Analysis of the *RET*, *GDNF*, *EDN3*, and *EDNRB* genes in patients with intestinal neuronal dysplasia and Hirschsprung disease

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Abstract

***Background*—Hirschsprung disease (HSCR) is a frequent congenital disorder with an incidence of 1 in 5000 live births, characterised by the absence of parasympathetic intramural ganglion cells in the hindgut resulting in intestinal obstruction in neonates and severe constipation in infants and adults. Intestinal neuronal dysplasia (IND) shares clinical features with HSCR but the submucosal parasympathetic plexus is affected. IND has been proposed as one of the most frequent causes of chronic constipation and is often associated with HSCR.**

***Methods*—We examined 29 patients diagnosed with sporadic HSCR, 20 patients with IND, and 12 patients with mixed HSCR/IND for mutations in the coding regions of the *RET*, *GDNF*, *EDNRB*, and *EDN3* genes. The entire coding regions were analysed by single strand conformational polymorphism and DNA sequencing.**

***Results*—Only three *RET* mutations were detected in patients with HSCR. In patients with IND or a mixed HSCR/IND phenotype, no mutations in these genes were observed. While HSCR and HSCR/IND showed over representation of a specific RET polymorphism in exon 2, IND exhibited a significantly lower frequency comparable with that of controls.**

***Conclusions*—The mutation frequency found in our sporadic HSCR patients (10%) and the allelic distribution of RET polymorphisms are comparable with earlier published data. A significantly different allelic distribution in an established HSCR associated polymorphism argues against common genetic pathways for HSCR and IND.**

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Keywords: Hirschsprung disease; intestinal neuronal dysplasia; *RET*; *GDNF*; *EDNRB*; *EDN3*

Hirschsprung disease (HSCR) is associated with congenital absence of ganglion cells in both the myenteric and submucosal plexus along variable lengths of the distal gastrointestinal tract. According to the spatial extent of aganglionosis, long segment forms (20%) are distinguished from short segment forms (80%). Typically, HSCR presents in neonates or early childhood with symptoms ranging from chronic constipation to acute ileus but late manifestations in adults have occasionally been described.[1] HSCR occurs in approximately 1 in 5000 live births. There is a male predominance with a sex ratio of 3.5–4.0 males to 1 female and an overall risk to siblings of 4%,[2 3] suggesting genetic factors. The disorder, which mostly occurs sporadically, is considered multifactorial with major and modifying genes. Genetic mapping in multiplex families and mutational analysis of candidate genes has led to the definitive identification of seven genes, which contribute to HSCR risk. Different forms of inheritance can be distinguished. Families with long segment as well as those with short segment HSCR mostly follow an autosomal dominant form of inheritances with incomplete penetrance.[4] Sporadic HSCR variants are thought to arise as a result of multifactorial inheritance,[3] whereas some HSCR variants being associated with distinct malformations, such as Waardenburg syndrome,[5 6] are mostly autosomal recessive or due to chromosomal abnormalities, as is the case in the association with Down's syndrome.[7] Molecular analyses have identified mutations in the *RET*,[8–14] *GDNF*,[15–17] *EDN3*[18–20] and *EDNRB*[21–24] genes segregating with HSCR. Common to these genes is a role in the development, migration, and survival of neural cells. Germline mutations in the receptor tyrosine kinase *RET* were most common and detected in 10–20% of sporadic HSCR patients[8–12] although population based studies demonstrated that only 3% of isolated HSCR harboured germline *RET* mutations.[14] Mutations in *GDNF*, a ligand for *RET*, seem to occur less frequently and may not suffice to induce the clinical picture of HSCR.[15–17] *EDNRB* mutations may account for 5–15% of HSCR cases.[22 23] The *EDBRB* ligand *EDN3* has been described as being mutated in only a few cases.[13 18–20] In addition, mutations in *NTN*,[25] which encodes another *RET* ligand, account for rare HSCR cases. Furthermore, heterozygous mutations in *SOX10* encoding the Sry related transcription factor have recently been reported in a few Shah-Waardenburg patients.[26] An *ECE1* gene mutation was found in a HSCR patient, who was also diagnosed with cardiac defects and autonomic dysfunction.[27]

Abbreviations used in this paper: IND, intestinal neuronal dysplasia; HSCR, Hirschsprung disease; SSCP, single strand conformational polymorphism.

