

Luo Yin<sup>a</sup>  
Virginia Barone<sup>a</sup>  
Marco Seri<sup>a</sup>  
Alessandra Bolino<sup>a</sup>  
Renata Bocciardi<sup>a</sup>  
Isabella Ceccherini<sup>a</sup>  
Barbara Pasini<sup>a</sup>  
Tatiana Tocco<sup>a</sup>  
Margherita Lerone<sup>a</sup>  
Sidney Cywes<sup>b</sup>  
Sam Moore<sup>c</sup>  
Jean Marie Vanderwinden<sup>d</sup>  
Marc Joël Abramowicz<sup>e</sup>  
Ulf Kristoffersson<sup>f</sup>  
Lars T. Larsson<sup>g</sup>  
Ben C. J. Hamel<sup>h</sup>  
Margherita Silengo<sup>i</sup>  
Giuseppe Martucciello<sup>a</sup>  
Giovanni Romeo<sup>a</sup>

- <sup>a</sup> Laboratorio di Genetica Molecolare, Istituto G. Gaslini, Genova, Italy;  
<sup>b</sup> Department of Paediatric Surgery, Institute of Child Health, Red Cross Children's Hospital, Rondebosch, South Africa;  
<sup>c</sup> Department of Paediatric Surgery, Faculty of Medicine, Tygerberg, South Africa;  
<sup>d</sup> Department of Pediatric Surgery, Hospital Universitaire des Enfants Reine Fabiola, U.L.B., Brussels,  
<sup>e</sup> Department of Genetics, University Hospital Erasme, U.L.B., Brussels, Belgium;  
<sup>f</sup> Department of Clinical Genetics, University Hospital, Lund,  
<sup>g</sup> Department of Pediatric Surgery, University Hospital, Lund, Sweden;  
<sup>h</sup> Department of Human Genetics, University Hospital Nijmegen, The Netherlands, and  
<sup>i</sup> Istituto di Scienze Pediatriche, Università degli Studi di Torino, Italy

## Heterogeneity and Low Detection Rate of RET Mutations in Hirschsprung Disease

### Key Words

Hirschsprung disease · Mutations in RET · Heterogeneity

### Abstract

Mutations in some exons of the RET proto-oncogene were recently observed in Hirschsprung patients. Using DNA polymorphisms and single-strand conformation polymorphism analysis for the whole coding sequence of the RET proto-oncogene, 82 unrelated Hirschsprung patients were screened systematically. A total of 4 complete deletions of RET and 12 point mutations were identified, each present in no more than one patient and distributed along the whole gene. De novo mutations could be documented in 4 patients. Southern blot and fluorescence in situ hybridization analysis carried out in a restricted number of patients did not reveal any deletion of RET. The low efficiency in detecting mutations of RET in Hirschsprung patients (20%) may originate mainly from genetic heterogeneity.

## Introduction

Hirschsprung disease (HSCR) is a relatively frequent genetic disorder (1 in every 5,000 liveborns) of neural crest development characterized by the absence of intramural ganglion cells in the hindgut. Approximately 80% of the HSCR cases are sporadic, while 20% show familial recurrence. An autosomal dominant HSCR gene has been genetically mapped on 10q11.2 [1, 2] and further localized in an interval of about 250 kb by physical characterization of three interstitial deletions [3]. This region contains the RET proto-oncogene whose mutations were recently reported in HSCR patients [4, 5].

Among 27 HSCR patients previously analysed by our group for 12 exons using polymerase chain reaction (PCR) amplification of genomic DNA and single-strand conformation polymorphism (SSCP), 4 point mutations were detected [4]. In the present work, 55 additional patients as well as the previous group of patients, for a total of 82 affected individuals, were screened by the following strategies: (1) Southern blot hybridization with a RET cDNA probe in order to identify deletions or/and rearrangements of RET; (2) polymorphism analysis searching for loss of heterozygosity (LOH) in HSCR patients which would indicate deletions of RET; (3) fluorescence in situ hybridization (FISH) using cosmids covering most of the RET proto-oncogene to detect deletions, and (4) SSCP analysis of the 20 exons of RET using primers already described by our group [6, 7], in order to detect point mutations.

