

Expression of PROKR1 and PROKR2 in Human Enteric Neural Precursor Cells and Identification of Sequence Variants Suggest a Role in HSCR

Macarena Ruiz-Ferrer^{1,2}, Ana Torroglosa^{1,2}, Rocío Núñez-Torres^{1,2}, Juan Carlos de Agustín³, Guillermo Antiñolo^{1,2}, Salud Borrego^{1,2*}

1 Unidad de Gestión Clínica de Genética, Reproducción y Medicina Fetal, Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Sevilla, Spain, **2** Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Sevilla, Spain, **3** Unidad de Gestión Clínica de Cirugía Infantil, Hospital Universitario Virgen del Rocío, Sevilla, Spain

Abstract

Background: The enteric nervous system (ENS) is entirely derived from neural crest and its normal development is regulated by specific molecular pathways. Failure in complete ENS formation results in aganglionic gut conditions such as Hirschsprung's disease (HSCR). Recently, PROKR1 expression has been demonstrated in mouse enteric neural crest derived cells and Prok-1 was shown to work coordinately with GDNF in the development of the ENS.

Principal Findings: In the present report, ENS progenitors were isolated and characterized from the ganglionic gut from children diagnosed with and without HSCR, and the expression of prokineticin receptors was examined. Immunocytochemical analysis of neurosphere-forming cells demonstrated that both PROKR1 and PROKR2 were present in human enteric neural crest cells. In addition, we also performed a mutational analysis of *PROKR1*, *PROKR2*, *PROK1* and *PROK2* genes in a cohort of HSCR patients, evaluating them for the first time as susceptibility genes for the disease. Several missense variants were detected, most of them affecting highly conserved amino acid residues of the protein and located in functional domains of both receptors, which suggests a possible deleterious effect in their biological function.

Conclusions: Our results suggest that not only PROKR1, but also PROKR2 might mediate a complementary signalling to the RET/GFR α 1/GDNF pathway supporting proliferation/survival and differentiation of precursor cells during ENS development. These findings, together with the detection of sequence variants in *PROKR1*, *PROK1* and *PROKR2* genes associated to HSCR and, in some cases in combination with *RET* or *GDNF* mutations, provide the first evidence to consider them as susceptibility genes for HSCR.

Citation: Ruiz-Ferrer M, Torroglosa A, Núñez-Torres R, de Agustín JC, Antiñolo G, et al. (2011) Expression of PROKR1 and PROKR2 in Human Enteric Neural Precursor Cells and Identification of Sequence Variants Suggest a Role in HSCR. PLoS ONE 6(8): e23475. doi:10.1371/journal.pone.0023475

Editor: Jan-Hendrik Niess, Ulm University, Germany

Received: February 25, 2011; **Accepted:** July 19, 2011; **Published:** August 12, 2011

Copyright: © 2011 Ruiz-Ferrer et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Fondo de Investigación Sanitaria, Spain (PI070080, PI1001290 and PI071315 for the E-Rare project), Consejería de Innovación Ciencia y Empresa de la Junta de Andalucía (CTS 2590) and Consejería de Salud de la Junta de Andalucía (PI0249-2008). The CIBER de Enfermedades Raras is an initiative of the ISCIII. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: salud.borrego.sspa@juntadeandalucia.es

Introduction

The enteric nervous system (ENS) is composed of a large number of neurons and glia, which are organised into interconnected ganglia distributed throughout the gastrointestinal tract. It is originated from neural crest cells (NCCs), that invade the foregut mesenchyme during embryogenesis and migrate in a rostrocaudal direction to extensively colonize the entire length of the gut [1]. Failure in these processes results in aganglionic gut conditions, such as Hirschsprung disease (HSCR) in humans. HSCR, with an incidence of 1:5000 live births, is the most common developmental disorder of the ENS, characterized by its incomplete formation and the absence of enteric ganglia in a variable segment of distal bowel. This leads to peristaltic misregulation and tonic contraction within the affected gut, causing intestinal obstruction [2]. HSCR most commonly presents sporadically and displays a complex pattern of inheritance with low, sex-dependent penetrance and variable expression [3].

The genetic complexity observed in HSCR could be explained by the complex nature of ENS development, which is regulated by an ever-increasing range of molecules and signalling pathways involving both the NCCs and intestinal environment [4]. Developmental biology studies have identified that the RET/GFR α 1/GDNF signalling pathway is the most critical player for enteric neurogenesis and the proper expression of these proteins have been demonstrated to be crucial for the normal development of the ENS. Recently, it was shown that Prokineticin-1 (Prok1) is expressed in the mucosa and mesenchyme of the mouse embryonic gut during ENS development and promotes the survival/proliferation and differentiation, but not migration, of enteric NCCs [5]. Prok1 crosstalks with GDNF/Ret signaling and probably provides an additional layer of signaling refinement to maintain proliferation and differentiation of enteric NCCs [6].

