Identification by array-Comparative Genomic Hybridization (array-CGH) of a large deletion of luteinizing hormone receptor gene combined with a missense mutation in a patient diagnosed with a 46,XY disorder of sex development and application to prenatal diagnosis

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Abstract. This paper reports the case of an infant presenting with sexual ambiguity at birth. The child presented with labia majora synechia, thready genital tubercle and perineal hypospadias. The karyotype was 46,XY. Low testosterone levels with no response to hCG administration, associated with high LH level for her age, high FSH level, high inhibin B levels and normal AMH indicated a lack of LH receptivity and prompted us to screen the \(LHCGR\) gene for mutations. A previously described missense mutation (p.Cys131Arg) was identified at homozygous state in the propositus and at heterozygous state in the mother. This variation, however, was not found in the father. Our attention was drawn by the presence of several single nucleotide polymorphisms (SNPs), identified at homozygous state without any paternal contribution from exon 1 to exon 10 of \(LHCGR\), suggesting a paternal deletion. Array DNA analysis was performed revealing a large deletion extending from 61,493 to 135,344 bp and including the \(LHCGR\) gene. Adequate genetic counselling was provided. This paper describes the first application of prenatal diagnosis in LHCGR deficiency for 46,XY disorders of sex development with the subsequent delivery of a normal baby.

Key words: 46,XY disorders of sex development, Inactivating mutation, \(LHCGR\) gene, Prenatal diagnosis, array-Comparative Genomic Hybridization

MALE sexual development in humans includes establishing gonadic sex and phenotypic sex. While gonadic sex is genetically controlled by and depends on the \(SRY\) gene located on the Y chromosome, establishment of phenotypic sex is regulated exclusively by sex-steroids. During the foetal period, male differentiation is an active phenomenon which requires anti-Müllerian Hormone (AMH) secretion, causing the regression of Müllerian ducts, and testosterone secretion by Leydig cells to induce the development of Wolff ducts in external genitalia. Male sexual differentiation is completed in two stages. The first, occurring during the eighth week of gestation, does not require gonadotropin (LH and hCG) secretion. The secreted testosterone stimulates the development of the Wolffian duct derivatives such as vas deferens, epididymis, prostate and the seminal vesicles. After week 9.5 of gestation, the development of male external genitalia is mediated through hCG-dependant testosterone secretion by Leydig cells [1].

hCG, produced by both the trophoblast and LH, is mediated by the same Luteinizing Hormone Receptor (LHGRP) [2]. LHGRP’s ability to stimulate the proliferation of Leydig cells was recognized when the phenotype of boys harbouring naturally occurring mutations was described [3, 4]. Three different clinical
phenotypes can result from altered LHCGR. The first is Familial Male-limited Precocious Puberty (FMPP) which occurs in males [5-8]. It is connected to gain of function mutations. For loss of function mutations in the LHCGR gene, the other phenotypes are Leydig Cell Hypoplasia (LCH) in males and primary Amenorrhea in females. Two types of LCH have been described [9].

Type I LCH is the most severe form and was originally described by Berthezene [10]. Patients present with 46,XY disorders of sex development (46,XY DSD) characterized by female external phenotype with primary amenorrhea and absence of breast development. Histological analyses revealed a few immature Leydig cells. This phenotype is caused by inactivating mutations which totally prevent hCG/LH transduction and thus testosterone production by the Leydig cells [3, 4, 11, 12].

Patients with type II LCH cover a broad phenotypic spectrum, presenting with sexual ambiguity with incomplete virilization of external genitalia and micropenis which may be associated with hypospadias or with delayed pubertal development. During puberty, partial virilization occurs and testicular size may be normal or slightly reduced, while penile growth is significantly impaired [13]. This milder phenotype is linked to loss of function mutations that partially impair the biological activity of LHCGR.

In humans, the LHCGR gene is located on chromosome 2p21 and spans approximately 80 kilobase pairs (kb). This gene comprises 11 exons. The first 10 exons encode the extracellular domain, while the remaining one encodes the seven-transmembrane and the cytoplasmic domains [2, 14].

