CLINICAL STUDY

Genetic analysis of the *INSL3* gene in patients with maldescent of the testis

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Abstract

Objective: Testicular maldescent is important because it is a common congenital disorder that is associated with an increased risk of infertility and testicular cancer. Murine studies indicate that testicular maldescent can result from disruption of insulin-like factor 3 (*INSL3*) activity and that it may be more severe when there is concurrent undermasculinisation. Therefore, the *INSL3* gene was screened for mutations and polymorphisms that may contribute to testicular maldescent in patients with undermasculinisation as well as those with isolated testicular maldescent.

Methods and Results: The patient groups consisted of individuals with isolated testicular maldescent ($n = 28$) and patients with undermasculinised genitalia and intra-abdominal ($n = 24$) or inguinal gonads ($n = 33$). The three control groups were: normal males ($n = 15$), males with undermasculinised genitalia and scrotal gonads ($n = 29$) and females ($n = 82$). SSCP/HA mutation screening detected eight variants, five of which were predicted to alter the protein sequence (A-1G, V19L, P25S, A36T, R78H). Three of the amino acid changes (A-1G, V19L, R78H) each occurred in a single control sample and one was identified in a male with undermasculinised genitalia and intra-abdominal testes (P25S). The A36T amino acid polymorphism was found in both patient and control groups at a similar frequency.

Conclusions: The evidence suggests that *INSL3* mutations and polymorphisms are not a major cause of testicular maldescent with or without associated undermasculinisation.

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Introduction

The failure of the testes to descend is a common congenital abnormality that has important long-term consequences including an increased risk of testicular cancer and reduced fertility (1, 2). Testicular descent is thought to be a biphasic process: there is an initial migration from a position near the lower pole of the kidney down to the lower abdomen, followed by a second phase through the inguinal canal to the scrotum (3). This relative transabdominal movement of the testis is believed to result from both the degeneration of the cranial suspensory ligament (CSL) and the development of the gubernaculum which are respectively attached to the cranial and caudal aspects of the testis (1). CSL degeneration is an androgen dependent process as androgen receptor (AR) deficient male mice have failure of the CSL to regress, along with maldescended testes (3, 4).

Gubernacular development appears to be controlled by insulin-like factor 3 (*INSL3*), a member of the insulin-like hormone superfamily (which also includes

relaxin) (5). In mice, the complete absence of *INSL3* (*Ins13* $-/-$) results in poor gubernacular development with intra-abdominal gonads and in contrast to AR deficient mice, there are normal internal and external male genitalia (6, 7). Furthermore, mice with absence of both *INSL3* and AR, have testes that remain at the embryonic 'starting' position, a more cranial position than occurs with the loss of only one of these genes (5). The additive effect of disrupting both the *INSL3* and androgen pathways suggests that 'mild' mutations or protein polymorphisms within *INSL3* (that may have no observable effect on their own) could contribute to testicular maldescent in the presence of reduced androgen action. This could account for the variation in the level of testis descent that occurs among patients with the same AR mutation that cannot be attributed to differences in the severity of genital undermasculinisation (8–10). Conversely, it is possible that subtle differences in androgenic activity arising from the mixed genetic background of the heterozygous *Ins13* ($+/-$) mice could have contributed to the variation in testicular descent at birth (from complete descent to

Table 1 Clinical details of the patient groups and control group 2.

