Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa

Shu-Huei Kao1, Hsiang-Tai Chao2 and Yau-Huei Wei1,3,4

1Department of Biochemistry, School of Life Science, National Yang-Ming University, and 2Department of Obstetrics/Gynecology, Veterans General Hospital-Taipei, and 3Center for Cellular and Molecular Biology, National Yang-Ming University, Taipei, Taiwan 112, Republic of China
4To whom correspondence should be addressed

Sperm motility is one of the major determinants of male fertility and is required for successful fertilization. In a previous study, we demonstrated that the occurrence and accumulation of the 4977 bp deletion of mitochondrial DNA (mtDNA) is associated with diminished fertility and motility of human spermatozoa. The possible relationship between multiple deletions of mtDNA and the decline of fertility and motility in human spermatozoa was further explored in 36 subjects including subfertile and infertile males in this study. Using long-range polymerase chain reaction (PCR), we confirmed the 4977 bp deletion and identified two novel large-scale deletions of the mtDNA. The results showed that the ratio of the deleted mtDNA in the spermatozoa with poor motility and diminished fertility were significantly higher than those in the spermatozoa with good motility and fertility. In addition, we found that the frequencies of the three large-scale deletions in the spermatozoa from patients with primary infertility and oligaasthenozoospermia were higher than those of the fertile males. Our findings suggest that mtDNA deletions may play an important role in some pathophysiological conditions of human spermatozoa.

Key words: deletion/fertility/mitochondrial DNA/motility/spermatozoa

Introduction

Defective sperm function is now recognized as one of the most important causes of human infertility. Sperm motility can be affected by a wide range of conditions, including abnormalities of the flagellar movement (Tredway et al., 1975; Aitken et al., 1985; Hull et al., 1985). Massive amounts of energy are consumed by the fast swimming of spermatozoa during fertilization. The mitochondria in the sperm midpiece are the energy generators and have been described as the combustion engine of the spermatozoa. Defects in the internal structure and organization of mitochondria in the midpiece have been observed in some patients with severe asthenozoospermia or necrospermia (Rao and Martin, 1989).

Like most human cells, spermatozoa also contain multicopy mitochondrial DNA (mtDNA) in addition to the nuclear DNA. The mammalian spermatozoon contains ~72–80 mitochondria in the mitochondrial sheath of the midpiece (Alcivar et al., 1989). Sperm mitochondria contain typical mtDNA, which is synthesized during meiosis and spermiogenesis (Alcivar et al., 1989). Furthermore, the mitochondrial genome remains active and is expressed in the ejaculated spermatozoa (Manfredi et al., 1997). Human mtDNA is a 16 569 bp double-stranded circular DNA molecule that codes for two rRNAs, 22 tRNAs, and 13 polypeptides that are essential for mitochondrial respiration and oxidative phosphorylation. Current concepts of the mitochondrial genetics embody five main features: (i) maternal inheritance, (ii) replicative segregation, (iii) high mutation rate, (iv) threshold in phenotypic expression of the mutant mtDNA, and (v) accumulation of somatic mtDNA mutations in ageing and degenerative diseases. Human mtDNA is compact (intron-less) and lacks the protection of histones or DNA-binding proteins (Shoffner and Wallace, 1994). It replicates rapidly without efficient proof-reading and DNA repair mechanisms (Fukunaga and Yielding, 1979; Yakes and van Houten, 1997), and thus has a mutation rate 10–100 times higher than that of nuclear DNA.

