Sperm Mitochondrial Mutations as a Cause of Low Sperm Motility

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ABSTRACT: We report the unique case of a 28-year-old man who, in spite of having a varicocele and a sperm concentration of 5 million/mL, of which 10% were motile and 20% had normal forms (oligoasthenoteratozoospermia [OAT]), was fertile. This was confirmed by paternity testing using 16 autosomal and 6 Y-chromosomal short tandem repeat (STR) loci. An analysis of mitochondrial genes that included cytochrome oxidase I (COI), cytochrome oxidase II (COII), adenosine triphosphate synthase6 (ATPase6), ATPase8, transfer ribonucleic acid (tRNA) serine I,

tRNA lysine, and NADH dehydrogenase3 (ND3) revealed, for the first time, 9 missense and 27 silent mutations in the sperm's mitochondrial DNA (mtDNA) but not in the DNA from the blood cells. There was a 2-nucleotide deletion in the mitochondrial COII genes, introducing a stop codon, which might be responsible for low sperm motility.

Key words: Infertility, oligoasthenoteratozoospermia, mitochondrial DNA, short tandem repeats.

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On the basis of the semen profile, male infertility is classified into 4 broad categories, namely azoospermia (complete absence of sperm), oligozoospermia (low sperm count), asthenozoospermia (low motility), and teratozoospermia (abnormal shape and size). An individual with a combination of the last 3 sperm abnormalities is said to have oligoasthenoteratozoospermia (OAT), and he is generally infertile (Tuerlings et al, 2002). Mitochondrial DNA (mtDNA) encodes for several genes whose products play an important role in oxidative phosphorylation resulting in the production of adenosine triphosphate (ATP) molecules, the energy currencies of the cell (Bafaluy et al, 2002). Mitochondria are important components of sperm and supply energy for its motility. Mutations in the mtDNA of sperm result in either functionless or malfunctioning proteins, hence affecting sperm motility. Mutations in mtDNA are found to be associated with a large number of diseases in humans. More than 100 different mtDNA point mutations as well as a large number of mtDNA deletions have been found to be associated with a large number of diseases (Graeber and Muller, 1998; Wallace, 1999). Several single nucleotide polymorphisms (SNPs) in the mitochondrial genes were

found to be associated with diabetes (Nomiyama et al, 2002), MELAS (mitochondrial encephalopathy, lactacidosis, and strokelike syndrome) (Hanna et al, 1998), neurological disorders (Cavelier et al, 2001), and male infertility (Holyoake et al, 2001; Spiropoulos et al, 2002). A sequence level difference between gametic and somatic cells is a very rare phenomenon. A few studies have demonstrated the existence of differences between somatic and germ cells (Chong et al, 1995). Here, we report the case of a fertile man with OAT having a mutation in the mitochondrial genes of sperm but not in the blood cells.

Materials and Methods

Case Report

The ages of the male and female partner of this study were 28 and 22 years, respectively, and they were first cousins. Because the female partner did not conceive after 1 year of marriage, both of them had undergone clinical examination. The results had shown that the female partner was normal. The male partner's scrotal Doppler study revealed that the size of the right testis was 39 × 17 mm and that the left side was 40 × 20 mm. The size, contours, and echo patterns bilaterally were normal, and no hydrocele was found. A varicocele was observed only in the left testis; epididymis/cord structures were normal. Results of semen analyses were as follows: quantity, 2.0 mL; count, 5 million/mL; motility, active—10%, sluggish—20%, and nonmotile—70%; white blood cell count, 12–15/high-power field; and morphology, normal form—20% and abnormal form—80% (Figure 1). With the above information, a clinician had informed the male partner that he could become a father only with assisted reproduction. However, after 8 months of clinical examination,
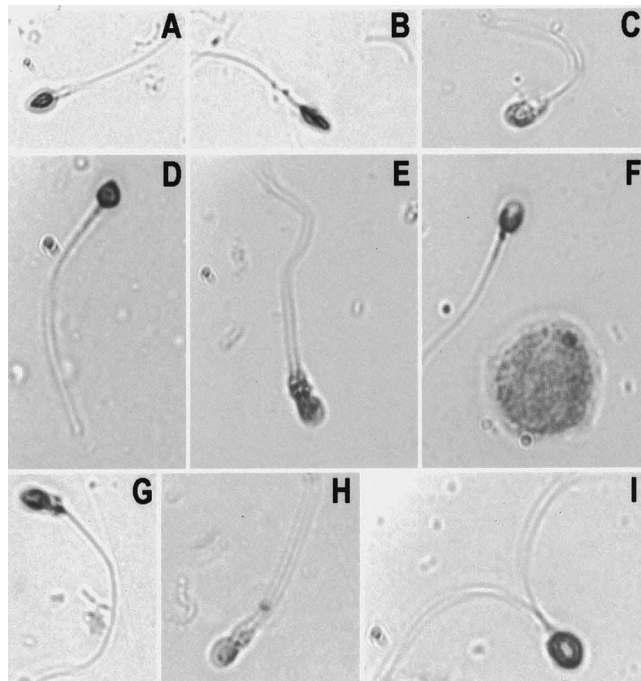
Figure 1. Microscopic view of semen. **(A)** Normal, **(B)** tapering head, **(C)** cytoplasmic droplet, **(D)** pin head, **(E)** pyriform head with defective midpiece, **(F)** pyriform head with blood cell, **(G)** small oval head with deformed midpiece, **(H)** cytoplasmic droplet attached to a normal head, and **(I)** Double-tailed.

