SEMA3A deletion in a family with Kallmann syndrome validates the role of semaphorin 3A in human puberty and olfactory system development

Jacques Young1,2,3,*, Corinne Metay4,5, Jerome Bouligand1,3,6, Bassim Tou1,3,6, Bruno Francou1,3,6, Luigi Maione1,2,3, Lucie Tosca7, Julie Sarfati2, Frédéric Brioude8, Blandine Esteva8, Audrey Briand-Suleau4,5, Sophie Brisset7, Michel Goossens4,5, Gerard Tachdjian7, and Anne Guiochon-Mantel1,3,6


*Correspondence address. Service d’Endocrinologie et des Maladies de la Reproduction or to Anne Guiochon-Mantel, Service de Génétique moléculaire, Pharmacogénétique et Hormonologie, both at Hôpital Bicêtre, 94275 Le Kremlin-Bicêtre, France. E-mail: jacques.young@bct.aphp.fr; anne.mantel@bct.aphp.fr

Submitted on November 20, 2011; resubmitted on December 27, 2011; accepted on January 12, 2012

BACKGROUND: Kallmann syndrome (KS) is a genetic disorder associating pubertal failure with congenitally absent or impaired sense of smell. KS is related to defective neuronal development affecting both the migration of olfactory nerve endings and GnRH neurons. The discovery of several genetic mutations responsible for KS led to the identification of signaling pathways involved in these processes, but the mutations so far identified account for only 30% of cases of KS. Here, we attempted to identify new genes responsible for KS by using a pan-genomic approach.

METHODS: From a cohort of 120 KS patients, we selected 48 propositi with no mutations in known KS genes. They were analyzed by comparative genomic hybridization array, using Agilent 105K oligonucleotide chips with a mean resolution of 50 kb.

RESULTS: One propositus was found to have a heterozygous deletion of 213 kb at locus 7q21.11, confirmed by real-time qPCR, deleting 11 of the 17 SEMA3A exons. This deletion cosegregated in the propositus’ family with the KS phenotype, that was transmitted in autosomal dominant fashion and was not associated with other neurological or non-neurological clinical disorders. SEMA3A codes for semaphorin 3A, a protein that interacts with neuropilins. Mice lacking semaphorin 3A expression have been showed to have a Kallmann-like phenotype.

CONCLUSIONS: SEMA3A is therefore a new gene whose loss-of-function is involved in KS. These findings validate the specific role of semaphorin 3A in the development of the olfactory system and in neuronal control of puberty in humans.

Key words: puberty / Kallmann / hypogonadotropic hypogonadism / GnRH / neuropilin / semaphorin 3A

Introduction

In mammals, GnRH secretion by hypothalamic neurons is a crucial element of the reproductive cascade, triggering the release of pituitary gonadotrophins, gonadal sex-steroid secretion, pubertal development and gametogenesis (Bouligand et al., 2010). Congenital hypogonadotropic hypogonadism (CHH) results from abnormal gonadotrophin secretion and is characterized by complete or partial pubertal failure (Brioude et al., 2010; Mitchell et al., 2011). CHH is mainly due to defective GnRH production or release by the hypothalamus or to
defective GnRH-receptor function in the pituitary (de Roux et al., 1997; Bouligand et al., 2009, 2010; Brioude et al., 2010; Mitchell et al., 2011). When CHH is associated with anosmia or hyposmia, it is known as Kallmann syndrome (KS, MIM 308700, 147950, 244200, 610628, 612370, 612702) (Dode and Hardelin, 2010; Mitchell et al., 2011). This form of CHH is due to developmental failure of GnRH neuron migration along olfactory axonal projections during fetal life (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010). KALI (MIM 308700), the gene responsible for the X-linked form, was identified 20 years ago (Legouis et al., 1991), leading to the identification of the first known factor in this developmental process. In the last 10 years, loss-of-function mutations in the genes encoding fibroblast growth factor receptor 1, FGFR1 (MIM 136350) (Dodé et al., 2003; Salenave et al., 2008), fibroblast growth factor 8, FGF8 (MIM 600483) (Falardeau et al., 2008; Trarbach et al., 2010), prokineticin 2, PROK2, (MIM 607002) and prokineticin receptor 2, PROKR2, (MIM 607123) (Dodé et al., 2006; Sarfati et al., 2010), CHD7 (MIM 608892) (Kim et al., 2008) and WRD11 (MIM 606417) (Kim et al., 2010) have been shown to cause several autosomal forms of the syndrome. However, these seven genes are mutated in fewer than 30% of KS patients (Dodé and Hardelin, 2010; Mitchell et al., 2011), indicating that other responsible genes remain to be discovered.

