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Androgen Receptor Genotyping in a Large Australasian Cohort with Androgen Insensitivity Syndrome; Identification of Four Novel Mutations

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ABSTRACT

We genotyped the androgen receptor (*AR*) gene in 31 Australasian patients with androgen insensitivity syndrome (AIS). The entire coding region of *AR* was examined including analysis of polymorphic CAG and GGN repeats in all patients. *AR* defects were found in 66.7% (6/9) of patients with complete AIS (CAIS) and 13.6% (3/22) of patients with partial AIS (PAIS). A novel deletion (N858delG) leading to a premature stop codon was found in CAIS patient PI. CAIS patient P2 has a novel deletion (N2676delGAGT) resulting in a stop at codon 787. These mutations would result in inactivation of AR protein. A novel insertion of a cysteine residue in the first zinc finger of the AR DNA-binding domain (N2045_2047dupCTG) was found in CAIS patient P3. PAIS patient P4 has a novel amino acid substitution (Arg760Ser) in the AR ligand binding domain, which may impair ligand binding. Five patients were found to have previously reported *AR* mutations and no mutations were identified in the remaining patients.

KEY WORDS

androgen insensitivity syndrome (AIS), androgen receptor (AR), mutation, human, paediatric

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INTRODUCTION

Androgen insensitivity syndrome (AIS) (incidence 1:20,000-64,000 male births) is an X-linked recessive disorder occurring in individuals with an XY karotype and varying degrees of impaired male genital development. Complete AIS (CAIS) is characterised by a complete absence of virilisation. Affected individuals have female external genitalia and breast development. Partial AIS (PAIS) comprises a wide phenotypic spectrum ranging from perineoscrotal hypospadias, bifid scrotum, and cryptorchidism, to marked undervirilisation including clitoromegaly and labio-scrotal fusion.

During embryogenesis androgens are critical for the virilisation of Wolffian duct structures and external genitalia. Androgen action is mediated by the androgen receptor (AR), a nuclear transcription factor. AR is encoded by a single copy gene located on a highly conserved region of the X chromosome (Xq11-12)[1]. *AR* mutations are the molecular cause of androgen insensitivity. Mutations have been identified throughout the coding region of the *AR* gene[2].

The AR is a member of the nuclear receptor family. Binding of androgen to the AR induces a conformational change that facilitates receptor dimerization, nuclear transport and interaction with target DNA, culminating in regulation of target gene transcription[3]. AR consists of three major functional regions, comprising the amino-terminal transactivation domain (TAD), a DNA-binding domain (DBD), and the carboxyl-terminal ligand-binding domain (LBD)[3,4]. Protein-protein interactions between domains and with co-regulatory proteins are also important in AR function.

Identification of *AR* mutations in patients with AIS, and *in vitro* molecular studies evaluating the functional effect of mutant AR for genotype/phenotype correlations are crucial for understanding this condition.

The TAD of the *AR* gene contains two polymorphic trinucleotide repeat regions. The glutamine (Gln) repeat region consists of a length of trinucleotide repeats located towards the 5′ end of exon A; (CAG)*x* CAA, *x* = #CAG, mean #CAG = 21, range #CAG = 9-38 (Caucasians)[5-7]. The glycine (Gly) repeat region consists of trinucleotide repeats located towards the 3′ of exon A; (GGN)*y* or [(GGT)3 GGG (GGT)2 (GGC)*x*], *x* = #GGC, *y* = #GGN, mean #GGN = 22, range #GGN = 10-31 (Caucasians)[8-10].

Abnormal expansion of the Gln repeat leads to X-linked spinal muscular dystrophy (SBMA), a neuromuscular disease associated with endocrinological abnormalities such as low virilisation, oligospermia or azoospermia, and testicular atrophy[11]. All patients with SBMA have an expanded Gln repeat (range 40-62 repeats)[12], and some patients show clinical signs of PAIS including elevated testosterone and/or LH levels[13]. The degree of endocrine dysfunction in SBMA is correlated with the number of Gln repeats[13].

It has also been shown that normal polymorphic size variation of the Gln repeat region is associated with undermasculinisation[*], male infertility[9,14-17], benign prostatic hyperplasia[18], prostate cancer[5,19] and endometrial cancer[20]. Normal polymorphic size variation of the Gly repeat is also associated with disease. Sasaki *et al.* report an increased risk of endometrial cancer with increased Gly repeats (GGC>17)[21], and there are reports of an association between prostate cancer and short Gly repeats (GGC<16)[5,19,22]. Some studies on the joint distribution of Gln and Gly repeats in male infertility[23] prostate cancer[5,22,24] and esophageal cancer[25] have been undertaken with no obvious trends in the data.

AR genotyping is important clinically. Characterisation of the molecular mechanisms of AR dysfunction is helpful in directing hormonal therapy. For example, the mutation Met807Thr disrupts testosterone binding of the receptor to a greater extent than dihydrotestosterone (DHT)[26]. Thus a patient with this mutation responds favourably to DHT treatment. This finding has been used to restore male genital development in an infant with PAIS[4,26]. Identification of *AR* mutations and characterisation of the inheritance of the mutant allele provide valuable information for genetic counselling of affected families.

We have used the method of genomic DNA PCR sequencing to analyse the *AR* genes of 31 patients with a provisional diagnosis of AIS. The 31 patients with AIS were referred from paediatric endocrinologists across Australia. As part of the study, we examined the length of the Gln repeat region in order to examine the role of *AR* Gln repeats in AIS. Gln repeat regions in family members were also examined in order to determine the pattern of inheritance of the mutant *AR* allele. We report here the spectrum of mutations identified in a large Australian cohort with clinical features consistent with AIS.

