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SHORT REPORT

Allele dosage-dependent penetrance of *RET* proto-oncogene in an Israeli-Arab inbred family segregating Hirschsprung disease

Lina Basel-Vanagaite*[,1], Anna Pelet[2], Zvi Steiner[3], Arnold Munnich[2], Yoram Rozenbach[4], Mordechai Shohat[1] and Stanislas Lyonnet[2]

[1]*Department of Medical Genetics, Rabin Medical Center, Beilinson Campus, Petah Tikva, Israel;* [2]*Université Paris-Descartes, Faculté de Médecine, Hôpital Necker-Enfant Malades, Paris, France;* [3]*Division of Pediatric Surgery, Hillel-Yaffe Medical Center, Hadera, Affiliated with the Rappaport Faculty of Medicine, The Technion, Haifa, Israel;* [4]*Division of Gastroenterology and Nutrition, Schneider Children's Medical Center of Israel, Petah Tikva, Israel*

Hirschsprung disease (HSCR) is characterised by intestinal obstruction resulting from an absence of ganglion cells in the intestinal tract. The mutations in the major gene, *RET,* associated with isolated HSCR, are dominant loss-of-function mutations with incomplete penetrance and variable expressivity. We have ascertained a large inbred Israeli-Arab family segregating HSCR. Sequencing of the *RET* gene showed a splicing mutation, IVS6 + 5G− > A, in the homozygous state in all the females with severe forms of HSCR and in the heterozygous state in the male patient with short-segment HSCR. The recently described hypomorphic-*RET* predisposing allele, rs2435357, was transmitted in the heterozygous state to the male patient, but was not transmitted to the three affected females. Although the heterozygous IVS6 + 5G− > A is of low-penetrance for short-segment HSCR disease, the homozygous state is fully penetrant for total aganglionosis or long-segment HSCR. As in other inbred populations segregating a weakly penetrant *RET* allele (Mennonite), our findings support the hypothesis that the penetrance of *RET* gene mutations for the HSCR phenotype depends on: (i) the nature of the mutation, (ii) the allele dosage and (iii) modifier-loci.
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Introduction

Hirschsprung disease (HSCR) is characterised by intestinal obstruction resulting from the absence of ganglion cells in a variable portion of the intestinal tract. The incidence of HSCR is 1:5000 live-births in Caucasian populations.[1] Symptoms range from abdominal distension and failure to pass stools in neonates, to chronic constipation and enterocolitis in childhood. In 80% of individuals, aganglionosis is restricted to the rectosigmoid colon (short-segment disease, S-HSCR), but in ~15% the aganglionosis extends proximal to the sigmoid colon (long-segment disease, L-HSCR). In ~5% of individuals, aganglionosis affects the entire large intestine (total colonic aganglionosis, TCA). Total intestinal aganglionosis (TIA) extending from the duodenum to the rectum is the rarest form and is usually fatal. The *RET* gene, located on chromosome 10q11.21, is the major gene in nonsyndromic HSCR.[2,3]

The vast majority of families with HSCR show linkage to the *RET* locus.[4] Heterozygous mutations within the *RET* gene coding sequence are identified only in 50% of (linked) families and 10–20% of sporadic cases[5–9] and are characterised by incomplete sex biased penetrance and a

***Correspondence: Dr L Basel-Vanagaite, Department of Medical Genetics, Rabin Medical Center, Beilinson Campus, Petah Tikva 49100, Israel. Tel: +972 3 937 7659; Fax: +972 3 937 7660; E-mail: basel@post.tau.ac.il**
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variable extension of aganglionosis.[10,11] The estimated penetrance is 72% in males and 51% in females.[5] Recently a major role of noncoding variations in intron 1 of *RET* (hypomorphic alleles) has been demonstrated by several studies.[9,12–15]

In this report, we describe an inbred Israeli-Arab family with HSCR where a splicing mutation segregates in affected family members either in the homozygous state resulting in TCA or L-HSCR (females) or in the heterozygous state resulting in S-HSCR (male).

Patients

The patients are all members of a large consanguineous Israeli-Arab family. In one branch of the family three female siblings have TCA (individuals III-5 (deceased), III-6 and III-9, Figure 1) and one female has L-HSCR (individual III-11, Figure 1), and in the second branch of the family one male has S-HSCR (individual III-2, Figure 1). Patients III-4, III-8 and III-11 also have congenital autosomal recessive ichthyosis, which is unrelated to the HSCR. The clinical features of the patients are summarized in Table 1. The research study was reviewed and approved by the Ethics Committee (CCPPRB approval 95-05-03, AP-HP, Paris).

