Association of large scale 4977-bp "common" deletions in sperm mitochondrial DNA with asthenozoospermia and oligoasthenoteratozoospermia

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ABSTRACT

OBJECTIVE: To determine the association of large-scale mitochondrial DNA (mtDNA) deletions with abnormal sperm or abnormal flagellar movement of human spermatozoa in asthenozoospermia and oligoasthenoteratozoospermia (OAT) subjects using percoll gradients fractionation and long-range polymerase chain reaction (PCR). **DESIGN:** We investigated sixty infertile men and thirty normal healthy fertile controls. Of sixty infertile men, 39 were asthenozoospermia and 21 were OAT. **MATERIALS AND METHODS:** Percoll gradients discontinuous technique was used for separation of spermatozoa on the basis of their motility. Long-range PCR was used for detection of "common" 4977-bp deletions, and primer shift technique was used for confirmation of deletions. **RESULTS:** Overall fourteen subjects (14/60; 23.3%) of which eight (8/39; 20.5%) asthenozoospermia and six (6/21; 28.6%) OAT had shown deletions of 4977-bp. Deletions were more common (23.3%) in 40% fraction than 60% (11.6%) and 80% (5%) fractions. Sequencing results had shown deleted region of mtDNA. **CONCLUSION:** Abnormal spermatozoa had more number of mtDNA deletions than normal sperm, and abnormal spermatozoa had lost genes for the oxidative phosphorylation. Our findings suggest that large-scale 4977-bp mtDNA deletions in the spermatozoa from the infertile subjects cause the asthenozoospermic and OAT pathophysiological conditions in infertile males.

KEY WORDS: Asthenozoospermia, deletion, infertility, mitochondrial DNA

INTRODUCTION

Male infertility is major important health problem. Global reports suggest that nearly 15% couples affected with infertility. Abnormal semen quality and quantity with low sperm motility (asthenozoospermia) or abnormal sperm morphology (teratozoospermia) is an important determinant of male infertility.[1] The deletions or mutations occurring in mitochondrial DNA (mtDNA) attract attention toward some of human disease either directly or associated with nuclear DNA mutations, especially affects the tissue or organ that have a high demand for respiratory energy.[2]

Spermatozoa needs energy for rapid motility after ejaculation and energy is produced by oxidative phosphorylation. Simultaneously, reactive oxygen species (ROS) and free radicals are formed as byproducts during oxidative phosphorylation. Sperm cells are susceptible to damage from oxidants because they lack endogenous antioxidants activity.[3] The molecular studies have revealed that mitochondrial genes ATPase 6, ATPase 8, COX 3, COX 2, CytB, ND3, ND4, ND5, and ND6 involved the formation of mature sperm and rapid flagellar movement after ejaculation.[4] The oxidative phosphorylation in mitochondria is essential for sperm

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motility. Any qualitative and quantitative abnormal changes in mtDNA may impair physiological functions of spermatozoa and diminish the motility of sperms.[5] High frequencies of nucleotide deletions and mutations in sperm mtDNA lead to aberrant changes in growth, development and differentiation of sperm and decreased flagellar movements.[6] More than 100 large scale deletions of mtDNA are associated with human diseases, and one of the deletions is 4977-bp deletion caused various pathological phenotypes in human. Such deletion accumulates during aging as well as by environmental factors.[7] The 4977-bp deletions have been known as the "common" deletions in mtDNA and used as an indicator for mtDNA damage. Kao *et al*. 1995 initially demonstrated that two 13 bp "direct repeats," present at nucleotide 8470–8482 and 13447–13459 bp position, which are prone to 4977-bp deletion in mtDNA.[8] Long chain PCR is a modified version of conventional PCR, and it amplifies a part or whole genome of mitochondria and allows to detection of multiple mtDNA deletions from a single sample. Number of studies investigated the relationship between multiple mtDNA deletions and sperm abnormalities, but they have produced conflicting data.[2,5,9] They also noted that deletions in mtDNA had significantly higher in asthenozoospermia and oligoasthenoteratozoospermia (OAT) when compared with normal males. It is quite common for deleted mtDNA molecules to coexist with wild-type mtDNA in mitochondria of the same cell known as "heteroplasmy." Such condition also revealed by the genetic as well as biochemical analysis of the target tissues.[10,11]