he approached us stating that, in contradiction to the above report, his partner was in the sixth month of pregnancy; hence, he wanted to test the paternity of the fetus. We had advised him to come after delivery.

Blood and Semen Samples

Two months after delivery, the couple along with the child came to our center for DNA profiling. Five- and 2.0-mL blood samples were collected from the couple and the child, respectively. The semen sample from the male partner was collected by masturbation after 5 days of sexual abstinence. Blood and semen samples of 5 fertile men were also collected as control samples. Semen samples were allowed to liquefy for 30 minutes at 37°C. All of the samples were collected with informed written consent.

DNA Extraction From Blood and Semen

DNA from the blood samples was extracted using a protocol described elsewhere (Thangaraj et al, 2002). The procedure used for isolating the DNA from the semen was as follows: the semen samples were centrifuged, and a pellet of each sample was washed with digest buffer (10 mM Tris HCl, pH 7.5, 10 mM NaCl, and 2% sodium dodecyl sulfate) and dissolved in 500 μL of digest buffer, which was followed by treating with proteinase K (0.3 mg/mL) at 56°C for 1 hour. The solution was centrifuged, and the pellet was washed with digest buffer 4–5 times and dissolved in deionized water to check for nonsperm cells using a Gram-modified Christmas Tree Stain (AmpF*l*STR Profiler Plus User's Manual). A smear of cells was made on a glass slide and stained with 1 drop of aqueous crystal violet (0.5% [wt/vol]) for

1 minute. The slide was rinsed with deionized water and treated with Gram iodine (0.33% iodine and 0.66% potassium iodide in deionized water) for 1 minute. The slide was then treated with nuclear fast red stain (2.5% of aluminum sulfate and 0.05% of Nuclear Fast Red) and then with picroindigocarmine stain (1.3% picric acid and 0.33% of indigo carmine). Finally, the slide was rinsed with 100% alcohol and observed under the microscope. After confirming the presence of only sperm cells (nonsperm cells will stain green with red nuclei, and sperm cells will stain red with green tails), the slide was treated with digest buffer with 40 mM of dithiothreitol and proteinase K and was incubated at 56°C for 2 hours. DNA was precipitated with ethanol after organic extraction with phenol-chloroform and chloroform-isoamyl alcohol.

Short Tandem Repeat Profiling and Genotyping

The father's, mother's, and child's DNA samples were amplified with 16 autosomal short tandem repeat (STR) loci (PowerPlex 16 system, Promega Corporation, Madison, Wis) per the manufacturer's instructions. Six Y-chromosome–specific STR loci (DYS19, DYS389I, DYS389II, DYS390, DYS391, and DYS393) were amplified in a multiplex reaction (Thangaraj et al, 1999). Amplified products were analyzed in an ABI 377 automated DNA sequencer (Perkin Elmer, Foster City, Calif) by mixing 1.0 μL of polymerase chain reaction (PCR) product, 1.5 μL of loading dye (formamide: blue dextrin; 5:1), and 0.5 μL of GS-ROX 500. After denaturation (94°C for 2 minutes), samples were electrophoresed in 5% acrylamide (Long Ranger, FMC Bioproducts, Rockland, Me) gel using an ABI 377 automated DNA sequencer (Perkin Elmer). Raw data were analyzed using GeneScan and Genotyper software (Perkin Elmer) to obtain the allele (repeat) size.

