**PROKR2 and PROK2 mutations cause isolated congenital anosmia without gonadotropic deficiency**

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**Abstract**

**Objective:** Isolated congenital anosmia (ICA) is a rare phenotype defined as absent recall of any olfactory sensations since birth and the absence of any disease known to cause anosmia. Although most cases of ICA are sporadic, reports of familial cases suggest a genetic cause. ICA due to olfactory bulb agenesis and associated to hypogonadotropic hypogonadism defines Kallmann syndrome (KS), in which several gene defects have been described. In KS families, the phenotype may be restricted to ICA. We therefore hypothesized that mutations in KS genes cause ICA in patients, even in the absence of family history of reproduction disorders.

**Design and methods:** In 25 patients with ICA and olfactory bulb agenesis, a detailed phenotype analysis was conducted and the coding sequences of **KAL1**, **FGFR1**, **FGF8**, **PROKR2**, and **PROK2** were sequenced.

**Results:** Three **PROKR2** mutations previously described in KS and one new **PROK2** mutation were found. Investigation of the families showed incomplete penetrance of these mutations.

**Conclusions:** This study is the first to report genetic causes of ICA and indicates that KS genes must be screened in patients with ICA. It also confirms the considerable complexity of GNRH neuron development in humans.

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**Introduction**

Olfactory disorders are very common in industrialized countries, where their prevalence is about 5% in the general population (1). The most common form is acquired anosmia related to trauma or upper respiratory tract infection (2). An association with neurodegenerative disorders has been reported (2). A small minority of patients with anosmia are classified as having congenital anosmia (CA), defined as absent recall of olfactory sensations since birth in the absence of any disease known to cause anosmia. CA may occur in various developmental disorders and often presents as a secondary phenotypic trait of a specific syndrome. The combination of anosmia due to olfactory bulb agenesis and hypogonadotropic hypogonadism defines Kallmann syndrome (KS), which is the most extensively studied form of syndromic CA. Isolated CA (ICA) is defined by a CA without any associated clinical sign. ICA may be due to developmental abnormalities such as olfactory bulb agenesis. It may also occur in the absence of developmental defect, possibly as a result of deficient intracellular signaling in the olfactory receptor pathway (2, 3, 4).

The reported prevalence of CA of one in 10 000 is probably an underestimation (1, 5). Most ICA cases are sporadic, but familial cases with dominant and reduced penetrance have been reported (3, 4). The mode of transmission of syndromic CA is mainly determined by that of the underlying syndrome. Genome mapping performed in two ICA Iranian familial cases with autosomal transmission and incomplete penetrance led to the mapping of a candidate region on chromosome 18 (4, 6). Recently, screening of three genes encoding the three major transduction components of primary sensory neurons failed to identify causative mutations in patients with ICA (3).

KS is characterized by a deficiency in gonadotrophin-releasing hormone (GNRH). GNRH neurons normally migrate from the olfactory placode to the hypothalamus along the olfactory tract during development. In the absence of olfactory bulb development, this neuronal migration process cannot occur. The result is a very small number of GNRH neurons in the hypothalamus,
deficient GNRH secretion, delayed puberty, and infertility in adulthood (7). In contrast to those with KS, patients with ICA have rarely been evaluated in terms of reproductive function.

Several genetic abnormalities have been identified in patients with KS (7). Mutations were first identified in the X-linked gene KAL1 (8). Subsequently, genes associated with autosomal dominant transmission of KS were described (FGFR1, FGF8, PROKR2, and PROK2) (9, 10, 11, 12, 13). All proteins encoded by these genes are directly involved in olfactory bulb development in mice. Detailed phenotype studies have established that family members of patients with KS may exhibit isolated anosmia without hypogonadism (14). Oligogenic transmission may contribute to explain the variable expression of KS (15). KS genes are thus valuable candidate genes for explaining ICA with olfactory bulb agenesis.

The aim of this study was to test the hypothesis that ICA with olfactory bulb agenesis and normal reproductive function may be due to mutations in KS genes. We studied 25 adults with complete anosmia since birth, abnormal olfactory bulb development, and normal reproductive function. Among them, 21 underwent sequencing of the coding sequences of KAL1, FGFR1, FGF8, PROKR2, and PROK2.

Materials and methods

The study was approved by the review board of the European Hospital Georges Pompidou Hospital and was conducted in accordance with the Declaration of Helsinki. All patients gave their informed consent for genetic testing.

