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Mutations in dynein genes in patients affected by isolated non-syndromic asthenozoospermia

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BACKGROUND: Asthenozoospermia (AZS) is a common cause of male infertility characterized by reduced forward motility (WHO grade A+B sperm motility <50% or A < 25%) or absent sperm motility in fresh ejaculate. AZS may exist as an isolated disorder, in combination with other sperm anomalies or as part of a syndromic association. Up to date, only a few genes, constituting the cilia/flagella structure, have been associated with isolated AZS in humans, whereas several other genes are known to be involved in syndromic form of AZS, including primary ciliary dyskinesia (PCD) and Kartagener syndrome (KS). Axonemal ultrastructural defects, including absent or shortened arms of dyneins, can be found in >50% of PCD/KS patients. Approximately 90% of KS male patients are affected by AZS. The majority of KS patients can be ascribed to dynein genes mutations. METHODS: Mutation screening of DNAI1, DNAH5 and DNAH11 genes was performed in 90 patients with isolated non-syndromic AZS and 200 controls. RESULTS: We found three mutations (one in each gene) specifically associated with AZS in seven patients (7.8%). Mutations are inherited from the mothers and may be found in familial clusters. No ultrastructural axonemal anomaly was detected in sperm. CONCLUSIONS: We report for the first time a possible association between mutations in dynein genes and isolated AZS. Male carriers of the mutations always exhibit AZS, whereas female carriers manifest no alterations in either fertility or pulmonary clearance.

Key words: asthenozoospermia; DNAI1; DNAH5; DNAH11; primary ciliary dyskinesia

Introduction

Asthenozoospermia (AZS) is a common cause of male infertility characterized by reduced forward motility [A+B sperm motility $<50\%$ or A $< 25\%$, World Health Organization (WHO) criteria (WHO, 1999)] or absent sperm motility in fresh ejaculate. AZS may exist as an isolated disorder, in combination with other sperm anomalies or as part of a syndromic association. AZS as an isolated disorder is found in as many as 24% of patients presenting for the evaluation of male subfertility and may be a significant factor in another 55% of patients with combined defects in sperm concentration, motility and morphology (Luconi *et al*., 2006). Decreased sperm motility may be caused by sperm dysfunction, prolonged periods of sexual abstinence, partial blockage of seminal tract, varicocele, infection or genetic factors (Gaur *et al*., 2007; Gdoura *et al*., 2007; Pasqualotto *et al*., 2008). To date, only a few genes, encoding the cilia/flagella structure (Baccetti *et al*., 2005; Zuccarello *et al*., 2008), have been associated with isolated AZS in humans, whereas several other genes (Guichard *et al*., 2001; Olbrich *et al*., 2002; Budny *et al*., 2006; Moore *et al*., 2006; Schwabe *et al*., 2008) are known to be involved in syndromic form of AZS, including primary ciliary dyskinesia (PCD) and Kartagener syndrome (KS).

PCD is an inherited rare disorder (prevalence of 1:16 000–1:60 000) characterized by recurrent respiratory infections caused by defective muco-ciliary clearance due to immotile or dyskinetic respiratory cilia, and reduced fertility due to AZS caused by sperm dysmotility (Zariwala *et al*., 2007). The combination of PCD and situs ambiguous (heterotaxy), exhibited by half of PCD patients, is referred to as KS. Both PCD and KS are considered cilia-related disorders (ciliopathies), linked to ultrastructural defects of the axoneme. The axoneme, a structure present in respiratory cilia, embryonic nodal cilia and sperm tail, has a $9 + 2$ arrangement consisting of nine microtubule doublets surrounding a pair of microtubule singlets (central pair). Radial spokes radiate from the central microtubules towards the peripheral microtubules. Nexin links connect the outer doublets and keep them intact and limit the range of microtubular sliding. Each microtubule A of the doublet contains motor proteins termed dynein and has two rows of dynein arms, termed the outer dynein arms (ODAs) and inner dynein arms (IDAs) (Luck, 1984).

