Association between the apolipoprotein B signal peptide gene insertion/deletion polymorphism and male infertility

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In male mice heterozygous for a null apolipoprotein B (apoB), allele infertility was noticed. These data led us to investigate a possible role of APOB gene polymorphism and male infertility in humans. In this case–control study, we searched for an association between the insertion/deletion (I/D) polymorphism of the APOB gene and male infertility in 560 Slovene Caucasian men. The study group consisted of 310 infertile patients: 115 with azoospermia and 195 with oligoasthenoteratozoospermia (OAT) and a control group of 250 fertile men. We found a statistically significant difference in the genotype distribution between the two groups (χ² = 6.315, P = 0.043). A separate analysis of azoospermic and OAT patients demonstrated that significant differences in genotype distribution were limited to the OAT group (χ² = 7.011, P = 0.030). The presence of the D allele (DD or ID genotypes) conferred a 1.6 risk (χ² = 6.089, P = 0.014, 95% confidence interval (95% CI) = 1.102–2.347) for male infertility in the OAT group of patients. We did not find a correlation between the I/D polymorphism genotypes and the clinical characteristics of infertile men: sperm concentration (P = 0.102), rapid progressive motility (P = 0.449), normal morphology (P = 0.085) and Johnsen score (P = 0.531). These data suggest that genetic variation in the signal peptide of the APOB gene (I/D polymorphism) might be a risk factor for the development of male infertility.

Key words: apolipoprotein B/azoospermia/male infertility/oligoasthenoteratozoospermia

Introduction

Male infertility affects 10% of the adult population. Disturbances in fertility are multifactorial in origin, i.e. genetic and environmental (Nieschlag et al., 2000). Genetic factors implicated in male infertility include autosomal and sex chromosome abnormalities, Y-chromosome microdeletions as well as several autosomal genes, e.g. genes controlling gonadotrophin secretion and action and the cystic fibrosis gene to name a few (Huynh et al., 2002; Krausz et al., 2003; Cram et al., 2004). An increasing number of genetically engineered animals provide insights into the genetic control of spermatogenesis (Anagnostopoulos, 2002; Huynh et al., 2002).

Huang et al. (1995) have reported that male mice heterozygous for a targeted mutation of the apolipoprotein B (apoB) exhibit male infertility in addition to neural tube defects, reduced rates of high-density lipoprotein (HDL) cholesterol ester and apoA-I transport and embryonic lethality in the homozygous state. Sperm from these mice failed to fertilize eggs both in vitro and in vivo (Huang et al., 1996). These experiments revealed that reduced sperm motility, survival time and sperm count also contributed to the infertility phenotype in the heterozygous animals. Moreover, when the genomic sequence encoding human APOB was introduced into genetically engineered animals, normal fertility was restored. These findings thus suggest that the APOB gene might have an important impact on fertility in male.

ApoB is one of the apolipoproteins, which plays an important role in lipoprotein metabolism. ApoB is the ligand for receptor-mediated removal of low-density lipoprotein particles from circulation (Kane and Havel, 1989). The gene, coding for human apoB, has been localized to chromosome 2p23-24. Substantial variability of the gene includes two alleles of the apoB signal peptide, coding for peptides of 27 and 24 amino acids in length (Visvikis et al., 1990).

The aim of this study was to test the hypothesis that genetic variability—insertion/deletion (I/D) polymorphism of the APOB gene is a risk factor for male infertility in humans. For this purpose, we analysed a group of 310 infertile men, ICSI candidates and a control group of 250 fertile men. Genotypes (I/I, I/D and D/D) were correlated with the clinical phenotype of infertility and with the findings of sperm analysis.

Materials and methods

Patients

We analysed male partners of infertile couples with an abnormal spermogram, ICSI candidates, attending the Andrology Centre, Department of Obstetrics and Gynecology, University Medical Centre Ljubljana. Patients with a history of testicular carcinoma, obstruction, congenital bilateral absence of vas deferens, cytogenetic abnormalities and Y chromosome microdeletions were excluded. The study group consisted of 310 patients: 115 with azoospermia and 195 with oligozoospermia or oligoasthenoteratozoospermia (OAT).

The control group of proven fertility consisted of 250 men with at least one offspring, but no andrological evaluation, informed consent was obtained from each patient. All patients were Slovene or of Slavic origin. The study was approved by the national medical ethics committee.
Clinical, sperm, hormonal and histological evaluations of infertile patients

In the 310 infertile men, sperm was assessed in terms of volume, concentration, rapid progressive motility and normal morphology using the World Health Organization (WHO) guidelines (WHO, 1999). The testicular volume was measured using a Prader’s orchidometer. Serum FSH was measured by Micro- particle Enzyme Immunoassay (AxSYM System, Abbott Laboratories, Chicago, IL, USA); the reference interval for FSH was 1–8 mIU/ml.

