Mutation screening of *SEMA3A* and *SEMA7A* in patients with congenital hypogonadotropic hypogonadism

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BACKGROUND: Congenital hypogonadotropic hypogonadism (HH), a rare disorder characterized by absent, partial, or delayed puberty, can be caused by the lack or deficient number of hypothalamic gonadotropin-releasing hormone (GnRH) neurons. *SEMA3A* was recently implicated in the etiology of the disorder, and *Sema7A*-deficient mice have a reduced number of GnRH neurons in their brains.

METHODS: *SEMA3A* and *SEMA7A* were screened by Sanger sequencing in altogether 50 Finnish HH patients (34 with Kallmann syndrome (KS; HH with hyposmia/anosmia) and 16 with normosmic HH (nHH)). In 20 patients, mutation(s) had already been found in genes known to be implicated in congenital HH.

RESULTS: Three heterozygous variants (c.458A>G (p.Asn153Ser), c.1253A>G (p.Asn418Ser), and c.1303G>A (p.Val435Ile)) were found in *SEMA3A* in three KS patients, two of which also had a mutation in *FGFR1*. Two rare heterozygous variants (c.442C>T (p.Arg148Trp) and c.1421G>A (p.Arg474Gln)) in *SEMA7A* were found in one male nHH patient with a previously identified *KISS1R* nonsense variant and one male KS patient with a previously identified mutation in *KAL1*, respectively.

CONCLUSION: Our results suggest that heterozygous missense variants in *SEMA3A* and *SEMA7A* may modify the phenotype of KS but most likely are not alone sufficient to cause the disorder.

Congenital hypogonadotropic hypogonadism (HH) is a rare disorder characterized by incomplete or absent puberty caused by the lack or deficient number of hypothalamic gonadotropin-releasing hormone (GnRH) neurons, disturbed secretion or action of GnRH, or both (1). Various patterns of inheritance have been observed in association with this condition, including X-linked, autosomal dominant, and autosomal recessive, as well as di- and oligogenic inheritance (2–4). Mutations in several genes, including *KAL1*, *FGFR1*, *FGF8*, *PROK2*, *PROKR2*, *CHD7*, *WDR11*, *GNRHR*, *GNRH1*, *KISS1R*, *KISS1*, *TAC3*, and *TACR3* (5,6), have been shown to cause the disorder. In the majority of cases, however, the molecular genetic cause remains unresolved, implying the existence of additional genes underlying the condition (7). We have described the phenotypic and genotypic features of Finnish patients with Kallmann syndrome (KS) (8). A molecular genetic cause was obtained for ~40% of Finnish KS patients, suggesting the presence of mutations in genes not yet implicated to underlie this syndrome.

Semaphorins are a class of secreted and membrane proteins that act as axonal growth cone guidance molecules. *Sema3A*- and *Sema7A*-mutant mice have a reduced number of GnRH neurons in their brains, with *Sema3A* being essential for the patterning of vomeronasal axons (9), whereas in *Sema7A* mutants, the olfactory system appears to remain unaffected (10). Thus, *SEMA3A* is a good candidate gene for KS and *SEMA7A* for normosmic HH (nHH). Two recent studies have suggested a role for *SEMA3A* mutations in the pathogenesis of KS in humans (11,12). However, studies on *SEMA7A* in patients with congenital HH have not yet been reported.

Our objective in the current work was to investigate whether mutations in these two candidate genes, *SEMA3A* and *SEMA7A*, are present in Finnish patients with congenital HH.

RESULTS

Overall, the mutation screening of *SEMA3A* and *SEMA7A* revealed few nonsynonymous variants. The nonsynonymous variants identified and the patients' phenotypes are presented in **Table 1**.

Among the 50 HH patients, we identified three previously reported heterozygous missense variants, c.1253A>G (p.Asn418Ser), c.458A>G (p.Asn153Ser), and c.1303G>A (p.Val435Ile) in *SEMA3A* in three patients with KS. The patients with the Asn418Ser and Asn153Ser variants also had previously identified mutations in *FGFR1*: c.1305_1306dupAT (p.Ser436Tyrfs*3) and c.1825C>T (p.Arg609*), respectively

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(8). In the patient with the Val435Ile variant, no mutations in the known KS genes (*KAL1*, *FGFR1*, *FGF8*, *PROK2*, *PROKR2*, *CHD7*, *WDR11*, or *NELF)* were found. All the identified *SEMA3A* variants have been reported in the Exome Variant Server (EVS; NHLBI GO Exome Sequencing Project (ESP), Seattle, WA) database, with minor allele frequencies 0.03% (Asn418Ser), 0.5 % (Asn153Ser), and 1.4 % (Val435Ile) in the European American population (**Table 1**). The frequency of the identified variants was not determined in Finnish controls.

