

# Novel mutations at RET ligand genes preventing receptor activation are associated to Hirschsprung's disease

Macarena Ruiz-Ferrer · Ana Torroglosa ·  
Berta Luzón-Toro · Raquel M. Fernández ·  
Guillermo Antiñolo · Lois M. Mulligan · Salud Borrego

Received: 30 July 2010 / Revised: 4 November 2010 / Accepted: 9 December 2010 / Published online: 5 January 2011  
© Springer-Verlag 2010

**Abstract** Hirschsprung disease (HSCR) is a developmental disorder characterized by the absence of ganglion cells along variable lengths of the distal gastrointestinal tract. The major susceptibility gene for the disease is the *RET* proto-oncogene, which encodes a receptor tyrosine kinase activated by the glial cell-derived neurotrophic factor (GDNF) family ligands. We analyzed the coding sequence of *GDNF*, *NTRN*, and, for the first time, *ARTN* and *PSPN* in HSCR patients and detected several novel variants potentially involved in the pathogenesis of HSCR. In vitro functional analysis revealed that the variant R91C in *PSPN* would avoid the correct expression and secretion of the mature protein. Moreover, this study also highlighted the role of both this variant and F127L in *NTRN* in altering RET activation by a significant reduction in phosphorylation. To support the role of *PSPN* R91C in HSCR phenotype, enteric nervous system (ENS) progenitors were

isolated from human postnatal gut tissues and expression of GFR $\alpha$ 4, the main co-receptor for PSPN, was demonstrated. This suggests that not only GDNF and NRTN but also PSPN might promote survival of precursor cells during ENS development. In summary, we report for the first time the association of *PSPN* gene with HSCR and confirm the involvement of *NRTN* in the disease, with the identification of novel variants in those genes. Our results suggest that the biological consequence of the mutations *NTRN* F127L and *PSPN* R91C would be a reduction in the activation of RET-dependent signaling pathways, leading to a defect in the proliferation, migration, and/or differentiation process of neural crest cells within the developing gut and thus to the typical aganglionosis of the HSCR phenotype.

**Keywords** Hirschsprung's disease · Enteric nervous system · Neural crest cells · RET proto-oncogene · GDNF · NRTN · PSPN · ARTN

M. Ruiz-Ferrer · A. Torroglosa · B. Luzón-Toro ·  
R. M. Fernández · G. Antiñolo · S. Borrego (✉)  
Unidad de Gestión Clínica de Genética, Reproducción y Medicina  
Fetal, Instituto de Biomedicina de Sevilla (IBIS), Hospital  
Universitario Virgen del Rocío/CSIC/Universidad de Sevilla,  
Avda. Manuel Siurot s/n,  
41013 Seville, Spain  
e-mail: salud.borrego.sspa@juntadeandalucia.es

M. Ruiz-Ferrer · A. Torroglosa · B. Luzón-Toro ·  
R. M. Fernández · G. Antiñolo · S. Borrego  
Centro de Investigación Biomédica en Red de Enfermedades  
Raras (CIBERER),  
Seville, Spain

L. M. Mulligan  
Department of Pathology and Molecular Medicine,  
Division of Cancer Biology and Genetics,  
Cancer Research Institute, Queen's University,  
Kingston, ON, Canada

## Introduction

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

to be involved in the pathogenesis of HSCR. Among them, the major susceptibility gene for the disease is the *RET* proto-oncogene, although traditional germline mutations in this gene only account for 15–20% of sporadic patients [2]. However, a common *RET* variant within the conserved transcriptional enhancer sequence in intron 1 has been shown to be associated with a great proportion of sporadic cases and could act as a modifier by modulating the penetrance of mutations in other genes and possibly of those mutations in the *RET* proto-oncogene itself [3].

*RET* encodes a receptor tyrosine kinase activated by the glial cell line-derived neurotrophic factor family ligands (GDNF family ligands, GFLs), a neurotrophic factor family comprising four members: GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN). The GFLs function as homodimers and activate *RET* through four different glycosyl phosphatidylinositol-linked co-receptors (GFR $\alpha$ 1–4). Extensive in vitro and in vivo studies has supported that each ligand has a preferred GFR $\alpha$  co-receptor to which the ligand binds with highest affinity and most potently activates RET [4, 5]. Although GFR $\alpha$ 4 has been considered the unique functional co-receptor for PSPN [6, 7], recent studies have demonstrated the ability of this ligand to interact also with GFR $\alpha$ 1 [8]. According to this, a clear cross talk between the different ligands and co-receptor pairs exists. The formation of such multi-subunit complexes promotes the transient dimerization of RET, leading to the autophosphorylation of specific tyrosine residues located in the intracellular domain and the subsequent activation of a wide spectrum of signaling pathways.

RET activation is crucial in ENS development, and both GDNF and NRTN have been demonstrated to promote the survival, proliferation, and differentiation of enteric neurons [9, 10]. Mice lacking *Ret*, *Gdnf*, or *Gfr $\alpha$ 1* share a similar phenotype, showing total intestinal aganglionosis caused by impaired migration of immature enteric neural crest-derived cells, whereas NRTN or GFR $\alpha$ 2 knockout mice show a middle phenotype with moderate deficit of enteric neurons [11–15]. In addition, recent studies suggest that cell death, and not migratory delay of neural crest cells, is the major cause of intestinal aganglionosis in *Ret* hypomorphic mice which reproduces phenotypic features of isolated HSCR in humans [16].

Since the activation of RET requires GFLs, the genes encoding such ligands are excellent candidates for HSCR. In fact, several heterozygous germline mutations in *GDNF* and only one in *NRTN* have already been described in HSCR patients, although generally in combination with *RET* mutations or other genetic alterations [17–22]. In the present report, we have screened the four genes encoding the RET ligands in a cohort of HSCR patients, thereby evaluating *ARTN* and *PSPN* for the first time as suscepti-

bility genes for the disease. We have also provided the first evidence that PSPN could be involved in ENS development as we have shown that GFR $\alpha$ 4 is expressed in human ENS precursors cells isolated from ganglionic gut samples.

