Journal of Andrology, Vol. 30, No. 6, November/December 2009

APOB Gene Signal Peptide Deletion Polymorphism Is Not Associated With Infertility in Indian Men

ARUN KHATTRI,* RAJEEV K. PANDEY,* NALINI J. GUPTA,† BAIDYANATH CHAKRAVARTY,† MAMTA DEENADAYAL,‡ LALJI SINGH,* AND KUMARASAMY THANGARAJ*

*From the *Centre for Cellular and Molecular Biology, Hyderabad, India; the †Institute of Reproductive Medicine, Kolkata, India; and the ‡Infertility Institute and Research Centre, Hyderabad, India.*

ABSTRACT: Apolipoprotein B (APOB) plays a key role in lipoprotein metabolism and plasma lipid transport. It has been shown that about two-thirds of male mice heterozygous for *ApoB* were infertile. Moreover, a 3-codon deletion polymorphism (rs11279109) in the signal peptide region of the *APOB* gene has been shown to be a risk factor for infertility in Slovenian men, but its association with infertility in Indian men has not been evaluated to date. Hence, in the present study, we have genotyped this polymorphism in 545 Indian men, including 294 infertile and 251 fertile men. Our results show that the distribution of this deletion polymorphism was consistent with the Hardy-Weinberg equilibrium in both infertile and fertile men. No statistically significant difference was observed in the distribution of the *APOB* signal peptide deletion polymorphism between infertile and fertile men ($\chi^2 = 0.156$, $P = .925$ for genotypes; $\chi^2 = 0.015$, $P = .903$ for alleles). Moreover, no significant difference was observed when infertile and fertile men were categorized on the basis of presence (D/D and D/W genotypes) or absence (W/W genotypes) of deletion (odds ratio, 0.955; 95% confidence interval, 0.644–01.418; $P = .820$). Our study concludes that the *APOB* gene deletion polymorphism is not a risk factor for the development of infertility in Indian men.

Key words: Spermatogenesis, apolipoprotein B.

J Androl 2009;30:734–738

Approximately 15% of human couples are infertile; about 50% of these cases are due to various factors or conditions affecting the male. Although we are familiar with the mechanisms of human reproductive physiology, the underlying causes of male subfertility are not fully elucidated (Meschede and Horst, 1997). Various causative factors include varicocele, obstruction of spermatic ducts, agglutination of sperm, impotence, hormonal imbalance, and genetic defect. Y-chromosome microdeletions in azoospermia factor (*AZF*) regions have been found in approximately 10% of infertile Indian men (Thangaraj et al, 2003a); deletion of *DAZ* gene copies and mutation in the autosomal gene *DAZL* have also been associated with male infertility (Reynolds and Cooke, 2005; Yang et al, 2005). Additionally, mutations in mitochondrial DNA have been associated with impaired sperm motility (Thangaraj et al, 2003b; Selvi Rani et al, 2006). However, the underlying genetic causes of infertility in the large proportion of infertile men remain largely unknown.

Correspondence to: Dr K. Thangaraj, Centre for Cellular and Molecular Biology, Uppal Rd, Hyderabad 500 007, India (e-mail: thangs@ccmb.res.in).

Received for publication March 9, 2009; accepted for publication June 16, 2009.

DOI: 10.2164/jandrol.109.007898

The B apolipoproteins APOB-100 and APOB-48 are derived from a common structural gene. They play central roles in lipoprotein metabolism and plasma lipid transport (Young, 1990; Kim and Young, 1998). APOB-100 is essential for the assembly of very–low-density lipoprotein in the liver, whereas APOB-48 is required for chylomicron formation in the intestine. Knockout studies on *ApoB* gene deletion have shown severely compromised fertility in *ApoB* heterozygous male mice (Huang et al, 1995, 1996), whereas another study found *ApoB* heterozygous male mice to be fertile (Farese et al, 1995). A 3–amino acid deletion polymorphism in the signal peptide region has been proven to cause inefficient translocation of the APOB protein (Sturley et al, 1994; Benhizia et al, 2001). The presence of the deletion variant resulted in a 66% reduction in endoplasmic transport compared with proteins containing the wild-type signal peptide (Sturley et al, 1994). Recent studies have associated this deletion polymorphism with oligoasthenoteratozoospermia in Slovene white men (Peterlin et al, 2006). Currently, there is no available report on the association of this polymorphism with infertility in Indian men, and studies on mouse models have yielded a controversial interpretation of the role of the *ApoB* gene in spermatogenesis. Our study evaluated the possibility of association of the *APOB* gene deletion polymorphism with infertility in groups of infertile and fertile Indian men.

