November 2006 · Vol. 8 · No. 11

A complex additive model of inheritance for Hirschsprung disease is supported by both RET mutations and predisposing *RET* haplotypes

Macarena Ruiz-Ferrer [1], Raquel M. Fernández, PhD[1], Guillermo Antiñolo, MD, PhD[1], Manuel López-Alonso, MD, PhD[2], Charis Eng, MD, PhD[3], and Salud Borrego, MD, PhD[1]

Purpose: The *RET* proto-oncogene is considered to be the major susceptibility gene involved in Hirschsprung disease. Traditional *RET* germline mutations account for a small subset of Hirschsprung disease patients, but several studies have shown that there is a specific haplotype of *RET* associated with the sporadic forms of Hirschsprung disease. We have investigated for *RET* germline mutations and analyzed the *RET* haplotypic distribution in carriers versus noncarriers of *RET* germline mutations. **Methods:** We have screened the coding region of *RET* in 106 Spanish Hirschsprung disease patients using dHPLC technology. Statistical comparisons of the distribution of *RET* haplotypes between sporadic patients with and without a *RET* germline mutation were performed. **Results:** Nine novel germline mutations and one previously described were identified. A significant over-transmission of the "Hirschsprung disease haplotype" was detected when comparing transmitted versus nontransmitted alleles in the group of Hirschsprung disease triads without mutation. However, no distortion of the transmission of alleles was found in the group of mutated families. **Conclusions:** These results would be concordant with a complex additive model of inheritance. The whole findings seem to suggest that low-penetrance mutations would be necessary but not sufficient and the additional presence of the "Hirschsprung disease haplotype" could contribute to the manifestation of the disease. ***Genet Med* 2006:8(11):704–710.**

Key Words: *Hirschsprung, haplotype, RET proto-oncogene, germline mutation, dHPLC*

Hirschsprung disease (HSCR, OMIM 142623) or aganglionic megacolon, occurring in approximately 1 in 5000 live births, is a neurocristopathy defined by the absence of intramural ganglion cells in the myenteric and submucosal plexuses along variable lengths of the gastrointestinal tract, resulting in functional intestinal obstruction. HSCR most commonly presents in sporadic cases, although it can be familial and may be inherited as autosomal dominant or autosomal recessive, with reduced penetrance, male predominance, and variability of the length of the aganglionic segment.[1,2] In addition HSCR is frequently associated with many other neurocristopathies and chromosomes abnormalities.[2]

To date, at least 10 genes have been described as associated with sporadic or syndromic forms of HSCR. These "HSCR genes" are generally related to the developmental program of neural crest cells, such as the *RET* proto-oncogene, glial cell line derived neurotrophic factor (*GDNF*), neurturin (*NTN*), endothelin 3 (*EDN3*), endothelin receptor β (*EDNRB*), endothelin converting enzyme 1 (*ECE1*), transcriptional factors *SOX10* and *PHOX2B*, Smad interacting protein 1 (*SIP1*) and *KIAA1279*.[2–5] However, undoubtedly, the major susceptibility gene for HSCR is the *RET* proto-oncogene (OMIM 164761), which encodes a receptor tyrosine kinase expressed in tissues and tumors derived from the neural crest and neuroectoderm.[2] This receptor consists of a 1114 amino acid transmembrane protein presenting an extracellular ligand-binding region, which includes a cadherin-like domain and a cysteine-rich region, and a conserved intracellular tyrosine kinase domain.[6] It has been shown that RET is the signaling component of a multisubunit complex acting as a receptor for growth factors of the GDNF family, including GDNF, neurturin (NTN), artemin (ARTN), and persephin (PSPN). All these factors activate RET via four different glycosyl phosphatidylinositol-linked GFRα receptors named GFRα 1 to 4 and encoded by the *GFRA1-4* genes, respectively.[7]

Loss of function germline mutations in *RET* have been reported to account for up to 50% of familial HSCR cases and between 3% and 35% of sporadic cases.[8–13] These mutations are distributed throughout different domains of RET and biochemical studies have shown variable consequences such as misfolding, failure to transport the protein to the cell surface, or even a complete abolished biological activity. In summary,

From the [1]Unidad Clínica de Genética y Reproducción, Hospitales Universitarios Virgen del Rocío, Seville, Spain; [2]Servicio de Cirugía Infantil, Hospitales Universitarios Virgen del Rocío, Seville, Spain; and [3]Genomic Medicine Institute, Department of Hematology and Medical Oncology, The Cleveland Clinic Lerner Research Institute, Cleveland, Ohio.

