Further insights into the role of T222P variant of RXFP2 in non-syndromic cryptorchidism in two Mediterranean populations

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ORIGINAL ARTICLE

Further insights into the role of T222P variant of *RXFP2* in non-syndromic cryptorchidism in two Mediterranean populations

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Summary

The aetiopathogenesis of isolated cryptorchidism remains largely unknown. Mutation screenings in the most relevant candidate genes for testicular maldescent lead to controversial data in the literature. In particular, the role of the T222P genetic variant of the *RXFP2* gene is still debated. Given the controversies, the aim of this study was to provide further data on this genetic variant in two Mediterranean populations. A total of 577 subjects from Spain and 550 from Italy (with and without a history of cryptorchidism) were analysed. The T222P substitution was found in both unilateral and bilateral cases and in a total of 12 controls. These data exclude a clear-cut cause–effect relationship between T222P variant and testicular maldescent. The T222P variant was found at a similar frequency in both cases and controls in the Spanish population, whereas in Italy, the frequency of T222P resulted significantly higher in the cryptorchid group ($p = 0.031$). The observed difference between the two countries and the highly variable phenotypic expression of the T222P variant may depend on the genetic background or on environmental conditions. The haplotype analysis of the *RXFP2* gene in T222P carriers and their parents showed that this variant is linked to the previously inferred C-C-G-A-13 haplotype and consequently provides further support to the 'founder effect' hypothesis. In conclusion, our data indicate that T222P is a frequent variant in the Spanish population with no pathogenic effect. Although in Italy it seems to confer a mild risk (odds ratio = 3.17, 95% confidence interval: 1.07–9.34) to cryptorchidism, the screening for this variant for diagnostic purposes is not advised because of the relatively high frequency of control carriers (1.4% of Italian men without a history of cryptorchidism).

Introduction

Cryptorchidism is the most frequent congenital birth defect in male children and can occur as an isolated disorder or in association with other congenital anomalies (syndromic cryptorchidism) (Toppari *et al.*, 2006). Our understanding of the physiology of testis descent has greatly advanced in the last few years (Ivell & Hartung, 2003; Virtanen & Toppari, 2008). It has become evident that a major role in testis descent is played by two major hormone/receptor systems: testosterone and its receptor AR (androgen receptor) and the INSL3/RXFP2 (former

LGR8) system, insulin-like factor 3 (INSL3) and its G-protein-coupled receptor, RXFP2 (relaxin family peptide 2). INSL3/RXFP2 are essential for the first phase of testicular descent, whereas androgens are involved in the inguinal phase (Foresta *et al.*, 2008). It was predicted that mutations in genes coding for the two hormone/receptor systems should cause cryptorchidism. Since the first mutation screening of the *INSL3* gene in cryptorchid men (Krausz *et al.*, 2000), a large number of polymorphisms and putative mutations have been described (Adham & Agoulnik, 2004; Ferlin *et al.*, 2006). Of all reported mutations, only two missense mutations, the P49S and V18M, have a deleterious effect on the ability of INSL3 to activate its receptor (Bogatcheva *et al.*, 2003; El Houate *et al.*, 2007). However, the role of these mutations (all heterozygous) in the pathogenesis of cryptorchidism remains to be established. Concerning the *RXFP2* gene, only one missense mutation at codon 222 (T222P) has been reported. According to the first two studies, this mutation was present in heterozygosity exclusively in men with history of testicular maldescent (Gorlov *et al.*, 2002; Ferlin *et al.*, 2003). In addition, functional analysis showed a severely reduced receptor surface expression of the mutant protein further supporting its causative role in the pathogenesis of cryptorchidism (Bogatcheva *et al.*, 2007). However, the proposed clear-cut cause–effect relationship between heterozygous mutation and cryptorchidism has been recently challenged by a multicentre study, which has clearly demonstrated that this genetic variant may not be considered further as a pathogenic mutation as it can be also found in men with no history of testicular maldescent (Nuti *et al.*, 2008). In this study, it was also demonstrated that the T222P variant is not restricted to the Mediterranean region, as it was observed also in a Central European country. Another recent study from Morocco has also reported a high frequency for this variant in the range of polymorphism in both cryptorchid men and non-cryptorchid men (El Houate *et al.*, 2008). Despite these controversies, the T222P variant has been reported as a genetic alteration associated with persistent bilateral cryptorchidism in a recent study on newborn children from Italy (Ferlin *et al.*, 2008).

