Lack of association between polymorphisms in the testis-specific angiotensin converting enzyme gene and male infertility in an Asian population

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Angiotensin converting enzyme (ACE) is a membrane-bound dipeptidyl carboxy-peptidase that generates vasoconstricting angiotensin II and inactivates vasodilating bradykinin. The ACE gene encodes two isozymes: the somatic isozyme (sACE) is found in many tissues including vascular endothelial cells, whereas the testis-specific isozyme (tACE) is expressed exclusively in developing spermatids and mature sperm. Thus, ACE might have physiological functions in addition to blood pressure regulation. Male mice lacking tACE activity show reduced fertility, indicating its importance in male fertility. In this study, we screened five recently defined tACE gene polymorphisms in 90 Singapore Chinese men with infertility and 84 fertile controls using PCR-based restriction fragment length polymorphism and DNA sequencing. However, only one of these polymorphisms was identified in both patient and control groups, the frequency of which was not significantly different in patients and controls. Thus, these ACE gene polymorphisms are unlikely to contribute to the pathogenesis of male infertility in the Singapore Chinese population.

Key words: angiotensin converting enzyme (ACE)/male infertility/polymorphisms/PCR–RFLP/testis-specific

Introduction

Approximately 8–10% of couples experience some form of infertility, of which male factors may account for up to 50% (World Health Organization, 1993). Conditions known to cause impaired fertility in the male include environmental factors, endocrine disorders, varicocele and genetic factors (Feichtinger, 1991; Thielemans et al., 1998). However, in a great number of cases of male infertility caused by inadequate spermatogenesis or sperm defects the origin of the problem remains unexplained.

Angiotensin converting enzyme (ACE; EC 3.4.15.1), which is a ubiquitously distributed dipeptidyl carboxypeptidase in mammalian organisms, catalyses the conversion of angiotensin I to the physiologically active octapeptide angiotensin II. Angiotensin II is one of the important components in the renin–angiotensin system (RAS), which controls fluid-electrolyte balance and systemic blood pressure (Peach, 1977; Cambien et al., 1992; Jeunemaitre et al., 1991). ACE also catalyses the cleavage of C-terminal dipeptides from several substrates including vasodilating nonapeptide bradykinin (Corvol et al., 1995). Elevated ACE activity has been implicated in cardiovascular diseases, and its inhibitors have been found to be effective in treating hypertension and congenital heart failure (Patchett and Cordes, 1985; Soubrier et al., 1988).

Two isoforms of ACE are known to exist in mammals. The larger isoform, somatic ACE (sACE), is found in blood and several other tissues including the vascular endothelial cells, renal epithelial cells and testicular Leydig cells (Chai et al., 1987; Sibony et al., 1993), whereas the testis-specific form of ACE (tACE) is expressed only in post-meiotic spermatogenic cells and sperm (Langford et al., 1993; Sibony et al., 1994).

Interestingly, both isoforms are encoded by the same gene consisting of 26 exons and spanning over ~24 kb (~16% coding sequence) with two homologous regions (exons 1–12, 14–26) on chromosome 17. Somatic ACE is encoded by the entire gene, whereas tACE is controlled by a 91 bp promoter in intron 12 driving expression of the testis-specific exon 13 and the second half of the gene (exons 14–26) (Soubrier et al., 1988; Bernstein et al., 1989; Howard et al., 1990; Hubert et al., 1991). The tACE protein has a unique N-terminal sequence determined by the testis-specific exon, while its remaining sequence is identical to the C-terminal domain of sACE (Ehlers et al., 1989; Kumar et al., 1989; Lattion et al., 1989).

Unlike sACE, which has been widely recognized as having an important role in RAS to regulate blood pressure, the function of tACE remains unknown. However, its importance in male reproduction has been recently demonstrated by ACE.
knock-out studies, where male mice lacking tACE have reduced fertility due to a defect in sperm migration within the oviducts and a decreased ability of sperm to bind to the zona pellucidae (Krege et al., 1995; Esther et al., 1996; Hagaman et al., 1998). This phenotype in the male was not an indirect effect of a blood pressure decrease due to the absence of sACE, because mutant males lacking only sACE have been found to be fertile (Hagaman et al., 1998). Furthermore, male fertility in the ACE null-mice can be restored by expression of tACE and not of sACE in the sperm (Ramaraj et al., 1998; Kessler et al., 2000). These studies clearly demonstrate that the expression of tACE in sperm is necessary for normal male fertility. Thus, molecular defects in the tACE gene may have a role in male infertility.