## Materials and Methods

### Patients

82 unrelated patients sequentially diagnosed as affected with HSCR according to criteria previously described [1] (35% with long- and 65% with short-seg-

ment HSCR) were included in this study. Of all patients, 63 apparently had sporadic HSCR, while in 19, a familial recurrence was reported (in more than one generation in 11 cases and in only 1 generation in 8). Linkage with RET had already been reported for 5 of these families [3], while no recombination with RET was observed in the remaining informative families (see Results). Genomic DNA was prepared either from blood samples or from lymphoblastoid cell lines.

### Southern Blot Analysis

In order to carry out deletion analysis in the HSCR patients by Southern blot, a full-length human cDNA clone of the short isoform [8, 9] was digested into three different fragments which encompass (1) the 5' untranslated region and the coding sequence up to exon 10; (2) exon 4 to exon 19, and (3) exon 12 to the untranslated 3' region of the short isoform. Genomic DNA from 28 HSCR patients was digested with three different restriction enzymes, electrophoresed on 0.8% agarose gel, and transferred onto Hybond N membranes (Amersham), which were then probed with the three fragments. The following bands (in parentheses) were obtained with each fragment used as probe and with the following restriction enzymes. Fragment 1: *Hind* III (10.5, 9.2, 7.1, 1.6 kb), *Bam*HI (12.5, 12.0, 7.9, 3.6, 1.75 kb), *Eco*RI (23.4, 14.7, 6.3, 1.95 kb); fragment 2: *Hind*III (9.2, 7.1, 4.8, 4.35, 3.4 kb), *Bam*HI (7.9, 7.8, 4.1, 3.65, 3.6, 1.75, 0.4 kb), *Eco*RI (14.7, 7.1, 6.9, 6.3, 1.95 kb), and fragment 3: *Hind*III (9.2, 4.8, 4.35, 3.4 kb), *Bam*HI (7.8, 4.1, 3.65, 0.4 kb), *Eco*RI (7.1, 6.9 kb).

### Polymorphism Analysis

Microsatellites sTCL2 [ref. 10; frequency of heterozygosity: 0.71] and D10S141 [ref. 11, 12; frequency of heterozygosity: 0.85] flanking the RET gene were used to genotype all the HSCR patients. Conditions for primer labelling, PCR amplification and electrophoresis are described elsewhere [1, 3].

Six intragenic RFLPs in exons 2, 3, 7, 11, 13 and 15 showed frequencies of heterozygosity as defined in Hearne et al. [13] equal to 0.41, 0.04, 0.41, 0.33, 0.38 and 0.33, respectively. They were revealed by restriction enzymes *Eag*I, *Mbo*II, *Bsm*I, *Ban*I, *Taq*I and *Rsa*I, respectively [7]. Four additional polymorphisms revealed by SSCP, two in exon 2 (frequencies of heterozygosity: 0.08 and 0.21), one in exon 4 (frequency of heterozygosity: 0.41) and one in exon 11 (frequency of heterozygosity: 0.39), were also analysed in all 82 patients.

The parents of those patients who were apparently homozygous for all the above 12 polymorphisms were genotyped for the same polymorphisms in order to detect possible LOH in their affected offspring.

**Table 1.** Deletions identified in 82 HSCR patients

Deletions		Occurrence	Pheno- type	
Cytogenetically visible deletion [3]	del10q11.2-21.2	complete deletion of RET	de novo	long form
	del10q11.2-21.2	complete deletion of RET	sporadic <sup>a</sup>	long form
Cytogenetically invisible deletion [3]	microdeletion in 10q11.2	complete deletion of RET	familial	long form
	<i>microdeletion in 10q11.2</i>	<i>complete deletion of RET</i>	<i>familial</i>	<i>long form</i>

The mutation not previously reported is shown in italics. The 2 cytogenetically invisible microdeletions were detected through LOH. de novo = Parents of the patient studied but negative for the mutation.

<sup>a</sup> Sporadic occurrence of the disease; parents of the patient not available for study.

### FISH

Cosmids No. 4 and 13 which cover most of the RET proto-oncogene [B. Pasini, in prep.] were utilized to detect a possible deletion in the patient who was homozygous for all polymorphisms described above and for whom the cultured cells (fibroblasts) were available. Cosmid DNA was labelled by nick translation (Boehringer kit) with biotin-16-dUTP incorporation. Metaphase chromosomes of the fibroblast cell line were prepared by routine methods. Hybridization and washing conditions were as described elsewhere [14, 15]. The signals were amplified once and the slides were stained with propidium iodide and 4,6-diamidino-2-phenylindole.