Testicular biopsy with histological assessment was performed in 47 azoospermic men. The histological diagnoses were as follows: 23 men were affected by Sertoli cell-only syndrome, 15 with hypospermatogenesis and 9 by early and late maturation arrest. Moreover, testicular histology was evaluated according to Johnsen’s modified classification (Holstein et al., 1994) from score 1 (no cell in sclerotic seminiferous tubules) to score 10 (spermatozoa present).

Molecular genetic methods

Genomic DNA was prepared from the peripheral blood samples using standard procedures. We analysed a three-codon I/D polymorphism in the signal peptide region of the apoB gene by PCR (Boerwinkle and Chan, 1989). The reaction mixture of 10 μl included 100 ng of DNA sample, 1x PCR buffer, 1.5 mM MgCl2, 200 μM deoxy-nucleotidetriphosphate (NTPs); 1 μM of each primer pair, 1/10 V dimethylsulphoxide (DMSO) and 0.5 U Taq DNA polymerase (Promega, Madison, WI, USA). The sequences of the primers used were forward primer: 5′-CAGCTGGGATGGACCCGCCGGA-3′ and reverse primer: 5′-ACGCCCTTGGCCGCGGACCA-3′ and yielded products of 84 bp/93 bp. The reactions were performed in a thermal cycler (MJ Research, Inc., Watertown, MA, USA). After an initial denaturation step at 94°C for 5 min, cycle parameters were 30 cycles at 94°C for 60 s, 62°C for 80 s and 72°C for 60 s. The programmes were followed by the final extension step at 72°C for 7 min. The reaction products were then analysed by electrophoresis on 7% V gel containing ethidium bromide (0.1 mg/ml) and visualized under UV light. 4% agarose gels (Sigma Chemical, St. Louis, MO, USA) containing ethidium bromide (0.1 mg/ml) and visualized under UV light. To confirm the accuracy of the I/D analysis on Spreadex gels and for sequencing the PCR products, we designed primers 5′-CTCCTCAGCCCTTCCATC-3′ and 5′-GGATGGCCCT-TCTCCTGGTTG-3′ which amplified longer PCR products of 366 bp/375 bp. The same reaction conditions as for the shorter PCR products were used. The PCR products were analysed on Spreadex® EL800 gels (Elchrom Scientific AG, Cham, Switzerland) for 180 min at 55°C and 120 V, stained by SYBR Gold (Molecular Probes, Leiden, the Netherlands) and visualized under UV light, as described previously (Kunej et al., 2004). The sizes of PCR products of APOB gene I/D polymorphism were determined in comparison with molecular weight markers (M) (Elchrom Scientific AG) (Figure 1) and with the fragments with known lengths obtained by sequencing, using Perkin-Elmer ABI Prism 310 Genetic Analyzer (PE ABI, Foster City, CA, USA).

Statistical analysis

Computations were carried out with the Statistics Package for Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA). Allele and genotype frequencies were compared between the groups using the χ2-test. The odds ratio (OR) was calculated to measure the strength of association between allele frequencies and infertility. Allele frequencies for the I/D polymorphism of the APOB gene in the infertile patients were correlated to clinical characteristics: sperm concentration, rapid progressive motility, normal morphology and Johnsen score by using Kruskal–Wallis test. P was considered significant if <0.05.

![Figure 1](image-url)  
**Figure 1.** The PCR products for APOB insertion/deletion (I/D) polymorphism on Spreadex gel EL800. The sizes of the PCR products were determined in comparison with molecular weight marker M3 (Elchrom Scientific AG). I/I, homozygosity for 375-bp allele; D/D, homozygosity for 366-bp allele; I/D, heterozygosity.

Results

Clinical, sperm and hormonal characteristics of the 310 infertile patients are summarized in Table I.

Genotype distributions of the apoB signal peptide I/D polymorphism of the 310 infertile men and 250 fertile controls are shown in Figure 2.

We found a statistically significant difference in the genotype distribution between the groups of infertile and fertile men ($\chi^2 = 6.315$, $P = 0.043$). A separate analysis of azoospermic and OAT patients revealed that significant differences in genotype frequencies were limited to the OAT group ($\chi^2 = 7.011$, $P = 0.030$). The presence of DD or ID genotypes conferred a 1.6 risk ($\chi^2 = 6.089$) for male infertility in the OAT patients (Table II).

We did not find any correlation between the I/D polymorphism genotypes and the clinical characteristics of infertile men: sperm concentration ($P = 0.102$), rapid progressive motility ($P = 0.449$), normal morphology ($P = 0.085$) and Johnsen score ($P = 0.531$).