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Intestinal neuronal dysplasia (IND), which was first described in 1971,[28] is a heterogeneous disorder of the enteric nervous system. Estimates on the frequency of IND range from 5% to 60% of all patients biopsied for suspected motility disorders. Up to 20% of patients diagnosed with IND show the full morphology of HSCR distally.[29] The most prominent histological features of IND are giant ganglia containing more than seven nerve cells.[30] The aetiology of IND is unresolved, with diverse pathomechanisms, including developmental disturbances, reactive changes, or inflammatory diseases being discussed.[31] The existence of IND as a clearly defined clinical and pathological entity is still a matter of intense debate.[32 33] In a recent study, a high interobserver variation with regard to the diagnosis of IND was described.[34]

Both HSCR and IND result in motility disturbances and constipation. A malfunction in the development of the enteric nervous system seems to play an important role in the aetiology of these clinically similar diseases.[35 36] Because IND and HSCR occur in combination, it may be possible that the molecular defects occurring in patients with HSCR may also be causative for IND. *RET* is viewed as the major susceptibility gene for HSCR but two IND families did not show linkage to *RET* on chromosome 10.[37] However, because of the problematic clinical characterisation of IND, possibly resulting from heterogeneity, and the existence of additional HSCR associated genes, common molecular pathways in the genesis of HSCR and IND cannot be excluded.

To investigate the potential role of the HSCR associated *RET*, *GDNF*, *EDNRB*, and *EDN3* genes in the development of IND, we performed mutation analysis of these genes in a series of 29 patients diagnosed with HSCR, 20 with IND, and 12 with combined HSCR/IND.

Materials and methods

PATIENTS AND CONTROL SAMPLES

Peripheral blood samples were obtained from 61 unrelated sporadic patients and 14 first degree relatives (11 parents and three sibs from eight patients) treated at the University Hospitals Bonn, Dusseldorf, Freiburg, Heidelberg, Mainz, Munich, Children's Hospitals Lippstadt, Mannheim, Protestant Jung-Stilling Hospital Siegen, Protestant Hospital Oberhausen, and Marien-Hospital, Bonn. HSCR was diagnosed in 29 patients, IND in 20, and a combined HSCR/IND phenotype in 12. Each case was classified by members of the German Hirschsprung Reference Group (WC, HM). The diagnosis was based on guidelines agreed on at a consensus meeting.[30] Blood DNA serving as control samples was obtained from a pool of anonymous donors. DNA was extracted according to standard protocols. All patients and controls consented to molecular analyses.

SSCP ANALYSIS AND DIRECT SEQUENCING

The primers and conditions for polymerase chain reaction mediated amplification of the exons of the *RET*,[38] *GDNF*,[39] *EDNRB*,[40] and *EDN3*[20] genes have been published previously. Polymerase chain reaction was performed in a final volume of 10 µl containing 10 ng of DNA, 50 mM KCl, 10 mM Tris HCl, pH 8.3, 200 mM of each dNTP, 0.1% gelatin, 20 pmol of each primer, and 0.25 units of Taq polymerase. $MgCl_2$ concentrations ranged from 1.0 to 2.0 mM depending on the primer pair. Initial denaturation at 95°C for three minutes was followed by 30 cycles on an automated thermocycler (Omnigene; Hybaid Ltd, Ashford, Middlesex, UK). These included denaturation at 95°C for 30 seconds, annealing at temperatures of 52–58°C for 40 seconds, and extension at 72°C for 30 seconds. A final extension step of 10 minutes at 72°C was added. Single strand conformational polymorphism (SSCP) analysis was carried out on long acrylamide gels (6–14% acrylamide, 0–10% glycerol, 1× TBE) on a sequencing apparatus (Pokerface; Hoefer Scientific, San Francisco, California, USA) at room temperature or at 4°C with 2–20 W. For detection of amplified products, a silver stain protocol was used.[41] Variant SSCP bands were excised and DNA was extracted followed by reamplification and direct sequencing. All aberrantly migrating fragments were sequenced bidirectionally. Sequence analysis was carried out with a semiautomated sequencer (Applied Biosystem, Foster City, California, USA, model 373A) and a Taq cycle sequencing kit.