### SSCP Analysis and Sequencing of the Conformational Variants

All 20 exons of the RET proto-oncogene were amplified using conditions and primers designed from the flanking intronic sequences [7] and PCR products of each exon were analysed by SSCP. Conformational variants thus revealed were sequenced using the Sequenase 2.0 kit (USB) directly from the purified PCR products.

Once a mutation had been detected in any of these patients, all the available relatives were analysed using SSCP and/or restriction enzyme analysis if the latter could be performed (see below).

### Restriction Analysis of the Mutations

When a point mutation created or abolished a restriction site, 15–25 µl of the PCR product was digested with the appropriate restriction enzyme, and the sizes of the fragments thus generated were visualized in non-denaturing 8 or 12% polyacrylamide gels.

## Results

All the RET mutations identified to date by our group in a total of 82 HSCR patients are summarized in table 1 and 2, with those previously unreported (1 microdeletion and 8 point mutations) indicated in italics.

### Microdeletions in HSCR Patients

No deletion or rearrangement was detected by means of Southern blot hybridization in the 28 patients for whom we had enough genomic DNA to carry out this analysis.

LOH in one HSCR pedigree (fig. 1) was revealed by the microsatellite sTCL2 which is 35–45 kb distal to the 3' end of the RET proto-oncogene [10; Pasini et al., in prep.], by the

**Table 2.** Point mutations identified in 82 HSCR patients

Point mutations			Amino acid change	Occurrence	Phenotype
<i>Exon 2</i>	<i>T119 → C</i>	<i>eliminates Alu<sup>I</sup> site</i>	<i>Leu40 → Pro</i>	<i>de novo</i>	<i>long form</i>
<i>Intron 5</i>	<i>G<sup>+9</sup> → A<sup>+9</sup></i>	<i>creates AluI site</i>	<i>possible splicing alteration</i>	<i>sporadic<sup>a</sup></i>	<i>short form</i>
<i>Exon 6</i>	<i>C1094 → A</i>	<i>creates MaeI site</i>	<i>Ser365 → stop codon</i>	<i>sporadic<sup>b</sup></i>	<i>long form</i>
Exon 6 [4]	delG1120		frameshift after the first 373 aa; stop codon 39 aa downstream	sporadic <sup>a</sup>	long form
<i>Exon 6</i>	<i>C1196 → T</i>	<i>eliminates MspI site</i>	<i>Pro399 → Leu</i>	<i>sporadic<sup>a</sup></i>	<i>short form</i>
<i>Exon 8</i>	<i>T1623 → A</i>		<i>Cys541 → stop codon</i>	<i>familial</i>	<i>short form</i>
<i>Exon 12</i>	<i>G2284 → C</i>	<i>eliminates KpnI site</i>	<i>Glu762 → Gln and possible splicing alteration<sup>c</sup></i>	<i>sporadic<sup>b</sup></i>	<i>long form</i>
<i>Intron 12</i>	<i>C<sup>+19</sup> → T<sup>+19</sup></i>	<i>creates RsaI site</i>	<i>possible splicing alteration</i>	<i>de novo</i>	<i>long form</i>
Exon 13 [4]	T2293 → C	eliminates MnlI site	Ser765 → Pro <sup>c</sup>	de novo	long form
Exon 15 [4]	G2690 → A	eliminates AvaI site	Arg897 → Gln <sup>d</sup>	sporadic <sup>b</sup>	long form
Exon 17 [4]	A2914 → G	creates Sau 96I site	Arg972 → Gly <sup>c</sup>	familial	long form
<i>Exon 17</i>	<i>C2918 → T</i>	<i>eliminates HaeIII site</i>	<i>Pro973 → Leu<sup>d</sup></i>	<i>familial</i>	<i>long form</i>

The mutations not previously reported are italicized. All these mutations were identified with SSCP followed by direct sequencing. aa = Amino acid; de novo = parents of patients studied but negative for the mutation.

<sup>a</sup> Sporadic occurrence of the disease with one parent found to be carrier of the mutation.

<sup>b</sup> Sporadic occurrence of the disease; parents of patients not available for study.