## Materials and methods

### Ethical approval

Ethical approval and fully informed consent were obtained from all the participants for surgery, clinical, and molecular genetic studies. The study conformed to the tenets of the Declaration of Helsinki.

### Mutational analysis

A total of 217 patients diagnosed with HSCR (23% women, 77% men) were included in the mutational analysis. One hundred ninety were sporadic cases, while 27 were familial cases belonging to 13 different families. In addition, we have also analyzed a group of 150 normal controls comprising unselected, unrelated, race, age, and sex-matched individuals. Genomic DNA was extracted from peripheral blood leukocytes from patients and healthy controls using standard protocols. The mutational screening of the complete coding sequence of *GDNF*, *NTRN*, *ARTN*, and *PSPN* was carried out by denaturing high-performance liquid chromatography in a WAVE DNA Fragment Analysis system (Transgenomic, Omaha, NE, USA). Those exons with aberrant profiles were subjected to sequence analysis using an ABI Prism<sup>®</sup>3730 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) and the SeqScape<sup>®</sup> v2.5 software (Applied Biosystem).

To predict the putative pathogenic role of novel variants at the protein sequence level, we selected the SIFT, Polyphen, and DiANNA tools (<http://blocks.fhcrc.org/sift/SIFT.html>; <http://genetics.bwh.harvard.edu/pph/>; <http://clavius.bc.edu/~clotelab/DiANNA/>). Novel variants located within the non-coding region were submitted to several Splice Sites and Transcription Factors Binding sequences prediction interfaces such as [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html); [http://www.fruitfly.org/cgi-bin/seq\\_tools/promoter.pl](http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl); and <http://www.ebi.ac.uk/asd-srv/wb.cgi>.

### Generation of human neurospheres

Human postnatal tissues from 13 ganglionic full-thickness gut or five endoscopic gut biopsy samples were obtained at Hospital Universitario Virgen del Rocío of Sevilla from either HSCR children undergoing gut resection surgery or endoscopic investigation for other gastrointestinal disorders.

These samples were incubated in a solution of 0.26 mg/mL trypsin collagenase, 5 mg/mL dispase, 0.28 mg/mL hyaluronidase, 3.3  $\mu$ /mL elastase, and 0.6 mg/mL collagenase in phosphate-buffered saline (PBS) for up to 30 min at 37°C. Digested tissue was triturated and washed, and the cells were cultured in a six-well ultra-low cluster plate. The culture medium was Dulbecco's modified Eagle's medium (DMEM, 1 mg/mL glucose) containing 100 U/mL penicillin, 100 g/mL streptomycin, supplemented with 2 mM L-glutamine (Gibco, Life Technology, Carlsbad, CA, USA), 0.05 mM 2-mercaptoethanol, 1% (v/v) N1 (Sigma Aldrich, Poole, Dorset, UK), 10% (v/v) human serum, 20 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, and 10 ng/mL GDNF (Pepro- tech, London, UK). Experiments were performed between passage 1 and 3.

### Immunocytochemistry

For immunocytochemical studies, neurospheres were seeded onto coverslips fibronectin/poly-D-lysine-coated, and fixed with 4% (w/v) paraformaldehyde in 0.1 M PBS. They were incubated for 1 h in 2.5% (w/v) bovine serum albumin in PBS and with primary and secondary antibodies. After washing, the coverslips were mounted on slides with Fluoro-Gel (EMS, Hatfield, PA, USA) and fluorescent signals were detected using a Leica Spectra confocal microscope. The primary antibodies used were GFR $\alpha$ 4 (goat polyclonal, 1:50; Santa Cruz Biotechnology, Inc.) and RET receptor (rabbit polyclonal, 1:250; Santa Cruz Biotechnology, Inc.). The secondary antibodies used were anti-rabbit IgG labeled with Alexa Fluor 568 (Life Technology) and anti-goat IgG labeled with Cy5 (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA).

### RNA isolation and RT-PCR

Total RNA was isolated from human postnatal gut tissues using  $\mu$ MACS<sup>TM</sup>mRNA isolation kit (Milteny Biotec, Bergisch Gladbach, Germany) and reverse transcription polymerase chain reaction (RT-PCR) was performed using  $\mu$ MACS<sup>TM</sup>One-step cDNA kit (Milteny Biotec). RNA from SK-N-BE(2), SK-N-MC, and HEK293 cells was isolated with TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). One microgram of RNA was used for RT-PCR using SuperScript<sup>TM</sup> RNA Amplification System (Invitrogen).

PCR amplification for human GFR $\alpha$ 4 cDNA was performed using specific primers previously described [23] and human *PSPN* cDNA was amplified using the primers: PSPN-F 5'-cgggatccgtggggcctcctggctgcagggg-3' and PSPN-R 5'-ggaattgcactcactgctgctgccccag-3'.

### Cloning of full-length human PSPN, NRTN, and ARTN

Full-length human unspliced *PSPN* cDNA was obtained from SK-N-BE(2) cells using the primers PSPN-F and PSPN-R. Full-length human *NRTN* and *ARTN* cDNAs were obtained from the RZPD clone collection (German Science Centre for Genome Research) and OriGene Technologies (Rockville, MD, USA), respectively. All cDNAs were cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen). The mutations A67T, A96S, and F127L in *NRTN*; R72H in *ARTN*; and R91C in *PSPN* were generated through PCR-mediated site-directed mutagenesis and confirmed by direct sequencing.

