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ORIGINAL ARTICLE

Genetic analysis of the human insulin-like 3 gene in pediatric patients with testicular torsion

Anna Paola Capra[1] · Elisa Ferro[1] · Maria Angela La Rosa[1] · Silvana Briuglia[1] · Tiziana Russo[2] · Salvatore Arena[2] · Carmelo Salpietro Damiano[1] · Carmelo Romeo[2] · Pietro Impellizzeri[2]

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Abstract

Purpose Testicular torsion (TT) mainly affects boys under 18 years old. To avoid orchiectomy, TT requires an immediate operative management. The etiology of TT is still controversial. Observed familiar recurrence suggests the presence of a genetic involvement. The INSL3 gene consists of two exons, and it is specifically expressed in fetal and adult Leydig cells. In transgenic mice, deletion of this gene was observed an increased testicular mobility and testicular torsion. We have hypothesized the possible involvement of the INSL3 gene as a predisposing factor of human TT.

Methods We performed genetic analysis in 25 pediatric patients with unilateral and intravaginal TT (left, $n = 13$, 56%; right, $n = 12$, 48%). The age of the patients ranged from 1 to 16 years (median age $n = 10.4 \pm 5.46$ years). In this study, we included two first male cousins affected by TT. Venous peripheral blood samples was obtained after parental written informed consent.

Results The Thr60Ala polymorphism was detected in exon 1 of INSL3 gene and other 2 rarer variants (rs1047233 and rs1003887) were identified in the 3′ untranslated region. These variants are prevalent in patients with TT instead of healthy subjects.

Conclusions Additional studies in a larger population are needed to better understand the clinical consequence of the INSL 3 variations founded. This would allow in the future to identify the patients at risk of TT to improve clinical management.

Keywords Testicular torsion · Insulin-like 3 gene · Pediatric · Adolescent · Genetic analysis

Introduction

Torsion of the spermatic cord or testicular torsion (TT) represents a common surgical emergency in pediatric age, affecting, above all, prepubertal and young adult males [1, 2].

The annual incidence of TT is estimated at 3.8 per 100,000 (0.004%) for boys under 18 years old [3].

To avoid orchiectomy, TT requires an accurate, timely diagnosis; operative management within 6 h after onset of symptoms [4] allows a better salvage rate of testis [4, 5].

✉ Pietro Impellizzeri
impellizzerip@unime.it

[1] Department of Human Pathology of Adult and Childhood "Gaetano Barresi", Unit of Emergency Pediatrics, University of Messina, Via Consolare Valeria, 1, 98125 Messina, Italy

[2] Department of Human Pathology of Adult and Childhood "Gaetano Barresi", Unit of Pediatric Surgery, University of Messina, Via Consolare Valeria, 1, 98125 Messina, Italy

TT may occur during sleep or following direct trauma, increased physical activity, or sudden contraction of the cremasteric muscle, as a response to mechanical, sexual, or thermic stimulation [6].

An anatomical predisposing condition, wherein a lack of normal fixation of the testis and epididymis to the scrotum, also known as "bell clapper deformity", continues to be the most commonly described risk factor for intravaginal TT [4].

Furthermore, a positive familiar history has been found in about 10% of children affected by TT, and it can affect multiple relatives and generations, particularly in cases of bilateral torsion [7, 8].

This evidence would suggest the presence of a genetic cause, with or without the contribution of other anatomical and environmental factors [9].

Insulin-like factor 3 (Insl3) has been characterized as a key human testicular hormone. In this regard, Insl3 and its receptor, the relaxin family peptide receptor 2 (Rxfp2), influence gubernaculum development and thickening, and are essential for trans-abdominal testis translocation during the

first phase of the testicular descent process [10–12]. Two independent studies have demonstrated that transgenic mice with targeted deletion of INSL3 showed defects in gubernaculum growth, resulting in bilateral cryptorchidism, as well as other sexual abnormalities [13, 14].

Moreover, studies in humans have investigated the possibility that naturally occurring mutations in the INSL3 gene interfere directly with male fertility or are associated with cryptorchidism, but there was no clear contribution of INSL3 variants [11, 15–30].

In INSL3 mutant mice, increased testicular mobility and subsequent testicular torsion were found in a gene dose-dependent manner, suggesting that Insl3 is a candidate-signaling molecule involved in human torsion events [31].

In this study, we explored the potential involvement of the INSL3 gene as a predisposing risk factor inducing human TT. To test this hypothesis, we performed direct sequencing of the INSL3 gene in a group of pediatric patients who presented with unilateral and intravaginal TT. In addition, our study carried out INSL3 genotyping in a patient who reported familial testicular torsion, and thus, the analysis was extended to the first-degree family members.

