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Novel MLPA procedure using self-designed probes allows comprehensive analysis for CNVs of the genes involved in Hirschsprung disease

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Abstract

Background: Hirschsprung disease is characterized by the absence of intramural ganglion cells in the enteric plexuses, due to a fail during enteric nervous system formation. Hirschsprung has a complex genetic aetiology and mutations in several genes have been related to the disease. There is a clear predominance of missense/nonsense mutations in these genes whereas copy number variations (CNVs) have been seldom described, probably due to the limitations of conventional techniques usually employed for mutational analysis. In this study, we have looked for CNVs in some of the genes related to Hirschsprung (*EDNRB*, *GFRA1*, *NRTN* and *PHOX2B*) using the Multiple Ligation-dependent Probe Amplification (MLPA) approach.

Methods: CNVs screening was performed in 208 HSCR patients using a self-designed set of MLPA probes, covering the coding region of those genes.

Results: A deletion comprising the first 4 exons in *GFRA1* gene was detected in 2 sporadic HSCR patients and *in silico* approaches have shown that the critical translation initiation signal in the mutant gene was abolished. In this study, we have been able to validate the reliability of this technique for CNVs screening in HSCR.

Conclusions: The implemented MLPA based technique presented here allows CNV analysis of genes involved in HSCR that have not been previously evaluated. Our results indicate that CNVs could be implicated in the pathogenesis of HSCR, although they seem to be an uncommon molecular cause of HSCR.

Background

Hirschsprung disease (HSCR, OMIM 142623) is a congenital malformation characterized by the absence of intramural ganglion cells in the myenteric and submucosal plexuses along a variable portion of the distal intestine, due to a defect of craniocaudal migration of neuroblasts originated from the neural crest [1,2]. HSCR presents an estimated incidence of 1/5000 live births, and has a non mendelian inheritance with reduced penetrance, variable expression and male predominance. Although familial forms exist, the vast majority of cases are sporadic. In addition, the disease can present as an isolated trait, although in a 30% of the cases it is associated with chromosomal abnormalities, neurodevelop-

ment disorders and a variety of additional isolated anomalies and syndromes [2].

HSCR has a complex genetic aetiology with several genes being described as associated with isolated or syndromic forms. *RET* proto-oncogene is considered the major causal gene in HSCR and has been extensively studied in different HSCR series worldwide. Both traditional *RET* coding mutations and a common non-coding *RET* variant within a conserved enhancer-like sequence in intron 1, have been reported to be associated with a great proportion of HSCR cases [2-4]. Other genes associated with HSCR encode for receptors, ligands (especially those participating in the *RET* and *EDNRB* signaling transduction pathways), and transcriptional factors, such as *SOX10* and *PHOX2B*, among others, that are usually involved in the neural crest cell development and migration [2].

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Interestingly, many recent reports point out the implications of altered gene dosage in diagnosis, prognosis and therapy in different human diseases [5]. Nonetheless, it does not seem to be apparently the case of HSCR, with the current data supporting a predominance of missense/nonsense mutations, although small deletions/insertions have been occasionally observed (Human Gene Mutation Database of the Institute of Medical Genetics in Cardiff, <http://www.hgmd.cf.ac.uk/ac/index.php>). In fact, no duplications and only one gross deletion affecting the entire sequence of *RET* have been reported [6,7]. To date, only 2 studies have been reported investigating gene dosage anomalies in HSCR patients based on MLPA technique (Multiple Ligation-dependent Probe Amplification) [8,9], which has an optimal performance to detect alterations of gene dosages [10]. Both of them used MLPA MRC-Holland commercial kit for HSCR, that analyses a limited number of genes (*RET*, *ZEB2*, *EDN3* and *GDNF*), and revealed no CNVs associated to HSCR in those genes [8,9]. In additions we have performed a *SOX10* deletion screening on our HSCR patients [11] based on a previously reported QMF-PCR method (Quantitative Multiplex Fluorescent PCR), obtaining negative results [12]. Nevertheless, studies in other "HSCR genes" are necessary to rule out the potential implication CNVs in the pathogenesis of HSCR.