In this study, we analyzed presence of 4977-bp sperm mtDNA deletions in high- and low-density percoll fractions of spermatozoa from different patients, by long-range polymerase chain reaction (PCR) with primer shift techniques, to know the association between sperm mtDNA deletions with asthenozoospermia and OAT.

MATERIALS AND METHODS

Collection and preparation of human spermatozoa

The study included sixty infertile men and thirty normozoospermic sperm samples. Out of them, asthenozoospermic subject were 39 and 21 were OAT. Recruitment of patients and fertile men was made according to guidelines established by World Health Organization 2010.[12] Sample has been obtained by masturbation or coitus interruptus method after 3–4 days abstinence. All the semen samples were collected into sterile container; then the sample were allowed to liquefy at 37°C for 30 min. Semen analysis was performed according to WHO standard guidelines. Semen sample was collected after routine diagnosis of sperm profile including motility, characteristic, concentration, and morphology. In our laboratory, sample with <15% normal morphology spermatozoa was considered abnormal as teratozoospermia. Sample with <20% normal motility spermatozoa was also considered as asthenozoospermic. Informed consent form was obtained from each patient and study was approved by Mahatma Gandhi Institute of Medical Sciences Institutional Ethical Committee.

Fractions of sperm sample

Fractionation of human sperm was done by using three steps (80%, 60%, and 40%) discontinuous percoll gradients. Percoll gradients were prepared by mixing 100% percoll solution (Sigma, CA, USA) with Ham's F-10 medium (Sigma, CA, USA) at different volume ratios. Freshly ejaculated spermatozoa were gently washed and 500 µL sperm sample was layered on top of percoll gradient and incubated at 37°C for 1 h 30 min in 5% CO_2 incubator.[13] After incubation, the spermatozoa migrated into different percentage of percoll gradients according to their motilities [Figure 1]. Fractionated spermatozoa were separated from Percoll gradients in fresh vials. Fractionated sperm samples were used for mtDNA extraction.

Sperm DNA extraction

Spermatozoa were collected before DNA extraction, and sperm was incubated with 50 mM Tris-HCL buffer (pH 6.8) at 8°C for 20 min to avoid contamination of lymphocytes and epithelial cells by osmotic shock method. Those sperm cells resist to this treatment were then collected by centrifugation and subjected to DNA extraction according to the method described.[8] Sperm cells digested at 55°C for 2 h in 1.5 mL tubes; lysate was extracted with phenol-chloroform. The aqueous layers were separated and precipitated with ethanol and 3 M sodium acetate (pH 5.6) and DNA precipitated was washed with 75% ethanol and dried. The sperm mtDNA was finally dissolved in 10 mM Tris-EDTA buffer, pH 8.5.

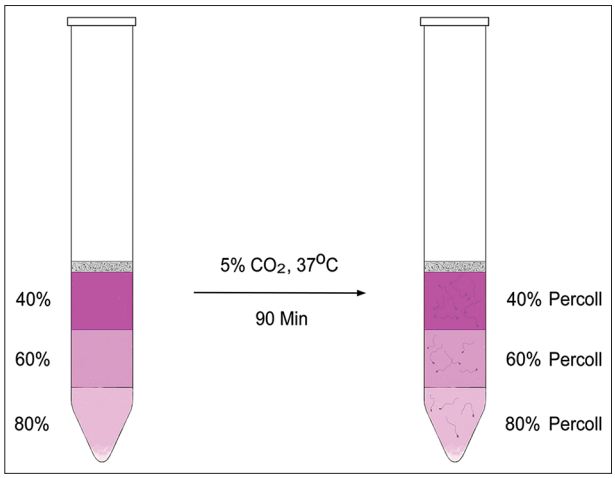

Figure 1: Fractionation of human sperm by self-migration into continuous percoll gradients layer according to their motilities of spermatozoa

