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Large deletion in the *KAL1* gene in two related patients with hypogonadotropic hypogonadism: diagnostic usefulness of cytogenetic and molecular methods

Rozległa delecja w genie *KAL1* u dwóch spokrewnionych pacjentów z hipogonadyzmem hipogonadotropowym: użyteczność diagnostyczna technik cytogenetycznych i molekularnych

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Abstract

Background: Kallmann syndrome type 1 (KS1) is a heterogeneous disorder where hypogonadotropic hypogonadism (HH) associated with an impaired sense of smell is observed. The aim of this study was to investigate the usefulness of the multiplex ligation-dependent probe amplification (MLPA) technique for differential diagnosis in comparison with molecular cytogenetics — fluorescence *in situ* hybridisation (FISH) or traditional PCR analysis and propose a diagnostic approach for patients with KS.

Material and methods: Karyotype and PCR analysis in two related patients and other family members were performed, followed by MLPA dosage sensitive analysis.

Results: In the proband and his maternal uncle, the PCR allowed the detection of a large deletion within the *KAL1* gene, from exon 4 to 14 (c.469-?_6314+?del). The deletion was also diagnosed in three female carriers in the presented family. These results were proved by the MLPA technique. Moreover, we traced the presence of the region located downstream and upstream to the *KAL1* gene on Xq22.32. However, FISH analysis failed to reveal any deletion in the critical region for KS. Simultaneously, we report difficulties connected with the PCR technique based on the primers for *KAL1* amplification presented in the literature. We designed primers that are specific to the X chromosome and bypass pseudogene *KALY* amplification.

Conclusions: FISH analysis is a convenient screening technique, but in the presented family it failed to detect the deletion. Therefore, in the face of a distinctive manifestation of KS, a subsequent molecular assay should be introduced. The MLPA is a useful technique for differential diagnosis in patients with HH combined with smell impairment. **(Pol J Endocrinol 2011; 62 (3): 224–229)**

Key words: *hypogonadotropic hypogonadism (HH), Kallmann syndrome (KS),* KAL1 *gene mutation, FISH, MLPA*

Streszczenie

Wstęp: Zespół Kallmanna typu 1 (ZK1) jest zaburzeniem heterogennym, w którym cechy hipogonadyzmu hipogonadotropowego mogą współistnieć z upośledzeniem węchu. Choroba ta może występować sporadycznie lub rodzinnie. Celem pracy była ocena użyteczności metody amplifikacji multipleks zależnej od ligacji sond (MLPA) w porównaniu z technikami cytogenetyki molekularnej i PCR w diagnostyce różnicowej ZK oraz zaproponowanie schematu postępowania diagnostycznego w jego przypadkach. Wczesne rozpoznanie ZK umożliwia leczenie objawowe z korzystnym efektem dla rozwoju cech płciowych i płodności u części chorych.

Materiał i metody: Badania przeprowadzono u 2 spokrewnionych chorych i członków ich rodziny. Oceniano kariotyp za pomocą technik cytogenetyki klasycznej i molekularnej — fluoroscencyjnej hybrydyzacji *in situ* (FISH). W badaniach DNA wykorzystano PCR, a następnie MLPA pod kątem jego analizy jakościowej i ilościowej.

Wyniki: U probanta i jego wuja za pomocą PCR wykryto delecję obejmującą eksony od 4 do 14 (c.469-?_6314+?del) genu *KAL1*. U trzech kobiet z rodziny pacjentów stwierdzono nosicielstwo tej delecji. Nie uwidoczniono jej za pomocą FISH z sondą specyficzną dla regionu krytycznego obejmującego *KAL1*. Obecność delecji potwierdzono, stosując MLPA. Udokumentowano jednocześnie obecność sekwencji położonych poniżej i powyżej genu *KAL1* na wysokości prążka Xq22.32. W pracy zilustrowano również trudności związane z amplifikacją *KAL1* przy zastosowaniu starterów opisanych w literaturze. Zaprojektowano własne sekwencje starterów, wykluczające jednoczesną amplifikację pseudogenu *KALY*.