In the present study we have analyzed the presence of CNVs for *EDNRB*, *NRTN*, *GFRA1* and *PHOX2B* in our patient series, using self-designed MLPA probes, as no commercial kit is available for those genes, and none of them has been previously evaluated for mid-size deletions/duplications using a high-throughput technique. The present self-design set of probes for MLPA analysis, together with the available MLPA commercial kit for HSCR, would lead to the complete analysis of CNVs within coding region of the most prevalent genes in HSCR.

Methods

Patients and Control Subjects

In this study, a total of 208 HSCR patients have been included (22% female, 77% male). 188 out of the 208 patients were sporadic cases, while 20 were familial cases belonging to 13 different families. In addition, 6 of those patients presented with associated Down's syndrome, and 1 presented with Waardenburg's Syndrome type 4. In order to define the exact HSCR phenotype in our patients, we have used the criteria recommended by Chakravarti and Lyonnet [1]. Following these criteria, 137 cases were catalogued as short-segment HSCR (S-HSCR, 81%), 21 cases as long-segment (L-HSCR, 12%), and 12 cases presented as total colonic aganglionosis (TCA, 7%). Data were not available for the remaining 38 cases.

We have also used a group of 100 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.

Genomic DNA was extracted according to standard protocols and 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 and was approved by the Hospitales Universitarios Virgen del Rocío IRB.

MLPA analysis

Gene dosage variations on *EDNRB*, *GFRA1*, *NRTN* and *PHOX2B* were analysed by MLPA technology. The selection of the genes was based on their implication in ENS development and HSCR disease [13]. More specifically, we have selected *EDNRB*, the second major gene for HSCR, which has a considerably higher mutational incidence than *EDN3*, *GDNF* or *ZEB2* [2], included in the commercial kit. Since *RET* and *GDNF* are already included in the commercial MLPA kit for HSCR, we decided to include *NRTN* and *GFRA1*, as they are implicated in the same signaling pathway and have been previously associated to HSCR [14,15]. In addition and due to the implication of *PHOX2B* deletions in human pathologies and syndromes than frequently present with HSCR (CCHS) [16], we have also included this gene in the present study. Following MRC-Holland recommendations, we designed 31 sets of probes to detect deletions and duplications in one or more exons of these 4 genes (Table 1). In addition, we designed 3 control fragments hybridizing to different genome regions, that have never been associated with HSCR before and have been reported to contain no CNVs. Probes and EK-1 kits were supplied by Sigma (Sigma-Aldrich, St. Louise, MO) and MRC-Holland (MRC-Holland, Amsterdam, Netherlands) respectively.

Capillary electrophoresis analysis was performed using an ABI PRISM[®] 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and for data analysis we used GeneMarker v 1.75 (Softgenetics L.L.C, State College, PA). We normalized the samples by peak height comparing patients with 10 controls. These 10 control individuals had been confirmed to have no duplications or deletions in the studied genes, by a previous analysis using Affymetrix Genome-Wide Human SNP Arrays 6.0. In addition as a positive control we included a patient harbouring a *GFRA1* deletion in heterozygosis (patient HSCR-5, presenting with TCA-total colonic aganglionosis), which had been previously characterized by southern blot and lost of heterozygosity of STRs [15]. This individual not only was useful as positive control, but also confirmed the validity of our method to detect deletions in the genes

Table 1: Self-designed MLPA probes used in the molecular analysis of *EDNRB*, *GFRa1*, *NRTN* and *PHOX2B* CNVs of 208 HSCR patients.

