Analysis of KAL1, FGFR1, GPR54, and NELF copy number variations by multiplex ligation dependent probe amplification in male patients with idiopathic hypogonadotropic hypogonadism

Yağış BAŞARAN1, Hilmi Umut ÜNAL2, Şinasi Erol BOLU1, Rahşan İLİKÇİ SAĞKAN1, Mustafa ÇAKAR2*, Abdullah TAŞLIPINAR1, Taner ÖZGÜRTAŞ1, Uğur Hacı MUŞABAK3
1Department of Endocrinology, Gülhane Military Medical Academy, Ankara, Turkey
2Department of Internal Medicine, Gülhane Military Medical Academy, Ankara, Turkey
3Department of Allergy and Immunology, Gülhane Military Medical Academy, Ankara, Turkey
4Department of Clinical Biochemistry, Gülhane Military Medical Academy, Ankara, Turkey

Aim: Hypogonadotropic hypogonadism is a production failure of gonadal hormones due to a lack of gonadotropin secretion. Here, we aimed to determine the prevalence of genomic rearrangements in the KAL1, FGFR1, GPR54, and NELF genes in patients diagnosed with hypogonadotropic hypogonadism.

Materials and methods: The study included 86 male patients with idiopathic hypogonadotropic hypogonadism (76 diagnosed with normosmic idiopathic hypogonadotropic hypogonadism and 10 with Kallmann syndrome). Additionally, 95 healthy controls were recruited to investigate rearrangements in the KAL1, FGFR1, GPR54, and NELF genes, using multiplex ligation dependent probe amplification.

Results: From the 86 patients, 3 patients with Kallmann syndrome had heterozygous deletions in exon 9 of the KAL1 gene (probe target sequence: 5941-L05940), and 1 of these patients also had a duplication in exon 11 of the same gene (probe target sequence: 4427-L03813). Additionally, 1 patient with nIHH had a duplication in exons 14 and 18 of the FGFR1 gene (probe target sequences: 4440-L03826 and 4441-L03827, respectively). No deletions/duplications were identified in the GPR54 and NELF genes and no genomic rearrangement was detected in the control subjects.

Conclusion: To improve our understanding of this complex condition and also for better genetic counseling and directing therapy, defining the genetic basis of these disorders is essential.

Key words: Normosmic idiopathic hypogonadotropic hypogonadism, Kallmann syndrome, KAL1, FGFR1, GPR54, NELF, multiplex ligation dependent probe amplification

1. Introduction
Hypogonadism is caused by a lack of gonadal hormone production. It may be due to inadequate gonadotropin secretion (hypogonadotropic) or primary testicular failure (hypergonadotropic). Levels of serum gonadotropin concentrations in these disorders assist in differential diagnosis.

Testosterone levels below normal are compatible with hypergonadotropic hypogonadism if the serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations are elevated, and the condition is hypogonadotropic hypogonadism if the serum LH and FSH concentrations are normal or reduced.

Kallmann syndrome (KS) is a clinically and genetically heterogeneous disease. It is associated with idiopathic hypogonadotropic hypogonadism (IHH), which may be associated either with anosmia or hyposmia, or with a normal sense of smell (normosmic hypogonadotropic hypogonadism, nIHH). Several mutations have been identified as the underlying cause of the condition to date. Generally, these are only responsible for the molecular basis of approximately 10%–20% of all patients with IHH.

These mutations are generally classified into 4 main groups according to the specific target they affect. Each of them is characterized by phenotypic and hormonal variations.
1. Gonadotropin-releasing hormone (GnRH) neuron migration and function mutations
2. GnRH synthesis and secretion mutations
3. Hypophyseal response to GnRH mutations
4. Gonadotropin biosynthesis mutations

In this study, we aimed to assess the prevalence of sequence variations within the genes (including KAL1, FGFR1, GPR54, and NELF) playing a role in the pathogenesis of IHH and to determine the association between genotype and phenotype.

2. Materials and methods

2.1. Patients and controls
The study was conducted between May 2006 and October 2009 at the Department of Endocrinology, Gülhane Military Medical Academy, Ankara, Turkey. The patients recruited for the study included 86 males with IHH (76 with nIHH and 10 with KS) and 95 eugonadal males with the same ethnic origin.