The role of T222P variant in Spain has not been fully investigated and only a small study population (77 patients and 48 controls) was screened in a multicentre study (Nuti *et al.*, 2008). In the aforementioned study, the frequency of T222P mutation was 0% in the patient and 4% in the control groups, showing an inverse situation to that observed in the Italian population in which 5% of patients had the mutation vs. 1.8% of the controls.

Given the controversies in the literature and the apparent sharp contrast between Italy and Spain, we sought to investigate further into the clinical significance of this genetic variant in the two Mediterranean populations by screening a total of 386 cases and 741 controls. Moreover, we also aimed to clarify the founding mutation issue by performing the *RXFP2* gene haplotype analysis in the mutation carriers and in their parents.

Materials and methods

Study populations

A total of 577 subjects of Spanish ancestry were analysed for the T222P variant of *RXFP2*. Among them, 187 patients had a history of cryptorchidism, whereas the remaining 390 were controls with no history of testicular maldescent at birth. The cryptorchid group included 113 with unilateral (1 of them had 'retractile' testis), 74 with bilateral cryptorchidism (6 of them had 'retractile testis'). Part of this study population has been already screened in the multicentre study by Nuti *et al.* (2008) (77 patients and 44 controls). The Italian study population included 550 subjects from Central Italy (Italian ancestry): among them, 199 had a history of testicular maldescent at birth, whereas 351 were controls. The cryptorchid group included 132 with unilateral and 67 bilateral cryptorchidism. Also in this case, part of the study population has been analysed in the previous multicentre study (Nuti *et al.*, 2008): 159 patients and 275 controls.

Molecular analysis of *RXFP2* gene

DNA was extracted from peripheral blood in all the participants in this study with the exception of the Italian controls for whom, depending on sample availability, the genomic DNA was isolated either from the peripheral blood or from the frozen semen.

Exon 8 of the *RXFP2* gene (RefSeq: NM_130806.3) containing the T222P variant, was amplified using the following primers: forward: 5′-GGGGAGGCAGGTTT TATTTC-3′; reverse: 5′-AAGCTAGTGCTAGATGTCATT GC-3′. The resulting DNA fragments were analysed by direct bidirectional sequencing using automated sequencer (ABI PRISM 3100; Applied Biosystems, Foster City, CA, USA). For *RXFP2* haplotype analysis, a total of five *RXFP2* polymorphisms were analysed in nine carriers of the T222P mutation. For the analysis of exon 12–intron 12 polymorphisms (exon 12: 957G>A, 993A>G; intron 12: INV12(-2)A9 > A13), the primers were described by Ferlin *et al.* (2003). Primers for the intron 7 [INV7(-351)A>C] and intron 9 polymorphisms [INV9(+380) A>C] and polymerase chain reaction conditions were described by Nuti *et al.* (2008). The resulting DNA fragments were sequenced using automated sequencer (ABI PRISM 3100; Applied Biosystems).

Statistical analyses

For statistical analysis of genotype distribution, test for deviation of Hardy–Weinberg Equilibrium (HWE) or two-point association studies, we employed tests adapted from Sasieni (1997). Odds ratio (OR) estimates were computed from 2 × 2 tables. These calculations were performed on the online resource facility at the Institute for Human Genetics, Munich, Germany (http://ihg.gsf.de) and using the Statistical Package for the Social Sciences software (SPSS, Evanston, IL, USA).

Results

Sequencing analysis of exon 8 of the *RXFP2* gene

Spanish study population

The T222P genetic variant was found in both cryptorchid and control subjects. The variant frequency observed in the two groups was almost the same; in the cryptorchid group 3/187 (1.6%), whereas in the controls 7/390 (1.8%).

Italian study population

The variant was significantly more frequent in the patient group (9/199, 4.5%) than in the control (5/351, 1.4%), with an OR = 3.17 [95% confidence interval (CI): 1.07–9.34, $p = 0.031$]. Data for both study populations are reported in Table 1.