No.	Patient group 1		Patient group 2			Patient group 3			Control group 2		
	RT (AoD)	LT (AoD)	RT	LT	Genitalia	RT	LT	Genitalia	RT	LT	Genitalia
1	D(9)	D(9)	AG	AG	FG	IG	IG	BS,PH,MP	LG	LG	FG
2	O	SG	AG	AG	BS,PH,MP	IG	IG	MP	LG	LG	FG,CM
3	O(12)	O(12)	AG	AG	FG#	IG	IG	BS,PH,MP	LG	LG	FG#
4	SG	IG	AG	AG	BS,PH	IG	IG	FG#	LG	LG	FG,CM
5	SG	IG	AG	AG	PH,MP	IG	IG	FG#	SG	SG	BS,PH,MP
6	D(8)	D(10)	AG	AG	FG#	IG	IG	BS,PH,MP#	SG	SG	BS,PH,MP
7	O(10)	D	AG	AG	BS,PH,MP#	IG	IG	FG◇	SG	SG	PH
8	SG	D(14)	AG	AG	FG,CM,U	IG	IG	BS,PH,MP#	SG	SG	PH,MP
9	SG	O(10)	AG	AG	FG	IG	IG	BS,PH	SG	SG	PH,MP
10	O(13)	O(13)	AG	AG	FG#	IG	IG	BS,PH,MP	SG	SG	BS,PH,MP
11	SG	H(10)	AG	AG	BS,Pen,MP◇	IG	IG	BS,PH	SG	SG	BS,PH,MP
12	SG	D	AG	AG	FG,CM◇	IG	IG	BS,PH,MP	SG	SG	BS,PH,MP
13	SG	O(14)	AG*	AG*	Glan,MP	IG	IG	BS,PH,U	SG	SG	BS,Pen
14	IG	SG	AG*	AG*	PH,MP	IG	IG	BS,PH,MP,U	SG	SG	BS,PH,MP
15	SG	D(7)	AG*	AG*	FG	IG	IG	FG	SG	SG	BS,PH,MP
16	IG	IG	AG*	AG*	FG,U	IG	IG	FG	SG	SG	BS,PH
17	D(Sc)	SG	AG*	AG*	BS,PH,U	IG	IG	FG	SG	SG	Pen,MP
18	AG	IG	AG*	AG*	Glan	IG	IG	FG	SG	SG	BS,PH,MP#
19	O(27)	SG	AG*	AG*	BS,PH	IG	IG	FG	SG	SG	BS,PH,MP#
20	H(P)	SG	AG*	AG*	FG,CM,U	IG	IG	FG#	SG	SG	Glan#
21	O(10)	O(10)	AG*	AG*	BS,PH,MP,U◇	IG	IG	FG#	SG	SG	BS,PH
22	IG	AG	AG*	IG	BS,PH◇	IG	IG	FG#	SG	SG	BS,PH,MP
23	O(P)	O(P)	IG	AG	FG	IG	IG	FG,CM,U	SG	SG	BS,PH
24	D(7)	D(7)	IG	AG	FG#	IG	IG	BS,PH	SG	SG	BS,PH,MP
25	D(P)	SG				IG	IG	BS,PH,MP	SG	SG	BS,PH,MP
26	D(15)	SG				IG	IG	FG#	SG	SG	BS,PH,MP
27	O(14)	SG				IG	IG	FG#	SG	SG	BS,PH
28	D(P)	SG				IG	IG	FG#	SG	SG	Glan,Gyn#
29						IG	IG	PH,MP#	SG	SG	BS,PH,MP#
30						IG	IG	Glan,MP			
31						IG	IG	BS,PH,MP			
32						IG	IG	Glan,MP			
33						SG	IG	BS,PH			

Patient group 1, isolated testicular maldescent; Patient group 2, undermasculinised genitalia and abdominal testis; Patient group 3, undermasculinised genitalia and inguinal testis; Control group 2, undermasculinised genitalia and bilateral scrotal testes. The left testis (LT) and right testis (RT) position are shown for each sample: SG, scrotal; IG, inguinal; AG, abdominal; AG*, direct visualisation of the testis within the abdominal cavity; LG, labial. The age of descent (AoD) into the scrotum is given in years of age (values in parentheses) where known (otherwise P, puberty; Sc, school age) for spontaneous delayed descent (D), hormonal treatment (H) or orchidopexy (O). Clinical features of undermasculinisation for each sample are shown: BS, bifid or unfused scrotum; PH, perineal hypospadias; MP, micropenis; Glan, glandular, hypospadias; Pen, penile shaft hypospadias; FG, female external genitalia; CM, clitoromegaly; U, uterus; Gyn, gynaecomastia. #Androgen receptor mutation present; ◇Other severe congenital anomalies.

bilateral maldescent) (7). The objective of this study was to examine the *INSL3* gene for mutations and polymorphisms that may cause or contribute to testicular maldescent in patients with undermasculinisation as well as in those with isolated testicular maldescent.

