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1 **Isolated Cryptorchidism: No Evidence for Involvement of Genes**

2 **Underlying Idiopathic Hypogonadotropic Hypogonadism**

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14 **Abstract**

15 Mutations in *FGFR1*, *GNRHR*, *PROK2*, *PROKR2*, *TAC3*, or *TACR3* underlie isolated hypogonadotropic
16 hypogonadism (IHH) with clinically variable phenotypes, and, by causing incomplete intrauterine activation
17 of the hypothalamic – pituitary – testicular axis, may lead to cryptorchidism. To investigate the role of defects
18 in these genes in the etiology of isolated cryptorchidism, we screened coding exons and exon-intron
19 boundaries of these genes in 54 boys or men from 46 families with a history of cryptorchidism. Control
20 subjects (200) included 120 males. None of the patients carried mutation(s) in *FGFR1*, *PROK2*, *PROKR2*,
21 *TAC3* or *TACR3*. Two of the 46 index subjects with unilateral cryptorchidism were heterozygous carriers of
22 a single *GNRHR* mutation (Q106R or R262Q), also present in male controls with a similar frequency (3/120;
23 $p = 0.62$). No homozygous or compound heterozygous *GNRHR* mutations were found. In conclusion,
24 cryptorchidism is not commonly caused by defects in genes involved in IHH.

25 **Keywords:** Cryptorchidism, *FGFR1*, *GNRHR*, *PROK2*, *PROKR2*, *TAC3*, *TACR3*

26 **Abbreviations:** *FGFR1*, fibroblast growth factor receptor 1; *GNRHR*, gonadotropin-releasing hormone
27 receptor; *PROK2*, prokineticin 2; *PROKR2*, prokineticin receptor 2; *TAC3*, tachykinin 3; *TACR3*, tachykinin
28 receptor 3; IHH, isolated hypogonadotropic hypogonadism; *AR*, androgen receptor; *INSL3*, insulin-like
29 factor 3; *RXFP2*, relaxin/insulin-like family peptide receptor 2

30 1. Introduction

31 Cryptorchidism is one of the most common malformation in full-term boys with an estimated prevalence of
32 2-8% (Virtanen et al., 2007a), and an increasing incidence (Boisen et al., 2004). Testicular development and
33 descent is a multifaceted process affected by anatomical, hormonal, environmental, and genetic factors
34 (Damgaard et al., 2008; Foresta et al., 2008; Main et al., 2010; Skakkebaek et al., 2001; Virtanen et al.,
35 2007a; Virtanen et al., 2007b). Mutations in the androgen receptor gene (*AR*) appear a rare cause of isolated
36 congenital cryptorchidism (Ferlin et al., 2008). Recently, variation in the genes encoding insulin-like factor 3
37 (*INSL3*) that regulates the first, transabdominal phase of testicular descent (at least in mice) and in its
38 receptor, *RXFP2* (*LGR8*), has been described in patients with congenital cryptorchidism (Ferlin et al., 2008;
39 Foresta et al., 2008; Virtanen et al., 2007b). However, the reported role of these variants in the etiology of
40 cryptorchidism is not consistent because of contradictory results (Ars et al., 2010; El Houate et al., 2008;
41 Koskimies et al., 2000; Krausz et al., 2000; Lim et al., 2001; Nuti et al., 2008; Roh et al., 2003). Thus,
42 relatively little is known about the genetic factors regulating testicular descent in humans.