Long-range polymerase chain reaction

The desired long segment of targeted sequences of mtDNA was amplified from 100–150 ng of sperm mtDNA in a 50 μL reaction mixture, containing 200 mM of each dNTP (Merck Biosciences, Bangalore), 1 mM of each light and heavy strand primers (IDT Technologies, USA), 1.5 U of herculase DNA polymerase, 50 mM KCL, 2 mM $Mgcl_2$, 25 mM Tris[hydromethyl] methyl-3-aminopropanesulphonic acid (Agilent Technologies, USA), 1 mM – mercaptoethanol, and 10 mM Tris-HCL (pH 8.5). PCR was carried out for 25 cycles in two steps in first 12 cycles using the denaturation at 94°C for 1 min, annealing at 55–58°C for 1 min, and primer extension at 72°C for 6 min and remaining 13 cycles denaturation and annealing condition were same but primer extension at 68°C for increasing 10 s to each reactions.[14] The long-range PCR products were then separated on a 1% ethidium bromide containing agarose gel for 1.5 h and observed on gel documentation system (Uvi-Teck, UK).

Polymerase chain reaction and sequencing

Primer-shift PCR method was used to determine the amplified DNA fragment was not the product of mis-annealing of primers from the DNA template. It prevents unwanted amplification in the detection of mtDNA deletions. We could obtain PCR products of 372, 572, 526 bp, by using primer pair MTL1-MTH1, MTL1-MTH2, MTL1-MTH3 [Table 1].[8,11] These reactions allowed to consider the presence of 4977-bp mtDNA deletions in spermatozoa with poor motility. By shifting of the primer pair, we could assure that the amplified PCR products were not due to mis-annealing of the primers to the DNA template but to the presence of large-scale deletions in sperm mtDNA. PCR products showed 4977-bp deletions in primer shift were used only for sequencing.

RESULTS

We have analyzed sperm mtDNA from sixty samples of infertile men with asthenozoospermia (39) and OAT (21) and thirty control semen samples from fertile males for deletion mapping of mtDNA. Human spermatozoa with a differing degree of motility were separated by 40%, 60%, and 80% discontinuous percoll gradient. Percoll fractions contain spermatozoa were fractionated after incubation on the basis of their sperm motility. The spermatozoa obtained from the 40% fractions of low-density percoll gradients exhibited poor motility. Thirty samples from control normozoospermic males were analyzed for mtDNA deletions from all three fractions of percoll. Results had shown no significant number of 4977-bp common deletions in mtDNA of fertile males except two males.

Using the long-range PCR techniques and the primer sets MTL1-MTH1, we first screened for the deletions of large scale 4977-bp deletions of mtDNA in spermatozoa from sixty infertile males. Out of sixty patients, 14 (23.3%) patients had showed deletions of 4977-bp (np 8470 bp to np 13447 bp). The common deletions of 4977-bp results revealed that 40%, 60%, and 80% percoll fractions of spermatozoa had 14 (23.3%), 7 (11.6%), and 3 (5%) deletions in mtDNA, respectively of infertile males using MTL1-MTH1 primer set [Table 2]. The 5349 bp band was the full-length PCR product from the wild-type mtDNA and the approximately 354–393 bp bands were generated from the 4977-bp-deleted mtDNA [Figure 2]. Using the primer-shift PCR technique, we obtained the PCR products with sizes in accordance with the shift in the distance of different primer pairs. MTL1 primer was set with another two primer sets MTL1-MTH2 and MTL1-MTH3 for primer shift and we obtained the PCR products of 572bp and 526 bp respectively from the 4977-bp deleted mtDNA [Table 3]. Heteroplasmic mtDNA is a combination of wild-type and deleted mtDNA (mixed populations of normal and deleted DNA). In our results, all 14 semen samples (14/60) of asthenozoospermia and OAT showed heteroplasmic mtDNA, i.e. a combination of normal and deleted mtDNA [Figure 3].