Results

ALTERATIONS IN *RET*

Three germline sequence variants were detected in *RET*. Of these, one was a 3 bp deletion in exon 3 affecting the sequence TCTCCTT at nucleotides 635–642, allowing for deletion of CTC, TCC, or CCT, all resulting in loss of a serine moiety at codon 148. (Base pair positions relative to the *RET* coding sequence are numbered from the transcription start site, as defined by Itoh and colleagues[42] and Kwok and colleagues[43].) A germline missense mutation was found in exon 15 at nucleotide 2813 G→A resulting in a R873Q exchange in codon 873. The third alteration affected intron 14 and was characterised by a G→A exchange localised at the putative branch site 24 nucleotides in front of exon 15. All three mutations occurred in patients with HSCR. Parental or sibling DNA from these three patients was not available. No *RET* mutations were seen in patients with IND or mixed HSCR/IND. None of these alterations was detected in 300 alleles from 150 unaffected control individuals. Thus these three alterations were interpreted as disease causing mutations.

POLYMORPHISMS

Conservative nucleotide polymorphisms were detected in exons 2, 3, 7, 13, and 15 of the *RET* protooncogene (table 1). These alterations have been described previously.[38] Allele 390 A of the exon 2 polymorphism has previously been shown to be over represented in patients with HSCR.[44 45] Allel 390 A in exon 2 exhibited a frequency of 0.770 in the patient group.

Table 1 Polymorphisms in RET and EDNRB genes

	Alleles in controls		Alleles in patients				
Gene	*No*	*Frequency*	*No*	*Frequency*	*Position*	*Nucleotide*	*Amino acid*
RET	29	0.141	94	0.770	Exon 2	390 A	45 A
RET	177	0.859	28	0.230		390 G	45 A
RET	—	—	121	0.992	Exon 3	570 C	125 V
RET	—	—	1	0.008		570 A	125 V
RET	—	—	77	0.631	Exon 7	1491 G	432 A
RET	—	—	45	0.369		1491 A	432 A
RET	—	—	75	0.615	Exon 13	2502 C	769 L
RET	—	—	47	0.385		2502 G	769 L
RET	—	—	92	0.754	Exon 15	2907 C	904 S
RET	—	—	30	0.246		2907 G	904 S
EDNRB	54	0.931	106	0.883	Exon 1	ATG-148A	5' UTR
EDNRB	4	0.069	14	0.117		ATG-148G	5' UTR
EDNRB	48	0.857	109	0.924	Exon 1	c169G	57G
EDNRB	8	0.143	9	0.076		c169A	57S
EDNRB	49	0.875	96	0.828	Exon 4	c818A+c875T	273K+292F
EDNRB	7	0.125	20	0.172		c818G+c875G	273R+292C

Allele frequencies, nucleotides affected, and amino acid exchange are given for the observed polymorphisms of the respective genes. — , not examined.

However, the frequency for 390 A differed between patients with HSCR (f=0.750), HSCR/IND (f=0.916), and IND (f=0.250). There was no significant difference between HSCR and HSCR/IND patients but a significant difference was found between patients with HSCR and IND (Fisher's exact test; $p<0.01$), and between patients with HSCR/IND and IND (Fisher's exact test; $p<0.01$). A control group exhibited 390 A with a frequency of 0.141. In *EDNRB*, four polymorphisms were detected: one in the untranslated region of exon 1, one in the coding portion of exon 1, and two in exon 4. The polymorphisms in exon 4 were in linkage disequilibrium. No sequence changes indicated by abnormal SSCP patterns were found in the *EDNRB*, *EDN3*, or *GDNDF* genes. All *EDNRB* polymorphisms were seen in a panel of control individuals exhibiting similar frequencies as the patients.

Discussion

In the present study, we investigated germline DNA from 29 patients with sporadic Hirschsprung disease (HSCR), 20 with intestinal neuronal dysplasia (IND), and 12 with a mixed HSCR/IND phenotype for mutations in the *RET*, *GDNF*, *EDNRB*, and *EDN3* genes. Three *RET* mutations were detected in HSCR patients whereas no such alterations were seen in IND and HSCR/IND patients.

