Insulin-like 3/Relaxin-Like Factor Gene Mutations Are Associated with Cryptorchidism*

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ABSTRACT

Cryptorchidism is a common anomaly of male sexual differentiation. Two phases of testicular descent are recognized, transabdominal and inguinoscrotal. With evidence that androgens and Müllerian inhibitory hormone were not completely responsible for testicular descent, the existence of a third testicular hormone mediating testicular descent was postulated. Insulin-like 3 (INSL3) [also known as relaxin-like factor (RLF) and Leydig insulin-like protein (LEY I-L)] is a member of the insulin/relaxin hormone superfamily that is highly expressed in Leydig cells. The phenotype of transgenic mice with targeted deletion of the *Insl3* gene was bilateral cryptorchidism with morphological evidence of abnormal gubernacular development.

With this implicit evidence that *Insl3* mediates testicular descent in mice, we performed mutation detection analysis of the coding regions of the 2 exon *INSL3* gene in genomic DNA samples obtained from 145 formerly cryptorchid patients and 36 adult male controls. Single-strand conformational polymorphism analysis was used for the mutation detection studies. Two mutations, R49X and P69L, and several polymorphisms were identified. Both mutations were located in the connecting peptide region of the protein. The frequency of *INSL3/RLF* gene mutations as a cause of cryptorchidism is low, because only 2 of 145 (1.4%) formerly cryptorchid patients were found to have mutations. (*J Clin Endocrinol Metab* **85:** 4013–4018, 2000)

N**ORMAL MALE SEXUAL** differentiation is a complex process requiring testicular development, stabilization of the Wolffian ducts, regression of the Müllerian duct derivatives, and testicular descent into the scrotum. Although many of the genetic and hormonal factors involved in the process of sexual differentiation have been established (1), the molecular mechanism underlying the sexually dimorphic position of the gonads remains unknown. Incomplete testicular descent, cryptorchidism, is a common anomaly of male sexual differentiation affecting approximately 3% of human males at birth. By 1 yr of age, the prevalence rate declines to 1% because of spontaneous testicular descent (2). Consequences of cryptorchidism include the need for surgical intervention and increased risks to develop oligo/azoospermia and testicular tumors (3).

Two phases of testicular descent have been recognized (4). In the first (or transabdominal) phase, the testes descend to the inguinal region. During this phase, rapid gubernacular cell proliferation occurs concurrently with regression of the cranial suspensory ligament. This first phase of testicular descent occurs between 10–15 weeks gestation in humans

and around 15.5–17.5 days post coitum in mice. During the second (or inguinal-scrotal) phase, the testes descend from the inguinal region into the scrotum by regression of the gubernaculum. This second phase occurs at 28–35 weeks gestation in humans and 2–3 weeks after birth in mice (5).

Thus, testicular descent requires a series of dynamic changes in the gubernaculum and the cranial suspensory ligament. Hormones such as androgens and Müllerian inhibitory hormone (MIH) have been investigated as factors that control testicular descent (6, 7). Current evidence suggests that transabdominal descent is not androgen-dependent (8). Although cryptorchidism is the most common presenting feature for persistent Müllerian duct syndrome, the aberrant positioning of the testes in this disorder has been attributed to anatomic obstruction, because the testes are typically attached to the Müllerian duct derivatives with variable positioning ranging from intraabdominal to transverse testicular ectopia. Persistent Müllerian duct syndrome, caused by either MIH or MIH type II receptor mutations, is a rare genetic cause of cryptorchidism (9–11). However, normal testicular descent in MIH-deficient knockout mice suggests that, at least in mice, the role of MIH in testicular descent is minimal (12).