Table 1: Sequences of primers used for primer shift technique to amplify mitochondrial DNA for detection of "common" 4977 bp deletion

Primers	Primers sequences	Nucleotide positions (bp)
MTL1	5'-AATTCCCCTAAAAATCTTTGAAAT-3'	8224-8248
MTH1	5'-AGTAATAGATAGGGCTCAGGC-3'	13572-13552
MTH2	5'-ACCTTCCGCCTACGCCTTAGC-3'	13772-13751
MTH3	5'-GCTTATCCGAAGGCCGACGGT-3'	13726-13705

Table 2: The occurrence of the 4977 bp deletions of mitochondrial DNA in the Percoll gradients fractionated spermatozoa from 14 males with asthenozoospermia and oligoasthenoteratozoospermia

Patients number	Age (years)	Semen type	4977 bp deletions			Percentage of motility
			40%	60%	80%	
P7	26	Asthenozoospermia	+	+	+	16
P9	30	OAT	+	–	–	-
P14	34	OAT	+	+	–	18
P19	27	Asthenozoospermia	+	+	–	15
P24	31	Asthenozoospermia	+	+	–	-
P27	27	OAT	+	–	–	11
P32	29	Asthenozoospermia	+	+	–	21
P37	29	Asthenozoospermia	+	+	+	-
P39	35	Asthenozoospermia	+	–	–	15
P42	31	Asthenozoospermia	+	–	–	-
P45	28	OAT	+	–	–	-
P49	39	OAT	+	–	–	20
P53	24	Asthenozoospermia	+	–	–	17
P59	27	OAT	+	+	+	19
	29.8±4.03		14	7	3	
			23.3	11.6	5	

OAT=Oligoasthenoteratozoospermia

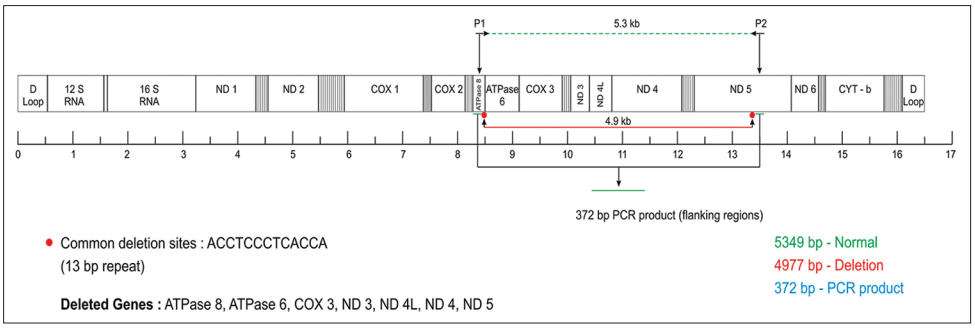

Figure 2: A scheme illustrating the strategy used for the confirmation of large scale 4977-bp deletion of mitochondrial DNA, 13 bp repeat common deletions sites and deleted gene on mitochondrial DNA