	Exon	Probe Oligo Sequence*	bp	
EDNRB	EDNRBen1Δ3	LPO TCTGGCGGTGATTGATGGGAAG RPO GGATGAATGAATAAAAGTACTTGTCTGATGGCACCC	100	
	EDNRBex1	LPO TCTACAAGAACAAGTGCATGCGAAACG RPO GTCCCAATATCTTGATCGCCAGCTTGCCATCAATCGCCATTGCA	112	
	EDNRBex2	LPO GGCAGAGGACTGGCCATTGGAG RPO CTGAGATGTGTAAGCTGGTGCCTTT	90	
	EDNRBex3	LPO CGACAGCAGTAGAAAATGTTTTGATTTGGGTG RPO GTCTCTGTGGTTCTGGCTGTCCCTGAAGAGGTT TTGTGTACGGACCTAAAGTTC	128	
	EDNRBex4	LPO TGGCATGCAGATTGCTTTAAATGATCAC RPO CTAAGCAGGTAAGAAAATACAAATATTGAGAGGGACACGGCG	113	
	EDNRBex5	LPO GTGGCCAAAACCGTCTTTTGCCTG RPO GTCCTTGTCTTTGCCCTCTGCTGGCTT	93	
	EDNRBex6	LPO CGATGCTATTACATAACCCAATTGCTCTGTATTTGGTGAG RPO GTGAGCAAAAGATTCAAAAAGTCTCTTGGAGGAAGTCGAGGAGTAC	130	
	EDNRBex7	LPO GCAGTCGTGCTTAAAGTTCAAAG RPO CTAATGATCACGGATATGACAACCTTTGCTGAGTG	99	
	GFRa1	GFRa1ex1	LPO CCTAGCGCAGATAAAGTGAGCCCGAAAG RPO GGAAGGAGGGGGCGGGACACCATTGCTATAGA CGTAGCTGTGAGTACCAACCGAATAGCAATC	135
		GFRa1ex2	LPO CAACGACTAGAGAGGCACCATGTTCTGGCGACC RPO GATGGAGCTGAACTTTGGGCGGCCAGTGACT GCCTGAAGGTCTCACGGCT	127
GFRa1ex3		LPO GGAGAAGAAGTGCCTGCGCATTACTGGAG RPO CATGTACCAGAGCCTGCAGGGTAC	96	
GFRa1ex4		LPO GGAGGATCCCATATGAACCAGTTAACAG RPO CAGATTGTCAGATATATTCCGGGTGGTC	100	
GFRa1ex5		LPO CAGGCACTTGAGGATTTCCAGGTAGGACCTCTAGTTGCAG RPO GAAAACAAGGTCAGGGCTGCCACTGGTTCTATAATACAATGGAGACG	131	
GFRa1ex6		LPO GGAACAAGTGCCTGGATGCAGCGAAGGCCTG RPO CAACCTCGACGACATTTGCAAGAAGTACCCGGTAT	108	
GFRa1ex7		LPO TGCCAGCCAGAGTCAAGGTCTGTCAG RPO CAGCTGTCTAAAGGAAAACCTACGCTGA	95	
GFRa1ex8		LPO CCATACATCACCGCATTCTCGCAGAAGAGCCTC AGTGTGGCCCATGGTGTGACTG RPO CAGCAACAGTGGGAACGACCTAGAAGAGTGCTTG	132	
GFRa1ex9a		LPO GAGGAAGTTCAATGGCTCCGATGTGACCCTGTGGCAG RPO CCAGCCTTCCAGTACAGACCACCACTGC	108	
GFRa1ex9b		LPO CCAATTGACCACCTATGGGCCAGTCC RPO CCAGTCAGGTCAAAGAAGAGGGTTTACGTAGACCTCA GTTCACTGGAGTCTGCTGTTGCTCTATACTCAC	140	
GFRa1ex9c		LPO GGTTCAAGGCAACACAGAGACAAAG RPO CATCTTCAGGGGGAGCAGGTAGAGG	91	

Table 1: Self-designed MLPA probes used in the molecular analysis of *EDNRB*, *GFRa1*, *NRTN* and *PHOX2B* CNVs of 208 HSCR patients. (Continued)