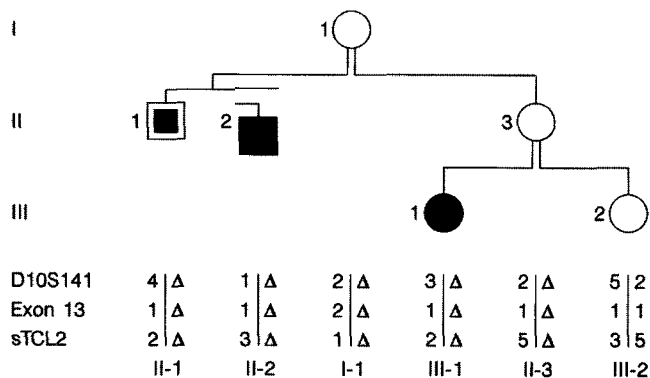
<sup>c</sup> Amino acid residues not conserved in receptor tyrosine kinases [28].

<sup>d</sup> Amino acid residues conserved in 100% of receptor tyrosine kinases [28].

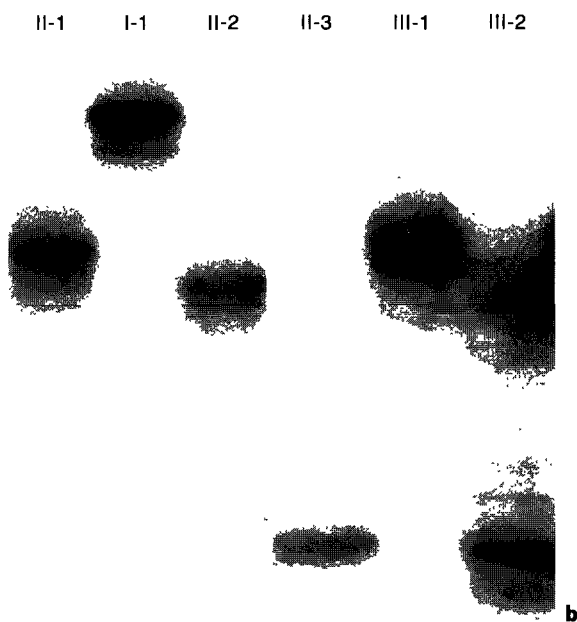
RFLP in exon 13 of the RET proto-oncogene [7] and by D10S141 located upstream of RET and about 150 kb centromeric to sTCL2 [10–12]. In all the patients (with either long- or short-segment HSCR) as well as in the asymp-

tomatic carriers of this pedigree (fig. 1), one allele of RET is deleted.

Four additional patients were apparently homozygous for all the polymorphisms analysed (see Materials and Methods). The prob-



**Fig. 1. a** Haplotype reconstruction in a HSCR pedigree in which a microdeletion of RET was detected through LOH. Filled symbols represent long-segment HSCR, the half-filled symbol represents short-segment HSCR. The alleles for D10S141, proximal to RET [10, 11], for the intragenic RFLP in exon 13 [7] and for sTCL2, distal to RET [10] are shown. Δ = Deletion. **b** LOH revealed by microsatellite sTCL2 (RET). The individual numbers correspond to those in the pedigree. The deletion was observed in both long- and short-segment patients, as well as in the two asymptomatic obligate carriers. This is in agreement with the autosomal dominant mode of inheritance with incomplete penetrance and with the linkage studies which showed that the same gene causes long- or short-segment HSCR in the same pedigrees (variable expressivity) [1–3].



ability of true homozygosity for all 12 polymorphisms in any one patient was calculated as 0.11% based on the frequencies of heterozygosity reported in Materials and Methods. No LOH could be proven, because 1 case was not informative while in 3 cases the parents of the patients were not available. FISH was carried out as described in Materials and Methods in 1 of these 4 patients, for whom the cul-