Both in PCD/KS (Sirvanci *et al*., 2008) and in isolated AZS patients (Wilton *et al*., 1992; Chemes *et al*., 1998; Courtade *et al*., 1998; Ishijima *et al*., 2002), several axonemal ultrastructural defects have been found by transmission electron microscopy (TEM), including absent or shortened ODA and/or IDA, and defects in radial spokes, nexin links or central pairs. For example, a structural alteration in dynein arms can be detected in >50% of PCD/KS patients (Afzelius, 1981).

AZS can be found in ~90% of PCD/KS male patients, the majority of them presenting dynein genes mutation (Storm van's Gravesande and Omran, 2005; Zariwala *et al*., 2007). To date, three of the several dynein arms subunits have been found to be mutated in individual patients and/or in several families affected by PCD/KS. These genes encode for three proteins belonging to the axonemal dynein cluster (ODA), particularly expressed in testis and trachea: dNAI1 (axonemal dynein, intermediate chain 1) (Pennarun *et al*., 1999; Guichard *et al*., 2001; Zariwala *et al*., 2001; Noone *et al*., 2002; Zariwala *et al*., 2006), DNAH5 (axonemal dynein, heavy chain 5) (Olbrich *et al*., 2002; Kispert *et al*., 2003; Bush and Ferkol, 2006; Hornef *et al*., 2006) and DNAH11 (axonemal dynein, heavy chain 11) (Bartoloni *et al*., 2002; Schwabe *et al*., 2008). For this reason, we screened 90 patients with isolated non-syndromic AZS for mutations in these three genes.

Materials and Methods

Subjects

We evaluated 90 consecutive subjects (age range 20–42 years) affected by idiopathic isolated AZS (A + B sperm motility <50% or A < 25% in fresh ejaculate) confirmed in three different semen analyses over 9 months. Exclusion criteria were medication, fever in the previous month, seminal infections, varicocele, systemic diseases, history of cryptorchidism or orchitis, manifest pulmonary symptoms. Moreover, in all subjects, the presence of antisperm autoantibodies was excluded by the SpermMAR-IgG and IgA test (FertiPro, Beernem, Belgium).

Controls (200 age-matched men) were proven fertile males with normal sperm parameters recruited from the husbands of wives 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 of 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 of abstinence. Routine semen analysis was performed after liquefaction of the semen within 1 h according to WHO Guidelines (WHO, 1999). For each sperm sample, motility was assessed by counting 200 spermatozoa from at least 10 individual fields using oil immersion with a magnification of ×100 under bright-field illumination. Sperm motility is classified in four grades: rapid and linear forward progression (A), slow or non-linear forward progression (B), non-progressive motility (C) and non-motility (D). Viability of sperm was assessed by eosin Y exclusion (normal value ≥50%). Sperm morphology was assessed after staining with May-Grunwald-Giemsa (Cincik *et al*., 2007). The percentage of sperm with normal morphology was assessed according to WHO criteria. For the purpose of this study, patients with isolated AZS were selected, thus excluding teratozoospermia and necrospermia.

Mutation detection

Mutation analysis of DNAI1 (RefSeq NM_012144, localized on 9p21-p13), DNAH5 (RefSeq NM_001369, localized on 5p15-p14) and DNAH11 (RefSeq NM_003777, localized on 7p21) was performed by PCR amplification and direct sequencing, using a set of oligonucleotide primers designed using available genomic sequence information and covering the entire gene region and flanking intronic sequence (Supplementary Table). 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 and 1 min extension at 72°C. An initial denaturation step of 5 min at 95°C before the first cycle and a final extension step of 7 min at 72°C after the last cycle were added. PCR products were separated by electrophoresis on 2% agarose gel. For direct sequencing, PCR products were enzyme-purified and both strands of the DNA fragment were sequenced using the same primers as for PCR. Sequence analysis was performed using the gap4 software of the Staden Package available at the Staden Package homepage. All the mutations were confirmed using two separate blood samples.