### Expression of wild-type and mutant proteins in HEK293 cells

HEK293 cells were transiently transfected with the *NRTN*, *ARTN*, or *PSPN* expression vectors using Lipofectamine<sup>TM</sup> 2000 Reagent (Invitrogen). Six hours after transfection, complete medium was changed to serum-free DMEM supplemented with insulin, transferrin, and selenium (Gibco). Two days later, conditioned medium was harvested and subsequently concentrated using the Amicon Ultra-15 10k centrifugal filter devices (Millipore, Carrigtwohill, Ireland). The secretion of wt and mt proteins in the conditioned medium was estimated by 10% SDS-PAGE, loading standards of purified recombinant human NRTN, ARTN, and PSPN, and detected by specific anti-human Neurturin, Artemin, and Persephin antibodies (Pepro- tech Inc., Rocky Hill, NJ, USA). Quantitative analysis was performed by densitometry using Image J (JAVA).

### RET phosphorylation assays

RET phosphorylation analysis was performed in human neuroblastoma SK-N-MC cells stably transfected with *RET* cDNA. In addition, 24 h before testing wt and mt *PSPN* conditioned medium, cells were also transiently transfected with a human GFR $\alpha$ 4 expression vector [23]. Cells were serum starved for 12 h and then stimulated for 15 min with 150 ng/mL of wt or mt NRTN and PSPN conditioned medium. Recombinant human protein standards and concentrated medium from mock-transfected cells were used as positive and negative controls, respectively. Thereafter, cells were solubilized with NP-40 lysis buffer with protease and tyrosine phosphatase inhibitors (Complete and Phos- Stop cocktail tablets, Roche Diagnostics, Mannheim, Germany). The whole cell extract was incubated with rabbit anti-RET polyclonal antibody (c19, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and immunoprecipitated with protein A/G PLUS-Agarose (Santa Cruz Biotechnology Inc.). Samples were separated by 7% SDS-

PAGE and transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). RET phosphorylation was analyzed by immunoblotting with an anti-phosphotyrosine monoclonal antibody (1:500, Santa Cruz Biotechnology Inc.) and a biotinylated secondary antibody (1:10,000, Jackson IR, Suffolk, UK). The same membrane was subsequently stripped and reprobbed with a polyclonal anti-RET antibody (H300, 1:1,000, Santa Cruz Biotechnology Inc.). Western blots were quantified by densitometry using ImageJ and Quantity One softwares (JAVA and BioRad, respectively).

### Statistics

Data are shown as the mean  $\pm$  SEM of values obtained from a minimum of five independent experiments. Comparisons between wt and mt proteins were analyzed by Student's *t* test. Differences were considered significant when  $p < 0.05$ .

## Results

### Mutational analysis

The mutational screening of *GDNF*, *NRTN*, *ARTN*, and *PSPN* genes in HSCR patients revealed a total of 13 heterozygous sequence variants (Table 1). Four of those variants, one detected in *NRTN* and the other three in *GDNF*, had been previously reported to be associated with HSCR. They were absent in the 150 control individuals tested, and it is worth mentioning that three of the patients carrying *GDNF* variants also presented with Down syndrome.

Regarding the nine novel changes, our results show that two of the variants in the *ARTN* gene were present in 15 patients, but also in 12 of the 150 healthy controls tested, leading us to consider them as novel polymorphisms rather than mutations associated with the HSCR phenotype. The remaining seven novel variants were completely absent in controls, although they had been inherited from one of the patient's parents and also were present in their healthy siblings. None of the patients carrying these changes presented any mutation in the coding sequence of several other "HSCR-associated" genes, namely, *RET*, *EDNRB*, *EDN3*, *NTF3*, *NTRK3*, *SOX10*, or *PHOX2B* [24–28] (unpublished data). All these patients were found to be homozygous or heterozygous for the common *RET* variant within the transcriptional enhancer in intron 1, most of them inheriting the *RET* susceptibility allele and the variants in GFL genes from different parents. Only the patient carrying the variant R91C in *PSPN* was observed to inherit this variant from the same parent from which they inherited the *RET* allele.

In silico analysis of the novel variants were performed to firstly examine their potential pathogenicity. Silent and intronic variants were submitted to several splice sites and transcription factors binding sequences prediction programs, but no biological effect was predicted for any of them. To evaluate the novel missense variants, we used both Polyphen and SIFT biotools. Based on the conserved status of the sequence and the physical properties of amino acids, only the PSPN protein carrying the variant R91C was predicted to be potentially deleterious according to both programs (Table 2). Moreover, the inclusion of a new cysteine residue in the protein sequence would induce a change in the three-dimensional structure of the protein by means of the formation of new disulfide bonds. DiANNA predicted that the presence of an additional cysteine residue at position 91 might induce the formation of a new disulfide bond with the residue 154, as well as lead to the destruction/generation of different bonds in comparison to the wt protein.

### Functional analysis of mutant proteins in vitro

We next investigated the functional significance of those novel variants at the protein sequence level in order to evaluate them as causative mutations for HSCR. We have also included the analysis of the mutation A96S, previously described in *NRTN*, since no functional study have been reported so far. We generated expression vectors for *NRTN*, *ARTN*, and *PSPN*, and the mutants A67T, A96S, F127L and double A67T/A96S in *NRTN*, R72H in *ARTN*, and R91C in *PSPN* were obtained by site-directed mutagenesis. HEK293 cells were transiently transfected with the corresponding expression vectors, and Western blot analysis of the conditioned medium revealed completely processed mature wt and mt proteins, with the expected molecular weight in comparison with the corresponding standards (Fig. 1). Medium from mock-transfected cells did not contain detectable amounts of either peptide. In addition, we observed that the *NRTN* and *ARTN* mt proteins could be recovered from HEK293 conditioned medium at levels comparable with the wt proteins (Fig. 1a, b), indicating that these alterations do not significantly affect either the polypeptide folding or the proteolytic processing. Interestingly, analysis of the mutation R91C in *PSPN* showed a significant reduction ( $57 \pm 8\%$ ) of the expression levels of the correctly processed peptide in comparison with the wt *PSPN* protein. Since *PSPN* expression vector was generated from the unspliced *PSPN* transcript, we also verified the presence of the spliced transcript in the transfected HEK293 cells by RT-PCR, and no differences in detection levels were observed for both wt and mt *PSPN* transcripts (unpublished data). This fact indicates that reduction in expression levels is not due to