One heterozygous missense variant, c.442C>T (p.Arg148Trp), was found in *SEMA7A* in a male patient with congenital nHH. The variant was predicted to be "probably damaging" by PolyPhen-2 (score: 1.00). A heterozygous nonsense variant, c.1167C>A (p.Cys389*) in *KISS1R* (NM_032551.4), a gene implicated in autosomal recessive nHH, was identified previously in the same patient, but no other mutation was found when the coding region, promoter region, and the cDNA of *KISS1R* were Sanger sequenced. Neither of the mutations was present in 200 controls, and both were reported once in the EVS database: *SEMA7A* Arg148Trp 1/12986 and *KISS1R* Cys389* 1/10952 chromosomes. Another missense variant in *SEMA7A*, c.1421G>A (p.Arg474Gln), was identified in a KS patient who also had a *KAL1* mutation deleting the last base of exon 8 and the first three bases of the following intron (g.2357_2360delAgta), thus disrupting a splice site (8). The Arg474Gln variant was predicted by PolyPhen-2 to be "probably damaging" with a score of 0.999. The variant is absent in the EVS database, but several other amino acid substitutions have been reported in the same position (p.Arg474Leu, p.Arg474Pro, and p.Arg474Trp), suggesting that some variation is tolerated in this amino acid position.

DISCUSSION

KS patients with *SEMA3A* mutations were recently reported by two groups: Young *et al.* (11) identified a heterozygous deletion of 213 kb deleting 11 of the 17 exons in *SEMA3A* in a family with KS, whereas Hanchate *et al.* (12) identified one small deletion (Asp538fs*31) and several heterozygous missense variants in *SEMA3A* in KS patients. To further elucidate the contribution of *SEMA3A* mutations to the pathogenesis of KS, we investigated a cohort of well-characterized Finnish patients with HH.

We identified three missense changes (Val435Ile, Asn153Ser, and Asn418Ser) in *SEMA3A* in three probands with KS. Two of these variants (Val435Ile and Asn153Ser) were also reported by Hanchate *et al.* (12). The Asn153Ser variant has been shown to cause reduced signaling activity in the GN11 cell line, whereas the Val435Ile variant results in impaired secretion of semaphorin 3A, suggesting that these two mutations have a pathogenic effect (12). Notably, two probands with *SEMA3A* variants (Asn153Ser and Asn418Ser) also had previously described truncating mutations in *FGFR1* (8). Di-/oligogenic inheritance of HH has been continuously reported (2–4), and it seems possible that *SEMA3A* mutations might have a role in modifying the KS phenotype, although conclusive evidence is still lacking. For example, Young *et al.* (11) proposed that the heterozygous *SEMA3A* deletion might be sufficient to cause the disease phenotype because no other mutations were detected and the deletion cosegregated in the family with the apparently autosomal dominantly transmitted KS phenotype. On the other hand, Hanchate *et al.* (12) suggested that monoallelic mutations in *SEMA3A* are not sufficient to cause the disease phenotype based on the finding that all the missense variants they detected were reported in the EVS database, some were also found in healthy controls, and some of the patients also carried mutations in other genes implicated in KS. In none of the patients in our study, did mutations in *SEMA3A* seem to be the sole cause of the disorder as we only found heterozygous variants that are also present in healthy control individuals. The third patient, with the Val435Ile variant in *SEMA3A* and without other detected mutations in the known HH genes, most likely still has at least one unidentified mutation contributing to the disorder.

