New roles of *EDNRB* and *EDN3* in the pathogenesis of Hirschsprung disease

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Purpose: Hirschsprung disease is characterized by the absence of intramural ganglion cells in the myenteric and submucosal plexuses within distal intestine, because of a fail in the enteric nervous system formations process. Endothelin-3-endothelin receptor B signaling pathway is known to play an essential role in this process. The aim of this study was to evaluate the implication of the *EDN3* and *EDNRB* genes in a series of patients with Hirschsprung disease from Spain and determinate their mutational spectrum. **Methods:** We performed the mutational screening of both genes in 196 patients with Hirschsprung disease using denaturing high-performance liquid chromatography technology. A case-control study using TaqMan Technology was also carried out to evaluate some common polymorphisms and haplotypes as susceptibility factors for Hirschsprung disease. **Results:** Besides several novel mutations in both genes, we found a truncating mutation in an alternative isoform of *EDNRB*. Interestingly, we obtained an overrepresentation of a specific *EDN3* haplotype in cases versus controls. **Conclusions:** Our results suggest that the isoform *EDNRBΔ3* might be playing an essential role in the formation of enteric nervous system. In addition, based on the haplotype distribution, *EDN3* might be considered as a common susceptibility gene for sporadic Hirschsprung disease in a low-penetrance fashion. ***Genet Med*** **2010:12(1):39–43.**

Key Words: *Hirschsprung disease, enteric nervous system, EDNRB, EDN3, complex disease*

Hirschsprung disease or aganglionic megacolon (HSCR, OMIM 142623) is a developmental disorder of variable penetrance and expressivity, male predominance, with an incidence of 1 of 5000 newborn human infants.[1,2] It is characterized by the absence of intramural ganglion cells in the myenteric and submucosal plexuses within a variable portion of the distal intestine. The most widely accepted etiopathogenetic hypothesis for HSCR is based on a defect of craniocaudal migration of neuroblasts, originated from the neural crest, to the developing gut, followed by cell proliferation and differentiation. This process is crucial for the proper enteric nervous system (ENS) formations.

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There are two major signaling pathways involved in the ENS formation, which are known to be involved in HSCR: the REarranged during Transfection (RET)-cell line-derived neurotrophic factor and the endothelin-3 (EDN3)-endothelin receptor B (EDNRB) systems.[2] Although mutations in the coding and/or noncoding regions of *RET* seem to be a necessary feature in a great portion of cases with HSCR,[2–4] *EDNRB* is regarded as the second major causal gene for HSCR.[1,2] In contrast, the gene encoding its ligand, *EDN3,* has been demonstrated to play a minor although significant role in HSCR, with a total of nine mutations being reported so far associated to either isolated or syndromic HSCR (visit HGMD, http://www.hgmd.cf.ac.uk/ac/all.php). *EDN3* gene is more likely to play a major role in syndromic forms of HSCR, such as Shah-Waardenburg syndrome (WS4, OMIM 277580), and to act as a rare susceptibility locus in non-syndromic HSCR.[5–7]

In this study, we report a *EDNRB* and *EDN3* complete molecular analysis in a series of cases with HSCR from Spain which, with the available data from our previous analysis of both *RET* coding mutations and haplotypes,[8,9] means a more comprehensive view of the genetic basis of HSCR disease in our cohort of patients.

MATERIALS AND METHODS

Patients and control subjects

In this study, we have included a total of 196 patients presenting with HSCR (22% female and 78% male patients) and their parents when available. One hundred seventy-six were sporadic cases, whereas 20 were familial cases belonging to 13 different families. Six of those patients presented with associated Down syndrome.

In addition, we have also analyzed a group of 150 normal controls comprising unselected, unrelated, race, age, and sex-matched individuals. An 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.

Mutational screening and analysis of sequence variants

Mutational screening was carried out by denaturing high-performance liquid chromatography in a WAVE DNA Fragment Analysis system (Transgenomic, Omaha, NE). Primers, polymerase chain reaction, and denaturing high-performance liquid chromatography conditions are available on request. Those samples with aberrant wave profiles were subjected to sequence analysis using an automated sequencer ABI 3730 and the software SeqScape Ver.2.5 (Applied Biosystems, Foster City, CA).

