Contribution of RET, NTRK3 and EDN3 to the expression of Hirschsprung disease in a multiplex family

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TITLE PAGE

Title: Contribution of *RET, NTRK3* and *EDN3* to the expression of Hirschsprung disease in a multiplex family.

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SUMMARY

Hirschsprung disease (HSCR) is a developmental disorder due to a defect of neural crest neuroblasts migration process. It is considered as the paradigm of complex disorders, with many loci contributing to the manifestation of the disease. Although HSCR commonly appears as a sporadic trait, approximately 20% of HSCR cases are familial, with complex patterns of inheritance. Here we report a multiplex HSCR family with an additive model of inheritance, in which the contribution of 3 genes (*RET, NTRK3, EDN3*) leads to HSCR phenotype. Our findings suggest that both *RET* and *NTRK3* mutations acting together would be necessary and sufficient for the appearance of the disease, while the *EDN3* mutation would act as a phenotype modifier factor in the context of this family as 2 different HSCR phenotypes are seen among the affected members: a short segment form, and a total colonic aganglionosis. The present results therefore support the complex additive model of inheritance previously proposed for Hirschsprung disease.

INTRODUCTION

Hirschsprung disease or aganglionic megacolon (HSCR, OMIM142623) is a developmental disorder of variable penetrance and expressivity, male predominance, and an incidence of 1/5000 newborn human infants [1, 2]. It is 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 neural-crest-derived neuroblasts. HSCR phenotype can be classified into 2 main groups: short segment forms (S-HSCR), which include patients with aganglionosis as far as the splenic flexure, and long segment forms (L-HSCR), when aganglionosis extends beyond there. A minority of cases present total colonic aganglionosis (TCA), the most severe form of the disease. Although HSCR commonly appears as a sporadic trait, approximately 20% of HSCR cases are familial, with complex patterns of inheritance, and a recurrence risk in relatives up to 200 times increased depending on the length of the aganglionosis [1].

HSCR is regarded as a complex and multifactorial disorder, in which the contribution of several different *loci* acting in an additive manner is usually required to cause the disease. Undoubtedly, the *RET* proto-oncogene is considered the major disease causing locus in HSCR with traditional coding mutations accounting for up to 50% of familial cases [1, 2]. In addition, most of the families without an identifiable *RET* coding mutation are compatible with linkage at the *RET* locus [3]. Interestingly several genes and loci have been found to modulate *RET* mutations penetrance or to interact with *RET* in HSCR. Gabriel et al, described two loci at 3p21 and 19q12 acting as RET-dependent modifiers under a multiplicative model of inheritance, showing again that HSCR susceptibility is accounted not only by *RET* mutations, but also by additional genetic events modulating *RET* expression [4].

Endothelin-B receptor (EDNRB) is regarded as the second major gene for HSCR [5], and the interaction between EDN3 and EDNRB is largely known to be essential for normal development of enteric ganglia [6, 7]. *EDN3* had been demonstrated to play a minor although significant role in both syndromic and nonsyndromic HSCR, acting as a rare susceptibility locus in nonsyndromic forms of the disease [8, 9, 10].

Besides the two mayor signalling pathways involved in the enteric nervous system (ENS) formation, that are known to be involved in HSCR, the NTF3-NTRK3 pathway has recently showed evidences of being also related to HSCR, since mutations in both *NTF3* and *NTRK3* genes were identified in isolated patients [11, 12].

Here we report a multiplex HSCR family with two affected members, who presented with aganglionosis extending to the descending colon and to the ileum respectively. Coding germline mutations in *RET* and *NTRK3* genes had been already detected in both patients [12, 13]. Therefore, we have sought to perform a mutational analysis of other candidate genes such as *GDNF, NTNR, PSPN, ARTN, NTF3, SOX10, PHOX2B,* *EDNRB* and *EDN3* genes with the aim of identifying additional genetic events that could explain the different phenotypes observed in both patients.