Methods

DNA was isolated by standard methods. Linkage analysis to the *RET* gene locus was performed using polymorphic genetic markers D10S141, RET INT-5 and StCl2, followed by sequencing of the SNP rs2435357 (IVS1-C>T). Mutation screening of the coding sequence of the *RET* gene was performed with primers designed for exons and the flanking splice sites. PCR products were directly sequenced in both directions on an ABI PRISM 3100 DNA sequencer (Perkin Elmer-Applied Biosystems) using the Big Dye Terminator method according to the manufacturer's instructions.

Results

Linkage to the *RET* gene locus was established. The haplotype 165-C-184-144 segregated with the HSCR phenotype (Figure 1). A splice-mutation, IVS6+5G−>A, was identified; this was not found in 120 control chromosomes. After the complete sequencing of the *RET* gene, no other sequence changes were found. The mutation IVS6+5G−>A involves a known canonical splice site, where the intronic nucleotide +5 is known to participate in the donor splice site processes. When the IVS6+5G−>A mutation was tested with a programme (http://www.fruitfly.org/seq_tools/splice.html), which is designed to predict the efficiency of splice sites, the score of the wild donor site sequence decreased from one to 0.54.

All the patients with TCA and the patient with L-HSCR were homozygous for the mutation (Figure 1). The same mutation in heterozygous state was found in the boy with

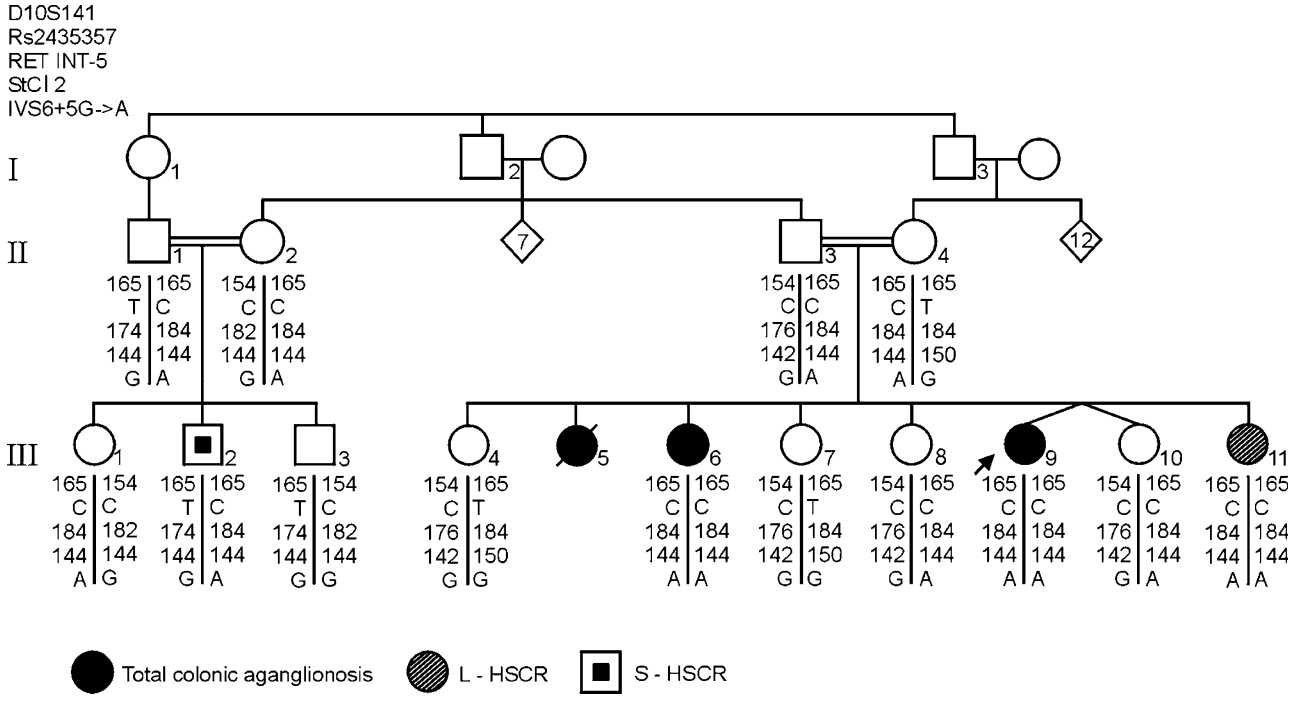

Figure 1 The haplotypes and mutation analysis in the affected and unaffected family members in the families with HSCR. The order of the genetic markers analysed is shown in the upper left-hand corner. The arrow indicates the proband.