Participants with a chronological age over 18 years and symptoms of hypogonadism were recruited into the patient group. They had no history of recent or previously taken medication and had normal testosterone levels with normal or reduced serum LH and FSH concentrations, a suppressed gonadotropin response to GnRH stimulation test, a normal anatomical view of the hypothalamic–pituitary axis in magnetic resonance imaging (MRI), and no pathologic finding other than bilateral testicular atrophy on the scrotal ultrasound. Subjects with a normal hypothalamic–pituitary–testicular axis were taken into the control group. All participants were tested in terms of their olfactory senses. The patient group underwent MRI of the olfactory bulbs and tracts. Subjects having a decreased sense of smell or anosmia were accepted as having KS.

After venipuncture, blood samples were collected into non-anticoagulated tubes (Grainer, Australia) and centrifuged at 5000 rpm for 10 min at room temperature. All serum specimens were stored at –80 °C prior to examination. A chemiluminescence immunoassay method was used to determine total testosterone (T), FSH, and LH levels by using an automated immunoassay analyzer (E170, Roche, Hitachi Corporation, Osaka, Japan). The radioimmunoassay kit DSL-4900 (Diagnostic System Laboratories, Webster, TX, USA) was used to measure free T levels.

2.3. Genetic analysis
DNA was extracted from the white blood cells of all patients and controls using the BioRobotGenoVisionGeno M-6 and EZ1 DNA Blood Kit (QIAGEN, Germany). A total of 125 ng of DNA in 5 µL units for each multiplex ligation dependent probe amplification (MLPA) reaction was used.

2.3.1. DNA denaturation
Five microliters of DNA in 0.2-mL polymerase chain reaction (PCR) tubes were dissolved in TE (10 mM Tris-HCl pH 8.2 + 0.1 mM EDTA). The tubes were put in a thermocycler, and DNA samples were denatured for 5 min at 98 °C and immediately cooled to 25 °C before opening the thermocycler.

2.3.2. Hybridization reaction
A hybridization master mix (1.5 µL MLPA buffer + 1.5 µL probemix) in quantities of 3 µL was added to each sample tube and mixed well. The thermocycler program was continued for 1 min at 95 °C, and then for 16 h at 60 °C.

2.3.3. Ligation reaction
A ligation master mix (3 µL Ligase-65 buffer A + 3 µL Ligase-65 buffer B + 25 µL dH2O) was added and mixed well by pipetting gently up and down. Then 1 µL of Ligase-65 was added and the solution was mixed again. The thermocycler was paused at 54 °C and 32 µL of the ligation master mix was added to each reaction tube. The program was continued for 15 min at 54 °C, followed by 5 min at 98 °C for heat inactivation of the Ligase-65 enzyme, and then paused at 15 °C.

2.3.4. PCR reaction
A PCR buffer mix (4 µL SALSA PCR buffer + 26 µL dH2O) was prepared and mixed briefly by turning. Then the 30 µL of the PCR buffer mix was added to new tubes for the PCR reaction and 10 µL of each ligation product was transferred to its corresponding PCR tube at room temperature. A polymerase master mix (2 µL SALSA PCR primers + 2 µL SALSA enzyme dilution buffer + 5.5 µL dH2O + 0.5 µL SALSA polymerase) was prepared and stored on ice until use. The thermocycler was heated to 60 °C. The PCR tubes were placed in it and 10 µL of polymerase mix was added to each tube. After mixing by pipetting gently up and down, the thermocycler program was continued immediately. The thermocycler ran for 35 PCR cycles (30 s at 95 °C; 30 s at 60 °C; 60 s at 72 °C) and ended with 20 min of incubation at 72 °C. It was then paused at 15 °C.

2.3.5. Fragment separation
Following the PCR reaction, a mix (0.75 µL of the PCR reaction + 0.75 µL water + 0.5 µL size standard + 13.5 µL HiDi formamide) was incubated for 2 min at 94 °C and stored on ice. PCR products were separated by capillary
electrophoresis using a Beckman Coulter CEQ-8000 Genetic Analyzer.