Wnioski: Metoda FISH jest dogodną techniką przesiewową w diagnostyce mikrodelecji, jednak w przypadku prezentowanej w pracy rodzinie nie pozwoliła na wykrycie delecji wewnątrzgenowej. U chorych z objawami odpowiadającymi ZK należy zatem w podobnych przypadkach zastosować inne metody analizy molekularnej. Potwierdzono użyteczność techniki MLPA w diagnostyce różnicowej pacjentów z HH i zaburzeniami węchu. **(Endokrynol Pol 2011; 62 (3): 224–229)**

Słowa kluczowe: *hipogonadyzm hipogonadotropowy (HH), zespół Kallmanna (ZK), mutacja genu* KAL1*, FISH, MLPA*

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Introduction

Kallmann syndrome is a clinically and genetically heterogenous disease that presents a diverse phenotype spectrum. However, hypogonadotropic hypogonadism (HH) associated with an impaired sense of smell, either partial (hyposmia) or total (anosmia) is the most frequent feature. The prevalence of the disease has been estimated at 1/8,000 in males and 1/40,000 in females, although it might have been underestimated, especially in women [1]. The physiological base for HH is specified and localised in detail, unlike the molecular bases which are still under investigation. The clinical phenotype of HH includes a wide range of symptoms ranging from absolute default of sexual maturation with subsequent infertility, to partial puberty and an almost normal testicular size with a eunuchoidal body habitus. Hypogonadotropic hypogonadism (secondary hypogonadism) is the result of an isolated deficiency of gonadotropin-releasing hormone from the hypothalamus with pubertal delay and subsequent infertility. Some of the symptoms can be diminished by hormone replacement therapy with a subsequent improvement in the patient's physical and psychological condition. Early diagnosis can lead to endogenous sex-steroid secretion and even reproduction [2].

The following three modes of Kallmann syndrome inheritance have been described: X-chromosome linked, autosomal dominant, and autosomal recessive; nevertheless sporadic cases are most frequent [1–5]. Six causative genes responsible for KS manifestation have been found to date: KS 1 (mutations within *KAL1*), KS 2 (*FGFR1*), KS 3 (*PROKR2*), KS 4 (*PROK2*), KS 5 (*CHD7*), and KS 6 (*FGF8*). These are presented with their chromosomal position and a short clinical description in Table I.

However, it is believed that mutations in these genes account for only 30% of all KS cases [3]. The explanation behind the connection of hypogonadotropic hypogonadism and anosmia in KS 1 is the common origin of GnRH neurons and mitral cells (which produce the *KAL1* gene product — anosmin-1) from the olfactory placode. Anosmin-1 plays an essential role during foetal development in neuronal migration from the olfactory tract through the cribriform plate and the forebrain until it reaches the hypothalamus [6–9]. A mutation in the *KAL1* gene, therefore, manifests itself as hypogonadotropic hypogonadism combined with lack of smell (anosmia).

Among the causes of X chromosome-linked Kallmann syndrome, translocations and deletions within the fragile critical Xp22 region have been described. *KAL1* gene mutations include missense and nonsense mutation, splice site mutations, point (a single base) deletions, intragenic insertions and deletions, as well as submicroscopic chromosomal deletions involving the entire *KAL1* gene [10]. Here, we report the case of a deletion of exons 4 to 14 (c.469-?_6314+?del) within the *KAL1* gene in two related patients and in three female carriers among the members of the presented family.

Materials and methods

The study group

The patients and their four relatives included in the study gave their written consent for participation in the genetic analysis (the medical research being scheduled in accord-

Table I. ***Types of Kallmann syndrome (KS). Elaboration based on 'Online Mendelian Inheritance in Man' (OMIM) from database of National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/omim)***

Tabela I. ***Typy zespołu Kallmanna (ZK). Opracowano na podstawie bazy „Online Mendelian Inheritance in Man" (OMIM) dostępnej na stronie*** **National Center for Biotechnology Information** ***(www.ncbi.nlm.nih.gov/omim)***