Identification of genes responsible for KS has led to the identification of unexpected factors and neuronal signaling pathways (Legouis et al., 1991; Dodé et al., 2003) involved in the development of the olfactory system and GnRH neurons, and has extended to humans the role of signaling pathways discovered in murine models (Ng et al., 2005; Dodé et al., 2006; Matsumoto et al., 2006; Pitteloud et al., 2007).

Several genes involved in KS have been identified through the detection of deletions (Legouis et al., 1991; Hardelin et al., 1993; Dodé et al., 2003). Here we used a pan-genomic approach (Miecznikowski et al., 2011) to detect such microrearrangements and thus to identify new genes involved in KS.

Materials and Methods

The study was approved by the Paris Sud University Hospital ethics committee and complied with human research guidelines as stated in the Declaration of Helsinki. All the patients gave their written informed consent before genetic analysis and hormone studies.

From a cohort of 120 patients with KS, we selected 48 patients (44 men and 4 women) with no mutations in known responsible genes and with no chromosomal anomalies on standard karyotyping. Genomic DNA was extracted from peripheral blood lymphocytes of the patients (Bouligand et al., 2009) and a reference male subject, and was labeled respectively with Cy5-dUTP and Cy3-dUTP. The Agilent 105A human oligonucleotide array (Agilent, Santa Clara, CA, USA) was used to detect chromosomal microarray abnormalities in the patients. All microarray and data analyses were performed according to the manufacturer’s instructions. The microarrays were scanned with an Agilent microarray scanner. Captured images were processed with Feature Extraction 10.7.3.1 software and data were analyzed with Genomics Workbench 5.0 (Agilent Technology). Copy-number variations were considered significant if they were defined by three or more oligonucleotides spanning at least 50 kb, contained at least one gene, and were not listed in the Database of Genomic Variants (Miecznikowski et al., 2011) and in the French BACH genomic database.

Finally, we studied 520 subjects without KS with the same comparative genomic hybridization (CGH) array chip as used in this family.

Quantitative PCR

We used real-time qPCR to test the relative copy number of SEMA3A. Primers (Supplementary data, Table SI) were designed using the third version of Primer Express (Applied Biosystems). Foster City, CA, USA). Albumin exon 12 was used as genomic reference. All the primers were controlled using BLAST on the NCBI website, using the Human Genomic Plus Transcript database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Amplifications were performed using a 7900HT Applied Biosystems real-time thermal cycler (Applied Biosystems). Reactions were performed in a final volume of 20 µl containing 10 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 1 µl of forward primer and 1 µl of reverse primer (final concentration 500 nM) and 8 µl of DNA at 1 ng/µl solution in pure water (8 ng of tested DNA per well). Amplification was performed as recommended by Applied Biosystems. The PCR program was started with 2 min at 50°C, 10 min at 95°C, followed by 50 cycles consisting of 95°C for 15 s and 60°C for 1 min. Dissociations curves, 95°C for 15 s, 60°C for 15 s and 95°C 15 s, were performed at the end of each run to control the specificity of the amplification with the presence of a unique and reproducible melting temperature. Standards curves, with various genomic DNA amount per well from 0.8 to 80 ng, were generated for each amplicon. These standards curves define the relation between the input DNA amount and the Cq value, i.e. number of cycles to reach the significant threshold of fluorescence. For each standard curve, PCR efficiency (E) and linear regression coefficient (R²) were determined. Experiments were validated for each standard curve when E was ≥90% and R² was ≥0.95.

SEMA3A sequencing

Genomic DNA was extracted from white blood cells. The entire SEMA3A coding region (exons 1–17) and intron–exon junctions were amplified and sequenced with primers described in Supplementary data, Table SI. PCR and sequencing products were purified on a Biomek® NXP-96 Laboratory Automation Workstation (Beckman Coulter, Villepinte, France) with Agencourt Ampure XP® and Agencourt Cleanseq® (Beckman Coulter Genomics, Danvers, MA, USA). Sequencing products were analyzed with an automated capillary sequencer (ABI PRISM 3130x1 Genetic Analyzer, Applied Biosystems). Electropherogram-derived sequences were compared with NCBI references for the SEMA3A gene (NG_011489.1Ref SeqGene and NM_006080.2 transcript).

