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Endothelin-B receptor mutations in patients with isolated Hirschsprung disease from a non-inbred population

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Hirschsprung disease (HSCR), or aganglionic megacolon, is the most common cause of congenital intestinal obstruction. Two different loci have been found to be tightly linked to HSCR on chromosomes 10 and 13, respectively. Recently, mutations in the RET protooncogene on chromosome 10q11.2 were identified in several HSCR patients. In addition, a missense mutation in the endothelin-B receptor (EDNRB) gene on chromosome 13q22 was found in an inbred Mennonite kindred affected by HSCR and associated abnormalities, demonstrating the involvement of EDNRB in HSCR pathogenesis. To test whether mutations in the EDNRB gene could account for Hirschsprung in patients from non-inbred populations, we analysed DNA samples from 17 probands of Italian origin with HSCR. We have identified two novel EDNRB mutations: a missense mutation in a sporadic case, S305N, which leads to a change of a serine to an asparagine, disrupting a putative phosphorylation site; and a single nucleotide deletion in a familial case, N378I, resulting in a truncated protein. Both mutations were found in one of the healthy parents, and neither of these mutations were found in any of the normal individuals tested. These data confirm the involvement of EDNRB in HSCR pathogenesis and demonstrate that EDNRB mutations could contribute to HSCR disease in non-inbred populations.

INTRODUCTION

Hirschsprung disease (HSCR), or aganglionic megacolon, is the most common cause of congenital intestinal obstruction with an incidence of 1/5000 live births. Early symptoms range from complete acute neonatal obstruction, characterized by vomiting, abdominal distention and failure to pass stool, to chronic constipation in the older child (1). Histologically, this developmental disorder is characterized by the absence of parasympathetic ganglion cells in intestinal segments of variable length, as a consequence of premature (5–12th week of gestation) arrest of cranio-caudal migration of neural crest cells toward the distal part of the colon.

A number of different disorders can be associated with congenital intestinal aganglionosis, such as trisomy 21 or Shah-Waardenburg syndrome (2). Although more than 80% of HSCR cases are sporadic (3), several pedigrees have been described in which the disease segregates as an autosomal dominant trait, with incomplete penetrance. In some of these families, genetic linkage to markers located in the pericentromeric region of chromosome 10 was found (4,5), followed by the identification of mutations in the RET protooncogene on 10q11.2, in HSCR patients (6,7). Recently, a second locus for HSCR was mapped to chromosome 13q22 in a Mennonite kindred, a large inbred family with high incidence of the disease (8). A missense mutation (W276C) in the endothelin-B receptor gene (EDNRB), mapping to 13q22, was identified in several affected individuals from this kindred, most of whom were homozygous for the mutated allele, although some heterozygotes were also described. The mutation present in the Mennonite kindred showed incomplete penetrance, as some non-affected individuals from this family were also found to be homozygous for the mutation. In addition, some affected individuals did not carry the mutation, suggesting the presence of additional susceptibility loci contributing to HSCR inheritance in this kindred (9).

To test whether or not mutations at this locus could account for HSCR disease in non-inbred populations, we studied 17 probands of Italian origin. Here, we report on two novel EDNRB mutations which suggest that this gene plays a significant role in HSCR disease pathogenesis.

RESULTS

Single strand conformation polymorphism analysis (SSCP) of the seven exons containing the entire coding region of EDNRB was performed on genomic DNA from 14 unrelated sporadic cases with HSCR and from five patients belonging to three different multicase families. Oligonucleotide primers corresponding to each exon of the EDNRB gene were designed from the previously published sequences of the intron-exon junctions (10). We

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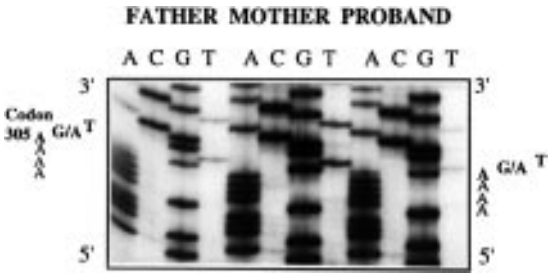

Figure 1. Identification of a missense mutation (S305N) in exon 4 of the EDNRB gene. Nucleotide sequence analysis of exon 4 PCR products from proband 20's family. Both the father and the affected son are heterozygous for a G→A transition in codon 305 (shown in bold characters to the side). Sequence analysis of the mother's sample appears normal.

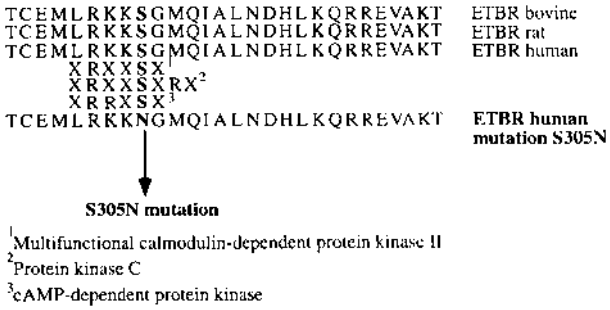

Figure 2. Amino acid structure of the EDNRB third cytoplasmic loop. Alignment of the amino acid sequence of the human EDNRB third amino acid loop with the corresponding sequence of the rat and bovine orthologues, with three serine-threonine protein kinases recognition motifs and with the S305N mutated receptor.

identified two mobility shifts in exons 4 and 6, from patient 20 and patient 18, respectively (data not shown).