Salud Borrego, MD, PhD, Unidad Clínica de Genética y Reproducción Hospitales Universitarios Virgen del Rocío Avenida Manuel Siurot s/n 41013 Seville, Spain.

Submitted for publication March 13, 2006.

Accepted for publication August 14, 2006.

DOI: 10.1097/01.gim.0000245632.06064.f1

the results derived from in vitro analyses suggest that haploinsufficiency is the most likely mechanism for the *RET* mutations associated to HSCR. It is hypothesized that this haploinsufficiency might produce a decrease of all RET-dependent signaling pathways, leading to defects of enteric nervous system development during embryogenesis, resulting in the HSCR phenotype.

Because traditional *RET* germline mutations accounted for such a small subset of HSCR, we sought to determine if other more common susceptibility factors existed which would predispose to the majority of HSCR. When the variant A45A (exon 2), was found to be highly associated with a large subset of nonfamilial HSCR,[14,15] it prompted us to hypothesize that this was the associated variant itself or that A45A was in linkage disequilibrium with the putative low penetrance susceptibility locus that would account for the majority of HSCR. Subsequently, we showed that A45A anchors ancestral haplotypes in linkage disequilibrium with such a putative common founding susceptibility locus estimated to be 22–50 kb upstream.[16] This was the starting point which led to the identification of a predisposing haplotype located at the 5′region of *RET*, whatever the ethnic background, with some functional data suggesting a putative role of the promoter variants −200A>G and −196C>A.[17–22]

In the present report, we report for the first time a mutational screening in the *RET* proto-oncogene in a series of HSCR patients from Spain, and with the available data derived from previous studies,[19] we have analyzed and compared the *RET* haplotypic distribution in carriers versus noncarriers of traditional *RET* germline mutations.

MATERIALS AND METHODS

Patients and control subjects

In this study, we have included a total of 106 HSCR patients from Spain (17% female, 83% male). Eighty-three of these patients were sporadic cases, while 23 were familial cases belonging to 13 different families. In order to define the exact HSCR phenotype in our patients, we have used the criteria recommended by Chakravarti et al.[2] Following these criteria, 47 cases were catalogued as short-segment HSCR (52%), 20 cases as colonic-segment forms (22%), 14 cases as long-segment (15%), and 10 cases presented with total colonic aganglionosis (11%). For subsequent analyses we included the colonic-segment cases into the group of long-segment forms.

In addition, we have also analyzed a group of 100 normal controls comprising unselected, unrelated, race, age, and sex-matched individuals. All of them were healthy voluntary donors, who came to the Hospital for other reasons and did not present any symptom suggestive of HSCR.

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.

PCR and dHPLC analysis

Genomic DNA was extracted according to standard protocols.[23] We screened all the *RET* exons, including intron/exon boundaries. Primers used for polymerase chain reaction (PCR) amplification and annealing temperatures are summarized in Table 1.

The dHPLC analyses were carried out on an automated HPLC device equipped with a DNA separation column using the WAVE DNA Fragment Analysis system (Transgenomic). PCR products were denatured for 3 min at 95°C and then gradually reannealed by decreasing sample temperature from 95°C to 65°C over a period of 30 min. Five μL of PCR products were then injected onto the DNASeq cartridge and eluted at a constant flow rate of 0.9 mL/minute through a linear acetonitrile gradient. DNA fragments were detected by the system's UV detector and analyzed as chromatograms. Oven temperature for optimal heteroduplex separation under partial DNA denaturation was determined for each amplicon using the WAVEMarker software (Table 1).

