et al., 1996). Tektins A and B are thought to form core protofilaments of tektin filaments, and tektin C is thought to form homodimers assembled onto periphery of these core protofilaments or to form a second separate tektin filament (Pirner and Linck, 1994). Tektins have axial periodicities that match the complex spacing of axonemal complex, e.g. dynein arms, radial spokes and interdoublet nexin links. These finding led to the hypothesis that tektin filaments act as molecular rulers to direct the spatial organization of these axonemal components, the precise three-dimensional organization of which is required for motility (Lindemann, 2003).

For all these reason, when tektin-t was cloned in human and its specific expression demonstrated in sperm tail, our attention was focused on it.

Moreover, recently Tanaka *et al.*, (2004) generated a tektin-t deficient mouse which shows male infertility caused by debilitating sperm motility and functionally defective tracheal cilia. Female mice were fully fertile. Even if a direct correlation

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**Table II.** Results of functional tests in fresh (1 h) and 4-h-old sperm.

<table>
<thead>
<tr>
<th>Time</th>
<th>Sperm/ml</th>
<th>Viability</th>
<th>Motility</th>
<th>Mitochondrial function</th>
<th>Apoptosis (annexin-V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A + B</td>
<td>D</td>
<td>Live sperm</td>
</tr>
<tr>
<td>1 h</td>
<td>73 million</td>
<td>88%</td>
<td>25%</td>
<td>35%</td>
<td>80%</td>
</tr>
<tr>
<td>4 h</td>
<td>73 million</td>
<td>60%</td>
<td>15%</td>
<td>62%</td>
<td>47%</td>
</tr>
</tbody>
</table>

Normal values for these functional studies performed on normozoospermic men showed non significant alterations of parameters after 4 h of observation (Lachaud *et al.*, 2004).
between null-mice and men with the heterozygous mutation is not possible, this animal data suggest an important involvement of tektin gene in spermatogenesis and/or sperm function.

After screening of a large cohort of AZS patients we found the mutation A229V in one patient, and we demonstrated that it was maternally inherited from the fertile mother. By protein alignment of different species, we observed that the a.a. in position 229 was strongly conserved (Fig. 1B), and it is completely conserved in the three sea urchin tektins (A1, B1 and C1) which have been more extensively studied to date (Fig. 1C). This observation led us to assert that a.a 229 holds some important role in the tektins’ functions, since it is located in the second coiled-coil strand of protein, i.e. involved in the three-dimensional structure of tektin filaments. Moreover, it is known that tektin filaments are the linker that closes the gap between tubules A and B, constituents of the external MT doublet of 9 + 2 axonemal structure, and even if it is still unknown how the other different proteins (nexin, dyneins, etc.) that form the axoneme bind the tektins, it is reasonable to suppose that such a conserved protein position may exert a crucial role. The mutation was present in our patient in heterozygous condition, suggesting an incomplete autosomal dominant inheritance, with a non-completely dominant allele, or a haplo-insufficiency. This model could also explain why not all spermatozoa were affected by axonemal anomalies, assuming that a residual, even if reduced, normal tektin-t protein production was still present (in agreement with 47% of residual mitochondrial function after 4 h). It is that AZS is the intermediate phenotype of heterozygote, even if the clinical consequences of homozgyosis in humans is still unknown, because we did not find a homozygous sequence change. We could suppose that the homozgyotic phenotype may have AZS and a major pulmonary involvement, as in PCD and KS patients. However, the non-syndromic AZS probably has multiple origins, mainly of unknown nature, and, even if tektin-t mutations are probably involved in some cases, the finding of 1 in 90 patients excludes this gene as a major origin of this condition.

Similar to tektin-t deficient mice, our patient also showed a diminished flagellar motility and an ineffective molecular propulsion machinery, as shown by the functional tests performed on patient’s spermatozoa.

In particular, the tests performed to assess the viability and the mitochondrial function (JC-1 and annexin-V test), showed alterations in our patient (Table II), demonstrating a possible early mitochondrial energy depletion linked to scarce productivity of sperm movement.

Moreover, when we extended the motility observation to 4 h, the A + B motility was very precociously reduced, with a selective increase of C (unproductive movement) parameter with respect to the others (data not shown).

Interestingly, Roy et al., (2007) have recently published the effects of absence of tektin-4 in male mice, describing some features very similar to our findings. Tektin-4 is a member of the tektin protein family expressed in spermatozoa that shows high homology with human tektin-4, which shares ~50% of homology with human tektin-t (data not shown). Furthermore, the position of a.a. 229 is conserved both in tektin-t and tektin-4. The Tekt4-null mice exhibit drastically reduced forward progressive velocity and uncoordinated wave form propagation along the flagellum, which is grossly normal, as revealed by TEM analysis. The ineffective flagellar strokes lead to very high consumption of intracellular ATP and null spermatozoa rapidly lose progressive motility when incubated for >2 h. Even if the tektin gene examined by us is different, the similarity with our observations is strong, as expected by two proteins belonging to the same family which are evolutionarily conserved.

Also our findings at TEM analysis could be involved in the deficit of energy metabolism found in Tektin-t and Tektin-4 null mice. In fact, we found several anomalies, including mitochondrial disorganization in the tail midpiece with intracrystae space dilatation and rarefaction, whereas tails showed axonemal anomalies, with severe microtubular disorganization represented by central MTs absence, peripheral MT reduction and a variable percentage of supernumerary outer dense fibers. No specific dynein arm anomalies were reported. These findings are indicative of the importance of tektin-t in the sperm flagella, suggesting that axially repeating tektin filaments are required for its periodic organization.

Finally, a peculiar morphology was evident from the observation of patient’s spermatozoa, consisting in of a high percentage of angled-neck sperm, ranging from 15 to 40%. It is possible that, since tektins are also components of the basal body, an alteration of protein sequence can modify the connection between neck and sperm tail and produce angled-neck sperm.

Undoubtedly, many aspects of the AZS phenotype remain unexplained, particularly the presence in our patient of recurrent respiratory infections and bronchitis, which could be correlated with a general impairment of bronchial cilia, even if not still proved (data in progress). Similarly, the young age of our patient give us no information about his real fertility, so we cannot exclude that he will run into a precocious fertility decline with increasing age, as shown in Tektin-4 aged null mice (Roy et al., 2007).

However, this is the first description of human pathology linked to a tektin-family gene, since only murine models are currently available for these genes.

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References


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