

## Study on KAL1 Gene Mutations in Idiopathic Hypogonadotropic Hypogonadism Patients with X-Linked Recessive Inheritance

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Idiopathic hypogonadotropic hypogonadism (IHH) is a condition caused by low doses of hypothalamic gonadotropin-releasing hormone (GnRH) leading to absence or incomplete sexual maturation. One of the disorders leading to IHH is Kallmann syndrome which is characterized by GnRH deficiency with anosmia or hyposmia. This disorder generally occurs as a hereditary syndrome with X-linked recessive inheritance pattern. However, autosomal dominant or recessive and sporadic cases have also been reported. *KAL1* is the most common mutated gene among these patients. The aim of this study was to determine the mutation spectrum of *KAL1* gene in twenty patients. *KAL1* exons were amplified by PCR method and the products were assessed by high resolution melting (HRM) technique. In addition, for one of the patients, all coding exons of the *KAL1* gene were sequenced. Deletion of exons 4, 5 and 6 were evident in 5%, 10%, and 10% of patients, respectively. Furthermore, HRM results showed hemizygous mutation of exon 12 with more than 95% probability in 25% of patients. Finding these mutations could be helpful in the early diagnosis and presymptomatic treatment of Kallman syndrome.

**Key words:** Idiopathic hypogonadotropic hypogonadism, kallmann syndrome, *KAL1* gene, X-linked recessive, GnRH

Idiopathic hypogonadotropic hypogonadism (IHH) is characterized by delayed sexual development as well as infertility caused by gonadotropin-releasing hormone (GnRH) production deficiency from the hypothalamus (1, 2). IHH affects about 1 per 10,000 male and 1 in 50,000 female (1).

Hypogonadotropic hypogonadism (HH) can occur as either acquired or congenital form.

Acquired HH may be caused by a variety of environmental factors such as drugs (for example: sex steroids and gonadotropin-releasing hormone analogues), head trauma, infiltrative or infectious pituitary lesions, endocrinopathies such as hyperprolactinemia, pituitary or brain radiation, exhausting exercise, and alcohol abuse.

The congenital HH is divided into two subtypes based on the presence of the sense of

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smell, which consist of congenital normosmic isolated HH (IHH) and anosmic HH or Kallmann syndrome (KS) (3).

KS may have a wide variety of clinical manifestations such as underdeveloped or absent olfactory bulbs, optic atrophy, deafness, cleft lip, renal malformations, cryptorchidism and neurological anomalies (4). KS constitutes approximately two-thirds of congenital HHs (3). This syndrome shows a wide genetic heterogeneity since various modes of inheritance, including: X-linked recessive, autosomal recessive, autosomal dominant as well as sporadic condition have been observed (5). Several genes have so far been shown to be involved in disease occurrence including *KAL1*, *FGF8*, *FGFR1*, *PROK2*, *PROKR2* and *WDR11* (4, 6). The criteria for the diagnosis of KS include absent or incomplete sexual puberty (hypogonadism), anosmia or hyposmia, infertility (almost all untreated patients are infertile), decreased muscle strength and diminished aggressiveness and drive (in men), lack of breast development in women, small penises (<8 cm long in adults) as well as low levels of serum testosterone (less than 100 ng/dL) and decreased prostate size in men.

*KAL1* is located at Xp23.3 and is the most common mutated gene causing KS in 10% of patients (4, 5). This gene encodes for anosmin-1 which is an embryonic component of the extracellular matrix and is involved in GnRH induced olfactory neurons migration from the olfactory placode to the hypothalamus during embryonic life (5, 7-9). Absence of olfactory nerve cells extension to the olfactory bulb will impair or suppress the sense of smell (4, 5, 10, 11). GnRH controls the production of several hormones leading to sexual development before birth and during puberty (4).

Mutations in *KAL1* also induce severe reproductive phenotypes including absent puberty

and high frequency of cryptorchidism or micropallus. The aim of this study was to investigate *KAL1* mutations among Iranian patients.