Prokineticins (PROK1 and PROK2) belong to the AVIT protein family, a recently identified family of cysteine-rich secreted protein that share the identical amino terminal sequence crucial

for their biological activities [7,8]. These proteins are known to bind and activate two closely related Gprotein-coupled receptors, PROKR1 and PROKR2, leading to the mobilization of calcium, the stimulation of phosphoinositide-3-kinase turnover, and the activation of the mitogen activated protein kinase (MAPK) signalling pathway [9–11]. Prokineticins were first identified in the gastrointestinal tract as a potent agents mediating smooth muscle contraction [7]. However, they also act as survival/mitogenic factors for endothelial cells, neurons, lymphocytes and hematopoietic stem cells.

This report provides the first evidence that PROKR2 could be involved in ENS development. We show that not only PROKR1, as it was previously suggested [5,6], but also PROKR2 are present in human enteric NCCs since both receptors are expressed in ENS progenitors isolated from ganglionic gut samples of patients diagnosed with HSCR. In addition, we have also performed a mutational analysis of *PROKR1*, *PROKR2*, *PROK1* and *PROK2* genes in a cohort of HSCR patients, evaluating them for the first time as susceptibility genes for the disease.

Results

Characterization of Cell Cultures

Postnatal ganglionic gut tissues from HSCR patients and controls were dissociated into near single cell suspension and plated in a medium supplemented with EGF, bFGF and GDNF that promotes the growth of isolated ENS progenitors. Over the next 3–4 days, floating neurospheres could be observed (Figure 1). After multiple (3–4) subcultures, part of the neurospheres derived cells still generated new neurospheres with similar characteristics, which indicates the existence of cells with self-renewal properties. To characterize the cells that form the neurospheres, they were immunostained using a neural stem cells marker (Nestin), neuronal marker (TuJ1), glial markers (GFAP, S100) and smooth muscle marker (SMA) (Figure 2). As a result, neurospheres were constituted by Nestin-positive cells ($77\% \pm 2$), and most of them also contained cells that expressed the neuronal marker ($11\% \pm 3$) (Figure S1). However, staining with antibodies against S100 and SMA showed only a small fraction of positives cells ($3\% \pm 1$ and $2\% \pm 0.4$, respectively), while immunofluorescence for GFAP demonstrated a lack of reactivity. RET was also expressed by neurosphere cells and confocal analysis revealed the presence of this receptor in all Nestin-positive neurosphere cells. Taken together, these results suggest that the neurosphere-like bodies contained a mixture of neural and non-neural cells representing different stages of differentiation. In addition, no differences were

observed between neurospheres derived from ganglionic gut from children with or without HSCR.

Human ENS-Derived Cells Expressed PROKR1 and PROKR2 receptors “in vitro”

The expression of prokineticins receptors in human neurospheres was examined. Immunocytochemical detection using specific antibodies revealed that both receptors, PROKR1 and PROKR2, were expressed in floating neurospheres obtained from the ganglionic gut from HSCR patients (Figure 3A and B, Figure S2). Cells PROKR1 and PROKR2 positives were also Nestin positive, which demonstrate that both receptors are expressed by undifferentiated enteric neural precursor cells (Figure 3C and E). In addition, we have also observed co-expression of PROKR1 with TuJ1 (Figure 3D), suggesting that this receptor is still expressed in cells already committed to neuronal fate. Analysis of mRNA by RT-PCR was also performed to confirm the presence of both PROKR1 and PROKR2 transcripts in human ENS-derived cells (Figure 4). cDNA from the neuroblastoma cell line SK-N-MC and human endometrium tissue were used as positive control and cDNA from lymphocytes as negative control (data not shown).

Mutational analysis

The mutational screening of *PROKR1*, *PROK1*, *PROKR2* and *PROK2* genes revealed a total of 13 heterozygous sequence variants in 16 unrelated HSCR patients (Table 1, Figure 5). Four of those variants, detected in *PROKR2*, had been previously reported as mutations associated to Kallmann Syndrome [12–14]. However, no clinical signs or symptoms of Kallmann syndrome were observed in HSCR patients carrying *PROKR2* variants. Only the variants R85C in *PROKR2* and the novel G54G in *PROK1* were present in control individuals, while the remaining variants were absent in 150 control individuals tested. In addition, when genomic DNA from other family member was available, we have also analysed them and we found that all the variants had been inherited from one of their parents. Five out of the 16 patients carrying these changes (32%) also presented a mutation in the coding sequence of *RET* or *GDNF* [15,16]. No mutations were detected in any other HSCR related gene, namely *NRTN*, *ARTN*, *PSPN*, *NTF3*, *NTRK3*, *EDNRB*, *EDN3*, *SOX10* or *PHOX2B* [16–21, unpublished results].