Kallmann syndrome type	OMIM	Gene map locus	Gene	Most distinctive features apart from hypogonadotropic hypogonadism
Kallmann syndrome type 1	308700	Xp22.32	*KAL1*	Anosmia/hyposmia, bimanual synkinesia (mirror movements), renal agenesis, pes cavus, high-arched palate, and cerebellar ataxia. Rarely, colour blindness
Kallmann syndrome type 2	147950	8p11.2-p11.1	*FGFR1*	Anosmia/hyposmia. Midline cranial anomalies (cleft lip, cleft palate and imperfect fusion)
Kallmann syndrome type 3	244200	20p13	*PROKR2*	Wide spectrum of olfactory and reproductive dysfunction without any distinctive manifestations
Kallmann syndrome type 4	610628	3p21.1	*PROK2*	Wide spectrum of olfactory and reproductive dysfunction without any distinctive manifestations
Kallmann syndrome type 5	612370	8q12.1	*CHD7*	Cleft lip and palate. Hearing disorders. Clinical manifestations reflecting CHARGE syndrome
Kallmann syndrome type 6	612702	10q24	*FGF8*	Varied degrees of gonadotropin-releasing hormone deficiency and olfactory function disability

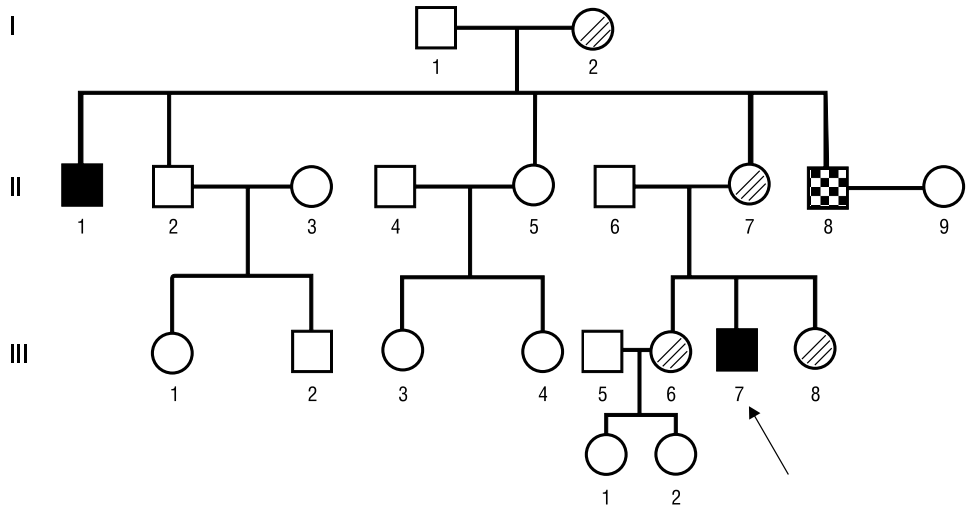

Figure 1. *The pedigree of the described family. The proband LG471 is indicated with an arrow (position III-7). LG471 and ZL567 (II-1) are the carriers of the detected KAL1 gene deletion (black square). Hemizygous female carriers (half-hatched circle) are: the proband's mother II-7, and sisters: III-6 and III-8. Proband's grandmother (I-2) deceased, but from the pedigree analysis we may infer that she was also the deletion carrier. II-8 — suspicion of KS (more details in text)*

Rycina 1. *Rodowód badanej rodziny. Probant LG471 wskazany jest strzałką (pozycja III-7). Pacjenci (czarne kwadraty) LG471 oraz ZL567 (II-1) są nosicielami wykrytej delecji w genie KAL1. Hemizygotycznymi nosicielkami żeńskimi (oznaczonymi zakreskowanym do połowy kołem) są: matka probanta (II-7) oraz siostry: II-6 i III-8. Babka probanta (I-2) zmarła, jednakże na podstawie analizy rodowodu można wnioskować, że również była nosicielką wykrytej delecji. II-8 — podejrzenie ZK (więcej szczegółów w tekście)*

ance with the World Medical Association Declaration of Helsinki). The family pedigree is presented in Figure 1.

