Abstract

Context. Mutations in the androgen receptor (AR) gene result in androgen insensitivity syndrome (AIS). In milder forms of AIS, male infertility appears as primary or even sole symptom. Identification of such mutations is fundamental for accurate diagnosis and for appropriate genetic counseling.

Objectives. To determine the prevalence of known point mutations in the AR gene causing male infertility in Syrian azoospermic men.

Design. 15 known point mutations in the AR gene were screened in a cohort of 110 Syrian infertile men.

Subjects and methods. The study involved 173 Syrian infertile men suffering from non-obstructive azoospermia. Chromosome aberrations and Y microdeletions were excluded in 110 patients, which were further tested for point mutations in the AR gene by real time PCR or DNA sequencing.

Results. The prevalence of AR mutations in our cohort was 3.6% (4/110). We found two patients with the Ala474Val mutation and one patient bearing the Pro390Ser mutation. Furthermore, one patient had a new mutation, del 57Leu, described for the first time in an infertile man. None of the 50 fertile Syrian men had this mutation, indicating that it is not a sequence polymorphism in the Syrian population.

Conclusion. The del 57Leu mutation in the AR gene is a possible cause of idiopathic male infertility. Furthermore, the Ala474Val and Pro390Ser mutations (previously found in several infertile men in
Italy) might be significant markers for male infertility in Mediterranean populations.

**Key words:** Idiopathic male infertility, androgen receptor gene, point mutations.

**INTRODUCTION**

Idiopathic male infertility accounts for more than 30% of all male infertility cases (1). Among these cases, genetic analysis revealed a variety of causes, mostly chromosome aberrations or mutations in functional genes (2). Investigation of these causes is a major step in diagnosis and management of infertile men, and is crucial to prevent passing genetic defects to offspring in future generations by *in vitro* fertilization procedures.

The androgen receptor (AR; OMIM No. 313700) is essential for development and maintenance of the male phenotype and spermatogenesis. It is a transcription factor that, upon binding androgens, translocates to the nucleus, dimerizes and triggers complex molecular events that regulate the expression of target cognate genes known as androgen-responsive genes (3). The AR protein consists of four functional domains: the N-terminal transactivation domain (TAD), the DNA-binding domain (DBD), the hinge region and the ligand binding domain (LBD). It is encoded by a single copy gene on the X-chromosome at Xq11–q12. The AR gene consists of eight exons: exon 1 encodes the TAD, exons 2 and 3 encode the DBD, and exons 4-8 encode the hinge region and the LBD (4).

Mutations in the AR gene cause various forms of male pseudohermaphroditism known as androgen insensitivity syndrome (AIS; OMIM No. 300068). To date, more than 900 mutations have been documented (5). AR mutations that severely impair the amount, structure or function of the AR cause the complete AIS (CAIS), in which 46 XY individuals are completely feminized at birth, e.g. have normal female external genitalia with absence of female internal genitalia (testicular feminizing syndrome) (6). Mutations that do not completely disrupt AR function cause partial AIS (PAIS) in which various degrees of ambiguous genitalia occur, including partial labial-scrotal fusion, hypospadias, bifid scrotum and gynaecomastia (7). On the other hand, subtle mutations that result in minimal AR dysfunction lead to mild AIS (MAIS) where depressed spermatogenesis occurs without any abnormalities in secondary male sexual characteristics. MAIS has a mild presentation that often goes unnoticed and untreated. Even with semenological, clinical and laboratory data, it can be difficult to distinguish between men with and without MAIS, and thus a diagnosis of MAIS is not usually made without confirmation of an AR gene mutation (8).

Recent studies have established a link between specific AR mutations and idiopathic male infertility. The AR gene mutations database (5) contains at least 27 such mutations, 9 of which have been identified independently in unrelated infertile individuals, suggesting that some of these mutations may represent low frequency sequence variants rather than *de novo* mutations. For example, the Gln58Leu mutation has been identified in two unrelated infertile Finnish men (9).
and in at least one Italian man (10, 11). Furthermore, the Gln798Glu mutation has been identified in one Australian infertile man (12), in one infertile German man (13), and in at least one infertile Italian man (11). Identification of such mutations is fundamental for accurate diagnosis and for appropriate genetic counseling. The aim of this study was to investigate the prevalence of some of these frequently reported AR mutations among Syrian infertile men in an effort to provide preliminary information about which of these mutations is common in Middle Eastern populations.