In the same vein, we wanted to screen in our patients another semaphorin gene, *SEMA7A*, which has recently been shown

Table 1. Clinical characteristics and molecular genetic findings in patients with KS or nHH with nonsynonymous variants in *SEMA3A* or *SEMA7A*

Proband	Sex	Diagnosis	Other phenotypic features	Family members with KS or nHH	Gene	Nucleotide and amino acid change	MAF (%)[a]	Mutations in other screened genes[b]
1	M	KS	No	No	*SEMA3A*	c.1303G>A (p.V435I), (rs147436181)	1.4	Not detected
2	F	KS	Cleft lip and dental agenesis	KS: sister, father, and paternal aunt	*SEMA3A*	c.458A>G (p.N153S), (rs139295139)	0.5	c.1825C>T (p.R609*) (*FGFR1*), (rs121909639)
3	F	KS	No	KS: son and daughter	*SEMA3A*	c.1253A>G (p.N418S), (rs142967103)	0.03	c.1305_1306dupAT (p.S436Yfs*3) (*FGFR1*)
4	M	nHH	No	No	*SEMA7A*	c.442C>T (p.R148W), (rs200895370)	0.01	c.1167C>A (p.C389*) (*KISS1R*), (rs371771794)
5	M	KS	Micropenis, cryptorchidism, and synkinesia	KS: maternal uncle	*SEMA7A*	c.1421G>A (p.R474Q[c])	N/A	g.2357_2360delAgta (*KAL1*)

Rs-numbers of variants/mutations are given when available.
F, female; KS, Kallmann syndrome; M, male; MAF, minor allele frequency; nHH, normosmic hypogonadotropic hypogonadism.
[a]MAF in the European American population in Exome Variant Server (EVS) database. [b]Screened genes: *FGFR1*, *FGF8*, *PROK2*, *PROKR2*, *CHD7*, *WDR11* (all patients), *KAL1* (KS patients only), *GNRHR*, *GNRH1*, *KISS1R*, *KISS1*, *TAC3*, and *TACR3* (nHH patients only). All mutations are heterozygous (except for the hemizygous mutation in *KAL1*). [c]Not found in the NCBI Short Genetic Variations database dbSNP or EVS database, but several other reported amino acid substitutions at the same position (p.R474L, p.R474P, and p.R474W).

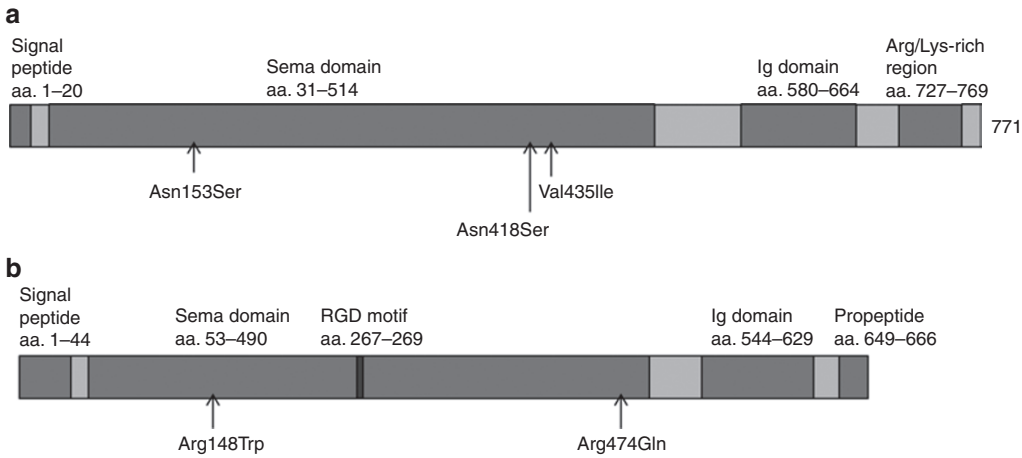

Figure 1. Functional domains of SEMA3A and SEMA7A and the identified variants. The named domains are shaded in darker gray. The predicted beginning and end of each domain according to UniProtKB protein knowledge database are indicated as amino acids (aa) below the name of the domain. (**a**) Functional domains of SEMA3A and the three identified missense variants. Arg/Lys-rich region, basic region rich in arginine and lysine; Ig domain, Ig-like domain. (**b**) Functional domains of SEMA7A (isoform 1) and the two identified missense variants. RGD motif, Arginine–glycine–aspartate motif that is recognized by certain integrin receptors. The potential propeptide is cleaved in the mature form of the protein.