Hence, existence of a third testicular hormone was postulated. Using an *in vitro* system, a low-weight molecular substance, known as descendin, derived from testicular extracts was found to stimulate porcine gubernacular cell proliferation; whereas ovarian extracts, testosterone, dihydrotestosterone, MIH, and inhibin, did not (13, 14). Recently another testicular hormone, insulin-like 3 (INSL3), also known as Leydig insulin-like protein (LEY I-L) or relaxin-like

factor (RLF) (15) has been identified. The gene and its protein product have been characterized in several species, including man, pig, sheep, and marmoset monkey (16–19). Similar to insulin and relaxin, it consists of a B chain, a connecting peptide region, and an A chain. As anticipated for a hormone involved in testicular descent, sexually dimorphic expression was observed in mice, with expression of *Insl3* first noted at postcoital day 13.5 in male embryos and not until postnatal day 6 in females (20).

Transgenic mice with targeted deletion of the *Insl3* gene showed bilateral cryptorchidism, normal virilization of the external genitalia, and normal androgen-dependent behavior. Morphological evaluation of the homozygous knockout males revealed developmental abnormalities of the gubernaculum (21, 22). Development of the Wolffian duct structures was normal, and Müllerian duct structures were absent, indicating appropriate testosterone and MIH secretion. Thus, these findings excluded the possibility that cryptorchidism in the *Insl3* knockout mice was secondary to androgen deficiency or the loss of the MIH-mediated activity.

Of interest, the phenotype of heterozygous male mice differed according to the age at which the animals were examined. At birth, approximately 75% of transgenic mice heterozygous for *Insl3* deletions had partial unilateral or bilateral undescended testes. But, all adult heterozygotes showed full testicular descent. Thus, the phenotype of the *Insl3* heterozygotes is similar to the human situation in which spontaneous resolution of cryptorchidism often occurs. With this implicit evidence that *Insl3* mediates testicular descent in mice, we sought to determine whether mutations in *INSL3/RLF* could be associated with human cryptorchidism.

Materials and Methods

Subjects

Blood samples for extraction of genomic DNA were obtained from 145 males who had previously undergone surgical correction for cryptorchidism and from 36 adult male controls. Ages of the subjects ranged from 1–54 yr; and for the controls, 26–54 yr. This protocol is approved by the Human Rights Committee of the Children's Hospital of Pittsburgh. Written informed consent was obtained from all adult participants and from parents of all children.

Mutation detection studies

Genomic DNA was extracted from peripheral blood leukocytes. Mutation detection studies were performed using single-strand conformational polymorphism (SSCP) analysis. Exon 1 was amplified using 5'-tgggagaaaggctctggcac-3' and 5'-ctgggctcatgcatgcaaac-3' primers (Fig. 1). For exon 2, the sequences of the primers used were 5'-tgcatgcatgagt-gtttggtggg-3' and 5'-atcagtagggacagagggtc-3'. The PCR reactions consisted of 0.125 μg genomic DNA, 0.15 μL of each of 20 pmol primers, 1.25 μL 10× PCR buffer, 0.07 μL *Taq* polymerase (Display TAQ FL), 0.09 μL ^{32}P deoxy-ATP (10 mCi/mL, NEN Life Science Products, Boston, MA), and 8.3 μL sterile water in a total vol of 12.5 μL. PCR thermocycler conditions for exons 1 and 2 were 94 C for 30 sec, 64 C for 30 sec, and 72 C for 1 min, for 30 cycles, with a final extension at 72 C for 7 min. Three distinct gel conditions were used for each PCR product: 1) MDE gel (FMC BioProducts, Rockland, ME) with 0.6× Tris-Boric Acid-0.5 M EDTA (TBE) buffer at 7 W, at room temperature for 18 h (exon 1) and 15 h (exon 2); 2) 5% acrylamide with 0.5× TBE buffer at 30 W, at 4 C for 4.5 h (exon 1) and 4 h (exon 2); and 3) 5% acrylamide with 5% glycerol and 0.5× TBE at 20 W, at room temperature for 6 h. Using these three different gel conditions, we have typically detected 85–90% of sequence variants (23).