MATERIALS AND METHODS

Subjects of study

The affected patients were two siblings belonging to the same HSCR family; they were the only affected members in the family, as no features suggestive of HSCR were found in none of the rest of relatives. No consanguinity in any degree was documented for this family. The whole pedigree is shown in Figure 1. Isolated HSCR was diagnosed in the two patients based on findings from anorectal manometry, and absence of enteric plexuses from histological examination of biopsy material. Following the criteria recommended by Chakravarti and Lyonnett to define HSCR phenotype [1], patient III1, with aganglionosis extending to descending colon, was catalogued as S-HSCR, while his brother III2 was catalogued as TCA.

Mutational analysis

Genomic DNA was extracted from peripheral blood leukocytes from all the available family members and healthy controls, using standard protocols. Primers were designed for the mutational screening of the *GDNF, NTNR, PSPN, ARTN, NTF3, SOX10, PHOX2B, EDNRB* and *EDN3* coding regions, the intron/exon boundaries and the untranslated regions. Primers and PCR conditions are available on request.

The mutational screening was carried out on an automated dHPLC device as previously described [13]. Those samples with aberrant wave profiles were subjected to sequence analysis using an automated sequencer ABI 3730 (Applied Biosystems, Foster City, CA) and sequences were compared to wild type EDN3 sequence (Genebank accession nº NC_000020.9).

RESULTS

One of the most interesting finding in this particular pedigree is that the two affected members also carry the *RET* mutation IVS6+2T>A [13], and the *NTRK3* mutation R645C [11] (Figure 1), both of them previously published as related to HSCR phenotype, while only one of them harbour the *EDN3* mutation.

Mutational screening of the selected genes, revealed the presence of heterozygous variant c.560insA at exon 4 of *EDN3* in the TCA patient (Figure 1, individual III.2). The mutation was found to be inherited from his unaffected mother, was present in other 3 healthy relatives, and absent in 300 control chromosomes tested. This insertion is located in the EDN3-like domain of the preproendothelin, but not in the mature endothelin, and it is predicted to generate changes at the protein level in two out of three of the EDN3 isoforms. In isoform 1 the insertion generates a premature stop codon, resulting in a prepreprotein of 197 residues length. By contrast, on isoform 2 this mutation, rather than generating a premature stop codon, results in an aberrant protein of 250 amino acids residues length. Of note, the *EDN3* mutation is only present in the patient with the more aggressive phenotype. As it is shown on Figure 1, there are healthy carriers of *EDN3* mutation, *NTRK3* mutation and *EDN3*+*RET* mutations. By contrast, only HSCR patients were found carrying the combination of *RET*+*NTRK3* mutations.

DISCUSSION

The individual contribution of mutations at *RET* and *NTRK3* was proposed to be necessary but not sufficient to cause the disease, as both of them were present in other unaffected members of the family. The fact that they appeared together only in the two affected members of the family [12], indicates that it is their additive effect which leads to the manifestation of the disease.

The *EDN3* gene encodes a large inactive preproendothelin-3 precursor that yields a biologically active 21-amino-acid mature peptide produced by a two-step proteolytic cleavage at two furin and one endothelin-converting enzyme 1 cleavage sites respectively. The *EDN3* mutation here reported leads to the aberration of a part of the protein which is cleavaged off during this process. This mutation had been previously described in a patient presenting with Idiopathic congenital central hypoventilation syndrome (CCHS, OMIM 209880) a disease which in rare cases is found associated with HSCR [10]., and in two HSCR patients [14]. The functional assay performed by Bolk et al. for the c.560insA mutation failed to demonstrate a deleterious effect for the protein when mutant cDNA was transfected into Chinese hamster ovary cells and levels of produced EDN3 peptides were measured [15]. However, the authors pointed out that the method used might be inappropriate to test this particular sequence change and the test was not predicting what would happen *in vivo*. In fact, the presence of this mutation alone has no consequences in the normal ENS development as we could observe at I1 and II1 family members, suggesting that the mutant protein is retaining part of the functionality or gene dosage is not crucial in this process. However, presence of such aberrant polypeptide coil in the protein could, even in a slightly manner, affect translation of the EDN3 precursor, polypeptide folding and/or proteolytic processing resulting in a less effective cleavage. We could then postulate that this subtle change could alter the development of the enteric nervous system and modulate the penetrance of mutations in other genes known to be responsible for the disease or modifying HSCR phenotype.