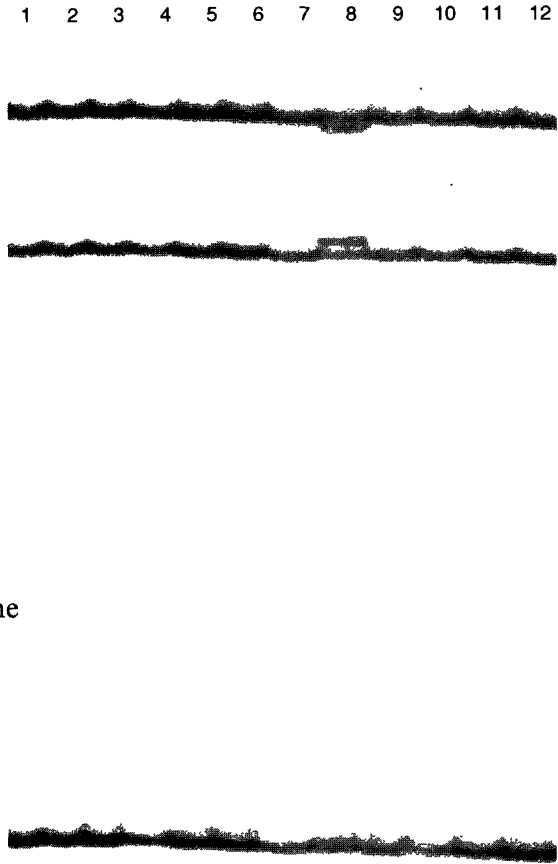
tured cells (fibroblasts) were available, but no deletion could be demonstrated.

All the remaining patients were heterozygous for at least one of the 10 intragenic polymorphisms or for the microsatellites sTCL2 and/or D10S141, indicating that no large deletion of the RET gene was present in their genomic DNA.

*Point Mutations in the RET  
Proto-Oncogene in HSCR Patients*

In addition to the 4 point mutations already reported [4], 21 conformational variants were observed during the SSCP analysis. An example of such a variant, which was subsequently characterized as a mutation of the last nucleotide of exon 12 (see below), is illustrated in figure 2. Direct sequencing, confirmed whenever possible by restriction enzyme cleavage, indicated that 8 of the variants represent non-conservative amino acid changes or may affect the normal RNA splicing (table 2). The remaining 13 variants represent common intragenic polymorphisms (see polymorphism analysis in the Materials and Methods section) or private silent mutations [7].

The 8 new point mutations identified in <sup>proline</sup> different patients (see table 2) include one C-A transversion at codon 365 and one T-A transversion at codon 541, which create a termination codon in place of a serine and a cysteine residue, respectively; one G<sup>+</sup>-A<sup>+</sup> transition in the donor splicing site of intron 5, one C<sup>+</sup>-T<sup>+</sup> transition in the possible donor splicing region of intron 12 and one G-C transversion affecting the last nucleotide of exon 12. This latter mutation predicts a Glu<sup>762</sup>-Gln non-conservative amino acid substitution in addition to a possible splicing error which would be analogous to that produced by a G-A substitution at the last nucleotide of exon 10 of the glucocerebrosidase gene in a Gaucher patient [16]. However, no direct proof for these three possible splicing errors has been obtained, since RET is not expressed in any type of cultured cells from patients. The remaining mutations are: one Leu40-Pro change and two missense mutations resulting in the substitution of a leucine for a proline residue at codons 399 and 973, respectively, in 2 different patients. All the available relatives of the probands were analysed and the results are shown in table 2 under 'occurrence'



**Fig. 2.** An example of a conformational variant detected through SSCP analysis. The PCR samples were loaded onto a 6% acrylamide gel with 5% glycerol, electrophoresed at 4°C and visualized by silver staining. No. 1-7 and 9-12 are normal samples, while No. 8 is the variant caused by a G-C transversion of the last nucleotide of exon 12 (see table 2) as demonstrated by direct sequencing (see text).

of the mutation. None of the mutations reported in this table was found in at least 120 normal control samples. Although all the patients were thoroughly screened by SSCP in all 20 exons, no second mutation was found in any patient with a deletion or with an already identified point mutation.

## Discussion

By combining four different approaches (SSCP and LOH analyses in all cases, plus Southern and FISH analyses whenever possible), we have been able to detect mutations in 20% of the patients studied (16 out of 82). Two cytogenetically visible deletions [17, 18] and two cytogenetically invisible microdeletions (one previously reported and one identified in the present study, both detected through LOH) have been observed (table 1). The 12 point mutations identified by SSCP analysis in the patients are equally distributed between the extracellular (exons 2, 5, 6, 8) and intracellular (exons 12, 13, 15, 17) domains. No single mutation is observed in more than one patient. As shown in tables 1 and 2, 2 deletions and 3 point mutations are observed in patients with familial recurrence of HSCR, while 2 deletions and 9 point mutations were identified in the patients with sporadic occurrence of the disease. Mutations were detected in 4 out of 13 families informative for two microsatellites (sTCL2 and D10S141) closely linked to RET, in which no recombination with RET was observed. Among the remaining 9 families, the highest lod score of 0.7 (at  $\theta = 0$ ) was observed in pedigree No. 1 already described in a previous report [3]. One point mutation in exon 8 (see table 2) was identified in a family among the 6 non-informative for the above markers.

