Molecular studies on the role of the androgen receptor in male infertility have thus far concentrated solely on exonic regions of the androgen receptor gene. We have therefore screened for the first time the androgen receptor gene 5' untranslated region (nucleotides –153 to +237) in 240 males with idiopathic infertility for lesions which could potentially impair spermatogenesis. This region encompasses the androgen receptor gene promoter. DNA was extracted from blood leukocytes and the polymerase chain reaction was used to amplify the promoter region as two overlapping products. Single strand conformational polymorphism analysis was carried out on these products to screen for mutations. This analysis did not reveal the presence of any gross deletions or mutations. Our results thus preclude aberrations in the promoter region of the androgen receptor gene as a common factor in the aetiology of idiopathic male infertility.

Key words: androgen receptor/gene/promoter/infertility

Introduction

The development and maintenance of the male phenotype, including sperm production, are dependent on the androgens, testosterone and dihydrotestosterone, whose physiological effects are mediated by the androgen receptor (AR). The AR is a ligand-activated transcriptional regulatory protein belonging to the superfamily of nuclear receptors, members of which include the glucocorticoid, progesterone, oestrogen, and retinoic acid receptors. The main functional domains of nuclear receptors are the transactivation, the DNA-binding, and the ligand-binding domains. Androgens bind with high affinity to the steroid-binding pocket of the AR causing nuclear translocation and interaction with specific DNA response elements of target genes, thereby regulating their transcription (Beato, 1989; Quigley et al., 1995).

The absolute requirement of androgens for spermatogenesis suggests that subfunctional ARs could exist in cases of infertility where normal serum androgen concentrations are observed. Systematic examination of the androgen receptor in infertile males has revealed that long polyglutamine tracts in the transactivation domain of the AR increase by up to 4-fold the risk of defective spermatogenesis (Tut et al., 1997). Studies on the androgen receptor coding regions of infertile males have indicated that long polyglutamine tracts in the transactivation domain of the AR increase by up to 4-fold the risk of defective spermatogenesis (Tsukuda et al., 1997; Aiman and Griffin, 1982; Morrow et al., 1987), that mutations of the promoter region of the AR gene could hinder the transcription machinery thereby reducing the total amount of AR protein in the testes.

In the present study, we have screened for the first time the promoter region of the AR gene in a large cohort of infertile males to investigate the susceptibility of this region to potentially pathogenic mutations.

Materials and methods

Patients and controls

Recruitment of patients (n = 240) was from the infertility clinic of the National University Hospital, which is a tertiary referral centre for the region. For each patient, two independent samples taken two months apart were used for measurement of sperm density. Blood samples were also taken for measurement of testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, and where indicated for chromosomal analysis. Individuals displaying chromosomal disorders, hyperprolactinaemia, hypopituitarism, infective or obstructive syndromes of the genital tract were excluded. Table I depicts the patient clinical data. Healthy males with proven fertility (n = 30) were used as control subjects.

Extraction of genomic DNA and polymerase chain reaction (PCR)

Genomic DNA was extracted from leukocytes of peripheral blood from patients and controls as described previously (Yong et al., 1994). Nucleotides –153 to +237 of the AR 5’ untranslated region (UTR) were amplified as two overlapping fragments using PCR. The first fragment encompassed nucleotides –153 to +123 and was amplified using the primer pair: 5’ TCTCCAAAGCCACTAGGCAG 3’ (sense) and 5’ GTAGACGACTTGGAGC 3’ (antisense). The PCR reactions contained 100 ng genomic DNA, 50 pmol each primer, 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl2, 50 mM KCl, 1 unit Taq polymerase (Pharmacia, Uppsala, Sweden) and 5% glycerol (v/v). The second fragment encompassing nucleotides +23 to +237 was amplified by the primer pair: 5’ GGAGCCAGCTTGCTGGGAGAG 3’ and 5’ TGTACAGCACTGGAGC 3’. The PCR reactions contained 100 ng genomic DNA, 50 pmol each primer, 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl2, 50 mM KCl, 1 unit Taq polymerase (Pharmacia, Uppsala, Sweden) and 5% glycerol (v/v). The second fragment encompassing nucleotides +23 to +237 was amplified by the primer pair: 5’ GGAGCCAGCTTGCTGGGAGAG 3’ and 5’ TGTACAGCACTGGAGC 3’.
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Figure 1. The androgen receptor gene promoter region (nucleotides –153 to +237). Paired arrows denote respective positions of polymerase chain reaction (PCR) primers used to amplify the region as two overlapping DNA fragments. Highlighted are the positions of the homopurine stretch and GC-box (Faber et al., 1992), transcription-initiation sites I and II (TIS I and TIS II, Faber et al., 1990) helix loop helix DNA binding protein site 2 (HLH2, Takane and McPhaul, 1996), and the sequence predicted to form a stem-loop structure (Mizokami and Chang, 1994).

Table I. Patient clinical data

<table>
<thead>
<tr>
<th>Testosterone (ng/ml)</th>
<th>FSH (IU/l)</th>
<th>LH (IU/l)</th>
<th>Sperm concentration (× 10^6/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>3.2–9.5</td>
<td>0.8–4.7</td>
<td>2.0–6.9</td>
</tr>
<tr>
<td>Reference range</td>
<td>5.2 ± 3.94</td>
<td>6.44 ± 6.54</td>
<td>7.32 ± 5.69</td>
</tr>
</tbody>
</table>

3′ (sense) and 5′ ACCGAAGAGGAAAAGGCGACCTC 3′ (antisense). The reaction mixture was similar to that for the first fragment except that glycerol was added to 10% (v/v). Both fragments were amplified using the thermal profile: 98°C for 5 min, 60°C for 30 s, 72°C for 2 min (1 cycle), followed by 30 cycles of 96°C for 1 min, 60°C for 30 s, 72°C for 1.5 min. Amplified fragments were visualized by electrophoresis on a 1.4% agarose gel.