## Materials and methods

### Patients

Twenty unrelated patients including 12 females and 8 males, aged from 15 to 29 years old suffering from KS were referred to endocrine research center (MUMS) and were enrolled into this study. Patients with the existence of secondary sexual and abnormal MRI were excluded from the study.

The study design was approved by the Ethics Committee of Mashhad University of Medical Sciences and informed consent was obtained from patients.

### Molecular analysis

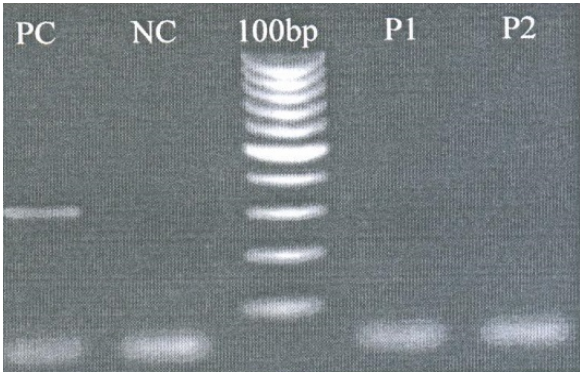
Five ml of peripheral blood was collected into EDTA containing tubes. DNA was extracted according to Enghelabifar et al. (12). Each of 14 exons of the *KAL1* gene was amplified by separate PCR reaction using specific primers listed in table 1. PCR was performed in 25 µl reaction containing 1X PCR buffer, 1-2 mM MgCl<sub>2</sub>, 0.2-1 mM of each dNTPs, 0.4 µM of each primer, 1-2.5 units of Taq DNA polymerase and 100 ng of template DNA. PCR reactions were performed in 2720 thermal cycler (Applied Biosystems, USA) with a cycling program of initial denaturation at 95 °C for 5 min followed by 30-35 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 50 s; and a final extension step at 72 °C for 5 min. PCR products were electrophoresed on 2% agarose gel and were visualized under UV light after ethidium bromide staining.

PCR products were also screened for mutation using high resolution melting (HRM) experiments using evagreen HRM mastermix. Melting analysis was performed by Q- Rotor instrument (Qiagen,

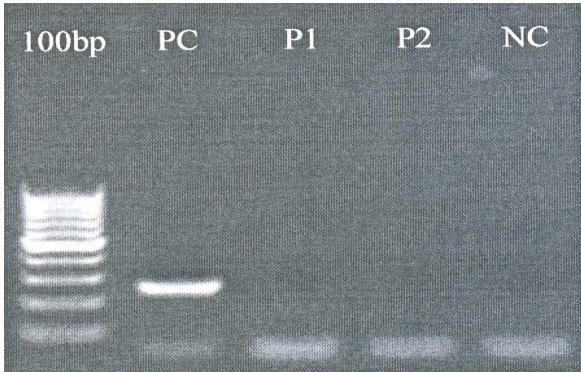
Germany) by heating samples gradually from 50 °C toward 95 °C, 0.1 °C each step.