2.3.6. MLPA Data analysis

The P132 Kallmann-1 kit (MRC Holland, Amsterdam, the Netherlands) contains MLPA probes for each of the 14 KAL1 exons, and the P133 Kallmann-2 kit (MRC Holland) contains MLPA probes for most of the exons of the FGFR1 gene (11 of the 18 exons). The P133 Kallmann-2 kit also contains probes for some of the NELF gene (3 of the 16 exons), the GPR54 gene (3 of the 5 exons), the GnRH1 gene (3 of the 4 exons), and for each of the GnRHR, PROK2, and PROKR2 genes.

Data were evaluated using Genotyper 2.0 (Applied Biosystems, Foster City, CA, USA). For the analysis of the data, Coffalyser software (an Excel-based program) was used. Peak areas for each exon were converted into an Excel file and the relative DNA copy number ratios of each fragment were compared to the same fragments from 2 or 3 healthy subjects.

3. Results

The Table shows the hormonal and genetic results of the patient group. Heterozygous deletions in exon 9 of the KAL1 gene (probe target sequence: 5941-L05940) occurred in 3 patients with KS, and 1 of these patients also had a duplication in exon 11 of the same gene (probe target sequence: 4427-L03813) (Figures 1–3). One of the nIHH patients had a duplication in exons 14 and 18 of the FGFR1 gene (probe target sequences: 4440-L03826 and 4441-L03827, respectively) (Figure 4). No deletions/duplications were identified in the GPR54 and NELF genes and no genomic rearrangement was detected in the control subjects.

In summary, using MLPA for the described genes, 5 genomic rearrangements were present in 4/86 (4.6%) patients with IHH. If only patients with nIHH were considered, 1/76 (1.3%) had a previously described duplication, and if only KS patients were considered, 3/10 (30%) had rearrangements in the KAL1 gene.

Table. Hormonal and genetic results of the patient group.

<table>
<thead>
<tr>
<th>P. No.</th>
<th>Free testosterone (pg/mL)</th>
<th>Total testosterone (ng/dL)</th>
<th>FSH (mIU/mL)</th>
<th>LH (mIU/mL)</th>
<th>Established mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>2.30</td>
<td>5.00</td>
<td>0.423</td>
<td>0.100</td>
<td>*Hdel on KAL1.</td>
</tr>
<tr>
<td>52</td>
<td>2.05</td>
<td>17.82</td>
<td>0.530</td>
<td>0.160</td>
<td>**Dup on FGFR-1.</td>
</tr>
<tr>
<td>81</td>
<td>2.90</td>
<td>48.12</td>
<td>1.080</td>
<td>0.788</td>
<td>***Hdel+Dup on KAL1</td>
</tr>
<tr>
<td>84</td>
<td>3.40</td>
<td>49.60</td>
<td>0.476</td>
<td>0.112</td>
<td>*Hdel on KAL1</td>
</tr>
</tbody>
</table>

*Heterozygous deletion on KAL1 gene.
**Heterozygous duplication on FGFR1 gene.
***Heterozygous deletions and duplication on KAL1 gene.

Figure 1. Duplication on FGFR1 gene in patient 52: target sequences 4440-L03826 of the 14th and 4441-L03827 of the 18th exons.
Figure 2. Heterozygous deletion on KAL1 gene in patient 29: target sequence 5941-L05940 of the 9th exon.

Figure 3. Heterozygous deletion on KAL1 gene in patient 81: target sequences 5941-L05940 of the 9th and 4427-L03813 of the 11th exons.

Figure 4. Heterozygous deletion on KAL1 gene in patient 84: target sequence 5941-L05940 of 9th exon.
When the genes are considered separately, KAL1 genomic rearrangements were found in 3/86 (3.4%) patients with IHH, in 0/76 (0%) patients with nIHH, and in 3/10 (30%) patients with KS; FGFR1 genomic rearrangements were found in 1/86 (1.1%) patients with IHH, in 1/76 (1.3%) patients with nIHH, and in 0/10 (0%) patients with KS.