In this preliminary study, we examined the relationship of five recently described polymorphisms in the human ACE gene (Rieder et al., 1999) with male infertility in Singapore Chinese men. Three of these polymorphisms, C10514T, T10527C and A10578G, are located in the testis-specific exon 13, and the remaining two, C23152A and G23202A, are in the last exon 26. Polymorphisms T10527C, A10578G and C23152A result in amino acid substitutions Ser to Ala, Ser to Gly and Ser to Arg respectively. The C10514T polymorphism is in absolute linkage disequilibrium with the Alu insertion/deletion (I/D), and the G23202A is a silent polymorphism (Rieder et al., 1999).

Materials and methods

Patients

Ninety Chinese men with infertility participated in this study. Their ages ranged from 24 to 65 years (35.6 ± 6.0; mean ± SD). Their semen parameters ranged from azoospermia to oligospermia. Two patients with azoospermia due to varicocele (obstruction of sperm transport occurring in conjunction with varicocele) presented a normal testicular size and consistency and possible swelling of the epididymal head, as well as a normal FSH level in the blood, whereas spermatogenic cells were absent from the ejaculate after varicocelectomy. Individuals displaying chromosomal disorders, hyperprolactinaemia, hypotuitarism, infective or obstructive syndromes of the genital tract were excluded. A total of 84 fertile Chinese men were recruited as control subjects for this study. Their ages ranged from 23 to 48 years (34.3 ± 5.6; mean ± SD). Their semen parameters were normal, and fertility was positively established 2 years before the trial; they fathered full-term pregnancies with normal infants. The study was approved by the Hospital Ethical Committee. Informed consent was obtained from all subjects for using their semen samples in this study.

Routine investigations included a complete haemogram, urine analysis and chest roentgenogram, and determination of fasting blood sugar, blood urea nitrogen, serum creatinine, serum bilirubin and alkaline phosphatase.

Specific investigations performed were semen analysis, urological evaluation, testicular biopsy, hormone estimations and scrotal exploration with vasography (where indicated). Semen samples were analysed according to the method described in the World Health Organization manual (World Health Organization, 1993). Azoospermia was defined as total absence of sperm in the semen, while oligospermia was indicated by a sperm density of <20×10^6/ml. Asthenozoospermia was defined as <50% spermatozoa with forward progression or <25% spermatozoa with rapid progression. Teratozoospermia was defined as reduced percentage (<30%) of morphologically normal spermatozoa, and oligoasthenoteratozoospermia was signified by disturbance of all three variables.

Semen cultures were carried out in relevant cases. Genital infections with Chlamydia or Mycoplasma species were particularly sought and patients with such infections were excluded from the study. Testicular biopsy was done for patients with an abnormal semenogram and who had normal gonadotrophin values and no obvious cause for semen abnormality.

Plasma levels of FSH, LH, prolactin and testosterone were analysed by their specific radioimmunoassays using reagents provided by the World Health Organization under the Matched Reagent Programme (Goh et al., 1979). Three blood samples were taken from each patient in the morning at intervals of 20 min, and the sera were pooled and stored at −20°C until assayed.

DNA extraction and PCR

Genomic DNA was extracted from peripheral blood using standard procedures. Specific primers for PCR amplification of exons 13 and 26 of the ACE gene are given in Table I. Primers 1 and 2 were used to amplify testis-specific exon 13, and primers 3 and 4 were for exon 26. The PCR amplification was carried out in a total volume of 30 µl reaction mixture containing 1.5 mmol/l of MgCl2, 0.2 mmol/l of each dNTP, 50–200 ng of genomic DNA, 15 pmol of each primer, and 1.5 IU of Taq DNA polymerase (Gibco BRL, Gaithersburg, MD, USA). The cycling profile consisted of denaturation at 94°C for 1 min, annealing at 65°C for 30 s and extension at 72°C for 40 s, except for the first cycle where denaturation was extended to 5 min. After PCR, 5 µl of the amplified product was examined for fidelity by electrophoresis on a gel containing 2% agarose.

Restriction fragment length polymorphism (RFLP) analysis and direct DNA sequencing

RFLP analysis was employed to detect the presence of individual polymorphisms as described previously (Sundarrajan et al., 1999). A total of 8 µl of the PCR product was used for each RFLP analysis. The restriction enzymes used to detect different polymorphisms and the expected size of DNA fragments upon digestion in each genotype are listed in Table I. After digestion, the restriction fragments were separated by electrophoresis on a 2–4% agarose gel, depending on the size of fragments. The subjects were classified into three groups according to their polymorphisms. The presence or absence of polymorphisms was confirmed by PCR-mediated direct DNA sequencing using a DNA Sequencer (ABI Prism TM 377, Perkin-Elmer) with Big Dye Cycle Sequencing Ready Reaction Kit (PE Biosystem, Norwalk, CT, USA).