	GFRa1ex9d	LPO GTGTCCACTTGTTTTACGCAG RPO CTGACTTTACTGGACATTATTCAGACCAGTGGTTGGGTGCGCTCG	108
	GFRa1ex10	LPO GCTGAAATCCAATGTGTCGGGCAATACACAC RPO CTCTGTATTTCCAATGTAAGTATGGGCGTGGACTCGCAT	112
	GFRa1ex11	LPO CCAAAGTGTGCGCGTGTGCATAAGGTTCCGTTCCCG CCCACTGCTGGTCTGTGGTAACC RPO GCTCTGTCCACCCTATTATCTTTAACAGAAACA	136
NRTN	NRTNex1	LPO CGTTCAAAGTCAAAGGCCCCACACTGAGTC RPO CTGGCCAGCGCCTGTGCCGTTGGCTGACGA GAAGTACGGAATCGAATCTATAGTGACCTAT	136
	NRTNex2	LPO CGAATTAGAGATTTAACTTCTCCCCTCGCAGACCGTGCACTC RPO CTGCAGGGGGCCCCGGATGCGATGGAGCTGTAA TACGACTCACTATAGGAGTA	139
PHOX2B	PHOX2B5'UTR	LPO CTTAAATCATGGGGCCACTGAAGTC RPO CACACTGCTCGTCTTTGT	92
	PHOX2Bex1A	LPO CCTCAATTCCTCTGCTACGAGTC RPO CTGTATGGCTGGGATGGACACC	88
	PHOX2Bex1B	LPO CCAGTGCTTCCAGTATAACCCGATAAGGAC RPO CACTTTTGGGGCCACGTCCGGCT	96
	PHOX2Bex2	LPO CTGTCATACTCTAGTTCCTTACAACTCTTCAC RPO GGACCACGGCGGCTCAACGATGCAAGCGACAT	108
	PHOX2Bex3A	LPO TCTTCTTCTCCCCCTGCTTACCGTCTCTC RPO TTCCGTCTTGGGCCAGGTGTGGTTCCAGAATTTACTAA	112
	PHOX2Bex3B	LPO CGTCCTATCTCGCTCAAAGACCCAACG RPO GTGCCAAAGCCGCTTAGTGAAGAGCAGT	100
	PHOX2B3'UTR	LPO CTTTTTCATTGAGGGCCTAAAGTAATCGCGCTAAGAATAAAG RPO GGAAAACGGCGTCCGCCCTCATTTGCAAAGTGTGCGGGTGTGC	126
Reference genes	TOR1A	LPO GCACCGGCAAAAATTTTCGTCAGCAAGATCAT RPO CGCAGAGAATATTTACGAGGGTGGTCTGAAC	104
	EPO	LPO GCCTCAGCTGCTCCACTCCGAACAATCACTG RPO CTGACACTTTCCGCAAACCTTCCGAGTCTAGATAGTTCCAACAA	117
	SS18	LPO CGACAGCATTACGAAGCACAGCAGCCACCTATGGGAATGATG RPO GGTCAAGTTAACCAAGGCAATCATATGATGCGATATGC	122

*Sequences do not included the universal primers located at the 5' end of LPO and 3' end of RPO.

analysed. Following manufacturer recommendations, dosage quotients under 0.5 or over 1.3 were considered as indicating potential deletions or duplications respectively, and were confirmed in 3 independent assays.

Results

With the aim to analyse anomalies in the gene dosage of several genes described as associated to HSCR (*EDNRB*, *GFRa1*, *NRTN* and *PHOX2B*), but never previously ana-

lyzed by MLPA, we designed specific synthetic MLPA D-probes, following MRC-Holland recommendations. The hybridization, ligation and amplification of the MLPA probes were performed in 4 different probemixes of 8-10 probes each, together with the 3 control probes. Signal peaks height of the amplified products observed after electrophoresis, were as homogeneous as expected for self-designed probes, and peak normalization was suc-

cessfully fulfilled between the patient samples and controls in all the probemixes tested.

After the validation of our probes, we screened a total of 208 HSCR patients and found a deletion in *GFRA1* gene (c.(?-555)_431+?del; Figure 1) that affects exons 1a, 2a, 3 and 4 in isoform NM_005264 and exons 2b, 3 and 4 in isoform NM_145793. This deletion was detected in a heterozygous state, in a sporadic and isolated male HSCR patient presenting with short-segment HSCR (patient HSCR-115), and was not found in 100 control individuals tested. This deletion was inherited by his unaffected father, and was found to be absent in other healthy members of the family. There are 2 CNVs described for *GFRA1* gene annotated in the Database of Genomics Variants <http://projects.tcag.ca>, Variant_48418 and Variant_48004. The first variant is a 2.5Kb deletion located in the 3' untranslated region of the gene, while the other consisted on a 36 Kb deletion the genomic region containing exons 7, 8 and 9 of *GFRA1*. Therefore, the available data support that we are describing a novel deletion. Interestingly, this is the second time this deletion has been found in our HSCR patient series, in a patient not related with the one previously reported [Figure 1; 15].