| Table 1. PCR primer sequences |                          |                           |                   |
|-------------------------------|--------------------------|---------------------------|-------------------|
| Exon                          | Primer Sequences 5' → 3' |                           | Product size (bp) |
| EX1                           | F                        | TTGAACTTTCCGGCTCAGTC      | 363               |
|                               | R                        | GCAGCCCCAGAAAGAACC        |                   |
| EX2                           | F                        | GTGTAGCTTTCTAATGGATCA     | 218               |
|                               | R                        | ATTGGTGGAAACTGGGCATA      |                   |
| EX3                           | F                        | CAGGCATTGAAAAAGCAACA      | 225               |
|                               | R                        | TGACCCACGTAAGCATAGTC      |                   |
| EX4                           | F                        | TCAGACTTCATGTGTCTTTAATGGA | 345               |
|                               | R                        | CTCCCTAGGCACACACAGA       |                   |
| EX5                           | F                        | GTTCTTCCTCAACTTTTACTTCA   | 303               |
|                               | R                        | CAGACACTACCTCCAGGATGA     |                   |
| EX6                           | F                        | GATCCAACATAACATGTCGGAAT   | 250               |
|                               | R                        | GTGTGCCTGGTAGCAAGGAT      |                   |
| EX7                           | F                        | CAATGCTTCACGTGTTGACC      | 340               |
|                               | R                        | CCCTCTGTGGGAATAACAATC     |                   |
| EX8                           | F                        | TTGCAATGAAGATGAGAGACG     | 285               |
|                               | R                        | CTCCATTGTGCCTTGTGTG       |                   |
| EX9                           | F                        | CCATCTTGCCCAGGAATCTA      | 299               |
|                               | R                        | TGGCTTGACATTTACTTCTTCAA   |                   |
| EX10                          | F                        | TACCTGGAATGTAACATCCA      | 278               |
|                               | R                        | CTTCCATCAAGTCATTACTCC     |                   |
| EX11                          | F                        | ATCCTTGTTGGATGGAATATG     | 349               |
|                               | R                        | TGAAACGCAGTTTGACAAGG      |                   |
| EX12                          | F                        | ACACCTTCTCCAGTCGCCTA      | 319               |
|                               | R                        | AGCAGTAGATACCAATGACACA    |                   |
| EX13                          | F                        | CGGTGAGCATGCTCTTTTATG     | 232               |
|                               | R                        | TTTCTCTATGTCCACAAGACCTG   |                   |
| EX14                          | F                        | AGGAACATTTGCCAGGGTCT      | 245               |
|                               | R                        | TCTGGAAGTGTGCATGTCTC      |                   |

(F=Forward and R=Reverse)



**Fig. 1.** Representative agarose gel (2%) indicating Exon 5 deletion. Length of presented band is 303 bp. 100bp DNA size marker was used to estimate PCR product bands. PC= Positive control. NC= Negative control. P1= Patient no 1. P2= Patient no 2.



**Fig. 2.** Representative agarose gel (2%) displaying Exon 6 deletion. Length of presented band is 250 bp. 100 bp DNA size marker was used to estimate PCR product bands. PC= Positive control. NC= Negative control. P1= Patient no 1. P2= Patient no2.

Fluorescent detection lasted 10 s in each step. Data were normalized against heterozygous control samples. Samples with more than 90% probability of presence of mutation were considered as positive results.

In addition, all exons of the gene were sequenced in one patient, using Sanger dideoxy method and were analyzed through Chromas software.

## Results

### Molecular detection of exon deletions by PCR

Upon PCR analysis we detected 5 male patients with hemizygous mutation. Deletion in exon 4 (1 case), exon 5 (2 cases) (figure 1), as well as complete deletion of exon 6 (2 cases) were

detected as three distinct abnormalities (figure 2).

### High resolution melting (HRM) analysis

HRM reaction was performed for all patients using specific primers of all 14 exons. In all reactions, negative controls were applied in order to exclude any contamination. However, no point mutation was evident (figures 3, 4; table 2).

After sequencing, Chromas software allowed the detection of a hemizygous point mutation in exon 12 of *KAL1* gene causing a change from Asn (AAC) to Ile (ATC) in codon 623 (figure 5). Four patients from both genders had heterozygous or hemizygous mutation in exon 12, at this position with a probability higher than 95% according to HRM analysis (figures 6, 7; table 3)

