CLINICAL STUDY

Evidence that luteinising hormone receptor polymorphisms may contribute to male undermasculinisation

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Abstract

Background: The luteinising hormone receptor (LHR) is necessary for the stimulation of androgen production and male genital development. It contains three protein polymorphisms: a leucine and glutamine insertion between codons 8 and 9 (LQ+) and two amino acid substitutions (N291S, N312S). **Objectives:** To determine whether these LHR polymorphisms are associated with male genital undermasculinisation or the androgen receptor polyglutamine repeat polymorphism (AR(Q)n), which contributes in some cases to the cause of genital undermasculinisation.

Methods: The LHR polymorphisms were assessed by PCR amplification of genomic DNA, followed by restriction enzyme analysis. The frequency of the LHR polymorphisms were compared between an undermasculinised male group ($n = 75$) and a control group ($n = 55$).

Results: LQ+ was not independently associated with the undermasculinised group ($P = 0.09$), but it was associated with increased AR(Q)n within the undermasculinised group ($P = 0.02$), particularly for AR(Q)n lengths ≥ 26 ($P = 0.002$). In the undermasculinised group, homozygosity for N291 (872A/A) was more frequent ($P = 0.05$), whereas homozygosity for N312 (935A/A) was less frequent ($P = 0.05$). The combination of the presence of 872A/A and the absence of 935A/A showed a stronger association with the undermasculinised group than either polymorphism independently ($P = 0.006$). The odds ratio of this genotype compared with any other, between the undermasculinised and control groups was 3.28 (95% confidence interval (CI) 1.33 to 8.08).

Conclusion: LHR polymorphisms may contribute to genital undermasculinisation.

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Introduction

The formation of the male external genitalia requires androgens acting via the androgen receptor (AR). Androgen production during this period of development is stimulated by human chorionic gonadotrophin (hCG) binding to the luteinising hormone receptor (LHR), which is the same receptor that mediates the action of luteinising hormone (LH) (1). LHR-inactivating mutations have been described in cases of varying severity, ranging from an individual with completely female external genitalia (with internal male genitalia and testes with Leydig cell agenesis/dysplasia) to those with micropenis or perineal hypospadias (2, 3). Recent LHR 'knock-out' studies have generated mice with a phenotype similar to that of the mild LHR-inactivating mutations in humans, although the genital abnormalities are not apparent until post-natal genital development (4, 5).

Activating mutations within the LHR can result in precocious puberty. However, unlike inactivating

mutations, which are widely distributed throughout the LHR, activating mutations have been identified only in the transmembrane domain and the third intracellular loop (6). In contrast to mutations of the LHR, the only described case of an inactivating LH mutation had no effect until puberty, indirectly suggesting that hCG is the primary ligand, or is able to compensate for missing pituitary LH, for the LHR during genital development (7). Reduced androgen responsiveness to hCG/LH and the absence of breast development can help distinguish, clinically, between individuals with LHR inactivating mutations and those with AR mutations (6).

The majority of individuals with male genital undermasculinisation do not have identifiable mutations within the genes involved in androgen action and production, including the LHR and AR. However, a subset of these cases of undermasculinisation without an apparent cause have an increased number of glutamines (but within the normal range of 11–31 repeats) within the AR polymorphic polyglutamine tract

(AR(Q)n) compared with cases with AR mutations and the normal population (8, 9). It is probable that polymorphisms in other factors involved in androgen action and production, such as the LHR, can also contribute to the multi-factorial aetiology of undermasculinisation (10).

At the commencement of this study, three LHR protein polymorphisms had been described:

the insertion of 5' CTGCAG 3' encoding leucine and glutamine (LQ) between codons 8 and 9 (11, 12);

a nucleotide substitution at 872(A → G) causing an asparagine to serine substitution at residue 291 (N291S) and the potential loss of an N-glycosylation site (12), and

a nucleotide substitution at 935(G → A) causing an asparagine to serine substitution at residue 312 (N312S) (12).

The biological effect of these polymorphisms on LHR function is unclear. However, their location in the extracellular domain might alter hCG, LH or LH variant binding, perhaps to a degree that is undetectable by *in vitro* studies. Therefore, the aim of this study was to determine whether polymorphisms in the LHR can also contribute to male undermasculinisation and, if so, whether they are associated with the AR(Q)n polymorphism.