Unique conformers were excised from the dried gels and eluted into 100 μL 0.1× Tris-HCl-EDTA. A 5-μL aliquot of each eluted conformer was reamplified using the original primer pair and thermocycling conditions. The PCR products were sequenced in both sense and antisense orientations, either manually using the Sequitherm Excel II DNA sequencing kit (Epicentre Technologies, Madison, WI) or using an ABI automated sequencer 377R according to the manufacturer's protocol. When nucleotide alterations altered restriction sites, restriction fragment digestion of PCR-amplified genomic DNA was performed to confirm the presence of the sequence variant (see *Results*).

Statistical analysis

AbSTAT statistical software (Release 1.94, Anderson-Bell, Boulder, CO) was used to perform χ-square analyses.

Results

Exon 1

SSCP analysis of exon 1 showed two distinct conformer patterns (Fig. 2). The conformer patterns were identified both among subjects with cryptorchidism and among controls. Conformers were excised from subjects who were homozygous for each pattern. On sequence analysis, eluted bands from one conformer pattern showed the normal sequence. Sequence analysis of eluted bands from the second conformer pattern showed two nucleotide changes. The first change was G→A at nucleotide position 1266, which
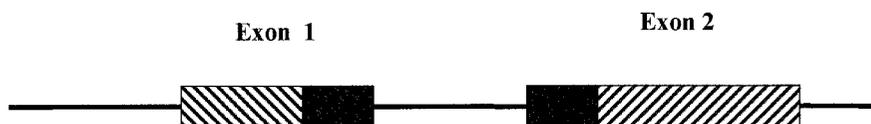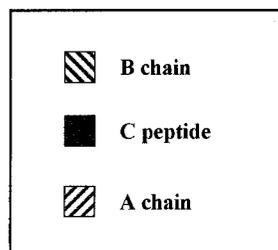
Exon 1   **Exon 2**

FIG. 1. Cartoon of *INSL3/RLF* gene.
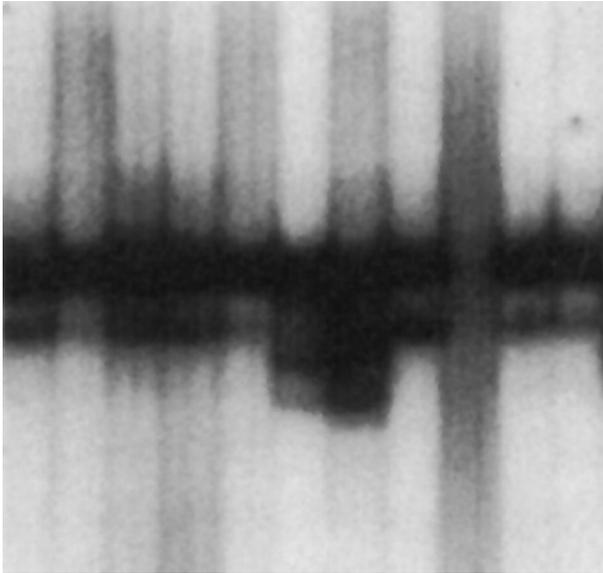
▨ B chain

■ C peptide

▨ A chain

FIG. 2. Autoradiograph of exon 1. Two conformer patterns were detected in formerly cryptorchid patients and control subjects.

TABLE 1. Exon 1 variant. Distribution of the variants, identified by SSCP analysis of exon 1, is listed below

	Patients	Control subjects
Wild-type	69	19
A36T	19	4
A36/A36T	57	13

mapped to codon 18 of the B chain of exon 1. This variant did not change the amino acid, leucine. The second change was G→A at nucleotide position 1318. The predicted consequence of this variation is to change codon 36 from alanine to threonine in the C peptide region of the protein. Because the conformer pattern showed homozygosity, both nucleotide changes were shown to be located on the same allele.

Among the patients treated for cryptorchidism (Table 1), 69 were homozygous for the normal allele, 19 were homozygous for two nucleotide changes, 1266G→A and 1318G→A. Fifty-seven subjects were heterozygous; 1 allele carried the normal sequence and the other allele carried both nucleotide changes. Among the control subjects, 19 men were homozygous for the normal allele, 4 men showed only the variant allele, and 13 men were heterozygous. Allelic frequency was comparable among patients and controls ($P > 0.05$).