In the past few years, more than 24 mutations of mtDNA have been identified and proved to be associated with human diseases (Wallace, 1994; Sherratt et al., 1997). Most of these mtDNA mutations are causally related to distinct neuromuscular and neurodegenerative diseases. Since sperm movement requires a large amount of ATP to propel the flagellar apparatus, a defect in mitochondrial respiratory function will cause a decline in motility and fertility. Indeed, we have demonstrated the association of the 4977 bp deletion of mtDNA with diminished fertility and motility of human spermatozoa (Kao et al., 1995). In this study, we further analysed sperm mtDNA deletions by long-range polymerase chain reaction (PCR) techniques and found two novel types of 7345 and 7599 bp deletions in the mtDNA of spermatozoa with poor motility. The nucleotide sequences flanking the breakpoints of both deletions were determined and the ratios of the mutant mtDNAs in spermatozoa of different motility scores were estimated by a semi-quantitative method. The role that
multiple mtDNA deletions may play in the pathophysiology of spermatozoa is discussed.

Materials and methods

Preparation and characterization of human spermatozoa

Semen collection

We collected 36 semen samples from nine healthy donors who had normal semen characteristics and from 27 subfertile or infertile patients at the Veterans General Hospital, Taipei, Taiwan. The latter included six males with unexplained infertility who had married fertile females, 12 males with primary infertility, seven with secondary infertility, one with oligozoospermia, and one with asthenozoospermia. Among the 12 patients with primary infertility, one has been infertile for 8 years, and all the others have been infertile and have not used any means of contraception for >3 years since their marriage. The six patients with secondary infertility had been infertile for >2 years since the last pregnancy of their wives. All the semen samples were obtained by masturbation after 3–4 days of abstinence. After the semen sample was liquefied at room temperature, the spermatozoa were fractionated by self-migration into a discontinuous Percoll gradient (Kao et al., 1995). Leukospermia and viscous semen were excluded. The semen was examined for sperm motility characteristics by computer-assisted semen analysis (CASA) using an HTM-2000 motility analyzer (Hamilton Thorn Research Inc. Danvers, MA, USA) and phase-contrast microscopy before and after the spermatozoa were fractionated by the Percoll gradient.

Sperm preparation

Spermatozoa were separated from seminal plasma by centrifugation at 300 g for 10 min at 25°C. Spermatozoa were fractionated by self-migration into a discontinuous Percoll gradient according to the method described by Aitken and Clarkson (1988) with some modifications. The sperm pellet was resuspended in phosphate-buffered saline (PBS; Dulbecco Oxoid, UniPath Ltd, Hants, UK; pH 7.3) and the final sperm count was adjusted to ~2–4 × 10^9 spermatozoa/ml. An aliquot of the sperm suspension was layered on the top of a tube containing 4 ml of 50–80% Percoll gradient in Ham’s F10 medium, and was incubated in an atmosphere of 5% CO₂/95% air at 37°C for 90 min. After incubation, the spermatozoa in the different Percoll gradients were collected and washed with PBS before centrifugation at 300 g for 10 min. The sperm pellet was then suspended in Ham’s F10 medium at a concentration of 6–8 × 10^9 spermatozoa/ml for the CASA assessment of sperm motility characteristics (Kao et al., 1995).

Osmotic shock

In order to avoid contamination of spermatozoa with the other cells such as lymphocytes and epithelial cells, we removed the contaminant cells by an osmotic shock method (Miller et al., 1994) before extraction of DNA. The sperm sample was incubated with 50 mM Tris–HCl buffer (pH 6.8) at 8°C for 20 min to lyse the contaminated cells. Spermatozoa that were resistant to this treatment were then collected by centrifugation at 1000 g for 5 min. The sperm pellet was immediately subjected to DNA extraction.

Sperm DNA extraction

Total DNA of human spermatozoa was extracted according to the method of Kao et al. (1995). An aliquot of 3–5 × 10^9 spermatozoa was incubated at 56°C for 2 h in a lysis buffer containing 2% sodium dodecyl sulphate (SDS), 10 mM dithiothreitol, 100 µg/ml proteinase K, and 50 mM Tris–HCl (pH 8.3). After digestion, the lysate was extracted with phenol, followed by phenol/chloroform (1:1, v/v), and chloroform. The aqueous layers were pooled and DNA was precipitated with isopropanol (1:1, v/v) and one-tenth volume of 3 M sodium acetate (pH 5.6), and incubated at ~30°C overnight. After washing with 75% ethanol (v/v), the pellet was dried by speed vacuum, and the DNA was finally dissolved in 10 mM Tris–HCl, pH 8.3.