**Table 1** GFL gene variants detected in HSCR patients

Gene	Nucleotide change	Amino acid change	Familial/ sporadic	Gender	Inheritance	Length of aganglionosis	Protein location	Reference
<i>GDNF</i>	c.152-73C>T		Sporadic	Male	Not available	Short segment		Novel
	c.277C>T	R93W	Sporadic	P1: Male	Father	Short segment	Mature protein	9, 11
				P2: Male	Mother	Short segment		
				P3: Male	Father	Long segment		
				P4: Female	Mother	Long segment		
				P5: Male	Mother	Short segment		
c.300A>G	P100P	Sporadic	Male	Not available	Short segment		Novel	
c.429G>A	R143R	Sporadic	Female	Father	TCA	Mature protein	10, 12, 13	
c.449G>A	D150N	Sporadic	Male	Father	Short segment	Mature protein	11, 12	
<i>NRTN</i>	c.199G>A	A67T <sup>a</sup>	Sporadic	Male	Mother	Short segment	Propeptide	Novel
	c.286G>T	A96S <sup>a</sup>	Sporadic	Male	Father	Short segment	Propeptide cleavage site	14
<i>PSPN</i>	c.381C>G	F127L	Sporadic	Male	Father	Short segment	Mature protein	Novel
	c.149-18C>T		Sporadic	Male	Father	Short segment		Novel
<i>ARTN</i>	c.271C>T	R91C	Sporadic	Male	Mother	Short segment	Mature protein	Novel
	c.215G>A	R72H	Sporadic	Female	Mother	Short segment	Propeptide	Novel
	c.-104T>C		Familial/ sporadic	Female/ male		Short segment/long segment		Novel polymorphism
	c.149-8C>T		Familial/ sporadic	Female/ male		Short segment/long segment		Novel polymorphism

TCA total colon aganglionosis

<sup>a</sup> Mutations detected in the same patient

differences in transfection efficiency or the mRNA splicing process. Taken together, these results may suggest that this alteration in PSPN generates unstable precursor molecules that lead to a reduced peptide secretion (Fig. 1c).

On the other hand, the variants F127L in NRTN and R91C in PSPN are located in the mature region of the corresponding proteins and could affect the ability of these ligands to induce RET autophosphorylation on tyrosine residues. To assess the ability of wt and mt proteins to activate the receptor, we evaluated RET tyrosine phosphorylation in SK-N-MC cells expressing RET. Serial concentrations of NRTN and PSPN were tested (50, 100, and 150 ng/mL), but receptor activation was only detected after incubation with 150 ng/mL of both protein standards and wt conditioned medium. RNA extraction and RT-PCR were

carried out and no expression of GFR $\alpha$ 4, the preferred co-receptor for PSPN, was observed in those cells. Thus, for the PSPN assay, we also transiently transfected the cells with a GFR $\alpha$ 4 expression vector to assure detectable RET phosphorylation levels.

We observed a significant decrease of RET phosphorylation level in the presence of F127L-NRTN mt protein in comparison with NRTN wt protein (20 $\pm$ 17%). Regarding the novel variant of PSPN, the functional analysis also showed a significant reduction in RET phosphorylation between R91C-PSPN mt and PSPN wt proteins (57 $\pm$ 7%) when cells were incubated with the same amount of wt and mt proteins in the conditioned medium (Fig. 2).

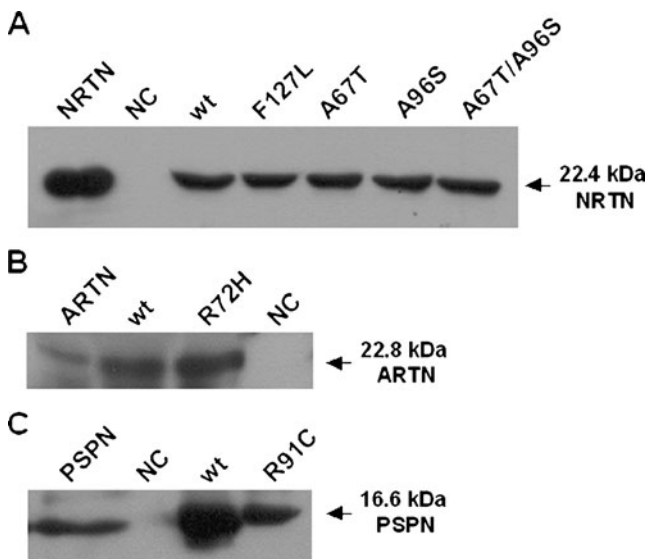
To assure that the effect was due to the mutations and not to variations in expression levels of the receptor, RET

**Table 2** Polyphen and SIFT analysis

Gene	Amino acid change	Polyphen analysis	SIFT analysis
<i>NRTN</i>	A67T	Benign functional effect	Not tolerated
	A96S <sup>a</sup>	Benign functional effect	Not tolerated
	F127L	Benign functional effect	Tolerated
<i>PSPN</i>	R91C	Probably damaging	Not tolerated
<i>ARTN</i>	R72H	Benign functional effect	Tolerated