In order to establish the pathogenic relevance of those variants, we have performed *in silico* analysis using different bioinformatic tools. Based on the conserved status of the sequence and the physical properties of amino acids, Polyphen and SIFT programs predicted

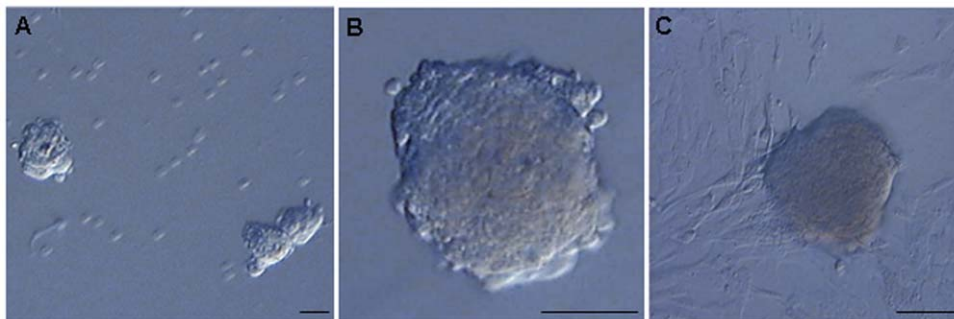


Figure 1. Generation of neurospheres from human neural precursors cells isolated from ENS. Phase contrast images showing characteristic neurospheres generated from freshly dissociated HSCR ganglionic gut tissue cells after 7 days in culture (A, B). Floating neurospheres were seeded onto coverslip and grown adhered using the same culture conditions (C). Scale bars 50 μm . doi:10.1371/journal.pone.0023475.g001

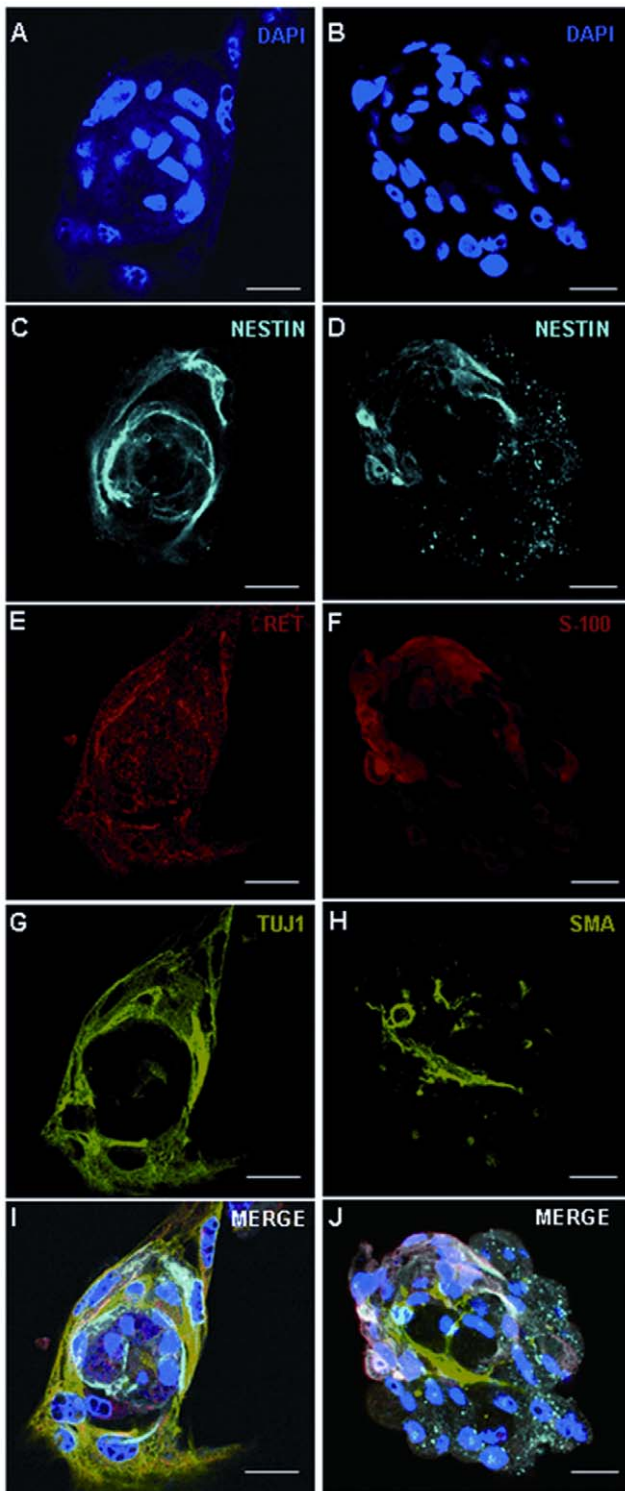


Figure 2. Characterization of neurospheres from human neural precursors cells isolated from ENS. Confocal microscopy images of floating neurospheres immunostained with antibodies against Nestin (blue), TuJ1 (green), SMA (green), S100 (red) and RET (red) and counterstained with DAPI (4,6-diamidino-2-phenylindole). Scale bars 25 μ m. doi:10.1371/journal.pone.0023475.g002

that, with the exception of the variant V297I in PROKR2, all the changes could generate a probably damaged protein. Moreover, DiANNA software also predicted that the inclusion of additional cysteine residues at positions 85 and 268 in PROKR2 could lead to

a different distribution of the disulfide bonds in comparison to the wild type protein, that may induce a change in the three-dimensional structure of the protein. Finally, after analysis using ScanProsite interface we observed that the residues R85, R268, P290, Y292 and V297 in PROKR2 were distributed in the functional domains of the receptor, suggesting that an amino acid change at these position could affect its function.