Determination of the incidence and proportion of deleted mtDNA in human spermatozoa

Synthesis of oligonucleotide primers

Oligonucleotide primers used for amplifying target sequences of mtDNA were chemically synthesized by Bio-Synthesis Inc. (Lewisville, TX, USA). The nucleotide sequences and sizes of the PCR products obtained from nine primer pairs are summarized in Table 1.

Polymerase chain reaction

The desired target sequence of mtDNA was amplified from 15–20 ng of DNA in a 50 µl reaction mixture containing 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of Taq DNA polymerase (Perkin-Elmer Cetus, Roche Molecular System Inc., Branchburg, NJ, USA), 50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris–HCl, pH 8.3. PCR was carried out for 35 cycles in a DNA thermal cycler (Perkin-Elmer/Cetus) using the thermal profile of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and primer extension at 72°C for 40 s.

Long-range PCR

A desired large segment (>8 kb) of mtDNA was amplified from 100 ng of sperm DNA in a 50 µl reaction mixture containing 200 µM of each dNTP, 1 µM each of L3 and H6 primers (Figure 1 and Table 1), 1 IU of Ex-Taq DNA polymerase (Takara Shuzo Co, Shiga, Japan), 50 mM KCl, 2 mM MgCl₂, 25 mM Tris(hydroxymethyl)methyl-3-aminopropanesulphonic acid (TAPS), 1 mM β-mercaptoethanol, and 10 mM Tris–HCl, pH 8.3. PCR was carried out for 35 cycles using the thermal profile of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 8 min. One more cycle of 20 s extension at 72°C was added for each of the last 15 cycles. The long-range PCR products were then separated on a 1.5% agarose/neutral gel (Diversified Biotech, Newton Center, MA, USA) (5:1, wt/wt) at 150 V for 1.5 h and stained with 1 µg/ml ethidium bromide at 25°C for 10 min.

Primer-shift PCR

In order to avoid artefacts in the detection of mtDNA deletions, a primer-shift PCR method was employed to ascertain that the amplified DNA fragment was not due to misannealing of the primers to the DNA template (Lee et al., 1994). By using primer pairs L5–H4, L6–H6, and L6–H5 (Table 1), we could obtain PCR products of 555, 354, and 295 bp respectively; and with primers L2–H3, L3–H5, and L4–H5, we obtained 756, 702, and 406 bp PCR products respectively (Figure 2). This allowed us to confirm the presence of the 7345 and 7599 bp-deleted mtDNAs respectively were cloned by cohesive ligation to a PCR™ II vector (Invitrogen Co, NV Leek, The Netherlands). A total of 52 positive clones from the 7345 bp-deleted mtDNA and 65 positive clones from the 7599 bp-deleted mtDNA were collected after cloning. The plasmid DNA was prepared from each of the positive
Table I. Oligonucleotide primers used for the analysis of the 4977, 7345 and 7599 bp deletions in the mitochondrial DNA (mtDNA) of human spermatozoa

<table>
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<tr>
<th>Primer pair</th>
<th>Amplified position (nucleotide position)</th>
<th>Length of PCR product amplified from normal mtDNA (bp)</th>
<th>Length of PCR product amplified from deleted mtDNA (bp)</th>
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<tr>
<td>L1–H1₂</td>
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<td>756</td>
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<td>8150–16450</td>
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<td>702</td>
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<td>L4–H3₅</td>
<td>8251–16255</td>
<td>8005</td>
<td>406</td>
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<td>7900</td>
<td>555</td>
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<td>L₃–H₆</td>
<td>8150–16509</td>
<td>8360</td>
<td>3383₂, 1015₅, 761₄</td>
</tr>
</tbody>
</table>

₂The primer sets used for the determination of the total mtDNA.
₃The primer sets used for the determination of the 4977 bp-deleted mtDNA.
₄The primer sets used for the determination of the 7345 bp-deleted mtDNA.
₅The primer sets used for the determination of the 7599 bp-deleted mtDNA.
₆The primer sets used for long-range polymerase chain reaction (PCR).