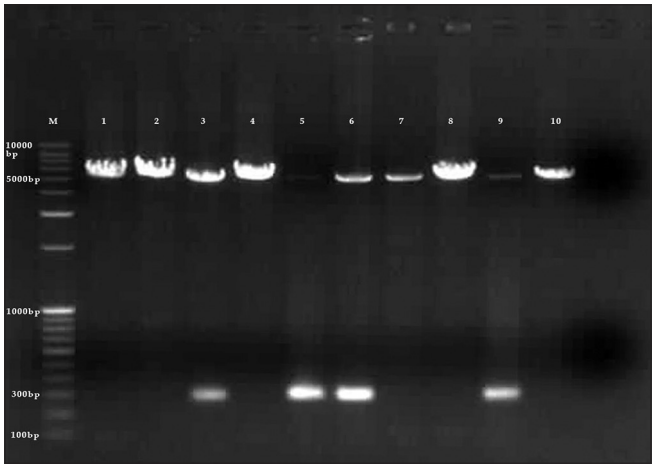

Figure 3: Detection of mitochondrial DNA molecules with the 4977-bp deletions by polymerase chain reaction method. Using primer pair MTL1-MTH1 we obtained the deleted and wild type mitochondrial DNA in lane 3, 5, 6, and 9. The polymerase chain reaction product size flanking the deleted part is 372 bp

Table 3: Nucleotide primer pairs used for the analysis of the 4977 bp deletions in the mitochondrial DNA of human sperm

Primer pair	Amplified region	PCR product length (normal mtDNA) (bp)	PCR product length (in deleted mtDNA) (bp)
MTL1-MTH1	8224-13572	5349	372
MTL1-MTH2	8224-13772	5549	572
MTL1-MTH3	8224-13726	5503	526

OAT=Oligoasthenoteratozoospermia, mtDNA=Mitochondrial DNA, PCR=Polymerase chain reaction

Sequencing the 372 bp PCR products of the regions flanking the breakpoint of 4977-bp deleted sperm mtDNA sequences have been done. The nucleotide sequences flanking the breakpoints of the deletions of 4977-bp of mtDNA in human spermatozoa are depicted in Figure 2. Upon close examination of the 4977-bp deletion, we found two "13 bp direct repeats" in the junction sites from np 8470 to 8482 and from np 13447 to 13460 bp on the mtDNA. We sequenced the 12 PCR products of 372 bp generated from 4977-bp deletions of mtDNA. Sequencing results indicated that maximum breakpoints for 4977-bp deletions were present in the flanking regions of deleted segments and around the direct repeat of 13 bp (ACCTCCCTCACCA) but rarely within the direct repeats. Thus, they generate different types of PCR products. The result confirms that two direct repeats of 13 bp cause or influence the mtDNA deletions in infertile men.

DISCUSSION

Sperm mitochondria play a crucial role in human fertilization, and defective sperm mtDNA is one of the causes of male infertility. Sperm dysfunction may be caused by various conditions such as the inability to carry out sperm-oocyte fusion, failure to sperm-zona recognition, and abnormalities of flagellar movement.[15] To known the etiology of the decreased motility and diminished fertility of the spermatozoa and to develop suitable therapeutic approaches, the molecular study of these abnormalities must be explained.

In this study, we have investigated 4977-bp large scale deletions of sperm mtDNA in sixty asthenozoospermic and OAT men. We used percoll gradient to fractionate sperm with different grade of motility. Long-range PCR was used for screening large scale deletion of mtDNA in spermatozoa with decline motility and deletions were confirmed by primer shift technique. We found large deletions of 4977-bp in 40% percoll fraction in 14 (23.3%) infertile men and in 60% percoll gradient had same deletion in seven patients. However, in 80% percoll gradients only three infertile males had shown 4977-bp deletions. Out of 14 mtDNA deleted infertile males, eight males had asthenozoospermia and six males had OAT [Table 4]. These deletions further confirmed by primer shift using another two primer sets combination. Nucleotide sequencing of PCR products flanking the deleted mtDNA revealed thirteen base-pair direct repeats present at the flanking sites of mtDNA, which are generally vulnerable sites for large-scale DNA deletions. The break-points of large scale deletions were found in or around the two direct 13 bp repeats.[16] Although, the 4977-bp deletion was commonly seen in 40% fraction of spermatozoa with poor motility;

Table 4: Proportion of the "common" deletion in 40%, 60%, 80% Percoll fractions of asthenozoospermia, oligoasthenoteratozoospermia and normal fertile males