METHODS

Patients

All patients were genotyped by the Mater Molecular Genetics Laboratory, Mater Pathology, Mater Misericordia Hospital, South Brisbane, QLD. The Mater Molecular Genetics Laboratory is accredited by the National Australia Testing Authority (NATA). Patient genotyping by the Mater Molecular Genetics Laboratory was approved by the Mater Health Services Human Research Ethics Committee (MHSHREC). All patients undergo testing as part of routine clinical diagnosis and assessment; clinicians obtain consent from patients prior to genetic testing.

Eleven patients with a provisional diagnosis of CAIS and 23 patients with a provisional diagnosis of PAIS were referred to our diagnostic service for detection of *AR* gene mutations (Table 1, patients P1 to P31). All patients were Caucasians from various regions of Australasia and were referred over a period of approximately 8 years from 1996 to 2004. At that time we were not aware of any other Australian diagnostic service performing *AR* genotyping in cases of AIS. Clinical data for 31 index patients with AIS (P1-P31) and three affected family members (A2, A6 and A25) are summarised

in Table 1.

Several methods of grading the clinical forms of AIS are in use. One method devised by Prader classifies the genital phenotype on a grade of 1-7 in order of increasingly female phenotype and thus severity of AIS[3]. A more recent grading system, devised by Ahmed *et al.*[27], was used to assess the phenotype of the patients reported herein. In this system specific clinical features are assessed resulting in a masculinisation score, a score of 12 representing normal male development (see Table 1).

An XY karyotype was recorded in all 34 affected patients. All patients have had endocrine investigations demonstrating normal testosterone production.

Patients P2, P6 and P10 had a family history of AIS. No other patients had a family history of AIS at the time of testing. In many cases family members were not available for testing. All genetic results from available family members and carriers are described in the Results section.

Genomic DNA PCR sequencing

Genomic DNA was extracted from whole blood (peripheral leukocytes)[28] and subjected to PCR analysis. *AR* gene specific primers used for PCR amplification and DNA sequencing are described in Table 2.

Exons B-H of the *AR* gene were PCR amplified and sequenced; regions amplified included exonic sequences, splice sites and the 3′-untranslated region (UTR). If a deleterious mutation was found, the Gln repeat region in exon A was PCR amplified and sequenced, followed by reporting of results. For patients tested more recently the method was modified so that the exon A Gly repeat region was also PCR amplified and sequenced, in addition to the Gln repeat region.

If no deleterious mutation could be detected in exons B-H, then the entire coding region of exon A was amplified and sequenced, including Gln/Gly repeat regions, splice sites and the proximal promoter region (-160 base pairs [bp] to the transcription start site [TSS]).

Exon B-H PCRs were performed using TaqF1 DNA Polymerase System (Cat#TAQ-1, Fisher Biotec, Sydney, Australia). Exon B-H PCRs were sequenced using the same primers as used for PCR amplification. Exon A PCRs were performed using GC-Rich PCR System (Cat#2140306, Roche/ Boeringer Mannheim, Mannheim, Germany). Exon A PCRs were sequenced using the same primers as used for PCR amplification in addition to nested sequencing primers. See Table 2 for details of PCR conditions. All PCR reactions contained 0.2 μM nucleotide mix and 0.5 μM of each primer .

PCR products were purified with High Pure PCR Product Purification Kit (Cat#1732668 Roche/Boeringer Mannheim, Mannheim, Germany), then sequenced using Big-Dye-Terminator-Cycle-Sequencing-Ready-Reaction (Cat#4303149, Applied Biosystems, Scoresby, Victoria, Australia). Analysis was performed at the Griffith University Sequencing Facility (Griffith University, Nathan, QLD, Australia). All sequences were compared to consensus sequences for *AR* genomic and mRNA sequences (Genbank Refseq NM000044).

All identified mutations were confirmed by repeating the entire procedure (DNA extraction, PCR and sequencing) on a second blood sample obtained from the patient.

RT-PCR sequencing

In three patients genital skin fibroblasts were available for tissue culture. Total RNA was extracted from cultured genital skin fibroblasts using RNeasy Mini Kit (Cat#74104, Promega, Sydney, Australia). Reverse transcription was carried out using the gene specific primer AR-H-3202-21-AS located in the 3′-UTR of the *AR* mRNA transcript. Reverse transcription was performed using the Reverse Transcription System (Cat#A3500, Promega, Sydney, Australia) according to the manufacturer's instructions. PCR amplification of cDNA was performed as described above using TaqF1 DNA Polymerase System (Cat #TAQ-1, Fisher Biotec). See Table 2 for details of primers and PCR conditions. The region of cDNA analysed included the 3′ end of exon A, exons B-H and the 3′-UTR. PCR products were purified and sequenced as described above using the same primers as used for PCR amplification in addition to nested sequencing primers.

TABLE 1

Clinical and genetic data for 34 patients with androgen insensitivity syndrome (AIS) and three affected family members