Among the sporadic cases, de novo mutations could be documented in 4 out of 11 cases through the analysis of both parents who did not show either the deletion or the point mutation observed in their affected child. While in 3 cases, the mutation (without any phenotype) was observed in one of the parents, in the remaining 4 patients the analysis could not be carried out in both parents (table 2). The proportion of new mutations for HSCR in the RET proto-oncogene is there-

fore quite high (at least 4 out of the 16 identified mutations).

lod  
Mutations of the RET proto-oncogene in the extracellular cysteine-rich domain (exons 10 and 11) had been previously identified in patients with multiple endocrine neoplasia (MEN) type 2A and familial medullary thyroid carcinoma (FMTC) [19, 20], while a single Met918-Thr substitution in the tyrosine kinase domain (exon 16) was found to be responsible for the development of MEN 2B and sporadic medullary thyroid carcinoma (MTC) [21-23]. Besides RET, there are at least 7 other proto-oncogenes and anti-oncogenes whose mutations may be associated with developmental disorders [24]. In addition, there are well over 100 other genes whose mutations may cause two or more diverse clinical disorders (phenotypic diversity due to allelic series) [24].

Unlike the situations in MEN 2A, MEN 2B, FMTC and MTC, the point mutations causing HSCR are distributed throughout different domains of RET and their detection rate is quite low, namely 20%. One explanation for this low detection efficiency may reside in the peculiar GC-rich nucleotide composition of the RET gene, which can render the mutated sequences indistinguishable from the normal ones during SSCP analysis. This difficulty might be overcome by utilizing in parallel other approaches such as denaturing gradient gel electrophoresis and chemical cleavage mismatch procedures. An additional technical problem may result from the positions of some intronic primers. Due to the peculiar nucleotide composition of RET, and in particular of some intronic regions, some primers had to be designed close to the exon-intron boundaries [7]. In these cases splicing errors might be missed. Furthermore, we have not yet analysed the 3' and 5' non-coding sequences, which might play important roles in controlling the expression of the RET pro-

to-oncogene. However, the most likely explanation for the low detection rate of RET mutations is genetic heterogeneity of HSCR.

The hypothesis of genetic heterogeneity among the patients analysed in the present study is supported by the following finding. While long-segment HSCR is usually observed in not more than 20% of patients, it is present in 35% of our 82 patients (probably because of a bias in clinical ascertainment). However, among those patients showing mutations in RET (tables 1, 2), 81% of the cases (13/16) have long-segment HSCR. Thus genes other than RET are likely to be mutated in short-segment HSCR. However, one should keep in mind that within the same family the same mutation can be associated with short- and long-segment HSCR, as observed in the pedigree of figure 1. This suggests the presence of modifier gene(s) [24]. The high rate of association of HSCR with other genetic diseases such as Down syndrome and Waardenburg syndrome, the chromosomal abnormalities in chromosomes 2, 13, 21 and 22 repeatedly observed in HSCR patients [25], the recent localization of a recessive gene for HSCR on human chromosome 13q22 in a large inbred Mennonite kindred [26] and, finally, the existence of three distinct murine mutations located on chromosomes 2, 14 and

15 [27] all causing aganglionosis in the mouse support the concept that HSCR is a heterogeneous and complex genetic disorder. The exclusion of the RET proto-oncogene as a candidate for total colonic aganglionosis in the spotting lethal (sl) rat strain [I. Ceccherini et al., submitted], which shows histopathological features strikingly similar to the human disease, further confirms the genetic heterogeneity of HSCR.