43 Androgens have a pivotal role in testicular descent (Foresta et al., 2008). We have previously found reduced
44 androgen bioactivity in 3-month-old boys with severe persistent cryptorchidism (Raivio et al., 2003),
45 although immunoreactive testosterone levels did not show any differences between cases (all types included)
46 and controls (Suomi et al., 2006). Expectedly, defects in the hypothalamic-pituitary-testicular (HPG) axis
47 typically lead to cryptorchidism, a relatively common phenotypic feature in patients with isolated
48 hypogonadotropic hypogonadism (IHH) (Pitteloud et al., 2002). It appears unlikely, however, that gene
49 defects underlying IHH would account for isolated cryptorchidism, because these patients usually do not
50 present with micropenis and may have slightly elevated rather than low gonadotropin levels during the
51 minipuberty of infancy (Suomi et al., 2006). On the other hand, some IHH patients carrying mutation(s) in
52 *FGFR1*, *GNRHR*, *PROK2/PROKR2* or *TAC3/TACR3* have displayed recovery of their hypogonadotropism
53 (Gianetti et al., 2010; Pitteloud et al., 2005; Raivio et al., 2007; Sinisi et al., 2008), a phenomenon described
54 also in a pediatric patient with an *FGFR1* mutation: this proband had bilateral cryptorchidism without

55 micropenis, but underwent spontaneous puberty as a teenager (Zenaty et al., 2006). It is currently unknown
56 whether such reversal of hypogonadotropism could occur earlier, already during the first few months of life.
57 Very recently, Caronia et al. (2011) found six heterozygous rare variants in genes underlying IHH (two in
58 *FGFR1* (G260E and R765H), two in *PROKR2* (R85H and L173R), one in *GNRHR* (R262Q), and one in
59 *KALI* (V371I)) in women with hypothalamic amenorrhea, and suggested that these variants may cause at
60 least partial susceptibility to HPG axis disorders. We recently reported a relatively high carrier frequency of
61 two *GNRHR* mutations (Q106R and R262Q), both leading to partial inactivation of the receptor (de Roux et
62 al., 1997; Layman et al., 1998), in Finnish population (altogether 5/200; 2.5%) (Vaaralahti et al., 2011). We
63 therefore hypothesized that some patients with homozygous or compound heterozygous *GNRHR* mutations,
64 or with rare variants in other genes underlying IHH, could present with cryptorchidism as a phenotypic sign
65 of partially deficient GnRH signaling.

66 We investigated 54 Finnish boys or men from 46 families with a history of cryptorchidism for mutations in
67 *FGFR1*, *GNRHR*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*. The roles of *INSL3* and *RXFP2* in cryptorchidism
68 have been previously studied in Finnish population, but only common polymorphisms were found with
69 similar frequencies in cases and controls (Koskimies et al., 2000; Roh et al., 2003).

70 **2. Subjects and Methods**

71 *2.1. Subjects*

72 Altogether 54 subjects were included from 46 families. Of these 54 subjects, 13 had bi- and 39 had unilateral
73 cryptorchidism, and information on the type of cryptorchidism in 2 cases was unavailable. Familial form of
74 cryptorchidism, i.e. a cryptorchid first degree relative, was reported in 19 of the 46 participating families. Of
75 the 54 subjects, 34 boys were followed up from birth and 33 of them had cryptorchidism diagnosed at birth
76 (in preterm cases the diagnosis was verified at the expected date of delivery), and one boy was diagnosed at
77 the age of 18 months. Boys were clinically examined after birth and at a mean age of 3 months, and were
78 followed up until the age of 18 months. None of them had micropenis. The familial cases included 5 boys
79 with bilateral (two of them were brothers) and 4 with unilateral cryptorchidism. Of the 34 subjects followed

80 up from birth, 19 boys of whom we had hormonal data at minipuberty all had normal gonadotropin and sex
81 steroid levels during the minipuberty of infancy (Suomi et al., 2006) and puberty has started in 16 boys. Of
82 the remaining 15/34 boys, 12 have by far displayed normal onset of puberty excluding classical IHH. Six
83 boys, of whom 3 had normal hormone levels at minipuberty, are still too young to determine the onset of
84 puberty, but they are being actively followed up. During the course of the follow-up, spontaneous descent of
85 the testis was observed in 12 of the 34 boys.