Results

SEMA3A deletion in a family with KS

In one KS propositus (Subject III-2 in Fig. 1A and Table I), we detected a heterozygous deletion of 213 kb at the 7q21.11 locus, removing the last 11 of the 17 SEMA3A exons (MIM 603961) (Fig. 1B). This heterozygous deletion was confirmed by quantitative real-time PCR with two primer pairs located in exons 10 and 15 (Fig. 1C). Real-time PCR showed the same SEMA3A deletion in the heterozygous state (Fig. 1C) in two KS-affected relatives of the propositus (Fig. 1A, Subjects II-2 and III-1), showing that this SEMA3A deletion cosegregated with the KS phenotype. In addition, we sequenced the non-deleted SEMA3A allele and found no mutation. Finally, this 7q21.11 deletion
was not detected in 520 control subjects without KS evaluated with the same CGH array chip as in this family.

We also found 14 others copy number variations in the cohort studied that did not cosegregate with the KS and/or did not included either encoding gene regions or relevant genes (Supplementary data, Table SII).

**Phenotype associated with SEMA3A deletion**

The propositus of this non-consanguineous family had consulted for pubertal failure at age 17. Physical examination showed features of complete hypogonadism, with a mean testicular volume of 2.5 ml (normal range 12–30 ml). He also had right cryptorchidism but not micropenis (Table I). His height was 167 cm and his weight 63 kg.

Olfactometry (Massin et al., 2003) showed hyposmia. Mirror movements, neurogenic deafness and midline abnormalities were absent (Dode and Hardelin, 2010). The detailed neurological examination showed no other anomalies (pyramidal syndrome, cerebellar syndrome, sensory or ophthalmological disorders, etc.). His intelligence quotient was normal. Magnetic resonance imaging (MRI) of the olfactory bulbs showed bilateral hypoplasia. MRI of the hypothalamus, pituitary and brain was normal. Computed tomography of the semicircular canals (Jongmans et al., 2009) was normal. The ocular fundus was normal. No bone abnormalities were found at radiography. Ultrasonography showed two normal kidneys. Echocardiography and cardiac auscultation were normal.

Laboratory tests showed a normal ferritin level and normal transferrin saturation coefficient. The serum testosterone, LH and FSH levels were very low, and the two gonadotrophins responded only weakly to GnRH challenge (Table I).

The sister of the propositus consulted at age 18 years for primary amenorrhea and pubertal failure (Tanner breast stage 1). Hormone assays showed a very low serum estradiol (E₂) level (Bry-Gauillard et al., 2010; Shaw et al., 2011), as well as low LH and FSH levels (Table I). Estrogen-progestin combination therapy induced normal breast development and menses. On re-examination at age 23 years, 6 months after withdrawal of hormone therapy, amenorrhea had recurred and E₂ and gonadotrophins levels were still very low (E₂: 8 pg/ml; LH: 0.6 IU/l; FSH: 0.9 IU/l, for normal values, see Table I).

The father of the propositus had been seen initially at 19 years in the Endocrinology and Reproductive Health Department of Bicêtre Hôpital in 1985 for pubertal failure associated with hypogonadotropic hypogonadism and hyposmia. His testosterone and gonadotrophin levels were very low at that time (Table I). At the age of 21 years, he received a 3-week course of subcutaneous pulsatile GnRH administered via a pump (5 µg/pulse every 60 min). His gonadotrophin and testosterone levels normalized on this treatment (LH: 4.2 IU/l; FSH: 3.9 IU/l; testosterone: 4.6 ng/ml, for normal values see Table I).
Table 1 Clinical and hormonal characteristics of the three kindreds with a SEMA3A deletion.