### Acknowledgements

We thank Dr. M. Takahashi for a RET cDNA probe, Francesco Caroli, Donatella Concedi and Monica Scaranari for their technical help, Dr. Monica Bozzano and Dr. Paola Ghisellini for participating in part of this work, which was supported by the AIRC, the Italian Telethon, PF 'Ingegneria Genetica', the Italian CNR (Progetto Finalizzato Ingegneria Genetica), the Italian Ministry of Health and the EU (PL910027). Y.L. and B.P. are supported by fellowships from Telethon and from Associazione Italiana per la Ricerca sul Cancro, respectively. The following clinicians have contributed blood from patients included in this study in whom we could not identify any RET mutations: Prof. S. Auricchio (Naples, Italy), Dr. I. Baric (Zagreb, Croatia), Prof. A. Schinzel (Zürich, Switzerland), Dr. J.M. Casasa (Barcelona, Spain), Dr. C. Monteiro (Lisbon, Portugal), Dr. L. Cutrone (Campobasso, Italy), Dr. I. van der Burgt (Nijmegen, The Netherlands), Dr. Albert Szent (Szeged, Hungary), Dr. M. Rady (Cairo, Egypt) and Dr. L. Van Maldergem (Loverval, Belgium).

---

### References

- 1 Lyonnet S, Bolino A, Pelet A, Abel L, Nihoul-Fékété C, Briard ML, Mok-Siu V, Kääriäinen H, Martucciello G, Lerone M, Puliti A, Yin L, Weissenbach J, Devoto M, Munnich A, Romeo G: A gene for Hirschsprung disease maps to the proximal long arm of chromosome 10. *Nat Genet* 1993;4:346-350.
- 2 Angrist M, Kauffman E, Slaugenhaupt SA, Matisse TC, Puffenberger EG, Washington SS, Lipson A, Cass DT, Reyna T, Weeks DE, Sieber W, Chakravarti A: A gene for Hirschsprung disease (megacolon) in the pericentromeric region of human chromosome 10. *Nat Genet* 1993;4:351-356.
- 3 Yin L, Ceccherini I, Pasini B, Matera I, Biccocchi MP, Barone V, Bocciaardi R, Kääriäinen H, Weber D, Devoto M, Romeo G: Close linkage with the RET proto-oncogene and boundaries of deletion mutations in autosomal dominant Hirschsprung disease. *Hum Mol Genet* 1993;2:1803-1808.