<sup>a</sup> Variant previously described but with no functional study reported





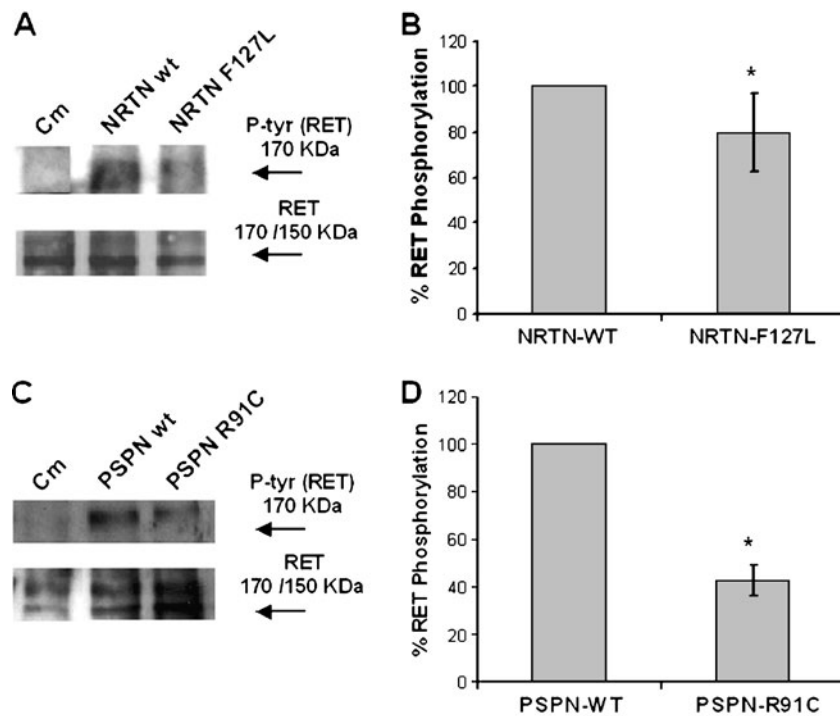
**Fig. 1** Expression of wild-type and mutant proteins in HEK293 cells. HEK293 cells were transiently transfected with the NRTN (a), ARTN (b), or PSPN (c) expression vectors and the secretion of wt and mt proteins in the conditioned medium estimated by Western blot. Standards of purified recombinant human NRTN, ARTN, and PSPN were used as positive controls. Conditioned medium from mock-transfected cells was used as a negative control (NC)

phosphorylation data were normalized with their respective total RET. No phospho-RET signal was obtained in untreated cells or cells treated with concentrated medium from mock-transfected HEK293.

Expression of PSPN and GFR $\alpha$ 4 in human postnatal gut and ENS precursor cells “in vitro”

To examine whether PSPN could be involved in ENS development, supporting its association with HSCR, we analyzed the expression of both GFR $\alpha$ 4 and PSPN in human postnatal gut tissues by RT-PCR. However, no detectable levels of the co-receptor and the splice PSPN transcript encoding the functional protein were observed in postnatal gut tissue samples. The unspliced PSPN transcript was verified by direct sequencing. The presence of the three transcripts corresponding to different splice isoforms identified in human GFR $\alpha$ 4 was observed in human thyroid gland tissue cDNA used as a positive control.

To rule out that these results were due to a dilution effect caused by the presence of many different cell types in the extracts, ENS precursor cells were isolated from human postnatal gut samples and the expression of the GFR $\alpha$ 4



**Fig. 2** RET tyrosine phosphorylation by F127L-NRTN and R91C-PSPN mutant proteins. SK-N-MC cells stably transfected with RET were serum starved for 12 h and then exposed to 150 ng/mL wt and mt of both NRTN and PSPN for 15 min. Concentrated medium from mock-transfected cells was used as control (Cm). RET was immunoprecipitated from whole cell extract and then was processed for SDS-PAGE and immunoblotting using an anti-phosphotyrosine (P-Tyr) antibody. Representative immunoblot showing phosphorylated and

total RET in cells after exposure to NRTN (wt and mt proteins) (a) and in GFR $\alpha$ -4 transiently transfected cells after exposure to PSPN (wt and mt proteins) (c). The graphs show the densitometric quantification of RET phosphorylation after 15-min incubation with NRTN (wt and mt proteins) (b) and PSPN (wt and mt proteins) (d), relative to total RET. Results were expressed as a percentage of the phosphorylation obtained after incubation with the wt proteins. Quantitative data were calculated as means  $\pm$  SEM,  $n=5$  independent experiments.  $*p<0.05$

co-receptor was examined in neurosphere-forming cells by immunocytochemical detection. Interestingly, using a specific antibody against the N-terminal domain of GFR $\alpha$ 4, we observed that the co-receptor together with the RET receptor were expressed by adhered cells from neurospheres, indicating a possible role of PSPN during ENS development (Fig. 3).