The proband LG471 (position III–7; Figure 1) is the second child born to healthy, non-consanguineous parents. He was admitted to the Department of Endocrinology and Metabolic Diseases, as well as to the genetic counselling centre, on account of delayed puberty and hypogonadism symptoms. His height was 181 cm and his weight was 87 kg. A eunuchoid profile, slight gynecomastia, small penis and scrotal testes with the dimensions: right — 19 × 12 × 11 mm and left – 20 × 12 × 12 mm and slightly enlargement nipples were noted. On examination, involuntary upper limb mirror movements were observed. The patient declared an impairment of smell, but no olfactory tests were formally performed. Serum levels of hormones were as following: LH — 0.217 U/l [normal range: 1.7–8.6 IU/l], FSH — 0.718 IU/l [normal range: 1–14 IU/l], estradiol — 16.88 pg/ml [normal range: 7.63–42.6 pg/ml] and testosterone — 0.292 ng/ml [normal range 2.8–8.0 ng/ml]. Magnetic resonance imaging of the pituitary gland revealed normal homogeneous structure of the parenchyma with an intact hypothalamic infundibulum. Cytogenetic analysis revealed a 46,XY karyotype.

The proband's maternal uncle ZL567 (II-1) was admitted to the Department of Genetics after his nephew was diagnosed with Kallmann syndrome type 1, which aroused suspicion of familial expression of the disease. Hormonal evaluation showed low testosterone level [0.322 ng/ml] and LH level [< 0.100 IU/l]. The patient was diagnosed with chronic arthritis of the femoral joint. He is unmarried and has no children. Cytogenetic analysis showed a normal 46,XY karyotype.

The other maternal uncle (II-8) of proband LD471 objected to genetic counselling. Historic data suggests he is also a carrier of the detected deletion. He is married but has no children, only an adopted one.

Cytogenetic evaluation and fluorescence in situ *hybridisation (FISH)*

The karyotype of peripheral blood lymphocytes (leucocytes) was analysed, applying standard culture techniques and GTG-banding of the chromosomes. The FISH was carried out on lymphocyte metaphase spreads with LSI Kallmann's (KAL Spectrum Orange/CEPX) Region Probe (Vysis, Abbott Molecular, USA) and *DXZ1* (spectrum Green) probe for the X-centromere as internal signal control following the manufacturer's protocol.

Genomic DNA extraction

Genomic DNA was isolated from 5 ml of the whole peripheral blood by means of the QIAamp DNA blood mini kit (Qiagen, USA), according to the enclosed protocol. Additionally, genomic DNA from a healthy male was isolated as a control.

PCR amplification

PCR amplification (reaction profile and primers sequences) conditions were as published by Hardelin et

al. with modification [11] but the primers for exons 1, 11, 12 and 14 were designed individually.

These are: exon 1, **F:**AGGTCGGCGAGGAGGGTC and **R:**GGAAGAGGCTGGAAAGGAGAGTT; exon 11, **F:**GCATATCCCGCAGCCATG and **R:**TCCTACATCTCTTCCCTCCACAT; exon 12, **F:**ATCCCTTGGGCACACCTTCTC and **R:**ACTCAATAGTGCAGCACACAGAAT; exon 14, **F:**AAAGGAGAGAAAGAGTGTGGTTGTA and **R:**GCTTGTAGGGAACTGGTGTCTG.

PCR reactions were carried out in a total reaction mixture volume of 25 μl, containing 1 μg of genomic DNA [concentration 10–50 ng/μl], 1 unit FastStart Taq DNA Polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 100 pM of the primers. PCR amplifications were performed for 32 cycles on a BioRad C1000 Thermal Cycler. The PCR products were visualised on 2% agarose gel with ethidium bromide addition and registered by means of the Photodocumentation System DP.-001.FDC (Vilber Lourmat, France).