L1 (3304–3323) 5'-AACATACCACTGGCACAACCT-3'
L2 (7901–7920) 5'-TGAGCCCTAGGATACCCG-3'
L3 (8150–8166) 5'-CCCGGGGTATACGGTGCTA-3'
L4 (8251–8270) 5'-GCCCGATTTACCTATACGG-3'
L5 (8531–8530) 5'-ACGGAAAATCGTTCGTCC-3'
L6 (8811–8830) 5'-CACCCAACTATCTA-3'
H1 (3717–3698) 5'-GGCCTACTGTCGGCAGTGCG-3'
H2 (3836–3817) 5'-GGCAGGAGTAACCATAGG-3'
H3 (16255–16236) 5'-CTTTGGAGATTCGCTTCGG-3'
H4 (16430–16411) 5'-TGGCGGATATGTTACGTAC-3'
H5 (16450–16431) 5'-CGAGAGGTAGCTGACTTTC-3'
H6 (16509–16490) 5'-AGGACCAGATGTGCAGTAC-3'

Figure 1. A scheme illustrating the strategy used to determine multiple mtDNA deletions of human spermatozoa using the long-range polymerase chain reaction (PCR) technique. Using the primer pair (L₃–H₆, 8150–16509), four types of PCR products were generated. The 8360 bp fragment was the full-length PCR product from the wild-type mtDNA, the 3383 bp fragment was produced from the 4977 bp-deleted mtDNA, the 1015 bp fragment was generated from the 7345 bp-deleted mtDNA, and the 761 bp fragment was amplified from the 7599 bp-deleted mtDNA, respectively.
Figure 2. A scheme illustrating the strategy used for the confirmation of large-scale deletions of mtDNA by the primer-shift polymerase chain reaction (PCR) method. By using the primer pairs L6–H5, L6–H6, and L5–H4, the PCR products of 295, 354, and 555 bp could be amplified from the 7345 bp-deleted mtDNA. Similarly, the PCR products of 406, 702, and 756 bp could be generated from the 7599 bp-deleted mtDNA using primer sets of L4–H3, L3–H5, and L2–H3, respectively. The change in sizes of the PCR products should be consistent with the shift of the length between primer sequences if the deletions are authentic.

separated on a 6% polyacrylamide gel containing 8 M urea, and the gel was dried and subjected to autoradiography.

**Construction of the calibration curve of the semi-quantitative PCR method**

We constructed a calibration curve in order to validate the semi-quantitative PCR method developed in this laboratory (Lee et al., 1994). We mixed different concentrations of the two plasmid DNAs (PCR™ II vector) containing similar sizes of inserted DNA sequences flanking the deleted mtDNA and wild-type mtDNA respectively. The plasmid DNA used for the quantification of the 7345 bp-deleted mtDNA quantification was constructed to encompass the sequence flanking the breakpoints from nucleotide position (np) 8531 to np 16 430 (555 bp), and the plasmid DNA for the quantification of total mtDNA encompassed the sequence from np 3304 to np 3836 (533 bp). The molar ratio between the plasmid DNA containing the deleted mtDNA and that containing the wild-type mtDNA fragment was set at 1:10^6, 1:10^5, 1:10^4, 1:10^3, 1:10^2, 1:10, and 1:1 respectively. The L1–H2 primer pair was used for quantification of total mtDNA and the L5–H4 for the 7345 bp-deleted mtDNA. Similarly, the plasmid DNA for the quantification of the 7599 bp-deleted mtDNA was constructed to encompass the sequence containing the breakpoint from np 8251 to np 16 255 (406 bp), and the plasmid DNA for the quantification of total mtDNA included the sequence from np 3304 to np 3717 (414 bp). The L1–H1 primer set was used for the quantification of total mtDNA and the L4–H3 for the 7345 bp-deleted mtDNA. Similarly, the plasmid DNA for the quantification of the 7599 bp-deleted mtDNA was constructed to encompass the sequence containing the breakpoint from np 8251 to np 16 255 (406 bp), and the plasmid DNA for the quantification of total mtDNA included the sequence from np 3304 to np 3717 (414 bp). The L1–H1 primer set was used for the quantification of total mtDNA and the L4–H3 primers for the 7599 bp-deleted mtDNA. The differentially mixed plasmid DNAs were serially diluted two-fold with 10 mM Tris–HCl (pH 8.5) and then subjected to PCR. Amplified DNA fragments were separated on 2% agarose gels at 150 V for 1.5 h and stained with 1 µg/ml ethidium bromide at 25°C for 10 min. The proportion of either 7345- or 7599 bp-deleted mtDNAs was determined by the ratio between the highest dilution fold that allowed the PCR product from the deleted mtDNA to be visible in the gel and that allowed the PCR product of the wild-type mtDNA to be clearly visualized (Kao et al., 1995).