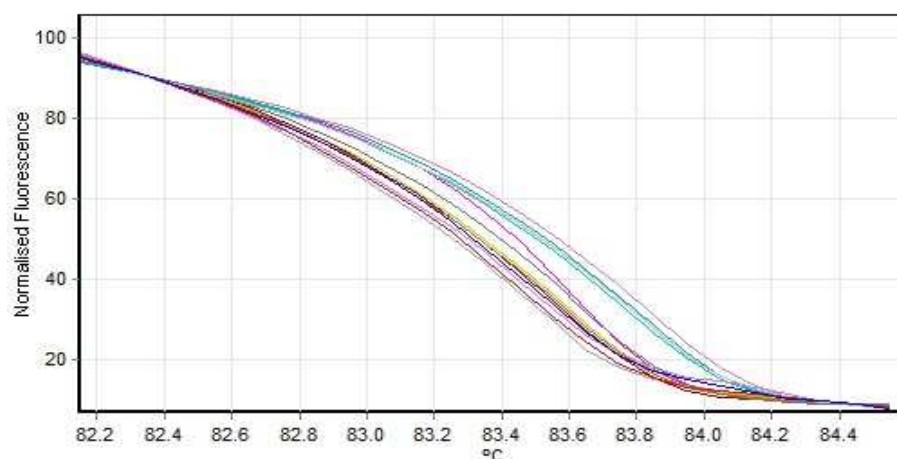


Fig. 3. Normal analysis HRM graph exon no 2.

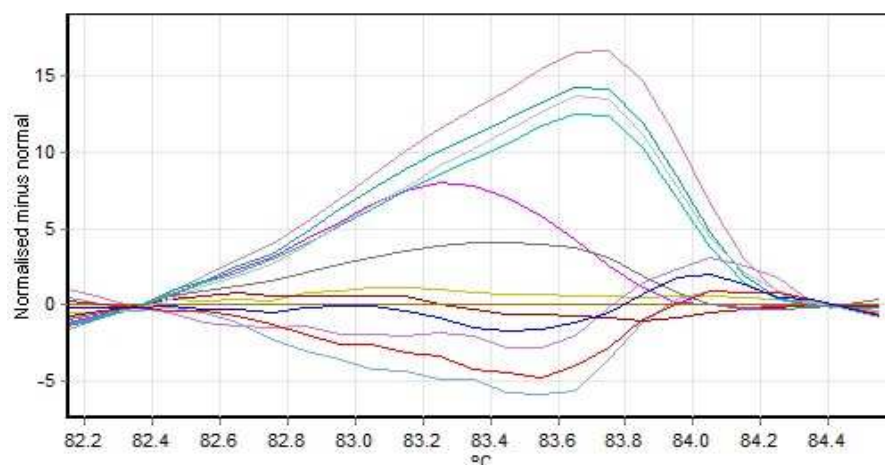


































Fig. 4. Normal graph exon no 2 (median line= no mutation, ascending line= homozygous, descending arc= heterozygous)

**Table 2.** Color, name = patient code, genotype, and confidence % of analysis of samples for exon 2.

| No | Colour  | Name | Genotype  | Confidence % |
|----|---|------|-----------|--------------|
| 2  |  | 2    | Normal    | 98.71        |
| 10 |  | 6    | Variation | 66.58        |
| 12 |  | 7    | Variation | 34.48        |
| 16 |  | 9    | Variation | 78.35        |
| 18 |  | 10   | Normal    | 91.42        |
| 19 |  | 11   | Variation | 13.92        |
| 20 |  | 12   | Normal    | 99.02        |
| 21 |  | 13   | Normal    | 100.00       |
| 23 |  | 14   | Variation | 23.96        |
| 29 |  | 17   | Variation | 29.18        |
| 31 |  | 18   | Variation | 87.55        |
| 33 |  | 19   | Variation | 87.19        |
| 35 |  | 100  | Normal    | 97.12        |

**Table 3.** Color, name = patient code, genotype, and confidence % of analysis of samples for exon 12.

| No | Colour  | Name | Genotype  | Confidence % |
|----|---|------|-----------|--------------|
| 1  |  | 101  | Variation |              |
| 2  |  | 102  | normal    | 91.32        |
| 4  |  | 103  | Variation |              |
| 6  |  | 104  | hetro     | 99.57        |
| 8  |  | 105  | hetro     | 92.36        |
| 10 |  | 106  | normal    | 94.60        |
| 12 |  | 107  | Variation |              |
| 14 |  | 108  | Variation |              |
| 16 |  | 109  | Variation |              |
| 18 |  | 110  | hetro     | 99.75        |
| 20 |  | 112  | Variation |              |
| 21 |  | 113  | hetro     | 100.00       |
| 23 |  | 114  | normal    | 100.00       |
| 25 |  | 115  | hetro     | 99.46        |
| 27 |  | 116  | Variation |              |
| 29 |  | 117  | Variation |              |
| 31 |  | 118  | Variation |              |
| 33 |  | 119  | Variation |              |
| 35 |  | 100  | Variation |              |