Sample classification	Number of sample	Percoll fractions			Sample-wise percentage of subjects with deletions (in 40% Percoll)
		40%	60%	80%	
Asthe	39	8	5	2	8/39 (20.5)
OAT	21	6	2	1	6/21 (28.6)
Total	60	14 (23.3)	7 (11.6)	3 (5)	14/60 (23.3)
Normal	30	2	2	0	2/30 (6.6)

OAT=Oligoasthenoteratozoospermia

many studies demonstrated that the 4977-bp large scale deletions had present in somatic tissues along with mtDNA of infertile male spermatozoa.[17] The mechanism by which these large-scale mtDNA deletions occur in still unclear but several studies have been proposed large scale DNA deletion by slipped-mispairing, illegitimate recombination, oxidative stress created by free radicals, and topoisomerase or DNA recombinase-mediated DNA breaks.[16]

mtDNA deletions are frequently found in the tissues of different patients and also in mitochondrial myopathy.[18] The 4977-bp deletion of mtDNA remove multiple structural genes such as ATPase 6, ATPase 8, COXIII, ND3, ND4L, ND4, five *t*-RNA genes, and the deletions results in multiple respiratory chain deficiencies. Defective respiratory enzymes enhanced free radical production, resulting in more intense oxidative damage.[19,20] Previous studies established that mitochondrial dysfunction may be caused by mtDNA mutation. Actually, respiratory chain continuously produces ROS in mitochondria and such oxidative stress, exogenous, and endogenous free radicals damage mtDNA.[21] Kumar *et al*. showed an increased frequency of nucleotide changes in the mitochondrial gene, including ATPase6, ATPase8, ND2, ND3, ND4, ND5 in the semen of the OAT infertile men. They suggested that excess ROS and low antioxidant levels in the semen might cause mutation/deletions in mtDNA and impair the fertilizing capacity of spermatozoa.[22] Kao *et al*. had shown a positive correlation between the intracellular content of 8-hydroxy-2-deoxyguanosine, as a biomarker of oxidative DNA and the level of mtDNA with the 4977-bp deletions in spermatozoa.[8] They suggested ROS-mediated oxidative stress to DNA might be a causative agent to large-scale deletions of mtDNA of Spermatozoa. Another report suggested that deletions or mutations in the mtDNA of sperm/egg cells made by continuously change the physiological environment of germ cells during human ageing.[23] In human sperm cells spectacular segregational and morphological changes occur during spermatogenesis, in which mtDNA mutations may be introduced. During spermatogenesis, mtDNA deletions/mutations may occur and accumulate in the original spermatid and ultimately damage respiratory function and sperm motility.[24]

In the process of intracytoplasmic sperm injection, we avoid the natural selection process of sperm and allows insemination by spermatozoa with low energy production, which means that sperm cells with considerable numbers of mtDNA 4977-bp deletions molecule do not produce enough energy for their movement thus oocyte fertilization frequency is low.[25] Such observation suggested that "common" deletions in a sperm mtDNA does not affect embryonic development rather than it reduces fertilization efficiency of spermatozoa which caused infertility in males. These results correlate between mtDNA integrity and fertilization efficiency of spermatozoa.[26]

CONCLUSION

Direct repeats present in mtDNA may introduce the break sites at the time of replication or recombination events. Another strong possibility is that for sperm motility continuous energy is required and during the energy production free radical molecules are produced and increases oxidative stress. In the absence of antioxidant activity in mitochondria, the increased oxidative stress caused DNA damage. These genetic alterations could be pathological mutations or common mtDNA deletions only affect male fertility because mtDNA is maternally inherited. Indeed, our results indicated the association of the 4977-bp deletions of mtDNA with diminished fertility and motility of human spermatozoa. The deletions responsible for decrease sperm motility have been associated with spermatozoa dysfunction and male infertility. Our finding suggests that there is a relationship between large-scale deletions of 4977-bp mtDNA in spermatozoa with poor motility patients with asthenozoospermia and OAT.

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Conflicts of interest

There are no conflicts of interest.

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