TEM analysis

To analyse the sperm tail ultrastructure, sperm from patients with mutations were characterized by TEM. Axoneme and mitochondria were analysed on 50 cross-sections of the tails for each patient with a mutation. Mitochondria were also analysed on longitudinal sections. For each axoneme, the number of outer doublet microtubules, central singlet microtubules, ODAs, IDAs and radial spokes were assessed.

For TEM analysis, ejaculates were immediately washed in Ham's F-10 medium, pH 7.3 (GIBCO, Life Technologies Ltd, Paisley, UK), centrifuged at 380*g* for 20 min, and pellets were resuspended in cold 100 mmol cacodylate buffer (per litre, 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). Ultrathin 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) (Francavilla *et al*., 2006).

Results

Initially, we analysed 20 exons from DNAI1 and, given their considerable expanse, 9 out of 79 exons from DNAH5 and 2 out of 82 from DNAH11. This screening procedure was chosen on the basis of previously reported mutations in patients affected by PCD and KS.

By direct sequencing, we identified four novel heterozygote sequence changes: r663C in DNAI1, E1756K and E2666D in DNAH5 and I3040V in DNAH11. Moreover, a further three known non-synonymous sequence changes were detected: a8S and V335I in DNAI1 and R3004Q in DNAH11 (Table I). When we tested 200 controls (normospermic men),

Table I. Summary of sequence variants found in 90 AZS patients.

Exon	Base	Amino acid	Type	Frequency in patients	Frequency in controls
DNAI1 gene					
19	CGC>TGC	**R663C**	unknown transversion	3/90	0/200
1	GCT>TCT	A8S	known SNP	7/90	19/200
11	GTC>ATC	V335I	known SNP	3/90	12/200
DNAH5 gene					
32	GAA>AAA	**E1756K**	unknown transition	1/90	2/200
48	GAG>GAT	**E2666D**	unknown transversion	1/90	0/200
28	CGG>CGT	R1458R	known SNP	8/90	np
32	GCC>GCT	A1724A	known SNP	3/90	np
53	ACG>ACA	T2966T	known SNP	14/90	np
62	ACA>ACG	T3491T	known SNP	11/90	np
75	GCC>GCT	A4357A	known SNP	4/90	np
DNAH11 gene					
55	ATT>GTT	**I3040V**	unknown transition	3/90	0/200
55	CGG>CAG	R3004Q	known SNP	3/90	4/200

In bold are novel mutations detected in this study. np, not performed.

we found the sequence variation E1756K with a frequency of 1%; R3004Q (2%); A8S (9.5%); V335I (6%). The presence of E1756K, R3004Q, A8S and V335I in control subjects (without manifest pulmonary symptoms) suggests they are common polymorphisms. We did not find mutations R663C, E2666D and I3040V in any of the control subjects. Moreover, to date, mutations in these three genes (DNAI1, DNAH5, DNAH11) have been linked to recessive PCD or KS. For this reason (to asses a compound heterozygosity), we extended the mutation screening to whole DNAH5 (79 exons) and DNAH11 (82 exons) genes in our patients with mutations. We did not find more than one mutation in any of our patients. In summary, we found three mutations (one in each gene) specifically associated with AZS, with a frequency of 7.8% (7 patients out of 90).

Mutation R663C in DNAI1 (c.2180 C>T) is located in exon 19 and encodes for the WD5-repeat domain (critical for beta-propeller structure assembly); mutation E2666D in DNAH5 (c.8040 G>T) is located in exon 48 and encodes for the AAA-3 domain; mutation I3040V in DNAH11 (c.9118 A>G) is located in exon 55 downstream to the AAA-4 domain. By performing protein alignment for different species, we observed that all these amino acids are evolutionarily strongly conserved, indicating their crucial role in the function of the dynein arm core (Fig. 1).