A large-scale genotyping of *EDNRB* single nucleotide polymorphism (SNPs) c.484-4125C>T (rs3027111), I187I (rs5349), and c.*1985G>A (rs4885491) and *END3* SNPs c.365 + 7474T>C (rs6064764), c.366-3935C>G (rs171969), and c*231 + 236G>A (rs260741) was performed in our HSCR trios and controls, using Taqman-based techniques for allelic discrimination (TaqMan® SNP Genotyping Assay, Applied Biosystems) and according to manufacturer's recommenda-

Table 1 *EDNRB* sequence variants detected in this study

Nucleotide change	Amino acid change	Exon/intron	Novel/previously described	Allelic frequency in control population (%)
c.-111del C[a]			Novel	0.48
c.-74C>T[a]			Novel	0
c.-67C>A[a]			Novel	0
c.43A>T[a]	K15X*		Novel	0
c.42_45del	L15PfsX29	1	Novel	0
c.159A>G	L53L	1	Novel	0
c.167A>C	K56T	1	Novel	0
c.169G>A	G57S	1	Previously described rs1801710	0
c.343G>A	G115R	1	Novel	0
c.360C>T	S120S	1	Novel	0
c.466C>T	P156S	1	Novel	0
c.552C>T	S184S	2	Previously described rs5348	0
c.561C>T	I187I	2	Previously described rs5349	1
c.731C>A	T244N	3	Previously described rs5350	0
c.732G>A	T244T	3	Novel	0.49
c.802-139A>G		3	Novel	0
c.802-122T>C		3	Previously described rs9530703	6.7
c.831A>G	L277L	4	Previously described rs5351	56.5
c.914G>A	S305N	4	Previously described rs5352	2.25
c.928G>A	A310T	4	Previously described	0
c.951 + 31G>C		4	Novel	0
c.951 + 82C>T		4	Novel	0
c.952-19A>G		4	Novel	0
c.1239C>G	S413S	7	Novel	0
c.*50A>T		7	Novel	0

[a]Annotated from the *EDNRBΔ3*.

tions, in a 7500 Fast Real-Time PCR System (Applied Biosystems). From these results, allelic, genotypic and haplotypic frequencies, and distributions were compared between patients with HSCR and controls, using the SPSS Ver15.0 for Windows. In each analysis, statistical significance was calculated using Pearson's χ^2 test, with statistical significance set at $\alpha = 0.05$. Haplotypes comprising the *EDNRB* and *EDN3* polymorphisms analyzed were generated based on the results of the complete triads when available (patient, father, and mother), which allowed us to reconstruct and compare the transmitted versus nontransmitted alleles. With these results, we proceeded to compare the distribution of haplotypes between the patients with HSCR and controls. Statistical estimates were calculated using the same method explained earlier.

RESULTS

EDNRB mutational screening

The 25 sequence variants found in the mutational screening of *EDNRB* gene are reported in Table 1. Seven of them consisted of noncoding or polymorphic nucleotide substitutions and another seven were silent changes in the coding region, leading to synonymous amino acidic substitutions. More interesting was the finding of six missense *EDNRB* mutations, all of them appearing in heterozygosis (Table 2). Three of these mutations had been previously reported to be responsible for the HSCR phenotype—G57S,[10] A310T,[11] and S305N[12]—although it currently seems that S305N is a rare polymorphism not related to HSCR. The remaining three missense changes were novel variants located in Exon 1: K56T, G115R, and P156S. All the information regarding the phenotype, sex, occurrence, and parental origin of these mutations is provided in Table 2. We have used Polymorphism Phenotyping (http://genetics.bw-h.harvard.edu.pph) and Sorting Intolerant From Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html) tools to examine the potential pathogenicity of these four novel missense *EDNRB* mutations. Both programs predicted a benign effect for the variant K56T, based in the not conserved status of the sequence among different species in this particular position. In contrast, the substitutions of

Table 2 Presumably pathogenic *EDNRB* and *EDN3* mutations[a] detected in isolated HSCR patients