PCR Assay for Y-Chromosome Microdeletion

Sequence-tagged site (STS) markers in the azoospermic factor (*AZF*) regions (*AZFa:* sY84, sY86, sY740, sY741, sY742, and DFFRY; *AZFb:* sY127, SY134, sY138, and sY142; and *AZFc:* sY254, sY255, and sY158) on the long arm of the Y chromosome were screened by PCR. Primers were synthesized using an ABI 392 oligosynthesizer (Perkin Elmer). Each marker was amplified separately in a 0.2-mL thin-wall tube using 50.0 ng of DNA, 10 pmol of each primer, 200 μM of diethylnitrophenyl thiophosphates (dNTPs), and 1× PCR buffer containing 1.5 mM MgCl$_2$ and 2 U of AmpliTaqGold (Perkin Elmer). PCR conditions of STS markers were as follows: initial denaturation (95°C for 10 minutes) and subsequent denaturations (94°C for 45 seconds) were the same for all the samples. The extension was 65°C for 1 minute for sY127, sY134, and sY255, whereas for the remaining STS markers, the extension was 72°C for 2 minutes. Different annealing temperatures that were used for different STS markers were as follows: 60°C for 15 seconds for sY254; 60°C for 1 minute for sY740, sY741, *DFFRY*, and sY742; 55°C for 30 seconds for sY86; 53°C for 30 seconds for sY84 and sY158; and 58°C for 30 seconds for sY127, sY134, and sY255. After every PCR, amplified products were analyzed by 2% agarose gel electrophoresis.

PCR Assay and Sequencing of Mitochondrial Genes

mtDNA genes such as cytochrome oxidase I (COI), cytochrome oxidase II (COII), adenosine triphosphate synthase6 (ATPase6), ATP synthase8 (ATPase8), transfer ribonucleic acid (tRNA) serine I, tRNA lysine, and NADH dehydrogenase3 (ND3) from the sperm and blood cells of the man with OAT as well as 5 fertile men were amplified and sequenced. Primer sequences for the above genes were obtained elsewhere (Rieder et al, 1998). PCR was carried out in a 0.2-mL thin-wall tube using 50.0 ng of DNA, 10 pmol of each primer, 200 μM of dNTPs, 1× PCR buffer containing 1.5 mM MgCl₂, and 2 U of AmpliTaqGold (Perkin Elmer). The amplification conditions used for the above genes were as follows: 95°C for 10 minutes, 35 cycles at 95°C for 1 minute, 59°C for 1 minute, and 72°C for 2 minutes. Amplified products were quantified by 2% agarose gel electrophoresis. PCR products were purified by treating with Exonuclease I and Shrimp Alkaline Phosphatase (Amersham, Piscataway, NJ) at 37°C and 80°C for 15 minutes each. Sequencing of PCR products was carried out using 100.0 ng (2.0 μL) of PCR product and 4 pmol (1.0 μL) of primer (forward and reverse separately), 4.0 μL of BigDye Terminator ready reaction kit (Perkin Elmer), and 3.0 μL of double-distilled water to adjust the volume to 10.0 μL. Cycle sequencing was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer) employing the following conditions: 30 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Extended products were purified using the protocol described by Thangaraj et al (1999). Purified samples were dissolved in 10 μL of 50% Hi-Di formamide and analyzed in an ABI 3700 automated DNA analyzer (Perkin Elmer). The sequences obtained were aligned with a reference sequence (Anderson et al, 1981) using the AutoAssembler to look for mutations.

