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 delay 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, neurodevelopment 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].
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 dosages 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 Waarbenburg’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 Rocio 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, GFRα1, NRTN and PHOX2B CNVs of 208 HSCR patients.

| Exon                        | Probe Oligo Sequence*              | bp |
|-----------------------------|------------------------------------|----|
| EDNRB EDNRBen1Δ3            | LPO TCTGGCCGGTGATGGGAGAAG          | 100|
|                             | RPO GGATGAAATGAATAAAAGTACCTTGCTGATGGCACCC |    |
| EDNRBex1                    | LPO TCTACAGAAACAAGTGCATGGCGAAACG   | 112|
|                             | RPO GTCACAATATCTGATGCGCCACTTGGCTGCTAATGCGCCATTCTG |    |
| EDNRBex2                    | LPO GGCAGAGGACTGGCCATTGGAG         | 90 |
|                             | RPO CTGAGATGTGAAGCTGGTGGTGCCTG     |    |
| EDNRBex3                    | LPO CGACAGCAGTGAATAATGGTATTGGTGG  | 128|
|                             | RPO GTCTCTGTGGTCCTGCTGCTGAAAGAGTT |    |
|                             | TTGTTAAGGACCTAAGGTC              |    |
| EDNRBex4                    | LPO TGGCACTGAGATTGCTTTAAATGATCAC | 113|
|                             | RPO CTAAGACAGTAAGAATAATACATTAGGAGGACCGGACCGGGGAGGAGGAGGAGGAGGAGG |    |
| EDNRBex5                    | LPO GTGGGCAAACGGTTTTTGGCT         | 93 |
|                             | RPO GTCCCTGTCTTTGCCCCCTCTGCTG     |    |
| EDNRBex6                    | LPO CGATGCTTCCATAAACCACATTTTCGCTG | 130|
|                             | RPO GTGAGCAAAAGATTACAAATATGCTGCTGAGAATGCGCTGCGGACGGAGGAGGAGGAGG |    |
| EDNRBex7                    | LPO GCAGTCGTGCTTTAAGGGCAAAG     | 99 |
|                             | RPO CTAATGATCGCAGGATATGCAACTTTTGCTGAGT |    |
| GFRα1 GFRα1ex1              | LPO CCTAGCGCAGATAAAGTGGAGGCGGGAAAG | 135|
|                             | RPO GGAAGGAGGGGGGGAGGACCCATTGCTATAGA|    |
|                             | CTAAGCAGATGATAGAAGGAGGACTTGCCCTG |    |
| GFRα1ex2                    | LPO CAACCGACTAGAAGAGCCAGCATTGCTGCTGCC | 127|
|                             | RPO GATGGAGCTGAACTTTGGGCGCCAGTGACT |    |
|                             | GCCTGAAAGGTCTCAGGGCT             |    |
| GFRα1ex3                    | LPO GGGAAGAAGACTGCTGGCGATTACTGGAG | 96 |
|                             | RPO CATGTACAGACGCTGCAGGTAC       |    |
| GFRα1ex4                    | LPO GGAGGATTCCCCCATATGAAACCGATTAAACAG | 100|
|                             | RPO CAGATGCTCAGATATTCAGGGGTTGTC  |    |
| GFRα1ex5                    | LPO CACGCCACTTGGAGATTCCCTCCAGGTAGGCCTG | 131|
|                             | RPO GAAAACAGGCTGAAGGGCTCCCTGCTTAC |    |
|                             | CTGATGCAAAATATACCTAAGGAGGAGGAGG |    |
| GFRα1ex6                    | LPO GGAAACACTGCTGCTGTAAGGCAGGGCGCT | 108|
|                             | RPO CAACCTCAGACGATTTGGCAAGAAGGTACCCGGGTAT |    |
| GFRα1ex7                    | LPO TGCCAGCCAGTCAAGGCTCTGCTCAG  | 95 |
|                             | RPO CACTGCTCAAAGGAAAACTAGCGCTGA  |    |
| GFRα1ex8                    | LPO CCATACATACCAGAGCAGCCCTCCACGAGCAGCGGCAGGCGTCTGTACE  | 132|
|                             | RPO AGTGGGCCCCATGGTGGTACTG       |    |
|                             | RPO CAGCAACAGTGGGAGCCACTAGAGAGGAGTGCTTG  |    |
| GFRα1ex9a                   | LPO GAGGAGAATGCTATGTCGGAATGGGCAGGGCAGG | 108|
|                             | RPO CACGCCTTCCACAGACACCACCAGCTC  |    |
| GFRα1ex9b                   | LPO CCAATTTATGCGCCGAGCAGCTTCAAGGGCGAGG | 140|
|                             | RPO CCATGCTAAGACAGAAGAGGGATTTAGCAATGAGCCTCA  |    |
|                             | GTCTCAGTTGATGCTGGTGCTCTATATACCTCAG |    |
| GFRα1ex9c                   | LPO GGTTCCAGGACACAGAGAAGGAAG    | 91 |
|                             | RPO CACTTCTAGGGGAGGAGTAAAGG      |    |
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 analyzed 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 successful.