Pat ID	AIS Diag	Clinical presentation of AIS	Sex of rearing	Masc score /12	*SF* /3	*MP* /3	*UM* /3	*RG* /1.5	*LG* /1.5	AR gene mutn[7]	Gln #CAG =?	Gly #GGN =?	Abs. value #CAG - #GGN
P1	C	Amniocentesis XY, phenotype at birth female, cleft palate. 21*dpp* bilateral inguinal hernia repair.	F	1	0	0	0	0.5	0.5	*N*858delG	25	23	2
P2	C	Presented at birth, bilateral testes palpable over pubis.	F	2	0	0	0	1	1	*N*2676delGAGT	20	–	–
A2	C	Presented 17 yr, primary amenorrhoea. Laparoscopy revealed lack of uterus, intra-abdominal testes. (Mat. aunt of P2)	F	1	0	0	0	0.5	0.5	*N*2676delGAGT	20	–	–
P3	C	Presented 4 weeks, osteomyelitis of ankle. Bilateral inguinal hernia and bilateral palpable gonads noted. Ultrasound revealed lack of Müllerian structures.	F	2	0	0	0	1	1	*N*2045_2047dupCTG	25	24	1
P4	P	Presented at birth, ambiguous genitalia.	M	5.5	3	0	0	1	1.5	R760S	27	23	4
P5	C	Presented 10 mo, inguinal masses. Ultrasound revealed lack of Müllerian structures. Bilateral testes removed.	F	2	0	0	0	1	1	P892L	21	–	–
P6	C	Presented 17 yr, primary amenorrhoea. Ultrasound revealed lack of Müllerian structures. Inguinal testes removed.	F	2	0	0	0	1	1	R752Q	19	–	–
A6	C	Presented 7 mo, inguinal hernia. 19 mo, labial/vaginal adhesions. (Niece of P5)	F	2.5	0.5	0	0	1	1	R752Q	19	–	–
P7	P	Presented 3*dpp*, ambiguous genitalia. Ultrasound revealed lack of Müllerian structures.	M	4.5	2	1	0	0.5	1	G568W	22	–	–
P8	P	Presented at birth, ambiguous/undermasculinised genitalia.	M	N/A						R871G	26	24	2
P9	C	Presented 3 months, mild facial dysmorphology. Karyotype analysis XY. Examination revealed lack of uterus and abdominal testes.	F	1	0	0	0	0.5	0.5	Y571C	26	24	2
P10	C	Tested due to family history of AIS.	F	N/A							29	10	19
P11	P	Presented at birth, microphallus and hypospadias.	M	8	3	1	1	1.5	1.5		34	19	15
P12	P	Presented at birth, hypospadias.	M	9	3	2	1	1.5	1.5		29	24	5
P13	C	Clinical details unavailable.	F	N/A							23	15	8
P14	P	Hypospadias, scrotal fusion.	M	6	0	3	0	1.5	1.5		22	16	6
P15	P	Presented at birth, ambiguous genitalia.	M	6	3	1	1	0.5	0.5		19	24	5
P16	P	Presented at birth, microphallus and hypospadias	M	5.5	1	1	0.5	1.5	1.5		19	24	5
P17	C	Presented 7 yr, clitoromegaly.	F	3	0	1	0	1	1		21	24	3

P18	P	Presented at birth, ambiguous genitalia and bilateral testes	F	**4**	0	1	0	1.5	1.5	**26**	**23**	3
P19	P	Presented at birth, ambiguous genitalia.	M	**5.5**	2	1	0	1.5	1	**22**	**24**	2
P20	P	Presented at birth, severe penioscrotal hypospadias.	M	**8.5**	2.5	2.5	0.5	1.5	1.5	**21**	**23**	2
P21	P	Ambiguous genitalia.	F	7	2	1	1	1.5	1.5	**20**	**23**	3
P22	P	Microphallus and hypospadias.	M	7	2	1	1	1.5	1.5	**20**	**19**	1
P23	P	Ambiguous genitalia.	M	7.5	2	2	1	1	1.5	**22**	**24**	2
P24	P	Ambiguous genitalia.	M	9	3	2	1	1.5	1.5	**22**	**23**	1
P25	P	Presented at birth, bilateral undescended testis and microphallus	M	9	3	1	3	1	1	**24**	**25**	1
A25	P	(Twin of P25, same presentation)	M	9	3	1	3	1	1	**24**	**25**	1
P26	P	Presented at birth, undervirilised genitalia.	M	9	3	2	1	1.5	1.5	**24**	**23**	1
P27	P	Presented at birth, ambiguous genitalia.	M	**9.5**	3	2	1.5	1.5	1.5	**25**	**23**	2
P28	P	Micropenis.	M	8	0	2	3	1.5	1.5	**26**	**24**	2
P29	P	Presented at birth, ambiguous genitalia.	M	8	2.5	2	0.5	1.5	1.5	**23**	**24**	1
P30	P	Hypospadias, scrotal fusion.	M	6	0	3	0	1.5	1.5	**22**	**23**	1
P31	P	Presented 11 yr, tall stature, micropenis.	M	7	0	1	3	1.5	1.5	**21**	**19**	2

AIS Diag = AIS diagnosis, complete AIS (CAIS) (C) or partial AIS (PAIS) (P).
Sex of rearing, male (M) or female (F). Note that all 34 patients had 46,XY karyotype.
Masc. score = masculinisation score, score/12 (sum SF + MP + UM + RG + LG) Five individual criteria are assessed and individual scores assigned as shown. These are added to give a masculinisation score (/12)[27].
SF = scrotal fusion, score/3: 0 = no, 3 = yes.
MP = microphallus, score/3: 0 = yes, 3 = no.
UM = urethral meatus, score/3: 0 = perineal, 1 = penile, 2 = glandular, 3 = normal.
RG = right gonad, score/1.5: 0 = absent, 0.5 = abdominal, 1 = inguinal, 1.5 = scrotal.
LG = left gonad, score/1.5: 0 = absent, 0.5 = abdominal, 1 = inguinal, 1.5 = scrotal.
Gln #CAG =?: exon A Gln repeat, (CAG)x CAA.
Gly #GGN=?: exon A Gly repeat, (GGT)3 GGG (GGT)2(GGC)x.
Abs. value, #CAG - #GGN = absolute value of the difference in #CAG and #GGN.
(Note that the Gly repeats were only routinely assayed for patients tested later in the study.)
N/A = data not available. Detailed phenotypic data were not provided by the referring clinician.
[T] The five AR gene mutations in bold are novel mutations.