Gene	Patient	Nucleotide change	Amino acid change	Ocurrence[b]/sex	Parent origin of the mutation	Presence in other family members[c]
EDNRB	HSCR 19	c.43A>T[d]	K15X[e]	Sporadic/M	Father	No
	HSCR 6	c.42_45del[d]	L15PfsX29	Sporadic/F	Mother	Yes
	HSCR 140	c.167A>C[d]	K56T	Sporadic/M	Mother	No
	HSCR 61	c.169G>A	G57S	Sporadic/M	Father	No
	HSCR 194	c.343G>A	G115R	Familial/2M	Father	No
	HSCR 113	c.466C>T[d]	P156S	Familial/2M	Father	No
	HSCR 82	c.928G>A	A310T	Sporadic/M	Father	No
EDN3	HSCR 9	c.560insA	T189NfsX63	Familial/M	Mother	Yes
	HSCR 130	c.572_573del[d]	K191RfsX60	Sporadic/F	Father	No

[a]All mutations found were in heterozygous state.
[b]The term "familial" is applied when more than one affected member with Hirschsprung disease is present in the same family. Otherwise, when there is no family history, the cases are considerer as sporadic.
[c]"Presence in other family members" refers to additional family members besides the parent from who the patient inherits the mutation.
[d]Novel mutation.
[e]Annotated from the *EDNRBΔ3* protein sequence.

hydrophobic glycine and proline for basic arginine and polar serine at Positions 115 and 156, respectively were predicted as not tolerated changes in the protein, probably having damaging effects on the protein function.

We have also found a novel frameshift mutation, consisting of a four nucleotides deletion (c.42_45del), which results in a premature stop codon at amino acid position 43, generating a protein with functional domain totally abolished.

We have also performed the mutational screening of an additional exon in the 5′ direction from Exon 1,[13] never previously analyzed in the context of HSCR. The only difference between conventional EDNRB and this transcript variant (*EDNRBΔ3*) would be the presence of 89 additional amino acids at the N terminus region of the protein. We detected three sequence variants at the 5′ untranslated region of this additional exon (Table 1). The most striking finding has been the detection of the coding mutation K15X (c.43A>T) in a sporadic HSCR patient. None of the variants described in this additional exon had been previously reported.

EDN3 mutational screening

Regarding the *EDN3* gene, 11 sequence variants were detected in the screened patients (Table 3). Besides the previously described *EDN3* mutation (c.560insA) found in a multiplex isolated HSCR family,[14] we have identified the variant c.572_573del in Exon 4, which at the protein level is located in the EDN3-like domain of the preproendothelin, but not in the mature endothelin. It was absent in 300 control chromosomes tested and results in an aberrant protein of 249 amino acids residues length, with an anomalous carboxy terminus sequence from Position 190 to the end. The novel mutation c.572_573del was detected in a sporadic nonsyndromic HSCR patient.

Table 3 *EDN3* sequence variants detected in this study

Nucleotide change	Amino acid change	Exon/intron	Novel/previously described	Allelic frequency in control population (%)
c.-248G>A		1	Novel	0.5
c.53–57C>T		2	Novel	4.0
c.365 + 23G>A		2	Previously described rs11570257	4.0
c.366-56G>C		3	Previously described rs11570340	3.3
c.542 + 119C>T		3	Previously described rs11570341	0
c.560insA	T189NfsX63	4	Previously described rs11570344	0
c.572_573del	K191RfsX60	4	Novel	0
c.621-80T>C		5	Previously described rs11570349	0
c.621-30insT		5	Novel	0
c.*240_241del		3′UTR	Previously described rs34516274	28.5
c.*240delC		3′UTR	Previously described rs11475273	15.7

Analysis of the distribution of *EDNRB* and *EDN3* variants and haplotypes in the different groups

We analyzed the allelic and genotypic distribution of three *EDNRB* SNPs in HSCR and controls, and haplotypes comprising them were generated in each group as previously described.[8] No statistical differences were found in the distribution for any of these variants or the haplotypes comprising them. Of note, regarding c.561C>T SNP, no 561T allele was detected among our patients with HSCR+ Down syndrome, in contrast with the previously published data.[15]

Regarding *EDN3*, no statistical differences were found in the allelic or genotypic distribution for SNPs c.366-3935C>G or c.*231 + 236 G>A, neither for the haplotypes comprising them. However, we found a statistically significant association of the c.366 + 7474T allele to HSCR when comparing the allelic distribution in cases versus controls ($\chi^2 = 4.90$, $P = 0.027$). A further analysis on haplotypic data revealed that this difference in allelic distribution seems to be due to an overrepresentation of a specific *EDN3* haplotype (c.365 + 7474T, c.366-3935C, c.*231 + 236A) in the patients group ($\chi^2 = 4.84$, $P = 0.028$).