Methods

Samples

Three different patient groups and three control groups were included in this study (described in Results and Table 1). The female samples that formed one of the control groups were randomly selected from the UK. All undermasculinised subjects (Table 1 – Patient groups 2 and 3, and Control group 2) were selected from the Cambridge intersex database which has previously

been described (further details available on request) (11). Since undermasculinisation, irrespective of the defect in the androgen–AR pathway can cause testicular maldescent, no distinction was made between samples with a sex steroid biosynthetic defect, an AR mutation or an unknown aetiology. Local ethics committee approval was obtained for the use of patient samples as part of a sexual development disorders program.

SSCP and heteroduplex analysis (SSCP/HA)

The coding sequence and exon–intron boundaries of the *INSL3* gene were amplified from genomic DNA by the polymerase chain reaction (PCR); using two primer sets (F1/R1 and F2/R2) for exon 1, and two primer sets (F7/R4 and F3/R3) for exon 2 (Fig. 1). Automated DNA sequencing of the PCR products confirmed the

Figure 1 Diagrammatic representation of the *INSL3* gene and the location of the oligonucleotides used in this study. The start of the opening reading frame (Start ORF) and the stop codon (Stop ORF) are shown.

amplification of the correct gene. Primer sequences are as follows: F1, 5' GAAGAATGTTCTGTCCCTTCTCCAA 3'; R1, 5' GCGCACCAGCGCGGTACGAA 3'; F2, 5' CCCAGAGATGCGTGAGAAGTTGT 3'; R2, 5' GCATCTGCGCTACGTGCACCT 3'; F3, 5' TGGAGAGACGACA TCTGCTCCAT 3'; R3, 5' CCTCAGGAGCTCACCAGAC CAGA 3'; F7, 5' GGTAGCATGTCCTCTGTCGTTTCC 3'; R4, 5' GGACAGAGGGTCAGCAGGTCTTGTT 3'. Each PCR contained (per μ l) 2.5×10^{-2} units Taq DNA polymerase (Gibco BRL, Paisley, UK), ~ 4 ng of each primer (MWG AG Biotech, Ebersberg, Germany), 1–10 ng genomic DNA and 2.5% v/v formamide (BDH, Dorset, UK). For all primer sets, thermal cycling involved an initial 240 s at 95 °C followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 150 s. SSCP/HA was performed using a modified version of an established protocol involving the addition of 6 μ l formamide loading buffer to 3 μ l of PCR product, denaturation at 95 °C for 5 min and placement on ice for ~ 30 min to encourage heteroduplex formation (12). Electrophoresis was through MDE™ (Flowgen, Staffordshire, UK) on a Protean II apparatus with 20 cm plates (BioRad, Hertfordshire, UK) at 10 °C for 12–13 h at 200 V. DNA fragments were visualised by silver staining (12).

Genotyping of the A36T polymorphism using the F2TaqI/R2 primer set

PCR amplification using the F2TaqI (5' tccaccgaagc caggccttcg 3')/R2 primer set generated a 107 bp product. The PCR reagents and thermal cycling conditions were the same as for the other primer sets. From each PCR (10 μ l), 3 μ l (15 ng) was incubated overnight according to the manufacturer's instructions with 1 unit of *EagI*, and another 3 μ l with 1 unit of *TaqI*, each in a total volume of 10 μ l (all buffers and enzymes from New England Biolabs, Hertfordshire, UK). Half of each restriction enzyme reaction was electrophoresed through a 10% w/v polyacrylamide (19:1 bis:acrylamide) (Anachem, Luton, UK) with 10% glycerol (Sigma, Dorset, UK) using the Protean II apparatus with 20 cm plates at 150 V, 10 °C and 14 h. DNA fragments were visualised by silver staining (12). The A36T genotype frequencies were analysed using the χ^2 test and Fisher's exact test was used for the analysis of allele frequencies (13, 14).