The present study describes a patient diagnosed with sexual ambiguity at birth. Screening for mutations in the LHCGR gene using DNA sequencing revealed an apparent homozygous mutation. Further investigation using polymorphism studies and array-CGH, evidenced a large deletion inherited from the father, covering exons 1 to 10 of the LHCGR gene. Finally, during a second pregnancy, prenatal diagnosis was performed on chorionic villi at the 13th week of gestation which excluded the LHCGR mutation.

### Subjects and Methods

**Case report**

The patient was born as the first child of unrelated healthy parents. The child presented with sexual ambiguity at birth with labia majora synechia, thready genital tubercle and perineal hypospadias. Gonads were palpable in the inguinal regions. The baby had a normal male karyotype (46, XY) and Fluorescence In Situ Hybridation (FISH) confirmed the presence of the SRY gene. Genitography showed blind-ending vagina, and no Müllerian structures were detected by ultrasound. Hormonal evaluation was performed at birth, at three months and at 2yrs 7months (Table 1). Serum testosterone levels were very low, coupled with high LH level for her age, high FSH level, high inhibin levels and normal AMH. Three intramuscular injections of recombinant human chorionic gonadotropin (hCG) failed to elicit testosterone, dehydroepiandrosterone or any response from delta4androstenedione, thus eliminating partial androgen insensitivity syndrome or testicular steroidogenesis disorders. The pituitary response of LH and FSH to GnRH was preserved. This hormonal feature suggested a lack of LH receptivity and prompted us to screen the LHCGR gene for mutations. Because of the severity of the ambiguity, the patient has been raised as a girl. Coelioscopy found neither uterus nor prostate. Two vas deferens were present.

<table>
<thead>
<tr>
<th></th>
<th>T (ng/mL)</th>
<th>E2 (pg/mL)</th>
<th>LH Basal (mU/mL)</th>
<th>LH Peak (mU/mL)</th>
<th>FSH Basal (pg/mL)</th>
<th>FSH Peak (pmol/mL)</th>
<th>Inhibine B (pg/mL)</th>
<th>AMH (pmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal range</td>
<td>0.03-0.52</td>
<td>&lt;10</td>
<td>0.05-1.3</td>
<td>0.1-1.6</td>
<td>17-94</td>
<td>150-1500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At birth</td>
<td>0.24</td>
<td></td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
<td>92</td>
<td>164</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 weeks</td>
<td>0.05</td>
<td>&gt;4</td>
<td>5.1</td>
<td>4.7</td>
<td>335</td>
<td>906</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 yrs 7 months</td>
<td>0.02</td>
<td>4.3</td>
<td>3.2</td>
<td>4.1</td>
<td>108</td>
<td>931</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 yrs 4 months*</td>
<td>10.8</td>
<td></td>
<td>118</td>
<td>&lt;15</td>
<td></td>
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</tr>
</tbody>
</table>

E2, estradiol; T, testosterone. The GnRH test was performed by intravenous administration of 100µg of GnRH and the highest values observed for plasma LH and FSH are reported. References hormone values were obtained from 46,XY age-matched individuals. * Values obtained after gonadectomy.

