Single nucleotide polymorphic alleles in the 5' region of the *RET* proto-oncogene define a risk haplotype in Hirschsprung's disease

M Sancandi, P Griseri, B Pesce, G Patrone, F Puppo, M Lerone, G Martucciello, G Romeo, R Ravazzolo, M Devoto, I Ceccherini

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Hirschsprung's disease is a congenital disorder characterised by intestinal obstruction caused by the absence of parasympathetic intrinsic ganglion cells along variable lengths of the colon.¹ The high proportion of sporadic cases (80–90%), the variable expressivity, the incomplete sex dependent penetrance, and the involvement of several genes, most of which are yet to be identified, show a complex pattern of inheritance for this disorder.^{2–4} The *RET* proto-oncogene is the major gene involved in Hirschsprung's disease, accounting for a high proportion of both familial (about 50%) and sporadic cases (10–15%). Five to ten per cent of patients show alterations in other genes such as the glial cell line derived neurotrophic factor (*GDNF*), neurturin (*NTN*), endothelin 3 (*EDN3*), endothelin B receptor (*EDNRB*), endothelin converting enzyme 1 (*ECE1*), transcriptional factor *SOX10*, and Smad interacting protein 1 (*SIP1*).^{1–5} The small number of affected patients with known mutations confirms the involvement of modifier genes or additional genetic risk factors, some of which are already mapped,^{3–6} in the aetiology of the disease.

According to what was expected for a complex inheritance pattern, several common polymorphisms of the *RET* proto-oncogene have been associated with a variable risk of developing Hirschsprung's disease. Moreover, specific *RET* haplotypes have been found to have either protective or predisposing effects, or to modulate the severity of the resulting phenotype.^{6–12} In particular, specific haplotypes comprising the rarer allele of a single nucleotide polymorphism (SNP) of exon 2 (A45A) have been strongly associated with Hirschsprung's disease, whereas the haplotype including the rarer allele of exon 14 SNP (S836S) has shown a low penetrant protective effect against the disease.^{11–12} Recently, Borrego *et al* have extended the genetic analysis of the SNP2 associated predisposing haplotype for Hirschsprung's disease, hypothesising the existence of a very ancient, low penetrant founder locus 20 to 30 kb upstream of SNP2, and suggesting that a 5' specific variant is related to the transcriptional activity of the *RET* proto-oncogene.¹³

To identify *RET* sequence variants of the gene promoter that might impair its expression or determine anomalous gene regulation—thus predisposing to Hirschsprung's disease—we have analysed the *RET* basal promoter region^{14–15} in 46 Italian patients with sporadic Hirschsprung's disease, 36 with sporadic medullary thyroid carcinomas, and 50 population matched controls. We report the identification of two novel *RET* SNPs, located at –5 and –1 base pairs (bp) from the transcription start site of the gene, and provide genetic evidence for a predisposing role of a low penetrant haplotype defined by these two markers in the pathogenesis of Hirschsprung's disease but not of medullary thyroid carcinoma.

Key points

- In an attempt to identify sequence variants possibly predisposing to Hirschsprung's disease, the whole basal promoter region of the *RET* gene was investigated in 46 sporadic Italian cases of Hirschsprung's disease, 36 sporadic cases of medullary thyroid carcinoma, and 50 population matched controls.
- Two novel single nucleotide polymorphisms (SNP) were located at –1 base pair (bp) and –5 bp from the *RET* transcription start site (–5G>A and –1C>A), the allelic frequencies of which were significantly different between the patients with Hirschsprung's disease and the controls.
- Haplotypes reconstructed using two additional SNPs—c135G>A (A45A) and c2307T>G (L769L), located in exons 2 and 13, respectively—also showed a different distribution in Hirschsprung's disease than in the controls. In particular, combinations ACAG and ACAT together accounted for 62.0% of haplotypes in Hirschsprung's disease and 21.8% in controls.
- The ACA combination of the two promoter and SNP2 alleles may represent the core haplotype associated with Hirschsprung's disease, acting as a modifying risk allele in the development of the condition.