Multiplex Ligation-dependent Probe Amplification analysis (MLPA)

Gene dosage analysis was performed using the SALSA® MLPA kit Kallmann-1 P132 (MRC-Holland, the Netherlands). The kit includes 16 probes that attach to *KAL1* gene sequence on chromosome Xp22.32 (one probe per exons 2 to 13, two probes per exons 1 and 14) and 17 control probes that hybridise to sequences located upstream and downstream, according to *KAL1* locus. The assay was performed according to the manufacturer's manual. After the PCR, amplicons were run on an ABI 3130xl Genetic Analyser (Applied Biosystems, USA) using ROX 500 size standard. Analysis of obtained peaks (measurement of each peak area) was performed by GeneMarker software V 1.90 (SoftGenetics LLC, USA) and normalised by dividing an individual peak by the combined area of all peaks in the lane. The patients' dosage histograms and ratio plot were compared to those obtained from healthy individuals by visual examination. Simultaneously, any visual disagreements were confirmed by information displayed in the report table (where peak ratio or simple loss/gain information was presented). Any minimum dosage quotient beyond the range < 0.75 or > 1.30 was considered abnormal and corresponded to deletion and duplication, respectively. Whenever we found a sample with an abnormal dosage quotient, the sample was re-analysed by the MLPA. Only the samples showing consistent results between the two tests were taken into consideration.

Results

To the assay, we subjected the proband LG471 (III-7) and his relatives: mother (II-7), father (II-6), two sisters (III-6; III-8) and uncle ZL567 (II-1). The molecular analysis of the proband LG471 and maternal uncle ZL567 by means of the PCR showed a large deletion within *KAL1* (Figure 2) that encompasses exons from 4 to 14 (c.469-?_6314+?del) and does not extend beyond *KAL1* since we detected signals for *STS* and *HDHD1* genes (both are on Xp22.32) located downstream to the *KAL1* gene. However, the FISH analysis with the use of a specific probe, LSI KAL1 that hybridises to the *KAL1* gene, failed to confirm this deletion (data not shown).

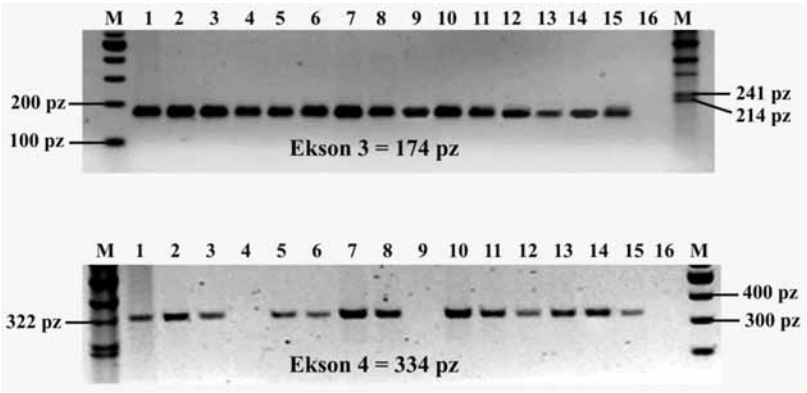

Figure 2. *Examples of electrophoregrams of amplification products obtained from PCR with the use of primers that are complementary to the exons 3 and 4 of the* KAL1 *gene. M: size marker; 1: positive control (healthy male); 2–15: patients' samples; 4: proband (LG471; III-7); 5: proband's sister (III-6); 6: proband's sister (III-8); 7: proband's mother (II-7); 8: proband's father (II-6); 9: proband's uncle (ZL567; II-1); 16: negative control (PCR reagents without DNA template). Absence of the amplification products in lanes 4 (proband) and 9 (proband's uncle) for exon 4 is observed. The same results for exons 5–14 were obtained in both patients*

Rycina 2. *Przykładowe elektroforegramy produktów PCR uzyskanych przy użyciu starterów komplementarnych do sekwencji 3. i 4. eksonu genu* KAL1. *M: marker wielkości; 1: kontrola pozytywna (zdrowy mężczyzna); 2–15: próbki pacjentów; 4: probant (LG471; III-7); 5: siostra probanta (III-6); 6: siostra probanta (III-8); 7: matka probanta (II-7); 8: ojciec probanta (II-6); 9: wuj probanta (ZL567; II-1); 16: kontrola negatywna (odczynniki PCR bez DNA matrycowego). Takie same wyniki u obu pacjentów otrzymano dla eksonów 5–14*