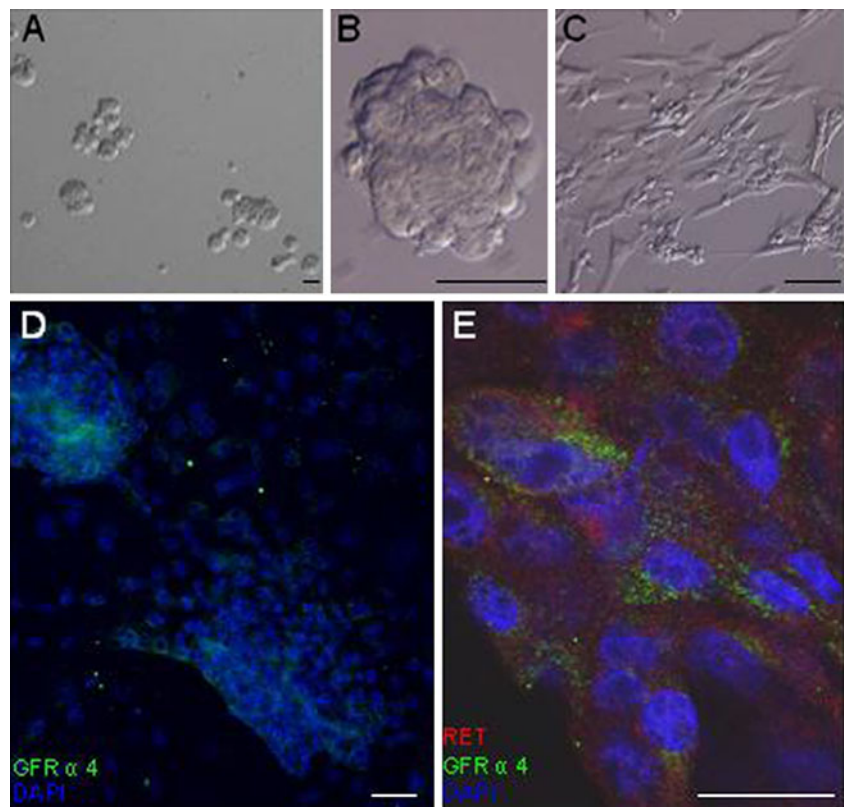
## Discussion

The relevance of RET signaling pathway during ENS development and the prominent role of the receptor in the pathogenesis of HSCR suggest that the genes encoding the GFLs could be excellent candidates to be involved in the disease. However, most of the mutations previously reported in *GDNF*, and the only one mutation reported in *NRTN*, co-segregated with mutations in the *RET* proto-oncogene and/or other genetic disorders, indicating that those mutations could be neither necessary nor sufficient to cause HSCR [16–21]. In addition, subsequent analysis of the functional role of mutations located in the mature region of *GDNF* demonstrated no effect on RET phosphorylation, even when a reduction in the binding affinity to GFR $\alpha$ 1 was observed [29, 30]. Taking these findings into account, such mutations could be regarded as genetic changes with a

modulatory effect that could contribute to the disease via interaction with other susceptibility loci.

In the present study, we have evaluated the four genes encoding RET ligands and novel variants in *NRTN*, *ARTN*, and *PSPN* potentially involved in the pathogenesis of HSCR were detected. Two of those novel variants were located in the propeptide region of *NRTN* (A67T) and *ARTN* (R72H), and they could be affecting the efficient biosynthesis and secretion of the mature and biologically active protein since deletions of amino acid sequences in this region were demonstrated to decrease the protein secretion levels [31]. In addition, the mutation A96S previously reported in *NRTN* led to the substitution of the first amino acid in the mature peptide, modifying the position +1 of the propeptide cleavage site, which could alter the proteolytic processing of the propeptide into the mature protein. However, we did not detect a significant difference in the ability of these three mutants to produce the correctly processed peptides. It is worth mentioning that the role of the A67T variant will also be influenced by the effect of the mutation A96S since both of them were inherited in the same allele. In this case, the double mt protein showed the same secretion level in comparison with the corresponding single mt and the wt *NRTN* protein. Therefore, we postulated that these mt proteins would have a subtle effect, if any, on the secretion of correctly

**Fig. 3** GFR $\alpha$ 4 expression in adhered neurosphere-derived cells. **a, b** Phase contrast images showing characteristic neurospheres generated from freshly dissociated gut tissue cells after 7 days in culture. **c** Floating neurospheres were adhered onto coverslips and grown as a monolayer, then fixed and processed as described in “Materials and methods.” **d, e** Confocal microscopy images of adhered neurospheres immunostained with antibodies against GFR $\alpha$ 4 (green) and RET (red). Scale bars, 50  $\mu$ m (**a–d**), 25  $\mu$ m (**e**)



processed peptides *in vivo*, below the detection threshold of this *in vitro* assay.

The most interesting result derived from the analysis was the identification of the novel variants F127L and R91C in the mature regions of NRTN and PSPN, respectively. A considerable decrease in mature protein secretion was observed for the R91C mutant. It would be probably due to the insertion of a novel cysteine two residues before the second cysteine-knot motif [32], which was predicted to generate new disulfide bonds affecting the proper folding of the polypeptide. The generation of an unstable precursor would decrease the efficiency of signal peptide cleavage and would result in a greatly reduced secretion of the mature peptide. In addition, we performed *in vitro* functional assays and demonstrated that both variants (F127L and R91C) significantly reduced the ability of NRTN and PSPN proteins to induce RET tyrosine phosphorylation. Comparison of GFL protein sequences from different species revealed that the variants were located in the same protein region, affecting conserved residues critical for the receptor binding [32, 33]. According to this, we suggest that phenylalanine at position 127 in NRTN could be involved in the ligand–receptor interaction. The amino acid change would reduce the binding affinity of the mutant protein for the RET receptor, leading to a decrease in RET dimerization and therefore in the level of tyrosine phosphorylation when compared to the wt protein. Moreover, a greater reduction in RET phosphorylation was obtained for the R91C-PSPN mt protein. The altered three-dimensional structure of the mutant PSPN could also prevent protein dimerization and affect RET binding more severely, thus explaining the more significant decrease in phosphorylation. Accordingly, the biological consequence of NRTN F127L and PSPN R91C mutants would be a reduction in the activation of the RET-dependent signaling pathways, leading to a defect in the proliferation, migration, and/or differentiation process of neural crest cells in the developing gut during embryogenesis and to the aganglionosis typically observed in HSCR patients.

On the other hand, PSPN has been demonstrated to promote the survival of ventral midbrain dopaminergic neurons, motor neurons, and also sympathetic neurons in culture [8, 23, 34, 35], but to date, there was no evidence about its requirement for the survival of enteric neurons. Extensive analysis of PSPN expression revealed that the unspliced PSPN transcript was present at relatively similar levels in all human tissues examined, whereas the functional spliced mRNA could be detected only at low levels in some specific tissues, but not in the gut [23]. According to this, functional PSPN could be widely expressed at developmental stage and the regulation of mRNA processing might be an important mechanism to regulate the production of the protein in selective tissues [23, 34]. In

contrast, expression of GFR $\alpha$ 4, the preferred co-receptor for PSPN, has been mainly detected in the adult human thyroid glands. In agreement with previous results, no expression of both the co-receptor and the spliced PSPN transcripts were detected in human postnatal gut tissues in the present study. However, since undifferentiated precursor cells are present within the gastrointestinal tract not only during embryonic development but also into early postnatal life, ENS progenitors were isolated from those tissues. Interestingly, using these neurosphere cultures, we could show the expression of GFR $\alpha$ 4 in enteric neural precursors cells, which suggests that PSPN signaling via GFR $\alpha$ 4/RET could be involved in ENS development. Moreover, a recent study has demonstrated that PSPN could also signal through the GFR $\alpha$ 1 co-receptor, which is known to play a role in neuronal survival during ENS development [8]. These findings suggest that not only GDNF and NRTN but also PSPN might promote survival of precursor cells during ENS development, supporting the role of PSPN R91C in HSCR phenotype.