Results

DNA profiling of the man with OAT, his partner, and the child using a 16 loci STR kit revealed an inheritance of 50% of the alleles from each STR loci from the man with OAT by the child. Y-chromosome STR profiling of the man with OAT and the child revealed the inheritance of all the alleles from the man with OAT. This suggests that the man with OAT is the biological father of the child. Analyses of the azoospermic factor regions (*AZFa, AZFb,* and *AZFc*) on the Y chromosome using STS markers did not show deletions in any of the azoospermic factor regions. The mitochondrial genes COI, COII, ATPase6, ATPase8, tRNA serine I, tRNA lysine, and ND3 were amplified and sequenced from sperm and blood cells of the man with OAT. A comparison of the sequences of the above genes with a reference sequence revealed a total of 36 nucleotide substitutions in the sperm mtDNA but not in the DNA from the blood cells (Figure 2A). Of the 36 substitutions, 8 were in COI, 13 were in COII, 5 were in ATPase8, and 10 were in ATPase6 (Figure 3). Of 36 substitutions, 9 were missense mutations (3 each in COI and
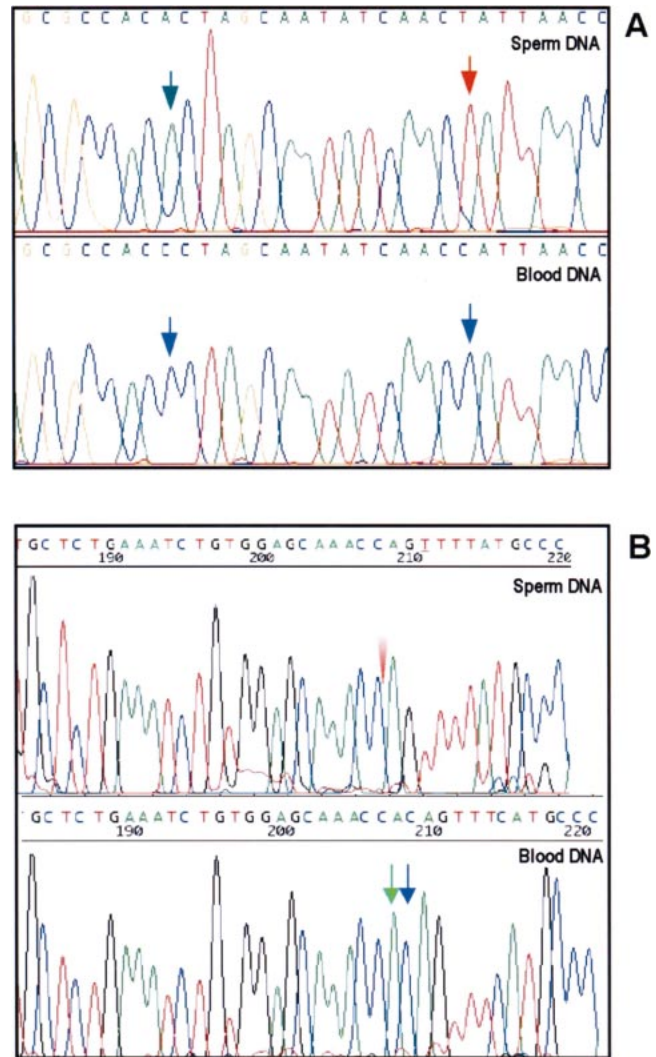

Figure 2. **(A)** Electropherogram of a partial sequence of the mitochondrial gene showing nucleotide substitutions in sperm. **(B)** Electropherogram of a partial sequence of the mitochondrial gene showing a 2-bp deletion in sperm (shown by arrow).

COII, 1 in ATPase8, and 2 in ATPase6), while the rest were silent mutations. Interestingly, there was a 2-bp deletion (at nucleotide positions 8195 and 8196) in the COII gene of sperm mtDNA (Figure 2B), which created a stop codon (AGA) at position 8216 that might have given rise to a truncated protein (Figure 3). A similar analysis of the blood cells from his 2-month-old son did not show any such mutation. An analysis of the DNA from the blood cells and sperm of 5 fertile men showed no variations between their 2 tissue types.

Discussion

Mitochondrial mutations are known to be associated with a large number of diseases (Wallace, 1999). Recent stud-

	Gene	Cytochrome oxidase I (CO I)								Cytochrome oxidase II (COII)															ATPase 8					ATPase 6									
MtDNA	Nucleotide position	6241	6260	6344	6382	6771	7266	7309	7315	7649	7704	7809	7867	7890	7911	8020	8064	8139	8151	8166	8195	8196	8202	8253	8391	8454	8460	8502	8544	8654	8675	8677	8700	8717	8859	8942	9059	9074	9167
	Reference	C	A	T	G	A	C	T	G	C	T	C	C	C	G	A	G	C	G	T	A	C	C	C	G	C	C	T	G	C	A	A	A	A	A	C	C	C	C
	Blood	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Sperm	T	C	C	A	C	G	A	A	T	C	T	T	T	A	G	A	T	A	C	-	-	T	T	A	T	T	C	A	T	T	C	G	G	G	T	A	T	T
Amino acid	Reference	*	*	F	*	H	*	I	*	T	*	L	*	*	*	I	*	*	*	*					*	*	*	*	A	*	*	K	*	*	T	*	*	*	*
	Sperm	*	*	L	*	P	*	M	*	I	*	F	*	*	*	V	*	*	*	*					*	*	*	*	T	*	*	Q	*	*	A	*	*	*	*

	H	S	F	M	P	I	V	L	E	L	I	P	L	K	I	F	E	M	G	P	V	F	T	L
	Q	F	Y	A	H	R	P	**Z**	?															