The sequence variations at positions 1266 and 1318 generate conservative amino acid changes and were found in comparable distribution in both affected subjects and controls. Thus, these variants most likely represent neutral linked polymorphisms and do not influence INSL3/RLF activity.

Exon 2

SSCP analysis of exon 2 revealed four different conformer patterns (A, B, C, and D). The majority of subjects (142 patients and 36 control subjects) showed the same pattern. Sequence analysis of conformer pattern A showed the normal sequence. Three different conformers (B, C, and D) were

identified in 3 formerly cryptorchid subjects (Fig. 3). All three individuals were heterozygous for a sequence variant with the presence of a unique conformer in addition to the common conformer pattern noted in the majority of patients and control subjects.

The propositus from whom conformer B was isolated presented with an incarcerated right inguinal hernia at 6 weeks of age and, at that time, was found to have a right undescended testis. He subsequently underwent right inguinal herniorrhaphy and orchiopexy. Sequence analysis of conformer B showed a C→T mutation at nucleotide 2450. The consequence of this mutation is that arginine at codon 49 is changed to a termination codon (R49X). This mutation also caused the loss of a *Bsma*1 site. Restriction fragment digestion confirmed the results of the SSCP analysis that this patient was heterozygous for the 2450C→T mutation. Restriction fragment digests of genomic DNA samples obtained from the maternal grandparents, mother, half-brother, and half-sister of the propositus were also examined (Fig. 4). The propositus, his mother, and maternal grandfather were heterozygous for the R49X mutation. Because this patient was African-American, 90 genomic DNA samples (180 chromosomes) from healthy control African-American males were studied. None showed the 2450 C→T variant.

The patient from whom conformer C was isolated presented at 8 months of age with a nonpalpable right testis. At the time of surgery, his right testis was located intraabdominally. Sequence analysis of unique conformer C showed a C→T change at nucleotide 2511. The consequence of this mutation is that codon 69 is changed from proline to leucine (P69L). This variation did not alter restriction sites. None of the 36 healthy control subjects show this variant. Genomic DNA samples from an additional 69 males were evaluated; none were found to carry the 2511 C→T variant.

The African-American subject from whom conformer D was isolated presented with unilateral cryptorchidism. At the time of surgical correction, his right testis was found to be within the right inguinal canal. Sequence analysis of unique conformer D showed a C→T nucleotide change at nucleotide 1259, which is located in the intron. No consensus splicing signals are altered. This conformer was identified in 16 African-American control subjects and in 0 of the 105 Caucasian control subjects.