Figure 1 shows nucleotide sequence analysis of exon 4 PCR products from patient 20's family: both the affected child and his father are heterozygous for a missense G→A transition in exon 4, the mother being homozygous for the normal allele. This mutation leads to a substitution of an Asn residue for a Ser at position 305, located within the third intracellular loop (Fig. 2). We screened a total of 50 unrelated, normal controls by SSCP analysis of the same exon; none of the 100 chromosomes examined had the mobility shift (data not shown).

SSCP analysis of exon 6 identified a mobility shift in patient 18, whose brother died during the first week of life after being diagnosed with HSCR disease. The same mobility shift was found in a proband's healthy brother, who does not exhibit any of the features of HSCR disease (data not shown). Sequence analysis of the proband PCR product revealed a single nucleotide deletion resulting in a frameshift mutation (Fig. 3A). We subcloned the PCR products from the proband and his unaffected brother into pBluescript SK- vector and confirmed the deletion by sequencing both normal and mutant alleles (data not shown). The deletion abolishes an *Eco*RI recognition site (Fig. 3A), therefore, digestion of the normal allele produces two fragments (57 and 72 bp) , while the mutant allele remains uncut (129 bp). Figure 3B shows *Eco*RI digestion pattern of exon 6 in the proband family; while the father is homozygous for the normal allele, the mother and her two sons have both normal and mutant alleles. We performed *Eco*RI

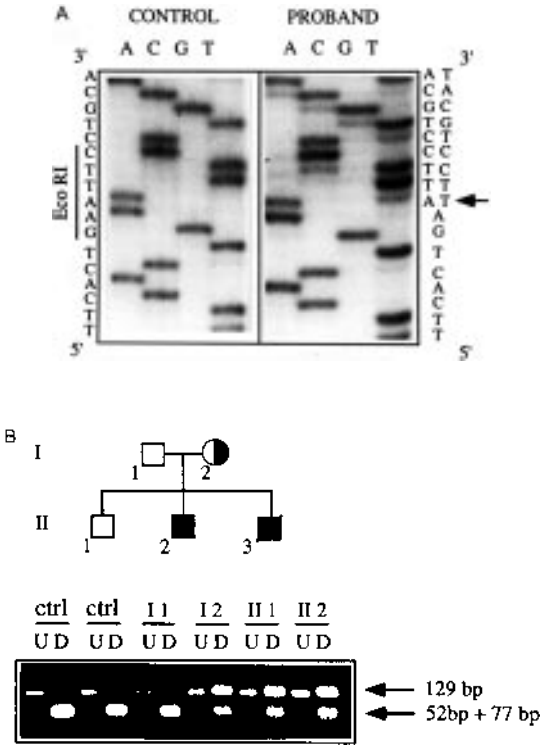

Figure 3. Identification of a single nucleotide deletion in exon 6 of the EDNRB gene. (**A**) Nucleotide sequence analysis of exon 6 PCR products from a control individual (left) and from proband 18 (right). An *Eco*RI recognition site is indicated on the left. Arrow on the right part of the picture indicates the deletion's position; from the arrow on, the abnormal allele sequence is clearly one nucleotide behind the normal allele. (**B**) *Eco*RI digestion pattern of exon 6 PCR products from two controls (ctrl) and from proband 18's family (II-2, solid square). 'U' lanes contain undigested samples; 'D' lanes contain digested samples. Arrows on the right indicate position of the fragment corresponding to the deleted uncut allele (129 bp) and to the normal allele (52 + 77 bp).

digestion of exon 6 PCR products from 50 unrelated controls: none of the 100 chromosomes tested had the deletion (data not shown). The frameshift mutation produces a change of 11 amino acid residues in the seventh transmembrane receptor domain and results in a truncated protein lacking the entire intracellular COOH tail. No mutations were found in the EDN3 gene, encoding one of EDNRB ligands, in any of the patients studied, while the study of the RET protooncogene is still in progress.

DISCUSSION

We identified two novel mutations in the EDNRB gene from patients with HSCR. EDNRB belongs to the G protein-coupled heptahelical receptors superfamily and is expressed in human cerebral cortex, cerebellum, placenta, lung, kidney, adrenal, as well as in colon, duodenum, and myenteric ganglion neurons (11–13). Binding of EDNRB with its ligands, endothelin 1, 2 and 3 (EDN 1, 2 and 3), triggers intracellular signal transduction events which lead to the activation of phospholipase Cβ, plasmamembrane calcium channels, and non receptor tyrosine kinases (14–16). This series of events mediates a number of biological functions, including vasoregulation, contraction of airway and intestinal smooth muscles, and fibroblast cell proliferation (17–19).