TABLE 2

Oligonucleotide primers and PCR conditions

Exons	Primer label *	Sequence 5′–3′	PCR product size (bp)	PCR annealing temp (*x*°C)	PCR cycling times (sec ["]) 94°C; *x*°C; 72°C x 34 cycles	PCR [Mg^{2+}] (mM)	PCR [GC-rich resn. soln.] (M)
Genomic DNA PCR amplification							
A	AR-A-172-25-S	GCACGAGACTTTGAGGCTGTCAGAG	1046	60	60"; 60"; 60"	2	1
	AR-A-1218-20-AS	CCAATGGGGCACAAGGAGTG					
A	AR-A-953-24-S	GGAAGCAGTATCCGAAGGCAGCAG	755	65	60"; 60"; 60"	2	1
	AR-A-1708-23-AS	CCACACGGTCCATACAACTGGCC					
A	AR-A-1410-27-S	GCACTGGACGAGGCAGCTGCGTACCAG	627	60	60"; 60"; 60"	2	1.5
	AR-A-2037-23-AS	CCAGAACACAGAGTGACTCTGCC					
A	AR-A-172-25-S	GCACGAGACTTTGAGGCTGTCAGAG	554	70	60"; 60"; 60"	2	0.5
	AR-A-726-21-AS	CCAGGGCCGACTGCGGCTGTG					
B	AR-B-(IntronA)-24-S	GCCTGCAGGTTAATGCTGAAGACC	379	60	60"; 60"; 60"	1.5	
	AR-B-(IntronB)-26-AS	CCTAAGTTATTTGATAGGGCCTTGCC					
C	AR-C-(IntronB)-23-S	GTTTGGTGCCATACTCTGTCCAC	413	60	30"; 60"; 60""	2	
	AR-C-(IntronC)-25-AS	CTGATGGCCACGTTGCCTATGAAAG					
D	AR-D-(IntronC)-23-S	GGAGTTTAGAGTCTGTGACCAGG	456	60	30"; 60"; 60"	2	
	AR-D-(IntronD)-23-AS	GATCCCCCTTATCTCATGCTCCC					
E	AR-E-(IntronD)-25-S	CAACCCGTCAGTACCCAGACTGACC	284	60	60"; 60"; 60""	1.5	
	AR-E-(IntronE)-21-AS	GCTTCACTGTCACCCCATCAC					
F	AR-F-(IntronE)-25-S	CTCTGGGCTTATTGGTAAACTTCCC	294	60	60"; 60"; 60"	1.5	
	AR-F-(IntronF)-23-AS	GTCCAGGAGCTGGCTTTTCCCTA					
G	AR-G-(IntronF)-22-S	CTTGGTGCTTTGTCTAATGCTC	276	60	60"; 60"; 60"	1.5	
	AR-G-(IntronG)-24-AS	CTCTATCAGGCTGTTCTCCCTGAT					
H	AR-H-(IntronG)-24-S	GAGGCCACCTCCTTGTCAACCCTG	289	60	60"; 60"; 60"	1.5	
	AR-H-3202-21-AS	GGAGTAGTGCAGAGTTATAAC					
cDNA PCR amplification							
A to G	AR-A-1778-20-S	CGAGGCGGGAGCTGTAGCCC	1116	55	60"; 60"; 60"	1.5	
	AR-G-2894-21-AS	GCATGCAATGATACGATCGAG					
C to H	AR-C-2218-25-S	GTTATGAAGCAGGGATGACTCTGGG	984	55	60"; 60"; 60"	1.5	
	AR-H-3202-21-AS	GGAGTAGTGCAGAGTTATAAC					
Additional sequencing primers							
A	AR-A-431-24-S	CCAGAATCTGTTCCAGAGCGTGCG					
A	AR-A-533-26-AS	CAGCAGCAGCAAACTGGCGCCGGGAG					
A	AR-A-623-21-S	GCAGCAGGGTGAGGATGGTTC					
A	AR-A-1153-19-S	GATTGCATGTACGCCCCAC					
A	AR-A-1469-22-AS	CAGAGCCAGTGGAAAGTTGTAG					
A	AR-A-1598-23-S	GCATGGCGCGGGTGCAGCGGGAC					
A	AR-A-1798-20-S	CCTACGGCTACACTCGGCCC					
B	AR-B-2033-20-S	GACCTGCCTGATCTGTGGAG					
C	AR-C-2244-25-AS	CCCAGAGTCATCCCTGCTTCATAAC					
D	AR-D-2244-20-S	GCCCGGAAGCTGAAGAAACT					
E	AR-E-2559-21-S	CAGATGGCTGTCATTCAGTAC					
E	AR-E-2675-23-AS	CATTGAAAACCAGATCAGGGGCG					
F	AR-F-2753-20-S	CCAAATCACCCCCCAGGAAT					
H	AR-H-3177-20-AS	GAAGACATCTGAAAGGGGGC					

Details of PCR amplifications and sequences of oligonucleotide primers used for PCR/DNA sequencing. Some primers are listed more than once.

* Primer labels: e.g. AR-B-(2033)-20-S, gene-exon-(position relative to published sequence)-size [bp]-antisense [AS] or sense [S].

PCR-RFLP

PCR products containing the region of interest were amplified from patient, carrier and/or wildtype DNA as described above. After purification PCR products were digested with Apa1 or Mwo1. Digestion products were visualised on 2.5% agarose gel.

RESULTS

Clinical and genetic data for 31 patients with AIS, P1 to P31, and three affected family members, A2, A6 and A25, are summarised in Table 1. Further details of genotyping results and carrier testing are presented in Table 3. Mutations are reported in a manner consistent with the *AR* mutations database available online, the McGill database[29]. All nucleotide and amino acid numbering of reported mutations is identical to the McGill database[29]. Figure 1 shows a sequencing chromatogram of the one of the novel mutations detected in this study.

CAIS patient P1 was found to have a novel deletion at nucleotide 858, N858delG (codon Gly166, exon A). This deletion leads to a premature stop at codon 172. A novel deletion N2676del GAGT (codon Glu772, exon E) resulting in a stop at codon 787 was found in CAIS patients P2 and A2. Both of these frameshift mutations would result in inactivation of AR protein. CAIS patient P3 was found to have a novel insertion of a cysteine residue in the first zinc finger of the AR DNA-binding domain (DBD) (N2045_2047dupCTG). A novel amino acid substitution (Arg760Ser) in the AR ligand binding domain (LBD) was found in PAIS patient P4.