Table 1 T222P *RXFP2* variant in patients with a history of testicular maldescent and in controls with no history of cryptorchidism

	No. of mutated/ total men	T222P variant frequency (%)	Statistical analysis, *p*-value [odds ratio (95% CI)]
Spanish			
Cryptorchid	3/187[a]	1.6	0.8 [0.89 (0.2–3.4)]
Unilateral	2/113	1.7	
Bilateral	1/74	1.3	
Control	7/390[a]	1.8	
Italian			
Cryptorchid	9/199	4.5	0.031
Unilateral	5/132	3.7	[3.17 (1.07–9.34)]
Bilateral	4/67	5.9	
Control	5/351	1.4	

[a]A total of 77 patients and 48 controls from Spain and 159 patients and 275 controls of Italian origin, from a previous multicentre study are included (Nuti *et al.*, 2008).

RXFP2 gene haplotyping

The analysis of five exonic and intronic polymorphisms allowed the discrimination of different possible haplotypes on the carriers (Table S1). The analysis of Spanish triplets (mother, father and son) available for two patients and of one control/mother pair also indicated that the T222P is in linkage with the C-C-G-A-13 haplotype (Fig. 1). In all three carriers, the variant had been transmitted from the father. Patient 08-202 shared with his father the C-C-G-A-13 haplotype, whereas patient sw09-9 shared the C-C-G-A-12 allele. In the case of the control carrier (07-276), only the mother was available for the analysis and she did not carry the variant. The haplotype in linkage with the variant in this subject is C-(C/A)-G-(A/G)-13, which potentially includes the C-C-G-A-13 haplotype.

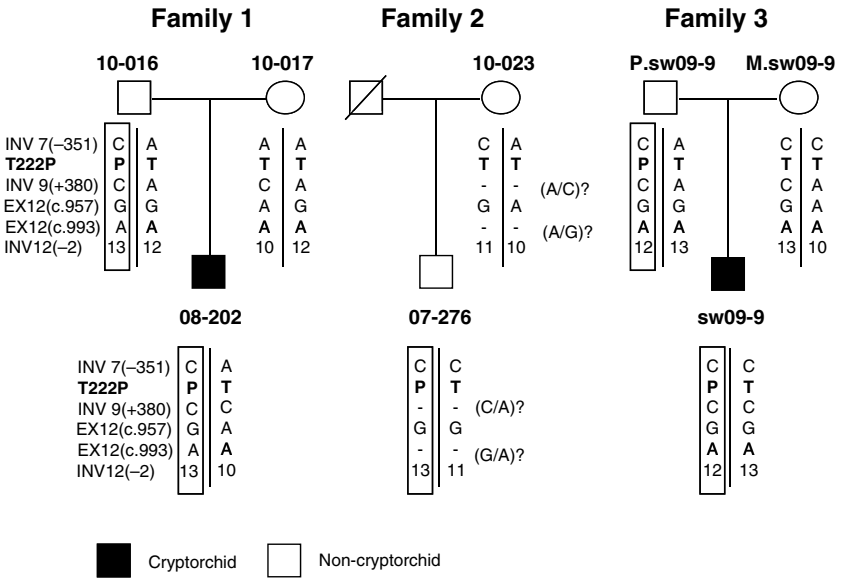

Figure 1 Haplotype analysis of the three Spanish families carrying the T222P variant of the *RXFP2* gene. The haplotype linked to the T222P variant is boxed. In all three carriers, the variant is inherited from the father.

Genotype/phenotype correlation

In all 12 cases and 12 controls, the T222P variant was found in heterozygosity. The phenotypic features of T222P variant carriers were heterogeneous in both geographical groups. In fact, the phenotypic expression of this variant also includes normal testicular descent in 12 cases (7 from Spain and 5 from Italy). This finding further excludes a causative relationship between the heterozygous variant and cryptorchidism. The testicular phenotype of the 12 cryptorchid patients was unilateral cryptorchidism in seven patients and bilateral cryptorchidism in five. In contrast to a previous study (Ferlin *et al.*, 2008), the T222P variant was not predominantly present in persistent bilateral cryptorchid patients. In all cases, orchidopexy was performed during infancy. Concerning the three Spanish T222P carriers, whose parents' DNA was available, two of them were cryptorchidic (08-202, sw09-9) and the third one had normal testicular descent (07-276). In all three subjects, the variant was transmitted from the father and all of them had normal testes descent. In the Italian group, DNA from parents was available only for one carrier, code MMP122 (already published in Nuti *et al.*, 2008), and in this case, the variant was transmitted by the mother.