Discussion

Consequences of cryptorchidism include impaired spermatogenesis and testicular neoplasms, conditions that may influence reproductive competence. It could, thus, be anticipated that the process of testicular descent involves multiple genes, some with redundant function, to ensure proper testicular descent and fertility. Nevertheless, cryptorchidism is a common disorder affecting 3% of male infants at birth. Familial cryptorchidism has been described, but seems to be uncommon, ranging from 1–6% (24). Among patients with cryptorchidism, testicular position shows phenotypic heterogeneity with locations ranging from intraabdominal to the inguinal canal (25, 26). In some cases of unilateral cryptorchidism, abnormalities can occur in the contralateral scrotal testis, suggesting the possibility that an intrinsic testicular
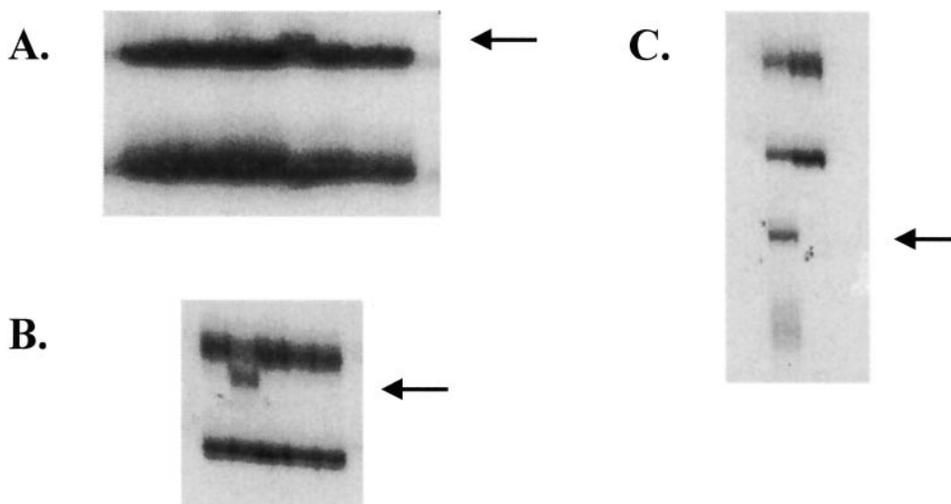
FIG. 3. Autoradiograph of exon 2 conformers. A, Conformer which showed 2450C→T variant; B, conformer which showed intron variant; C, conformer which showed 2511C→T variant.
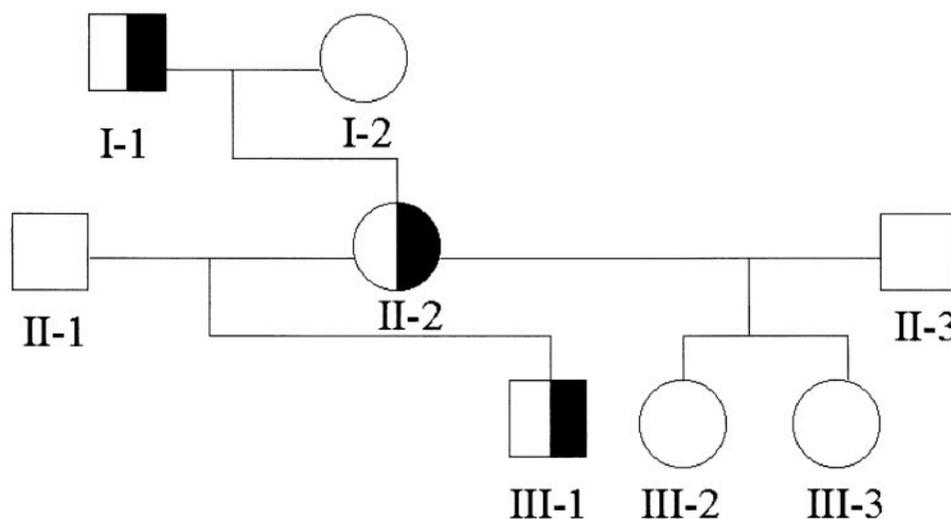

FIG. 4. Restriction fragment length polymorphism analysis. *Upper portion*, Diagram of family pedigree; *lower portion*, results of *Bsma*I digest. The 2450C→T variant causes the loss of a *Bsma*I site. Carrier status is indicated by the *shaded markers* in the pedigree diagram.

abnormality may be present in some affected individuals (27).

Based on the phenotype of the knockout mouse, one gene that might be associated with cryptorchidism in humans is *INSL3/RLF*. INSL3/RLF, a member of the insulin-like hormone superfamily, is expressed predominantly in the Leydig cells of the fetal and adult testes and in the theca cells of the postnatal ovaries and seems to be a marker of Leydig cell differentiation status (28–30). Transcription of the *INSL3/RLF* gene is mediated by steroidogenic factor-1, an orphan nuclear receptor essential for fetal pituitary, adrenal, and gonadal differentiation (31–33). Recently, an INSL3/RLF receptor has been identified (34).