Discussion

Several studies have demonstrated that undifferentiated precursors cells are present within the gastrointestinal tract not only during embryonic development but also into early postnatal life [22–26]. In the present report, ENS progenitors were isolated from the ganglionic gut from childrens diagnosed with or without HSCR, using full-thickness gut resection specimens or gut biopsy samples, respectively. Immunocytochemical analysis of neurosphere-forming cells showed that the majority of the ENS progenitors in culture were undifferentiated neural stem cells, expressing most of them the RET receptor. A subset of neuronal cells was also observed, including TuJ1 positive cells co-expressing nestin that still retain multipotent characteristics but are competent to differentiate along the differentiated phenotype. However, only a small fraction of S100 positives cells could be identified, indicating an initial step for glial lineage differentiation in the neurospheres. In this sense, it has been demonstrated that neural diversity lineages strongly depends on the cell-intrinsic differences in their responsiveness to factors and gut NCCs are more responsive to neurogenic factors than gliogenic factors, giving rise primarily to neurons [27]. Moreover, diverse environmental conditions was suggested to play a role in regulate differentiation, and the neurosphere microenvironment in culture could be not appropriate enough for the differentiation of glial cells [24,28]. On the other hand, it was possible to dissociate primary derived neurospheres and generate secondary and tertiary neurospheres, supporting the presence of self-renewing progenitors in culture [24].

Using these neurospheres cultures, we investigated the expression of PROKR1 and PROKR2 in human enteric NCCs. Our results show that not only PROKR1 is present in neural stem cells and neuronal precursors, but also PROKR2 receptor is observed. PROKR1 expression was previously demonstrated in mouse enteric neural crest derived cells and Prok-1 was shown to work coordinately with GDNF in the development of the ENS [5,6]. Firstly, both GDNF and Prok-1 share common downstream elements, prominently the MAPK and Akt pathways, which provide multiple points of insertions between these two factors and lead them to exhibit similar biological functions [5]. In addition, GDNF potentiate the proliferative and differentiation effects of Prok-1 by up-regulating PROKR1 expression in enteric NCCs [6]. This functional redundancy of PROKR1/Prok-1 and RET/GFR α 1/GDNF signalling supports the idea that Prok-1/PROKR1 provides a compensatory pathway to ensure the proper development of ENS.

On the other hand, our results show for the first time the expression of PROKR2 in human enteric neural crest derived cells, which confirm that expression profiles of prokineticin receptors in mouse are slightly different from that in human [29]. We suggest that PROKR2 would have a relevant role by inhibiting apoptosis of enteric neuronal precursors, as it was previously described in neural crest-derived neuroblastoma cells [30]. Therefore, PROKR2 could mediate neuronal protection or survival not only in the central nervous system [31], but also during the ENS development.

According to that, *PROKR1*, *PROK1*, *PROKR2* and *PROK2* were evaluated as susceptibility genes for HSCR, based on the

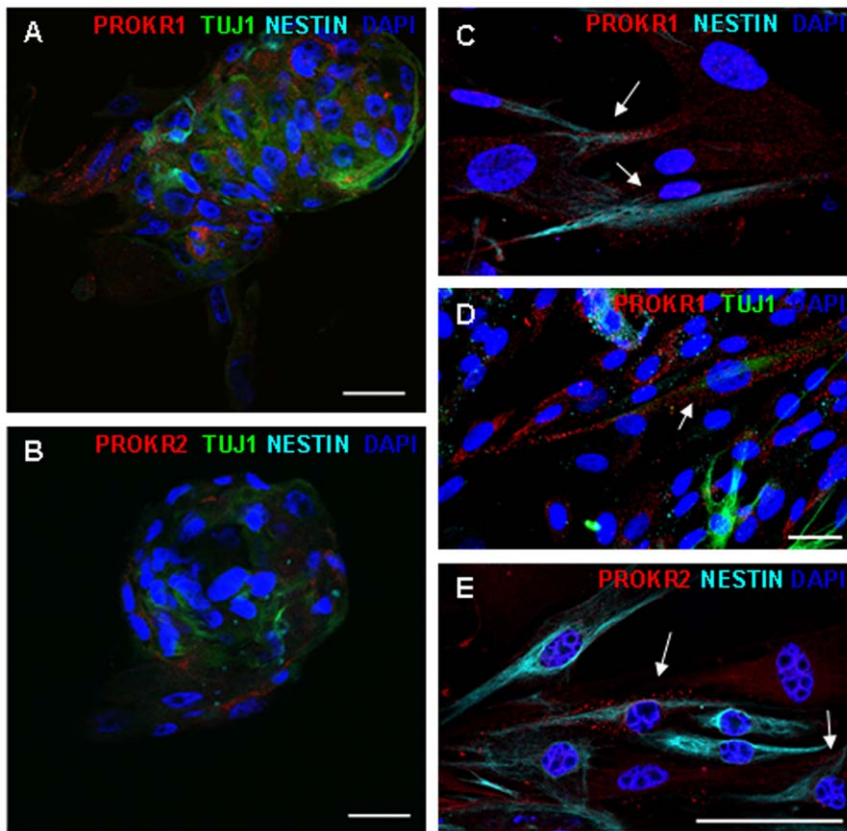


Figure 3. PROKR1 and PROKR2 expression in neurosphere cultures. Confocal microscopy images of floating neurospheres (A, B) and adhered neurosphere derived cells as described in Figure 1C (C–E) immunostained with antibodies against PROKR1 (red), PROKR2 (red), Nestin (blue) and TuJ1 (green). Scale bars 25 μ m.
doi:10.1371/journal.pone.0023475.g003