METHODS

Haplotypes analysis

We selected 46 patients with Hirschsprung's disease on the basis of their Italian origin and sporadic disease occurrence. Twenty seven of these had already been screened for mutations in the 21 exons of the *RET* proto-oncogene, and three nucleotide changes had been found: one missense mutation of exon 15 (R897Q), a 4 bp insertion in exon 10, and a silent mutation of exon 11 (I647I) already reported to interfere with correct *RET* mRNA processing or stability.¹⁶ We also investigated 36 Italian patients with medullary thyroid carcinoma and 50 controls (who have already been described¹⁰).

Amplification of 392 bp encompassing the *RET* basal promoter was carried out using primers P1F (5'-CCC GCA CTG AGC TCC TAC-3') and P1R (5'-GGA CGT CGC CTT CGC CAT-3'), in the presence of 50 μM 7-deaza-dGTP, for 35 cycles, each consisting of one minute at 96°C, one minute at 59°C, and one minute at 72°C, and a final elongation step of seven minutes at 72°C. Direct sequencing was undertaken using an ABI Prism 377 DNA sequencer (PE Biosystems, Foster City, California, USA).

Transfection and CAT assay

Transfection was performed in IMR32 neuroblastoma cell line with polyethylenimine (PEI) as previously described.¹⁵ In brief, 10 µg of DNA, diluted in 300 µl of medium devoid of serum and antibiotics, was mixed with 30 µl of 10 mM PEI (Sigma-Aldrich). After 10 minutes of incubation at room temperature, the DNA/PEI mixture was diluted by 3 ml of complete medium and delivered to the cells. After two hours, the transfection mixture was removed, the cells washed, and fresh medium added. Transfected DNA consisted of 6 µg of the four promoter constructs. Total DNA was kept constant at 10 µg by adding pBluescript DNA (Stratagene, La Jolla California, USA). Two days after transfection, cells were collected and transfection efficiency determined by a spectrophotometric quantitation of the β-galactosidase activity in cell lysates. Equivalent amounts of protein extracts were employed in CAT reactions, using acetyl-CoA and ¹⁴C-chloramphenicol. For quantitative analysis, reactions were extracted with xylene and quantified by scintillation counting. We measured activity above background, where background was the CAT activity in protein extracts from cells transfected with the pCAT Basic vector (Promega, Madison, Wisconsin, USA). Each transfection was repeated three times.

Statistical analysis

Haplotypes and their frequencies were estimated by means of the EHPlus software¹⁷ and homogeneity in haplotype distributions between cases and controls was tested by means of the non-parametric test implemented in the same program.

RESULTS

RET promoter SNPs

Analysis of the RET segment from -147 to +53 with respect to the transcription start site allowed us to identify two novel RET SNPs: a transversion C>A of nucleotide -1 and a transition G>A of nucleotide -5. Allele frequencies, calculated in 46 patients with Hirschsprung's disease and in 50 population matched healthy control individuals, showed a statistically significant difference at both loci (table 1). Allele A of SNP-5 had a frequency of 70% in Hirschsprung's disease and 24% in controls (Fisher's exact test, p<0.00001), while allele C of SNP-1 had a frequency of 83% in Hirschsprung's disease and 60% in controls (Fisher's exact test, p<0.0009).

As RET mutations have been found in association with multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B) and medullary thyroid carcinoma,¹⁸ the analysis of SNP-5 and SNP-1 was also undertaken on 36 Italian patients with sporadic medullary thyroid carcinoma. Allele frequencies were similar to those of the control population, but very different from the Hirschsprung's disease sample

(the A allele of SNP-5 was found at a frequency of 18%, while the C allele of SNP-1 was found at a frequency of 51%). In all three groups of individuals, genotype frequencies fit the expectations of the Hardy-Weinberg equilibrium.

Reconstruction of RET promoter haplotypes

We further analysed our Hirschsprung, medullary thyroid carcinoma, and control panels and reconstructed haplotypes at the two RET promoter SNPs. Haplotypes of double heterozygotes, not deducible by family analysis, were reconstructed by comparing the pattern of migration of the corresponding polymerase chain reaction (PCR) products in 12% polyacrylamide gel supplemented with 8% glycerol with that of individuals known to be homozygous for specific alleles at the two SNPs. Figure 1 gives examples of the band migration of short amplification products including the two promoter SNP loci and corresponding to different allelic combinations (see legend for details).