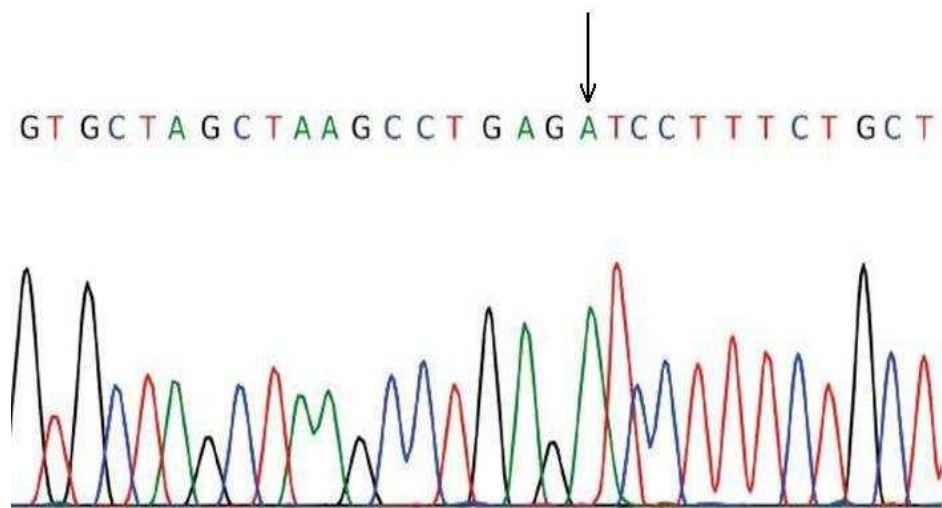


Fig. 5. Hemizygous mutation of exon 12

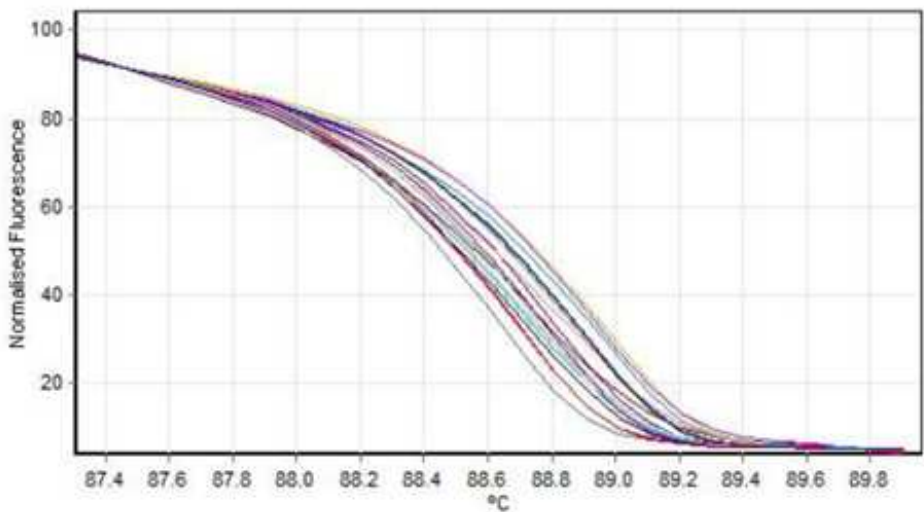


Fig. 6. heterozygote mutation of exon 12

Table 4. Mutations identified in Sato et al. study

| <i>KAL1</i> mutation | Nucleotide change  | Amino acid change | position |
|----------------------|--------------------|-------------------|----------|
| One missense         | 488G>A             | C163Y             | Exon 4   |
| Three nonsense       | 1270C>T            | R424X             | Exon 9   |
|                      | 1891C>T            | R631X             | Exon 13  |
|                      | 571C>T             | R191X             | Exon 5   |
| Three frameshift     | 100–101del CG      | ---               | Exon 1   |
|                      | 262–269delGAGCCCTG | ---               | Exon 3   |
|                      | 714–715del GA      | ---               | Exon 5   |
| Splice donor site    | IVS4+1G>T          | Splicing error    | Exon 4   |