In order to preliminarily examine the potential damaging effect of this deletion on *GFRA1* expression and functionality, we used InterProScan and AlternativeSplicing tools from EBI and Transec from EMBOSS. We verified that the critical translation initiation signal in the gene was abolished; subsequently no wild-type (WT) protein was expected to be expressed from the deleted copy of the gene. In addition, we checked *in silico* whether the deleted allele could produce any protein with similar functional capacity as GFR α 1. We found that an alternative peptide could be translated from deleted isoform NM_005264 with the same carboxyl-terminus amino-acidic sequence. This putative protein would maintain one of the 3 GDNF/GAS1 domains in the WT protein, but would also lack the localization signal in the N-terminal region. Although this deletion is well refined in its 3' end, we failed to establish the boundaries in the 5' end where all transcription and translation signals are located. Therefore it seems unlikely that the aberrant protein could be expressed, and in that case it would have a very limited, or even null, functionality.

Discussion

HSCR has a complex genetic aetiology and point mutations in several genes have been reported to be implicated in a portion of isolated and syndromic HSCR forms [2]. It is tempting to speculate that other genetic events different from point mutation, such as CNVs, have a functional role in the pathogenesis of HSCR. Very little is known in this field for HSCR since typical screening methods based

in conventional PCR are only able to detect small deletions/duplications (a few base pairs), and cytogenetic techniques can exclusively detect alterations in the order of megabases. Those techniques are neither powerful nor adequate to detect CNVs [10], so that those types of rearrangements would be missed. In this way, it would be possible that such mid-size deletions/duplications in several HSCR genes have been underreported. In addition, traditional techniques used to detect mid-size deletions/duplications, such as southern blot, are expensive, time consuming and not suitable for high-throughput results. For this reason we planned to perform CNVs screening in a large series of HSCR patients using MLPA technology, which can be performed in a large number of individuals within a short period time, in order to determine if it is a reliable technique suitable for a routine CNVs screening. Despite the negative results previously reported for HSCR MLPA commercial kit [8,9], we have obtained positive results with the finding of a deletion affecting the 4 first exons in *GFRA1*. This deletion was previously identified in a sporadic HSCR patient, but its actual implication in the pathogenesis of this disease remained unknown [15]. The finding of the same deletion in an independent patient with the same phenotype and its absence in the control population, support that this deletion at the *GFRA1* locus is a mutational event potentially related to HSCR. In addition, the implementation of MLPA technique for midsize deletion detection leads us to refine the deleted region at *GFRA1* locus. The protein GFR α 1 is one of the four co-receptors of the RET tyrosine kinase receptor. The binding of RET to GFR α 1 is required for the specific recruitment of GDNF and the subsequent phosphorylation of RET. Therefore, the presence of such a deletion in GFR α 1 would avoid the expression of the protein, presumably preventing RET phosphorylation and affecting the correct development of the ENS. The presence of this mutation in unaffected members of the family suggest that it could be necessary but not sufficient to produce the phenotype, and additional unidentified genetic events might be acting in this HSCR patient. In this sense, no point coding mutations were detected in this patient, or in the previously described patient harbouring the same deletion, in other HSCR-related genes tested such as *RET*, *GDNF*, *NRTN*, *PSPN*, *ARTN*, *EDNRB*, *EDN3*, *NTF3*, *NTRK3*, *SOX10* or *PHOX2B*. The present results indicate that CNVs are not a common molecular cause of HSCR, although they should be taken into account for further studies.

Conclusions

One of our goals was to provide a simple, reliable, economic and fast method for CNVs screening in HSCR related genes, and the present study has successfully validated the self-designed MPLA probes for CNVs analysis.

