Case Report

Novel Compound Heterozygous Variants in the LHCGR Gene in a Genetically Male Patient with Female External Genitalia

Short title: Novel variants in the LHCGR Gene

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Key words: Disorder of sexual differentiation (DSD); Leydig cell hypoplasia (LCH); LHCGR gene; novel variants

What is already known on this topic?
Both loss and gain of function mutations of the LHCGR gene can cause human diseases. Inactive LHCGR variant causes type 1 Leydig cell hypoplasia (LCH), which is characterized by the complete absence of male differentiation. To date, 77 variants have been reported,
including 49 missenses, 11 nonsenses, 5 gross deletions, 4 small insertions, 4 small deletions, 3 splicing variants, and 1 gross insertion.

What this study adds?

In this study, we identified two novel heterozygous variants in the \textit{LHCGR} gene (c.349G\textgreater{}A, p.Gly117Arg and c.878C\textgreater{}A, p.Ser293*) causing type 1 LCH in a 2-year-9-month old patient presenting with female external genitalia and bilateral testis tissues in the inguinal region.

Abstract

\textbf{Objectives}: The \textit{LHCGR} gene encodes a G-protein coupled receptor that plays a pivotal role in sexual differentiation in males, ovarian development in females, and in fertility via its interaction with luteinizing hormone (LH) and chorionic gonadotropin (CG). Inactive variants of the \textit{LHCGR} gene cause Leydig cell hypoplasia (LCH), which is a rare disease and one of the causes of disorder of sexual differentiation (DSD) in males. The aim of this work is to study the clinical and molecular characteristics of a 2-year-9-month old patient with type 1 LCH.

\textbf{Methods}: Whole-exome sequencing was performed for the patient family, and variants in the \textit{LHCGR} gene were validated by Sanger sequencing. Pathogenicity of the missense variant was evaluated by multiple in silico tools.

\textbf{Results}: The Chinese patient, who exhibits DSD, has female external genitalia (normal labia majora and minora, external opening of urethra under the clitoris and blind-ended vagina) and bilateral testis tissues in the inguinal region. Genetic sequencing revealed compound
heterozygous variants in the *LHCGR* gene in the patient, including a novel missense variant in exon 4 (c.349G>A, p.Gly117Arg) and a novel nonsense variant in exon 10 (c.878C>A, p.Ser293*). The missense variant is in the first leucine-rich repeat (LRR) domain of the LHCGR protein, which is predicted to affect ligand recognition and binding affinity and thus protein function.

**Conclusions**: The patient is molecularly and clinically diagnosed with type 1 LCH, which is caused by novel, compound heterozygous variants of the *LHCGR* gene. This report expanded the genotypic spectrum of *LHCGR* variants.

1. **Introduction**

The human luteinizing hormone/chorionic gonadotropin receptor (*LHCGR; OMIM #52790*) gene belongs to the G-protein coupled receptor (GPCR) 1 family. The *LHCGR* gene encodes a shared receptor for both luteinizing hormone (LH) and chorionic gonadotropin (CG), and the receptor plays a critical role in male sexual differentiation, female ovarian development and fertility (1). *LHCGR* is located on chromosome 2p21, which contains 12 exons. The *LHCGR* gene encodes a 699 amino acids protein that consists of an N-terminal cysteine-rich region, a tandem leucine-rich repeats (LRRs) region and a C-terminal cysteine-rich region (2, 3). In males, the N-terminal region and the LRR1-LRR7 repeats are essential for the high affinity binding of human CG (hCG), which stimulates the production of testosterone and maturation of fetal Leydig cells during early embryogenesis. In addition, the interaction between LH and LHCGR maintains postnatal testosterone level that is required for male secondary sex characteristics and spermatogenesis during puberty (4, 5).
Both loss and gain of function mutations of the *LHCGR* gene can cause human diseases. In males, germline activation of *LHCGR* is associated with inherited, autosomal dominant precocious puberty (OMIM#152790). Biallelic inactivation of the *LHCGR* causes Leydig cell hypoplasia (LCH, OMIM#238320) that leads to male disorder of sexual differentiation (DSD). Constitutively inactive *LHCGR* variant causes type 1 LCH, which is characterized by the complete absence of male differentiation. Partially inactive *LHCGR* variant results in type 2 LCH that features hypogonadal phenotypes with variable severity (6, 7). In females, inactivated *LHCGR* gene has no effect on the primary and secondary sex characteristics, but it causes amenorrhoea and infertility due to aberrant follicular maturation and ovulation (8).