Materials and methods

Venous peripheral blood samples were obtained for genetic analysis after parental written informed consent was obtained from 25 pediatric patients with unilateral TT (left, $n = 13$, 56%; right, $n = 12$, 48%). Patient ages ranged from 1 to 16 years (median age $n = 10.4 \pm 5.46$ years).

All individuals included in this study were Caucasian, except for one of Asiatic origin, and none were family relatives; patients with other ipsi or contralateral testicular abnormalities, affected by endocrinal alterations, with a diagnosed "bell clapper deformity" or with TT as a consequence of a testicular trauma were excluded from the study. Each participant underwent surgical exploration and received contralateral testis fixation. After derotation of the spermatic cord, orchidopexy was performed in 11 patients (44%), and orchiectomy was performed in the other 14 (56%). As a patient had a familial history of TT (two first cousins), eight members of this family were included in the study for INSL3 genotyping, after written informed consent.

Control blood samples were obtained from 30 volunteers of the same age, without TT or any other abnormalities of the male genitalia, after their parents gave written informed consent. The study was approved by the local Ethical Committee of the University Hospital of Messina.

Genomic DNA was extracted from peripheral blood leukocytes with an NLM AA1001 kit (Nuclear Laser Medicine, Settala, MI, Italy) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) primers were designed for the human gene INSL3 (reference gene ID: 3640), to cover the sequence of the two exons, as well as the intron exon boundaries. The exon 1 primer sequences were 5′-AAA GACTCGTTGCCCAGTG-3′ and 5′-CACACTCCAGTG CGGACC-3′; the exon 2 forward primer was 5′-ATGAGT GTTTGGTGGGTTAC-3′; and the exon 2 reverse primer was 5′-TGCCTCTCTAGTTATCAAGC-3′. The PCR reactions consisted of 40 ng genomic DNA, 1 µl of each of 10 µM primers, 1 µl dNTPs (10 mM), 2.5 µl 10X reaction buffer with 15 mM MgCl2, 0.2 µl StoS Taq polymerase (5 U/µl) (GeneSpin S.r.l., Lodi, Italy), and 17.8 µl sterile water in a total volume of 25 µl. The PCR thermocycler profile for exon 1 amplification was: 94 °C for 4 min; 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min, for 35 cycles; with a final extension at 72 °C for 4 min. PCR conditions for exon 2 amplification were: 94 °C for 4 min; 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min, for 35 cycles; with a final extension at 72 °C for 4 min.

PCR products generated were, respectively, 557 and 774 bp. The purified PCR fragments were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, TX, USA) using an ABI PRISM 3130 automated sequencer (Applied Biosystems, Monza, Italy) according to the manufacturer's instructions.

Statistical analyses were carried out using Statistica 10.0 StatSoft. Fisher's exact test was used to compare genotype frequencies of TT patients group versus healthy subjects. *P* values of less than 0.05 were considered significant. Hardy–Weinberg equilibrium was determined using Courtlab calculator (downloaded from http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls).

To estimate the linkage disequilibrium (LD) between polymorphisms, LD coefficients (r^2 and D') were calculated using the online tool LDlink developed by the Division of Cancer Epidemiology and Genetics (DCEG) of the National Cancer Institute (NCI), one of the National Institutes of Health (NIH) (https://analysistools.nci.nih.gov/LDlink/?tab=ldmatrix).

Results

Variations found in the INSL3 gene and clinical features of the 25 TT patients are listed in Table 1. Three common polymorphisms were detected in exon 1. Two of those are synonymous variations, so are probably not pathogenic: Ala9= (rs22866663, MAF/MinorAlleleCount: 0.1228/615) and Leu42= (rs1047233, MAF/MinorAlleleCount: 0.2572/1288). The other one was a nucleotide substitution: A to G in the C-peptide region, resulting in a threonine (Thr) to alanine (Ala) change at codon 60 (rs6523). Thr60Ala was