<table>
<thead>
<tr>
<th>Subject (see Fig. 1A)</th>
<th>II-2</th>
<th>III-1</th>
<th>III-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex/age at diagnosis (years)</td>
<td>M/19</td>
<td>F/18</td>
<td>M/17</td>
</tr>
<tr>
<td>Height (cm)/weight (kg)</td>
<td>193/103</td>
<td>178/70</td>
<td>167/63</td>
</tr>
<tr>
<td>Reproductive phenotype at diagnosis</td>
<td>Hypogonadism</td>
<td>Primary amenorrhea, absent breast development (SI)</td>
<td>Hypogonadism</td>
</tr>
<tr>
<td></td>
<td>Penis: 6 cm</td>
<td>Mean TV: 4 ml</td>
<td>Penis: 5 cm</td>
</tr>
<tr>
<td></td>
<td>Mean TV: 2.5 ml</td>
<td></td>
<td>Mean TV: 2.5 ml</td>
</tr>
<tr>
<td>Sense of smell</td>
<td>Hyposmia</td>
<td>Anosmia</td>
<td>Hyposmia</td>
</tr>
<tr>
<td>Mirror movements</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Midline defects</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Testosterone (T: ng/ml)/estradiol (E2: pg/ml)</td>
<td>0.15/–</td>
<td>–/7</td>
<td>0.12/–</td>
</tr>
<tr>
<td>FSH* (IU/l) basal→stimulated</td>
<td>1.2→2.8</td>
<td>1.1→2.5</td>
<td>0.5→2.6</td>
</tr>
<tr>
<td>LH* (IU/l) basal→stimulated</td>
<td>0.9→3.8</td>
<td>0.1→2.2</td>
<td>0.2→2.4</td>
</tr>
<tr>
<td>Other anterior pituitary dysfunctions</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Encephalic MRI (corpus callosum, cerebelum)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Kidney sonography</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Olfactory bulbs MRI</td>
<td>Bilateral hypoplasia</td>
<td>Bilateral aplasia</td>
<td>Bilateral hypoplasia</td>
</tr>
</tbody>
</table>

M, male; F, female; TV, mean testicular volume; MRI, magnetic resonance imaging.
*aOlfactometry as reported in Pitteloud et al. (2007).
*bCleft palate or lip, arquate, cleft lip.
*cNormal range in adults, basal LH: 2.8–7.1 IU/l; basal FSH: 2.4–7.0 IU/l; testosterone in men: 2.8–9.0 ng/ml; E2 in woman: 24–90 pg/ml (early follicular phase).
*dGnRH:100 µg intravenous.

The deletion reported here removes exons 7–17 which encode the critical domain required for the signal transduction within the cell (Cariboni et al., 2011). In line with this murine model, re-establishment of GnRH neurons, leading to hypogonadism (Cariboni et al., 2011). The deletion reported here removes exons 7–17 which encode the secreted protein that has chemorepulsive properties for neuropilin-1 expressing axons (Schwarting et al., 2000; Cariboni et al., 2011). Semaphorin 3A contains 771 amino-acids and interacts with neuropilin-1, which is a crucial step for interaction with plexin, a protein necessary for the signal transduction within the cell (Cariboni et al., 2011). In this animal model, GnRH neurons accumulate above the cribriform plate (Cariboni et al., 2011), as also previously described in human Kallmann fetuses (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010). In line with this murine model, re-establishment of the deletion reported here removes exons 7–17 which encode the secreted protein that has chemorepulsive properties for neuropilin-1 expressing axons (Schwarting et al., 2000; Cariboni et al., 2011). Semaphorin 3A contains 771 amino-acids and interacts with neuropilin-1, which is a crucial step for interaction with plexin, a protein necessary for the signal transduction within the cell (Cariboni et al., 2011). In this animal model, GnRH neurons accumulate above the cribriform plate (Cariboni et al., 2011), as also previously described in human Kallmann fetuses (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010). In line with this murine model, re-establishment of the deletion reported here removes exons 7–17 which encode the secreted protein that has chemorepulsive properties for neuropilin-1 expressing axons (Schwarting et al., 2000; Cariboni et al., 2011). Semaphorin 3A contains 771 amino-acids and interacts with neuropilin-1, which is a crucial step for interaction with plexin, a protein necessary for the signal transduction within the cell (Cariboni et al., 2011). In this animal model, GnRH neurons accumulate above the cribriform plate (Cariboni et al., 2011), as also previously described in human Kallmann fetuses (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010). In line with this murine model, re-establishment of...
pituitary gonadotrophin secretion after pulsatile administration of exogenous GnRH to Subject II-2 of the family described here shows that CHH in this genetic form of KS is linked to GnRH deficiency, as in other genetic forms of KS (Salenave et al., 2008). Thus, in patients with SEMA3A deletions, as in sema 3A − / − mice, semaphorin signaling alteration could lead to abnormal migration of GnRH neurons to the hypothalamus, which is the anatomical site compatible with GnRH release into the hypothalamo-pituitary portal flow. This could therefore explain the absence of stimulation of pituitary gonadotropic cells leading to hypogonadotropic hypogonadism. In KS patients with SEMA3A deletion, the response to exogenous GnRH also shows that the sensitivity of pituitary gonadotrope cells to GnRH is not significantly affected as in patients with others causes of GnRH deficiency (Bouligand et al., 2009; Brioude et al., 2010).

