LETTER TO THE EDITOR

Genetic evidence to exclude the androgen receptor-polyglutamine associated coactivator, ARA-24, as a cause of male undermasculinisation

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Defects in the development and function of the male reproductive system are common (1). Abnormalities range from completely female external genitalia to isolated hypospadias or infertility (1). The underlying basis of the abnormalities is mostly attributable to a reduction in androgen (testosterone or 5- α -dihydrotestosterone (DHT)) action, referred to as undermasculinisation. In some cases of undermasculinisation, abnormalities of androgen production have been demonstrated, and in others there are identifiable defects in the function of the androgen receptor (AR), which mediates the effect of the androgens (2).

The AR is a member of the nuclear hormone receptor superfamily (3). It contains three functional domains: an N-terminal ligand-independent activation function, a central DNA-binding domain, and the C-terminus, which includes residues crucial for androgen binding, receptor dimerisation and transactivation. The N-terminal domain includes a polymorphic region of 11–31 glutamine repeats (AR[Gln]_n) (4). Mutations in the AR have been shown to cause complete genital undermasculinisation (complete androgen insensitivity syndrome, CAIS) and the less clinically severe partial androgen insensitivity syndrome (PAIS). Although PAIS is the most common diagnosis of undermasculinised genitalia in 46XY males (1), for most cases there is no identifiable cause (2).

There is evidence that expansions of the AR polyglutamine repeat contribute to undermasculinisation. Excessively long AR[Gln]_n tracts (40–62 repeats) occur in the rare X-linked spinal and bulbar muscular atrophy (SBMA), which often has the associated features of male infertility, testicular atrophy and gynaecomastia (5). Smaller increases in polyglutamine repeat length (remaining within the normal range) may contribute to moderate to severe undermasculinised male genitalia (6). In the latter disorder, AR[Gln]_n length is clearly affecting at least one other factor that is sensitive to its length and is therefore probably interacting directly with this region of the AR. Therefore, in the many patients with undermasculinisation who have no identifiable defect in either androgen production or the AR, it is necessary to investigate other factors that interact with the AR.

The recruitment of multiple coactivator proteins is essential to enhance AR-mediated androgen-dependent transactivation (7). There is evidence that one of these AR coactivators, ARA-24 (8) (also known as RAN) can contribute to reduced AR function. It was demonstrated that expansion of the AR[Gln]_n tract length diminishes the physical interaction of ARA-24 with the AR, resulting in decreased coactivation (8). Consequently, mutations or polymorphisms that reduce the action, interaction, or both, of ARA-24 with the AR would be expected to contribute to reduced androgen-dependent transactivation and lead to undermasculinisation. There is now compelling evidence that some cases of undermasculinisation may be due to disruption of coactivator function (9, 10). Therefore the aim of this study was to investigate whether mutations or polymorphisms within the coding region of ARA-24 are a common cause of undermasculinised genitalia in 46XY males with normal androgen biosynthesis, normal AR-androgen binding characteristics and AR[Gln]_n within the normal range.

The patient group was composed of 25 men with normal 46XY karyotypes (Table 1). All patients' samples were obtained from the Cambridge Intersex Database (1). Local ethics committee approval was obtained for the use of patient samples as part of a sexual development disorders research programme. All patients had a normal gonadotrophin profile and a testosterone and DHT response to human chorionic gonadotrophin stimulation consistent with a diagnosis of androgen insensitivity syndrome. Furthermore, all patients had AR-binding studies performed on genital skin fibroblasts and all had normal receptor androgen affinity and binding capacity (1) as measured using methods described previously (11). AR[Gln]_n was determined as described previously (6) or by direct sequencing.

RNA was extracted from the primary genital skin fibroblast cultures of patients' skin biopsies using either the Chomczynski method (12) or the Trizol reagent (Life Technologies, Paisley, Scotland, UK). cDNA was synthesised using standard methods (Superscript II, Life Technologies). The polymerase chain reaction (PCR) was used to amplify the coding region of the ARA-24 transcript, using primer sequences of 5'-ggcgcttctggaaggaacgccc-3'

Table 1 Patients' clinical features. All patients' samples were obtained from the Cambridge Intersex Database (1). Androgen receptor binding studies that measured receptor affinity (K_d) ($\times 10^{-10}$ mol/l) and binding capacity (B_{max}) ($\times 10^{-18}$ /mg DNA), were performed on genital skin fibroblast cultures as described elsewhere (11). Exons B–H of the *AR* gene, where >90% of PAIS-causing mutations are found (www.mcgill.ca/androgendb), were screened by direct sequencing. AR[Gln]_n was determined as described previously (7) or by direct sequencing. Physical features of undermasculinisation are: UF, unfused scrotum; BS, bifid scrotum; MP, micropenis; PSH, perino-scrotal hypospadias; PH, perineal hypospadias; GH, glandular hypospadias.