In addition to these functions, EDNRB and its ligand EDN3 have an essential role during embryogenesis (see ref. 20 for review). Functional nullisomy for either EDNRB or EDN3 in mice results in megacolon and coat color spotting, demonstrating that both genes are involved in the development of two neural crest derived-cell lineages, enteric ganglia and epidermal melanocytes (21,22). This complex phenotype is reminiscent of that observed in HSCR patients from a large inbred Mennonite kindred, carrying a missense mutation in the EDNRB gene (9). The finding of this mutation in an inbred family raised the important issue of whether or not mutations at the EDNRB locus represent a significant pathogenetic factor in HSCR patients from general, non-inbred, populations.

We have identified a G→A transition at nucleotide position 914 of EDNRB cDNA in a sporadic case with HSCR. This mutation leads to the substitution of an Asn for a Ser (S305N) located within the receptor third intracellular loop. The entire third cytoplasmic loop, as well as the serine at position 305, is highly conserved among several species and in addition, this Ser residue is located within a putative serine-threonine kinase recognition sequence motif (see Fig. 2) (23), suggesting that this residue plays an important role in receptor mediated signal transduction.

In addition to the missense mutation, we have also identified a single nucleotide deletion in exon 6 of the EDNRB gene in a multicase HSCR family. This mutation produces a frameshift in the EDNRB coding region, generating a stop codon 12 codons downstream. The resulting product is a truncated protein lacking part of the seventh transmembrane domain as well as the entire carboxy-terminal region of the EDNRB protein, which contains several putative sites of post-translational modification (12).

In both cases, the mutations that we have identified were present in the heterozygous state in affected individuals and were absent in 50 unrelated, normal controls (100 chromosomes). Both homozygotes and heterozygotes for the W276C mutation were found in the previously described Mennonite kindred segregating HSCR, the HSCR penetrance being higher in homozygous individuals (9). Our data is consistent with the concept that, due to the polygenic inheritance of HSCR disease, affected individuals from non-inbred populations are more likely to be heterozygous for specific mutations.

Incomplete penetrance in polygenic diseases can be due to the presence of modifier genes. Our data clearly implicate EDNRB in HSCR pathogenesis, however, mutations at other predisposing loci, such as the RET protooncogene (6,7,24,25) or the EDN3 gene (22), may act in a synergistic way with EDNRB mutations to produce the HSCR phenotype. To test this hypothesis, we examined the EDN3 gene in our patients and could not detect any mutations (data not shown). Identification of additional predisposing genes, such as those encoding proteins involved in EDNRB- or RET-mediated signal transduction and in EDN3 processing, will further elucidate the mechanisms leading to this complex developmental disorder.

MATERIALS AND METHODS

Study population

The population enrolled in the study consisted of 17 families in which one or more children had HSCR. Three families had two affected sibs. A total of 19 children (mean age 6 years; age range 1–17 years; 16 boys), who had undergone surgical treatment, were enrolled in this study. HSCR was diagnosed by findings from a barium enema, anorectal manometry, and suction biopsy or deep rectal biopsy. In 10 of 19 children, including patients 18 and 20, a barium enema revealed a narrow segment that was limited to the rectum and rectosigmoid (short form). In five patients, this segment extended to the transverse colon (long form); and in the remaining four, colonic dilatation extended to the anus without an apparent narrowed segment (ultrashort form). In each patient, rectal inhibitory reflex was absent by anorectal manometry and ganglion cells were not found on rectal biopsy specimen. Four probands had other phenotypes associated with HSCR, including white forelock in three cases and Down's syndrome in one case.

Mutation detection

Patients' genomic DNA was isolated from whole blood by a phenol-chloroform extraction protocol described elsewhere (26). Sequences of primers necessary to amplify all seven EDNRB exons were obtained from a previously published source (10). Numbering of EDNRB nucleotides and amino acids is based on this source. DNA samples were amplified by polymerase chain reaction (PCR), using $\gamma[^{32}P]$-ATP labeled primers for 35 cycles (at 94, 55 and 72°C, each step for 50 s) after an initial 'hot start' cycle (at 94°C for 4 min). Sequences of primers for EDN3 exons were kindly provided by Dr A. Chakravarti (Dept of Genetics, Case Western Reserve University, Cleveland, OH, USA). PCR products were analysed by conventional SSCP analysis (27). For each sample in which mobility shifts were detected, PCR amplification and SSCP analysis were repeated in order to confirm the pattern. For these samples, PCR products were purified on Qiagen PCR spin-column (Chatsworth, CA) from residual single-stranded primers and deoxynucleotide triphosphates; sequence analysis was performed with Sequenase 2.0 (US Biochemical, Cleveland, OH), according to manufacturer's instructions. All products were sequenced from both directions using PCR primers. Where indicated, PCR products were subcloned into pBluescript SK- vector according to standard techniques (26) and manually sequenced with Universal and Reverse primers, as described above. Exon 6 PCR products from proband, relatives and controls were subjected to *Eco*RI digestion and electrophoresed on agarose gels.

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