Statistical analysis

Statistical tests of significance and χ² analyses and Fisher’s exact test (two-sided) were carried out using Statistics Package for Social Sciences for Windows, version 10.0. A P-value <0.05 was considered to be statistically significant.

Results

Patients’ clinical data

Out of 90 patients analysed, 11 (12.2%) had azoospermia, 20 (22.2%) asthenozoospermia, 10 (11.1%) teratozoospermia, six (6.7%) oligospermia, seven (7.8%) asthenoteratozoospermia, 10 (11.1%) oligoasthenozoospermia, three (3.3%) oligoteratozoospermia and 23 (25.6%) oligoasthenoteratozoospermia. Among them, 52 (57.8%) had idiopathic infertility. The mean serum testosterone was within the normal range. The mean
ACE polymorphisms and male infertility

Table I. Primers and restriction enzymes used for PCR–RFLP analysis of tACE gene polymorphisms

<table>
<thead>
<tr>
<th>Exon</th>
<th>Polymorphism</th>
<th>Restriction enzyme</th>
<th>RFL (bp)</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>C10514T</td>
<td>NheI</td>
<td>167, 167, 145, 22</td>
<td>WP: Exon 13, 1: 5'-GGGCCACCCCTCTGCTGGTCGC-3'</td>
</tr>
<tr>
<td>13</td>
<td>T10527C</td>
<td>BanI</td>
<td>162, 5</td>
<td>HTP: Exon 13, 2: 5'-CCCTGCCCCCCTCCCTGAT-3'</td>
</tr>
<tr>
<td>13</td>
<td>A10578G</td>
<td>HaeIII</td>
<td>113, 54, 113, 87, 54, 26</td>
<td>HMP: Exon 13, 3: 5'-CCGGCTCTTCAGCATCCGGAC-3'</td>
</tr>
<tr>
<td>26</td>
<td>C23152A</td>
<td>BanI</td>
<td>90, 17</td>
<td>Exon 26, 1: 5'-GCCGACCCAGCCGGGT-3'</td>
</tr>
<tr>
<td>26</td>
<td>G23202A</td>
<td>AluI</td>
<td>73, 34</td>
<td>Exon 26, 2: 5'-GCCGACCCAGCCGGGT-3'</td>
</tr>
</tbody>
</table>

The primer has a nucleotide transition from C to G (underlined) in order to incorporate the NheI recognition site in the presence of 10514T. Primers 1 and 2 were used to amplify exon 13, and 3 and 4 exon 26. RFL = restriction fragment length; WP = without polymorphism; HTP = heterozygous polymorphism; HMP = homozygous polymorphism.

Table II. Patient clinical data

<table>
<thead>
<tr>
<th></th>
<th>FSH (IU/l)</th>
<th>LH (IU/l)</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE</td>
<td>3.79 ± 0.91</td>
<td>4.37 ± 0.78</td>
<td>4.64 ± 0.29</td>
</tr>
<tr>
<td>Reference range</td>
<td>0.8–4.7</td>
<td>2.0–6.9</td>
<td>3.2–9.5</td>
</tr>
</tbody>
</table>

Table III. Incidence of the C10514T polymorphism in patients and controls

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Genotypes</th>
<th>Frequency of T allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>Patients (n = 90)</td>
<td>9 (10%)</td>
<td>43 (48%)</td>
</tr>
<tr>
<td>Controls (n = 84)</td>
<td>10 (12%)</td>
<td>40 (48%)</td>
</tr>
</tbody>
</table>

The C allele is in absolute linkage disequilibrium with the Alu deletion, and T allele with the Alu insertion.

Physiological functions. The existence of a local RAS in female as well as in male reproductive tissues is now known (Speth et al., 1999). Gonadotrophins regulate the activity of all the components of the RAS, and reproductive tissues possess angiotensin II receptors. There is now a body of evidence implicating the RAS in pathophysiologies associated with reproductive function. However, the overall significance of the RAS for normal reproductive function remains questionable (Speth et al., 1999).

Testicular ACE possesses properties that are biochemically, catalytically and immunologically distinct from those of sACE (Velletri, 1985) because of different transcriptional regulations of the ACE gene (Soubrier et al., 1988; Ehlers et al., 1989; Lattion et al., 1989). Testicular ACE is under androgenic control and is associated with germinall cells (Velletri, 1995).