4. Discussion

Hypogonadism is a condition resulting from deficient gonadal functions, with high or low serum gonadotropin levels due to several reasons. Primary gonadal deficiencies or pituitary defects may be the causes of this disorder (1). IHH has been known as a hereditary disease, but studies pointing towards mutations in 30% of cases lead to a newly emerging genetic basis of the disease and suggest other genes to be discovered. KS is a congenital form of IHH and these patients have absent (anosmia) or diminished (hyposmia) sense of smell (2). The pathogenetic mechanism depends on the aplasia or hypoplasia of the olfactory tracts and bulbs (3). A GnRH deficiency leads to hypogonadism (4), which most probably results from a failure of the embryonic migration of neuroendocrine GnRH cells (5). During fetal development, these cells migrate from the olfactory epithelium to the hypothalamus (6) and this neuronal movement is modulated by anosmin, a 680-amino acid neural cell adhesion molecule encoded by KAL1 (7). Patients with KS may present with midline craniofacial abnormalities (like cleft palate, cleft lip, or choanal atresia), involuntary upper limb mirror movements (bimanual synkinesis), dental or renal agenesis, sensorineural deafness, oculomotor deficits, and other less-documented developmental disorders (8). KS is a genetically heterogeneous disease and males predominantly have this disorder. The vast majority of cases are sporadic, but recessive-X-linked, autosomal dominant and autosomal recessive modes of inheritance have also been described, and several loss-of-function mutations in different genes have been identified in this disorder: 1) KAL1 gene mutations on chromosome Xp22.3, responsible for the X-linked form (9); 2) FGFR1 (KAL2) gene mutations on chromosome 8p11.2-12; 3) GPR54 gene mutations on chromosome 19p13; and 4) mutations in NELF genes on chromosomes 9q34.3. These genes are all responsible for the autosomal recessive forms of the disease (10).

In apparent sporadic forms of IHH, with or without anosmia, the prevalence of KAL1 mutations has been variably reported to range from 3.1% to 27.8% (11,12). Bhagavath et al. reported that the overall KAL1 mutation prevalence might not be that much higher than previously estimated. The prevalence reported in 138 IHH patients (109 males and 29 females) was 3.7% for normosmic males, 6.3% for anosmic/hyposmic males, and no mutation was found in female patients (13). In the present study, 3 of 86 IHH patients (76 with nIHH and 10 with KS) had a heterozygous deletion in exon 9 of the KAL1 gene (probe target sequence: 5941-L05940) and 1 of these patients also carried a duplication in exon 11 of the same gene (probe target sequence: 4427-L03813). The prevalence of KAL1 gene mutations was found to be 3.4% among the patient group (3.9% among the patients with nIHH and 30% among the patients with KS). This mutation frequency is comparable to that reported by Bhagavath et al.

In KS, FGFR1 mutation is often due to an autosomal dominant transition pattern, and hypogonadism may additionally be associated with cleft palate and dental agenesis. The degree of hypogonadism and associated anomalies depends on the incomplete penetration. Different from KAL1 mutations, FGFR1 mutations have been identified in patients with hypogonadism that is not accompanied by anosmia. The genomic defects in patients with KS are due to tyrosine kinase or single amino acid change mutations occurring in an immunoglobulin-like ring. Asp224His and Gly237Asp changes were detected in the second immunoglobulin-like ring, and Tyr339Cys, Gln274Gly, and Ser346Cys were changes observed in the third ring. Gly703Arg, Asn724Lys, and Pro722Ser changes were detected in the tyrosine kinase region. These mutations reduce the activity of tyrosine kinase and receptor affinity, lead to retention of proteins within the endoplasmic reticulum, and reveal the pathology. This study examined in detail the FGFR1 copy number variations in hypogonadal patients and their families. When the study results are evaluated, the same FGFR1 defect is reported to lead to a heterogeneous picture with varying degrees of pubertal development in patients and their family members. This broad spectrum suggests that many anomalies can be detected in endogenous GnRH secretion. Genetic defects in the FGFR1 may take a role in a wide range of disorders, such as delayed puberty or hypothalamic amenorrhea. In this study, the close relationship between IHH and dental anomalies or cleft palate was confirmed in the patient with FGFR1 duplications, suggesting that FGFR1 plays a key role in the development of teeth and palate.