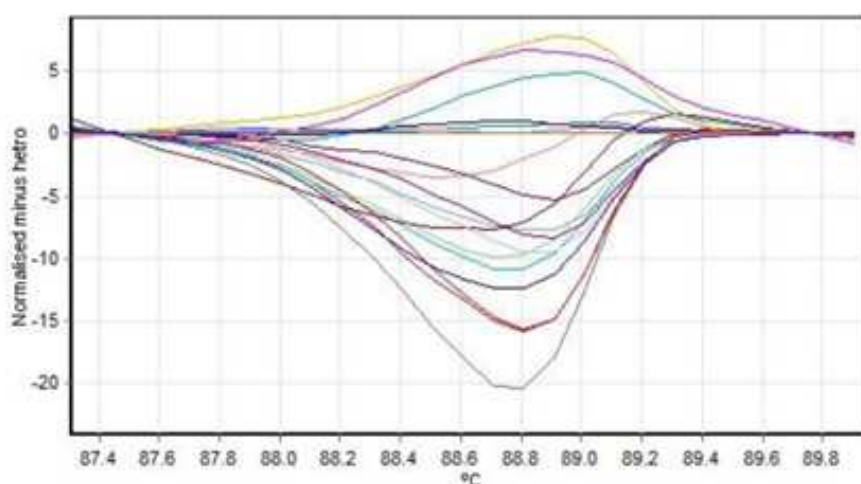


Fig.7. Normal graph exon no 12 (median line= no mutation, ascending line= homozygous, descending arc= heterozygous)

## Discussion

KS may be caused by mutations in *KAL1*, *FGFR1*, *PROKR2* and *PROK2* genes leading to types 1 to 4 KS respectively, among which type 1 is the most common form. Due to adverse conditions such as absent puberty, infertility and the impact of these conditions on the affected person's social and health life, investigation on this disease is important for the diagnosis and treatment of patients before clinical manifestations.

In the present study, twenty patients with X-linked pattern of inheritance were investigated for *KAL1* gene mutations. The molecular study showed that exonic deletions constituted almost 25% of genetic etiology of X-linked KS patients analyzed.

Deletion of exon 4 occurred within the region encoding the whey acidic protein domain of the *KAL1* protein, which is involved in axonal targeting process (13). Deletions in exons 5 and 6 occurred within the region encoding the first fibronectin type III-like repeat of the *KAL1* protein, which is involved in neuronal migration and axonal targeting processes (14). These mutations are different from those reported previously in other populations. In 2004 Sato et al. studied twenty-eight KS patients by whole exome sequencing of *KAL1* gene and detected eight point

mutations. The mutations consisted of one missense, three nonsense and three frameshift mutations and one splice donor site (table 4) (15).

Laitinen et al. in 2011 investigated thirty patients affected with KS. They analyzed 7 identified KS genes (*KAL1*, *FGFR1*, *FGF1*, *PROK2*, *PROKR2*, *CHD7* and *WDR11*). But only three male patients showed mutation in *KAL1* gene, whereas, nine patients had mutation in *FGFR1* gene. *KAL1* mutations were nonsense mutation R262X, the frameshift mutation S158WfsX45, and a deletion of the last nucleotide of exon 8 and the first three nucleotides of intron 8 (g.2357\_2360delAGta) abolishing the splice site (16). The results of this study was in contrast with the present investigation. This difference can be due to the selection of patients, with X-linked recessive pedigree in our cases, while Laitinen et al. investigated KS patients with different inheritance patterns.

Newbern et al. in 2014 identified a mutation in homeobox gene expressed in embryonic stem cells (*HESX1*) involved in HH including incomplete forms and also the incomplete forms of KS (17).