etiopathogenesis of the disease. Several missense variants in *PROKR1*, *PROK1* and *PROKR2* genes were detected, most of them affecting highly conserved amino acid residues of the protein and located in functional domains of both receptors, which suggests a possible deleterious effect in their biological function. It is also worth of mentioning that four of the *PROKR2* mutations were previously described associated to Kallmann syndrome [12–14], another

congenital disorder defined by hypogonadotropic hypogonadism and olfactory abnormalities, often associated with renal agenesis and other developmental defects. In these patients, hypogonadism is due to a failure of embryonic migration of gonadotropin-releasing hormone-synthesizing neurons from the olfactory epithelium to the forebrain, and insufficient prokineticin signalling through *PROKR2* seems to play a critical role. The analysis of the functional effect of these mutations by measuring intracellular calcium release upon ligand binding has demonstrated a decreased signalling activity of the receptor [32]. Specifically, P290S impaired cell surface-targeting of the receptor and R85C, R85H and R268C presumably impaired G protein-coupling. However, when both the wild-type and mutant receptors were co-expressed, none of the mutant receptors affected the properly signalling activity provided by the wild-type receptor. Therefore, these results argue against a dominant negative effect of these mutations *in vivo*, supporting the current hypothesis of an autosomal recessive inheritance accepted for the disease. Interestingly, we observed that mutations in *RET* proto-oncogene or *GDNF* are frequently associated to the presence of sequence variants in these genes in our cohort of HSCR patients, contributing to the manifestation of the more severe

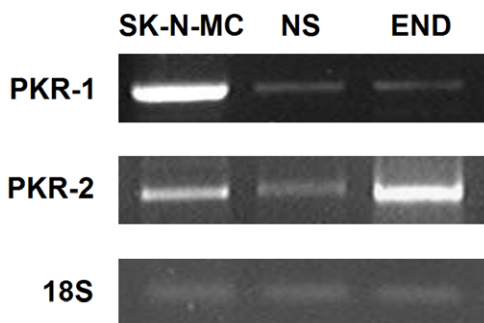


Figure 4. RT-PCR analysis of PROKR1 and PROKR2 expression in human ENS-derived cells. Total RNA was isolated from neurospheres (NS) and both *PROKR1* and *PROKR2* transcripts were detected by RT-PCR. cDNA from the neuroblastoma cell line SK-N-MC and human endometrium tissue (END) were used as positive control. Ribosomal 18S was used as the internal control.
doi:10.1371/journal.pone.0023475.g004

Table 1. *PROKR1*, *PROK1*, *PROKR2* sequence variants detected in HSCR patients.

Gene	Nucleotide Change	Amino Acid Change	Familial/Sporadic	Inheritance	Segment length	In silico analysis	Mutations in <i>RET</i> or other HSCR genes	Novel/Previously reported
<i>PROKR1</i>	c.387C>T	p.S129S	Sporadic	Not Available	S-HSCR	No effect		Novel
	c.1062A>T	p.K354N	Sporadic	Father	S-HSCR	Possibly damaging		Novel
	c.1121G>A	p.L374L	P1: Sporadic P2: Familial	P1: Not Available P2: Mother	TCA	No effect		Novel
<i>PROK1</i>	-45G>A		Sporadic	Mother	S-HSCR	No effect	P953L in <i>RET</i> from mother	Novel
	c.142C>T	p.R48W	Sporadic	Not Available	L-HSCR	Probably damaging		rs62623571
	c.162G>T	P.G54G*	P1: Sporadic P2: Sporadic	P1: Mother P2: Father	TCA S-HSCR	No effect	P1: P992L in <i>RET de novo</i>	Novel
<i>PROKR2</i>	c.202G>A	p.G68S	Sporadic	Mother	L-HSCR	Probably damaging		Novel
	c.253C>T	p.R85C*	P1: Sporadic P2: Familial	P1: Father P2: Father	S-HSCR S-HSCR	Probably damaging		Cole LW, 2008
	c.254G>A	p.R85H	Sporadic	Mother	NA	Probably damaging	V145L in <i>RET</i> from mother	Dodé C, 2006
	c.802C>T	p.R268C	Sporadic	Father	TCA	Probably damaging	G593X in <i>RET</i> from father	rs78861628
	c.868C>T	p.P290S	Sporadic	Mother	L-HSCR	Probably damaging	R93W in <i>GDNF</i> from father	Dodé C, 2006
	c.876 C>T	p.Y292Y	Sporadic	Father	S-HSCR	No effect		Novel
	c. 889G>A	p.V297I	Sporadic	Father	S-HSCR	Tolerated		Novel

S-HSCR: short segment; L-HSCR: long segment; TCA: total colonic aganglionosis; NA: not available data.