We found the mutation R663C (DNAI1) in two unrelated patients (#2616 and 1530) and two brothers (#4900 and 4900/4; the second one not included in the initial screening of the 90 AZS patients); E2666D (DNAH5) in one patient (#1935) and I3040V (DNAH11) in one patient (#3305) and two first cousins (#2659 and 3109) (Table II).

For consanguineous patients (#4900–4900/04 and #2659–3109), we rebuilt the pedigree, analysing the family members who were available (Fig. 2a and b) and showed that in each case the mothers of patients were carriers of the mutation. This finding suggests that the mutation is always associated with AZS when carried by a male, whereas no manifest alterations of fertility and pulmonary clearance are present in female carriers.

No ultrastructural anomaly was detected in sperm by TEM, and all the samples showed classical 9 + 2 doublet

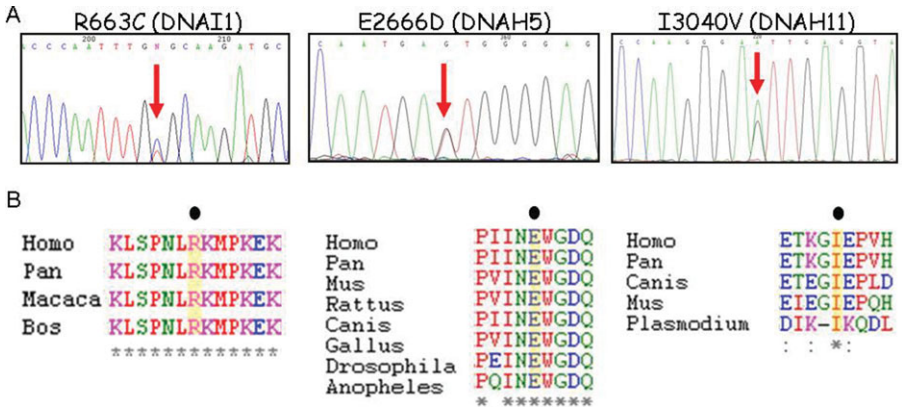

Figure 1: Mutational and protein sequence analysis results.
(**A**) Chromatogram of the mutation R663C in DNAI1 (c.2180 C>T); mutation E2666D in DNAH5 (c.8040 G>T); mutation I3040V in DNAH11 (c.9118 A>G). Red arrows indicate the position of the base substitution. (**B**) Results of CLUSTAL W computational analysis of dyneins alignment. We performed CLUSTAL W alignment analysis to compare the human DNAI1 (699 aa, RefProt NP_036276), DNAH5 (4624 aa, RefProt NP_001360), DNAH11 (4523 aa, RefProt NP_003768) protein sequences with the dynein sequences of several different species. Black dots indicate the position of the reported substitution.

Table II. Summary of clinical phenotype of the patients with mutations.

Patient	Mutation	Gene	Pedigree	A+B motility[a] (%)	Sperm count[a] (millions/ml)	Viability[a] (%)	Age (years)
#2616	R663C	DNAI1	unrelated	16	33	78	41
#1530	R663C	DNAI1	unrelated	12	82	67	42
#4900	R663C	DNAI1	Family 001	6	29	75	33
#4900/04	R663C	DNAI1	Family 001	13	20	60	29
#1935	E2666D	DNAH5	unrelated	14	76	68	40
#3305	I3040V	DNAH11	unrelated	2	23	72	34
#2659	I3040V	DNAH11	Family 002	14	36	66	40
#3109	I3040V	DNAH11	Family 002	10	21	58	35

Normal values: A+B motility >50%; sperm count: >20 millions/ml; viability: >50%. [a]Mean of the three different sperm evaluations.

microtubular configuration, as well as normal dynein arms and radial spokes.