CAIS patient P5 was found to have the mutation Pro892Leu, previously reported in CAIS[29,30]. A similar CAIS mutation Pro892Ser is associated with reduced AR binding capacity and a diminished AR transactivation property[31].

CAIS patients P6 and A6 were each found to have the mutation Arg752Gln. This mutation is located in the AR LBD, and has been previously reported in CAIS[29,32]. The Arg752Gln mutation results in diminished androgen-binding capacity[29,32].

PAIS patient P7 was found to have the mutation Gly568Trp, previously reported in PAIS[29,33]. This

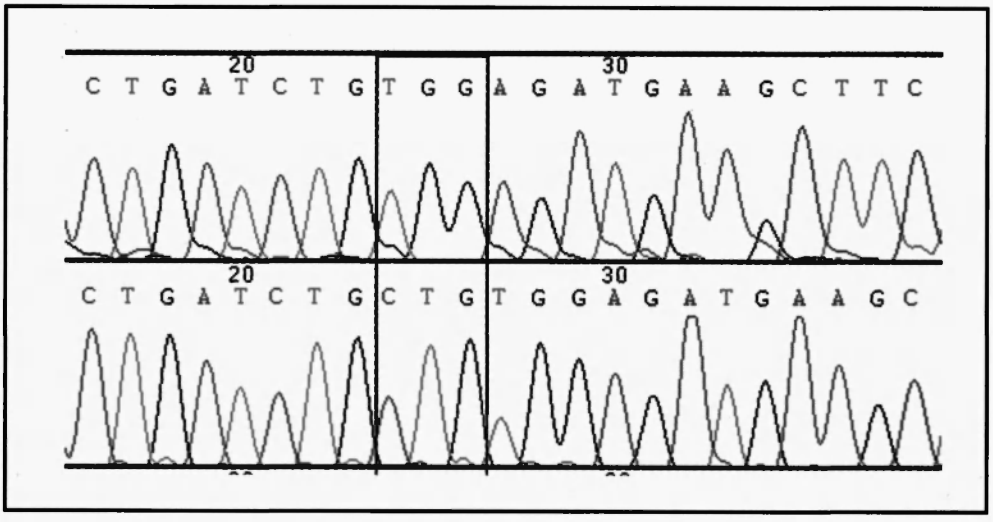

Fig. 1: N2045-2047dupCTG mutation. Lower panel: Sequencing chromatogram depicting hemizygous 2045_2047dupCTG mutation in patient P3. The CTG duplication is indicated by the black box. Upper panel: Sequencing chromatogram depicting wildtype sequence.

TABLE 3

Details of genotyping results and carrier testing for patients P1 to P9 and affected family members A2 and A6

Pat ID	Hemizygous *AR* gene mutation Nucleotide [Y]	*AR* gene mutation Protein/amino acid [Y]	AIS pheno-type	AR functional domain	*AR* Exon	Carrier/family testing
P1	**N858delG**	**Frameshift, stop at codon 172**	CAIS	Deletion of TAD, DBD, LBD	A	RFLP analysis using *Apa1* showed that mother of P1 is heterozygous for mutation
P2	**N2676delGAGT**	**Frameshift, stop at codon 787**	CAIS	Deletion of LBD	E	Sequencing analysis showed that mother of P2 is heterozygous for mutation
A2	**N2676delGAGT**	**Frameshift, stop at codon 787**	CAIS	Deletion of LBD	E	See pedigree (Fig. 2)
P3	**N2045_2047dupCTG**	**Insertion C after C562**	CAIS	DBD	B	RFLP analysis using *Mwo1* showed that mother of P3 is heterozygous for mutation
P4	**N2642G-T**	**R760S**	PAIS	LBD	E	Sequencing analysis showed that mother of P4 is heterozygous for mutation
P5	N3037C-T	P892L	CAIS	LBD	H	Sequencing analysis showed that sister of P5 does not have the mutation
P6	N2617G-A	R752Q	CAIS	LBD	E	Sequencing analysis showed that mother of P6 is heterozygous for mutation
A6	N2617G-A	R752Q	CAIS	LBD	E	See pedigree (Fig. 3)
P7	N2064G-T	G568W	PAIS	DBD	B	Sequencing analysis showed that mother of P7 is heterozygous for mutation
P8	N2973A-G	R871G	PAIS	LBD	H	Sequencing analysis showed that sister of P8 does not have the mutation
P9	N2074A-G	Y571C	CAIS	DBD	B	Sequencing analysis showed that mother of P9 is heterozygous for mutation

[Y] The five *AR* gene mutations in bold are novel mutations.

mutation has an effect on transrepression of androgen-induced activation by activator protein-1 (AP-1), a transcription factor known to modulate AR action[34]. Gly568Trp may result in disruption of cross talk between the androgen signalling pathway and other growth factor pathways.

The mutation Arg871Gly was identified in PAIS patient P8. This mutation has been previously reported in PAIS[29,35]. Shkolny *et al*[35] report that the Arg871Gly mutation results in discordant androgen binding in transfected COS-1 cells and in genital skin fibroblasts from the patient.

CAIS patient P9 was found to have the mutation Tyr571Cvs which has previously been reported in CAIS[29,36]. This mutation is located in the first zinc finger of the AR DBD. The Tyr571Cys mutation may affect the 3-dimensional structure of the DBD zinc finger leading to altered DNA-binding activity of the AR protein.

Sequencing of the entire *AR* gene exons A-H failed to reveal any deleterious mutations in patients P10 to P31 or family member A25.

In three of the 34 patients and family members, genital skin fibroblasts were available for tissue culture. RT-PCR on genital skin fibroblast tissue culture from patients P21, P23 and P24 showed that exons B-H of *AR* cDNA were intact.