**Statistical analysis**

Linear regression analysis was used to determine the statistical significance of the relationship between sperm motility and the proportion of mtDNA with the different deletions.

**Results**

**Identification of the 7345 and 7599 bp deletions of mtDNAs in human spermatozoa**

Human spermatozoa with differing degrees of motility were separated by a 50–80% discontinuous Percoll gradient. After self-migration of spermatozoa into the Percoll gradient, we obtained five fractions of spermatozoa from 36 semen samples according to their motility as measured by the CASA system. The spermatozoa obtained from the fraction of low density Percoll gradient exhibited poor motility (data not shown).

Using the long-range PCR techniques and the primer sets L3–H6, we first screened for the existence of large-scale deletions of mtDNA in human spermatozoa from 36 males with diminished fertility. The results revealed four major bands (with lengths of ~8360, 3383, 1015, and 761 bp, respectively) on the gel after ethidium bromide staining (Figure 3). The 8360 bp band was the full-length PCR product from the wild-type mtDNA, the 3383 bp band was generated from the 4977 bp-deleted mtDNA, the 1015 bp band was obtained from the 7345 bp-deleted mtDNA, and the 761 bp band was amplified from the 7599 bp-deleted mtDNA. Spermatozoa in lanes 1–5
Multiple mtDNA deletions in human spermatozoa

Figure 3. Demonstration of large-scale deletions of mtDNA in infertile and subfertile male patients by long-range polymerase chain reaction (PCR). Using the primer sets L3–H6 and long-range PCR, we detected large-scale deletions of mtDNA in human sperm with poor motility. The 8360 bp band was amplified from the wild-type mtDNA, the 3383 bp band was amplified from the 4977 bp-deleted mtDNA, the 1015 bp band was generated from the 7345 bp-deleted mtDNA, and the 761 bp band was from the 7599 bp-deleted mtDNA. Spermatozoa in lanes 1–5 had the motility scores of 5.0, 10.0, 22.0, 0.5, 25.0% respectively. Lane 6 is the blank, in which the sperm DNA was omitted from the reaction mixture. Lane M is the DNA size marker (a 200 ng mixture containing HindIII-restricted Lambda phage DNA and HaeIII-restricted φX174 phage DNA).

Figure 4. Detection of mtDNA molecules with the 7345 and 7599 bp deletions by the primer-shift polymerase chain reaction (PCR) method. By using the primer pairs L6–H5 (lane 1), L6–H6 (lane 2), and L5–H4 (lane 3), we obtained the PCR products of 295, 354, and 555 bp respectively, from 7345 bp-deleted mtDNA (panel A). Similarly, we obtained the PCR products of 406, 702, and 756 bp from the 7599 bp-deleted mtDNA using primer sets of L4–H3 (lane 1), L3–H5 (lane 2), and L2–H3 (lane 3) respectively (panel B).