Table 1 Hormonal evaluation performed in the propositus.
Table 2  Primer sequences used to amplify the LHCGR gene. (F) forward (R) reverse.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Exon</td>
<td>5'-GGT CAA GGC AGA GCA GAC TC-3' (F)</td>
<td>5'-GTG GGG GAA ATT TGA TAG GG-3' (R)</td>
</tr>
<tr>
<td>2 Exon</td>
<td>5'-CCT CAG CCT GAA TCC AGT TC-3' (F)</td>
<td>5'-GCA TTC AGT CAT TTG AAG CAA-3' (R)</td>
</tr>
<tr>
<td>3 Exon</td>
<td>5'-GCC CAT TGC TCC TAA AG-3' (F)</td>
<td>5'-GGA TTT CTC CCA GAG TC-3' (R)</td>
</tr>
<tr>
<td>4 Exon</td>
<td>5'-TCC TCC CAA GCA TAT GAA GG-3' (F)</td>
<td>5'-GGT ATG TAA AAG CAC CCA GCT C-3' (R)</td>
</tr>
<tr>
<td>5 Exon</td>
<td>5'-CAT CTG CCA CAT GA-3' (F)</td>
<td>5'-CCA GTG AGT GAG GAA TGT GG-3' (R)</td>
</tr>
<tr>
<td>6 Exon</td>
<td>5'-GGA AAA ATG TGT TTC CAA ACT G-3' (F)</td>
<td>5'-TAG CCA GCC AGT TGC CTA GT-3' (R)</td>
</tr>
<tr>
<td>7 Exon</td>
<td>5'-CAT CAT GCA CAT AGC TTG GTC-3' (F)</td>
<td>5'-GAT GTG GAG GGA GAC CCT AA-3' (R)</td>
</tr>
<tr>
<td>8 Exon</td>
<td>5'-GAT CCT GCC ACT GCA CTC-3' (F)</td>
<td>5'-GGG TGA ATC AAC TAT TTG GTA GC-3' (R)</td>
</tr>
<tr>
<td>9 Exon</td>
<td>5'-GCT GGA AGC ACT GCA CTC-3' (F)</td>
<td>5'-GCA ACA GCT CCG TAA CCA AG-3' (R)</td>
</tr>
<tr>
<td>10 Exon</td>
<td>5'-TGG ATG GAG GGA CAC CCT AA-3' (F)</td>
<td>5'-GAC GGT GAG GGT GTA GAC AG-3' (R)</td>
</tr>
<tr>
<td>11 Exon</td>
<td>5'-GAC TTT TGC ATG GGG CTC TA-3' (F)</td>
<td>5'-GTG CCA TGC AGG TGA AAT C-3' (R)</td>
</tr>
<tr>
<td>11c Exon</td>
<td>5'-TCA ATG TGG TGG CCT TCT TC-3' (F)</td>
<td>5'-TGC CAT GTA ACA ATG ACA AAT AG-3' (R)</td>
</tr>
</tbody>
</table>

Bilateral gonadectomy was performed when the patient was three years and 4 months old. Histological evaluation revealed testes with rete testis, some epididymal structures and residual adrenal structures. It showed the presence of Sertoli cells with germinal cells and Leydig cells hypoplasia.

**DNA analysis**

Appropriate informed consent was obtained from the parents in order to perform genetic studies. DNA extracted from peripheral blood was amplified by PCR with the following primer pairs (Table 2). PCR products were sequenced using CEQ™ 8000 Genetic Analysis System Beckman Coulter (Roissy, France).

**Identifying polymorphisms**

We studied 12 microsatellite markers, spanning from chromosome 1 to chromosome 16, using Genetic-print kits GenePrint™ Fluorescent STR Systems (Promega, Charbonnières, France). Each kit (γSTR, CTTv, FFFL) was used to amplify 4 different microsatellite markers. The PCR fragments were checked using Abi Prism™ 3100 Genetic Analyzer and alleles for each microsatellite marker were identified.

**Microarray analysis**

High molecular weight genomic DNA was extracted from the patient’s peripheral blood lymphocytes using the QIAtamp DNA Blood Midi kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. DNA concentration was determined with NanoDrop ND-1000 spectrophotometer and software (NanoDrop Technologies, Berlin, Germany). Detection of gene copy number was performed by array-CGH experiments in accordance with standard practice and the manufacturer’s recommendations (Agilent™, Agilent Technologies, Santa Clara, Ca) using 44,000 oligo probes at approximately 40-100 kb intervals across the genome (Human Genome CGH microarray 44B kit, Agilent™). Female genomic DNA (Agilent™) was used as a reference in hybridizations which were analyzed with CGH-analytics software by applying a Z-score segmentation algorithm to identify chromosome aberrations.

**Results**

**Sequence analysis of the LHCGR gene**

We found a T→C transition at nucleotide 391 (c.391T>C) in exon 5 changing cysteine at codon 131 to arginine (p.Cys131Arg) (Fig. 1A). This substitution was identified in homozygous state in the propositus (Fig. 1B and 1C1). It was found at heterozygous state in the mother (Fig. 1B and 1C3). We did not find this variation in the father (Fig. 1B and 1C2).