In this study, we report a rare pediatric patient of type 1 LCH due to novel, compound heterozygous mutations in the *LHCGR* gene. Our findings expanded the spectrum of genotype-phenotype correlation in the *LHCGR* variants.

2. Materials and Methods

Clinical presentation of the patient

The proband was a 2-year-9-month old whose social gender was female. The child was taken to our hospital due to the absent of vagina since her birth. The patient was born full term by natural delivery, and she is the second child of healthy parents of non-consanguineous marriage. Her birth weight was 3,900 g, and her current weight is 17 kg (96.8th percentile) and her height is 97 cm (73.5th percentile). Physical examination showed that the patient exhibited predominantly female external genitalia, with normal bilateral labia majora, bilateral labia minora, and external opening of urethra under the clitoris. However, she had a blind-ended vagina without external opening. The patient showed absence of scrotum and
penis. Abdominopelvic ultrasound examination detected bilateral testis tissues in inguinal region (size, left 2.0cm×0.7cm×0.9cm; right 1.7cm×0.7cm×0.9cm). Uterus or other Mullerain structure was not observed. Laboratory examination results showed that she had extremely low serum testosterone and dihydrotestosterone (DHT) levels (0.01nmol/L), which could not be stimulated by hCG. Serum levels of LH and follicle stimulating hormone (FSH) were in normal range (3.84 IU/L and 9.09 IU/L, respectively), and both of them were hyper-responsive (24.48 IU/L and 22.33 IU/L, respectively) to stimulation with 2.5 μg/kg of luteinizing hormone releasing hormone (LHRH). Thyroid hormones, estradiol, prolactin, blood chemistry, and complete blood count were all normal. **Primary genetic analysis revealed that the patient’s karyotype was 46, XY and no pathogenic variant was identified in SRY gene.** The patient was primarily diagnosed with male pseudohermaphroditism.

All procedures followed were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000, and the protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval no:XJMU-FAHIRB-2017005). Informed consent was obtained from the patient’s family.

**Whole exome sequencing and data analysis**

The genomic DNA was isolated from peripheral blood samples of the patient and her parents using the QIAamp Blood DNA Mini kit® (Qiagen GMBH, Hilden, Germany). A total of 3 μg of genomic DNA was sheared to obtain DNA fragments with sizes between 150 bp and 200 bp. The capture library was prepared using SureSelect Human All Exon V6 kit (Agilent
Technologies Inc., Santa Clara, CA, US) following the manufacturer's protocol. Next, clusters were generated by isothermal bridge amplification with an Illumina cBot station and sequencing was performed by an Illumina X10 System (Illumina, CA, USA). Base calling and quality assessment of sequence reads were performed using Illumina Sequence Control Software (SCS) with Real Time Analysis (RTA). Alignment of sequence reads to the reference human genome (Human 37.3, SNP135) was performed using the NextGENe® software (SoftGenetics, PA, USA). All single nucleotide variants (SNVs) and indels were saved in a VCF format file, which was then uploaded to Ingenuity® Variant Analysis™ (Ingenuity Systems, CA, USA) for biological analysis and interpretation.

**Sanger Sequencing Verification of the LHCGR Gene**

The primers for amplifying the *LHCGR* gene (GenBank accession no. NM_000233.3) were designed using Primer 3 online software (http://primer3.ut.ee/). The designed primers for exon 4 of the *LHCGR* gene were as follows: forward, 5′-AGCCAGCAACTTCTGGTGAC-3′ and reverse, 5′-TCCAACCTTTTCCTTGTTTTG-3′, and the primers for amplifying exon 10 were: forward, 5′-GACGCACAGTCAGGTTTAGC-3′; reverse, 5′-GCTGATAATAAGGTGCACACAG-3′. Both exons and exon-intron boundaries were amplified using polymerase chain reaction (PCR). The reaction mixture for each amplification contained 1x Premix Taq (Takara Biotechnology Co., Ltd., Dalian, China). The products were examined on a 1% agarose gel and purified with the QIAquick Gel Extraction kit (Qiagen GmbH). The resulting DNA was sequenced using the ABI3730XL sequencer (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the forward and reverse primers. The sequence data were
then analyzed using Mutation Surveyor® DNA Variant Analysis Software (version 4.0.4; SoftGenetics, LLC.).