MATERIALS AND METHODS

Subjects
173 Syrian infertile men suffering from non-obstructive azoospermia (consulting the Orient Hospital in Damascus in the period 2008-2009) were involved in this study. Patients (age 22-58 yrs) were clinically evaluated and their FSH, LH and testosterone (T) levels were assayed by the Modular E170 platform electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). The androgen sensitivity index (ASI) was calculated by multiplying the T and LH values. Cytological analysis was accomplished by a testicular biopsy procedure under local or general anesthesia, as it allows evaluation of tubular status and permits identification of both germ and Sertoli cells. Patients were interviewed for the presence of any cases of infertility in their families.

Karyotype analysis was performed on peripheral lymphocytes, and microdeletions of the azoospermia factor (AZF) region on the Y chromosome were analyzed by multiplex-PCR. Chromosome aberrations and Y microdeletions were excluded in 110 patients, and only these were further subjected to AR gene analysis. 50 Syrian men of proven fertility were also included as control subjects. This study has been approved by the Institutional Review Board of the Atomic Energy Commission of Syria (AECS). Patients and controls were informed about the study and a written consent was signed by every participant for blood sampling.

Isolation of genomic DNA
Genomic DNA was isolated from 2 mL peripheral blood using a conventional phenol/chloroform procedure. Briefly, red blood cells were lysed by the addition of 3 volumes of RBC lysis buffer (5 mM MgCl₂, 10 mM NaCl, 10 mM Tris-HCl pH7) and eliminated by 3 consecutive washes in the same buffer. Leukocyte pellets were resuspended in 1 mL water and lysed by the addition of 20 µL 10% SDS. After addition of 80 µL proteinase K (1 mg/mL), cell lysates were incubated at 54°C for 30 min under shaking, and then extracted with an equal volume of Tris-saturated phenol (pH 8). After three extractions with chloroform, genomic DNA was precipitated by the addition of 2.2 mL of cold ethanol, washed with 75% ethanol, air dried and dissolved in 500 µL high quality water.

PCR amplification of exon 1 of the AR gene
Exon 1 (~1600 bp) is long and difficult to amplify as one single PCR fragment, especially because it contains two polymorphic amplification refractory GC-rich repeat regions ([CAG]ₙ at 5’ and [GGC]ₙ at 3’). Therefore, we separately
amplified this exon as 3 overlapping PCR fragments using a set of six primers (Table 1): fragment A (~288 bp, containing the [CAG]_n repeat), fragment B (744 bp) and fragment C (~702 bp, containing the [GGC]_n repeat).

### Multiplex-PCR amplification of exons 2-8 of the AR gene

We chose multiplexing PCR reactions in order to reduce labor and number of PCR tubes. The first multiplex-PCR reaction amplified exons 2, 3 and 6, whereas the second reaction amplified exons 4, 5, 7 and 8 of the AR gene. Each multiplex-PCR reaction (25 µl final volume) contained the following components (final concentrations): buffer (1x), MgCl₂ (3 mM), dNTPs (200 µM each), DMSO (5%), primers (300 nM each, see Table 2), Taq polymerase (1 unit GoTaq, Promega) and gDNA (30-100 ng). PCR cycling conditions were as follows: initial denaturation 94° C for 3 min, then 35 cycles of 94° C for 45 sec,