**Paternity identification**

As the mutation was not found in the father, paternity was confirmed by studying microsatellite markers from chromosomes 1 to 16. Examples of five representative markers (M1 to M5) are depicted on Fig. 2. For marker 1 (M1), the father presented two alleles of 311 and 315 bp in size whereas the mother was homozygous with an allele of 307 bp. The fact that the propositus presents markers 307 and 311 has led us to conclude that allele 311 was inherited from the father.
Fig. 1  A. Amplification of exon 5 and 6 of the LHCGR gene. S1 (3µL) and S2 (6µL) are the 100 bp ladder. C1, C2 and C3 are PCR products (558 bp), for the propositus, his father and his mother respectively. B. Sequence alignment of the family with the reference sequence. C is the reference sequence. C1, C2 and C3 are the sequence of the propositus, his father and his mother. This alignment shows that the father (C2) has the wild sequence (TGT), whereas the propositus and his mother present a T to C change at nucleotide 391 (CGT). C. The family sequences of exon 5 of the LHCGR gene. C1 is the patient’s sequence with a homozygous mutation due to a T→C substitution at nucleotide 391 of the LHCGR gene. C3 is the mother’s sequence which has a heterozygous mutation as one allele is TGT and the other CGT. C2 is the father’s sequence showing the wild sequence TGT at codon 131.

Fig. 2  Microsatellite markers of GenePrint™ Fluorescent STR Systems. Five representative microsatellite markers M1 to M5 are represented among the twelve tested. The size (in bp) of the different alleles is indicated at the bottom of the peaks. Informative markers are represented by an arrow. They indicate that the proband has inherited an allele from each parent. M4 is not informative because both parents have the same alleles.
homozygote (T/T) and (C/C) respectively, confirming the contribution from each of the parents. The proband, in contrast, displays only the same SNPs as his mother from exon 1 to exon 10. For example, in exon 10 (rs2293275), there is an apparent homozygous state for adenine (A) inherited from the mother, while the father presents only a guanine (G), thus excluding any paternal contribution. Interestingly, these SNPs were also found at an apparent homozygous state in the father along the LHCGR gene. Loss of heterozygosity (LOH) suggested that a partially-deleted LHCGR allele had been inherited from the father.

Array-CGH

Array-CGH analyses were performed on DNA proband to further characterize the size of the deletion. This revealed a 2p16.3 deletion, including two probes, A_14_P110765 and A_14_P200059, located at position 48,813,607 and 48,833,407 (hg18-build36) respectively (Fig. 4). These probes are thought to have hybridized with intron 1 of the LHCGR gene. Probe A_14_P119242 which was retained, is located at position 48,768,875, which is within exon 11 of the LHCGR gene (c.1565 to c.1624). Taking SNP and array-CGH data together, the proximal breakpoint was mapped between the heterozygous rs11125179 in exon 11 (position 48,769,375) and rs2293275, which displays LOH (position 48,774,879) and the distal breakpoint between rs10176989 (LOH, exon 1, position 48,836,372) and A_14_P110349 (undeleted probe, position 48,904,719) (Fig. 4). Thus, the
The estimated size of the deletion extends from 61,493 to 135,344 bp. With the exception of the *LHCGR* gene located between 48,774,867 and 48,836,384, no other known gene is included in this deletion.

**Prenatal diagnosis**

Conventional cytogenetic analysis revealed a male (46,XY). DNA sequencing of exon 5 showed a normal sequence. Moreover, the presence of the different SNPs at heterozygous state (Fig. 3) was also determined. These results confirm that the foetus has inherited the normal alleles from his parents. Finally, pregnancy and delivery at term were normal and the mother gave birth to a normal boy of 3.250kg and 51cm.

**Discussion**

Our understanding of the causes of sexual ambiguity has progressed from the determination of the hormonal etiologies to defining the genetic basis of intersex disorders. The localization of specific genes involved in the process of sexual differentiation has made it possible to determine the mutations and other molecular events that result in sexual ambiguity. With this information, some disorders can now be diagnosed before birth and possibly even treated *in utero*. Since the first description by Kremer *et al.* [3], several *LHCGR* inactivating mutations have been described in males with 46,XY DSD [4, 11, 12, 15-29]. Inactivating receptor gene mutations are found in homozygous or compound heterozygous states and the syndrome follows a pattern of autosomal recessive inheritance. In this paper, we have described a new compound heterozygous mutation, namely a missense mutation in exon 5 and a large deletion of the *LHCGR* gene.