Materials and Methods

Subjects and Controls

Blood samples were collected from men attending the Institute of Reproductive Medicine (Kolkata, India) and the Infertility Institute and Research Centre (Hyderabad, India). Informed written consent was obtained from all participants, and permission to proceed was granted by the Institute Ethics Committee. A total of 294 infertile men who did not exhibit any known medical or genetic cause of infertility, such as obstruction, injury, endocrinologic defect, or karyotypic abnormality, were included in the study. Men who exhibited deletion or mutation in our previous studies on the analysis of Y-chromosome microdeletions (Thangaraj et al, 2003a; and Thangaraj et al, unpublished results), *DAZ* copy deletions (Rani et al, unpublished results), and mitochondrial *ND4* gene analysis (Selvi Rani et al, 2006) were excluded. A total of 294 infertile men were further identified as 1 of 3 subtypes in accordance with World Health Organization criteria (World Health Organization, 2000): 137 azoospermatic (Azoo; no sperm in ejaculate), 55 oligoasthenozoospermatic (OA; $<20 \times 10^6$/mL sperm concentration and $<50\%$ motile sperm), and 102 oligoasthenoteratozoospermatic (OAT; $<20 \times 10^6$/mL sperm concentration, $<50\%$ motile sperm, and $<30\%$ sperm with normal morphology). A total of 251 ethnically matched men who, in accordance with WHO criteria (World Health Organization, 2000), exhibited normal semen parameters (25×10^6/mL to 130×10^6/mL sperm concentration, $>50\%$ motile sperm, and $>30\%$ with normal morphology) and possessed normal levels of inhibin B, testosterone, leutinizing hormone, and follicle-stimulating hormone were included in this study as controls. In addition, every fertile man in the control group had fathered at least one child. Paternity was verified using short tandem repeat–based DNA fingerprinting (AmpF/STR Identifiler kit; Applied Biosystems, Foster City, California).

Genotyping of APOB Deletion

To genotype the 3-codon deletion polymorphism (rs11279109) in the signal peptide region of the *APOB* gene, we designed and custom synthesized (Sigma-Proligo; Proligo Singapore Pty Ltd, Singapore, Singapore) primers as described previously (Peterlin et al, 2006). The sequences of the primers used were forward primer 5′-FAM-CTCCTCAGCCCCTCCATC-3′, and reverse primer 5′-GAGTGCCCTTCTCGGTTG-3′, which amplified polymerase chain reaction (PCR) products of 366/375 bp. PCR for each sample was performed in 0.2-mL, thin-walled tubes using 2.0 ng of DNA, 2 to 5 pm of each primer, 200 μM dinucleotide triphosphates, 10× PCR buffer, 1.5 mM $MgCl_2$, and 0.5 units of AmpliTaq Gold (Applied Biosystems). Because the guanine cytocine content of the amplified region is very high (76.8%), 20% (v/v), 5M Betaine (Sigma-Aldrich, St Louis, Missouri) was added as a PCR enhancer. The PCR reaction was carried out in a GeneAmp 9700 Thermal Cycler (Applied Biosystems) under the following conditions: 96°C for 10 minutes, 35 cycles at 96°C for 45 seconds, 66°C for 30 seconds, and 72°C for 1 minute, and a final extension at 70°C for 7 minutes. Amplified products were analyzed in an ABI 3700*xl* DNA Analyzer (Applied Biosystems) using GeneScan. For GeneScan, 0.5 μL of PCR product was mixed with 0.2 μL of GeneScan-500 LIZ Size Standard (Applied Biosystems) and 9.3 μL of HiDi Formamide (Applied Biosystems). Raw data were analyzed using GeneMapper Software, version 4.0 (Applied Biosystems). To reconfirm the results, we sequenced approximately 80% samples. Sequencing samples were reamplified using primers without florescent tags (FAM), and the sequencing reaction was performed as described previously (Khattri et al, 2009).

Statistical Analysis

Through statistical analysis, the risk of infertility in relation to the *APOB* gene signal peptide deletion polymorphism was estimated using the Statistics Package for Social Sciences, version 16 (SPSS Inc, Chicago, Illinois). In a χ^2 test for association, odds ratios at their 95% confidence intervals were calculated, and $P < .05$ was considered significant. The online resource facility of the Institute for Human Genetics (Munich, Germany; http://ihg2.helmholtz-muenchen.de/ihg/snps.html) was used to test for deviation of the Hardy-Weinberg equilibrium and evaluation of different models of association.