Strong allelic association was present between the two SNPs in all three groups of individuals, as expected from the very short distance (4 bp) separating them (table 2). The "AA" haplotype was very rare, being present only once among the Hirschsprung group and absent in controls and cases of medullary thyroid carcinoma, while the "AC" haplotype was very common in patients with Hirschsprung's disease (68.5%) in comparison with controls (26%) and MTC cases (18%). In the two latter groups, "GA" and "GC" were the most frequent haplotypes. The difference in haplotype frequencies was highly significant when comparing Hirschsprung's disease with controls ($\chi^2 = 37.0$, 3 df, p<0.00001) and Hirschsprung's disease with medullary

Figure 1 The migration pattern of different haplotype combinations at the two promoter SNPs reported on the top of each lane is shown. In particular, 66 base pair amplification products were obtained by using primers HIN1F (5'-TAC CCG CTC CTC CGG CGC-3') and HIN1R (5'-TGC GCG CCC TGC TTC GGT-3') for 35 cycles at the annealing temperature of 61°C. A 12% polyacrylamide SSCP gel supplemented with 8% glycerol was then run at 50 W for four hours at 4°C and DNA bands were finally revealed by silver staining.

Table 1 Description of five RET single nucleotide polymorphisms and their position with respect to the most proximal SNP (the locus at -5 nt of the basal promoter)

Single nucleotide polymorphism location	Distance from the most proximal SNP (nt)	Codon involved	Nucleotide substitution	Allele frequency (%)*		
				Hirschsprung's disease	Control	p Value
Basal promoter (-5)	0		G>A	30	76	<0.00001
Basal promoter (-1)	4		C>A	83	60	0.0008
Exon 2	23461	A45A	CCG>GCA	38	70	0.00001
Exon 11	37608	G691S	GGT>AGT	88	81	0.23
Exon 13	41332	L769L	CTT>CTG	53	79	0.0002

Allele frequencies in Hirschsprung's disease and control individuals are reported, along with the significance of their difference measured by Fisher's exact test.
*Allele frequencies are given for the most frequent allele in controls (underlined in previous column).

Table 2 Distribution of the haplotypes at the -5 and -1 *RET* promoter SNP loci in patients with Hirschsprung's disease, patients with medullary thyroid carcinoma, and controls

Haplotype (-5; -1)	HD	%	Control	%	MTC	%
AA	1	1.1	0	0	0	0
GA	15	16.3	38	38	35	48.7
AC	63	68.5	26	26	13	18
GC	13	14.1	36	36	24	33.3
TOT	92	100	100	100	72	100

Both absolute and relative (%) frequencies are reported.
HD, Hirschsprung's disease; MTC, medullary thyroid carcinoma.

thyroid carcinoma ($\chi^2 = 43.4$, 3 df, $p < 0.00001$), but not between medullary thyroid carcinoma and controls ($\chi^2 = 2.4$, 2 df, $p = 0.31$). Finally, the observed genotype combinations of haplotypes were in agreement with those expected on the basis of the frequencies of single haplotypes within each of the three groups of individuals (data not shown).

Functional analysis of *RET* promoter SNPs

It has already been shown that specific alleles or haplotypes in the promoter region of a gene may influence gene expression, thus predisposing to the development of a complex disease.¹⁹⁻²² To elucidate the possible transcriptional significance of the *RET* promoter haplotypes which could explain their different distribution among different patient groups, we undertook a functional analysis. Transient transfection experiments were carried out in human neuroblastoma IMR32 cells using four constructs of the *RET* basal promoter, each containing one of the four haplotypes (AA, GA, AC, GC) cloned upstream of the CAT reporter gene in the pCATbasic vector. CAT assay radioactive counts of cellular lysates, obtained from three repeated sets of transfections as already described,²³ were compared by analysis of variance (ANOVA). We did not find any significant difference in the expression of the reporter gene among the four promoter constructs (table 3). Exclusion from the ANOVA of the AA haplotype also resulted in a non-significant difference among promoter constructs GA, GC, and AC, which were the most represented in the controls and patients with Hirschsprung's disease (data not shown).