*Sequence variants detected in normal controls.

doi:10.1371/journal.pone.0023475.t001

phenotypes. In summary, we show *PROKR1* and *PROKR2* expression in human enteric NCCs, which suggests that both prokineticin receptors might mediate a complementary signalling to the *RET/GFR α 1/GDNF* pathway supporting proliferation/survival and differentiation of precursor cells during ENS development. These results, together with the detection of sequence variants in *PROKR1*, *PROK1* and *PROKR2* genes associated to HSCR and in some cases in combination with *RET* or *GDNF* mutation, provide the first evidence to consider them as susceptibility genes for HSCR.

Materials and Methods

Ethical approval

Approval from the Hospital Universitario Virgen del Rocío of Sevilla Health Ethics Subcommittee and fully written 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, as well as the requirements established in the Spanish law (Ley 14/2007, from 3 July 2007, “Ley de Investigación Biomedica”).

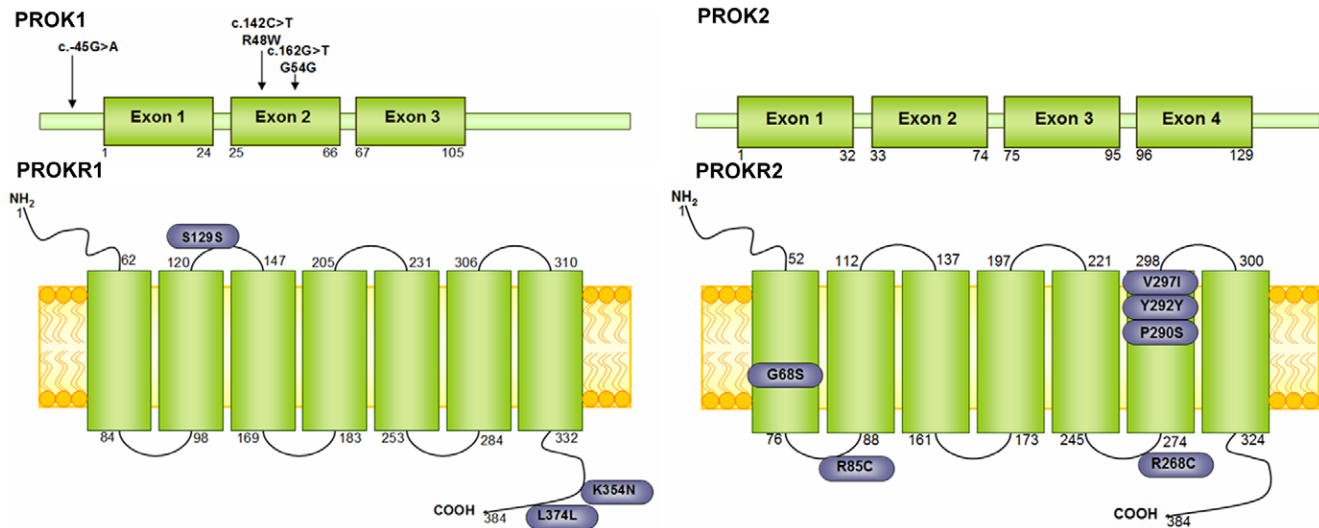


Figure 5. Schematic representation of *PROKR1*, *PROK1*, *PROKR2* and *PROK2* proteins. Distribution of the missense variants identified in our cohort of HSCR patients with respect to the predicted proteins structure using the SOSUI secondary structure prediction program. doi:10.1371/journal.pone.0023475.g005

Generation of Human Neurosphere

Human postnatal tissues of ganglionic full-thickness gut were obtained from 13 HSCR neonates (3 female, 10 male) undergoing gut resection surgery at Hospital Universitario Virgen del Rocío in Sevilla. In addition, 5 endoscopic gut biopsy samples (1 female, 4 male) from patients investigated for other gastrointestinal disorders were used as controls. From both HSCR patients and controls, age were comprised between 6 and 24 months.

