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ORIGINAL ARTICLE

Mitochondrial DNA haplogroup associated with sperm motility in the Han population

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In this study, we aimed to determine whether the main mitochondrial DNA (mtDNA) haplogroups of the Han people have an impact on spermatozoa motility. We recruited 312 men who were consecutively admitted to two affiliated hospitals of College of Medicine, Zhejiang University from May 2011 to April 2012 as part of fertility investigations. Semen and whole blood samples were collected from the men. We determined the mtDNA haplogroups by analysing the sequences of mtDNA hypervariable segment I and testing diagnostic polymorphisms in the mtDNA coding region with DNA probes. No significant differences were found in the clinical characteristics of the mtDNA haplogroup R and non-R ($P>0.05$). Our results suggest that mtDNA haplogroup R is a strong independent predictor of sperm motility in the Han population, conferring a 2.97-fold (95% confidence interval: 1.74–4.48, $P<0.001$) decreased chance of asthenozoospermia compared with those without haplogroup R.
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INTRODUCTION

Currently, approximately one of six couples are subfertile, with half of these cases resulting from male-factor infertility.[1] Furthermore, approximately 15% of male infertility cases may have a latent genetic basis.[2]

The mitochondrial genome encodes 13 oxidative phosphorylation (OXPHOS) subunits and is essential for the production of adenosine triphosphate,[3] which is vital for sperm motility.[4] Male subfertility may be partially due to genetic polymorphisms in the mitochondrial genome. The geographic origin of an indigenous population can significantly impact sequence polymorphisms within their mitochondrial DNA (mtDNA). These mtDNA variants create clusters of related mtDNA haplotypes known as haplogroups.[5] A considerable body of data suggests that mtDNA haplogroups have functional importance, being associated with respiratory-chain activity[6] and disease susceptibility, such as Alzheimer's disease,[7] Parkinson's disease[8] and severe sepsis.[9] Several studies have also investigated mtDNA and sperm function in humans. The most famous study of mtDNA haplotype variation on sperm motility was reported by Ruiz-Pesini *et al.*[6] The authors showed that haplotype H was underrepresented and haplotype T was overrepresented in men with asthenozoospermic ejaculates, and a subsequent study provided some support for these findings.[10] However, other studies failed to identify an association between mtDNA and either sperm motility[11,12] or cellular bioenergetic parameters.[13] It is worth noting that all follow-up studies have been conducted with the common European mitochondrial DNA haplogroups. The Han people constitute the largest ethnic group of China and of the world, constituting approximately 93% of the Chinese population and nearly 20% of all humankind. Phylogenetic analysis of Han mtDNA shows that most of the lineages can be allocated to specific subhaplogroups of Eurasian founder haplogroups M, N and R (which is itself a subhaplogroup of N shared between Europe and East Asia).[14] In the present study, we prospectively studied a cohort of men undergoing diagnostic semen analysis as part of fertility investigations to determine whether the main mtDNA haplogroups of the Han people affect spermatozoa motility.

MATERIALS AND METHODS

Subjects

A total of 312 participants were consecutively recruited from May 2011 to April 2012 on admission to the Department of Reproductive Endocrinology, Women's Hospital, School of Medicine, Zhejiang University and Andrological Clinic of the First Affiliated Hospital of the College of Medicine, Zhejiang University, as part of fertility investigations with their partners. Men were excluded from the study if they (i) had a recent history of receiving gonadotoxic therapy or occupational chemical exposure; (ii) were undergoing a vasectomy or vasectomy reversal procedures; (iii) were unable to provide an antegrade ejaculate on-site by self-masturbation; or (iiii) had a recent history of infection or hot bath. All of the men completed a questionnaire that recorded the details of their lifestyles, and semen samples and whole blood samples were subsequently collected.

This study was approved by the Institutional Ethics Committee of Zhejiang University, and all participants gave their informed consent prior to their inclusion in the study.

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Semen collection and analysis

All ejaculates were provided by masturbation after 2–7 days of sexual abstinence. The samples were assessed according to the WHO recommendations (1999). After liquefaction at 37 °C and within 1 h of ejaculation, the volume of the ejaculate was measured, and the number and percentage of motile spermatozoa were evaluated. In particular, sperm-motility analysis was performed at room temperature; a minimum of four microscopy fields were scanned systematically, and sperm motility was graded as follows: a (rapidly progressive), b (slowly progressive), c (non-progressive) or d (immotile).