Sequences flanking the breakpoints
The nucleotide sequences flanking the breakpoints of the novel deletions of 7345 and 7599 bp of mtDNA in human spermatozoa are depicted in Figures 5 and 6 respectively. Upon closer examination of the 7345 bp deletion, we found a dinucleotide (5’-GG-3’) in the junction sites from np 9009 to 9008 or from np 16 354 to 16 353 on the heavy strand of mtDNA. In the 7599 bp deletion, there was a seven nucleotide direct repeat (5’-GTAGTTG-3’) located in the junction sites from np 8643 to 8637 or from np 16 242 to 16 236 on the heavy strand of mtDNA.

Construction of the calibration curve of the semi-quantitative PCR method
We validated the semi-quantitative PCR method used in this study by constructing a calibration curve (Figure 7). There was no significant difference between the experimental line and theoretical correlation line. This suggests that the proportion of the mtDNA with each of the two deletions was determined reliably by the semi-quantitative PCR method.
Occurrence of the 7345 and 7599 bp deletions of mtDNA in human spermatozoa

We also examined the frequency of occurrence of the 7345- and 7599 bp-deleted mtDNAs in the spermatozoa with different motility and fertility. Higher incidence was found in the spermatozoa with poor motility and diminished fertility in comparison with the spermatozoa with good motility and fertility (Table II). The frequencies of occurrence of the 7345 bp-deleted mtDNA were 61.1, 55.6, 36.1, 33.3, and 16.7% for the spermatozoa in the residual fractions (<50%), and the 50, 60, 70, and 80% fractions of the Percoll gradient. The average incidences of the 7599 bp-deleted mtDNA were 61.1, 47.2, 30.5, 13.9, and 5.6% for spermatozoa in the residual fraction (<50%), 50, 60, 70 and 80% of the Percoll gradient respectively. The frequencies of occurrence of the 7345 bp-deleted mtDNA were 11.1% (1/9, the number of subjects with the indicated deletion/total number of subjects in each tested group), 83.3% (15/18), 57.1% (4/7), 100% (1/1), and 100% (1/1) for the study subjects with normal fertility and males with primary infertility, secondary infertility, asthenozoospermia, and oligozoospermia respectively. The frequencies of occurrence of the 7599 bp-deleted mtDNA were 11.1% (1/9), 83.3% (15/18), 42.8% (3/7), 100% (1/1), and 100% (1/1) for the subjects with normal fertility and males with primary infertility, secondary infertility, asthenozoospermia, and oligozoospermia respectively. These data suggested a higher incidence of mtDNA deletions in the male with primary infertility and oligozoospermia. It is noteworthy that no significant correlation was found between the frequency of occurrence of the large-scale mtDNA deletions and the age of the study subjects.

Proportions of the 7345- and 7599 bp-deleted mtDNAs in human spermatozoa

The average proportions of the 7345 bp-deleted mtDNA were 0.003 ± 0.002%, 0.028 ± 0.003%, 0.050 ± 0.002%, 0.150 ± 0.005% and 0.22 ± 0.005% for the spermatozoa in the 80, 70, 60, 50%, and residual fractions (<50%) of the Percoll gradient (Figure 8). Linear regression analysis revealed that the amount of mtDNA with the 7345 bp deletion was negatively associated with sperm motility ($P < 0.01$). The average proportion of the 7599 bp-deleted mtDNA were 0.005 ± 0.001%, 0.010 ± 0.008%, 0.042 ± 0.015%, 0.070 ± 0.023% and 0.230 ± 0.055% for spermatozoa in the 80, 70, 60, 50%, and residual fractions (<50%) of the Percoll gradient (Figure 8). This finding indicates that the proportion of the mtDNA with the 7599 bp deletion was negatively associated with sperm motility ($P < 0.05$).