Discussion

AZS is a common cause of male infertility and most cases are of unknown origin. This is the first report assessing the presence of mutations in DNAI1, DNAH5, DNAH11 genes in patients affected by isolated AZS. Apart from normal variants detected in both patients and controls, we found three mutations (one in each gene) specifically associated with AZS, with a frequency of 7.8% (7 out of 90). All these mutations caused substitution of amino acids that are strongly conserved in the superior species, indicating their important role in the protein structure. In particular, all these three proteins are located in the axonemal ODA, permanently attached to the A tubule of each outer microtubule doublet and transiently attached to the B tubule of the adjacent microtubule doublet, to generate a sliding motion.

The intermediate chain 1 (DNAI1) is a WD repeat protein, containing five WD repeat (Trp–Asp di-peptide) domains (mutation R663C is located in WD5 domain), which forms a

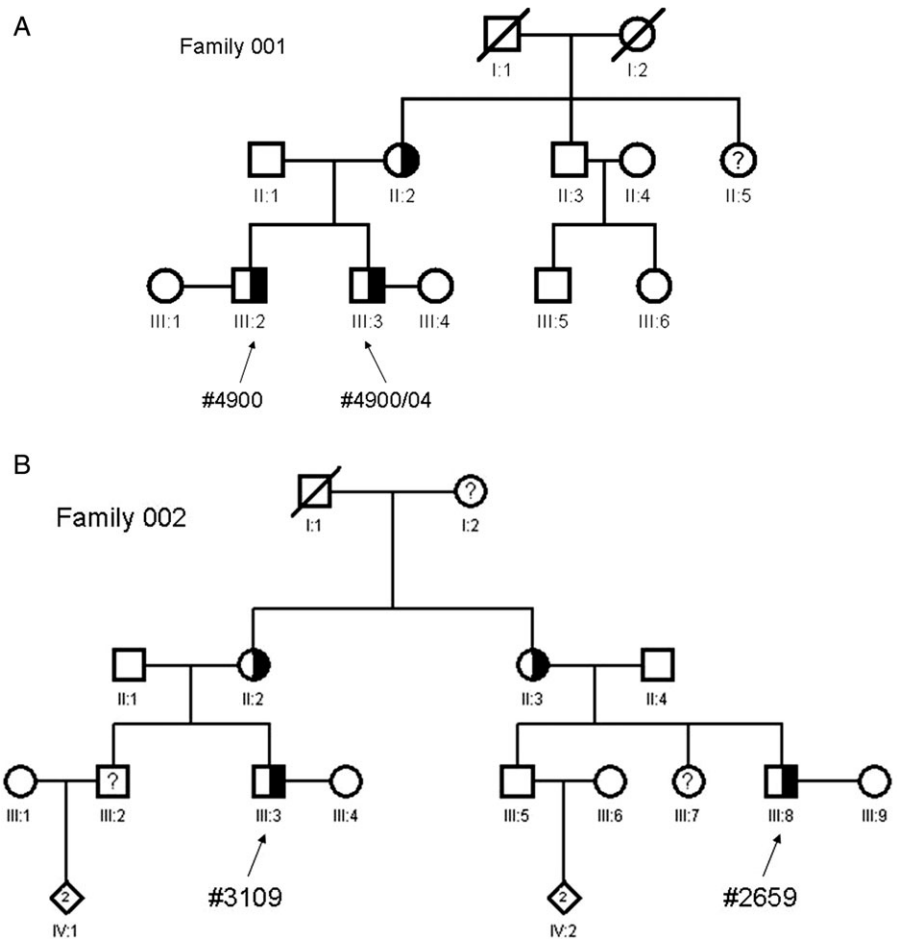

Figure 2: Pedigree of Family 001 (mutation R663C in DNAI1) and Family 002 (mutation I3040V in DNAH11).
(**A**) Family 001. Black arrows indicate the patients with mutation (III-2: patient #4900; III-3: patient #4900/04). Subject II-5 did not accept the screening. (**B**) Family 002. Black arrows indicate the patients with mutation (III-3: patient #3109; III-8: patient #2659). Subjects I-2 and III-7 were not available. Subject III-2 did not accept the screening, but he adopted two sons (IV-1). He never conceived by spontaneous fertilization.

beta-propeller structure with several blades that serves as a stable rigid scaffold platform to which proteins can bind either stably or reversibly, crucial for protein–protein interaction.