**Pathogenicity predictions of the identified variant**

The potential pathogenicity of the missense variant was analyzed by using MultAlin (http://multalin.toulouse.inra.fr/multalin/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), CADD (http://cadd.gs.washington.edu/), and MutationTaster (http://www.mutationtaster.org/).

**Statistical Analysis**

Not applicable

3. Results

**Identification of the causal variants**

To obtain a rapid and accurate clinical genetic diagnosis, trio-WES was used to screen for causal variants. For the patient, WES yielded a total of 103,509,228 reads, and the mean target coverage was 133 reads with 95.52% having 20× coverage and 99.83% having 1× coverage. The candidate variants were first filtered by the following parameters: (1) minor allele frequency (MAF) under 1% in genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/); (2) the benign variants, including synonymous and harmless missenses predicted by Ingenuity and those predicted to have no impact on splicing by MaxEntScan. Subsequently, clinical symptoms of male pseudohermaphroditism were used as filtering indexes to analyze the candidate variants. As a result, we identified a compound alteration with two heterozygous variants within the *LHCGR* gene, which we believe to have contributed to the patient's condition. Of the two variants, one is a novel missense variant in
exon 4 (c.349G>A, p.Gly117Arg), and the other was a novel nonsense variant in exon 10 (c.878C>A, p.Ser293*). We have further confirmed the compound heterozygous variants by Sanger sequencing, and we also found that the patient's father is heterozygous for the nonsense variant and the patient's mother is heterozygous for the missense variant (Fig. 1A).

**Pathogenicity Predictions for c.349G>A (p.Gly117Arg)**

To evaluate the pathogenicity of the novel variant c.349G>A, we first analyzed the conservation of Gly117 using MultAlin software. As shown in Fig. 1B, results from MultAlin show that the amino acid glycine (Gly) at codon 117 is highly evolutionarily conserved. Next, we used three in silico prediction software to evaluate the impact of the variant on protein function. The PolyPhen-2 score of the variant is 0.96, indicating that the variant is probably damaging. The MutationTaster score is 1, which implies that the variant is likely disease causing. The CADD score is 25.4, which suggests that the variant can be damaging. To better understand the missense variant, the WT and variant amino acid at codon 117 were modeled into the three-dimensional structure of the LHCGR protein (9) (Fig. 2). Based on the structure (9) and domain information of the LHCGR wild-type protein obtained from Uniprot (http://www.uniprot.org/), the amino acid substitution at the 117th position (p.Gly117Arg) was predicted to disrupt the first leucine-rich repeat (LRR) domain, which may affect recognition and binding affinity of LHCGR to hCG and/or other ligands. Taken together, our analysis results indicate that the c.349G>A (p.Gly117Arg) variant is likely harmful to the protein function.

4. Discussion
In current study, we report a socially defined female Chinese patient presenting with DSD, who has normal labia majora and minora, external opening of urethra under the clitoris, but blind-ended vagina. The patient has a karyotype of 46, XY, and bilateral testis tissues in the inguinal region. **We first excluded harmful variant in the SRY gene.** By performing WES, we identified a compound heterozygous variants in the patient, with a novel missense variant (c.349G>A, p.Gly117Arg) and a novel nonsense variant (c.878C>A, p.Ser293*) in her LHCGR gene that contributed to the patient’s condition. The missense and nonsense variants were inherited from the unaffected heterozygous father and mother, respectively. According to the variant interpretation guidelines from the American College of Medical Genetics and Genomics/the Association for Molecular Pathology (10), the nonsense variant is classified to be pathogenic (PVS1+PM2+PP4), and the missense variant is likely pathogenic (PM2+PM3+PP3+PP4). Therefore, the patient was molecularly and clinically diagnosed with type 1 LCH.

Gender assignment for LCH patients can be difficult, which is influenced by genital appearance, surgical options, fertility potential, and the views of the family (11). Timing of gender assignment can be controversial, especially when the psychological age is taken into consideration. The social gender of our patient was female, whose psychological gender was also female as we assessed. Physically, the patient’s abnormal testis tissues showed no function in provocation test using hCG. Based on medical advice from experts and discussions with the parents, the patient underwent bilateral orchidectomy. Testicular histology revealed that the seminiferous tubules were lined only by a few Sertoli cells. The interstitial region appeared to have only a few fusiform cells that appear to be immature.
Leydig cells (Fig.3), which is consistent with LCH phenotypes and confirmed the diagnosis of LCH. Interestingly, several previously reported cases showed that delayed orchidectomy after adolescence might result in primary amenorrhea and breast underdevelopment (12,13). For these reasons, orchidectomy was performed in our patient right after the gender assignment, and normal secondary female characteristic development is expected in the future.