RET mutations constitute the most frequently observed genetic alteration in patients with HSCR disease. The frequency ranges from 0% to 50% in sporadic cases.[10–12 14 46] In general, studies on sporadically occurring HSCR yielded lower frequencies of *RET* mutations than studies on familial disease. The frequency of *RET* mutations in the present study was 10% in patients diagnosed with HSCR, matching values in previous studies. No *RET* mutations were detected in patients with IND or mixed HSCR/IND. While the difference in the number of *RET* mutations between HSCR patients and those diagnosed with IND or mixed HSCR/IND was not significant, it indicates that this mutation is not a major factor in the pathogenesis of IND. There are several possible explanations for our relatively low detection rate. *RET* mutations were frequently seen in up to 50% of patients with familial forms of HSCR. In our study, no patient with a positive history for familial HSCR was enrolled, possibly accounting for the reduced number of mutations in the *RET* gene. While the sensitivity of SSCP may account for varying detection rates, we identified several polymorphisms previously described.[38] However, a frequent polymorphism (G691S) was not detected by our assay, emphasising the limitations of SSCP analysis.

Recent studies revealed that allele 390 A of the exon 2 polymorphism is over represented in patients with HSCR.[44 45] Our data support these findings as both groups (HSCR and HSCR/IND patients) exhibited allele 390 A in a comparable frequency. In contrast, IND patients had a frequency for 390 A in the range of that detected in a control group. Both HSCR and HSCR/IND patients carried this allele significantly more often than IND patients. Our findings may suggest that IND is genetically different from HSCR. This hypothesis is further supported by a recent study challenging IND as a defined pathological process and suggesting that IND may in part represent a normal variant of development.[34]

Occasional mutations in the *GDNF* gene were observed in HSCR patients.[15–17] *GDNF* has been identified as a ligand of a multicomponent receptor system consisting of GFRA1 and *RET*.[47–50] In our series, no *GDNF* mutations were seen in HSCR, IND, or mixed HSCR/IND patients. A recent study based on 269 patients, including the patients in this series, revealed no mutations in GFRA1.[51] One study suggested that germline mutations of *GDNF* are not sufficient to cause HSCR but may play a role as a modulating factor with other susceptibility loci such as the *RET* gene.[17] Therefore, a concept of genes synergistically cooperating in generating a HSCR phenotype was promoted.[22] This hypothesis is supported by the incomplete penetrance in familial HSCR which may result from the presence of modifier genes.[52 53]

Besides a missense mutation of *EDNRB* detected in the Mennonite pedigree, further

variants have been identified in familial and sporadic cases of HSCR. However, they only account for a few cases. The homozygous status of *EDNRB* mutations is likely to be associated with syndromic HSCR whereas the heterozygous status appears to play a role in the development of non-syndromic HSCR.[54] In our series, no mutations were detected, stressing the minor role of *EDNRB* in HSCR. Also, *EDNRB* does not seem to be responsible for the pathogenesis of IND.

No mutations in the *EDN3* gene were detected in our series. The rate of mutations of *EDN3* in patients with HSCR is approximately 1%. Among the reported gene alterations, mutations in Shah-Waardenburg syndrome are homozygous, whereas heterozygous carriers in these families may only be affected by depigmentation and deafness with incomplete penetrance.[18] In sporadic non-syndromic HSCR, heterozygous mutations are found in this gene. However, mutations in the *END3* gene may not be sufficient or necessary to cause HSCR but may act more as modifier genes.[55] Whether other as yet unidentified genes are responsible for the development of HSCR is open to question as there are various reports on repeated chromosomal abnormalities.[56 57]

In conclusion, a set of patients including 29 with HSCR, 20 with IND, and 12 with mixed HSCR/IND was systematically analysed for genes involved in the pathogenesis of HSCR. The data support the fact that HSCR is a clinically well defined disease with *RET* mutations being the most frequently detected disease associated genetic alteration, with over representation of RET allele 390 A. However, the overall frequency of mutations detected in sporadic HSCR was low and allows for other, as yet unidentified, genetic mechanisms. In addition, our data indicate that IND may be genetically different from HSCR and may support the recently assumption[34] that IND does not constitute a clinically and histopathologically well defined disease.

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