In addition, those exons with an aberrant wave profile were also screened by dHPLC analysis in a group of 100 normal controls, in order to determine if the variants identified were mutations or just common polymorphisms.

Sequence analysis

Those samples with aberrant wave profiles were subjected to sequence analysis. PCR products were purified using the Quiaquik gel extraction kit (Quiagen) and sequenced using the DYEnamic™ ET Dye terminator cycle sequencing kit (Amersham Biosciences) and a MegaBACE™ automated sequencer (Amersham Bioscience). The alignment of our results with the sequences provided for *RET* (RefSeq NM_020975.3) was carried out using the software BioEdit Sequence Alignment Editor version 5.0.9.

When a novel *RET* variant was identified in a proband, all the available family members were sequenced for the appropriate exon in order to define their molecular status regarding such variant.

Statistical analysis

The distribution of *RET* haplotypes comprising the 2 variants within the promoter region (−200A>G and −196C>A) was analyzed and compared between sporadic patients with and without a *RET* germline mutation. In addition, we searched for a distortion of the transmission of alleles from parents to affected HSCR children, in both mutated and nonmutated groups. The genotyping data were available from previous reports.[19] Comparisons were performed using the χ^2 analysis with Yate's correction, with statistical significance at $P < 0.05$.

RESULTS

We have analyzed the coding region of *RET* in 106 Spanish HSCR patients using dHPLC technology. A total of 23 aberrant

Table 1
Amplification and dHPLC conditions for *RET* genomic sequence analysis

Exon/ Primers	Annealing Temperature (°C)	dHPLC Temperature (°C)	% B
1F CTAGCCGCAGTCCCTCCA	62	68.6	51.1
1R ACAGAAAGGCGATTCTGAAC			
2F CCTTATTCTCACCATCCCTC	56	60.5	56.8
2R AGTGTCAGCGGCTGTGATAA		64	
3F CCCCACAGACCTGACTTCTCT	67	62.8	56.6
3R AAGACCAGCAGTAGCAGGCA		63.9	
4F GCCCCTGTCTGCTTGGTGC	64	65	55.8
4R GGACACTAAACCGACCGAG		67.4	
5F ACTGACCAACGCCCTCTGC	60	63	53.9
5R GCACCTCATTTCCTGGGGG		65.3	
6F ATTGTTGTGCCCCTACCTG	58	64	54.3
6R CCCCAGACAGGCAATAGGTA			
7F TCTACCCTCAGGCCATTACA	56	61.7	58.3
7R AACCATTTACTGCTGGGTCA		63	
8F TGGTGCTGTTCCCTGTCC	62	65	53.5
8R CCACCGGTGCCATCGCCCCT			
9F AGCCTGCTGTGTGTCCTGTG	60	62	48.2
9R CCATGCCCTGATTAAACCCT		64	
10F GCGCCCCAGGAGGCTGAGTG	68	64.7	50
10R GGTGGTGGTCCCGGCCGCC			
11F TGCCAAGCCTCACACCAC	67	64	55.1
11R TCCCTCCCTGGAAGGCAG		64.8	
12F TTCTTCCTCCCCTGTCATCCT	62	59.3	54.9
12R TCTTCAGGGTCCCATGCTG		65	
13F GCAGGCCTCTCTGTCTGAACTT	65	63.5	54.6
13R GGAGAACAGGGCTGTATGGA			
14F AAGACCCAAGCTGCCTGAC	65	62	54.6
14R GCTGGGTGCAGAGCCATAT		65.6	
15F GTCTCACCAGGCCGCTAC	60	62.2	54.5
15R ATGGTGCACCTGGGATCCCT		64.4	
16F AGGGATAGGGCCTGGCCTTC	66	58.6	50.3
16R TAACCTCCACCCCAAGAGAG			
17F CACTGGTCCTTTCACTCTCT	59	62	52.2
17R GGGAGGGAATGCACACAGAT		63.4	
18F TGTGGTGGGCTGTCCTTCTG	63	61.6	52.4
18R CTGGGGTGAGGCTGGAGTCT		62.9	
19F AGTGACCGGCCATCTCTGT	64	58.1	52.2
19R ATAGTGCAAAGGGGACAGC		62.2	
20F AGTTTTGGTTCTTCAGTGC	56	58	53.6
20R GACTTTCCATTCTCAGCAT			
21F GCTTCTTGTCATTCTTCATTGC	61	61	53.7
21R CTTGGCCTCACAAAATGC			

wave profiles were detected and subsequent direct sequence analysis revealed 13 previously described polymorphisms and 10 different germline mutations.

