Mutational Spectrum of Semaphorin 3A and Semaphorin 3D Genes in Spanish Hirschsprung patients

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Abstract

Hirschsprung disease (HSCR, OMIM 142623) is a developmental disorder characterized by the absence of ganglion cells along variable lengths of the distal gastrointestinal tract, which results in tonic contraction of the aganglionic colon segment and functional intestinal obstruction. The RET proto-oncogene is the major gene associated to HSCR with differential contributions of its rare and common, coding and noncoding mutations to the multifactorial nature of this pathology. In addition, many other genes have been described to be associated with this pathology, including the semaphorins class III genes SEMA3A (7p12.1) and SEMA3D (7q21.11) through SNP array analyses and by next-generation sequencing technologies. Semaphorins are guidance cues for developing neurons implicated in the axonal projections and in the determination of the migratory pathway for neural-crest derived neural precursors during enteric nervous system development. In addition, it has been described that increased SEMA3A expression may be a risk factor for HSCR through the upregulation of the gene in the aganglionic smooth muscle layer of the colon in HSCR patients. Here we present the results of a comprehensive analysis of SEMA3A and SEMA3D in a series of 200 Spanish HSCR patients by the mutational screening of its coding sequence, which has led to find a number of potentially deleterious variants. RET mutations have been also detected in some of those patients carrying SEMAs variants. We have evaluated the A131T-SEMA3A, S598G-SEMA3A and E198K-SEMA3D mutations using colon tissue sections of these patients by immunohistochemistry. All mutants presented increased protein expression in smooth muscle layer of ganglionic segments. Moreover, A131T-SEMA3A also maintained higher protein levels in the aganglionic muscle layers. These findings strongly suggest that these mutants have a pathogenic effect on the disease. Furthermore, because of their coexistence with RET mutations, our data substantiate the additive genetic model proposed for this rare disorder and further support the association of SEMAs genes with HSCR.

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Introduction

Hirschsprung disease (HSCR, OMIM 142623) is a developmental disorder occurring in 1 of 5,000 live births. HSCR most commonly presents as isolated cases and displays a complex pattern of inheritance with low, sex dependent penetrance and variable expression. It is characterized by the absence of ganglion cells along variable lengths of the distal gastrointestinal tract, which results in tonic contraction of the aganglionic colon segment and functional intestinal obstruction. Such aganglionosis is attributed to a failure of neural crest cells (NCC) to migrate, proliferate, and/or differentiate during enteric nervous system (ENS) development in the embryonic stage [1,2].

The RET proto-oncogene (OMIM 164761) is the major gene associated to HSCR. RET has been extensively studied in HSCR patients and over 100 mutations have been identified along the gene (see Human Gene Mutation Database). However, mutations in the RET coding sequence account for only up to 50% or 7–20% of familial and sporadic cases, respectively [2]. The involvement of RET in the pathogenesis of HSCR is further supported by the existence of a specific haplotype, constituted by common RET polymorphisms, which seems to be responsible for the majority of sporadic forms [3,4,5]. This HSCR-associated RET haplotype is characterized by a common allele (c.73+9277T, rs2433357) within a conserved enhancer-like sequence in intron 1 (MCS9.7) [5,6], making a 20-fold greater contribution to risk than coding mutations [5]. It has been demonstrated a difference in ability of SOX10 to bind to MCS9.7 and transactivate RET depending on the bearing allele at such specific locus [6].

On the other hand, numerous molecular genetic studies have identified rare coding mutations in many other genes (GDNF, NRTN, PSPN, EDNRB, EDN3, ECE1, NTF3, NTRK3, SOX10, PHOX2B, LICAM, ZFHX1B, KIAA1279, TCF4, PROK1, PROK1R, PROKR2, GFRA1, NRG1 and SEMAs) related to HSCR [2,7–13]. However, the conventional mutations related to HSCR reported so far only explain around 5% of cases, being the vast majority of
them long segment HSCR and/or total colonic aganglionosis and syndromic forms of the disease [2].