- 4 Romeo G, Ronchetto P, Yin L, Barone V, Seri M, Ceccherini I, Pasini B, Bocciardi R, Lerone M, Kääriäinen H, Martucciello G: Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature* 1994;367:377-378.
- 5 Ederly P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, Nihoul-Fékété C, Ponder BAJ, Munnich A: Mutations of the RET proto-oncogene in Hirschsprung's disease. *Nature* 1994;367:378-379.
- 6 Ceccherini I, Bocciardi R, Yin L, Pasini B, Hofstra R, Takahashi M, Romeo G: Exon structure and flanking intronic sequences of the human RET proto-oncogene. *Biochem Biophys Res Commun* 1993;196:1288-1295.
- 7 Ceccherini I, Hofstra RMW, Yin L, Stulp RP, Barone V, Stelwagen T, Bocciardi R, Nijveen H, Bolino A, Seri M, Ronchetto P, Pasini B, Bozzano M, Buys CHCM, Romeo G: DNA polymorphisms and conditions for SSCP analysis of the 20 exons of the RET proto-oncogene. *Oncogene* 1994;9:3025-3030.
- 8 Takahashi M, Buma Y, Iwamoto T, Inaguma Y, Ikeda H, Hiai H: Cloning and expression of the *ret* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene* 1988;3:571-578.
- 9 Takahashi M, Buma Y, Hiai H: Isolation of *ret* proto-oncogene cDNA with an amino-terminal signal sequence. *Oncogene* 1989;4:805-806.
- 10 Lairmore TC, Dou S, Howe JR, Chi D, Carlson K, Veile R, Mishra SK, Wells SA Jr, Donis-Keller H: A 1.5-megabase yeast artificial chromosome contig from human chromosome 10q11.2 connecting three genetic loci (RET, D10S94, and D10S102) closely linked to the MEN 2A locus. *Proc Natl Acad Sci USA* 1993;90:492-496.
- 11 Gardner E, Papi L, Easton DF, Cummings T, Jackson CE, Kaplan M, Love DR, Mole SE, Mulligan LM, Norum RA, Ponder MA, Reichlin S, Stall G, Telenius H, Telenius-Berg M, Tunnacliffe A, Ponder BAJ: Genetic linkage studies map the multiple endocrine neoplasia type 2 loci to a small interval on chromosome 10q11.2. *Hum Mol Genet* 1993;2:241-246.
- 12 Mole SE, Mulligan LM, Healey CS, Ponder BAJ, Tunnacliffe A: Localisation of the gene for multiple endocrine neoplasia type 2A to a 480 Kb region in chromosome band 10q11.2. *Hum Mol Genet* 1993;2:247-252.
- 13 Hearne CM, Ghosh S, Todd JA: Microsatellites for linkage analysis of genetic traits. *Trends Genet* 1992;8:288-294.
- 14 Lichter P, Boyle AL, Gremer T, Ward DC: Analysis of genes and chromosomes by nonisotopic in situ hybridization. *Genet Anal Tech Appl* 1991;8:24-35.
- 15 Wada M, Little RD, Abidi F, Porta G, Labella T, Cooper T, Valle GD, D'Urso M, Schlessinger D: Human Xq24-Xq28: Approaches to mapping with yeast artificial chromosomes. *Am J Hum Genet* 1990;46:95-106.
- 16 Ohshima T, Sasaki M, Matsuzaka T, Sakuragawa N: A novel splicing abnormality in a Japanese patient with Gaucher's disease. *Hum Mol Genet* 1993;9:1497-1498.
- 17 Puliti A, Covone AE, Bicocchi MP, Bolino A, Lerone M, Martucciello G, Jasonni V, Romeo G: Deleted and normal chromosome 10 homologs from a patient with Hirschsprung disease isolated in two cell hybrids through enrichment by immunomagnetic selection. *Cytogenet Cell Genet* 1993;63:102-106.
- 18 Fewtrell MS, Tam PKH, Thomson AH, Fitchett M, Currie J, Huson SM, Mulligan LM: Hirschsprung's disease associated with a deletion of chromosome 10 (Q11.2Q21.2) - A further link with the neurocristopathies. *J Med Genet* 1994;31:325-327.
- 19 Mulligan LM, Kwok JBJ, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L, Ponder MA, Telenius H, Tunnacliffe A, Ponder BAJ: Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 1993;363:458-460.
- 20 Donis-Keller H, Dou S, Chi D, Carlson KM, Toshima K, Lairmore TC, Howe JR, Moley J, Goodfellow P, Wells SA Jr: Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet* 1993;2:851-856.
- 21 Hofstra RMW, Landsvater RM, Ceccherini I, Stulp RP, Stelwagen T, Yin L, Pasini B, Hoppener JWM, Van Amstel HKP, Romeo G, Lips CJM, Buys CHCM: A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature* 1994;367:375-376.
- 22 Carlson KM, Dou S, Chi D, Scavarda N, Toshima K, Jackson CE, Wells SA Jr, Goodfellow PJ, Donis-Keller H: Single missense mutation in the tyrosine kinase catalytic domain of the RET protooncogene is associated with multiple endocrine neoplasia type 2B. *Proc Natl Acad Sci USA* 1994;91:1579-1583.
- 23 Eng C, Smith DP, Mulligan LM, Nagai MA, Healey CS, Ponder MA, Gardner E, Scheumann GFW, Jackson CE, Tunnacliffe A, Ponder BAJ: Point mutation within the tyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. *Hum Mol Genet* 1994;3:237-241.
- 24 Romeo G, McKusick VA: Phenotypic diversity, allelic series and modifier genes. *Nat Genet* 1994;7:451-453.
- 25 Badner JA, Sieber WK, Garver KL, Chakravarti A: A genetic study of Hirschsprung disease. *Am J Hum Genet* 1990;46:568-580.
- 26 Puffenberger EG, Kauffman ER, Bolk S, Matisse TC, Washington SS, Angrist M, Weissenbach J, Garver KL, Mascari M, Ladda R, Slaugenhaupt SA, Chakravarti A: Identity-by-descent and association mapping of a recessive gene for Hirschsprung disease on human chromosome 13q22. *Hum Mol Genet* 1994;3:1217-1225.
- 27 Kapur RP: Contemporary approaches toward understanding the pathogenesis of Hirschsprung disease. *Pediatr Pathol* 1993;13:83-100.
- 28 Hanks SK, Quinn AM, Hunter T: The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* 1988;241:42-52.