In the family reported here, the KS phenotype seems to be transmitted in autosomal dominant manner. This suggests (i) that haploinsufficiency of semaphorin 3A in humans, as reported in KS patients with FGFR1 mutations (Dodé et al., 2003; Salenave et al., 2008), is sufficient to cause the Kallmann phenotype, contrary to model mice, in which only homozygous knock-out leads to a similar phenotype; or (ii) that there is a second genetic event, not revealed by our CGH-array approach, within the framework of digenic or oligogenic transmission (Dodé et al., 2006; Dodé and Hardelin, 2010; Sarfati et al., 2010; Sykiotis et al., 2010; Mitchell et al., 2011). This putative second event does not correspond to mutation of genes known to be involved in normosmic CHH or KS, as we screened for mutations in the coding parts of these genes in the family members, with negative results. Moreover, our pan-genomic approach revealed no deletions spanning these genes or in other genes associated with KS, such as NELF (Xu et al., 2011). This second genetic event, if it exists, may therefore consist of a point mutation in an as-yet-unidentified KS responsible gene.

Interestingly, none of the family members with KS reported here had any other clinical neurological abnormalities. This suggests that the role of semaphorin 3A in neuronal migration could be restricted, in humans to the olfactory system development and GnRH neurons migration, despite its expression in other neuronal and non-neuronal structures (Schwartz et al., 2000; Goshima et al., 2002; Carboni et al., 2011). This, however, needs to be confirmed by detailed phenotypic analysis of a larger number of KS patients with SEMA3A mutations.

Re-examination of Subject II-2 at age 54 years showed that his congenital gonadotrophin deficiency persisted. This suggests that GnRH deficiency does not appear to be reversible in this genetic form of KS, as reported in some patients with other genetic forms (Quinton et al., 1999; Raivio et al., 2007; Mitchell et al., 2011). Here again, studies of larger numbers of patients will be necessary to determine the prevalence of reversibility in cases of SEMA3A mutations.

Finally, we identified the SEMA3A deletion in one of 48 KS propotis, corresponding to a prevalence of 2.0%, but the precise frequency of mutations in this gene is being examined in an ongoing exhaustive analysis of point mutations and deletions in the same cohort.

In conclusion, we report cosegregation between the KS phenotype and a heterozygous SEMA3A deletion in a family with several affected members, clearly demonstrating the involvement of this gene in the development of the olfactory system and in the migration of GnRH neurons in humans. SEMA3A is therefore a novel actor necessary for the completion of human puberty and reproduction. This discovery of a new KS responsible gene will assist with genetic counseling for this syndrome that exhibits multiple modes of transmission.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

**Acknowledgements**

We thank Isabelle Boucly (Bicêtre Hospital), Valérie Delattre, Valérie Ortonne, Wilfried Verbeqc-Morlot (Henri Mondor Hospital) for their excellent technical assistance.

**Authors’ roles**


**Funding**

This work was supported in part by grants from Paris-Sud 11 University (Bonus Qualité Recherche 2009, Attractivité Univ Paris Sud 2010), INSERM KalGenopath 09-GENO-017, PHRC HYPOPROTEO P081212 and Fondation pour la Recherche Médicale. This work was supported in part by Agence Nationale de la Recherche (ANR KalGenopath 2010), Fondation pour la Recherche Médicale (FRM) and Agence Francaise de Lutte Contre le Dopage (AFLD).

**Conflict of interest**

None declared.

**References**


Carboni A, Davidson K, Rakic S, Maggi R, Parnavelas JG, Ruhrberg C. Defective gonadotropin-releasing hormone neuron migration in mice lacking SEMA3A signalling through NRPI and NRPII: implications for...
SEMA3A deletion in Kallmann