HSCR is regarded as a complex and multifactorial genetic disorder, in which the contribution of several different loci acting in an additive or multiplicative manner is usually required to cause the disease [3]. Based on this evidence, several HSCR-associated regions (9q31 [14], 19q12 [15], 3p21 [15,16], 16q23 [17], 21q21 [18], or 4q31.3-q32.3 [19]) have been identified by genome wide linkage and genome wide association studies (GWAS), although the genes underlying such associations have not been identified yet in most of the cases.

Through a GWAS performed by the International HSCR Consortium, in which our group takes part, a significant cluster of SNPs was identified in a region on chromosome 7, containing strong association with HSCR with allelic effects independent of RET [33], that fell downstream from the protein SEMA3D (7q21.11; OMIM 609907) and upstream from SEMA3A (7p12.1; OMIM 603961) ([S. Arnold et al., 2nd International Symposium: Development of the

Table 1. SEMA3A sequence variants detected in the current study.

| Nucleotide Change | Aminoacid Change | Novel/Described | Allelic frequency (%) in control population |
|-------------------|------------------|-----------------|------------------------------------------|
| c.112+52del       |                  | Novel           | 0                                        |
| c.112+52 C>G      |                  | Novel           | 0                                        |
| c.112+63 T>C      | rs13231702       | 0               |
| c.113-110 A>C     | rs12671857       | 52.2            |
| c.113-42 G>C      | rs17241389       | 0               |
| c.201T>C          | S67S             | Novel           | 1                                        |
| c.267A>G          | Q89Q             | Arnold et al., 2009 | 0                                      |
| c.270+96A>G       |                  | Novel           | 0                                        |
| c.333+84T>A       |                  | Novel           | 0                                        |
| c.333+92G>T       | rs6955597        | 0.3             |
| c.334-24delTT     |                  | Novel           | 0                                        |
| c.391G>A          | A131T            | rs143007146     |                                          |
| c.453+24A>G       | rs1990044        | 47.9            |
| c.547+167G>A      | rs2527039        | 15.8            |
| c.548-45C>T       |                  | Novel           | 0                                        |
| c.548-67T>C       |                  | Novel           | 1                                        |
| c.548-79G>C       |                  | Novel           | 0                                        |
| c.548-92 T>C      |                  | Novel           | 0                                        |
| c.668-199_201del   | Novel            | 2.5             |
| c.668-138A>T      |                  | Novel           | 0                                        |
| c.668-20 C>T      | rs2272221        | 10.5            |
| c.668-14 T>A      | rs227222        | 2.2             |
| c.705T>C          | S235S            | rs34541339      | 6.8                                      |
| c.732C>T          | Y244Y            | Novel           | 0                                        |
| c.811-139C>T      | Novel            | 0               |
| c.945C>T          | N315N            | Novel           | 0                                        |
| c.1140+46C>G      | Novel            | 0               |
| c.1302T>C         | I434I            | Novel           | 15                                       |
| c.1303G>A         | V435I            | Arnold et al., 2009 | 3                                      |
| c.1361-52T>A      | rs10250165       | 23.8            |
| c.1361-14A>G      | rs3735513        | 14.7            |
| c.1453-9deA       |                 | Novel           | 0                                        |
| c.1495+132A>C     | rs17246251       | 61.5            |
| c.1563G>C         | G521G            | rs10487665      | 0                                        |
| c.1652-8insTA     | Novel            | 0               |
| c.1653-6C>T       | rs701320         | 84              |
| c.1792A>G         | S598G            | Novel           | 0                                        |
| c.1860+30A>G      | rs7809708        | 26.5            |
| c.2151A>G         | T717T            | rs797821        | 32.5                                      |

Compilation of all the SEMA3A sequences variants found by dHPLC. Note that the missense variants are shaded.
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In such study, short-HSCR trios were analyzed with the 500K SNP array platform (Affymetrix) and the SEMA SNPs cluster was identified and subsequently refined. The two SEMA family III members demonstrated very similar temporo-spatial patterns of expression throughout the colon. Notably, they were co-expressed with RET in this tissue, supporting the possibility that any of them might modify RET function in the developing ENS.