Patients

We identified 25 patients seen over a 10-year period at the olfactory diseases outpatient clinic of our department. There were 12 males and 13 females ranging in age from 18 to 59 years, with a mean age of 31 years. A physical examination and nasal fibroscopy were performed routinely to eliminate known causes of acquired anosmia. The degree of anosmia was assessed by olfactory tests, and the congenital nature of the anosmia was established by olfactory bulb imaging.

Olfactory screening tests

To evaluate anosmia severity, we used the Biolfa olfactory test (Amplifon, CCA, Biodigital, Paris, France), which is highly specific and sensitive (16). This test uses two series of 30-ml glass vials containing odorous chemical substances. The first series of tests is quantitative and serves to determine the lowest concentrations at which three different substances (eugenol, aldehyde C14, and phenyl ethyl alcohol) are detected; for each of the three tests, a score is computed as the inverse of this threshold concentration. In the second series of tests, which is semi-qualitative, the quality of olfaction is evaluated by asking the patient to recognize specific odors among a broad panel commonly encountered in Southern European countries (16). For our study, we defined anosmia as a threshold concentration tending to infinity (score = 0) with a negative semi-qualitative test. The same investigator performed all tests.

Imaging

Rhinencephalon morphology was evaluated by magnetic resonance imaging (MRI) performed using a frontal coil in all patients. In addition to the medical history, the main criterion for establishing the congenital nature of the anosmia was the presence of morphological rhinencephalon abnormalities such as olfactory bulb agenesis or hypogenesis.

Clinical evaluation

A clinical evaluation was performed to look for clinical features suggesting anosmia associated with a known syndrome. Plasma levels of LH, FSH, testosterone, and estradiol (E2) were performed in all 25 patients. Renal ultrasonography was performed routinely. Dental agenesis and synkinesia, which are encountered in KS, were searched. A family history of anosmia was sought routinely.

DNA sequencing

Resequencing of KAL1 (NM_000216), FGFR1 (NM_023110), FGF8 (NM_33165), PROK2 (NM_001126128), and PROKR2 (NM_144773) was performed using genomic DNA from blood lymphocytes. PCR primers and PCR protocols were as reported elsewhere (9, 10, 12). Sequence analysis was performed using Seqscape Software (Applied Biosystems, Foster City, CA, USA).

Results

Phenotype

All 25 patients had complete anosmia with a score of 0 in the quantitative test and an inability to identify any odors in the qualitative test. Trigeminal chemosensory sensations were normal. All known causes of acquired anosmia were ruled out. MRI of the olfactory bulbs and tracts was obtained in all 25 patients and showed bilateral olfactory bulb agenesis in 22 patients, olfactory bulb agenesis on one side and hypogenesis on the other side in two patients, and bilateral olfactory bulb hypogenesis in one patient (Fig. 1). Bilateral olfactory tract agenesis was found in ten patients and morphologic abnormalities of the olfactory sulci in seven.
patients. MRI showed no other lesions of the CNS. Based on these findings, the 25 patients with complete anosmia and abnormalities of the olfactory bulbs or tracts were classified as having ICA due to abnormal olfactory bulb development.

Clinical signs of KS were sought and their hormonal status was investigated (Table 1). All 25 patients were adults at diagnosis. None had a history of infertility and nine had children at diagnosis. A 31-year-old male had delayed puberty (patient #20). There was no history of cryptorchidism or micropenis at birth in any of the males. In all 25 patients, the Tanner stage was 5, indicating normal puberty. Plasma levels of testosterone, E2, LH, and FSH were normal in all patients. Syndactyly and unilateral hand and foot polydactyly were noted in a 19-year-old male (patient #19). Another patient had a cleft palate, bilateral eyelid ptosis, and vesicoureteral reflux (patient #11). Developmental renal abnormalities were found in three patients, including two with solitary horseshoe kidneys (patients #11 and #14) and one with abnormal kidney rotation to the right (patient #7).

A family history of anosmia was reported for six patients (Fig. 2). In one family (F1), cases of anosmia were identified in the four previous generations and in the daughter of the index patient. In two other families (F2 and F3), anosmia was found in the dizygotic twin of the index patient but not in the parent. In another family (F4), a paternal grandparent had anosmia. Anosmia was also reported in paternal cousins in two additional families (F6 and F7). These results show that ICA with olfactory bulb agenesis may be transmitted as an autosomal dominant trait with incomplete penetrance.