DNA sequencing and protein alignments

Samples showing band shifts on SSCP/HA were PCR amplified directly from genomic DNA and sequenced by automated Taq Big Dye™ terminator cycle sequencing (10 μ l volume) using the forward and reverse PCR primers. Sequencing reactions were electrophoresed on an ABI 377 according to the manufacturer's instructions (Perkin Elmer, Warrington, UK).

Multi-species protein alignment using Pile-up (Genetics Computer Group, Madison, USA) included the following sequences (Genbank accession numbers in parentheses) (15): pig *INSL3* (X73636), cow relaxin-like factor (RLF) (AF094580), marmoset RLF (AJ011961), goat RLF (AF233686), mouse *INSL3* (X95603), RAT (AF139919), sheep Leydig insulin-like factor (16) and deer RLF (17). The *INSL3* DNA and protein sequence are numbered in accordance with Burkhardt and colleagues (18).

Results

Mutation analysis

SSCP/HA was performed on samples from male patients with: isolated testicular maldevelopment (Patient group 1, $n = 28$), undermasculinised genitalia and intra-abdominal cryptorchidism (Patient group 2, $n = 24$) and undermasculinised genitalia and inguinal testes (Patient group 3, $n = 33$). Three control groups were also screened by SSCP/HA (total $n = 125$): normal males (Control group 1, $n = 15$), males with undermasculinised genitalia and scrotal gonads (Control group 2, $n = 29$) and females (Control group 3, $n = 82$).

Four different nucleotide variants were detected within the patient groups, of which two altered the predicted protein sequence (Fig. 2 and Table 2). One of the nucleotide variants that altered the amino acid sequence was a known polymorphism involving a nucleotide 192G to A transition resulting in an alanine or threonine at codon +36 (A36T) (19). The other involved a heterozygous nucleotide 160C to T transition, causing the substitution of a proline (CCC) for a serine (CTC) at codon +25 (P25S). This latter change was unique to a patient (C110) with a 46 XY karyotype, completely female external genitalia, bilateral intra-abdominal testes, no uterus and normal male accessory sex glands. The gonadal histology following

Table 2 Nucleotide variants detected.

No.	Sample	Sample group	Phenotype	Nucleotide variant	Region	Site in protein sequence and codon (amino acid)
1	C164	Patient 2	Moderate U, IG	41A	Signal	−16 GCG (A) to GCA (A)
2	9H	Control 3	Control female	85G	Signal	−1 GCG (A) to GGG (G)*
3		Multiple		140A	B-chain	+18 CTG (L) to CTA (L)
4	C241	Control 2	Gyn, Infert, SG AR mutation	141C	B-chain	+19 GTG (V) to CTG (L)*
5	C336	Control 2	Moderate U, SG	152T	B-chain	+22 TGC (C) to TGT (C)
6	C110	Patient 2	Severe U, AG	160T	B-chain	+25 CCC (P) to CTC (S)*
7		Multiple		192A	C-peptide	+36 GCC (A) to ACC (T)*
8	4A	Control 3	Control female	1409A	C-peptide	+78 CGC (R) to CAC (H)*

Sample groups are as defined in the text. U, undermasculinisation; IG, inguinal gonads (bilateral); AG, abdominal gonads (bilateral); SG, scrotal gonads (bilateral); Gyn, gynaecomastia; Infert, infertility; *Nucleotide variant results in an amino acid change. AR, androgen receptor. Note that the initiation codon is numbered −24.

orchidectomy (age 10 years) was that of immature testis with Sertoli, Leydig and germ cells. The hormonal data and the surgical report describing the exact location of the testes at orchidectomy were unavailable. Sequencing of the entire coding region of the AR gene and all exon–intron boundaries did not detect a mutation (data not presented). Parental DNA was unavailable to determine whether the alteration (160C>T) occurred *de novo*.