Results

PCR amplification of deletion polymorphism rs11279109 in the signal peptide region of the *APOB* gene resulted in products of 375 bp (wild type [W]) or 366 bp (deletion [D]), which were confirmed by sequencing (Figure 1a) and GeneScan (Figure 1b). Analysis of the deletion polymorphism revealed that 24.3% of fertile and 23.47% of infertile men carried the deletion (D/D and D/W) genotypes (Table; Figure 2). This deletion polymorphism was in Hardy-Weinberg equilibrium in both infertile and fertile men. The heterozygosity index of this locus in our population was 0.213. No significant difference was observed when distribution of genotypes or alleles was compared between fertile and infertile men ($\chi^2 = 0.156$, $P = .925$ for genotypes; $\chi^2 = 0.015$, $P = .903$ for alleles). Furthermore, no significant difference was observed when any of the individual patient subgroups (Azoo, OAT, and OA) was compared either with another subgroup or with fertile men, or when categorizing fertile and infertile men (and their subgroups) based on the presence (D/D and D/W) or absence (W/W) of deletion (Table). The frequency of individuals carrying the deletion was, however, increased in azoospermic men.

Discussion

Sperm maturation involves a broad range of processes that occur during sperm migration through the seminif-

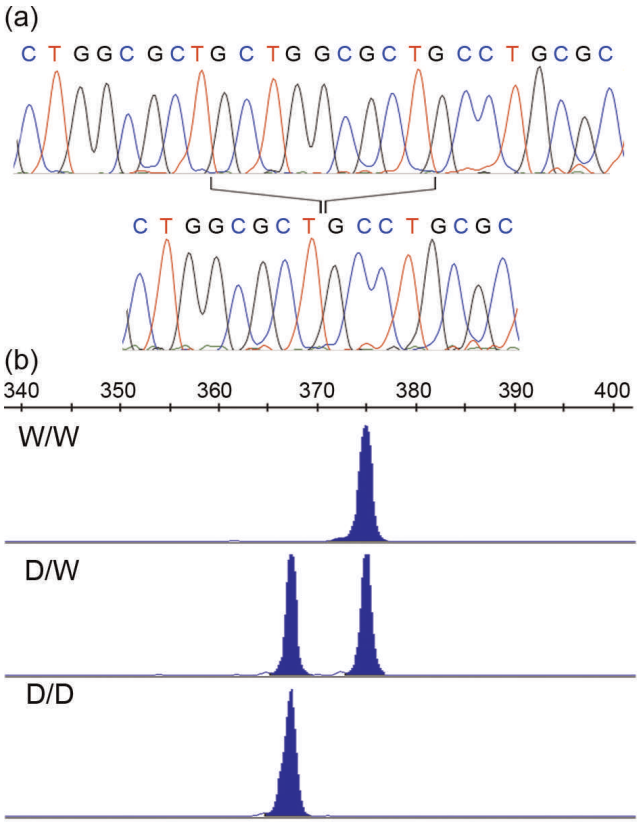

Figure 1. Alleles of *APOB* gene signal peptide deletion polymorphism. **(a)** Sequences of individuals with wild-type (W) and deletion (D) alleles. **(b)** GeneScan results of individuals with W/W, D/W, and D/D genotypes. Color figure available online at www.andrologyjournal.org.