Among the common polymorphisms detected, 7 were located in coding region, such as A45A (exon 2, rs1800858), V125V (exon 3, rs1800859), A432A (exon 7, rs1800860), G691S (exon 11, rs1799939), L769L (exon 13, rs1800861), S836S (exon 14, rs1800862) and S904S (exon 15, rs1800863); the remaining 6 were localized in intronic regions, such as IVS1 + 53G>A (intron 1, rs12267460), IVS2 + 9G>A (intron 2, rs2435351), IVS4 + 48A>G (intron 4, rs2435352), IVS12 + 47C>T (intron 12, rs760466), IVS14-24G>A (intron 14, rs2472737) and IVS19 + 48A>G (intron 19, rs2075912). All these polymorphisms had been previously evaluated as susceptibility factors for HSCR by cases-controls studies.[14,15,24]

Regarding the *RET* mutations identified, all of them were novel except for one that had been previously described by Wu et al.[25] Both their nature and location varied widely (Figs. 1 and 2) and consisted of 2 insertions (both in exon 12), 2 splicing mutations (introns 6 and 13), and 6 nucleotide substitutions which include five missense (exons 3, 4, 6, 13 and 19) and a double change resulting in a silent and a nonsense replacements (exon 10) (Table 2). All the mutations found were shown to be absent in the 100 healthy controls tested. As shown in Table 2, these 10 mutations were detected in 11 independent families, being one of them (A373V, exon 6) present in 2 different pedigrees.

Of the 13 families with familial cases, 3 were mutation positive (Fig. 3), which corresponds to a frequency of 23%. However and surprisingly, in one of these families (HSCR 3), we observed that only one of the two affected members harbored the mutation Y791N, which had been inherited from his unaffected mother and was also present in other apparently healthy family members. Because codon 791 is related to FMTC/MEN 2A syndromes, all the carriers of the Y791N mutation were examined for clinical and biochemical symptoms of MEN 2 but they were found to be negative.

Among the 83 patients with sporadic HSCR, 8 (10%) were shown to carry *RET* germline mutations. Only the 2 insertions detected (740insC and 756insT) were de novo mutations, while in the 6 remaining cases they were inherited from one of their unaffected parents and frequently appeared in other healthy relatives, which is a relatively common feature among complex diseases.

We analyzed a possible correlation between the presence of *RET* germline mutations and the length of aganglionic segment in our series. We verified that the frequency of *RET* mutations was higher among patients with either long-segment or total colonic aganglionosis than in patients with a short-segment form. More specifically, such *RET* mutations were found in 4/47 short-segment cases (8.5%), 4/34 long-segment cases (11.8%), and in 4/10 patients presenting with total colonic aganglionosis (40%).

Finally, with the available data from a study previously reported,[19] we proceeded to analyze the distribution of *RET* haplotypes, comprising variants at the promoter region of the

