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. 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. 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). 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, 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. 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 (S8365) has shown a low penetrant protective effect against the disease. 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 specific variant is related to the transcriptional activity of the RET proto-oncogene.

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 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. We also investigated 36 Italian patients with medullary thyroid carcinoma and 50 controls (who have already been described). Amplification of 392 bp encompassing the RET basal promoter was carried out using primers P1F (5′-CCC GCA CTC GCC CTT CAT-3′) and P1R (5′-GGA CTC GCC CTT GCC 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).

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Transfection and CAT assay

Transfection was performed in IMR32 neuroblastoma cell lines with polyethylenimine (PEI) as previously described. 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 spectro-photometric 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 EPHPlus software 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 transition 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<0.00009).

As RET mutations have been found in association with multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B) and medullary thyroid carcinoma, 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.

Table 1

<table>
<thead>
<tr>
<th>Single nucleotide polymorphism location</th>
<th>Distance from the most proximal SNP (nt)</th>
<th>Codon involved</th>
<th>Nucleotide substitution</th>
<th>Allele frequency (%)</th>
<th>Hirschsprung’s disease Control</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal promoter (–5)</td>
<td></td>
<td></td>
<td>–A</td>
<td>30 76</td>
<td>&lt;0.00001</td>
<td></td>
</tr>
<tr>
<td>Basal promoter (–1)</td>
<td></td>
<td></td>
<td>–A</td>
<td>83 60</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td></td>
<td>A45A</td>
<td>GCG –GCA</td>
<td>38 70</td>
<td>0.000001</td>
<td></td>
</tr>
<tr>
<td>Exon 11</td>
<td></td>
<td>G691S</td>
<td>GGT –AGT</td>
<td>88 81</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Exon 13</td>
<td></td>
<td>L769L</td>
<td>GT1–CTG</td>
<td>53 79</td>
<td>0.00002</td>
<td></td>
</tr>
</tbody>
</table>

Allele frequencies in Hirschsprung’s disease and control individuals are reported, along with the significance of their difference measured by Fisher’s exact test.

(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 (x² = 37.0, 3 df, p<0.00001) and Hirschsprung’s disease with medullary...
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 pCATH basic 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 patient groups, 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 the variant A allele of SNP14, under-represented 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,24 while specific haplotypes have been shown to have either protective or predisposing effects, or to modulate the severity of the resulting phenotype.16,17 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.25 Other studies have identified predisposing haplotypes for Hirschsprung’s disease characterised by the presence of the A variant of SNP2.18,19 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."

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, 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 SNP2 and 83% at SNP1), and they decrease in control individuals (24% at SNP2 and 6% at SNP1), and further in cases of sporadic medullary thyroid carcinoma (18% at SNP2 and 51% at SNP1). Such a tendency, further confirmed by the opposite distribution of the promoter SNP combinations between Hirschsprung’s disease and medullary thyroid carcinoma. This hypothesis is in line with that of the association between Hirschsprung’s disease and medullary thyroid carcinoma (GA, 16.3%; AC, 68.5%; 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).

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. 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 loci 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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