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence (5’--&gt;3’)</th>
<th>PCR fragment</th>
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<tbody>
<tr>
<td>AR-1F</td>
<td>TCCAGAATCTGTCTCCAGAGCGTGTC</td>
<td>A (~288 bp)</td>
</tr>
<tr>
<td>AR-2R</td>
<td>GTGTGAAGGTGGCTGTTCCTCAT</td>
<td></td>
</tr>
<tr>
<td>AR-3F</td>
<td>TCCCCAAGCCCATCGTAGAG</td>
<td>B (744 bp)</td>
</tr>
<tr>
<td>AR-6R</td>
<td>GTAGACGGCAGTTCAAGTGTCC</td>
<td></td>
</tr>
<tr>
<td>AR-7F</td>
<td>ACCAAAGGGCTAGAAGGCGA</td>
<td>C (~702 bp)</td>
</tr>
<tr>
<td>AR-12R</td>
<td>CCGAAAGGCACATTCTGGGAAG</td>
<td></td>
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</tbody>
</table>

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<tr>
<th>Primer ID</th>
<th>Sequence (5’--&gt;3’)</th>
<th>PCR fragment</th>
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</thead>
<tbody>
<tr>
<td>AR-201F</td>
<td>TTCAGTGACATGTGTGGCCATTGG</td>
<td>Exon 2 (260 bp)</td>
</tr>
<tr>
<td>AR-202R</td>
<td>GGTTAGTGCTCTCTCTGGGAAG</td>
<td></td>
</tr>
<tr>
<td>AR-301F</td>
<td>GTTGGTGCCATACCTGTCAC</td>
<td>Exon 3 (333 bp)</td>
</tr>
<tr>
<td>AR-302R</td>
<td>TCTGGTCTAAAGAGAGACTAG</td>
<td></td>
</tr>
<tr>
<td>AR-401F</td>
<td>TTTGGGTGGTACGGATATTTGGGATG</td>
<td>Exon 4 (634 bp)</td>
</tr>
<tr>
<td>AR-402R</td>
<td>ACTTGTAACAAATCCCCCTTCCCAAGG</td>
<td></td>
</tr>
<tr>
<td>AR-501F</td>
<td>GATCCTTGGGAGTCCGGAATAC</td>
<td>Exon 5 (330 bp)</td>
</tr>
<tr>
<td>AR-502R</td>
<td>CATCACCATCACCACCAACCAGGT</td>
<td></td>
</tr>
<tr>
<td>AR-601F</td>
<td>CCCTCATTCTTTTCTTCCTG</td>
<td>Exon 6 (196 pb)</td>
</tr>
<tr>
<td>AR-602R</td>
<td>GGCAATCCTCTGCACCTTCTAG</td>
<td></td>
</tr>
<tr>
<td>AR-701F</td>
<td>ACTTGACTCTCTTTCAGATCGGATCCA</td>
<td>Exon 7 (496 bp)</td>
</tr>
<tr>
<td>AR-702R</td>
<td>AAAGCCAGGGGGATGGAAGC</td>
<td></td>
</tr>
<tr>
<td>AR-801F</td>
<td>TTCAGTGACATGTGGTGGCATTTGG</td>
<td>Exon 8 (277 bp)</td>
</tr>
<tr>
<td>AR-802R</td>
<td>GGTTAGTGCTCTCTGGGAAG</td>
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58°C for 45 sec and 72°C for 55 sec (final elongation cycle at 72°C for 5 min). After electrophoresis in a 2% agarose gel containing ethidium bromide, the amplified products were visualized and photographed using a UV-transilluminator.

**Screening of point mutations in the AR gene by real time PCR**

Single nucleotide changes in the AR gene corresponding to 14 known mutations (Asn233Lys, Leu270Phe, Pro340Leu, Glu353Gln, Pro390Ser, Ala474Val, Gly506Asp, Leu547Phe, Arg607Gln, Ala645Asp, Phe747Ile, Gln798Glu, Leu821Val, Met886Val) were detected using 5'-FAM labeled Taqman MGB-probes (minor groove binding probes) designed to hybridize to AR DNA segments encompassing each one of the tested mutations. For each mutation tested, two Taqman MGB-probes were designed; one probe was fully identical with the sequence of the “wild type” allele, whereas the other probe was fully identical with the sequence of the mutant allele.