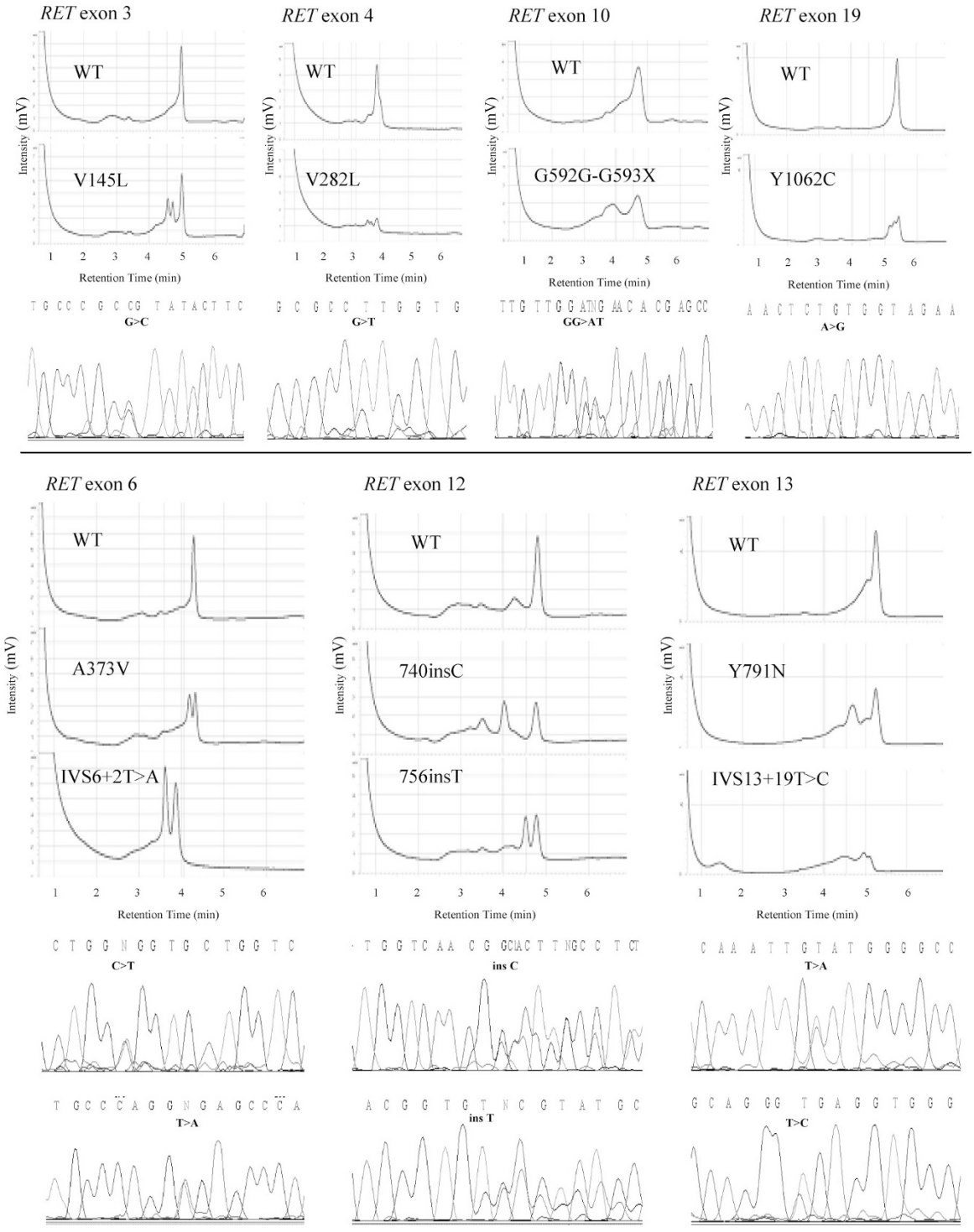

Fig. 1. Denaturating high performance chromatography elution profiles and sequences analysis of the *RET* mutations in HSCR patients.

gene (−200A>G and −196C>A). Based on the presence or absence of *RET* germline mutations, we divided our series of sporadic patients into two groups (those with traditional germline *RET* mutations and those without) and made comparisons between them. Although the frequencies of each of the 3 haplotypes found were slightly different in families with traditional *RET* mutations compared to those without mutations (AC: 50% vs. 62%, GC: 29% vs. 22%, GA: 21% vs. 15%), such differences did not reach statistical significance ($\chi^2 = 0.84$, $P = 0.55$).