All the samples were incubated in a solution of 0,26 mg/mL Trypsin Collagenase, 5 mg/mL Dispase, 0,28 mg/mL Hyaluronidase, 3,3 µg/mL Elastase and 0,6 mg/mL Collagenase in phosphate-buffered saline (PBS) for up to 30 minutes at 37°C. Digested tissue was triturated and washed, and the cells were cultured in 6-wells ultra-low attachment cluster plate. The culture medium was Dulbecco's modified Eagle medium (DMEM; 1 mg/mL Glucose) containing 100 U/mL penicillin, 100 g/mL streptomycin, supplemented with 2 mM L-glutamine (Gibco, Life Technology, California, 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 (bFGF), 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL glial cell derived neurotrophic factor (GDNF) (Peprotech, London, UK). Subcultures were performed every 7 days and 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% (wt/vol) paraformaldehyde in 0.1 M PBS. The primary antibodies used were β -III-tubulin (TuJ1) (1:2000; Promega Corporation, Madison), Nestin (1:200; Santa Cruz Biotechnology, Inc.), glial fibrillary acidic protein (GFAP) (1:1000; Dako), S100B (1:200; Dako), α -Smooth Muscle Actin (α SMA) (1:400; Sigma Aldrich), RET receptor (1:250; Santa Cruz Biotechnology), Prokineticin Receptor 1 (PKR1) (1:500) and Prokineticin Receptor 2 (PKR2) (1:1000); (Lifespan Biosciences, Inc., Seattle, WA). The secondary antibodies used were labeled with Alexa Fluor 568 (Life Technology), Cy5 and Cy2, (Jackson Immuno Research Laboratories, Inc., West Grove, PA). The coverslips were mounted on slides with Fluoro-Gel (EMS, Hatfield, PA, USA) and fluorescent signals were detected using a Leica Spectra confocal microscope. All of the secondary antibodies were adsorbed against several species to prevent undesired cross-reactions. Omission of primary antibodies resulted in no detectable staining in all cases.

RT-PCR

Total RNA was isolated from neurospheres and SK-N-MC cells [32] using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), according to manufacturer instructions. 1 µg of RNA was reverse-transcribed using SuperScriptTM RNA Amplification System and PCR reactions were performed using specific primers: PROKR1-F 5'-TGAGGATGTGACCAAT-TCCA-3', PROKR1-R 5'-GATGGTGAAGCCGTAGAAGG-3', PROKR2-F: 5'-CGGCAGCTCTCCTGGGAGCATGGC-3'; PROKR2-R: 5'-CGTCTGGAACCCAGGGACTGCC -3' and 18S-F: 5'-CAGCCACCCGAGATTGAGCA-3', 18S-R: 5'-TAGTAGCGACGGGCGGTGTG-3'. The estimated sizes of RT-PCR products were 769 for PROKR1, 432 bp for PROKR2 and 253 for 18S.

Mutational analysis

A total of 230 patients diagnosed with HSCR (23% female, 77% male) at Hospital Universitario Virgen del Rocío in Sevilla were

included in the mutational analysis. 208 were sporadic cases, while 22 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 and intron/exons boundaries of *PROKR1*, *PROK1*, *PROKR2* and *PROK2* was carried out by denaturing high performance liquid chromatography (dHPLC) in a WAVE DNA Fragment Analysis system (Transgenomic, Omaha, NE). In addition, those exons with aberrant profiles were subjected to sequence analysis using an ABI Prism[®]3730 Genetic Analyzer (Applied Biosystem, Foster City, CA) and the SeqScape[®] v2.5 software (Applied Biosystem, Foster City, CA).

When a novel change was detected, the appropriated DNA fragment was also screened in a group of 150 normal controls, in order to determine that such variant is not just a common polymorphism never previously described.

Bioinformatic tools

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/cgi-bin/seq_tools/promoter.pl; and <http://www.ebi.ac.uk/asd-srv/wb.cgi>. To predict the putative pathogenic role of a novel variant at the protein sequence level, we selected the SIFT, Polyphen and DiANNA tools (<http://blocks.fhcr.org/sift/SIFT.html>, <http://genetics.bwh.harvard.edu/pph/>, <http://clavius.bc.edu/~clotelab/DiANNA/>). The PROKR1 and PROKR2 protein sequences were submitted to ScanProsite (<http://expasy.org/tools/scanprosite/>) to scan for the occurrence of patterns, profiles and motifs stored in the PROSITE database.

Supporting Information

Figure S1 Histogram showing distribution of Nestin+ cells, TuJ1+ cells, S-100+ cells and SMA+ cells in neurospheres. At least 3 different preparations were assessed for each marker and 3–6 neurospheres were analysed per coverslip (~1.300 cells). Data are presented as percentage of each phenotype with the standard error of the mean.

(TIF)

Figure S2 Confocal microscopy images of floating neurospheres immunostained with antibodies against Nestin (blue), TuJ1 (green), PROKR1 (red), PROKR2 (red) and counterstained with DAPI (4,6-diamidino-2-phenylindole). Scale bars 25 µm.

(TIF)

Acknowledgments

We would like to thank the patients and families that participated in this study.

Author Contributions

Conceived and designed the experiments: MR-F SB. Performed the experiments: MR-F AT RN-T. Analyzed the data: MR-F AT RN-T SB. Contributed reagents/materials/analysis tools: AT RN-T. Wrote the manuscript: MR-F GA SB. Delineated the clinical presentation of the patients and recruited the families: JcDA SB.