In order to detect a mutation in a patient, two real-time PCR reactions were set up, one reaction contained a probe specific for the wild type allele, and the other contained a probe specific for the mutant allele. Each real-time PCR reaction (20 µl final volume) contained the following components (final concentrations): buffer (1x), MgCl₂ (4 mM), dNTPs (200 µM each), DMSO (5%), two primers (300 nM each) flanking the tested mutation, MGB-probe (120 nM each), 4 µl diluted (1:100) PCR products, Taq polymerase (1 unit GoTaq, Promega). Real-time PCR was performed using a Quantica instrument (Technne, England) with the following cycling conditions (30 cycles): 94°C for 15 sec, 60°C for 1 min (annealing and extension in one step). Fluorescence acquisition was performed in the FAM-channel at the end of each cycle. Data were analyzed using Quansoft software (Technne).

For each reaction tube, amplification curves were plotted against cycle number. The shape of both amplification curves were compared to each other. The presence of a mutation was suspected if the amplification curve corresponding to a “mutant” probe showed a significant increase in fluorescence while that of the

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Table 3. Endocrine data of the four azoospermic patients with AR mutations (ranges of normal hormonal values are indicated in parentheses)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>AR mutation</th>
<th>FSH (1.5-15 U/L)</th>
<th>T (8.3-38.2 nmol/L)</th>
<th>LH (1.4 -7.7 U/L)</th>
<th>ASI* (7-135 Uxnmol/L²)</th>
<th>Testes biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>#07</td>
<td>del 57Leu</td>
<td>24.9</td>
<td>20.2</td>
<td>4.83</td>
<td>97.6</td>
<td>SCOS**</td>
</tr>
<tr>
<td>#64</td>
<td>Pro390Ser</td>
<td>27</td>
<td>13.4</td>
<td>20.4</td>
<td>273.4</td>
<td>SCOS**</td>
</tr>
<tr>
<td>#32</td>
<td>Ala474Val</td>
<td>11.7</td>
<td>9.1</td>
<td>5.4</td>
<td>49.1</td>
<td>SCOS**</td>
</tr>
<tr>
<td>#74</td>
<td>Ala474Val</td>
<td>24.6</td>
<td>16.0</td>
<td>7.3</td>
<td>116</td>
<td>SCOS**</td>
</tr>
</tbody>
</table>

*Androgen sensitivity index (T x LH).
** Sertoli cell-only syndrome.
“wild type” probe did not increase significantly with increasing cycle number. Suspected mutations were retested by real-time PCR and results were confirmed by DNA sequencing using BigDye Terminator ddNTPs technology.

Screening for the Gln58Leu mutation in the AR gene by DNA sequencing

Since the nucleotide exchange (CAG/CTG) leading to the Gln58Leu mutation is situated at the first codon of the [CAG]_n repeat region, which is directly preceded by three consecutive CTG (Leu) codons, it is difficult to detect this mutation using DNA probes. Therefore, the Gln58Leu mutation was screened by direct DNA sequencing of a PCR product containing the CAG repeat region (fragment A, see PCR amplification of exon 1 of the AR gene).

PCR products were sequenced in the forward direction using primer AR-1F (situated 80 bp upstream of the first CAG codon of the CAG-repeat). Sequencing reactions were set up using BigDye Terminator chemistry (Applied Biosystems, version 3.1) following the manufacturer’s instructions.

RESULTS

Screening of 110 infertile Syrian men for 14 known mutations (see Materials & Methods) in the AR gene by real time PCR revealed the Pro390Ser mutation [c.1530 C>T(p.Pro390Ser)] in one patient ( #64), and the Ala474Val mutation [c.1783 C>T(p.Ala474Val)] in two unrelated patients (#32 and #74) (Fig. 1). The existence of these mutations was confirmed by DNA sequencing (Figs 2 & 3). None of the 14

Figure 1: Detection of Pro390Ser and A474V point mutations in the AR gene by real time PCR in patients #64 and #74. Amplification curves corresponding to “wild type” probes are in black, while those corresponding to “mutant” probes are in red. A and C: normal fertile control. B and D: infertile patients bearing the Pro390Ser and A474V mutations, respectively. Note the significant increase in fluorescence of mutant probes (red curves) in patients #64 and #74, indicating the presence of the Pro390Ser and A474V mutations in these patients, respectively.
tested mutations were detected in any of the remaining 107 patients.