Patients and methodology

Patients

A heterogeneous group of undermasculinised patients ($n = 75$) with normal AR binding studies, normal single-strand conformation polymorphism (SSCP)

screening of the AR gene or normal sequencing of the AR coding region were selected from the Cambridge intersex database (13). All samples with complete undermasculinisation (the group most likely to have AR mutations) had a normal AR coding region as determined by SSCP, sequencing, or both (previously described methodologies) (13). Control individuals ($n = 55$) who had either a characterised AR mutation or a diagnosis of Denys-Drash syndrome (as opposed to the undermasculinised group without an identifiable cause) were selected from the same database. The validity of using this control group is supported by the previous demonstration that the distribution of AR(Q)n lengths (which has geographic and ethnic variation) within this group is the same as that in the normal population (8). Local ethics committee approval was obtained for the use of patient samples as part of a sexual development disorders program.

Genotyping

A 215 bp region of exon 1 containing the 5' CTGCAG 3' (LQ) insertional polymorphism was PCR-amplified using oligonucleotides 114 (5' gacactggcaagccgcaagccc 3') and 115 (5' gctgtgtactcacagtcgagttag 3') (Fig. 1). The additional repeat creates a second PvuII restriction enzyme site that allows alleles with the additional 6 bp (three fragments of 27 bp, 65 bp and 128 bp), to be distinguished from those without the repeat (two fragments of 65 bp and 149 bp).

A 111 bp fragment of exon 10 containing both polymorphic sites (872A/G and 935A/G) was PCR-amplified with oligonucleotides 117 (5' cctcttctcttccagacaga 3') and 118 (5' catgcaaatacttacagtgttttgga 3'). The ApoI restriction enzyme was used to distinguish between 872A alleles containing an ApoI site (generating

Figure 1 Diagrammatic representation of the LHR gene and oligonucleotide positions. The boxes represent the regions of exon 1 and exon 10 that were amplified using oligonucleotide pairs 114/115 and 117/118 respectively. The location of the polymorphisms and the restriction enzymes used to discriminate between the alleles are shown.

Table 1 AR(Q)n, exon 1, A872G and A935G genotypes in undermasculinised (U) and control (CON) individuals.