86 In addition, 13 families including 20 male subjects participated via study on the familial incidence of
87 cryptorchidism. Six of these male subjects reported of having congenital cryptorchidism. Of the 20 males, 19
88 had been previously treated for cryptorchidism, and one boy had had a spontaneous recovery of
89 cryptorchidism. None of them displayed penile anomalies in infancy. In two families the fathers had
90 unilateral and the sons had bilateral cryptorchidism; in three families, both the fathers and the sons had
91 unilateral cryptorchidism; in one family, both brothers had unilateral cryptorchidism; and, in one family, the
92 son had bilateral cryptorchidism and the type of the father's cryptorchidism remained unknown. In addition,
93 three male subjects with unilateral cryptorchidism had a cryptorchid first degree relative. Unfortunately, the
94 presence or absence of hypogonadism and the data on pubertal development of these subjects and their
95 family members were unavailable. In one family, however, the pregnancy had been induced by
96 microinjection, and both son and the father had cryptorchidism and were included in this study. No assisted
97 reproductive techniques were used in 10 families, and in 2 families this information was unavailable.

98 Two hundred (120 males, 80 females) control subjects were included from the same geographical region.
99 DNA was obtained from a blood sample. The study was accepted by the joint ethics committee of Turku
100 University and Turku University Hospital, and informed consent was obtained from the participants or
101 guardians of the participating boys.

102 2.2. *Mutation screening of FGFR1, GNRHR, PROK2, PROKR2, TAC3, and TACR3*

103 Genomic DNA from peripheral blood leukocytes of the subjects was extracted, and the coding exons and
104 exon-intron boundaries of *FGFR1*, *GNRHR*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3* were PCR amplified and
105 screened by direct sequencing. In addition, exons 1-3 of the *GNRHR* were screened in control DNA in 200

106 (120 males, 80 females) subjects. PCR products were purified with ExoSAP-IT treatment (Amersham
107 Biosciences, Piscataway, NJ, USA), and sequenced using the ABI BigDyeTerminator Cycle Sequencing Kit
108 (v3.1) and ABI Prism 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA,
109 USA). The sequences were aligned and read with Sequencher® 4.9 software (Gene Codes Corporation, Ann
110 Arbor, MI, USA). All primer sequences and PCR conditions are available upon request.

111 2.3. *Statistical analysis*

112 All p -values are two-sided, and $p < 0.05$ was considered significant. Fisher's exact test was used to
113 determine statistical significance between cases and controls (Simple Interactive Statistical Analysis, SISA,
114 <http://www.quantitativeskills.com/sisa/>).

115 3. Results

116 3.1. *Mutation screening of FGFR1, GNRHR, PROK2, PROKR2, TAC3, and TACR3*

117 We investigated 54 subjects with a history of cryptorchidism from 46 families for mutations in the *FGFR1*,
118 *GNRHR*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3*. We did not identify mutations in the coding regions of
119 *FGFR1*, *PROK2*, *PROKR2*, *TAC3*, or *TACR3*. However, two unrelated subjects, both with a positive family
120 history of cryptorchidism in a first-degree relative, carried a heterozygous missense mutation, Q106R or
121 R262Q, in *GNRHR*. Both subjects had unilateral cryptorchidism and neither of them had a spontaneous
122 descent of a testis. The subject with R262Q mutation and his parents had normal onset of puberty, but the
123 father had been operated for cryptorchidism. The second subject carrying a Q106R mutation had normal
124 fertility, and his son had congenital cryptorchidism. Unfortunately, affected family members of these two
125 subjects were unavailable for the analysis. These heterozygous missense *GNRHR* mutations were also
126 present in 2 and 3 of 200 controls (2 and 1 of 120 males), respectively. Thus, the prevalence of the missense
127 mutations between 46 index subjects and male controls did not differ ($p = 0.62$).

128 **4. Discussion**

129 We hypothesized that some patients with isolated cryptorchidism might harbor defects in genes previously
130 shown to be indispensable for the intact function of the hypothalamic-pituitary-gonadal axis. Recently,
131 Sykiotis et al. (2010) showed that some patients with IHH also have testicular defects, and Hadziselimovic et
132 al. (2010) reported reduced expression of *FGFR1* in cryptorchid testis as compared to normally descended
133 testis analyzed in testicular biopsies. Carriers of *FGFR1* mutations may have wide a phenotypic spectrum
134 ranging from healthy carriers to severe IHH and Kallmann syndrome (IHH and decreased/absent sense of
135 smell) (Dode et al., 2003; Pitteloud et al., 2005). However, none of the boys in the current study displayed
136 mutations in the coding regions of *FGFR1*, suggesting that cryptorchidism is not commonly caused by IHH-
137 causing *FGFR1* mutations with incomplete penetrance. Because we used DNA obtained from peripheral
138 blood leukocytes, we cannot formally rule out, however, the possibility of tissue mosaicism of *FGFR1*
139 mutations, previously described in a family with an IHH proband (Sato et al., 2006).