Table 1 Characteristics of study subjects

Pattern ID	Age at surgery	Testis	Surgical outcome	Ala9= (*rs2286663*)	Leu42= (*rs1047233*)	Thr60Ala (*rs6523*)	G>A (*rs1003887*)	C>A (*rs17750642*)
TT1	15	Left	Orchiectomy	+/+		+/+	+/+	
TT2	9	Right	Orchiectomy				+/+	
TT3	11	Left	Orchiectomy	+/−		+/+	+/+	
TT4	15	Left	Orchiectomy		+/−	+/+	+/+	
TT5	13	Left	Orchiectomy		+/+	+/+	+/+	
TT6	14	Left	Orchiectomy				+/−	
TT7	1	Left	Orchiectomy			+/+	+/+	
TT8	15	Left	Orchiectomy			+/+	+/+	
TT9	2	Right	Orchidopexy				+/−	
TT10	15	Left	Orchidopexy				+/+	
TT11	14	Left	Orchidopexy					+/−
TT12	5	Right	Orchidopexy					
TT13	16	Right	Orchidopexy				+/−	
TT14	16	Right	Orchidopexy	+/−		+/+	+/+	
TT15	16	Right	Orchidopexy				+/−	
TT16	1	Right	Orchiectomy			+/+	+/+	
TT17	4	Right	Orchiectomy				+/−	
TT18	4	Right	Orchidopexy					
TT19	3	Right	Orchidopexy					
TT20	4	Right	Orchiectomy			+/+	+/+	
TT21	13	Left	Orchidopexy		+/+	+/+	+/+	
TT22	15	Left	Orchidopexy	+/−		+/+	+/+	
TT23	13	Right	Orchiectomy				+/−	+/−
TT24	13	Left	Orchiectomy				+/−	+/−
TT25	13	Left	Orchiectomy		+/+	+/+	+/+	

+/+ homozygosis; +/− heterozygosis; empty boxes represent wild type genotype for the variation in analysis

found in 12 TT patients in homozygosis (48%) and also in a total of 12 control subjects: 4 were homozygous (13.3%) and 8 were heterozygous (26.6%).

Two other variants were identified in the 3′ untranslated region (UTR) of the INSL3 gene. The first one is a transition G>A (rs1003887) and was present in 14 TT subjects in homozygosis (56%) versus 6 controls (20%), and this was also present in heterozygosis in 7 patients (28%) versus 18 controls (60%). The second variant was a C>A substitution (rs17750642) and is a more uncommon variant (MAF/MinorAlleleCount: 0.0144/72), which was found in only 3 TT patients in heterozygosis (12%).

The rs6523 polymorphism was in strong linkage disequilibrium with the rs1003887 polymorphism ($r^2 = 0.861$, $D' = 0.955$) and in weak linkage disequilibrium with the other exon 1 polymorphism: rs1047233 ($r^2 = 0.374$, $D' = 1$). In addition, rs1047233 and rs1003887 were in weak linkage disequilibrium ($r^2 = 0.328$, $D' = 0.964$).

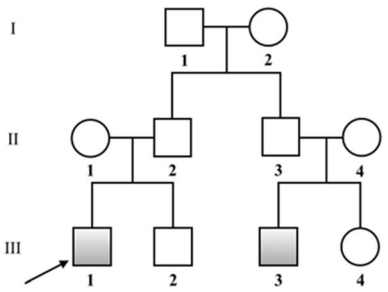
The statistical analysis for the last three variations described is reported in Table 2. Although Thr60Ala (rs6523) has been detected in homozygosis in significantly more TT patients compared to healthy subjects, the genotype frequencies fit the Hardy–Weinberg law only in the control group ($X^2 = 3.03$; $P = 0.08$) and not in the TT group ($X^2 = 25$; $P < 0.0001$). However, for rs1003887, the genotype distributions were in accordance with the equilibrium law in both the control ($X^2 = 1.20$; $P = 0.27$) and patient group ($X^2 = 2.77$; $P = 0.09$) and Fisher's test result is statistically significant.

In this study, we included two first male cousins affected by unilateral TT, respectively at age of 11 (Patient ID: TT3, see Table 1) and 13 years.

We performed sequence analysis of the INSL3 gene in all members of the family to search for the predisposing allele. The genealogical tree of the two generations is outlined, and the INSL3 genotype for each member of the pedigree is reported in Fig. 1. Familiar genetic testing showed that all subjects were homozygous for Thr60Ala and rs1003887, and they carried other synonymous variations.

Table 2 Association study of INSL3 polymorphisms and risk of testicular torsion (TT)

SNPID	MAF	Genotype/allele	Patients with TT *N* (%)	Controls *N* (%)	Fisher's exact test
rs6523	0.3139/1572	AA	13 (52)	18 (60)	
		AG	0 (0)	8 (27)	
		GG	12 (48)	4 (13)	
rs1003887	3321/1663	GG	4 (16)	6 (20)	
		GA	7 (28)	18 (60)	$X^2 = 8.05$; $p = 0.17$
		AA	14 (56)	6 (20)	
rs17750642	0.0144/72	CC	22 (88)	30 (100)	
		CA	3 (12)	0 (0)	
		AA	0 (0)	0 (0)	



	Ala9= *(rs2286663)*	Leu42= *(rs1047233)*	Thr60Ala *(rs6523)*	G>A *(rs1003887)*
II-1	-/-	-/-	+/+	+/+
II-2	+/-	-/-	+/+	+/+
II-3	-/-	+/+	+/+	+/+
II-4	-/-	-/-	+/+	+/+
III-1	+/-	-/-	+/+	+/+
III-2	+/-	-/-	+/+	+/+
III-3	-/-	+/-	+/+	+/+
III-4	-/-	+/-	+/+	+/+

Fig. 1 Pedigree analysis of a family with two cousins (III-1 and III-3) with a history of TT

Discussion

Testicular torsion is responsible for long-term effects on exocrine and endocrine functions, leading to reduced fertility and compromised spermatogenesis in adulthood [32].