Sample ID	Group	Severity	AR(Q)n	Exon 1	A872G	A935G	Sample ID	Group	Severity	AR(Q)n	Exon 1	A872G	A935G
1	U	A	29	1/1	A/A	A/G	67	U	D	22	1/1	A/A	A/G
2	U	A	21	1/1	A/G	A/G	68	U	D	25	1/2	A/A	A/A
3	U	A	24	1/1	A/A	A/G	69	U	D	23	1/2	A/A	A/G
4	U	A	24	1/2	A/A	A/G	70	U	D	22	1/2	A/A	A/G
5	U	A	25	1/1	A/A	G/G	71	U	D	21	1/1	A/A	A/G
6	U	B	24	1/2	A/A	A/G	72	U	D	21	1/1	A/A	G/G
7	U	B	21	1/1	A/G	A/G	73	U	D	22	1/1	A/A	A/G
8	U	B	20	1/2	A/A	A/G	74	U	D	22	1/2	A/A	A/G
9	U	B	27	1/2	A/A	A/G	75	U	D	23	1/1	A/A	G/G
10	U	B	24	1/1	A/A	G/G	76	U	D	23	1/1	A/A	G/G
11	U	B	22	1/1	A/A	A/G	66	CON	D	20	1/1	A/G	A/G
12	U	B	23	1/1	A/A	G/G	77	CON	D	19	1/1	A/A	A/G
13	U	B	26	1/1	A/A	A/G	78	CON	D	26	1/1	A/A	G/G
14	U	B	25	1/1	A/A	G/G	79	CON	D	20	2/2	A/A	G/G
15	U	B	26	1/2	A/A	A/G	80	CON	D	22	1/2	A/A	G/G
16	U	B	26	1/2	A/A	G/G	81	CON	D	23	1/1	A/G	A/G
17	U	B	30	1/2	A/A	G/G	82	CON	D	20	1/1	A/A	A/A
18	U	B	23	1/1	A/A	A/G	83	CON	D	21	1/1	A/A	A/A
19	U	B	23	1/1	A/A	A/G	84	CON	D	26	1/1	A/A	A/G
20	U	B	26	1/1	A/A	G/G	85	CON	D	18	1/1	A/A	A/G
21	U	B	25	1/1	A/A	A/G	86	CON	D	20	1/1	A/A	G/G
22	U	B	28	2/2	A/A	G/G	87	CON	D	21	1/1	A/G	A/G
23	U	B	25	1/1	A/A	G/G	88	CON	D	21	1/2	A/A	G/G
24	U	B	26	1/2	A/A	A/G	89	CON	D	22	1/1	A/A	A/A
25	U	B	28	1/1	A/G	A/G	90	CON	D	22	1/1	A/A	A/G
26	U	B	20	1/1	A/A	G/G	91	CON	D	22	1/1	A/A	A/A
27	U	B	27	1/2	A/A	G/G	92	CON	D	ND	1/2	A/A	A/G
28	U	B	28	1/1	A/A	A/A	93	CON	D	23	1/1	A/A	G/G
29	U	B	29	1/2	A/A	A/G	94	CON	D	24	1/1	A/A	A/G
30	U	B	30	1/2	A/A	A/G	95	CON	D	24	1/2	A/A	G/G
31	U	B	31	1/1	A/A	A/G	96	CON	D	25	1/1	A/A	G/G
32	U	B	29	1/2	A/A	G/G	97	CON	D	27	1/1	A/A	G/G
33	U	B	20	1/2	A/A	A/A	98	CON	D	28	1/1	G/G	A/A
34	U	B	24	1/1	A/A	A/G	99	CON	D	24	1/1	A/A	A/G
35	U	B	20	1/2	A/A	A/G	100	CON	D	20	2/2	A/A	A/A
36	U	C	20	1/1	A/A	A/G	101	CON	D	21	1/2	A/A	A/G
37	U	C	25	1/2	A/A	A/G	102	CON	D	22	1/1	A/A	G/G
38	U	C	25	1/1	A/A	G/G	103	CON	D	22	1/1	A/A	A/G
39	U	C	27	1/1	A/A	G/G	104	CON	D	22	1/1	A/A	A/G
40	U	C	30	1/2	A/A	A/G	105	CON	D	23	1/1	A/A	A/G
41	U	C	27	1/2	A/A	G/G	106	CON	D	24	1/2	A/A	A/G
42	U	C	23	1/1	A/A	G/G	107	CON	D	24	1/2	A/A	G/G
43	U	C	26	1/2	A/A	A/G	108	CON	D	25	1/1	A/G	A/G
44	U	C	19	1/1	A/A	A/G	109	CON	D	25	1/2	A/A	A/G
45	U	C	23	1/1	A/A	G/G	110	CON	D	28	1/2	A/A	A/G
46	U	C	24	1/1	A/G	A/A	111	CON	D	29	1/1	A/A	G/G
47	U	C	25	1/1	A/A	A/G	112	CON	D	29	1/1	A/G	A/G
48	U	C	23	1/1	A/A	A/G	113	CON	A	19	1/2	A/A	A/A
49	U	C	23	1/2	A/A	A/A	114	CON	A	24	1/1	A/A	A/A
50	U	C	29	1/2	A/A	G/G	115	CON	B	23	1/1	A/A	A/G
51	U	C	22	1/1	A/A	G/G	132	CON	B	ND	1/1	A/A	A/G
52	U	C	28	1/2	A/A	G/G	116	CON	C	30	1/1	A/A	A/G
53	U	C	27	1/2	A/A	A/G	117	CON	C	21	1/1	A/A	A/G
54	U	C	17	1/1	A/A	G/G	118	CON	C	22	1/2	A/A	A/G
55	U	C	30	1/1	A/A	A/A	119	CON	C	23	1/2	A/A	G/G
56	U	C	24	1/1	A/A	A/G	120	CON	C	23	1/2	A/A	G/G
57	U	C	27	1/1	A/A	A/G	121	CON	C	21	1/1	A/A	G/G
58	U	C	23	2/2	A/A	G/G	122	CON	C	25	1/2	A/A	G/G
59	U	C	19	1/2	A/A	A/G	123	CON	C	26	1/1	A/A	G/G
60	U	C	26	1/1	A/A	G/G	124	CON	D	21	2/2	A/A	A/A
61	U	C	22	1/1	A/A	G/G	125	CON	B	26	1/1	A/A	A/G
62	U	C	24	1/1	A/A	A/G	129	CON	D	ND	1/1	A/G	A/G
63	U	C	14	1/1	A/A	G/G	130	CON	D	ND	1/1	A/G	A/G
64	U	C	27	1/2	A/A	A/G	131	CON	D	ND	1/1	A/A	G/G
65	U	D	24	1/1	A/A	A/G	133	CON	D	ND	1/1	A/A	A/G