Haplotype frequencies at four *RET* SNPs

In order to correlate one or more specific allele combinations with an increased risk of developing Hirschsprung's disease, we reconstructed haplotypes using SNPs that showed associations with the disease in the single marker analysis,

Table 3 Comparison by analysis of variance (ANOVA) of radioactive counts obtained from three repeated experiments after a CAT assay in lysates of cells transfected with four different reported gene constructs, each corresponding to one of the four different *RET* promoter haplotypes

<i>RET</i> promoter haplotype	N	Mean	SD
AA	3	1120.72	728.74
AC	3	2022.44	498.50
GC	3	1525.67	1201.71
GA	3	1667.00	856.99
Total	12	1583.96	807.22

ANOVA: $F = 0.564$ [3;8 df], $p = 0.654$.

Table 4 Haplotype frequencies at four *RET* SNPs (SNP-5, SNP-1, SNP2, and SNP13) in patients with Hirschsprung's disease and controls

Haplotypes	Hirschsprung's disease	Controls
ACAG	0.3754	0.0992
ACAT	0.2442	0.1186
GAGT	0.1543	0.3443
GCGT	0.0957	0.2618
GCGG	0.0543	0.0717
ACGT	0.0384	0.0222
AAGG	0.0196	-
ACGG	0.0180	-
GAAT	-	0.0431
GCAG	-	0.0266
GAAG	-	0.0125

namely SNP-5, SNP-1, SNP2, and SNP13 (table 1), and compared their distribution in Hirschsprung's disease and controls. Haplotypes and their frequencies were estimated by means of the EHPlus software,¹⁷ and homogeneity in haplotype distributions between cases and controls was tested by means of the non-parametric test implemented in the same program. We observed a total of 11 haplotypes over the 16 expected,²⁴ eight in Hirschsprung's disease and nine in the normal controls, with six haplotypes being present in both groups (table 4). The frequencies of single haplotypes were quite different in the two groups and, overall, the difference between the two distributions was highly significant ($p < 0.0001$).

The two haplotypes most often observed among the Hirschsprung cases, ACAG and ACAT, together accounted for 62.0% of the haplotypes in Hirschsprung's disease and 21.8% of those in the controls. They harbour the AC combination of alleles at the promoter region already shown to be over-represented in Hirschsprung's disease (table 2), the variant A allele of SNP2, and differ only for the allele of SNP13. The polymorphic variants present at the first three loci in these haplotypes are the most common in Hirschsprung's disease, while the T and the G alleles at the fourth locus (SNP13) are equally frequent (see table 1). We believe ACA represents the core haplotype associated with Hirschsprung's disease, and propose a role of this haplotype as a modifying risk allele in the development of the disorder.

DISCUSSION

Hirschsprung's disease, the most common hereditary cause of intestinal obstruction, shows considerable phenotypic variation and complex inheritance. Loss of function *RET* mutations account for 10-15% of sporadic cases, while several common *RET* polymorphisms have been reported to show an allelic association with the disease. In particular, *RET* SNP alleles are present with different frequencies in different populations in patients with Hirschsprung's disease compared with controls,⁷⁻⁹ while specific haplotypes have been shown to have either protective or predisposing effects, or to modulate the severity of the resulting phenotype.¹⁰⁻¹³

We have recently described a *RET* haplotype comprising the T variant of SNP14, under-represented in Hirschsprung's disease and showing non-random transmission from healthy carrier parents to affected siblings. We hypothesised that this haplotype, which is conserved in a region of about 29 kb, confers a protective effect against the development of Hirschsprung's disease, probably by dysregulation of *RET* transcript expression.¹² Other studies have identified predisposing haplotypes for Hirschsprung's disease characterised by the presence of the A variant of SNP2.¹⁰⁻¹¹ Recently, Borrego *et al* have extended by 2 kb the genetic analysis of these

haplotypes with the addition of three SNPs located in the 3' portion of intron 1. Their statistical analysis suggests the existence of a low penetrant locus of susceptibility for Hirschsprung's disease at a distance of 20–30 kb upstream of SNP2.¹³