The heavy chains 5 and 11 (DNAH5 and DNAH11, respectively) are constituted by an N-terminal domain interacting with the other intermediate and light chains, a motor domain (core) arranged in heptameric AAA subdomains with ATP-ase function (mutations E2666D and I3040V are located in AAA-3 and AAA-4 domains, respectively), a linking stalk and a microtubule-binding domain.

As previously described (Schwabe *et al*., 2008) for DNAH11 mutations, no ultrastructural anomaly was detected, and all the sperm tail samples showed classical 9 + 2 doublet microtubular configuration, as well as normal dynein arms and radial spokes. Unfortunately, in the study of Schwabe *et al*., 2008, no sperm analysis was performed, so we have no details about the fertility status of those patients with mutations to compare with ours. Nevertheless, this result could be explained by the fact that probably a heterozygous mutation (as we found in our patients) is not sufficient to cause structural damage, but only a functional injury to the execution of flagellar movement. However, we have no explanation relating to the non-involvement of the bronchial cilia, and we do not know why the mutations affect only sperm motility but not muco-ciliary clearance. The absence of pulmonary symptoms does not justify the use of invasive medical procedures, such as bronchoscopy, to obtain appropriate samples for the analysis. The analysis of the mRNA of these three genes could provide information about the type of damage produced by the mutations. However, this is impracticable, since sperm are notoriously trascriptionally and translationally silent cells and hence contain very limited amounts of mRNA. In fact, amplification of DNAI1, DNAH5, DNAH11 mRNAs from total sperm RNA revealed very low expression of these genes. This result was expected, because the sperm tail proteins are assembled at an earlier stage of spermatogenesis and the small amount of RNA probably represents only a remnant.

Apart from a few families (Narayan *et al*., 1994; Alvarez González *et al*., 2006), PCD is usually inherited as an autosomal recessive disease (Sturgess *et al*., 1986) and therefore we expected to find two mutations also in our patients. We cannot exclude a compound heterozygosity with another axonemal structure gene, but we can also suggest that AZS is caused by haploinsufficiency or by dominant sex-specific mutations. This is supported by the fact that in our familial cases, the healthy mothers are only carriers of the mutation and have no fertility alteration or bronchial diseases, whereas the sons with the mutation are affected by AZS. The sons without mutations are fertile. Besides, this is not the first description of autosomal dominant inheritance through the maternal line in cases of PCD (Alvarez González *et al*., 2006). A third hypothesis that may explain the finding of an heterozygous state in our patients could be related to a second germinal mutation occurring early during development (gonocytes), in addition to the somatic mutation detected in the peripheral leukocytes. Thus primary spermatocytes might indeed be homozygous for the mutant allele ('second hit' hypothesis). Finally, another hypothesis is that isolated AZS is the mild phenotype of the PCD/KS complex. More studies, especially on the dynein function, are mandatory to confirm these data.

In conclusion, we report for the first time a possible association between mutations in dynein genes and isolated AZS. The present findings, if confirmed on larger samples and different ethnicity, have implications for a better diagnostic approach to patients with AZS. Furthermore, many AZS patients are involved in assisted reproductive programmes (Intra Cytoplasmatic Sperm Injection, ICSI), and thus may transmit the disease to their offspring. In fact, dominant AZS alleles could give rise to infertile sons, whereas recessive alleles could, in the next generations, increase the frequency of male infertility or, at worst, cause the transmission of PCD/KS complex via a carrier partner.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org.

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