erous tubules and the epididymis, including the remodeling of sperm membrane components (De Vriese and Christophe, 2003). The plasma membrane of sperm plays a very active role in its fertilization capacity and in spermatozoon-oocyte cross-talk. Its biochemical constitution is one of the main fields of interest in the study of sperm physiology and pathology (Lenzi et al, 1996). Because APOB is one of the apolipoproteins that play an important role in lipoprotein metabolism, there is a potential role for APOB in male infertility, because this polymorphism alters the structure of the signal peptide of the gene central to lipid metabolism (Visvikis et al, 1990). Moreover, some other genes affecting lipid metabolism have been associated with infertility in knockout mice, such as the apolipoprotein E receptor-2 (*Apoer2*; Andersen et al, 2003), acid sphingomyelase (*Asm*; Butler et al, 2002), and adenosine triphosphate–binding cassette transporter 1 (*Abca1*; Selva et al, 2004) genes. A prior study (Peterlin et al, 2006) has found significantly increased proportions of D/D and D/W genotypes among OAT men in the Slovene white population, but we did not find any significant difference in the deletion frequency (D/D and D/W genotypes) between fertile and infertile men or subgroups of infertile men (Azoo, OA, and OAT). Rather, the frequency of deletion was slightly decreased in OAT (20.59%) and OA (20.20%) men (Table). Although the authors of the Slovenian study reported the association of deletion within OAT, it was marginally significant ($P = .014$; at 95% significance level, when Bonferroni correction applied: $P < .05/2 = .025$). The difference between these 2 studies could very well be explained by the ethnic difference of the study populations. The most notable difference is the frequency with which D/D and D/W deletion genotypes occur: 52.8% of fertile men carried a deletion in the population studied by Peterlin et al (2006), whereas only 24.4% carried a deletion in our study. This difference was not surprising to us, knowing the unique ethnic origin of Indian populations. Earlier, we contrasted the results of the studies performed on Indian populations compared with other populations; for example, CAG repeat expansion in the androgen receptor was reported to be associated with male infertility (Patrizio et al, 2001), but our study did not find an association (Thangaraj et al, 2002). Similarly, the A386G polymorphism in the *DAZL* gene was not associated with male infertility in Indian males (Thangaraj et al, 2006), but it was found to be associated with severe spermatogenic failure in the Taiwanese popula-

Table. *Association of* APOB *gene signal peptide deletion polymorphism with infertility considering deletion (D/D and D/W) as a risk factor*

Genotype	Study Group				
	Fertile Men	Infertile Men	Azoospermia	OAT	OA
W/W	190 (75.70)	225 (76.53)	100 (72.99)	81 (79.41)	44 (80.00)
D/D and D/W	61 (24.30)	69 (23.47)	37 (27.01)	21 (20.59)	11 (20.20)
Odds ratio	...	0.955	1.152	0.808	0.779
95% confidence interval	...	0.644–1.418	0.717–1.853	0.461–1.414	0.379–1.601
P	...	.82	.559	.45	.489

Abbreviations: ..., not applicable; D, deletion; OA, oligoasthenozoospermia; OAT, oligoasthenoteratozoospemia; W, wild type.

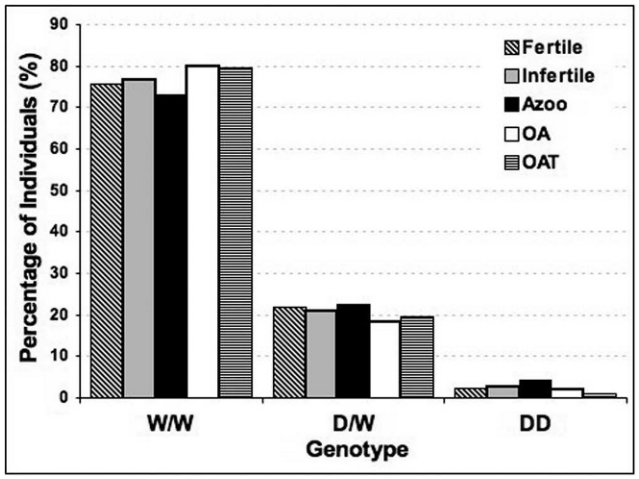

Figure 2. Distribution of genotypes for the *APOB* gene signal peptide deletion polymorphism in infertile and fertile men. Azoo indicates azoospermia; OAT, oligoasthenoteratozoospemia; OA, oligoasthenozoospermia; D, deletion; W, wild type.

tion (Teng et al, 2002). Similar patterns were observed in other genes involved in sex determination (Rajender et al, 2006), alcoholism (Bhaskar et al, 2007; Rao et al, 2007), mitochondrial disorders (Vanniarajan et al, 2006), and cardiomyopathies (Dhandapany et al, 2009).