DISCUSSION

In this report, we present a complete genetic analysis of *EDNRB* and *EDN3* in the context of HSCR in our series of 196 patients.

There are several features that led us to propose the novel *EDNRB* variants c.42_45del, K56T, G115R, and P156S as disease-causing mutations. Deletion c.42_45del causes a frameshift that originates a premature stop codon at an amino acid position 43. As a result, a truncated protein of 42 amino acids length would be generated, lacking all the seven transmembrane domains essential for the protein function. Mutation G57S has been shown to fail at the inhibition of adenylate cyclase, leading to failures in the protein-G–coupled signaling pathway responsible of neural cells differentiation in the bowel.[16] Regarding mutation K56T, located nearby G57S mutation, we think both could have a similar functional consequence for the protein, because of the physical proximity of the amino acids residues. Mutations G115R and P156S sequence comparison shows that the amino acids in both cases are conserved in mammalian EDNRB proteins, as well as the amino acids surrounding these positions, indicating an important role of those regions in the protein function. Interestingly, both mutations G115R and P156S were found in two different multiplex HSCR families and present in all affected members. In addition, for all those patients, we have observed the concurrence of an *EDNRB* mutation and the HSCR-susceptibility *RET* haplotype.[8] Thus, *RET* also determines the susceptibility to HSCR on those patients, supporting again that the presence of additional predisposing genetic events seems to be a necessary feature to cause megacolon.

Here we report, for the first time, a mutational screening of the extra region of this new *EDNRB* transcript variant. One of the most relevant findings has been the detection of the mutation K15X in an alternative spliced isoform of EDNRB protein. Tsutsumi et al.[13] described three different subtypes of cDNA for *EDNRB* gene as a consequence of alternative splicing in the 5′ region of the gene. One of those alternative isoforms (*EDNRBΔ3*) encodes an extra protein sequence of 89 amino acids at the 5′ extreme, but shares an identical coding region with the traditional protein. Tissue-specific expression and specific transcriptional regulatory sequences of *EDNRBΔ3* have to be clarified, but there is evidence of expression in the small intestine and colon.[13] The detection of this mutation, in a sporadic HSCR patient, let us to hypothesize that *EDNRBΔ3* might play an important role in the pathogenesis of HSCR disease, indicating a putative functional implication of this particular isoform in the ENS development, opening a new field of knowledge in this area.

With regard to *EDN3*, the novel mutation c.572_573del was detected in a sporadic HSCR patient also harboring the HSCR-susceptibility *RET* haplotype in heterozygosis, but no other point mutations in other HSCR-susceptibility genes (*GDNF, NTNR, PSPN, ARTN, NTF3, NTRK3, SOX10, PHOX2B, EDNRB,* and *EDN3*). The fact that both mutation on EDN3 and HSCR-susceptibility RET haplotype had been inherited from his healthy father suggests that other unidentified genes or additional factors may contribute to the occurrence of HSCR disease in this patient.

EDN3 is regarded as a rare susceptibility gene for HSCR, because of a limited number of patients who harbor point mutations at this locus, especially in the isolated forms. Interestingly, a reduced expression of EDN3 in the colon of sporadic HSCR patients, both ganglionic and aganglionic, versus controls pointed out the importance of *EDN3* in HSCR.[17] The absence of mutations at the *EDN3* locus in patients with such an aberrant EDN3 expression led authors to propose the existence of unknown factors regulating gene expression. Our results bring a new genetic clue to explain such phenomenon. It is plausible that the association of rs6064764, with HSCR disease might be indicating the existence of linkage disequilibrium with some functional, still unidentified allele of these genes. Another possibility is that this allele might be acting as a functional variant *per se*, leading to a higher predisposition for HSCR, although its precise molecular mechanism remains to be elucidated. In any case, our evaluation of *EDN3* as a susceptibility gene for HSCR using common polymorphisms suggests that this gene might be considered as a common susceptibility gene for sporadic HSCR in a low-penetrance fashion, more than a minor gene for this disease as it is currently considered.

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