Individuals with an overall impaired sperm motility of less than 50% or less than 25% of active motility were categorized as asthenozoospermic.[15] A moderate asthenozoospermic phenotype is defined by $\geqslant$25% and $<$50% progressive spermatozoa, while severe asthenozoospermic phenotype is determined as having $<$25% progressive spermatozoa.

DNA analysis and haplogroup determination

Genomic DNA was extracted from patient whole blood samples using the Whole DNA Extraction Kit (Sangon, Shanghai, China). All of the DNA samples were stored at −20 °C until examination.

After the clinical data were obtained, we determined mtDNA haplogroups by analysing sequences of the mtDNA hypervariable segment I and testing for diagnostic polymorphisms in the mtDNA coding region using DNA probes.[9] Eleven FAM-labelled oligonucleotide probes (Genecore, Shanghai, China) were designed to examine specific diagnostic polymorphisms, which were used to define haplogroups in the mtDNA coding region and listed in **Table 1**.[9] We performed PCR with fluorescence-labelled hybridisation probes using a Master Mix Kit (ABI, Foster City, CA, USA) on an Mx3005P real-time qPCR system (Stratagene, San Diego, CA, USA). The sequences of mtDNA from position 8196 to 8316 were amplified and sequenced to determine the presence of 9-bp deletion, the diagnostic marker for haplogroup B. The primers were forward-ACAGTTTCATGCCCATCGTC and reverse-ATGCTAAGTTAGCTTTACAG.

Finally, comprehensive analysis of the hypervariable segment I sequences and diagnostic polymorphisms in the mtDNA coding region were used to identify the haplogroups according to the updated East Asian mtDNA phylogenetic tree.[16]

Statistical analysis

A univariate comparison was performed to compare variables between the two groups by using the unpaired *t*-test for continuous variables and Fisher's exact test for categorical variables. For all comparisons, $P<0.05$ was considered to be statistically significant. Multivariate logistic regression analysis was applied to determine the independent contribution of the mtDNA haplogroup to the prediction of sperm motility. Odds ratios with 95% confidence intervals were used to estimate the association between independent and dependent variables.

RESULTS

The clinical characteristics of the investigations are summarized in **Table 2**. The mean age of the men in this investigation was 32.2±6.4 years. The mean seminal volume was 3.4±1.7 ml, and the mean seminal pH was 7.2±0.3. No significant differences were found between mtDNA haplogroup R and non-R ($P>0.05$). The mean sperm concentration was 46.3±28.5 million cells per ml. No significant differences were found in the sperm concentration, vital spermatozoa and abnormal spermatozoa between the mtDNA haplogroup R and non-R ($P>0.05$; **Table 2**).

The frequency of the main subhaplogroups of the Han population in the study cohort is shown in **Table 3**, which is consistent with previous reports of the distribution of the Han population.[14] Fisher's exact test indicated that the frequency of mtDNA haplogroup R was significantly lower ($P=0.023$; **Table 3**) in the asthenozoospermia group compared with that of the entire study cohort. A comparison of the clinical

Table 1 DNA primers and probes used to detect polymorphisms in the mtDNA coding region[a]

Haplogroups (DSNP)	*Primers*	*Probes*
F	F: CCTGGAGCCTCCGTAGACCTA	FAM-CCATCAATTTCATCACAAC
(6392)	R: GGGCGTTTGGTATTGGGTTA	
N9a	F: GATGAATAATAGCAGTTCTACCGTACAAC	FAM-CAGCACCACGACCCT
(5147)	R: AGCTTGTTTCAGGTGCGAGATAG	
Y	F: AACCCACACTCAACAGAAACAAAG	FAM-CTACAACCACGACCAAT
(14693)	R: GAGGTCGATGAATGAGTGGTTAATT	
A	F: GCTCACATCACCCCATAAACAAATA	FAM-TGGTCCTAGCCTTTC
(663)	R: GTGGTGATTTAGAGGGTGAACTCAC	
D	F: ACTACTCAACTTAAACTCCAGCACCAC	FAM-CCTGAAACAAGATAACATGAC
(5178A)	R: TGGGCAAAAAGCCGGTTAG	
G	F: CCTTTCACTTCTGAGTCCCAGA	FAM-CAAGGCACCCCTCT
(4833)	R: GGGGCTAGTTTTTGTCATGTGAG	
M7	F: CCCTTCACCATTTCCGACG	FAM-ACGGACTTCACGTCAT
(9824)	R: TGAAGCAGATAGTGAGGAAAGTTGA	
C	F: CATCAAAAAAATCGTAGCCTTCTC	FAM-CAAGTCAACTAGGACTC
(13263)	R: ACAGATGTGCAGGAATGCTAGGT	
Z	F: CGCAGACCTCCTCATTCTAACC	FAM-AGCTACCCTTTTACCATCA
(15784)	R: TTAGGATTGTTGTGAAGTATAGTACGGAT	
M8a	F: AACCGCATCCATAATCCTTCTAAT	FAM-TCCGGACAATGAA
(4715)	R:TTAATGATGAGTATTGATTGGTAGTATTGG	
M9	F: GCCCATACCCCGAAAATGT	FAM-CCCAACCCGTCATCTA
(4491)	R: GTGATGAGTGTGCCTGCAAAGA	