In an attempt to identify sequence variants of the *RET* gene promoter, possibly impairing its expression and thus predisposing to Hirschsprung's disease, we have found two novel SNPs, located at –1 bp and –5 bp from the *RET* transcription start site, the alleles of which have a different distribution in patients with Hirschsprung's disease compared with either healthy controls or patients with medullary thyroid carcinoma (table 1). On the other hand, allelic frequencies of *RET* promoter SNPs in patients with medullary thyroid carcinoma do not differ statistically from controls. They show a good agreement with previous reports describing *RET* haplotype distribution in a different population,¹³ and the lack of significance in our analysis could reflect the smaller size of our sample (36 v 50) or the different population background (Italian v Spanish). In any case, at both SNP loci, the highest frequencies are found in sporadic cases of Hirschsprung's disease (70% at SNP–5 and 83% at SNP–1), and they decrease in control individuals (24% at SNP–5 and 60% at SNP–1), and further in cases of sporadic medullary thyroid carcinoma (18% at SNP–5 and 51% at SNP–1). Such a tendency, further confirmed by the opposite distribution of the promoter SNP combinations between Hirschsprung's disease and medullary thyroid carcinoma (GA, 16.3% v 48.7%; AC, 68.5% v 18%; GC, 14.1% v 33.3%) (table 2), suggests a role for these two SNPs, or another variant in linkage disequilibrium, in the aetiology of colonic aganglionosis and thyroid cancer. This hypothesis is in keeping with the opposing pathogenic processes acting during development of Hirschsprung's disease (haploinsufficiency or loss of function of the *RET* gene) and medullary thyroid carcinoma (constitutive activation of the *RET* receptor).^{25–26}

Haplotypes reconstructed using two additional SNPs in exons 2 and 13 showed that two combinations, ACAG and ACAT, together account for 62.0% of Hirschsprung's disease haplotypes and 21.8% of control haplotypes, supporting the view that ACA may represent the core haplotype acting as the predisposing allele in the development of Hirschsprung's disease. In particular, a functional effect predisposing to Hirschsprung's disease of either the promoter SNPs, the exon 2 SNP, a combination of these, or another *RET* variant yet to be identified in linkage disequilibrium with them, can be postulated.

The strong association between the A variant of SNP2 and the AC haplotype of the two promoter SNPs, located 23.5 kb away from exon 2, is reminiscent of the hypothesis proposed by Borrego *et al* about the existence of a very ancient, low penetrant founder locus located, by statistical inference, 20–30 kb upstream of SNP2.¹³ It is tempting to speculate that one or both of the two promoter SNPs may in fact represent the same locus hypothesised in Borrego's analysis. In any event, our results provide experimental support for their hypothesis that a predisposing locus for Hirschsprung's disease may reside in this region of the *RET* gene.

A functional effect of the promoter SNPs predisposing to Hirschsprung's disease could not be confirmed by our gene reporter assay. The two promoter SNPs do not seem to interfere with the expression of the *CAT* gene, even if the hypothesis of a functional effect cannot yet be completely ruled out. Our in vitro model system might have been limited by the length of the promoter segment considered, which allowed us to test only the basal activity of the *RET* promoter and its possible tissue specificity. Alternatively, the existence of linkage disequilibrium between the two promoter SNPs

and another functional locus might explain their association with Hirschsprung's disease.

Conclusions

Overall, our data support the existence of a low penetrant variant of the *RET* gene lying within or in close vicinity to the ACA allele, exerting an effect on either transcription, splicing, or function of *RET*, and acting as a Hirschsprung's disease susceptibility allele. The possibility that such a functional variant is one of the alleles characterising the predisposing haplotype or a combination of them, cannot yet be excluded. A systematic search for additional variants in the crucial 23.5 kb encompassing basal promoter, exon 1, intron 1, and exon 2, and surrounding sequences, together with functional analyses of ACA associated *RET* splicing and *RET* expression, will allow the precise molecular mechanism for the association of this *RET* haplotype with Hirschsprung's disease susceptibility to be elucidated.

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Authors' affiliations

M Sancandi, P Griseri, B Pesce, G Patrone, F Puppo, M Lerone, I Ceccherini, Laboratorio di Genetica Molecolare, Istituto G Gaslini, Genova, Italy
G Martucciello, Divisione e Cattedra di Chirurgia Pediatrica, Istituto G Gaslini, Genova, Italy
R Ravazzolo, Laboratories di Genetica Molecolare, Istituto G Gaslini, Genova, Italy
G Romeo, Cattedra di Genetica Medica, Università di Bologna, Bologna, Italy
M Devoto, Department of Research, Nemours Children's Clinic, Wilmington, Delaware, USA

M Sancandi and P Griseri contributed equally to this work

Correspondence to: Isabella Ceccherini, Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini, L.go G Gaslini 5, 16148 Genova, Italy; isa.c@unige.it

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