Discussion

The findings of Huang et al. (1995, 1996) suggested that the apoB gene might have an important impact on fertility in male mice. In this case–control association study, we provide evidence that genetic variation in the APOB gene might be a risk factor for infertility in humans. We have found that the D/D and I/D genotypes are significantly more common in OAT patients than the I/I genotype compared with fertile controls. This I/D polymorphism in the signal peptide of the human apoB gene has a potential physiological significance as it alters the structure of the signal peptide of a gene central to lipid metabolism (Li et al., 1988; Visvikis et al., 1990); additionally, a direct role of the signal peptide variants in the regulation of apoB intracellular metabolism has been found (Benhizia et al., 2001). Moreover, it has been shown that the apoB gene is expressed in the testis and epididymis in mouse and that the expression of human APOB in the testis and epididymis contributes to the correction of the infertility phenotype in apoB +/- mice containing the human APOB transgene (Huang et al., 1996).

We did not find a correlation between the I/D polymorphism and clinical characteristics: sperm concentration, rapid progressive motility, normal morphology and Johnsen score. In apoB +/- mice, the sperm counts were reduced compared with wild-type controls. However, the percentage of motile sperm was markedly reduced in the apoB +/- mice, and survival time was significantly shorter than in controls. Moreover, it was shown that sperm from apoB +/- mice were unable to penetrate the zona pellucida and that sperm binding did not attenuate, even after pronuclei had clearly formed when zona-free mouse oocytes were inseminated (Huang et al., 1996).

In addition to the APOB gene, other genes affecting lipid metabolism have been associated with infertility in knockout mice, apolipoprotein (apo) E receptor-2 (apoER2) gene (Andersen et al., 2003), acid sphingomyelase (ASM) gene (Butler et al., 2002) and the ATP-binding cassette transporter 1 (ABCA1) gene (Selva et al., 2004). These data suggest an important role of lipid metabolism in male infertility. Lipids are important constituents of the male gamete membrane and liquid content. They are composed of polyunsaturated fatty acids, cholesterol, phospholipids, sphingomyelin and plasmalogens. The continuous changes in lipid membrane and liquid contents during sperm activation, capacitation and acrosome reaction guarantee sperm to be able to fertilize (Feki et al., 2004). Moreover, reactive oxygen species induce membrane lipid peroxidation and sperm DNA fragmentation, which results in the impairment of sperm motility and morphology and difficulty in fertilization (Aitken and Krausz, 2001). Nevertheless, the mechanism of a possible involvement of apoB in male infertility remains unclear. Further studies enrolling larger
Table 1. Clinical, sperm and hormonal characteristics of the 310 infertile men

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Azoospermia (n = 115)</th>
<th>Oligozoospermia (n = 195)</th>
<th>Asthenozoospermia (n = 9), teratozoospermia (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular volume (right/left) (ml)</td>
<td>11.7 ± 5.5/11.8 ± 5.8</td>
<td>12.6 ± 4.7/12.6 ± 4.6</td>
<td>12.9 ± 3.5/12.8 ± 3.1</td>
</tr>
<tr>
<td>Sperm concentration (×10^6 spermatozoa/ml)</td>
<td>0 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Sperm rapid progressive motility (%)</td>
<td>–</td>
<td>4.3 ± 1.6</td>
<td>6.3 ± 6.8</td>
</tr>
<tr>
<td>Sperm normal morphology (%)</td>
<td>–</td>
<td>6.3 ± 6.8</td>
<td>13.1 ± 11.5</td>
</tr>
<tr>
<td>Serum FSH level (IU/l)</td>
<td>19.0 ± 14.3</td>
<td>13.1 ± 11.5 (n = 45)</td>
<td>4.7 ± 0.5 (n = 3)</td>
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</table>

Values are given as means ± SD.

Figure 2. Distribution of genotypes for the APOB insertion/deletion (I/D) polymorphism in infertile men versus the controls.

Table II. Distribution of genotype frequencies of the APOB insertion/deletion (I/D) polymorphism in oligoasthenoteratozoospermia (OAT) versus the controls following a dominant model

<table>
<thead>
<tr>
<th>Genotype</th>
<th>OAT (%)</th>
<th>Control group (%)</th>
<th>Odds ratio (OR) [95% confidence interval (95% CI)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/D + I/D</td>
<td>115 (59.0)</td>
<td>118 (47.2)</td>
<td>0.014 (1.102–2.347)</td>
</tr>
<tr>
<td>II</td>
<td>80 (41.0)</td>
<td>132 (52.8)</td>
<td></td>
</tr>
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</table>

numbers of infertile men from different populations are needed to confirm our findings. Genetic association studies are namely prone to beta statistical error and population-specific genotype effects which make results difficult to replicate.

In conclusion, we provide evidence that the genetic variation (I/D polymorphism) in the human ApoB signal peptide modulates ApoB17 translocation. Biochem Biophys Res Commun 283,149–157.


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References