Figure 3. Sequence analysis of mitochondrial genes of the oligoasthenoteratozoospermic man showed 8 nucleotide substitutions in cytochrome oxidase I, 13 substitutions and 2-bp deletions in cytochrome oxidase II, 5 substitutions in adenosine triphosphate synthase 8 (ATPase8), and 10 substitutions in ATPase6 in sperm mitochondrial DNA (mtDNA). These substitutions created 9 missense mutations, while the rest were silent mutations (*). A 2-bp deletion in cytochrome oxidase II (8195 and 8196) introduced a stop codon (AGA) at 8216, which possibly resulted in a truncated protein.

ies have shown that mutations in the mitochondrial genome are associated with poor semen parameters, such as sperm maturation (Holyoake et al, 2001), sperm motility (Folgero et al, 1995; Kao et al, 1998), and other related disorders (St John et al, 2000). To our knowledge, we have observed a mutational difference in the gametic and somatic mitochondrial genome for the first time.

In the present study, we have observed 8, 13, 5, and 10 SNPs in COI, COII, ATPase8, and ATPase6, respectively, in the DNA from sperm, of which 9 were missense mutations (Figure 3). However, a similar study of the DNA from blood cells did not show any such abnormalities (Figure 2A). We have also observed a novel 2-bp deletion (nucleotides 8195 and 8196) in the COII gene (Figure 2B), which might have given rise to a truncated protein (Figure 3). Holyoake et al (2001) has found the 2 most common substitutions at 9055 and 11719 in men with a significantly higher frequency of reduced sperm motility. Since the genes of mtDNA are important for the motility of the sperm, the fact that, in the man with OAT, only 10% of the sperm are motile suggests that the above mutations in the mitochondrial genes, particularly in the 2-bp deletion, are responsible for the low motility. Our study also suggests that the man with OAT possessed tissue-specific mosaicism for mtDNA. The mosaicism was demonstrated by the fact that the somatic cells (blood cells) have a normal sequence, but the germ cells have mutated genes. Chong et al (1995) observed similar differences between 2 different tissues in the CAG (cytosine-adenine-guanine) repeat length of a patient with spinocerebellar ataxia type 1. The reason for having a higher probability of tissue-specific mosaicism in spermatozoa than in oogonia is because there is cell division in testicular tissue, which may result in an accumulation of mutations (Telenius et al, 1994; Chong et al, 1995).

The man with OAT was also found to have a varicocele at the left testis, which is often associated with male infertility (Redmon et al, 2002). However, this is the most treatable form of male infertility (Schlesinger et al, 1994). Several studies have demonstrated success in pregnancy following varicocelectomy (Schlesinger et al, 1994). However, pregnancy in the case presented in our study was successful without varicocelectomy. Also, a few studies demonstrate an association between varicocele and Y-chromosome microdeletion (Pryor et al, 1997). However, STS analysis in the present study did not show a Y-chromosome microdeletion.

Recent studies of semen profiles of fertile and infertile men have shown that the average sperm concentration in infertile men is less than 13.5×10^6/mL, that the number of motile sperm is less than 32%, and that less than 9% of the sperm have a normal morphology. The average semen concentration of the fertile men in the same study was more than 48.0×10^6/mL; motile sperm were more than 63%, and more than 12% of the sperm had a normal morphology (Guzick et al, 2001). Over the past 50 years, it has been found that the mean sperm concentration has decreased by 2.1%/y, the motile sperm by 0.6%/y, and the normal morphology by 0.5%/y (Auger et al, 1995). Accordingly, the World Health Organization changes the defined values for semen measurement in fertile and infertile men with every edition of their laboratory manual. However, recent studies suggest that current World Health Organization reference values for semen parameters should be reconsidered (Auger et al, 1995). Our study suggests that the average value defined may not be true for every case, since the man with OAT was fertile. It may be that semen profiles are different between populations. A systematic study of semen profiles of fertile men belonging to different ethnic populations would provide more precise values.

Acknowledgments

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