Neither the nucleotide 160T variant (P25S) nor the silent nucleotide 41A variant were identified in the control groups, therefore they are both unique. However, the polymorphisms found in the patient groups at nucleotide 192G>A (A36T) and nucleotide 140G>A were detected in control samples (Table 2). Four additional heterozygous DNA sequence variants were identified (each in a single sample) in the control groups, three of which altered the amino acid sequence (Fig. 2 and Table 2): (i) a nucleotide 141G>C transversion resulting in a valine instead of leucine at codon +19 (V19L) in an undermasculinised male with bilateral scrotal testes; (ii) a nucleotide 85C>G transversion at codon −1 causing the substitution of an alanine with a glycine in a female control (A-1G); and (iii) a nucleotide 1409G>A transition replacing an arginine for histidine at codon 78 (R78H) in a female control.

Comparison of amino acid changes with a multi-species protein alignment

A multi-species alignment of the INSL3 protein sequence was used to identify evolutionary conserved

regions and residues that are thus likely to be functionally important (Fig. 3). One of the conserved regions within the B chain contains a tryptophan residue (B27) that is important for high affinity binding to the putative INSL3 receptor (20). The P25S substitution occurs within the C-G-G-P-R (B22–26) amino acid sequence that is essential to the correct positioning of this tryptophan residue (20). *In vitro* replacement of the (L-) proline of this sequence with D-proline almost completely abolishes INSL3 activity and even the replacement with the similar alanine residue (also a neutral charged and hydrophobic) results in only 75% retention of wild-type activity (20). INSL3 binding is clearly sensitive to changes at the site of the hydrophobic proline residue therefore its replacement with a smaller, uncharged serine residue would be expected to substantially reduce activity.

The other conserved region in the B chain contains the R-A-L-V-R sequence that seems to be important for the binding of INSL3 to the relaxin receptor (21). However, it is the arginine (R) at either end of this sequence that is crucial and therefore the substitution of the valine for leucine (V19L), another neutral charged hydrophobic amino acid, is probably conservative. The other three amino acid changes (including the A36T polymorphism) occur at non-conserved sites and on their own would not be expected to impair INSL3 function sufficiently to cause testicular maldescent. In support of this, the A36T polymorphism was found in both patient and control samples. However, it is possible that these amino acid changes could contribute to maldescent in the presence of other disruptive influences on testis descent.

Figure 2 Nucleotide variants within the *INSL3* gene. Panels A–H show automated Taq polymerase Big Dye™ terminator cycle sequencing chromatograms for seven of the eight types of nucleotide variant found (the nucleotide 192A/G polymorphism is shown in Fig. 3). (A) Heterozygous for nucleotide 41G/A, (B) heterozygous for nucleotide 85C/G, (C) homozygous for nucleotide 140A, (D) homozygous for nucleotide 140G, (E) heterozygous for nucleotide 141G/C, (F) heterozygous for nucleotide 152 C/T, (G) heterozygous for nucleotide 1409G/A, (H) sequencing shows homozygosity for nucleotide 160T although SSCP analysis (not shown) and restriction enzyme analysis (I) shows the presence of the wild-type allele (colour chromatogram available on request). Panel I shows the ϕ x174/BsuRI marker (M) with the 194, 118 and 72 bp fragments indicated, the 184 bp undigested product (U), the PCR product from a normal male digested with Apal creating two fragments of ~122 and ~68 bp indicating the wild-type sequence (160C). The sample with the 160T variant allele (which is not cut by Apal) is shown to have the wild-type allele. The lesser intensity of the ethidium bromide stained bands of the cut allele is due to the smaller size of the fragments and heterodimer formation.