References

- Le Douarin NM, Kalchauer C (1999) *The Neural Crest*. New York: Cambridge University Press. pp 445.
- Heanue TA, Pachnis V (2007) Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. *Nat Rev Neurosci* 8: 466–469.
- Amiel J, Sproat-Emison E, Garcia-Barcelo M, Lantieri F, Burzynski G, et al. (2008) Hirschsprung disease, associated syndromes and genetics: a review. *J Med Genet* 45: 1–14.
- Tam PK, Garcia-Barcelo M (2009) Genetic basis of Hirschsprung's disease. *Pediatr Surg Int* 25: 543–558.
- Ngan ES, Lee KY, Sit FY, Poon HC, Chan JK, et al. (2007) Prokineticin-1 modulates proliferation and differentiation of enteric neural crest cells. *Biochim Biophys Acta* 1773: 536–545.
- Ngan ES, Shum CK, Poon HC, Sham MH, Garcia-Barcelo MM, et al. (2008) Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic factor (GDNF) to mediate proliferation and differentiation of enteric neural crest cells. *Biochim Biophys Acta* 1783: 467–478.
- Li M, Bullock CM, Knauer DJ, Ehlert FJ, Zhou QY (2001) Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Mol Pharmacol* 59: 692–698.
- Kaser A, Winklmayr M, Lepperdinger G, Kreil G (2003) The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. *EMBO Rep* 4: 469–473.
- Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, et al. (2002) Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *J Biol Chem* 277: 19276–19280.
- Soga T, Matsumoto S, Oda T, Saito T, Hiyama H, et al. (2002) Molecular cloning and characterization of prokineticin receptors. *Biochim Biophys Acta* 1579: 173–179.
- Negri L, Lattanzi R, Giannini E, Melchiorri P (2007) Bv8/Prokineticin proteins and their receptors. *Life Sci* 81: 1103–1116.
- Dodé C, Teixeira L, Levlillers J, Fouveau C, Bouchard P, et al. (2006) Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet* 2: 175.
- Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, et al. (2008) Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. *J Clin Endocrinol Metab* 93: 3551–3559.
- Abreu AP, Trarbach EB, de Castro M, Frade Costa EM, Versiani B, et al. (2008) Loss-of-function mutations in the genes encoding prokineticin-2 or prokineticin receptor-2 cause autosomal recessive Kallmann syndrome. *J Clin Endocrinol Metab* 93: 4113–4118.
- Ruiz-Ferrer M, Fernández RM, Antiñolo G, López-Alonso M, Eng C, et al. (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, Torroglosa A, Luzón-Toro B, Fernández RM, Antiñolo G, et al. (2011) Novel mutations at RET ligands genes preventing receptor activation are associated to Hirschsprung's disease. *J Mol Med* 89: 471–80.
- 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, et al. (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.
- 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.
- Sánchez-Mejías A, Watanabe Y, Fernández RM, López-Alonso M, Antiñolo G, et al. (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.
- Sánchez-Mejías A, Fernández RM, López-Alonso M, Antiñolo G, Borrego S (2009) Contribution of RET, NTRK3 and EDN3 to the expression of Hirschsprung disease in a multiplex family. *J Med Genet* 46: 862–864.
- Rauch U, Hänsgen A, Hagl C, Holland-Cunz S, Schäfer KH (2006) Isolation and cultivation of neuronal precursor cells from the developing human enteric nervous system as a tool for cell therapy in dysganglionosis. *Int J Colorectal Dis* 21: 554–559.
- Almond S, Lindley RM, Kenny SE, Connell MG, Edgar DH (2007) Characterisation and transplantation of enteric nervous system progenitor cells. *Gut* 56: 489–496.
- Lindley RM, Hawcutt DB, Connell MG, Edgar DH, Kenny SE (2009) Properties of secondary and tertiary human enteric nervous system neurospheres. *J Pediatr Surg* 44: 1249–1255.
- Metzger M, Caldwell C, Barlow AJ, Burns AJ, Thapar N (2009) Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. *Gastroenterology* 136: 2214–2225.
- Young HM, Bergner AJ, Muller T (2003) Acquisition of neuronal and glial markers by neural crest-derived cells in the mouse intestine. *J Comp Neurol* 456: 1–11.
- Bixby S, Kruger GM, Mosher JT, Joseph NM, Morrison SJ (2002) Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity. *Neuron* 35: 643–656.
- Kruger GM, Mosher JT, Bixby S, Joseph N, Iwashita T, et al. (2002) Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. *Neuron* 35: 657–669.
- Ngan ES, Tam PK (2008) Prokineticin-signaling pathway. *Int J Biochem Cell Biol* 40: 1679–1684.
- Ngan ES, Sit FY, Lee K, Miao X, Yuan Z, et al. (2007) Implications of endocrine gland-derived vascular endothelial growth factor/prokineticin-1 signaling in human neuroblastoma progression. *Clin Cancer Res* 13: 868–875.
- Melchiorri D, Bruno V, Besong G, Ngomba RT, Cuomo L, et al. (2001) The mammalian homologue of the novel peptide Bv8 is expressed in the central nervous system and supports neuronal survival by activating the MAP kinase/PI-3-kinase pathways. *Eur J Neurosci* 13: 1694–1702.
- Monnier C, Dodé C, Fabre L, Teixeira L, Labesse G, et al. (2009) PROKR2 missense mutations associated with Kallmann syndrome impair receptor signalling activity. *Hum Mol Genet* 18: 75–81.