This is the first study on Indian men and second study worldwide addressing the association of the *APOB* gene signal peptide deletion polymorphism with male infertility. Men with 1 of 3 different phenotypes of infertility (Azoo, OA, and OAT) were included in our study, but no association with the *APOB* signal peptide deletion polymorphism was detected in any of them. Our results suggest that the polymorphism is not associated with infertility in Indian men, although it has been shown to be associated with OAT in Slovene white men (Peterlin et al, 2006). Knockout mouse studies on the *APOB* gene also produced confusing results: two-thirds of male *ApoB* heterozygous mice were found infertile, despite normal genitourinary system function and mating behavior (Huang et al, 1995). Sperm from these heterozygous knockout males failed to fertilize eggs, either in vivo or in vitro. These sperm were able to fertilize eggs once the zona pellucida was removed, but they displayed persistent abnormal binding to the egg after fertilization. In vitro fertilization–related and other experiments revealed that reduced sperm count, motility, and survival time also contributed to the infertility phenotype. Furthermore, when the genomic sequence encoding human *APOB* was introduced into genetically engineered animals, normal fertility was restored (Huang et al, 1996). Contrary to the above studies, infertility was not reported in the study by Farese et al (1995) on *APOB* heterozygous male mice. Peterlin et al (2006) and our study have produced contrasting findings on the *APOB* gene signal peptide deletion polymorphism. Accordingly, we expect similar trends in other populations. Therefore, it is important to perform replicate studies on patients from diverse ethnic origins before either designating or excluding this polymorphism as a risk for male infertility.

Acknowledgments

We thank Dr Alkes Price, Assistant Professor, Harvard School of Public Health (Boston, Massachusetts) for going through the manuscript and editing it for English. This work is supported from the grant from the Indian Council of Medical Research and the Council of Scientific and Industrial Research, government of India, New Delhi, India.

References

Andersen OM, Yeung CH, Vorum H, Wellner M, Andreassen TK, Erdmann B, Mueller EC, Herz J, Otto A, Cooper TG, Willnow TE. Essential role of the apolipoprotein E receptor-2 in sperm development. *J Biol Chem*. 2003;278:23989–23995.

Benhizia F, Ginsberg HN, Humphries SE, Talmud PJ. Variation in the human ApoB signal peptide modulates ApoB17 translocation. *Biochem Biophys Res Commun*. 2001;283:149–157.

Bhaskar LV, Thangaraj K, Shah AM, Pardhasaradhi G, Praveen Kumar K, Reddy AG, Papa Rao A, Mulligan CJ, Singh L, Rao VR. Allelic variation in the NPY gene in 14 Indian populations. *J Hum Genet*. 2007;52:592–598.

Butler A, He X, Gordon RE, Wu HS, Gatt S, Schuchman EH. Reproductive pathology and sperm physiology in acid sphingomyelinase-deficient mice. *Am J Pathol*. 2002;161:1061–1075.

De Vriese SR, Christophe AB. *Male Fertility and Lipid Metabolism*. Urbana, IL: The American Oil Chemists Society; 2003.

Dhandapany PS, Sadayappan S, Xue Y, Powell GT, Rani DS, et al. A common MYBPC3 (cardiac myosin binding protein C) variant associated with cardiomyopathies in South Asia. *Nat Genet*. 2009;41:187–191.

Farese RV Jr, Ruland SL, Flynn LM, Stokowski RP, Young SG. Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc Natl Acad Sci U S A*. 1995;92:1774–1778.

Huang LS, Voyiaziakis E, Chen HL, Rubin EM, Gordon JW. A novel functional role for apolipoprotein B in male infertility in heterozygous apolipoprotein B knockout mice. *Proc Natl Acad Sci U S A*. 1996;93:10903–10907.

Huang LS, Voyiaziakis E, Markenson DF, Sokol KA, Hayek T, Breslow JL. apo B gene knockout in mice results in embryonic lethality in homozygotes and neural tube defects, male infertility, and reduced HDL cholesterol ester and apo A-I transport rates in heterozygotes. *J Clin Invest*. 1995;96:2152–2161.

Khattri A, Pandey RK, Gupta NJ, Chakravarty B, Deendayal M, Singh L, Thangaraj K. CA repeat and RsaI polymorphisms in ERbeta gene are not associated with infertility in Indian men. *Int J Androl*. 2009;32:81–87.

Kim E, Young SG. Genetically modified mice for the study of apolipoprotein B. *J Lipid Res*. 1998;39:703–723.

Lenzi A, Picardo M, Gandini L, Dondero F. Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as

markers of sperm function to possible scavenger therapy. *Hum Reprod Update*. 1996;2:246–256.

Meschede D, Horst J. The molecular genetics of male infertility. *Mol Hum Reprod*. 1997;3:419–430.

Patrizio P, Leonard DG, Chen KL, Hernandez-Ayup S, Trounson AO. Larger trinucleotide repeat size in the androgen receptor gene of infertile men with extremely severe oligozoospermia. *J Androl*. 2001;22:444–448.