Figure 3 The predicted amino acid residue changes found in the control and patient samples compared with the multi-species alignment of the INSL3 protein sequence. Boxes indicate regions of high sequence conservation.

Genotyping of the A36T polymorphism

All patient and control samples (excluding those with an altered amino acid sequence) were genotyped to determine if the relative frequency of the alleles encoding the A36T polymorphism differed between the groups. The migration of PCR products (F2/R2)

containing nucleotide 192G (encoding alanine) and 192A (threonine) could be distinguished by SSCP. Sequencing of the PCR product from samples showing only the slow, the fast or both the fast and slow migrating bands respectively demonstrated the 192A, 192G and both the 192A and G allele sequences. The validity of genotyping by the SSCP pattern was further

Table 3 Genotyping of the A36T polymorphism.

Group	Phenotype	A (%)	A/T (%)	T (%)	Total#	χ^2	P value	F test
Patient								
1	Isolated	12 (43%)	8 (29%)	8 (29%)	28	0.58	0.748	0.116
2	U-AG	11 (48%)	9 (39%)	3 (13%)	23*	N/A	N/A	0.092
3	U-IG	15 (45%)	10 (30%)	8 (24%)	33	0.23	0.891	0.118
Control								
1	Normal male	6 (40%)	6 (40%)	3 (20%)	15	N/A	N/A	0.161
2	U-SG	14 (50%)	9 (32%)	5 (18%)	28*	0.52	0.771	0.093
3	Female controls	34 (43%)	28 (35%)	18 (22%)	80*			
Subgroup								
	Complete U	14 (58%)	6 (25%)	4 (17%)	24	1.86	0.395	0.055
	Severe U	14 (44%)	11 (34%)	7 (22%)	32*	0.01	0.995	0.119
	Moderate U	10 (40%)	10 (40%)	5 (20%)	25*	0.21	0.900	0.131
	Mild U	3 (100%)			3	N/A	N/A	0.051

Patients with other amino acid changes are excluded from the genotype and allele frequency analysis of some groups (*). The actual number of samples as well as the percentage (values in parentheses) that are homozygous for alanine (A), homozygous for threonine (T) or heterozygous for alanine and threonine (A/T) are shown for each group. The groups for which genotyping and analysis were performed are defined in the text. U, undermasculinisation; AG, intra-abdominal gonad; IG, inguinal gonad; SG, scrotal gonad. N/A, χ^2 test is not applicable for groups where the expected value of the T genotype is less than 5. The P value refers to the χ^2 test. The P value from the analysis of the allele frequencies using Fisher's exact test is also shown (F test).

Figure 4 Genotyping the A36T polymorphism. Three representative samples show that the scoring of the fast (F, 192G) and slow (S, 192A) migrating alleles by SSCP is the same as by restriction enzyme digestion with *TaqI* (cuts 192A) and *EagI* (cuts 192G). The presence in the homozygous 192A (*TaqI* and *EagI*) of additional faint bands demonstrates the importance of controlling for partial digestion and overspill from adjacent lanes. In the heterozygous samples the greater intensity of the uncut fragment compared with cut fragment can be accounted for by heterodimer formation.

confirmed by a method that minimised the possibility of incorrect scoring due to partial or failed digestion. The F2*TaqI* oligonucleotide containing an alteration of the wild-type sequence at the third base from the 3' end, was incorporated into the PCR product and subsequent digestions with the *TaqI* and *EagI* restriction enzymes allowed the identification of the respective nucleotide 192A and 192G alleles. The genotyping of 19 samples (8 homozygous for 192G, 4 homozygous for 192A and 7 heterozygous samples) by this method was identical to that by SSCP (Fig. 4).

The genotype frequency within the population was determined from the randomly selected females: 0.43 were 192G homozygous, 0.35 were heterozygous and 0.22 were homozygous for 192A. Comparison of this genotype frequency to the patient groups (1, 2 and 3)

and Control group 2, showed no statistically significant difference (Table 3). The frequencies of the 192G (0.6) and 192A (0.4) alleles were calculated from the genotype frequencies of the female controls. These allele frequencies were compared with all three patient (1, 2 and 3) and control groups (1 and 2) using Fisher's exact test and were also not significantly different (Table 3).