to have a role in the migration of GnRH neurons in mice (10). SEMA7A is able to promote axon outgrowth by signaling through integrin receptors containing the β1 subunit (13) and promotes directional migration of GnRH neurons through β1-integrin–dependent cell adhesion (10). β1-integrin signaling was also recently shown to be involved in the development of GnRH neuron axonal projections to the median eminence in mice (14). *Sema7A*$^{-/-}$ adult mice have a 30% reduction of GnRH-1 cells in their brains compared with wild-type mice, and innervation of the median eminence by GnRH-1 neuron fibers is also clearly reduced (10). This phenotype seems to be the result of defective GnRH-1 neuron migration, which ultimately leads to reduced fertility in *Sema7A*$^{-/-}$ mice, whereas the development of the olfactory system appears to remain unaffected (10). We identified two rare variants in *SEMA7A*, both of which are predicted to be probably damaging by PolyPhen-2. Both of the variants are located in the conserved Sema domain of SEMA7A (**Figure 1**), which is responsible for receptor-binding specificity of the protein (15), although they are not located in the arginine-glycine-aspartate motif (amino acids 267–269) recognized by certain integrin receptors (16). It seems likely that the identified mutations alone are not sufficient to cause the disease phenotype, although they may contribute to it. Interestingly, one of the patients with a rare variant in *SEMA7A* also had a heterozygous nonsense mutation in *KISS1R*, a gene implicated in autosomal recessive nHH (17), bringing forth the possibility of di-/oligogenic inheritance. Whether or not this *KISS1R* variant is in homozygous or compound heterozygous state capable of causing HH, however, is still unclear.

Taken together, the role of defective semaphorin signaling in GnRH deficiency has recently gained interest. Our results show that mutations in *SEMA3A* and *SEMA7A* are rare among Finnish patients but might have a role in modifying the disease phenotype. Further studies in different populations are warranted.

METHODS

Fifty patients with HH who were previously diagnosed on a basis of (i) clinical signs and symptoms of hypogonadism (absent puberty, infertility, and/or decreased libido), (ii) low sex steroid levels in association with inappropriately low or normal gonadotropin levels, and subnormal or normal response to GnRH stimulation test, (iii) otherwise normal anterior pituitary function, (iv) normal radiological imaging of the hypothalamic and pituitary areas, and in case of KS, (v) defective sense of smell detected by formal testing (UPSIT score <5th percentile of age; University of Pennsylvania Smell Identification Test; Sensonic, Haddon Heights, NJ), and/or absent or rudimentary olfactory bulbs in magnetic resonance imaging, were screened for mutations in *SEMA3A* and *SEMA7A*. Patients were enrolled from all five university hospitals in Finland. Of them, 30 patients (27 men and 3 women), including 19 KS patients (18 men and 1 woman), 9 nHH patients (7 men and 2 women), and 2 men with adult onset HH, were not found to have mutations in known HH genes (8,18). In addition, 20 HH patients were previously described to have mutation(s) in *KAL1* (NM_000216.2; 3 patients), *FGFR1* (NM_023110.2; 12 patients), *GNRHR* (NM_000406.2; 4 patients), or *CHD7* (NM_017780.2; 1 patient) (8,18–21).

The coding exons and exon–intron boundaries of *SEMA3A* (NM_006080.2) and *SEMA7A* (NM_003612.3) were PCR amplified from the genomic DNA of the patients. PCR products were purified with ExoSAP-IT treatment (Amersham Biosciences, Piscataway, NJ) and bidirectionally sequenced using the ABI BigDyeTerminator Cycle Sequencing Kit (v3.1) and ABI Prism 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA). The sequences were aligned and read with Sequencher 4.9 software (Gene Codes, AnnArbor, MI). All primer sequences and PCR conditions are available upon request. Exon 4 of *SEMA7A* was also sequenced in 200 controls. The minor allele frequencies of the identified variants were checked in the EVS database, and the effects of the identified *SEMA7A* variants were predicted with the web version of PolyPhen-2 software (22).

This study was performed with appropriate permissions from the Ethics Committee (E7) of the Helsinki University Central Hospital. Written informed consents were obtained from the participants, and also from their guardian if the participant was less than 16 y of age.

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