To date, a total of 77 variants have been identified in the \textit{LHCGR} gene (Human Gene Mutation Database (HGMD): http://www.hgmd.cf.ac.uk/), including 49 missenses, 11 nonsenses, 5 gross deletions, 4 small insertions, 4 small deletions, 3 splicing variants, and 1 gross insertion. In contrast to the infrequency of activating variants, inactivating homozygous and compound heterozygous variants that alter structure of the \textit{LHCGR} protein and subsequently its function is more common. As shown in Table 1, we summarized the inactivating variants from literatures (14-25). Interestingly, although the frequency of these variants is extremely low in gnomAD database (most of them are 0), homozygous variants account for most gene variations in patients. Variants occur more frequently in exon 11 may be simply explained by the fact that it is the largest exon of the \textit{LHCGR} gene.

\textbf{Study limitations}

In our case, the nonsense variant (p.Ser293*) is a loss of function mutation, and our analysis shows that the missense variant (p.Gly117Arg) is mostly likely also a loss of function mutation, which lead to the inactivation of LHCGR. However, the functional analysis is lacking, which should be performed in the future study.

\textbf{Conclusion}
We report a 46, XY, DSD Uyghur patient with type 1 LCH with novel heterozygous compound variants in the *LHCGR* gene. Her clinical features correlated with the molecular diagnosis. She was treated after choosing her social gender to be female. This is one of only a few LCH cases that underwent gender assignment and treatment following molecular confirmation of clinical diagnosis.

**Conflict of interest statement**

The authors declare no conflict of interest related to this study.

**Acknowledgements**

We are deeply grateful to the patient and the patient’s family, for their participation in this study. This work was supported by the National Natural Science Foundation of China (Grant No. 81360139).

**References**


Figure 1. Genetic sequencing of the LHCGR gene. (A) Sanger sequencing confirmed a novel heterozygous missense variant in exon 4 (c.349G>A, p.Gly117Arg) and a novel heterozygous nonsense variant in exon 10 (c.878C>A, p.Ser293*) in the patient, which were inherited from the parents. (B) The referred amino acid of codon 117 (Gly) is highly evolutionarily conserved among species.
Figure 2. **Three-dimensional structure model of the LHCGR protein.** The indicated amino acid (p.117, colored arrow: black, wild-type; red, variant) is located in the first leucine-rich repeat (LRR) domain of the LHCGR protein.
Figure 3. Histologic analysis of the surgical testicular tissue samples (400x). Hematoxylin and eosin (HE) staining revealed that the seminiferous tubules were lined only by a few Sertoli cells, and the interstitial tissue appeared to have only a few fusiform cells that might be immature Leydig cells.
Table 1. Summarize of the inactivating variants of *LHCGR* gene

<table>
<thead>
<tr>
<th>Patient</th>
<th>Variant</th>
<th>AF</th>
<th>Position</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
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<tr>
<td>1</td>
<td>c.340A&gt;T; p.I114F (het)*</td>
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<td>Pseudohermaphroditism (46, XY)</td>
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<tr>
<td>2</td>
<td>c.391T&gt;C; p.C131R (hom)</td>
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<td>3</td>
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<tr>
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<td>8</td>
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<tr>
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<td>17</td>
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<tr>
<td>18</td>
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<td>12</td>
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<td>19</td>
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<td>25</td>
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<td>c.1660C&gt;T; p.R554* (hom)</td>
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<td>27</td>
<td>c.1757_1758del; p.S586Ffs*19 (het)</td>
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<td>28</td>
<td>c.34_60; p.K12_P20del (het)</td>
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<tr>
<td>31</td>
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<td>34</td>
<td>Del Exon10 (hom)</td>
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<td>Pubertal development, small testicles, and delayed bone maturation</td>
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AF, allele frequency in gnomAD (http://gnomad.broadinstitute.org/); het, heterozygous; hom, homozygous; del, deletion;
* only heterozygous of p.I114F was identified in the patient, there should be another heterozygous variant in the patient, such as exon deletion.

<table>
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<tr>
<th></th>
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<td>c.878C&gt;A; p.S293* (het)</td>
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<td>10</td>
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