The hormone pattern is that of a Leydig cell defect, with consistently undetectable testosterone levels. Above normal LH levels for the child’s age and the absence of a testosterone response to hCG stimulation suggest resistance to LH. In contrast, secretion of AMH and inhibin B by Sertoli cells were normal for the child’s age or even above normal as regards inhibin B. It is likely that the high inhibin B levels were the result of the increase in FSH secretion due to the lack of feedback from estradiol. It is well known that estradiol can be produced by the aromatization of testosterone in different sites of the brain (review in [30]). It has been shown that, in women, estradiol is the main inhibitor of FSH rise during the menstrual cycle and that this inhibitory effect is more pronounced upon FSH secretion than upon LH secretion [31]. In addition, it should be noted that the transient post-natal increase in gonadotropin secretion occurred normally in this patient, since FSH, LH and inhibin B levels were significantly higher at 10 weeks of age than at 2 years.

Two different mutations were found in this patient, a missense mutation p.Cys131Arg previously described at homozygous state [22] in a case of incomplete Leydig cell agenesia, and a large deletion in chromosome 2 removing exons 1 to 10 of *LHCGR* and thus inducing total LHCGR loss of function. The phenotype of the patient described in the present study appears to be more severe than in the homozygous p.Cys131Arg patient. Both patients present with sexual ambiguity, but the patient examined here has been raised as a girl because she presented with partial fusion of labioscrotal fold, hypertrophy of the genital tubercle and perineal hypospadias, whereas the other patient is closer to the male end of the spectrum with micropenis and hypospadias. The p.Cys131Arg mutation is located in the extracellular domain of the LH receptor and the mutant receptor exhibited marked impairment of hCG binding while some cAMP production could be observed at high hCG concentrations [22]. Similarly, partial masculinization of the external genitalia with hypoplastic phallus and hypospadias suggests that some production and biological action of testosterone/DHT must have occurred in order for the labioscrotal folds to fuse during pregnancy when the mutant receptor was stimulated by the high concentration of hCG. The difference between the two phenotypes could be related to the allelic combination of the mutations, the patient with the complete loss of function allele being more severely affected than the patient with homozygous p.Cys131Arg mutation.

To our knowledge, this is the first multi-exonic *LHCGR* deletion to be identified by array-CGH, in this case transmitted to our patient by the father. Identification of a large deletion is difficult when PCR is performed within the deletion, with potential misdiagnosis as regards the risk for subsequent offspring. Indeed, only the undeleted allele was studied and the mutation in the remaining allele was observed as being homozygous. Accordingly, using DNA sequencing, the patient was first identified as being homozygous for the p.Cys131Arg mutation. LOH along the *LHCGR* gene, without any paternal contribution, indicated a probable deletion prompting us to test the DNA using array-
CGH. The estimated size of the deletion is between 61,493 and 135,344 bp and no gene other than \textit{LHCGR} has been deleted. Array-CGH data are congruent with SNP genotyping and confirm the presence of exon 11 of \textit{LHCGR}. The deletion of exons 8 [12] and 10 [15] has previously been observed in 46,XY DSD. In this paper, the proximal breakpoint was found within intron 10 between positions 48,769,375 (rs1125179, ex11) and 48,774,879 (rs2293275, ex10). Due to its size, it has not been possible to determine the boundaries of the deletion in the present study.

Nonetheless, adequate genetic counselling was provided. On account of the severity of the ambiguity and after being granted the approval of the multidisciplinary center of prenatal diagnosis, the mother requested prenatal diagnosis for a second pregnancy. DNA extracted from chorionic villi at the thirteenth week of gestation were screened for \textit{LHCGR} mutations and two normal alleles were found. This result was confirmed by studying the different SNPs along the gene. A male infant of normal appearance was delivered.

As described above, a new compound mutation with a large deletion of \textit{LHCGR}, leading to a severe form of 46,XY DSD was identified using array-CGH. Adequate genetic counselling has been provided and this report describes the first application of prenatal diagnosis for 46,XY DSD associated with a \textit{LHCGR} mutation with the subsequent delivery of a normal baby.

\textbf{Declaration of interest}

The authors have nothing to declare.

\textbf{Acknowledgments}

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