Discussion

In recent years, male infertility has increased in the industrialized countries due to a decline in sperm counts and a rise
Multiple mtDNA deletions in human spermatozoa

Figure 7. Construction of the calibration curve for semi-quantitative determination of the deleted mtDNA. Plasmid DNAs containing the inserts representing the wild-type mtDNA and either 7345- or 7599-deleted mtDNA were mixed at different ratios. The DNA mixtures were then subjected to the semi-quantitative polymerase chain reaction (PCR) analysis (see Materials and methods). Each data point on the solid lines represent the intensity of the PCR-amplified DNA band on the gel for the indicated amount of the plasmid DNA containing the insert representing the 7345 bp-deleted (panel A) or 7599 bp-deleted (panel B) mtDNA. Log scales were used for construction of the two curves. The open circles on the broken lines represent the theoretical values for the ratios between the 7345 bp-deleted (panel A) or 7599 bp-deleted mtDNA (panel B) mtDNA and the wild-type mtDNA.

in testicular and sperm pathologies. Defective sperm function has been identified as the largest defined cause of male infertility, accounting for ~27% of all the couples attending infertility clinics (Hull et al., 1985). Sperm dysfunction may be caused by a wide range of conditions, including abnormalities of flagellar movement (Mortimer et al., 1986; Aitken et al., 1989), failure in sperm–zona recognition (Overstreet, 1983), and inability to carry out sperm–oocyte fusion (Aitken et al., 1982). In order to understand the aetiology of the decreased motility and diminished fertility of the spermatozoa and to develop an appropriate therapeutic strategy, the molecular basis of these defects must be elucidated.

In this study, we have investigated large-scale deletions of sperm mtDNA in 36 subfertile and infertile males. We used the Percoll gradient to fractionate spermatozoa with different motility, and then screened for large-scale deletions of mtDNA in spermatozoa with poor motility by using long-range PCR techniques. In addition to the previously reported 4977 bp deletion, we found two novel deletions of the 7345 and 7599 bp of mtDNA in the spermatozoa with low motility scores (Figure 3). These three large-scale deletions were found to occur either alone or in different combinations. Among them, the 4977 bp deletion was the most frequently seen in spermatozoa with poor motility. While the 4977 bp deletion was also present in the somatic tissues, we could only detect the 7345- and 7599 bp-deleted mtDNA in the spermatozoa from infertile or subfertile males. The mechanisms by which these large-scale mtDNA deletions occur in mitochondria remain unclear. In the past decade, several mechanisms have been proposed, including slipped-mispairing (Shoffner et al., 1989), illegitimate recombination (Mita et al., 1990), oxidative reactions elicited by free radicals (Poulton et al., 1993), and DNA strand break effected by a topoisomerase or DNA recombinase (Lestienne et al., 1995). Analysis of the nucleotide sequences flanking the breakpoints of the 7599 bp deletion revealed a seven nucleotide direct repeat, which is generally found in the large-scale deletions of mtDNA (Johns et al., 1989; Poulton and Holt, 1994). The seven nucleotide repeat (5'-GTAGTTG-3') is located in the breakpoints at np 8643/8637 and np 16 242/16 236 on the heavy strand (Figure 6). A GG dinucleotide was noted in the breakpoints of the 7345 deletion at np 9009/9008 and np 16 354/16 353 on the heavy strand respectively (Figure 5). The breakpoints of both 7345 and 7599 bp deletions are located in the hot-regions that are prone to large-scale deletions. The nucleotide sequences within these regions were found to assume unusual structures, such as bent DNA and anti-bent DNA (Hou and Wei, 1996). These regions may be thus more vulnerable to attack by free radicals or serve as recognition motifs for certain recombination machinery involved in the large-scale deletions (Mita et al., 1990). Analysis of the human mtDNA revealed five GTAGTTG direct repeats, which include np 5617–5611, np 8643–8637, np 13296–13290, np 16079–16073, and np 16242–16236 on the heavy strand. Except for np 5617–5611, four GTAGTTG direct repeats are located in the hot-regions of mtDNA. However, the possible large-scale deletions with breakpoints flanking np 13296–13290 and np 16079–16073 were not found in this study. Thus, some other factor(s) may be involved in eliciting the large-scale deletions of mtDNA.