Using SSCP analysis, we have identified three variants involving the coding region and one involving the intron in the *INSL3/RLF* gene. The 2450C→T mutation that changes codon 49 from arginine to a termination codon is clearly a deleterious mutation because it leads to a truncated protein product lacking the A chain. The 2511 C→T change that changes codon 69 from proline to leucine is likely deleterious

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HUMAN (C-PEPTIDE)    RRPAAGGDRELLQWLERRHLLHGLVADSNLTL-----GPGLQPLPQTSHHRHHR
MARMOSET             RRPVAAGDGELLQWLERRHLLYGLVANSEP----APGGPGLQPMPQTSHHRHRR
COW                  DGRPVAGGDRELLRWLEGQHLLHGLMASGDPVLVLAP-----QPLPQASRHHHHRR
SHEEP                 GRPAAGGDRELLRWLEGQHLLHGLMASGDPVLVLAP-----QPLPQASRHHHHRR
PIG                  GRAVAGGDRELLQWLEGQHLFHGLMASGDPMLVLAP-----QPPPQASGHHHHRR
MOUSE                TQPCGDPGRELLQWLEQRHLLHSLVADVDPALD----------PQLPRQASHARG
CONSENSUS            -----------------------------------------PQ------H-R
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Fig. 5. Comparison of protein sequences. Amino acid residues are indicated by the *single universal letter code*. The R residue at codon 49 and the P residue at position 69 are *underlined*.

because it leads to a nonconservative amino acid change, changes a highly conserved residue, and occurs in less than 1% of the population (Fig. 5). Both mutations occur in the connecting peptide region of the INSL3/RLF protein. The intron variant seems to be a rare polymorphism occurring preferentially in African-Americans. The linked L18L;A36T variant seems to be a common polymorphism because the frequency of the less common allele is high and is similar among the patients and control subjects (32.8% and 29.2%, respectively). This exon 1 variant was previously described (35, 36).

The propositus with the R49X mutation presented with incarcerated right inguinal hernia at 6 weeks of age and, at that time, was found to have a right undescended testis. He subsequently underwent right inguinal herniorrhaphy and orchiopexy. The maternal grandfather, who has the same mutation, does not recall being told that he had cryptorchidism and never required surgical repair of cryptorchidism. As to the grandfather's reported phenotype, we cannot exclude the possibility that he had cryptorchidism with spontaneous resolution during childhood or that the presence of an incarcerated inguinal hernia altered the natural history of cryptorchidism for the proband. Phenotypic heterogeneity, perhaps secondary to modifier loci, could account for the different clinical features. The mother of the propositus reported no fertility problems.

To the best of our knowledge, these are the first reported human mutations identified in *INSL3/RLF* that are associated with cryptorchidism. Finding mutations in 2 patients with cryptorchidism, 1 of whom had an intraabdominal testis, provides additional circumstantial evidence that *INSL3/RLF* is involved in testicular descent in humans. Both mutations were unique and were located in the connecting peptide region of the protein. Because both patients were heterozygous for *INSL3/RLF* mutations, haploinsufficiency is the likely mechanism. The frequency of *INSL3/RLF* mutations as a cause of cryptorchidism seems to be low because we only found 2 of 145 (1.4%) formerly cryptorchid subjects to have *INSL3/RLF* mutations. This low frequency of *INSL3/RLF* mutations may account for the negative findings reported in a cohort of 30 boys with cryptorchidism (36) and 31 men with cryptorchidism (37). Other candidate loci include the *INSL3/RLF* receptor, other downstream signaling intermediates, or *HOXA10* (38–41).

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NIH-NIDDK Study

At the National Institutes of Health (NIH) in Pheonix, Arizona, we are studying the neurophysiology of eating behavior in successful dieters (18 yr or older, healthy, nonsmoker), *i.e.* people who were very obese (BMI ≥ 35 kg/m^2), lost a significant amount of weight without the help of drugs or surgery, and have maintained a near-normal body weight (BMI ≤ 25 kg/m^2) for at least 6 months. The NIH Institutional Review Board approved the study. **We need referrals**.

We offer a monetary compensation for time and participation, reimbursement of travel expenses, and a free medical check-up. The study requires a 5-day hospital stay at the NIH Research Unit in the Phoenix Indian Medical Center, Phoenix, Arizona.

For more information, contact P. Antonio Tataranni, M.D. or Angelo Del Parigi, M.D., NIH-NIDDK. Phone: 602-200-5327; E-mail: adelpari@mail.nih.gov.