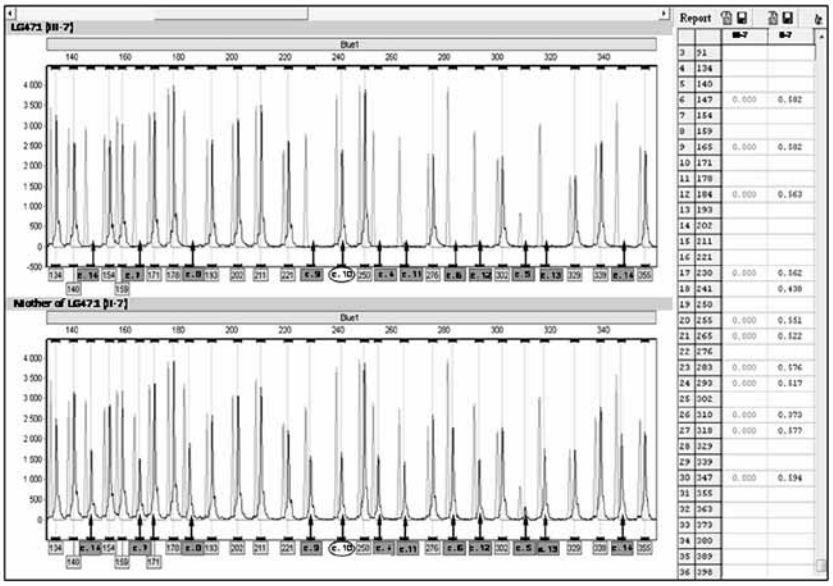

Figure 3. *Electrophoregram illustrating deletion of* KAL1 *detected by means of the MLPA. Multi-exon deletion extending from exon 4 to 14 (c.469-?_6314+?del) is indicated in the light grey boxes connected with black arrows showing absence (in the proband LG471; III-7) or lower peaks (in the proband's mother II-7) of KAL1 probes (dark grey) compared to the control peaks (light red). On the right in the Report Table, loss information is displayed together with the probe number and the basepair size of that probe (ranging from 91 to 398 bp). Probe size corresponds as follows: 147 — exon 14; 165 — exon 7; 184 — exon 8; 230 — exon 9; 241 — exon 10; 255 — exon 4; 265 — exon 11; 283 — exon 6; 293 — exon 12; 310 — exon 5; 318 — exon 11; 347 — exon 14. Note: probe size 241 (indicated in the ellipse) is not reliable according to the manufacturer's notes, since a signal via this probe might be also obtained on a chromosome Y sequence*

Rycina 3. *Elektroferogram przedstawiający delecję w* KAL1 *wykrytą za pomocą techniki MLPA. Wieloeksonową delecję obejmującą eksony od 4. od 14. (c.469-?_6314+?del) zaznaczono jasnoszarymi prostokątami ze strzałkami wskazującymi brak ciemnoszarych pików (probant LG471; III-7) lub obniżone piki (u matki probanta; II-7) dla sond genu KAL1, w odniesieniu do referencyjnych — jasnoszarych pików. Po prawej stronie ryciny w „Report table" przedstawiono wartość ubytku materiału genetycznego w próbie wraz z przyporządkowanym numerem sondy (który jednocześnie informuje o wielkości produktu). Wielkości sond odpowiadają następującym eksonom: 147 — ekson 14.; 165 — ekson 7.; 184 ekson — 8.; 230 — ekson 9.; 241 — ekson 10.; 255 — ekson 4.; 265 — ekson 11.; 283 — ekson 6.; 293 — ekson 12.; 310 — ekson 5.; 318 — ekson 11.; 347 — ekson 14. Sygnał dla sondy 241 (zaznaczonej elipsą) zgodnie z informacją podaną przez producenta zestawu nie jest wiarygodny, gdyż umożliwia również amplifikację odcinka pseudogenu chromosomu Y*

In further analysis, the MLPA technique with the use of the Kallmann 1 P132 kit confirmed the obtained PCR results. Moreover, thanks to a dosage-sensitive MLPA application, we detected carrier status in the proband's mother (II-7) as well as in his two sisters (III-6 and III-8) who possessed a hemizygous *KAL1* gene and did not manifest any signs of Kallmann syndrome (Figure 3).