Several studies have documented that tACE and not sACE activity is essential for normal male fertility (Krege et al., 1995; Hagaman et al., 1998). Mice lacking both sACE and tACE expression, prepared by gene targeting to disrupt the gene coding for both ACE isozymes, have profoundly low blood pressure and kidney dysfunction. In addition, the male homozygous mutants sire very few pups although they have normal testes, sperm counts, morphology and motility, whereas the mutant females are fertile. Spermatozoa from mice lacking both ACE isozymes show defects in transport within the oviducts and in binding to the zona pellucida of the oocyte. Males generated by gene targeting to lack sACE but retain tACE were observed to be normally fertile. Moreover, restoration of fertility in the ACE null-mice can be achieved by sperm-specific expression of the testicular isozyme (Ramaraj et al., 1998), but not by sACE expression (Kessler et al., 2000). However, angiotensin I might not be a substrate for tACE because male mice lacking angiotensinogen have normal fertility (Hagaman et al., 1998). Since tACE plays an important role in normal male fertility, it is possible that molecular defects in the tACE gene may be involved in male infertility.

Recently, some novel polymorphisms have been identified in the tACE gene in European–Americans and African–Americans (Rieder et al., 1999). In this study, we screened 90 Singapore Chinese men, with different degrees of infertility, for five single nucleotide polymorphisms (SNPs) in the tACE gene, three of which were in the testis-specific exon 13, and three of which resulted in amino acid replacement. More than half

Screening of the ACE gene polymorphisms

PCR–RFLP and direct DNA sequencing were carried out to screen all five polymorphisms previously identified in the testis-specific exon 13 and in exon 26 (Rieder et al., 1999). Four polymorphisms, T10527C, A10578G, C23152A and G23202A, could not be identified in the study subjects. Only one polymorphism, C10514T, was detected in both infertile and control groups without any significant difference in frequency between the groups (P = 0.915; \( \chi^2 = 0.177 \); Table III). Out of 90 patients, nine were non-polymorphic, 43 were heterozygous and 38 were homozygous for this polymorphism. Among 84 controls, the respective figures were 10, 40 and 34. The prevalence of the polymorphism and the occurrence of homozygous individuals were also not significantly higher in patients than in controls (P = 0.809 and 0.878 respectively). Furthermore, the difference in frequencies of the polymorphism between each group of patients, as described above, was also not statistically significant (data not shown).

Discussion

Although ACE has primarily been studied in the context of its role in blood pressure regulation, it may have many other
found between known polymorphisms in the tACE gene and promoter regions of the gene in all the exons/introns as well as in the transcription regulating regions to screen infertile men for mutations/polymorphisms of the ACE protein in spermatozoa. However, there was no significant difference in the frequency of this polymorphism between patients and controls. There was also no statistical difference in the frequency of the polymorphism between the subgroups of patients. The frequency of the C allele (34.8%), which is in linkage disequilibrium with the Alu deletion (Rieder et al., 1999), was lower than the reported frequency of 55% in Caucasians (Barley et al., 1994; Bloem et al., 1996), suggesting ethnic differences in the distribution of this polymorphism. Our results are also consistent with those of previous studies, which have found that the frequency of the Alu deletion is considerably lower in Asians than in Caucasians (Doi et al., 1996; Hsieh et al., 2000).

Among the many polymorphisms identified in the ACE gene, the Alu insertion/deletion (I/D) polymorphism in intron 16 has gained immense attention, since it was found to be strongly associated with circulating ACE levels (Rigat et al., 1992). There is also evidence to suggest that the Alu deletion increases the risk for cardiovascular diseases (Evans et al., 1994; Soubrier et al., 1994), such as myocardial infarction (Cambien et al., 1992; Tietre et al., 1993) and diabetic nephropathy, and subsequently to the progression of other types of glomerular disease (Doria et al., 1994; Marre et al., 1994; Costerousse et al., 1997). However, at the same time, considerable negative evidence exists on this issue (Keavney et al., 2000) and this may be due to geographical and ethnic differences in the distributions of the polymorphism and disease prevalence (O'Malley et al., 1999). Moreover, in a genetic study, no significant relationship was found between this I/D genotype and ACE levels in human seminal fluid (Williams et al., 1995).

In conclusion, in this preliminary study, no association was found between known polymorphisms in the tACE gene and male infertility in Singapore Chinese subjects. Extensive studies to screen infertile men for mutations/polymorphisms in all the exons/introns as well as in the transcription regulating and promoter regions of the tACE gene are warranted in order to determine further the status of a defective tACE gene in male infertility. It would also be interesting to study the association of the I/D polymorphism in the tACE gene with levels of the ACE protein in spermatozoa.

References


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