The variants in the GFL genes here described are incompletely penetrant since they were carried by other healthy members of the family. Our findings would completely fit with the additive model of inheritance previously proposed for HSCR in which the expression of the disease seems to depend on the contribution of different combinations of gene alleles acting in an additive or multiplicative fashion [36]. Following this model, those genetic variants could modulate the penetrance of mutations located in other genes or modify expressivity of the disease in affected individuals. Our results suggest that the variants here characterized may be contributing to the final phenotype acting in combination with additional mutational events in other genes, such as the common *RET* variant within the transcriptional enhancer in intron 1, since most of the patients have inherited these variants from different parents [3]. Presumably, none of these mutations are likely to cause HSCR independently, but the co-occurrence of both different mutational events in the same patient may have contributed to the manifestation of the phenotype. In this sense, only the mutation R91C in *PSPN*, with a demonstrated functional effect, was inherited from the same parent together with the *RET* susceptibility allele, suggesting that this variant may be contributing to the final phenotype, acting in combination with additional mutational events in other loci.

In summary, we report for the first time the association of *PSPN* with HSCR as well as confirm the involvement of *NRTN* in the disease, with the identification of novel variants in those genes. *In vitro* analyses revealed a decreased secretion level of correctly processed peptide for the variant R91C in *PSPN* and altered RET activation by a significant reduction of autophosphorylation also for



this variant and F127L in NRTN, supporting their effects on HSCR phenotype. To get insight into the role of PSPN R91C in HSCR, ENS progenitors were isolated from human postnatal gut tissues and expression of GFR $\alpha$ 4, the main co-receptor for PSPN, was shown, suggesting that PSPN might promote survival of precursor cells during ENS development.

**Acknowledgments** We would like to thank the patients and families that participated in this study. We also thank Dr. Borghini (from Dr. Ceccherini's lab, Instituto G Gaslini) for providing the SK-N-MC cell line stably transfected with RET cDNA, Dr. Saarna (University of Helsinki) for the GFR $\alpha$ 4 expression vector, and Dr. Bocciardi (Instituto G Gaslini) for technical assistance. This work was supported by Fondo de Investigación Sanitaria, Spain (PI070080 and PI071315 for the E-Rare project) and Consejería de Innovación Ciencia y Empresa (CTS 2590). The CIBER de Enfermedades Raras is an initiative of the ISCIII.