Raivo et al. investigated 134 IHH (22 females, 112 males) patients with a normal sense of smell and genotype–phenotype relationship in terms of FGFR1 gene mutation frequency, and they evaluated the sequence analysis of the mentioned gene (14). Heterozygous mutations in the FGFR1 gene occurred in 9 IHH patients (5 men and 4 women, 7%), and a wide range of clinical features from lack of pubertal development to reversible IHH was observed in both male and female patients. An important result of this study was the higher rate of FGFR1 mutations in the hypogonadal females.

In the present study, 86 HH patients were scanned and only 1 patient (numbered 52) had FGFR1 genomic
rearrangement. There was a duplication on the FGFR1 gene in target sequences 4440-L03826 of the 14th exon and 4441-L03827 of the 18th exon. KS cases due to mutations of FGFR1 have autosomal dominant penetration, and it is detected in about 10% of cases. However, our evaluation of the patient's family tree failed to show another case with hypogonadism; the patient was also found to have nIHH. Pathologies such as renal agenesis and synkinesis are observed more frequently in cases of KAL1 mutations than in the genetic form connected to FGFR1. In concordance, there was no phenotypic abnormality in our patient associated with FGFR1 gene defect. Understanding the role of the FGFR1 gene in the human reproductive system necessitates screening more patients with IHH.

The most commonly used smell identification test in the literature is the University of Pennsylvania Smell Identification Test (UPSIT) (15). Clinical assessment of olfactory function in the study population was achieved qualitatively by asking the subjects to recognize strong smells such as coffee, onion, and garlic. Since it is the most commonly preferred imaging technique, the olfactory tracts and bulbs of the patients were examined by MRI. However, MRI images of the olfactory pathways revealed no abnormalities in hypogonadal patients, including those with KS. Genetic defects in KS patients were sporadic, and therefore they had no abnormalities on MRI scans.

The GPR54 gene is also known as AOXR12 or HOT7T175. It encodes a G-protein-coupled heptahelical receptor that contains 398 amino acids and shows homology with the galanin and opioid receptor family (16). De Roux et al. reported that the GPR54 gene is localized in 19p13 and described a deletion in the GPR54 gene in 5 siblings (4 males, 1 female) with a history of IHH. The deletion was due to loss of receptor function as a result of a 155-bp deletion covering the intron 4 3’ and the end of exon 5 to 5’ (17).

Tenenbaum-Rakover et al. reported a homozygous mutation (T → C transition) in patients with IHH. This homozygous mutation results in a change of alanine to threonine in the 15th exon (21). Layman et al. investigated 54 IHH/KS patients in terms of KAL1 gene deletions and 100 IHH/KS patients in terms of FGFR1, GNRH1, GNRHR, GPR54, and NELF gene deletions using the MLPA kit. KAL1 deletions were presented in 7.4% of all patients, and when only the patients with KS were considered the percentage increased to 12.1%. Interestingly, no deletion in FGFR1, GNRH1, GNRHR, GPR54, and NELF genes was detected in the 100 IHH/KS patients (22). In our study, 10 KS and 76 IHH patients were screened in terms of NELF gene and no genomic rearrangement was found in the mentioned gene.

MLPA is a new technique and is used effectively in detecting genomic sequence deletion and duplications (23). Most of the prevalence studies have used PCR-based DNA sequencing for detecting point mutations and small deletions or duplications (23). KAL1 gene deletions were identified in 4 (7.4%) of 54 patients with IHH (14 with nIHH and 40 with KS) by Pedersen-White et al., which is nearly a similar percentage reported by previous studies based on the traditional PCR-based sequencing methods (23).

In conclusion, numerous genes involved in the maturity of the hypothalamic–pituitary–gonadal axis have been identified within the last few years. However, defects in these genes account for a small minority of cases. These data suggest that additional genes may play an important role in the pathogenesis of IHH. Further studies of genes that regulate hypothalamic–pituitary–gonadal development and functions are needed to clarify the additional genetic defects.

Finally, MLPA is advantageous over the traditional techniques because of its simplicity, relatively low cost, and efficiency for identifying genomic deletions and duplications.

Acknowledgments
The study was supported by the Gülhane Military Medical Academy Scientific Research Fund (Project No: AR-2008/69).
References