The groups with abdominal, inguinal and scrotal gonads samples were sorted according to whether the degree of undermasculinisation was complete (completely female genitalia, $n = 24$), severe (unfused scrotum, perineal hypospadias and micropenis or female genitalia with clitoromegaly, $n = 32$), moderate (micropenis or hypospadias and unfused scrotum, $n = 25$) or mild (isolated hypospadias, $n = 3$). The genotype and allele frequencies of these groups were not significantly different to that of the female controls indicating that the severity of undermasculinisation is not influenced by the A36T polymorphism (Table 3).

Discussion

In patients with isolated testicular maldescent, no mutations were identified in the coding region or the exon-intron boundaries of the *INSL3* gene. These patients had a relatively 'mild' phenotype with many having only delayed descent, consistent with the observed phenotype of mice with heterozygous *INSL3* mutations (more likely than homozygous mutations in sporadic isolated cryptorchidism) (7). The screening for *INSL3* variants in patients with undermasculinisation as well as testicular maldescent identified one individual with a substitution (P25S) in the small, highly conserved and functionally important region for *INSL3* binding. The absence of genital abnormalities in murine *Insl3* knockout mice ($-/-$ or $+/-$) suggests that this would not have contributed to the patient's undermasculinised genitalia (5, 6). It is possible that the amino acid change may have contributed to the failure of the testes to descend out of the abdomen and into the inguinal region (22). However, three other unique missense variants (A-1G, V19L, R78H) were identified in controls, and the P25S variant maybe another rare or 'private' polymorphism. Therefore, without functional studies and evidence that the mutation is only present in affected family members or occurred *de novo*, the relationship between the substitution and the patient's phenotype remains unresolved.

There appears to be no allele or genotype of the A36T polymorphism that confers susceptibility to testicular maldescent either in the absence or the presence of insufficient androgen action. However, because of the heterogeneity of the groups (which was desirable for a mutation screening study) it is possible that an association may have not been detected if the A36T polymorphism has a very weak effect on

testis descent. Future association studies would thus benefit from a larger number of samples and greater homogeneity of the groups.

As well as this study, two other studies have failed to find *INSL3* mutations in patients with isolated testicular maldescent (although one did not examine all sequence encoding the C peptide) (23, 24). One of these studies also showed no association between isolated testicular maldescent and the A36T polymorphism (24). Unfortunately, it is unclear in these other studies whether cryptorchidism refers to inguinal or intra-abdominal testes or both, thereby making it difficult to compare or combine the data with that of the present study (23, 24). However, together these data suggest that if the cause of testicular maldescent lies within the *INSL3* gene *per se*, then the mutations are located in regions that regulate *INSL3* expression (but outside the first 202bp promoter region which has already been examined) or affect post-transcriptional and post-translational modification (24).

Recent evidence shows that murine *INSL3* expression is substantially reduced by diethylstilbestrol (DES), a drug associated with a higher incidence of cryptorchidism and other genital anomalies following human intra-uterine exposure (25, 26). It will be interesting to determine whether this is part of the mechanism of action, of other environmental endocrine disruptors. Discovering how *INSL3* expression is regulated could be the key to understanding other genetic, neurological and physical influences on testis descent and perhaps shed some light on why the incidence of testicular maldescent appears to be increasing (2, 27).

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Note added in proof

Tomboc and colleagues (28) have identified heterozygous DNA sequence variants that are predicted to alter the *INSL3* protein (P69L and R49X). Unfortunately, the variant was not shown to be due to a *de novo* mutation in one case (P69L) and the other variant (R49X) was present in an unaffected grandfather. These results further demonstrate the difficulty in interpreting the phenotypic effect of *INSL3* variants and the need for further functional studies.

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