**Disclosure of potential conflict of interests** The authors declare no conflict of interests related to this study.

## References

- Chakravarti A, Lyonnet S (2002) Hirschsprung disease. In: Scriver CS et al (eds) *The metabolic and molecular bases of inherited disease*. McGraw-Hill, New York, pp 6231–6255
- Amiel J, Sproat-Emison E, Garcia-Barcelo M, Lantieri F, Burzynski G, Borrego S, Pelet A, Arnold S, Miao X, Griseri P, Hirschsprung Disease Consortium et al (2008) Hirschsprung disease, associated syndromes and genetics: a review. *J Med Genet* 45:1–14
- Emison ES, McCallion AS, Kashuk CS, Bush RT, Grice E, Lin S, Portnoy ME, Cutler DJ, Green ED, Chakravarti A (2005) A common sex-dependent mutation in a RET enhancer underlies Hirschsprung disease risk. *Nature* 434:857–863
- Takahashi M (2001) The GDNF/RET signaling pathway and human diseases. *Cytokine Growth Factor Rev* 12:361–373
- Airaksinen MS, Saarna M (2002) The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci* 3:383–394
- Enokido Y, de Sauvage F, Hongo JA, Ninkina N, Rosenthal A, Buchman VL, Davies AM (1998) GFR  $\alpha$ -4 and the tyrosine kinase Ret form a functional receptor complex for persephin. *Curr Biol* 8:1019–1022
- Masure S, Cik M, Hoefnagel E, Nosrat CA, Van der Linden I, Scott R, Van Gompel P, Lesage AS, Verhasselt P, Ibáñez CF et al (2000) Mammalian GFR $\alpha$ 4, a divergent member of the GFR $\alpha$  family of coreceptors for glial cell line-derived neurotrophic factor family ligands, is a receptor for the neurotrophic factor persephin. *J Biol Chem* 275:39427–39434
- Sidorova YA, Mätlik K, Paveliev M, Lindahl M, Piranen E, Milbrandt J, Arumäe U, Saarna M, Bespalov MM (2010) Persephin signaling through GFR $\alpha$ 1: the potential for the treatment of Parkinson's disease. *Mol Cell Neurosci* 44:223–232
- Taraviras S, Marcos-Gutierrez CV, Durbec P, Jani H, Grigoriou M, Sukumaran M, Wang LC, Hynes M, Raisman G, Pachnis V (1999) Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. *Development* 126:2785–2797
- Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E (2002) Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. *Development* 129:5151–5160
- Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* 367:380–383
- Moore MW, Klein RD, Fariñas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. *Nature* 382:76–79
- Cacalano G, Fariñas I, Wang LC, Hagler K, Forgie A, Moore M, Armanini M, Phillips H, Ryan AM, Reichardt LF et al (1998) GFR $\alpha$ 1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* 21:53–62
- Heuckeroth RO, Enomoto H, Grider JR, Golden JP, Hanke JA, Jackman A, Molliver DC, Bardgett ME, Snider WD, Johnson EM Jr et al (1999) Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron* 22:253–263
- Rossi J, Luukko K, Poteryaev D, Laurikainen A, Sun YF, Laakso T, Erikäinen S, Tuominen R, Lakso M, Rauvala H et al (1999) Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR  $\alpha$ 2, a functional neurturin receptor. *Neuron* 22:243–252
- Uesaka T, Enomoto H (2010) Neural precursor death is central to the pathogenesis of intestinal aganglionosis in Ret hypomorphic mice. *J Neurosci* 30:5211–5218
- Angrist M, Bolk S, Halushka M, Lapchak PA, Chakravarti A (1996) Germline mutations in glial cell line-derived neurotrophic factor (GDNF) and RET in a Hirschsprung disease patient. *Nat Genet* 14:341–344
- Ivanchuk SM, Myers SM, Eng C, Mulligan LM (1996) De novo mutation of GDNF, ligand for the RET/GDNFR- $\alpha$  receptor complex, in Hirschsprung disease. *Hum Mol Genet* 5:2023–2026
- Salomon R, Attie T, Pelet A, Bidaud C, Eng C, Amiel J, Sarnacki S, Goulet O, Ricour C, Nihoul-Fekete C et al (1996) Germline mutations of the RET ligand GDNF are not sufficient to cause Hirschsprung disease. *Nat Genet* 14:345–347
- Hofstra RM, Osinga J, Buys CH (1997) Mutations in Hirschsprung disease: when does a mutation contribute to the phenotype. *Eur J Hum Genet* 5:180–185
- Hofstra RM, Wu Y, Stulp RP, Elfferich P, Osinga J, Maas SM, Siderius L, Brooks AS, vd Ende JJ, Heydendael VM et al (2000) RET and GDNF gene scanning in Hirschsprung patients using two dual denaturing gel systems. *Hum Mutat* 15:418–429
- Doray B, Salomon R, Amiel J, Pelet A, Touraine R, Billaud M, Attie T, Bachy B, Munnich A, Lyonnet S (1998) Mutation of the RET ligand, neurturin, supports multigenic inheritance in Hirschsprung disease. *Hum Mol Genet* 7:1449–1452
- Lindahl M, Poteryaev D, Yu L, Arumae U, Timmusk T, Bongarzoni I, Aiello A, Pierotti MA, Airaksinen MS, Saarna M (2001) Human glial cell line-derived neurotrophic factor receptor  $\alpha$  4 is the receptor for persephin and is predominantly expressed in normal and malignant thyroid medullary cells. *J Biol Chem* 276:9344–9351
- Ruiz-Ferrer M, Fernández RM, Antiñolo G, López-Alonso M, Eng C, Borrego S (2006) A complex additive model of inheritance for Hirschsprung disease is supported by both RET mutations and predisposing RET haplotypes. *Genet Med* 8:704–710
- Ruiz-Ferrer M, Fernández RM, Antiñolo G, Lopez-Alonso M, Borrego S (2008) NTF-3, a gene involved in the enteric nervous system development, as a candidate gene for Hirschsprung disease. *J Pediatr Surg* 43:1308–1311
- Fernández RM, Sánchez-Mejías A, Mena MD, Ruiz-Ferrer M, López-Alonso M, Antiñolo G, Borrego S (2009) A novel point variant in NTRK3, R645C, suggests a role of this gene in the pathogenesis of Hirschsprung disease. *Ann Hum Genet* 73:19–25

27. Sánchez-Mejías A, Fernández RM, López-Alonso M, Antiñolo G, Borrego S (2010) New roles of EDNRB and EDN3 in the pathogenesis of Hirschsprung disease. *Genet Med* 12:39–43
28. Sánchez-Mejías A, Watanabe Y, Fernández RM, López-Alonso M, Antiñolo G, Bondurand N, Borrego S (2010) Involvement of SOX10 in the pathogenesis of Hirschsprung disease: report of a truncating mutation in an isolated patient. *J Mol Med* 88:507–514
29. Eketjäll S, Ibanez CF (2002) Functional characterization of mutations in the GDNF gene of patients with Hirschsprung disease. *Hum Mol Genet* 11:325–329
30. Borghini S, Bocciardi R, Bonardi G, Matera I, Santamaria G, Ravazzolo R, Ceccherini I (2002) Hirschsprung associated GDNF mutations do not prevent RET activation. *Eur J Hum Genet* 10:183–187
31. Suter U, Heymach JV Jr, Shooter EM (1991) Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. *EMBO J* 10:2395–2400
32. Baloh RH, Tansey MG, Johnson EM Jr, Milbrandt J (2000) Functional mapping of receptor specificity domains of glial cell line-derived neurotrophic factor (GDNF) family ligands and production of GFRalpha1 RET-specific agonists. *J Biol Chem* 275:3412–3420
33. Chen ZY, He ZY, He C, Lu CL, Wu XF (2000) Human glial cell-line-derived neurotrophic factor: a structure–function analysis. *Biochem Biophys Res Commun* 268:692–696
34. Milbrandt J, de Sauvage FJ, Fahrner TJ, Baloh RH, Leitner ML, Tansey MG, Lampe PA, Heuckeroth RO, Kotzbauer PT, Simburger KS et al (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron* 20:245–253
35. Yang J, Lindahl M, Lindholm P, Virtanen H, Coffey E, Runeberg-Roos P, Saarma M (2004) PSPN/GFRalpha4 has a significantly weaker capacity than GDNF/GFRalpha1 to recruit RET to rafts, but promotes neuronal survival and neurite outgrowth. *FEBS Lett* 569:267–271
36. Emison ES, Garcia-Barcelo M, Grice EA, Lantieri F, Amiel J, Burzynski G, Fernandez RM, Hao L, Kashuk C, West K et al (2010) Differential contributions of rare and common, coding and noncoding ret mutations to multifactorial Hirschsprung disease liability. *Am J Hum Genet* 87:60–74