**Mutation analysis**

Of the 25 patients, 21 underwent resequencing of the KAL1, FGFR1, FGFR8, PROK2, and PROKR2 coding sequences, using lymphocyte DNA. Three nonsense PROKR2 mutations were found in three unrelated ICA patients (Table 1). In patient #8 (family F8), a heterozygous C-to-T transition (c.253C>T) leading to substitution of a cysteine for arginine 85 (p.Arg85Cys) was found in exon 1 of PROKR2. Patient #21 (family F10) had substitution of a histidine for Arg85 (p.Arg85His) due to a heterozygous c.254G>A transition. In patient #11 (family F7), a heterozygous C-to-T transition at position 868 (c.868T>C) leading to substitution of a serine for proline 290 (p.Pro290Ser) was found in exon 2 of PROKR2. Exon 2 of PROKR2 in patient #20 (family F9) exhibited a heterozygous c.137G>T transversion leading to the p.Cys46Phe substitution. In family F8, the mutation was transmitted to the unaffected daughter of patient #8 (Fig. 2). In family F7, the mutation was identified in the unaffected mother of the index patient (Fig. 2). Family studies were not feasible in families F9 and F10.

**Discussion**

ICA is a rare disease defined by a lifelong inability to perceive odors. Despite the description of familial cases, no genetic abnormality has been identified so far in patients with idiopathic ICA. Since 2003, several new mutations have been identified in the autosomal dominant form of KS, with considerable variability in expressivity. Some family members of mutated patients with KS have ICA. Therefore, a reasonable hypothesis was that KS genes might be involved in ICA, even in the absence of KS in the family. We confirmed this hypothesis by identifying three PROKR2 mutations in three of 21 patients who had ICA due to developmental olfactory bulb abnormalities with no history of pubertal-onset disorders. In addition, we identified a PROK2 mutation in a patient with ICA and untreated delayed puberty. Our findings establish the considerable genetic heterogeneity of ICA and confirm the complex association between olfactory tract development and GNRH neuron migration.

To our knowledge, this study reports the largest cohort of patients with ICA and bilateral olfactory bulb agenesis or hypopгенesis. This cohort thus differs from others including patients with ICA and normal olfactory bulbs by MRI (3, 4). In addition, we included only patients with normal reproductive function and normal levels of gonadotropic hormones.

The phenotype analysis in families F7 and F8 showed that ICA was sporadic in both index patients. Therefore, whether the PROKR2 mutations identified in our study are pathogenic deserves discussion. The substitutions of the arginine 85 by a cysteine, a glycine, and a histidine have been reported in both familial and sporadic cases of KS (10, 12, 17, 18). These substitutions induce alterations in PROK2-induced PROKR2 activation in vitro (19). Phenotype severity varied widely in these cases, ranging from KS with mild olfactory bulb hypopгенesis to complete anosmia with severe...
Table 1 Clinical and MRI data in 21 patients screened for mutations in KAL1, FGFR1, FGF8, PROK2, and PROKR2.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Gender</th>
<th>Complaint</th>
<th>Olfactory test</th>
<th>MRI</th>
<th>Endocrine tests</th>
<th>Kidney</th>
<th>FGF8</th>
<th>FGFR1</th>
<th>KAL1</th>
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<td>NI</td>
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<td>Solitary horseshoe kidney</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>p.Arg85His</td>
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BA, bilateral agenesis; TA, tractus agenesis; NI, normal.
gonadotropic deficiency (10, 12, 17, 18). KS due to PROKR2 mutation was usually transmitted as a dominant trait but recessive transmission was also reported in a few cases (20). Based on these previous reports, it is reasonable to propose that ICA in the index patient of the families F8 and F10 was caused by Arg85Cys or Arg85His substitution in PROKR2 respectively. Pro290Ser is another substitution reported in KS due to a heterozygous (10) or homozygous (18) mutation. This substitution induces complete loss of receptor function in vitro (19). However, it has also been reported in unaffected individuals in the general population (10). These results confirm that penetrance of the PROKR2 heterozygous mutation is incomplete in adults. Conceivably, very mild pubertal delay may have gone undetected in the index patients of families F7, F8, and F10.