Parental haplotypes were examined in the context of the affected children´s haplotypes, so that transmitted and non-transmitted haplotypes were noted and their frequencies compared in each of the groups (Table 3). Similar distortion values

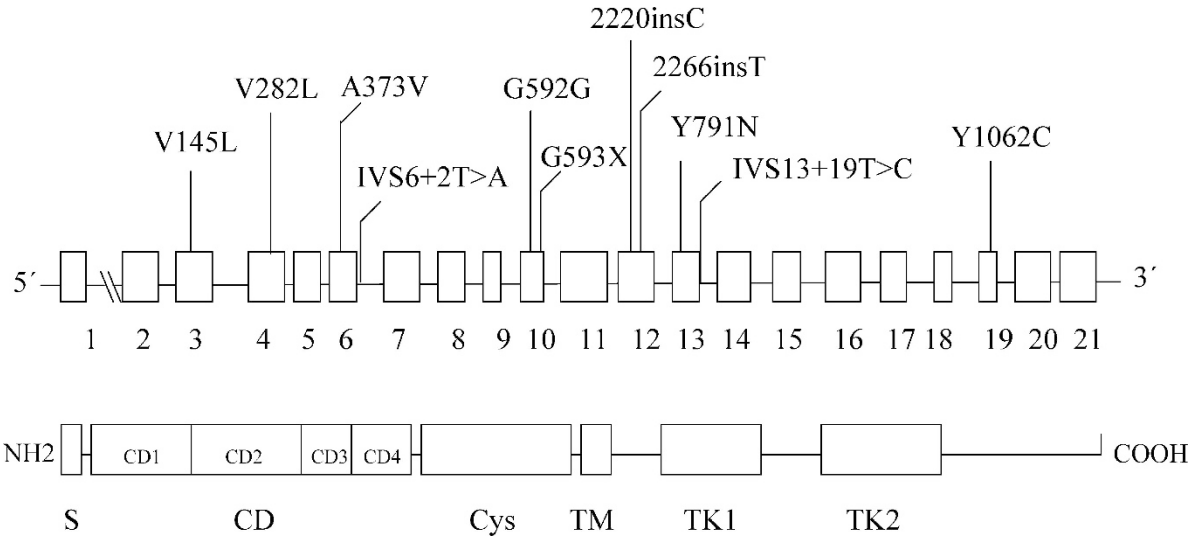

Fig. 2. Schematic representation of the *RET* gene, the encoded protein, and the HSCR mutations identified in this study. The corresponding functional domains of the RET protein are indicated: S, signal peptide; Cd, cadherin-like domain; TM, transmembrane domain; Cys, cysteine-rich domain; TK, tyrosine kinase domain 1 and 2.

Table 2
RET germline mutations identified in HSCR patients

Patient	Familial/ sporadic [a]	Exon/ intron	Nucleotide change	Amino acid change	Parent origin of the mutation	Presence in other family members[b]	Haplotype	Length of aganglionosis
HSCR 22	Sporadic	3	c433G>C	V145L	Mother	No	AC/AC	TCA
HSCR 128	Familial	4	c844G>T	V282L	Mother	No		Short-segment
HSCR 1	Sporadic	6	c1118C>T	A373V	Mother	No	AC/GC	Long-segment
HSCR 75	Sporadic	6	c1118C>T	A373V	Father	Yes	GC/GA	Long-segment
HSCR 9	Familial	6	IVS6 + 2T>A		Mother	No		Long-segment,TCA
HSCR 74	Sporadic	10	c1776 − 7GG>AT	G592G	Father	No	AC/AC	TCA
				G593X	Father	No		
HSCR 27	Sporadic	12	c2220insC	740insC	de novo	No	GC/GA	TCA
HSCR 39	Sporadic	12	c2266insT	756insT	de novo	No	GC/GA	Long-segment
HSCR 3	Familial	13	c2371T>A	Y791N	Mother	Yes		Not available
HSCR 8	Sporadic	13	IVS13 + 19T>C		Mother	Yes	AC/GA	Short-segment
HSCR 70	Sporadic	19	c3185A>G	Y1062C	Father	Yes	AC/GC	Short-segment

[a] 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 considered as sporadic.
[b] "Presence in other family members" refers to additional family members besides the parent from who the patient inherits the mutation.

to those previously published were obtained for the group of patients without *RET* mutation, supporting again the association of the AC haplotype with HSCR. However, interestingly no distortion of the transmission of alleles from parents to affected offspring was found in the group of triads with a *RET* mutation, which would support a major role for such mutations in the pathogenesis of the disease.