Single strand conformation polymorphism (SSCP) analyses

Amplified fragments were denatured by heating to 95°C for 5 min. After brief incubation on ice, samples were run on an 8% non-denaturing polyacrylamide gel containing 5% glycerol (v/v) at 12°C as described previously (Yong et al., 1994).

Results

Patient clinical data

Of the 240 patients analysed, 27% were azoospermic (no spermatozoa detectable), 44% were severely oligozoospermic (<5×10^6 spermatozoa/ml), 21% were moderately oligozoospermic (5–20×10^6 spermatozoa/ml) and 8% were normozoospermic (>20×10^6 spermatozoa/ml). The patients' hormonal parameters obtained are shown in Table I. Mean serum testosterone was within normal limits. The mean gonadotrophin concentrations (FSH and LH) were borderline high indicating some degree of testicular hypofunction in the patients.

Screening of the androgen receptor gene promoter region

Figure 1 shows the region of the AR gene promoter that we have screened for mutations in infertile men. This region was amplified in the form of two overlapping PCR products as smaller DNA fragments are more compatible for the efficient detection of mutations using the SSCP method (Sheffield et al., 1993). PCR amplification of the AR promoter region was successful for all 240 patients and 30 controls as judged by agarose gel electrophoresis, and did not reveal any gross aberrations in patients or controls (Figure 2). Using SSCP, no mobility shifts could be discerned between patients or controls (Figures 3A and 3B).

Discussion

Germline mutations in gene promoter/regulatory regions have been implicated in the pathogenesis of several disorders including β-thalassaemia, retinoblastoma, haemophilia B Leyden (reviewed in Cooper, 1992), coronary artery disease (Jansen et al., 1997), familial combined hyperlipidaemia (Yang et al., 1995) and atherosclerosis (Kontula and Ehnholm, 1996).
These mutations are typically associated with quantitative changes in transcriptional activity, attributable to altered binding of trans-acting protein factors to specific DNA sequences resident in the promoter region. The AR gene promoter region does not contain an easily distinguishable TATA or CAAT box, two common motifs recognized by components of the transcription machinery. It is characterized by a long (~90 bp) homopurine/homopyrimidine (pur/pyr) stretch located immediately upstream of a GC-box (nucleotides –59 to –32) which binds the constitutive activator SP1 (Faber et al., 1993). In addition to SP1, transcriptional activity is mediated by the cAMP-response element binding protein factor and AP2 (Mizokami et al., 1994). Transcriptional initiation occurs in two regions, AR-TIS I (transcription beginning at nucleotides +1, +2 or +3) and AR-TIS II (transcription-initiation at nucleotides +12 or +13) (Faber et al., 1991; Jenster et al., 1995). Deletion of the pur/pyr stretch has been shown to confer a 3-fold reduction in promoter activity in vitro (Mizokami et al., 1994). This could be due to the removal of multiple weak SP1 binding sites resident in this region, which may function as a reservoir channelling this transcription factor to the adjacent functional GC-box (Chen et al., 1997). Abolishment of the SP1 binding site by site-directed mutagenesis of the GC-box prevents transcription from AR-TIS II (Faber et al., 1993). Transcription from AR-TIS I is dependent upon unresolved sequences in the region –5 to +57 (Faber et al., 1993). Mizokami and Chang (1994) have identified a 181 bp region (+21 to +202) which they suggest plays an essential role in the induction of AR transcription. Particularly important was the sequence from +109 to +129 which is predicted to form a stem-loop secondary structure. More recently, Takane and McPhaul (1996) described two functional sequence elements that show similarity to the consensus binding site of the helix–loop–helix (HLH) family of proteins. These are located at positions –168 to –152 (HL1) and –19 to –14 (HL2) and their disruption leads to 40–60% reduction of promoter activity in vitro. Based on the above observations, we screened the 5’UTR (nucleotides –153 to +237) of the AR gene for mutations in the above sequence motifs which could possibly cause male infertility through reduction of transcriptional and/or translational competence. Using the PCR–SSCP technique, we were unable to detect mutations in this region. For the PCR fragment lengths screened in this study, detection of 70–95% of all mutations present would be expected (Orita et al., 1989; Michaud et al., 1992; Sheffield et al., 1993). Given the relatively large cohort screened in this study it is, however, most likely that mutations within the region examined are rare in infertile men. Additionally, this method was successfully used by Crocito et al. (1997) who detected two independent germline point mutations in the AR gene 5’ UTR (positions +2 and +214) of two men with prostate cancer. Our data thus supports the observation that promoter mutations, both in the AR gene (Crocito et al., 1997) and in general (Cooper, 1992) are infrequent. However, it is conceivable that mutations which result in subtle reduction of promoter activity are located in regions of the AR gene 5’ UTR not examined here. Mizokami et al. (1994) have identified numerous sequences located further upstream which bear similarity to the consensus DNA binding motifs for AP-1, cAMP response element binding protein, interleukin-6, NF-1 and retinoic acid receptor. A mutational analysis of these regions in the present cohort of patients is currently being undertaken.

References
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