In addition, based in these findings, next-generation sequencing technologies and functional analyses have allowed the identification of semaphorins class III genes (SEMAs) 3A and 3D mutations potentially involved in the pathogenesis of HSCR [13]. SEMAs represent the largest family of axonal guidance cues identified so far, that provides directional information to growing axons. The role of SEMAs patterning sensory projections in the peripheral [20] and central nervous system [21] is well known. Previous studies have also suggested a role for members of the SEMA family in NCC development, proliferation, migration, and/or differentiation, which are processes related with HSCR etiology [22–25]. More specifically, SEMA3A is expressed in the mesenchyme of distal large intestine and it also acts as a repulsive signal for neurites of Remak ganglia [26]. Although ENS precursors derived from sacral NCCs express the receptor Neuropilin1 (NP1), it remains to be demonstrated if SEMA3A acts on migration by a direct effect on the migrating ENS precursors, or by an indirect action over extrinsic axons that accompany those cells when colonizing the bowel, and are repelled if SEMA3A is present in the outer segments of colon mesenchyme [22]. More recently, different in vitro and in vivo approaches reinforce the important role of SEMAs and their receptors as key players in the immunological and the neurological systems [27–29].

Furthermore, it has been proposed that increased SEMA3A expression may be a risk factor for HSCR pathology in a subset of HSCR patients, based on the upregulation of this gene in the aganglionic smooth muscle layer of the colon [30]. In addition, the association between two SEMA3A polymorphisms (rs7804122 and rs797821) and the risk of HSCR in the Northeastern Chinese population has been validated, as it was previously demonstrated in Caucasian population [31]. Based on the evidences of the implication of SEMA class III genes in HSCR, we have performed a screening of the coding region of SEMA3A and SEMA3D genes in a series of 200 isolated Spanish HSCR cases, to determine their mutational spectrum in our population. Three mutations, A131T-SEMA3A, S598G-SEMA3A and E198K-SEMA3D were further studied using an immunohistochemical approach to evaluate their expression levels in the HSCR samples. All mutants presented an increased amount of the proteins in colon tissue sections in comparison with the normal proteins, indicating the pathogenic role of these mutations.

### Materials and Methods

#### Patients and Controls Subjects

In this study we have included a total of 200 Spanish HSCR patients (23% female, 77% male), and their parents when available. 180 were sporadic cases, while 20 were familial cases belonging to 13 different families. In addition, we have also analyzed a group of 200 normal controls comprising unselected, unrelated, race, age, and sex-matched individuals.

#### Ethics Statement

A written informed consent was obtained from all the participants for clinical and molecular genetic studies. The study conformed to the tenets of the declaration of Helsinki as

### Table 2. SEMA3D sequence variants detected in the current study.

| Nucleotide Change | Aminoacid Change | Novel/Described | Allelic frequency (%) in control population |
|-------------------|------------------|-----------------|--------------------------------------------|
| c-151-158_154del  |                 | Novel           | 1                                          |
| c.376-32G>A       | Novel            | 0               |
| c.496-12T>C       | Novel            | 0               |
| c.589+37G>A       | rs17159594       | 0               |
| c.590-33T>A       | Novel            | 3.5             |
| c.71B+C           | Novel            | 0               |
| c.636C>T          | D212D            | 0               |
| c.592G>A          | E198K            | Novel           | 0                                          |
| c.861+67_71Tdel   | rs5631427        | 4.8             |
| c.1545+91G>A      | rs7780132        | 28.3            |
| c.1546-9 G>A      | Novel            | 0               |
| c.1578C>T         | L526L            | 43.8            |
| c.1703+28G>C      | rs6468008        | 27              |
| c.1843C>A         | P61ST            | 0.5             |
| c.1901G>A         | R634Q            | TMP ESP_7:84636125* | 0    |
| c.1906+14T>C      | Novel            | 0               |
| c.2103G>T         | K701Q            | rs7800072       | 35.8                                       |

Compilation of all the SEMA3D sequences variants found by dHPLC. Note that the missense variants are shaded.