Severity of undermasculinisations A = mild (isolated hypospadias); B = moderate (hypospadias and unfused scrotum or micropenis); C = severe (female genitalia with clitoromegaly or unfused scrotum with perineal hypospadias and micropenis); D = complete (female external genitalia). The number of repeats (CTGCAG) encoded by exon 1 are designated as 1 or 2. The presence of an adenosine (A) or a guanine (G) at nucleotide 872 and 935 are shown. ND = not done.

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two fragments of 92 bp and 19 bp) and 872G alleles with no *ApoI* site. Alleles of the 872A/G polymorphism were also distinguishable using *Tsp509I*, which also cleaves only the 872A allele. Oligonucleotide 118 contains a mismatch (the most 3' guanine) that creates an *RsaI* site only in the 935G allele (generating two fragments of 85 bp and 26 bp).

All restriction enzyme incubations were performed according to the manufacturer's instructions (New England BioLabs, Hitchin, Hertfordshire, UK). DNA fragments were separated by electrophoresis and visualised by ethidium bromide staining. PCR was performed using standard conditions (further details available on request). Exon 1 PCR products with and without the 6-nucleotide insert and exon 10 PCR products containing the 872A, 872G, 935A and 935G nucleotides (as determined by restriction enzyme analysis) were directly sequenced and confirmed the validity of the genotyping methodology. The number of polyglutamine repeats in the undermasculinised and control (except four samples) groups had previously been determined by measuring the CAG repeat length within the AR (includes the terminal CAA codon encoding glutamine) (8).

Statistical analysis

Fisher's exact test was used to compare allele frequency between the undermasculinised and control groups. The unpaired *t*-test that was used to compare mean AR(Q)*n* length between groups and the odds ratio (OR) were calculated by standard methods. Statistical significance was regarded as $P \leq 0.05$.

Results

LQ insertion

The proportion of individuals possessing at least one allele encoding the additional LQ+ was greater in the undermasculinised group (40%) than in the control group (29%), although this difference did not attain significance ($P = 0.07$) (Table 1). However, undermasculinised individuals with LQ+ were found to have longer AR(Q)*n* lengths (mean = 25.4) compared with those without (mean = 23.6; $P = 0.02$). AR(Q)*n* repeat lengths of ≥ 26 showed the strongest association ($P = 0.002$) with possession of at least one allele for LQ+ within the undermasculinised group (Table 2). These relationships between LQ+ and AR(Q)*n* length were absent in the control group ($P > 0.05$ for all AR(Q) lengths).

N291S (A872G) and N312S (G935A)

The frequency of the 872A allele encoding N291 was significantly greater ($P = 0.03$) in the undermasculinised group ($n = 146$, 97.3%) than in the controls

($n = 101$, 91.8%). The greater proportion of homozygous individuals with the 872A allele (872A/A) in the undermasculinised group, compared with that of the control group ($n = 47$, 85%), accounted for most of the association ($P = 0.05$) (Table 1).

The frequency of the 935A allele in the undermasculinised group (53 of 150, 35.3%) was not significantly lower than that in the control group (46 of 110, 41.8%; $P = 0.06$). However, when the effect of two 935A alleles were considered, the undermasculinised group (6 of 7, 58%) had a significantly lower proportion than the controls (10 of 55, 18.2%; $P = 0.05$).