140 Two subjects with cryptorchidism carried a heterozygous missense change, Q106R or R262Q, in *GNRHR*.
141 These mutations have previously been described in homozygous or compound heterozygous state in patients
142 with IHH, delayed puberty, or with IHH but spontaneous pregnancy (Beranova et al., 2001; Caron et al.,
143 1999; de Roux et al., 1997; Dewailly et al., 2002; Lin et al., 2006), whereas heterozygous carriers of these
144 mutations were unaffected (Beranova et al., 2001; de Roux et al., 1997). On the other hand, R262Q was
145 recently found in heterozygous state in a woman with hypothalamic amenorrhea (Caronia et al., 2011).
146 However, we found that the frequency of heterozygous Q106R or R262Q *GNRHR* mutations did not differ
147 between cases and male controls, and we therefore conclude that these heterozygous *GNRHR* mutations do
148 not contribute to the prevalence of common cryptorchidism.

149 Neurokinin B and prokineticin signaling appears indispensable for HPG axis function in boys during late
150 gestation and minipuberty of infancy, as evidenced by high frequency of micropenis among male IHH
151 probands with *PROK2*, *PROKR2*, *TAC3*, or *TACR3* mutations (Abreu et al., 2008; Gianetti et al., 2010;
152 Sarfati et al., 2010; Topaloglu et al., 2009). Since none of our subjects had micropenis or reported penile

153 anomalies, it was somewhat predictable that none of the subjects carried mutations in *TAC3*, *TACR3*,
154 *PROK2*, or *PROKR2*.

155 The limitation of this study is a relatively small sample size. However, if mutation(s) in *FGFR1*, *GNRHR*,
156 *TAC3*, *TACR3*, *PROK2*, or *PROKR2* did cause 10% of cryptorchidism, we would have with 99% probability
157 found at least one case with conclusive mutation(s) among 46 index subjects. Similarly, if the prevalence of
158 the mutation(s) was 4%, the probability to detect at least one is still 85%. Finally, the familial occurrence of
159 cryptorchidism supports the genetic etiology for the condition (Weidner et al., 1999), and the risk for
160 cryptorchidism in male infant is increased if a brother or father is affected (Elert et al., 2003; Foresta et al.,
161 2008). In this study, 41% (19/46) of participating families had more than one cryptorchid family member,
162 but high familial incidence herein was due to specific selection of the familial cases.

163 In conclusion, our results show that IHH-causing *FGFR1* mutations with incomplete penetrance, or
164 homozygous/compound heterozygous *GNRHR*, *PROK2*, *PROKR2*, *TAC3*, or *TACR3* mutations are not
165 frequently encountered in patients with isolated cryptorchidism. It is therefore unlikely that mutations in
166 these genes would contribute significantly to the prevalence of isolated cryptorchidism. Despite this, the
167 familial cases suggest that there is a genetic susceptibility to common cryptorchidism.

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305 **Highlights**

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307 • We investigated the defects in *FGFR1*, *GNRHR*, *PROK2*, *PROKR2*, *TAC3*, and *TACR3* in 54
308 subjects with isolated cryptorchidism.

309 • Two subjects carried heterozygous *GNRHR* mutations (Q106R and R262Q), but these changes
310 were also present in controls with similar frequency.

311 • None of the subjects carried mutation(s) in *FGFR1*, *PROK2*, *PROKR2*, *TAC3* or *TACR3*.

312 • It is unlikely that mutations in these genes would significantly contribute to the prevalence of
313 isolated cryptorchidism.

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