DISCUSSION

Several studies in different populations have identified a variety of mutations in different domains of the *RET* proto-oncogene associated with HSCR. It was demonstrated that the molecular mechanism of RET dysfunction highly depends on the location of the mutation. While HSCR mutations detected in the extracellular domain of RET affected the protein folding, maturation and intracellular transport to the plasma membrane, mutations in the tyrosine kinase domain resulted in impairment of its kinase activity or interference with its binding to cytosolic proteins which would be critical to signaling events.[26] This is the first study in which a complete *RET* mutational screening is reported in a series of consecutive HSCR cases coming from Spain. We have identified 10 germline mutations among 106 patients, resulting in a mutation frequency

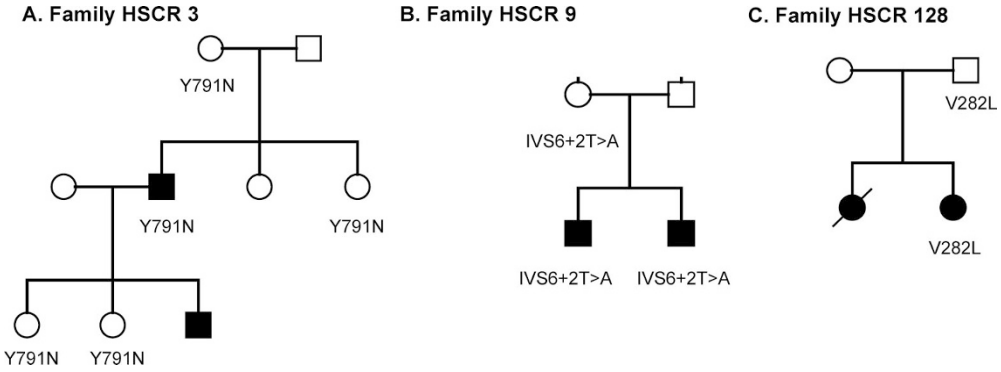

Fig. 3. Pedigrees of familial HSCR cases with mutations in the *RET* proto-oncogene.

Table 3
Distribution of *RET* transmitted and nontransmitted haplotypes in the groups of HSCR patients with and without traditionl *RET* mutations

	HSCR patients withouttraditional RET mutations		HSCR patients withtraditional RET mutations	
	Transmitted alleles	Nontransmitted alleles	Transmitted alleles	Nontransmitted alleles
AC	105	32	7	3
GC	36	64	4	8
GA	27	61	3	3
	$\chi^2 = 59.57, P < 10^{-9}$		$\chi^2 = 2.93, P = 0.23$	

Haplotypes were constituted by the combination of the two variants located within the promoter region of RET (−200A>G and −196C>A).

of 10% in sporadic patients and 23% in familial cases. Our frequencies are therefore concordant with previous studies that report values of up to 50% in familial forms and 3–35% in sporadic cases.[2,9,10,12,22]

We have found three missense mutations affecting the extracellular domain of *RET*, V145L, V282L and A373V, which might act affecting the protein folding, maturation or intracellular transport to the plasma membrane, as it has been previously proposed. Of note, V145 is one of the high conserved residues in the consensus sequence of the cadherin-like domain 1 (CLD1), having a structural role in one of the hydrophobic cores present in the extracellular region of the protein.[27] The rest of missense mutations are located in the intracellular tyrosine kinase domain, being especially interesting a replacement of tyrosine in position 1062 for cysteine. This mutation had been previously described by Wu et al. in identical twin brothers.[25] Tyrosine 1062 represents an autophosphorylation site of the RET protein and is a major binding site for the Shc adaptor proteins that are involved in the signaling pathways triggered by RET.[28] Consequently, several downstream pathways would be disturbed with the presence of the conservative mutation Y1062C.