Sequencing of the 5’ region of the CAG-repeat in AR exon 1 did not reveal the Gln58Leu mutation in any of the 110 patients. However, we discovered an unexpected sequence variant (c.532_534del CTG(p.Leu57del)) situated just before of the CAG-repeat in one infertile man (patient #7). This mutation (deletion of a CTG triplet) leads to the deletion of one leucine residue out of four consecutive leucines immediately preceding the polyglutamine tract of the AR receptor (del Leu57, Fig. 4). Sequencing of the same DNA region of 50 fertile Syrian men did not reveal this variation, indicating that del Leu57 is not a sequence polymorphism

Figure 2: Confirmation of the Pro390Ser mutation in patient #64 by DNA sequencing. (A) Normal fertile control. (B) Patient #64 showing a cytidine-to-thymidine substitution (CCG?TCG) at codon 390, predicatively altering the normally present proline to serine at amino acid position 390 within the transactivation domain of the AR protein. The arrow points to the mutated nucleotide.

Figure 3: Confirmation of the Ala474Val mutation in patient #32 by DNA sequencing. (A) Normal fertile control. (B) Patient #32 showing a cytidine-to-thymidine substitution (GCG?GTG) at codon 474, predicatively altering the normally present alanine to valine at amino acid position 474 within the transactivation domain of the AR protein. The arrow points to the mutated nucleotide. The same mutation was found and confirmed in patient #74 (data not shown).
in the Syrian population.

Of the four azoospermic patients in whom mutations within the AR gene were identified, patient #64 (bearing the Pro390Ser mutation) showed a clear MAIS phenotype (sparse facial and body hair with mild gynecomastia). The other 3 patients showed signs of MAIS, although to a lesser extent compared to patient #64. Table 3 summarizes clinical and endocrine data of these four patients. None of these four patients had a family history of male infertility.

DISCUSSION

We investigated the presence of 15 point mutations in the AR gene known to cause male infertility in a cohort of 110 infertile Syrian patients with no chromosome aberrations or AZF microdeletions. We found 3 different mutations in 4 unrelated patients, with a frequency of 3.6% (4/110). This is slightly higher than the generally accepted AR mutation frequency (~2%) in men with azoospermia or severe oligospermia (11, 13).

We are the first to report the del Leu57 mutation in the context of male infertility. It is highly unlikely that this mutation represents a sequence polymorphism in the Syrian population, as none of the 50 fertile Syrian men had this sequence variant. Moreover, none of numerous previous studies analyzing the same DNA region by DNA sequencing revealed this mutation in fertile controls (14-17).

The del Leu57 (as germline mutation) was previously reported only once in a patient suffering from testicular cancer (18). This patient reportedly sought only semen cryopreservation and was obviously fertile. Conversely, our patient (#7) bearing this mutation had no testicular cancer, but was azoospermic and
showed typical signs of MAIS. Fine needle aspiration (FNA) of this patient showed a maturation arrest at the spermatocyte level. Thus, we assume that the impact of the del Leu57 mutation on spermatogenesis can obviously vary in severity depending on the genetic background.

As shown in Fig. 4, the del Leu57 mutation removes one leucine residue out of a stretch of 4 consecutive leucines situated directly before the polyglutamine tract (CAG-repeat). Computerized analysis using the “PolyPhen-2” software (19) classified the del Leu57 as “benign”, i.e. predicted to have no or only a minor deleterious effect on the protein structure. This is consistent with the mild phenotype observed in the affected patient. Interestingly, insertion (instead of deletion) of a leucine at residue 57 of the AR protein (also equivalent to the Gln58Leu mutation) was previously reported to cause mild AIS and male infertility (9, 11). Taken together, these results indicate that the length of the (Leu)₄ stretch preceding the polyglutamine tract is critical for full AR functionality and that adding or deleting one of these four leucine residues can lead to mild AIS and male infertility.