Large-scale deletions of mtDNA are frequently found in the affected tissues of patients with mitochondrial myopathy (Holt et al., 1989) or elderly subjects (Yen et al., 1991). In a previous study (Kao et al., 1995), we first demonstrated that the 4977 bp deletion of mtDNA also occurs in the spermatozoa of infertile and subfertile males. The 4977 bp deletion of mtDNA causes the removal or truncation of multiple structural genes (ATPase 6/8, COIII, ND3, ND4L, and ND4) and five tRNA genes. The 7345 and 7599 bp deletions cause a loss or
Table II. The occurrence of the 7345 and 7599 bp deletions of mtDNA in the Percoll gradient fractionated spermatozoa from males with different fertility

<table>
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<tr>
<th>Patient no.</th>
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<th>7599 bp-deleted mtDNA Percoll gradient (%)</th>
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The spermatozoa were separated by self migration into Percoll gradients of 50%, 60%, 70%, and 80%, respectively. The 7345 bp- and 7599 bp-deleted mtDNA were then searched for in the spermatozoa from different Percoll fractions. + = presence of the indicated deletion; - = denotes its absence in the mtDNA of spermatozoa from each of the fractions.

N = normal fertile spermatozoa; 1* = primary infertility for >8 years; 1 = primary infertility for 3 years; 2 = secondary infertility; A = asthenozoospermia; O = oligozoospermia.

Truncation of the structural genes of ATPase 6/8, COIII, ND3, ND4L, ND4, ND5, ND6, Cyb, and eight tRNA genes. These mtDNA deletions may result in multiple respiratory chain deficiencies (Lee and Wei, 1997). Defective respiratory enzymes containing protein subunits encoded by the deleted mtDNA may further enhance free radical production, resulting in more profound oxidative damage. Spermatozoa are especially liable to oxidative damage because their plasma membranes are rich in poly-unsaturated fatty acids. Abnormalities in the ultrastructure of sperm midpiece were found to increase in the spermatozoa treated with lipoperoxides (Rao and Martin, 1989). It has been established that mitochondrial dysfunction may be caused by mtDNA mutation and oxidative damage caused by endogenous and exogenous free radicals (Yakes and van Houten, 1997). As reactive oxygen species (ROS) are continuously generated by the respiratory chain, they may cause significant oxidative damage to mtDNA if not efficiently eliminated. Fraga et al. (1996) reported that smoking, which depletes antioxidants and increases oxidative stress, induced a significant increase of 8-hydroxy-2’-deoxyguanosine in sperm DNA. In the presence of mtDNA mutations (especially multiple deletions), which may be caused by ROS or free radicals generated during normal aerobic metabolism, the spermatozoa are deprived of ATP (due to the defective respiratory functions of mitochondria) and will then run into a state of ‘energy crisis’ through a ‘vicious cycle’ as recently proposed by Wei (1998). This ‘vicious cycle’ may have catastrophic consequences and is accelerated by electron leakage from defective mitochondria; as such, it may play an important role in the pathophysiology of some male infertility and subfertility.

On the other hand, Kitagawa et al. (1993) reported that the 4977 bp-deleted mtDNA is accumulated in post-menopausal ovaries. The 4977 bp mtDNA deletion was detected in the...
Multiple mtDNA deletions in human spermatozoa

In summary, we demonstrated in this and the previous studies that the incidences and proportions of the mtDNA with the 4977, 7345, and 7599 bp deletions are negatively associated with motility and fertility. On the basis of these findings, we suggest that multiple large-scale deletions of mtDNA are associated with the decline in fertility and motility of spermatozoa and may play an important role in the pathophysiology of spermatozoa in some subfertile or infertile males.

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