*Source: Ensemble.

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## Table 3. SEMA3A and SEMA3D missense variants detected in isolated HSCR patients.

| Gene       | Nucleotide Change | Aminoacid Change | Protein domain | Length of aganglionosis | Inheritance | Gender | Proteinopathy | Length of aganglionosis | Inheritance | Genomic DNA from the patient | Patients information |
|------------|-------------------|------------------|----------------|-------------------------|-------------|--------|---------------|-------------------------|-------------|----------------------------|---------------------|
| SEMA3A     | c.391G            | A131T            | SEMA           | Male                    | Paternal    | Male   | Data not available | Male                    | Paternal    | RET enhancer mutation in homozygosis | Other mutational events in the patient (new) |
|            |                   |                  |                |                         |             |        |                |                         |             |                            |                     |
| SEMA3A     | c.391G            | A131T            | SEMA           | Male                    | Maternal    | Male   | De novo or paternal*1 | Male                    | Maternal    | RET enhancer from the mother |                     |
|            |                   |                  |                |                         |             |        |                |                         |             |                            |                     |
| SEMA3A     | c.1792A           | S598G            | Ig-like        | Female                  | Male        | Female | R53Q mutation*1    | Female                  | Male        | EDNRB KSX mutation inherited from the father |                     |
|            |                   |                  |                |                         |             |        |                |                         |             |                            |                     |
| SEMA3D     | c.592G            | E198K            | SEMA           | Male                    | De novo     | Male   |                  | Male                    | De novo     | RET enhancer mutation in homozygosis |                     |
|            |                   |                  |                |                         |             |        |                |                         |             |                            |                     |
| SEMA3A     | c.1901G           | R634Q            | Ig-like        | Male                    | Maternal    | Male   |                  | Male                    | Maternal    | RET enhancer in homozygosis |                     |
|            |                   |                  |                |                         |             |        |                |                         |             |                            |                     |
| SEMA3D     | c.592G            | E198K            | Ig-like        | Male                    | Maternal    | Male   |                  | Male                    | Maternal    | RET enhancer mutation in homozygosis |                     |
|            |                   |                  |                |                         |             |        |                |                         |             |                            |                     |

### Immunohistochemistry (IHC)

Formalin-fixed paraffin embedded (FFPE) colon tissue blocks were collected from patients with the following mutations: A131T-SEMA3A (both ganglionic and aganglionic sections), S598G-SEMA3A and E198K-SEMA3D (only ganglionic tissues). Unfortunately, no material from patients with R634Q-SEMA3D mutations was available. Four μm thick paraffin sections were dewaxed in xylene and rehydrated in a series of graded alcohols. Endogenous peroxidase activity was blocked with water containing 3% H₂O₂ for 30 minutes. Antigen retrieval was done by microwaving using citrate phosphate buffer (pH 6.0). Sections were incubated at 4°C overnight with the primary antibodies anti-SEMA3A (1:50 dilution, anti-rabbit, AntibodyBcn, Barcelona, Spain) and anti-SEMA3D (1:5 dilution, anti-rabbit, Novus Biologicals, Cambridge, UK). After several washes in Tris buffer, peroxidase-labelled secondary antibodies and 3,3′-diaminobenzidine were applied to develop immunoreactivity, according to manufacturer’s protocol (EnVision; Dako, Glostrup, Denmark). The slides were then counterstained with hematoxylin and mounted in DPX (BDH Laboratories, Poole, UK).

### Results and Discussion

Members of SEMA class III protein subfamily are inhibitory axon guidance molecules, which could help to determine the axonal projection patterns of several neurons and ganglia throughout the central and peripheral nervous system [32]. Moreover, SEMA3A has been related to HSCR through different approaches [13,30,31].