In summary, patients PI, P2 and A2 have novel *AR* mutations which result in inactivation of the AR protein. Patients P5, P6, A6, P7, P8 and P9 have previously reported deleterious *AR* gene mutations known to be associated with the AIS phenotype. Thus genotyping has provided a clear explanation of phenotype for these seven patients and two affected family members. Patients P3 and P4 have novel *AR* mutations of uncertain functional effect on AR protein. For these two patients further studies on the effect of the novel mutations will be required to account for the AIS phenotype.

Pedigree analysis for patient P2

Figure 2 shows the pedigree analysis of the N2676delGAGT mutation and polymorphic markers (exon A Gln repeat). All family members tested were screened for both the N2676delGAGT mutation and exon A polymorphic markers (exon A Gln repeat). Patient A2 is the 46,XY aunt of patient P2. The mother of P2 is a heterozygous carrier of the mutation. Another sibling of A2 has an AIS affected child; these family members were unavailable for testing. The maternal grandmother of P2 is deceased, but the presence of the mutation in more than one of her progeny suggests that she may have carried the N2676delGAGT mutation.

Pedigree analysis for patient P6

Figure 3 shows the pedigree analysis of patients P6 and A6. All family members tested were screened for both the Arg752Gln mutation and exon A polymorphic markers (exon A Gln repeat). The mother of P6 is a heterozygous carrier for the Arg752Gln mutation. The sister of P6 was shown to be an unaffected carrier who transmitted the mutation to her 46,XY daughter, CAIS patient A6. The absence of the Arg752Gln mutation in the aunt and grandmother of patient P6 indicates that the mother of P6 had acquired the mutation *de novo*. Mutations of the *AR* gene occurring *de novo* have been shown to occur at a rate of 26.7% in AIS[37].

Gln/Gly repeats

For all patients tested the number of *AR* Gln (CAG) and Gly (GGN) repeats were within the normal range. However, several patients have CAG or GGN repeat lengths that were at the upper or lower ends of the normal ranges (Table 1). For most of the patients the number of CAG and GGN repeats are similar and differ by a value of 3 or less. However, for several of the PAIS/CAIS patients with no deleterious mutation the number of CAG and GGN repeats differs by a value of 5 or more (patients P10 to P16). Patients P10 and P11 have a difference of 19 and 15, respectively.

DISCUSSION

We detected *AR* gene defects in 66.7% (6/9) of non-related CAIS patients, comparable to previous reports of 83% (57/69)[27]. The rate of detection of *AR* gene defects in PAIS has been reported as 28% (12/43)[27]. The lower detection rate for PAIS reported here of 13.6% (3/22) may be due to the small sample number.

Of the nine *AR* gene mutations identified in this study, five are previously identified *AR* mutations,

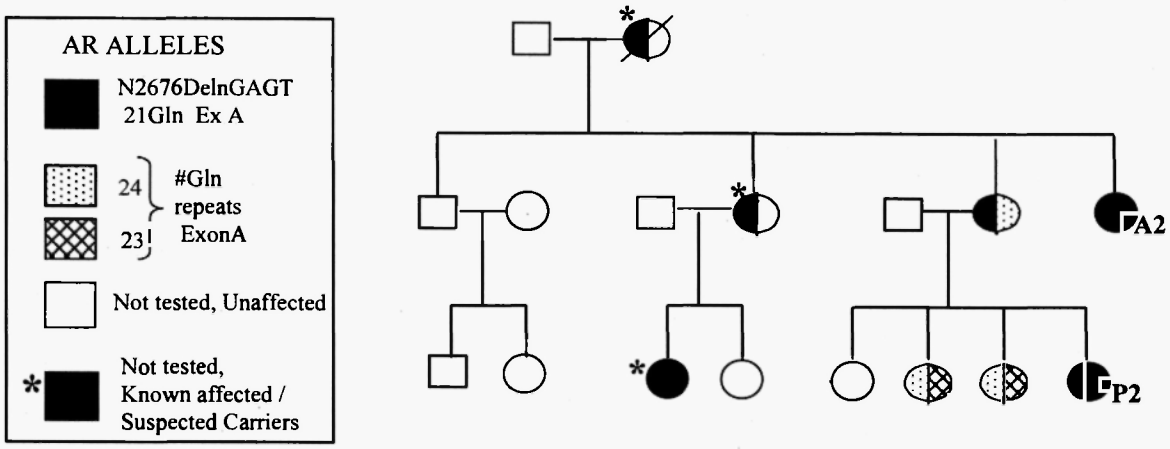

Fig. 2: **Pedigree for N2676delGAGT mutation.** Pedigree for family of patients P2 and A2. P2 and A2 both have CAIS and are hemizygous for the N2676delGAGT mutation. Pedigree analysis demonstrates that the deceased mother of A2 may have carried the mutation. A third affected patient is indicated in the pedigree, although this patient and her mother have not been tested for the mutation. (The marker used for pedigree analysis is the polymorphic AR Gln repeat region, the results for which are indicated in the pedigree.)