S-HSCR and in seven healthy family members (Figure 1). Individuals II-1, II-4 and III-2 carried the hypomorphic allele T (SNP rs2435357) in trans. The rs2435357 allele was not present in any of the females homozygous for the IVS6 + 5G− > A mutation.

Discussion

Heterozygous mutations of the *RET* proto-oncogene occur in families with TCA, L- or S-HSCR. [7,16–18] Homozygous *RET* gene mutations causing HSCR are extremely rare.

Only one patient with TIA and three with TCA owing to homozygous *RET* mutations have been reported in the literature (Table 2). In the family described by Geneste *et al*,[19] as in our patients, TCA was caused by a homozygous *RET* gene mutation, whereas in contrast to our family, L-HSCR in another set of their patients was caused by the same mutation in the heterozygous state.

In this study, we illustrate the effect of *RET* gene dosage on the penetrance and expressivity of the HSCR phenotype. Although the IVS6 + 5G− > A mutation in heterozygous state is of low penetrance for S-HSCR disease (less than 12.5% if the obligate untyped carriers in generation I are included), there is full penetrance (100%) in the homozygous state. Homozygous patients show little variability of expression (TCA with small bowel involvement and L-HSCR).

In a study describing homozygous mutation inheritance in the *EDNRB* in a large inbred Mennonite kindred with HSCR, most of the affected individuals were homozygous for the mutated allele, although some heterozygotes were also described.[20] Homozygotes and heterozygotes for the *EDNRB* mutation W276C had a 74 and 21% risk, respectively, of developing HSCR. The *EDNRB*-mutation showed incomplete penetrance, as some unaffected individuals from this family were also found to be homozygous. In addition, some affected individuals did not carry the mutation, suggesting the presence of additional susceptibility loci contributing to HSCR inheritance.[21] Even in an isolated population, such as the Mennonites, HSCR is a multigenically inherited disease involving interaction between the hypomorphic-*EDNRB* allele and one or more *RET* HSCR-susceptibility variants.[22] In our study, all the family members who are homozygous for the *RET* gene mutation have severe forms of HSCR. None of them carries the hypomorphic allele rs2435357, which we chose because of homology and evolutionary conservation between rodents and primates and because *in vitro* studies have highlighted an enhancer role for this region.[15,23]

Two males, II-1 and III-2, are heterozygotes for the splice mutation and the hypomorphic allele; however, individual

Table 1 Clinical characteristics of the patients

Patient	*Age*	*Age at onset of the disease*	*Clinical presentation*	*Age at operation*	*Extension of aganglionosis*
III-2	5 years	5 mo	Constipation	6 mo	S-HSCR
III-5	Died at the age of $2\frac{1}{2}$ years from liver failure	2 days	Abdominal distension, vomiting	15 mo	TCA
III-6	10 years	2 days	Abdominal distension	14 mo	TCA+6 cm of terminal ileum
III-9	4 years	2 days	Abdominal distension, bilious vomiting	7 mo	TCA+10 cm of terminal ileum
III-11	1 year	1 day	Abdominal distension, vomiting	9 mo	L-HSCR (2/3 of colon up to hepatic flexure)

mo = months.

Table 2 Reported patients with homozygous RET mutations and their heterozygous siblings

Mutation	*Genotype*	*Gender*	*Extension of aganglionosis*	*Reference*
R313Q	M/M	?	TCA with small bowel involvement	Seri *et al*[7]
A969T	M/M or M/−	Male	TIA	Inoue *et al*[17]; Shimotake *et al*[18]
L1061P	M/M	Female	TCA	Geneste *et al*[19]
	M/M	Female	TCA	
	M/wt	Male	L-HSCR	
IVS6+5G− > A	M/M	Female	TCA with small bowel involvement	This study
	M/M	Female	TCA with small bowel involvement	
	M/M	Female	L-HSCR	
	M/wt	Male	S-HSCR	

M = mutated allele, wt = wild-type allele, − = deletion of entire *RET* exon(s).

III-2 is affected (S-HSCR), whereas his haplo-identical father, II-1, is unaffected. Additional genetic changes are thought to be responsible for the variable expressivity of the disease in the homozygous and heterozygous patients described in this study.

As suggested in other inbred populations segregating a weakly penetrant *RET* predisposing allele, our findings support the hypothesis that the penetrance of *RET* gene mutations for the HSCR phenotype depend on: (i) the nature of the mutation, (ii) the allele dosage and (iii) the modifier-loci.

The results of this study emphasise the importance of ascertaining the molecular basis of HSCR in families with more than one affected individual, especially if they originate from a small-inbred population. The detection of a *RET* gene mutation allows the families to be offered genetic counselling and enables early disease detection in the homozygous individuals.

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