The substitution Cys46Phe found in PROK2 is also suspected to be deleterious. By similarity, Cys46 should form a disulfide bond with Cys34 (www.uniprot.org). The absence of this disulfide bond probably unfolds PROK2 and results in an inactive protein. We did not find any mutations in FGFR1, FGF8, and KAL1 genes in accordance with the higher penetrance of the gonadotropic axis deficiency in patients bearing mutations in those genes. Mutation screening in HS6ST1, WDR11, and NEFL genes was not performed due to the very low frequency of mutations in these three genes in KS.

This study confirms that the reproductive function may be normal in patients with complete congenital olfactory bulb agenesis. In KS, gonadotropic deficiency is due to absence of GNRH neuron migration from the olfactory placode to the mediobasal hypothalamus (21, 22). Several mechanisms might explain the normal development of the gonadotropic axis in ICA as compared with KS, despite olfactory bulb agenesis in both: i) the developmental defects in the olfactory tract are less severe in ICA than in KS; ii) MRI may lack sufficient sensitivity to detect very small olfactory tracts in ICA; iii) the causative genomic mutations differ between ICA and KS; and iv) the olfactory bulbs and tracts undergo involution after GNRH neuron migration to the hypothalamus in ICA but not in KS.

In our study, the main criterion for classifying anosmia as congenital was the presence of rhinencephalon abnormalities by MRI. Conceivably, patients with ICA may have very thin olfactory tracts that are not visible by MRI but that might be sufficient to ensure normal reproductive function. Indeed, in mice, the number of hypothalamic GNRH neurons necessary for normal gonadotropic function is <25% of the number in normal mice (23). In this study, we observed that the bilateral olfactory bulb agenesis was the most common abnormality; olfactory bulb asymmetry and bilateral olfactory bulb hypogenesis, in contrast, were less frequent. Thus, the developmental defects of the olfactory system vary in ICA from complete agenesis to mild hypogenesis. Similar variability in olfactory bulb developmental defects has been reported in KS due to FGFR1 mutations. The normal gonadotropic axis function in ICA is therefore not ascribable to diminished severity of the olfactory bulb developmental defect compared with KS.

Teixeira et al. (22) have reported the absence of GNRH neurons in the preoptic and periventricular areas of the hypothalamus of a KS fetus. In Medaka, KAL1 deletion results in GNRH neuron accumulation in the olfactory placode (24, 25). However, in other disorders with arhinencephaly, such as trisomy 13 and trisomy 18, the number of GNRH neurons in the periventricular area of the fetal hypothalamus was nearly 40% of the control (22), suggesting incomplete arrest of GNRH neuron migration. The arrest of GNRH neuron migration in arhinencephalic fetuses may be determined not only by olfactory tract agenesis but also by the genetic defect leading to this agenesis. In the oligogenic model recently

Figure 2 Pedigrees of familial cases with or without mutation. Index cases are indicated by a number (see Table 1 for the clinical description). circles: females; boxes: males; open symbols: unaffected patients; closed boxes: affected patients. Asterisks indicate patients with mutations.
proposed by Sykiotis et al. (15), several genes act together to cause KS. A similar model may be relevant to ICA. In the oligogenic model, PROKR2 would be one of the major disease-causing genes, but differences in additional genes or environmental factors would orient the phenotype toward ICA or KS. SEMA3A may be one of these genes orienting the phenotype toward KS (26).

Our phenotype analysis of 25 patients with ICA indicates a fairly high prevalence of associated developmental defects. Thus, 3/24 patients had developmental renal defects, in keeping with data obtained in KS (27). These results confirm that common pathways are involved in olfactory bulb and kidney development, as previously shown in studies on anosmin (28) and prokineticin 2 (29). Interestingly, renal agenesis has not been reported in patients with PROKR2 mutations (30). This study confirms the high phenotypic heterogeneity of disorders associated with PROKR2 and PROK2 loss of function mutations (31, 32).

In this study, we obtained the first evidence of a genetic cause of ICA with olfactory bulb agenesis by systematically analyzing four KS genes in a small group of patients. However, genetic defects were found in only four of 22 patients, indicating considerable genetic heterogeneity. This study indicates that patients with ICA must be routinely evaluated for endocrine disorders and other developmental defects. Further work is necessary to describe other genetic defects leading to ICA. Such genetic works may then be informative for KS.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
A Moya-Plana and O Lacourreye recruited patients. C Villanueva performed and analyzed DNA sequencing. P Bonifils recruited patients, designed the study, analyzed results, and wrote the manuscript. N de Roux designed the study, analyzed results and wrote the manuscript.

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