Also worthy of note is the finding of a double sequence variation in exon 10 (c.1776−7GG>AT) which generates two different replacements, the silent G592G and the nonsense G593X. Such nonsense mutation would result in the protein truncation at the transmembrane and tyrosine kinase domains, which may cause the loss of protein expression in the plasma membrane.

Finally two intronic mutations were also localized, IVS6 + 2T>A and IVS13 + 19T>C. The first mutation in intron 6 involves the second base following the exon, abolishing the canonical "gt" donor site necessary for correct splicing. On the other hand, in silico studies predicted that mutation in intron 13 also abolishes a donor splice site. Therefore, we have arguments enough to postulate that both are splice site mutations capable of causing aberrant splicing.

Analysis of parental DNA in sporadic cases showed that all these mutations previously mentioned are inherited from either the unaffected mother or father, and also appeared in apparently healthy siblings. Therefore, expression and penetrance of the disease in *RET* mutation carriers were variable within HSCR pedigrees, according to a complex model of inheritance. In contrast, the two insertions are both de novo mutations, thus suggesting that they could be directly related with the disease phenotype. The insertions occurred in exon 12 and are expected to abolish tyrosine kinase activity.

It is also interesting to point out that, at least 8/12 patients with *RET* mutations (67%) presented long-segment/TCA phenotype. This finding is in accordance with those previously reported where, mutations in *RET* are associated with long-segment phenotype more frequently than the short-segment phenotype.[8–13]

Because of the recent discovery of a predisposing haplotype for HSCR located at the 5′region of *RET* we have also proceeded to analyze the distribution in carriers and noncarriers of germline *RET* mutations. In a previous report we had shown an association of the combination −200A/−196C to the sporadic forms of HSCR in our series.[19] This association was supported not only by the results from comparative studies between our HSCR patients and normal controls, but also from the TDT analysis in the HSCR triads. Moreover, luciferase expression assays revealed a significantly depressed activity for the HSCR-linked haplotype at −200/−196 in comparison with other combinations associated with controls, which might be indicative of functional mechanism for both promoter variants. Our present results support the previous find-

ings since a significant over-transmission of the risk haplotype was detected when comparing transmitted versus nontransmitted alleles in the group of HSCR triads without mutation. However, no distortion of the transmission of alleles from parents to affected offspring was found in the group of mutated families. Moreover, of note, we have observed that the two patients with de novo mutations, did not present the risk haplotype, suggesting that the mutation alone could play a causative role in the development of the disease phenotype in those patients. By contrast, except for one patient, the remaining sporadic cases presented the *RET* mutations in trans with the risk haplotype.

On the other hand, the finding of association to HSCR across the *RET* intron 1 leaded to search for functional elements in such region.[29] Emison et al. showed that a common noncoding *RET* variant (rs2435357, IVS1 + 9277T>C) within a conserved enhancer-like sequence in intron 1 was significantly associated with HSCR susceptibility. They also found that the T-allele reduced in vitro enhancer activity markedly, decreasing *RET* transcription. Of note, such variant had been also found to be associated to HSCR in our series.[19] Moreover, we had observed that among the 13 different haplotypes generated in our previous study, the allele IVS1 + 9277T was exclusively present in the "HSCR haplotype" and in complete linkage disequilibrium with the −200A/−196C promoter combination. Therefore, all these data would be concordant with a complex additive model of inheritance in which the confluence of several factors, such as *RET* coding mutations or specific haplotypes, are necessary to produce the phenotype. In these terms, we could justify the presence of *RET* coding mutations in healthy members of the HSCR families: such low-penetrance mutations would be necessary but not sufficient to produce the phenotype, and the additional presence of the "HSCR haplotype" could contribute to the manifestation of the disease. Moreover, mutational events occurring in other candidate genes still unidentified, such those involved in the development of enteric nervous system, may have also a critical role.

ACKNOWLEDGMENTS

We would like to thank the families who participated in the study. This study was partially funded by grants PI040266 and PI051291 from the Fondo de Investigación Sanitaria, Spain.

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