During the ENS formation neural-crest-derived cells, originated at sacral and vagal levels, colonise the gut and proliferate in response to GDNF via RET transduction. Those proliferating precursors migrate in a rostro-caudal fashion and differentiate into enteric neurons and glia in response to a variety of neurotrophic factors within the microenvironment of the forming bowel. One of those neurotrophic factors is NTF-3, which exerts its function through the receptor NTRK3. NTF-3 has a late-acting role in the formation of the ENS, promoting survival and differentiation of a subset of enteric precursors into neurons and glia, and maintaining those differentiated cells during adulthood [16]. Thus, mutations that impair the function of RET and NTRK3 in the same patient would result in failure of proliferation and migration of enteric precursors to the most distal portion of the gut. This would lead to the absence of ENS plexuses on that region, together with a failure of differentiation and maintenance of a portion of those precursors into neurons and glia. EDN3 acts preventing early differentiation of neural-crest-derived precursors [17]. In this regard, the additional presence of an alteration in the EDN3 signalling pathway might lead to an increase in the length of the aganglionic region, as differentiation would abolish migration in an earlier state of ENS formation.

Therefore, our finding of an *EDN3* mutation in a multiplex family also harbouring mutations at *RET* and *NTRK3* genes support the hypothesis of an additive model of inheritance for HSCR. The presence of the *EDN3* c.560insA in the patient with the aganglionosis extending to the ileum, and not in his affected sibling with a milder phenotype (Figure 1), strongly suggests that the *EDN3* gene, would be acting as a phenotype modifier factor in this particular family and that the accumulation of gene variants predisposing to HSCR in the genetic background of the patient have a greater impact on the expression of the disease.

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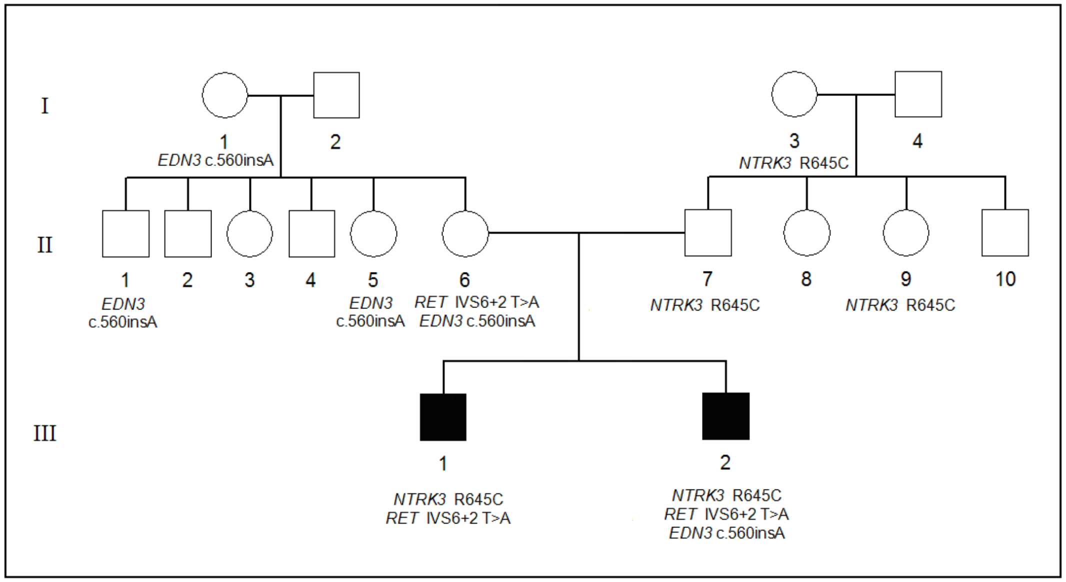
I
II
III
1
EDN3 c.560insA
2
3
NTRK3 R645C
4
1
EDN3 c.560insA
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3
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EDN3 c.560insA
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RET IVS6+2 T>A
EDN3 c.560insA
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