We have detected a total of 56 sequence variants in the mutational screening of SEMA3A and SEMA3D genes (Tables 1 and 2). The most interesting finding among these results was the detection of four missense variants (SEMA3A: A131T, S598G; SEMA3D: E198K, R634Q) in heterozygosis. Two of them were already described (A131T-3A with a MAF = 0.01 and R634Q-3D with a MAF = 0.0093) in public databases (1000 Genomes, EVS and NCBI) but the other two (S598G-3A and E198K-3D) were novel. All four variants are located in the coding region of both SEMA proteins and found exclusively in HSCR series but absent in the control population (Table 3). They were detected with a mutational frequency range of 0.005%–0.011%, which was comparable with the percentages of SEMA4 mutations previously described in Caucasian population (0.004%–0.011%) [13].
There are several evidences that led us to propose the four missense variants in two SEMA genes (SEMA3A: A131T, S598G; SEMA3D: E198K, R634Q) as mutations associated to HSCR. First, the mutations were detected in two main protein domains (Figure 1): the SEMA domain, which is important for protein-protein interaction (A131T-SEMA3A and E198K-SEMA3D) and the domain implicated in receptor binding (S598G-SEMA3A and R634Q-SEMA3D). Second, in silico predictions (SIFT, Polyphen) had shown the damaging effect of those four mutant proteins. Here, we have also partially characterized the mutations A131T-SEMA3A and S598G-SEMA3A and E198K-SEMA3D by immunohistochemistry. In histological analyses, both SEMA3A (Figure 2) and SEMA3D (Figure 3) were expressed in the ganglion cells of the myenteric and submucosal plexuses as well as in the smooth muscle layers of the ganglionic colon of our patients and in the control samples.

Regarding the A131T-SEMA3A mutation, we obtained both ganglionic and aganglionic segments from the patient. A highly expressed mutant SEMA3A protein in the smooth muscle layers in the aganglionic segment was detected. This increased amount of protein seemed to be higher than the increase found in both the corresponding ganglionic segment and the control sample (Figure 4). This is in accordance with previously published results for the wild type SEMA3A protein, that it is upregulated in the aganglionic colon tissue [30].

In the case of S598G-SEMA3A and E198K-SEMA3D mutants, a more intense immunostaining in all ganglionic tissue layers in comparison with the control sample was detected (Figure 4).

Based on the increased amount detected of both SEMA3A and SEMA3D proteins in colon tissue of HSCR patients, we propose that those variants results in the accumulation of SEMAs proteins leading to impaired enteric axonal projection during NCCs migration and/or differentiation, with functional consequences in ENS formation. Given the role of SEMAs as crucial modulators during embryonic development [32], abnormal SEMAs expression or function may have a pleiotropic effect, as it has been observed in mouse null mutants of genes encoding SEMA3A and its receptor NP1 [33]. This hypothesis fits with the lack of null mutations in our mutational screening, being more plausible that the missense mutations detected on SEMA3A and SEMA3D genes cause a more subtle effect on protein function than null mutations do. Thus, we propose that the genetic background of the individual in combination with the presence of those hypomorphic mutations in the SEMA genes would generate the specific phenotype observed in our patients, in accordance with the additive model previously proposed for HSCR [34]. We failed to detect any other coding mutation in the previously associated HSCR genes in the patients with SEMA3A mutations (data not shown), except for RET and EDNRB genes (Table 3), although we cannot discard the contribution of additional mutational events in still unidentified HSCR genes.

It is worth of mention the high incidence of co-occurrence of coding RET mutations and SEMA3A mutations in our series of patients, as 50% of the patients with mutations on this locus present coding mutations in RET. All patients carrying mutations either in SEMA3A or SEMA3D genes have inherited
Figure 2. Immunostaining of Semaphorin 3A in colon from controls and HSCR patients. The SEMA3A staining illustrated that the expression was present at smooth muscle (D, E, F) and submucous (G, H, I) layers, as well as in myenteric (J, K) and submucous plexuses (M, N) either in normal colon (A, D, G, J, M) and patients with A131T-3A (B, E, H, K, N) and S598G-3A mutations (C, F, I). The FFPE tissue block from patient with S598G had no all tissue layers. Scale bars: A–C = 200 μm and the rest of pictures = 10 μm.