Patient No.	Clinical feature	AR binding		AR exon	AR[Gln] _n
		K_d	B_{max}		
1	PH, MP	1.1	593	B–H	25
2	PH, MP	1.5	902	B–H	30
3	PH, BS	0.7	1747	B–H	25
4	MP, BS	0.9	1780	B–H	22
5	BS, PSH	0.9	775	B–H	23
6	PH, MP	1.2	1101	B–H	26
7	BS, PSH	1.1	540	B–H	24
8	BS, PSH, MP	1.0	308	B–H	20
9	BS, PSH	1.0	619	B–H	27
10	BS, PSH	1.6	1289	B–H	19
11	PH	1.4	989	B–H	24
12	PSH, MP	1.8	858	B–H	29
13	MP, PH	1.4	844	B–H	28
14	PSH	0.8	551	B–H	27
15	MP, GH	0.9	752	B–H	22
16	GH	1.9	1268	B–H	25
17	UF, PH	1.3	774	B–H	25
18	UF, PH, MP	0.9	914	B–H	20
19	UF, PH	2.5	1186	B–H	31
20	PH, MP	0.6	428	B–H	22
21	PH, MP	0.9	137	B–H	27
22	UF, PH	1.1	919	B–H	26
23	BS, PH	1.2	493	B–H	23
24	UF, PH, MP	0.9	416	B–H	21
25	MP	2.9	320	B–H	25

(forward) and 5'–gcaccgctgacatcacaggacag–3' (reverse). All products were sequenced directly in both directions using automated sequencing techniques (Applied BioSystems, Warrington, UK) and were consistent with the published sequence of *ARA-24* (GenBank accession No. AF052578).

The coding region of *ARA-24* was successfully screened by direct sequencing of RT-PCR products, thereby excluding absence (but not partial reduction) of *ARA-24* expression in the target tissue as the cause of undermasculinisation. In addition, this method was able to exclude genetic rearrangement or exonic deletions as causes of *ARA-24* inactivation in undermasculinised men. Significantly, no mutations or polymorphisms were found in *ARA-24* of any of the 25 patients.

More than 40 proteins have been shown to interact functionally with the AR *in vitro* (full references of all AR-interacting proteins are provided at the excellent AR database at <http://www.mcgill.ca/androgendb>). Mouse genetic models reveal that disruption of a general nuclear receptor coactivator (SRC-1) can result in

multiple nuclear hormone resistance syndromes (13). Furthermore, disruption of AR–coactivator interactions has been shown to cause reduced male fertility, Kennedy's syndrome (8) and PAIS (14). *ARA-24* is a member of a subset of coactivators known to be crucial for optimal AR functioning (7) and thus disruption of its functions could potentially contribute to undermasculinisation (15). However, we have shown, by the absence of *ARA-24* mutations in 25 well-characterised undermasculinised men with apparently normal androgen biosynthesis, AR, AR[Gln]_n and AR binding characteristics, that *ARA-24* mutations are not a common cause of moderate to severe undermasculinisation.

Longer AR[Gln]_n regions appear to contribute to undermasculinisation by a multifactorial aetiology (16). The apparent sensitivity of *ARA-24* activity to AR[Gln]_n length and its direct interaction with this region of the AR (8) suggest that it is a good candidate for involvement in undermasculinisation. It has yet to be determined whether a reduction in *ARA-24* activity is the mechanism by which longer AR[Gln]_n contributes to genital abnormalities. However, this study was also able to exclude both *ARA-24* protein and nucleotide polymorphisms as factors contributing directly to the multifactorial cause of male undermasculinisation. This does not exclude the possible involvement of *ARA-24* mutations in other disorders of abnormal androgen action which have yet to be studied, including prostate cancer or testicular maldescent. Future clinical studies may wish to examine these phenotypes, particularly when there is further *in vitro* evidence to delineate *ARA-24* function.

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