Peterlin B, Zorn B, Volk M, Kunej T. Association between the apolipoprotein B signal peptide gene insertion/deletion polymorphism and male infertility. *Mol Hum Reprod*. 2006;12:777–779.

Rajender S, Thangaraj K, Gupta NJ, Leelavathy N, Rani DS, Nambiar RG, Kalavathy V, Santhiya ST, Rajangam S, Gopinath PM, Chakravarty B, Singh L. A novel human sex-determining gene linked to Xp11.21-11.23. *J Clin Endocrinol Metab*. 2006;91:4028–4036.

Rao VR, Bhaskar LV, Annapurna C, Reddy AG, Thangaraj K, Rao AP, Singh L. Single nucleotide polymorphisms in alcohol dehydrogenase genes among some Indian populations. *Am J Hum Biol*. 2007;19:338–344.

Reynolds N, Cooke HJ. Role of the DAZ genes in male fertility. *Reprod Biomed Online*. 2005;10:72–80.

Selva DM, Hirsch-Reinshagen V, Burgess B, Zhou S, Chan J, McIsaac S, Hayden MR, Hammond GL, Vogl AW, Wellington CL. The ATP-binding cassette transporter 1 mediates lipid efflux from Sertoli cells and influences male fertility. *J Lipid Res*. 2004;45:1040–1050.

Selvi Rani D, Vanniarajan A, Gupta NJ, Chakravarty B, Singh L, Thangaraj K. A novel missense mutation C11994T in the mitochondrial ND4 gene as a cause of low sperm motility in the Indian subcontinent. *Fertil Steril*. 2006;86:1783–1785.

Sturley SL, Talmud PJ, Brasseur R, Culbertson MR, Humphries SE, Attie AD. Human apolipoprotein B signal sequence variants confer a secretion-defective phenotype when expressed in yeast. *J Biol Chem*. 1994;269:21670–21675.

Teng YN, Lin YM, Lin YH, Tsao SY, Hsu CC, Lin SJ, Tsai WC, Kuo PL. Association of a single-nucleotide polymorphism of the deleted-in-azoospermia-like gene with susceptibility to spermatogenic failure. *J Clin Endocrinol Metab*. 2002;87:5258–5264.

Thangaraj K, Deepa SR, Pavani K, Gupta NJ, Reddy P, Reddy AG, Chakravarty BN, Singh L. A to G transitions at 260, 386 and 437 in DAZL gene are not associated with spermatogenic failure in Indian population. *Int J Androl*. 2006;29:510–514.

Thangaraj K, Gupta NJ, Pavani K, Reddy AG, Subramainan S, Rani DS, Ghosh B, Chakravarty B, Singh L. Y chromosome deletions in azoospermic men in India. *J Androl*. 2003a;24:588–597.

Thangaraj K, Joshi MB, Reddy AG, Gupta NJ, Chakravarty B, Singh L. CAG repeat expansion in the androgen receptor gene is not associated with male infertility in Indian populations. *J Androl*. 2002;23:815–818.

Thangaraj K, Joshi MB, Reddy AG, Rasalkar AA, Singh L. Sperm mitochondrial mutations as a cause of low sperm motility. *J Androl*. 2003b;24:388–392.

Vanniarajan A, Rajshekher GP, Joshi MB, Reddy AG, Singh L, Thangaraj K. Novel mitochondrial mutation in the ND4 gene associated with Leigh syndrome. *Acta Neurol Scand*. 2006;114:350–353.

Visvikis S, Chan L, Siest G, Drouin P, Boerwinkle E. An insertion deletion polymorphism in the signal peptide of the human apolipoprotein B gene. *Hum Genet*. 1990;84:373–375.

World Health Organization. *WHO Manual for the Standardized Investigation, Diagnosis and Management of the Infertile Male*. Cambridge, United Kingdom: Cambridge University Press; 2000.

Yang XJ, Shinka T, Nozawa S, Yan HT, Yoshiike M, Umeno M, Sato Y, Chen G, Iwamoto T, Nakahori Y. Survey of the two polymorphisms in DAZL, an autosomal candidate for the azoospermic factor, in Japanese infertile men and implications for male infertility. *Mol Hum Reprod*. 2005;11:513–515.

Young SG. Recent progress in understanding apolipoprotein B. *Circulation*. 1990;82:1574–1594.