### Table 1: Self-designed MLPA probes used in the molecular analysis of *EDNRB*, *GFRA1*, *NRTN* and *PHOX2B* CNVs of 208 HSCR patients. (Continued)

| Gene        | Probe Name | 5' End | 3' End |
|-------------|------------|--------|--------|
| GFRa1ex9d   | LPO GTGTCAGCGCTGTGGTTTCAACGCA | 108    |
|             | RPO CTGCACTTTACTGACGACATTATTTGTGCAA | |
| GFRa1ex10   | LPO GCTGAAATCAGCTGTCGGAATACACACAT | 112    |
|             | RPO CCTGATTCGATATTTTCAATGTAAGTAGTGGGCTGGACTCGCAT | |
| GFRa1ex11   | LPO CCAAGCTGGCGCTGTAAGCTGTCGATAGTGGATACCC | 136    |
|             | RPO GCTGTGCGACTTACCTATATACGAGGATGAACAAACAGA | |
| NRTN        | NRTNex1    | LPO CGTCAAGATCCGAGGCGGACTGACAT | 136    |
|             | RPO CTGCCCCAGGCGGACTGACATGAACAAACAGA | |
|             | NRTNex2    | LPO CGAATTAGAGATATTATACGATCTGCTCAGGACCGTGTGACT | 139    |
|             | RPO CATTGAGGAGGGCGGACTGACATGAACAAACAGA | |
| PHOX2B      | PHOX2B5'UTR| LPO CTAAATCATGCGGCCACCTGAAGTCT | 92     |
|             | RPO CACACATGCGACACGCTCATTTGCTT | |
|             | PHOX2Bex1A | LPO CCTCAATTCCCTGCACTCCAGAGTCT | 88     |
|             | RPO CTGTAGGCTGAGGACCC | |
|             | PHOX2Bex1B | LPO CCGAGTGCCGCGTCGGACTGACATATGGAACCGAC | 96     |
|             | RPO CACTTGGGCGGACTGCGCTGCT | |
|             | PHOX2Bex2  | LPO CTGTCATACTCTGTTAGCTGCTATG | 108    |
|             | RPO GGCAGCGGGCGGCGCTCAAGGAGCCGAC | |
|             | PHOX2Bex3A | LPO TTCTTCTTCTTCTGCGTGTCATCTC | 112    |
|             | RPO TTTCGCGTCTGCTGAGGCTGAGGCAATTTACTAA | |
|             | PHOX2Bex3B | LPO CTGCTCACTTCCTGCTGACCTCAGAAGGCCACCTAAGGAACCG | 100    |
|             | RPO GTGCAAATCGCGCCTGATGGAAGGCAAG | |
|             | PHOX2B3'UTR| LPO CTGGGCCACCCGGCGCGGTGCAGCT | 126    |
|             | RPO GCAGGAGGTGCGGCGGGGG | |

**Reference genes**

| Gene | Probe Name | 5' End | 3' End |
|------|------------|--------|--------|
| TOR1A| LPO GACACGCAGAACATGACAT | 104    |
|      | RPO GCACGACAGAATATTTACGAGGGGTGCCTGAAG | |
| EPO  | LPO GCCCGTCCTCCTGCTGACCTGGAACATCAGT | 117    |
|      | RPO CTGACACATCGCGCAACCCATCTGCTGACCTGGAACATCAGT | |
| SS18 | LPO CGACACGAGCAAGAATATTTACGAGGGGTGCCTGAAG | 122    |
|      | RPO GTGCAAGTTCACCAAGGCAATCAGTGAATGGGCGGATG | |

*Sequences do not included the universal primers located at the 5' end of LPO and 3' end of RPO.*
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 2.5K 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 GFRα1. 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 functional capacity as GFRα1. 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 functional capacity as GFRα1. 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 functional capacity as GFRα1. 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 functional capacity as GFRα1.

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 GFRα1 is one of the four co-receptors of the RET tyrosine kinase receptor. The binding of RET to GFRα1 is required for the specific recruitment of GDNF and the subsequent phosphorylation of RET. Therefore, the presence of such a deletion in GFRα1 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.
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 \textit{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 \textit{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.

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