It is noteworthy that all three mutations found in our cohort are situated in the TAD of the AR receptor. In particular, amino acids Pro390 and Ala474 are situated in the transcription activation unit 5 (TAU-5) (4). Therefore, alterations in these amino acids are expected to affect protein-protein interactions of the AR receptor with coregulators, leading to deregulation of the expression of androgen responsive genes.

The Pro390Ser mutation was previously reported in at least one Italian and two German men affected with MAIS (11, 13), but it has never been demonstrated to cause CAIS. Moreover, the phenotype of our patient (#64) bearing the Pro390Ser mutation is also consistent with MAIS. Thus, we assume that this mutation leads generally to a mild phenotype and male infertility. Although the Pro390Ser mutation was previously reported in the context of CAIS (20), it is very unlikely that this mutation is the cause of the severe phenotype because the 46, XY female patient bearing this mutation had also a second mutation (Arg855Gly) in the LBD of the AR receptor probably causing inability to bind androgen. Since serine and proline are similar in size and have no electric charge, the Pro390Ser mutation could result in a minor defect of the AR protein. On the other hand, replacement of proline at the same site by arginine (Pro390Arg), which is much larger than proline and contains a basic group, leads to a severe phenotype of complete feminization (21).

To our knowledge, the Ala474Val mutation was previously reported only in Italy in four unrelated men with MAIS (10, 11). The mild phenotype of this mutation could be explained by the similar aliphatic nature of alanine and valine. The fact that we have found the same mutation (Ala474Val) in two independent infertile Syrian men in a relatively small cohort suggests that this mutation could represent a low frequency sequence variant rather than a
random mutation that occurred *de novo* through independent events. Unfortunately, maternal blood samples of both patients were not available, and we could not confirm that these patients inherited the mutation from their respective mothers. Furthermore, neither patient reported any family history of male infertility. It is noteworthy that both patients have the same CAG/GGN repeat numbers (19/23), although further investigation is needed to assess if their haplotypes surrounding the AR gene are identical too. Since the Ala474Val is obviously common among Italian infertile men (10, 11), and in view of well-documented exchanges between Mediterranean populations in History, it is possible that the Ala474Val mutation emerged once at some time point and spread by a “founder effect”. Therefore, it would be interesting to know if these infertile Italian and Syrian patients bearing the Ala474Val mutation also share the same haplotype surrounding the AR gene.

It has been suggested previously that an elevated androgen sensitivity index (ASI = the multiplication product of LH and testosterone) is an indication of androgen insensitivity (22). In milder forms, infertile men at risk for AR mutations may be distinguished on the basis of a high ASI, although this index lacks specificity and sensitivity (13). In our study, out of four patients found to carry AR mutations, only one patient (#64 carrying the Pro390Ser mutation) showed an elevated ASI, while the others had normal values (Table 3). This indicates that the ASI criterion is not a reliable marker to predict the existence of AR mutations in men with idiopathic infertility. Nevertheless, we can assume that specific AR mutations causing a more severe phenotype have a greater tendency to cause an elevated ASI. This could be the case for the Pro390Ser mutation, which has been found in two German infertile men who both showed, like our patient #64, elevated ASI values (13).

**In conclusion,** we have identified a new mutation in the AR gene (del Leu57) as a possible cause of male infertility. Moreover, the identification of the Ala474Val mutation in two unrelated infertile Syrian men suggests that this mutation (previously found in at least 4 unrelated infertile men in Italy) might be a significant marker for male infertility in Mediterranean populations. The same conclusion could also be valid for the Pro390Ser mutation previously found in several unrelated infertile men in Italy. Analysis of the whole coding region of the AR gene in a greater number of patients is still necessary in order to build a complete picture of AR mutations in Syrian men. This will provide the basis for appropriate molecular analysis of the AR gene and subsequent genetic counseling useful for infertile couples benefiting from assisted reproductive techniques.

**Acknowledgements**

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**Conflict of interest.**

The authors did not report any conflict of interest.
References