According to the present study, molecular analysis of *KAL1* gene can be helpful for the diagnosis of X-linked recessive KS. Detection of mutation in these conditions can help presympto-

matic diagnosis of members and therefore facilitate early therapies. Further exome sequencing of other patients should help to find other molecular defects in this gene.

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### Conflict of interests

The authors declared no conflict of interests.

### References

1. Zhao W, Ye H, Zhao X, et al. A network investigation on idiopathic hypogonadotropic hypogonadism in china. *Int J Endocrinol* 2013;2013:591012.
2. King TF, Hayes FJ. Long-term outcome of idiopathic hypogonadotropic hypogonadism. *Curr Opin Endocrinol Diabetes Obes* 2012;19:204-10.
3. Fraietta R, Zylberstein DS, Esteves SC. Hypogonadotropic hypogonadism revisited. *Clinics (Sao Paulo)* 2013;68 Suppl 1:81-8.
4. Entrala-Bernal C, Montes-Castillo C, Alvarez-Cubero MJ, et al. Genetic diagnosis of idiopathic hypogonadotrophic hypogonadism: a new point mutation in the KAL2 gene. *Hormones (Athens)* 2014;13:280-5.
5. Pitteloud N, Meysing A, Quinton R, et al. Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome with a wide spectrum of reproductive phenotypes. *Mol Cell Endocrinol* 2006;254-255:60-9.
6. Chen DN, Ma YT, Liu H, et al. Functional rescue of Kallmann syndrome-associated prokineticin receptor 2 (PKR2) mutants deficient in trafficking. *J Biol Chem* 2014;289:15518-26.
7. Pitteloud N, Acierno JS, Jr., Meysing AU, et al. Reversible kallmann syndrome, delayed puberty, and isolated anosmia occurring in a single family with a mutation in the fibroblast growth factor receptor 1 gene. *J Clin Endocrinol Metab* 2005;90:1317-22.
8. Kim SH, Hu Y, Cadman S, et al. Diversity in fibroblast growth factor receptor 1 regulation: learning from the investigation of Kallmann syndrome. *J Neuroendocrinol* 2008;20:141-63.
9. Topaloglu AK, Kotan LD. Molecular causes of hypogonadotropic hypogonadism. *Curr Opin Obstet Gynecol* 2010;22:264-70.
10. Versiani BR, Trarbach E, Koenigkam-Santos M, et al. Clinical assessment and molecular analysis of GnRHR and KAL1 genes in males with idiopathic hypogonadotrophic hypogonadism. *Clin Endocrinol (Oxf)* 2007;66:173-9.
11. Bonomi M, Libri DV, Guizzardi F, et al. New understandings of the genetic basis of isolated idiopathic central hypogonadism. *Asian J Androl* 2012;14:49-56.
12. Enghelabifar M, Allafan S, Khayatzaheh J, et al. Association of the maternal 14-bp insertion/deletion polymorphism in the histocompatibility leukocyte antigen G gene with recurrent implantation failure. *Iran J Reprod Med* 2014;12:641-6.
13. MacColl G, Bouloux P, Quinton R. Kallmann syndrome: adhesion, afferents, and anosmia. *Neuron* 2002;34:675-8.
14. Hynes RO, Lander AD. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell* 1992;68:303-22.
15. Sato N, Katsumata N, Kagami M, et al. Clinical assessment and mutation analysis of Kallmann syndrome 1 (KAL1) and fibroblast growth factor receptor 1 (FGFR1, or KAL2) in five families and 18 sporadic patients. *J Clin Endocrinol Metab* 2004;89:1079-88.
16. Laitinen EM, Vaaralahti K, Tommiska J, et al. Incidence, phenotypic features and molecular genetics of Kallmann syndrome in Finland. *Orphanet J Rare Dis* 2011;6:41.
17. Niederberger C. Re: Identification of HESX1 mutations in Kallmann syndrome. *J Urol* 2014;191:1081.