Discussion

The search for clinically relevant genetic factors involved in non-syndromic cryptorchidism has been largely unsuccessful (Giachini *et al.*, 2007; Foresta *et al.*, 2008). Paradoxically, not even the strongest candidate genes such as *INSL3/RXFP2* appear to be involved in the aetiopathogenesis of testicular maldescent. The *INSL3* gene has been the subject of a number of studies in which the entire coding region has been sequenced (for review, see Foresta *et al.*, 2008). In contrast, the *RXFP2* gene has been sequenced only in a small number of subjects (approx. 160 patients; Gorlov *et al.*, 2002). In fact, based on the study by Gorlov *et al.* (2002) which reported for the first time the T222P mutation in exon 8, all subsequent studies have focused on the screening of this specific variant. More than 4000 subjects from different geographical/ethnic origin have been analysed so far (Table 2). After the publication of the first studies, it became evident that this genetic variant is prevalent in the Mediterranean area (only two carriers have been described from Hungary in a later study) and is completely absent from other geographical areas such as Northern Europe and Asia. Previous studies suggested that all (except three) T222P carriers share a common inferred haplotype: C-C-G-A-13 (Ferlin *et al.*, 2003; El Houate *et al.*, 2008; Nuti *et al.*, 2008). This finding leads to the hypothesis about a common ancestor with the C-C-G-A-13 haplotype in the Mediterranean area. The haplotype analysis in our study population confirmed the presence of the common C-C-G-A-13 inferred haplotype in all (except one) Italian and all Spanish carriers (Table S1). The analysis of two Spanish triplets (mother, father and cryptorchid son) and a mother/son pair also supported the hypothesis of a linkage between the T222P variant and the C-C-G-A-13 haplotype in these cases: in one case, the variant is on the C-C-G-A-13 haplotype, whereas in the other, it is on the C-C-G-A-12 haplotype. The latter may also derive from the common C-C-G-A-13 through the contraction of the polyadenine stretch. Contraction and expansion of repeated sequences is a common phenomenon. In the control carrier, the markers are less informative (C-A/C-G-A/G-13) because of the non-availability of the DNA from the father. However, in this subject also, the T222P variant can be potentially linked to the C-C-G-A-13 haplotype.

Table 2 The results of T222P analyses published in the literature in cryptorchid patients and controls from different countries

Geographical origin	Total no.	No. of T222P variants (%)	References
Cryptorchid			
France	20	1 (5)	1
United States	13[a]	0 (0)	3
Mixed origin	41		1
Finland	23	0 (0)	2
Italy (Northern Italy)	1198	29 (2.4)	4, 5, 9
Italy (Central Italy)	**199**[b]	**9 (4.5)**	7
Hungary	70	2 (2.8)	7, cs
Japan	62	0 (0)	6
Morocco	109	3 (2.7)	8
Egypt	53	3 (5.6)	7
Spain	**187**[b]	**3 (1.6)**	7, cs
Control			
France	62	0 (0)	1
United States	31	0 (0)	1, 3
Finland	33	0 (0)	2
Italy (Northern Italy)	850	0 (0)	4, 5, 9
Italy (Central Italy)	**351**[b]	**5 (1.4)**	7, cs
Hungary	140	1 (0.7)	7
Germany	100	0 (0)	1
Japan	60	0 (0)	6
Morocco	250	4 (1.6)	8
Spain	**390**[b]	**7 (1.8)**	7, cs

[a]Patients with familial cryptorchidism.

[b]Includes 77 patients and 48 controls from Spain and 159 patients and 275 controls of Italian origin, from a previous multicentre study (Nuti *et al.*, 2008).

Current study population is shown in bold; cs, current study.

References: 1, Gorlov *et al.*, (2002); 2, Roh *et al.*, (2003); 3, Feng *et al.*, (2004); 4, Ferlin *et al.* (2003); 5, Bogatcheva *et al.*, (2007); 6, Yamazawa *et al.*, (2007); 7, Nuti *et al.*, (2008); ; 8, El Houate *et al.*, (2008); 9, Ferlin *et al.*, (2008).

These data clearly indicate that a founder effect seems to be the most plausible explanation for the geographical restriction of this variant and both Italian and Spanish carriers probably descend from a common ancestor.