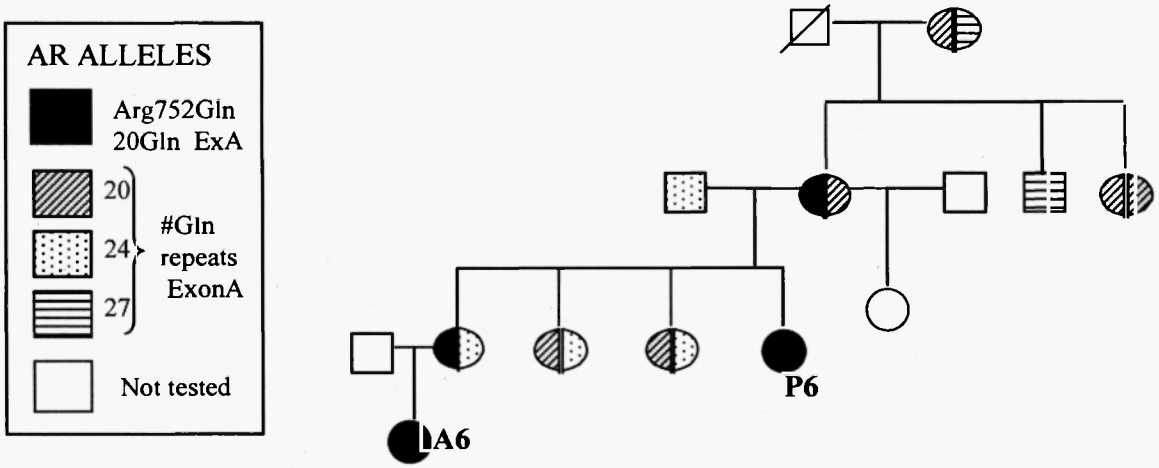

Fig. 3: **Pedigree for Arg752Gln mutation.** Pedigree for family of patients P6 and A6. Patients P6 and A6 both have CAIS and are hemizygous for the Arg752Gln mutation. Pedigree analysis demonstrates that the mutation occurred *de novo* in the mother of P6. The mothers of P6 and A6 are both heterozygous unaffected carriers for the Arg752Gln mutation.

two are novel mutations known to lead to inactivation of AR, and two are novel mutations of uncertain effect on AR function. The four novel mutations are discussed in detail.

The N858delG mutation found in PI has not been previously reported[29]. The deletion results in a frameshift that introduces a premature stop at codon 172. Other mutations have been described resulting in a stop at codon 172, and all but one are associated with CAIS[29]. One patient was reported as having PAIS, but it was shown that the PAIS phenotype was a result of somatic mosaicism[38].

The AR protein has been reported to have two isoforms, AR-A and AR-B. AR-A is an N-terminally truncated version of the full-length AR-B isoform. AR-A retains the steroid and DNA binding domains, but lacks a significant portion of the amino terminal transactivation region of AR-B. Transfection experiments have shown subtle differences in function between the AR-A and AR-B isoforms on selected response elements[39].

Wilson and McPhaul[40] showed that the AR-A isoform co-migrates with an expressed fragment that initiates from a second methionine at AR residue 189. It has been suggested that the AR-A isoform could arise *in vivo* as a result of translation from an internal start site at Met189[40,41]. In addition, Gao and McPhaul[39] report that the nucleotide sequence surrounding Met189 would cause it to be used inefficiently as a translation initiation site. *In vitro* proteolysis results of Gregory *et al.*[42] raise an alternative theory, i.e. that AR-A arises as a proteolytic fragment of full length AR-B. They suggest that observations of an AR-A isoform in tissue extracts and cell culture is due to proteolysis of AR-B during protein extraction. Thus the AR-A isoform is generated *in vivo* as a result of proteolysis of AR-B, rather than from initiation of translation at Met189. Further, Gregory *et al.*[42] propose that AR-A has no functional role *in vivo*.

Any AR mutations leading to a premature stop 5′ of codon Met189, such as the N858delG mutation reported here, would result in an inactive 3′-truncated version of AR-B. The severe phenotype of CAIS in these cases[29] suggests that AR-A is either not present or not functional in these patients, supporting the evidence of Gregory *et al.*[42] that AR-A is produced by proteolysis and not from translation at Met189. Genital skin fibroblasts from two patients with a premature stop 5′ of codon Met189, Gln60Stop[41], were observed to have residual androgen binding activity. This could be explained by translation of a truncated AR isoform from a putative start site at Met507. An AR isoform arising from Met507 would retain the ligand binding domain but would be inactive due to absence of the entire N-terminal domain.

The N2676delGAGT mutation found in CAIS patients P2 and A2 has not been previously reported. Pedigree analysis of the family of P2 shows that two of her sisters do not carry the N2676 delGAGT mutation (Fig. 2), clearly demonstrating the value of *AR* genotyping. The N2676delGAGT mutation results in a frameshift leading to a premature stop at codon 787. This would result in the loss of the ligand binding domain of the AR protein. Various mutations in the *AR* gene have been described leading to a premature stop at codon 787. A CAIS patient with a 13-bp deletion in exon D of the *AR* gene has been described, leading to a frameshift and a premature stop at codon 787[29,43]. This 13-bp deletion was reproduced in androgen receptor wildtype cDNA and transfected into mammalian cells. Western blot analysis demonstrated a smaller androgen receptor of 94 kDa for the transfected mutated cDNA instead of 110 kDa[43]. A lack of androgen binding was demonstrated in the mutated transfected cells. It was shown that the mutant could bind target DNA but was unable to transactivate a reporter gene[43]. A CAIS patient with a substitution leading to a stop at codon 786 has also been characterised; *in vitro* androgen binding studies report a B_{max} of zero, indicating that this mutant receptor is unable to bind ligand[29,44]. Various reports describe CAIS patients with a 1-bp deletion in codon Pro766 of the *AR* gene[27,29,44-46]. This 1-bp deletion in Pro766 would result in a frameshift leading to a premature stop at codon 787 (erroneously reported as leading to a stop in codon 807[29]).