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It is well established that activation of \textit{RET} is essential for proliferation, migration and differentiation of enteric neural precursors [35]. The vast majority of enteric neurons and glial cells arise from vagal NCCs, although sacral NCCs also give rise to some neurons and glia, principally in the distal hindgut, after the caecum [36,37]. Migration of enteric precursors derived from vagal NCCs, within the colon has been shown to depend mainly on the proliferation at the migration wave front, rather than on the presence of factors that promote migration [38]. For that reason, mutations in \textit{RET} cause a failure of those cells to colonise the distal colon in an appropriate number to form the enteric ganglia, leading to HSCR [2]. The expression of \textit{GDNF} in the hindgut is lower than in the caecum [39] and therefore many authors have proposed that migration and proliferation of sacral NCCs is less dependent on \textit{GDNF}. In addition, sacral NCCs are able to migrate through pre-caecum colon in a slower manner compared to vagal derived cells [22,39–42]. In such scenario, we propose that the absence of enteric ganglia in the distal part of the bowel, due to a fail of vagal NCCs, could be rescued by the migration and differentiation of sacral NCCs. It has been demonstrated that \textit{SEMA3A} regulates the entry of extrinsic axons into the hindgut and also sacral NCCs, as they accompany those axons when migrating to the colon. In fact, defects on \textit{SEMA3A} expression impair the entry of sacral enteric precursors in the hindgut [22]. For that reason, we suggest that a fail on sacral NCCs to colonize the hindgut in the appropriate moment, due to the presence of mutations in \textit{SEMA3A}, would results in a more dramatic phenotype in patients also harbouring mutations in \textit{RET}, as \textit{SEMA3A} has no effects on vagal NCCs [22]. In agreement with this hypothesis, we did not observe an enteric phenotype in family members of our HSCR patients with isolated mutations in \textit{RET} or \textit{SEMA3A}. Segregation analysis for these mutations in both genes together with the available data of mutations in other genes associated to HSCR, led us to speculate with the potential synergistic effect of mutations in both \textit{RET} and \textit{SEMA} genes. We believe that the major contributing mutation could be either on \textit{RET} or \textit{SEMA}, or in both genes at the same time alone or together with some other mutations in unknown genes, due to the complexity of the genetic basis of HSCR and the additive model proposed for the disease. Future analyses will be needed to clarify the role of both genes.

In summary, in this study we have analyzed three aminoacidic substitutions, A131T-\textit{SEMA3A}, S598G-\textit{SEMA3A} and E198K-\textit{SEMA3D}. These variants result in an increase of \textit{SEMA} proteins levels in the HSCR colon tissue. Our findings further support the functional implication of \textit{SEMA} as signalling molecules in the development of ENS and indicate a synergistic effect of mutations in both \textit{SEMA} and \textit{RET} genes, to influence the phenotype of our HSCR patients.

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Author Contributions

Conceived and designed the experiments: BLT RF AT SB. Performed the experiments: BLT RF AT. Analyzed the data: BLT RF AT DIS SB. Contributed reagents/materials/analysis tools: BLT AT JCdA. Wrote the paper: BLT RF AT CMV GA SB.

Figure 3. Immunostaining of Semaphorin 3D in colon from controls and HSCR patients. The SEMA3D staining illustrated was present at smooth muscle (C, D) and submucous (E, F) layers, as well as in myenteric (G, H) and submucous plexuses (I, J) either in normal colon (A, C, E, G, I) and patients with E198K-3D mutation (B, D, F, H, J). Scale bars: A, B = 200 µm and the rest of pictures = 10 µm.

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Figure 4. Immunohistochemical detection of A131T-SEMA3A, S598G-SEMA3A and E198K-SEMA3D mutant proteins in FFPE colon samples. Immunohistochemistry analysis of A131T-3A, S598G-3A and E198K-3D mutant proteins in smooth muscle layer of control samples (3A: A, D; 3D: H, K) and colon tissue from patients with A131T-3A (ganglionic: B, E; aganglionic: C, F), S598G-3A (G, J) and E198K-3D mutations (I, L) was performed. Scale bars: A, B, C, G, H, I = 200 μm and the rest of the pictures = 10 μm.

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