Abbreviations: DSNP, diagnostic single nucleotide polymorphism; F, forward; R, reverse.
[a] The position of the base alteration in degenerate oligonucleotide probes is underlined.

Table 2 Demographic and clinical characteristics of the men

	Total	*R cohort*	*Non-R cohort*	P[a]
Age (year)	32.2±6.4	31.8±6.1	32.5±6.5	0.597
Smoke (%)	69	19 (16.1)	50 (25.8)	0.050
Drink (%)	9	2 (1.7)	7 (3.6)	0.491
Seminal volume (ml)	3.4±1.7	3.4±1.7	3.4±1.6	0.815
Seminal pH	7.2±0.3	7.2±0.3	7.2±0.3	0.438
Cellular parameters				
Sperm concentration (million cells per ml)	46.3±28.5	47.1±29.1	45.9±28.4	0.798
Vital spermatozoa (%)	45.8±14.5	46.4±14.9	45.6±14.5	0.728
Abnormal spermatozoa (%)	50.5±9.5	49.6±9.8	50.8±9.8	0.432
Sperm motility				
AP (%)	178	49 (41.5)	129 (66.5)	<0.001
MAP (%)	79	21 (17.8)	58 (29.9)	0.022
SAP (%)	99	28 (23.7)	71 (36.6)	0.024

Abbreviations: AP, asthenozoospermic phenotype; MAP, moderate asthenozoospermic phenotype; SAP, severe asthenozoospermic phenotype.
[a] R cohort *vs.* non-R cohort.

characteristics between the R and non-R haplogroup cohorts showed no significant differences ($P>0.05$; **Table 2**). To avoid the potential influence of confounding risk factors, we subsequently performed logistic regression analysis using asthenozoospermia as the dependent variable and the following independent variables: age, seminal volume, vital spermatozoa, abnormal spermatozoa, and the presence or absence of mtDNA haplogroup R. mtDNA haplogroup R was a strong independent predictor of asthenozoospermia, conferring a 2.97-fold (95% confidence interval: 1.74–4.48, $P<0.001$) decreased chance of asthenozoospermia.

DISCUSSION

Asthenozoospermia is considered to be a major cause of untreatable male subfertility.[17] A growing body of evidence indicates that sperm motility strongly relies on adenosine triphosphate, which is provided by the mitochondrial OXPHOS system.[18] Four of the five enzymatic complexes constituting the OXPHOS system are partly encoded by mtDNA. Thus, mutations in mtDNA genes that impair the expression of one or more proteins encoded in the mtDNA can cause reduced sperm motility and, therefore, asthenozoospermia, by affecting the performance of mitochondrial adenosine triphosphate production. Furthermore, mtDNA is maternally inherited. Any mutations that impact male but not female fitness cannot be effectively removed from the population by natural selection.[19] Therefore, mutations that disrupt sperm motility but do not cause any remarkable effects in females can persist in populations and even reach appreciable frequencies because of genetic drift. The major conclusion drawn from our investigation provides direct evidence to support the opinion above. Our investigation found that mtDNA haplogroup R was a strong independent predictor for sperm motility, conferring a 2.97-fold decreased chance of asthenozoospermia compared with those negative for the R mtDNA haplogroup.