The N2045_2047dupCTG mutation found in CAIS patient P3 has not been previously reported. This mutation results in the insertion of a cysteine in the first zinc finger of the highly conserved AR DNA-binding domain between amino acids 562 and 563 (Fig. 4). The mutation is adjacent to one of

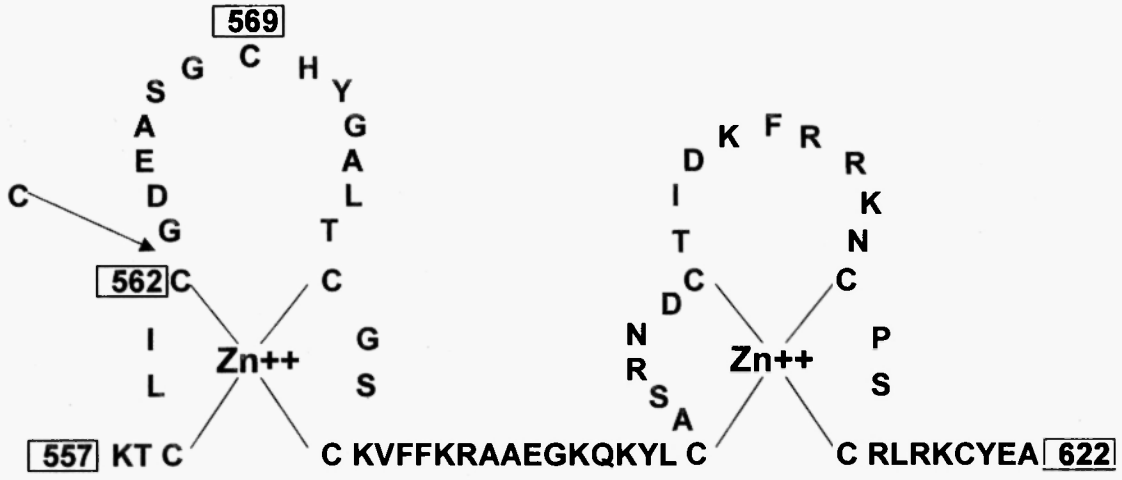

Fig. 4: AR Zn finger, N2045-2047dupCTG mutation.

four highly conserved cysteine residues which are required to bind a zinc ion. Binding of metal ion is essential for stabilisation of the active conformation of the domain, enabling the AR to interact with target DNA[47]. There is also a conserved cysteine residue at position 569 in the DBD which is likely to be involved in stabilizing the DBD but not in binding of the metal ion (Fig. 4)[48]. The additional cysteine residue may affect the 3-dimensional structure of the zinc finger causing inefficient binding of the mutant AR to target DNA sequences due to decreased stability, or may reduce the capacity of the bound receptor to activate transcription. This is the first report of amino acid duplication in the AR. Future work may involve *in vitro* studies to elucidate the role of the N2045_2047dupCTG mutation in AIS.

The Arg760Ser mutation found in PAIS patient P4 has not been previously reported. The arginine residue at position 760 is highly conserved in AR from various species, and is located in the highly conserved LBD of the AR. The Arg760 residue is located in LoopH4H5 in the predicted 2-dimensional structure of the AR[49]. The residue is located outside of the ligand-binding pocket and does not interact with the bound ligand directly[50], but is involved in maintaining the structure of the ligand-binding pocket. The region surrounding the Arg760Ser mutation is a known mutational cluster site of the AR[36]. The mutational cluster is located between codons 728 and 774 and is homologous to a region in the human thyroid receptor which is a known cluster site for mutations that cause thyroid hormone resistance[51]. This would indicate that this region plays an important role in the interaction between hormone and receptor. The conversion of the large basic, positively charged arginine residue at position 760 to the small, neutral, hydrophilic serine residue would be likely to have an effect on the 3-dimensional structure of the AR.

Patients P10 and P11 had no deleterious AR mutation but were found to have a large difference between the number of CAG and GGN repeats (Table 1). Although highly speculative, it is possible that this Gln/Gly repeat imbalance has a functional consequence. Long Gln repeats are associated with undermasculinisation, male infertility and defective spermatogenesis[8,9,14-17]. Short Gln repeats are associated with prostate cancer[5,19]. Expansion of the Gln repeat is associated with reduced expression of AR in COS cells[52]. Short Gly repeats are associated with benign prostatic hyperplasia[18] and prostate cancer[5,19,22,24,53] and long Gly repeats with endometrial cancer[21]. Contraction of the Gly repeat led to increased levels of AR protein in DU145 human prostate cancer cells[54].

The mechanism by which AR function may be affected by Gln/Gly repeat length can only be speculated upon. The Gln/Gly repeats are located in a region of AR protein known to bind to co-

regulators. This region is also important for intra molecular interaction between the N- and C-terminal ends of AR induced by ligand binding[55-58]. It was demonstrated that a normal N/C interaction occurs only for AR protein with 16-29 Gln repeats[59]. Recent *in vitro* work by Werner *et al.*[60], indicating that the combination of a short Gly repeat with a long Gln repeat influenced the effect of a mutation within the ligand binding domain, Ala645Asp, supports our hypothesis of a functional relationship between the AR polyglycine and polyglutamine segments.

The Gln/Gly repeat region is capable of forming stable RNA stem-loop structures, and the stability of these structures increases with repeat number[54,61]. Some cellular RNA-binding regulatory proteins bind to stem-loop motifs in target RNA[62]. It is possible that variation in the repeat length of AR mRNA molecules has an effect on the binding of RNA regulatory proteins, resulting in altered mRNA processing.

One hundred percent (9/9) of the mutations identified in this study were in exons B-H. The majority of *AR* gene mutations in AIS are located in exons B-H[29]. Thus our genotyping strategy is successful, i.e. to first sequence only exons B-H and then to test exon A if no deleterious mutation is found. No deleterious *AR* gene mutations were found in patients P10 to P31. The cause of abnormal virilisation in this significant proportion of patients remains unclear, as has been found in other studies[27]. It is possible that the AIS phenotype may be due to mutations in the *AR* promoter or in other genes involved in the androgen-response pathway. The next step in genotyping our patient cohort would be to examine the distal 5′-promoter regions of the *AR* gene, at least in the 3/9 CAIS patients found not to have a mutation. Mutations in the promoter may affect expression of the *AR* gene and/or binding to regulatory factors. In addition, genital skin biopsy from patients and subsequent cDNA analysis and/or expression studies could be undertaken. DNA analysis in AIS is an invaluable tool providing insights into the relationship between genital phenotype and *AR* mutation. In addition, it is vital clinically in terms of accurate diagnosis, carrier detection, pedigree analysis and genetic counselling.

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