In this paper, we substantiate a family's *KAL1* gene transmission with the same deletion which does not affect female fecundity within this family (on mother II-7, and sisters: III-6 and III-8). We observed that FISH analysis for the critical region in the absence of additional clinical manifestations for contiguous gene syndrome (CGD) is not an effective technique. It is essential to perform a complete diagnostic procedure in order to detect this rearrangement.

Discussion

Since the genetic ground of KS is very heterogeneous, a differential clinical picture of the disease can be observed. Phenotype-genotype correlation analyses of the disease show that the manifestation of KS is determined by alternative factors other than the mutation itself. These factors may encompass modifier genes, epigenetic and environmental factors.

Many cases are clearly familial with X-chromosome linked, autosomal dominant and autosomal recessive inheritance, but most cases are sporadic. The molecular analysis of KS to date has included mutations of the: *KAL1*, *FGRF1*, *PROKR2*, *PROK2*, *CHD7* and *FGF8* genes, which accounts for 30% of all KS cases, suggesting that there might be more genes involved in the development of the disease, or that the disease escapes the monogenic Mendelian heritable pattern.

Moreover, it seems that mutations in some genes show incomplete penetrance [2, 11–13]. In their extensive report concerning the complex genetics of IHH (idiopathic hypogonadotropic hypogonadism), Pitteloud et al. [12] argued that oligogenicity might be responsible for monogenic IHH disorders presenting variable phenotypes within and between families. The majority of *PROK2* and

PROKR2 mutations are monoallelic, but there are some cases where mutations on both alleles of either gene are detected [13]. Additionally, male patients with detected biallelic mutations in *PROK2* or *PROKR2* suffer from more severe disorders of the reproductive system [13]. Hardelin and Dode [3] recognized KS as the result of insufficient cell signalling through FGFR or PROKR2, therefore the mutation within *FGFR1* and *PROK2* disrupts the normal embryonic development (FGF signalling, fibroblast growth factor signalling) and the integration of intracellular signalling (prokinecitin signalling). Anosmin-1 (the product of *KAL1*) acts as an enhancer of FGF and, probably, of prokinecitin signalling as well.

Kallmann syndrome type 1 rarely arises from a deletion which encompasses only the *KAL1* gene (Xp22.32). The majority of mutations in KS 1 are point mutations and small deletions or duplications (1–2 exons). Large deletions are rare, and only a very few gross deletions have been reported due to the difficulty in detecting them using a PCR-based DNA sequencing [14].

We have managed to detect a large deletion that does not exceed the *KAL1* gene sequence and includes exons from 4 to 14 (c.469-?_6314+?del). Despite the magnitude of this deletion, FISH studies with DNA probes specific to the *KAL1* gene sequence did not detect it. Probably the rearrangement in the critical region must be much larger, and exceed the *KAL1* gene, to be detected by the FISH technique. Mutated *KAL1* gene participation in males in contiguous gene syndrome (CGS), including the loss of genes responsible for Leri-Weill syndrome and some cases of short stature, chondrodysplasia punctata, Duchenne dystrophy, ichthyosis, mental retardation or ocular albinism, is more common [2, 5].

Clinical features of KS in CGS apply on a more complex phenotype which is a combination of distinctive features for each disease entity. A diagnosis based solely on the PCR technique and sequencing data might be misleading, since the *KAL1* gene has its pseudogene *KALY* located on chromosome Y. Some amplicons obtained via the PCR may originate from *KALY* amplification, and sequencing analysis in males can be difficult to interpret [15, 16].

Conclusions

Screening for KS in patients with HH, especially if the condition is accompanied by anosmia/hyposmia, is crucial in making an accurate diagnosis. Concurrently, an early diagnosis of KS, symptomatic treatment and regular follow-up are beneficial for patients in order to prevent them from pernicious sequelae and improve their sexual development. Several molecular techniques should be applied to diagnose the rearrangements in the KS critical region. FISH failed to detect in this family the large intragenic deletion. MLPA seems to be a useful, rapid technique for differential diagnosis in patients with HH combined with smell impairment.

Conflict of interests

The authors declare that they have no competing interests. All authors read and approved the final manuscript.

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