The proportion of undermasculinised individuals having the combination of 872A/A and the absence of 935A/A (equivalent to the presence of 935G/G or 935G/A) (88%) compared with the control individuals (69%), was greater than the proportions having either genotype alone, and the association was much stronger ($P = 0.006$). The OR of having this genotype (presence of 872A/A and absence of 935A/A) rather than any other genotype in the undermasculinised group compared with the control group was 3.28 (95% CI 1.33 to 8.08). In contrast, alleles encoding LQ+ did not strengthen the association of the 872A/A ($P = 0.05$) or the 935A/A ($P = 0.06$) genotypes.

Discussion

This study demonstrates that the N291S and N312S polymorphisms are associated with male undermasculinisation of unknown aetiology. The greater proportion of undermasculinised individuals with the allele (872A) encoding asparagine (N291) compared with the control group indicates that it is the presence, and not the loss of, a potential N-glycosylation site (requiring the asparagine at 291) that contributes to the disorder (12). In contrast, the allele encoding asparagine at

Table 2 Comparison of AR(Q)*n* repeat length between undermasculinised individuals with the additional LQ repeat and those without.

AR(Q) <i>n</i> repeat length	Additional LQ*		<i>P</i>
	Present (no. (%))	Absent (no. (%))	
≤ 22	6	14	
≥ 23	24 (80)	31 (69)	NS (0.12)
≥ 24	21 (70)	23 (51)	NS (0.052)
≥ 25	19 (63)	16 (36)	0.012
≥ 26	17 (57)	10 (22)	0.0021
≥ 27	13 (43)	7 (16)	0.0068
≥ 28	8 (27)	5 (9)	NS (0.056)
≥ 29	6 (20)	3 (7)	NS (0.067)

*Includes both homozygous and heterozygous individuals with the allele encoding the extra LQ repeat.

codon 312 (935A/A) was more frequent within the control group, suggesting that it might 'protect' against undermasculinisation. The presence of a stronger association with the undermasculinised samples when both unfavourable genotypes for the exon 10 polymorphisms (presence of 872A/A and absence of 935A/A) are combined suggests that the effects of these polymorphisms are synergistic and biologically relevant.

The genotypes with at least one allele encoding LQ+ were strongly associated with increased AR(Q)n length in the undermasculinised group. The location of these two polymorphisms on different chromosomes excludes linkage disequilibrium. However, unlike AR(Q)n length, LQ+ was not shown to be independently associated with the undermasculinised group (although the *P* value was close to the threshold for significance). This may be because the independent biological effect of LQ+ is relatively small or, alternatively, it might be an indicator of longer repeat lengths at other loci.

The control individuals in this study were selected from the same database, to avoid selection bias for factors such as ethnicity or geography that may influence allele frequencies (12). However, the contribution of the LHR polymorphisms may be larger than observed in this study, because these polymorphisms may have also contributed to the aetiology in the control individuals with AR mutations and Denys-Drash syndrome (i.e. these controls favoured the null hypothesis of observing no effect). The lack of association between the exon 1 and exon 10 polymorphisms may indicate that there are separate multi-factorial pathways leading to undermasculinised genitalia. An additional LHR amino acid polymorphism (R124Q) has recently been described (14); it will be interesting to determine whether it contributes to undermasculinisation and, if so, whether it interacts with the exon 1 polymorphism, the exon 10 polymorphisms, or via an alternative pathway. Future studies are likely to require a large independent cohort with matched controls to assess how the LHR polymorphisms interact, the size of their effect, and the nature of their contribution to the aetiology, severity and modification of other clinical features (e.g. testicular maldescent) associated with undermasculinisation.

This study demonstrates not only the novel finding that LHR polymorphisms are associated with male genital development, but also that associations exist between polymorphisms in the LHR and AR genes in this disorder. Therefore the data support the previously proposed multi-factorial aetiology for undermasculinised genitalia, particularly in cases without single gene defects (9). Other disorders associated with the abnormal production and regulation of androgens, such as male infertility, testicular maldescent and isolated hypospadias, may also benefit from the investigation of polymorphisms in the LHR.

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