Table 3 Frequencies of the main subhaplogroups of the Han population of the study cohort

Haplogroup	*Total (%)*	*AP cohort (%)*	P[a]
B	56 (17.9)	25 (14.0)	0.312
F	58 (18.6)	21 (11.8)	0.055
R	118 (37.8)	49 (27.5)	0.023
N9	12 (3.8)	9 (5.1)	0.643
A	25 (8.0)	19 (10.7)	0.328
N	155 (49.7)	77 (43.3)	0.188
D	64 (20.5)	48 (27.0)	0.117
G	4 (1.3)	3 (1.7)	0.708
M7	36 (11.5)	27 (15.2)	0.263
M8	40 (12.8)	14 (7.9)	0.100
M9	4 (1.3)	2 (1.1)	>0.999
M	148 (47.4)	94 (52.8)	0.261
Other	9 (2.9)	7 (3.9)	0.600
Total	312	178	

Abbreviation: AP, asthenozoospermic phenotype.
[a] Total *vs.* AP cohort.

In the Han population, mtDNA haplogroup R, which is itself a subhaplogroup of N shared between Europe and Asia, can be subdivided into two common subgroups, F and B.[14] According to the updated mtDNA phylogenetic tree for East Asians, the defining feature of mtDNA haplogroup F is the specific polymorphism at nucleotide 6392 in the coding region, whereas mtDNA haplogroup B is characterized by a diagnostic marker consisting of a 9-bp deletion. In the present study, we determined the mtDNA haplogroups of 312 age-matched local Han men and found that the frequencies of the major Han mtDNA haplogroups (B, F, A, N9, D, G, M7, M8, M9) were similar to the published data.[20] This finding suggests that the lower frequency of mtDNA haplogroup R in asthenozoospermia patients is not related to geography and emphasizes the representation of the study cohort in the general population.

Although there are increasing studies of genetic variations in asthenozoospermia, data regarding mtDNA haplogroups are still very limited, especially for the Asian population. In the study by Ruiz-Pesini *et al.*,[6] mtDNA haplotype H was found to be underrepresented and haplotype T was overrepresented in men with asthenozoospermic ejaculates. Furthermore, significant differences in OXPHOS component activity were found between different haplotypes. Although there is no direct evidence, e.g., the mtDNA haplogroup H and T in Europeans, because mtDNA haplogroups are associated with mitochondrial function, especially respiratory-chain activity in the Asian population, the possibility should not be ignored. Our results indicate that mtDNA haplogroup R strongly impacts sperm motility, which may provide potential insight into the relationship between mtDNA haplogroups and mitochondrial function. The specific polymorphisms used to define mtDNA haplogroup R may imply a possible explanation. Nucleotide 12705 and 3970 are the specific polymorphism sites of mtDNA haplogroup R. The polymorphisms lie within the mtDNA gene coding for the subunits of complex I. Therefore, these polymorphisms may influence the functions of the complex, such as electron transport, electron leakage and reactive oxygen species production.[21]

A number of studies failed to identify associations between mtDNA and sperm motility. The discordance among haplotype studies is most likely a reflection of the difficulty in matching cases and controls and the existence of genuine variations in the genome of different populations.[22] Different investigations have focused on different populations. Even within Europeans, the frequencies of the main subhaplogroups are variable, e.g., the Spanish and United Kindom populations. The Han maternal gene pool largely belongs to three specific subhaplogroups of the Eurasian founder haplogroups, M, N and R, which is different from Europeans. Therefore, different conclusions can be drawn. We should pay attention to the probability that many different polymorphisms may militate against mitochondrial function together *vs.* single base differences. Ruiz-Pesini *et al.*[6] demonstrated the existence of variable complex IV-specific and cytochrome c oxidase activities between haplotypes H and T. Variable complex I-specific activities were included in our investigation perhaps due to different mtDNA haplogroup polymorphisms.

In summary, we studied a cohort of 312 men and found that mtDNA haplogroup R was a strong independent predictor of sperm motility, conferring a 2.97-fold decreased chance of asthenozoospermia compared with those without haplogroup R. Our results provide potential insights into the relationship between mtDNA haplogroups and mitochondrial function.

AUTHOR CONTRIBUTIONS

YMZ designed and supervised the study. GFF and JZ analysed the data and wrote the paper. LMF, LJL and NXS gathered the data. LMF and LJL edited the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages of the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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