Our results further support the relatively high incidence of T222P variant in the two Mediterranean countries. However, although in Spain the frequencies are similar in cases and controls, in Italy, the T222P variant appears to be a mild risk factor for crytorchidism. This later finding contradicts our previous conclusion on the Italian population (Nuti *et al.*, 2008) in which a higher but statistically non-significant ($p = 0.054$) difference in mutation frequencies was observed between cases and controls. The enlarged sample size allowed us to ascertain that this variant, although not causative, can be considered as a mild genetic susceptibility factor to cryptorchidism in Italy (OR = 3.17; 95% CI: 1.07–9.34). Similar to the Spanish population, a lack of association between the T222P variant and cryptorchidism has also been reported in another Mediterranean country, Morocco, where the frequency of T222P was 2.7% in patients and 1.6% in controls (El Houate *et al.*, 2007).

The T222P variant has been transmitted by the father in the three Spanish subjects and this is in contrast to data from Italy. In fact, both in Central and Northern Italy, the variant has been transmitted by the mother in all 10 triplets tested (Bogatcheva *et al.*, 2007; Nuti *et al.*, 2008). Interestingly, also in Morocco, in two of three families, a paternal transmission of the variant was reported (El Houate *et al.*, 2007). All carrier fathers had normal testis descent.

The observed marked geographical differences in allelic frequencies make it very difficult to combine data from different geographical areas. Even a meta-analysis restricted to the Italian studies is biased by the fact that in the Northern Italian studies, the T222P variant was absent in 850 controls, whereas in Central Italy, the frequency reached 1.4%. This controversy cannot be explained by differences in the methodology (both laboratories used direct sequencing) or in the selection criteria of controls (with the exception of 300 newborn subjects included as controls in the study of Ferlin *et al.* (2008); therefore, it remains an unsolved paradox. According to Ferlin *et al.* (2008), no cases with T222P variant (all heterozygous) showed spontaneous descent of the testis and were reported exclusively in association with bilateral cryptorchidism. The observed 7 unilateral carriers and 12 controls in our study population are in sharp contrast to this finding and strongly argue against considering the T222P variant as a pathogenic mutation with clear-cut cause–effect relationship with cryptorchidism.

The highly variable phenotypic expression in heterozygous carriers may reflect the presence of other genetic or environmental factors acting in combination with the T222P variant. Animal models indicate that the phenotypic manifestation of *Insl3/Rxfp2* knockout alleles is susceptible to genetic background with noted delay in testis descent in heterozygotes described in one report of an *Insl3* mutant (Nef & Parada, 1999), but not in another *Insl3* mutant (Zimmermann *et al.*, 1999) and in the *Rxfp2* (Gorlov *et al.*, 2002). Different environmental conditions, especially exposure to xeno-oestrogens, may also influence the phenotypic expression of heterozygote carriers. In this regard, it has been shown that exposure to mono-*N*-butyl phthalate, an endocrine disruptor with weak oestrogenic activities, impairs *Insl3* gene expression and affects testicular descent in rats (Wilson *et al.*, 2004; Shono *et al.*, 2005). Only heterozygous carriers have been identified so far; it remains therefore an open question whether homozygosity is strictly associated with cryptorchidism also in humans.

The different scenario in the two countries may be related to the accumulation of different genetic or environmental modifiers leading to a more mild or wild-type phenotype in heterozygotes of Spanish descent and to a more frequently observed pathological condition in Italy. This hypothesis is supported by a recent study which provides evidence about relevant genetic differences between populations belonging to different geographical areas within Europe (Novembre *et al.*, 2008).

As cryptorchidism is a significant risk factor for impaired sperm production and testis cancer, the identification of transmissible genetic factors is of considerable interest. We provide evidence that the T222P variant does not have any pathogenic effect on cryptorchidism in the Spanish population. Although in Italy it seems to confer a mild risk to cryptorchidism, the screening for this variant for diagnostic purposes is not indicated because of the relatively high frequency of control carriers (1.4% of Italian men without a history of cryptorchidism).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Haplotype analysis of *RXFP2* gene based on five intronic (introns 7 and 9) and exonic (exon 12) polymorphisms with all possible inferred haplotypes. The common inferred haplotype C-C-G-A-13 is indicated in bold.

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