Genetic, hormonal, and anatomical factors influence a complex molecular mechanism leading to male gonad development, but part of this process remains unexplained. Insl3 was first described in 1990 as a novel peptide and is expressed at high levels in the human testis [33, 34]. In all mammalian species examined so far, Insl3 is a major secretory product of mature interstitial Leydig cells and is also expressed by testes in the male fetus in mid-gestation [34].

Moreover, it has been reported that Insl3 plays a crucial role in rodent testicular descent by acting on Rxfp2 receptors expressed within the gubernacular ligaments, causing these to expand and anchor the testes in the inguinal region [35]. As a consequence, numerous human mutation analyses have sought to elucidate the possible involvement of Insl3 and its specific receptor Rxfp2 in cryptorchidism [35, 36].

The analysis of sporadic and possible familial cases of undescended testis revealed several mutations, some of which are common polymorphisms, found both in healthy and affected subjects.

All patients carrying INSL3/RXFP2 mutations were heterozygous, and thus, the mutation-dependent mechanism of testicular maldescent may be caused by reduced signaling [24, 26]. However, a definitive causative role for many of these mutations in humans is still lacking [37].

The etiology of TT is still unclear and the related risk factors remain controversial; some evidence suggests that the risk of TT, a rare condition, can be inherited, particularly in cases of bilateral torsion. Furthermore, experimental animal studies suggest that the mechanism predisposing patients to TT may involve the INSL3 gene, but until now, no study has tested this hypothesis in humans [7].

Therefore, we performed a genetic analysis of the INSL3 gene in 25 pediatric patients affected by unilateral TT for the first time. We confirmed that synonymous Ala9= and Leu42= variations are common polymorphisms found in both patients and healthy subjects, as previously reported in cryptorchid subjects, probably without any pathogenic significance [36].

Our results for the Thr60Ala (rs6523) mutation are not conclusive, despite this variation seeming to be more represented in the group of TT patients in homozygosis. However, this observation could not be statistically verified due to the small sample size in analysis. Nonetheless, this missense polymorphism in the N-terminal region of the Insl3 propeptide can have an effect on Insl3 binding to its receptor [38, 39].

The literature, based on a larger group of cryptorchid patients, suggests that Thr60Ala is a polymorphism present at high frequency in the general population, and is

probably unrelated to any particular phenotype of testicular maldescent [11, 17, 21, 24, 30, 36]. More recently, the rs6523 polymorphism of INSL3 showed significant association with increased risk of polycystic ovary syndrome in a well-characterized cohort of more than 400 Indian women [39]. Future studies in other populations are needed to establish whether Thr60Ala polymorphism in the INSL3 gene may contribute to the pathophysiology of male or female reproductive function.

The other two reported variants are less common, and both are located in the 3′ regulatory region. The G>A substitution (rs1003887) is prevalent in homozygosis in TT patients with statistical significance.

To evaluate the possible role of rs1003887 in a TT event, we analyzed the INSL3 gene in all members of a family in which two first cousins have a history of TT, and the proband (patient ID: TT3), included in our study, is homozygous for this genetic variant. The variation in analysis was found in all subjects of the family in homozygosis, in both unaffected males and females and in related affected subjects. The role of rs1003887 on Insl3 biological activity should, therefore, be better characterized.

The last variant, C>A substitution (rs17750642), was found in only three patients in heterozygosis and was not present in healthy subjects. To our knowledge, we have reported the presence of this variant for the first time, which has not been previously described in larger population screening. Additional studies are needed to confirm the possible association with suggestive phenotypes, and functional analysis is necessary to ascertain whether the mutated INSL3 is efficiently transcribed and processed as a stable mature hormone.

Limitations of this study are the small sample of patients and the lack of bilateral torsion cases, a very rare condition.

To define the role of INLS3 in the pathogenesis of TT, a multi-center study could be useful for screening a larger number of patients. This would allow to identify the patients at risk of TT and to improve future clinical management.

Furthermore, collecting more information on predisposing genotypes and clinical characteristics of suggestive phenotypes will be useful to better understand the role of endogenous factors, such as other genes involved in testicular descent and development, and possible interactions with exogenous disruptors to preserve male fertility in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study. No identifying information about participants is available in the article.

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