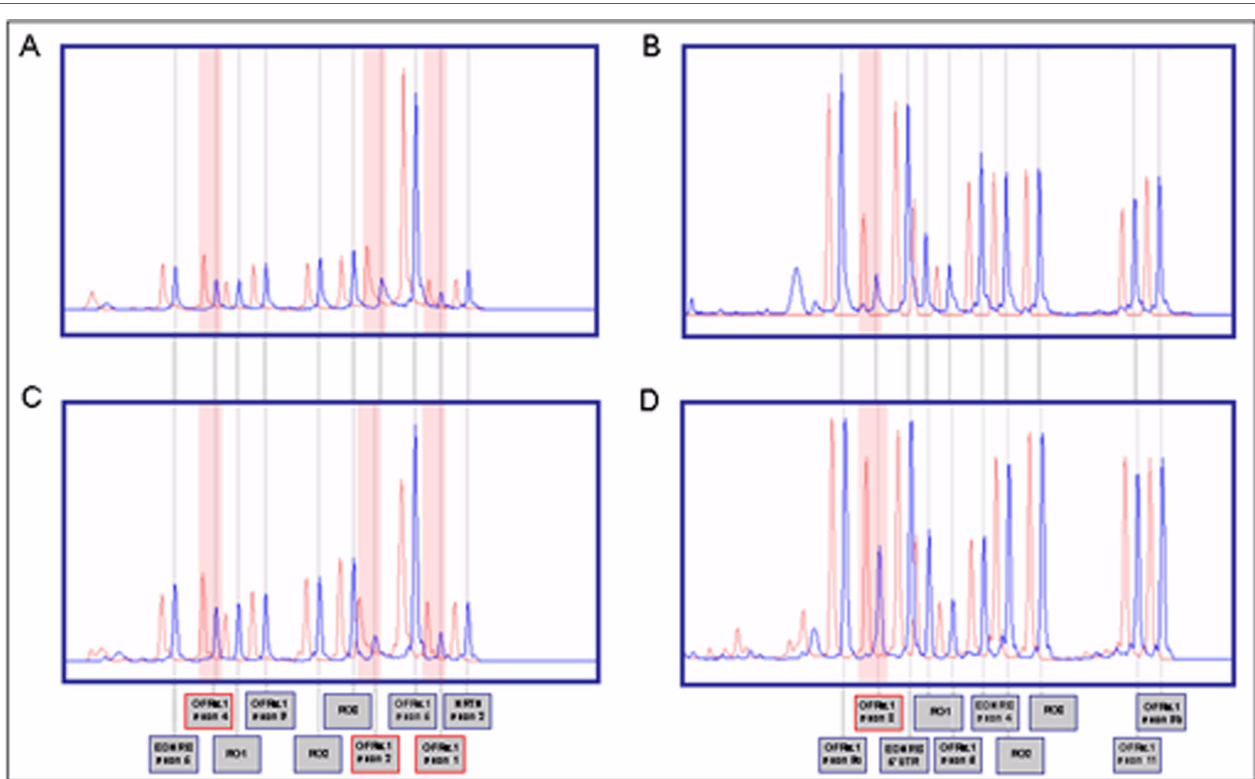


Figure 1 MLPA profiles for control individuals (red) and for the HSCR patient (blue). Two different probes-mixes are shown (A and C mix 1; B and D mix 2). For those HSCR patients (A and B, HSCR-115; C and D, HSCR-5), it was observed a decrease for the dosage of *GFRA1* exons 1, 2, 3 and 4, highlighted in red boxes and by arrows.

The design and validation of MLPA probes for additional genes represent an implementation for a technique that was restricted to the commercial production. In this sense, the present design, together with the commercial MLPA kit for HSCR, allows the complete analysis of CNVs in the coding region of the most prevalent genes for HSCR. In addition, the presence of a *GFRA1* deletion that seems to impair protein function, in an unrelated HSCR patient supports and confirms the idea that this specific deletion might participate in the development of HSCR. Despite the fact that CNVs seems to be an uncommon susceptibility factor leading to this disease, our results point out the importance of taking into account those molecular events in HSCR studies from now on, at least in *GFRA1* gene. Further screening of CNVs in additional series of patients would be necessary in order to completely address its real implications in the pathogenesis of HSCR.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS-M and RN-T carried out the molecular genetic studies and participated in the MLPA analysis. AS-M and SB participated in the design of the study and drafted the manuscript. RMF and GA helped to draft the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We would like to thank all the patients who participated in the study. This study was funded by Fondo de Investigación Sanitaria, Spain (PI070070 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 (PI-0249/2008). The CIBER de Enfermedades Raras is an initiative of the ISCIII. ASM is a predoctoral fellow founded by Instituto de Salud Carlos III, Spain.

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Received: 2 February 2010 Accepted: 11 May 2010

Published: 11 May 2010

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Pre-publication history

The pre-publication history for this paper can be accessed here:
<http://www.biomedcentral.com/1471-2350/11/71/prepub>

doi: 10.1186/1471-2350-11-71

Cite this article as: Sánchez-Mejías *et al.*, Novel MLPA procedure using self-designed probes allows comprehensive analysis for CNVs of the genes involved in Hirschsprung disease *BMC Medical Genetics* 2010, **11**:71

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