RET genotypes comprising specific haplotypes of polymorphic variants predispose to isolated Hirschsprung disease

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Abstract
Background—Hirschsprung disease (HSCR), which may be sporadic or familial, occurs in 1:5000 live births and presents with functional intestinal obstruction secondary to aganglionosis of the hindgut. Germline mutations of the RET proto-oncogene are believed to account for up to 50% of familial cases and up to 30% of isolated cases in most series. However, these series are highly selected for the most obvious and severe cases and large familial aggregations. Population based studies indicate that germline RET mutations account for no more than 3% of isolated HSCR cases. Recently, we and others have noted that specific polymorphic sequence variants, notably A45A (exon 2), are over-represented in isolated HSCR.

Purpose—In order to determine if it is the variant per se, a combination thereof, or another locus in linkage disequilibrium which predisposes to HSCR, we looked for association of RET haplotype(s) and disease in HSCR cases compared to region matched controls.

Methods—Seven loci across RET were typed and haplotypes formed for HSCR cases, their unaffected parents, and region matched controls. Haplotype and genotype frequencies and distributions were compared among these groups using the transmission disequilibrium test and standard case-control statistic.

Results—Twelve unique haplotypes, labelled A–L, were obtained. The distributions of haplotypes between cases and controls ($\chi^2=81.4, p<0.0001$) and between cases and non-transmitted parental haplotypes were significantly different ($\chi^2=53.1, p<0.0001$). Genotypes comprising up to 20% of haplotypes were formed for cases and controls. There were 38 different genotypes among cases and controls combined. Inspection of the genotypes in these two groups showed that the genotype distribution between cases and controls was distinct ($\chi^2=93.8, p<0.0001$). For example, BB, BC, BD, and CD, all of which contain at least one allele with the polymorphic A45A, are prominently represented among HSCR cases, together accounting for >35% of the case genotypes, yet these four genotypes were not represented among the population matched normal controls. Conversely, AA, AG, DD, GG, and GJ, none of which contains A45A, are commonly represented in the controls, together accounting for 43% of the control genotypes, and yet they are never seen among the HSCR cases.

Conclusions—Our data suggest that genotypes comprising specific pairs of RET haplotypes are associated with predisposition to HSCR either in a simple autosomal recessive manner or in an additive, dose dependent fashion.

(J Med Genet 2000;37:572–578)

Keywords: transmission disequilibrium test; chromosome 10; polymorphisms

The RET proto-oncogene, on 10q11.2, is a major susceptibility gene for Hirschsprung disease (HSCR, MIM 142623), a common disorder occurring in 1 in 5000 live births and characterised by the absence of the intramural ganglia of Meissner and Auerbach in the hindgut, which results in functional intestinal obstruction. HSCR most commonly presents as isolated cases although it can be familial and may be inherited as an autosomal dominant or autosomal recessive trait, with reduced penetrance and male predominance. Currently, at least seven related genes are believed to play some aetiological role in the pathogenesis of hereditary syndromic and non-syndromic HSCR.

The RET proto-oncogene encodes a receptor tyrosine kinase expressed in derivatives of the neural crest and neuroectoderm. Gain of function germline mutations in the RET proto-oncogene are associated with multiple endocrine neoplasia type 2 (MEN 2), an autosomal dominantly inherited cancer syndrome characterised by medullary thyroid carcinoma, phaeochromocytoma, and hyperparathyroidism. Interestingly, loss of function germline RET mutations have been found in HSCR. Depending on the series, up to 50% of familial HSCR cases and anywhere between 10 and 35% of sporadic cases were reported to be accounted for by loss of function germline RET mutations. However, these series were highly selected, usually for familial cases or severe presentations. The only population based series, however, estimates the frequency of germline RET mutation in 69 unselected HSCR cases to be 7% and only 3% of isolated HSCR cases in this population based cohort had germline RET mutations. Although several other putative HSCR susceptibility
genes have been proposed, including those that encode glial cell line derived neurotrophic growth factor (GDNF), one of the ligands for RET, and endothelin-3 and endothelin receptor-beta (EDNRB). Only germline heterozygous mutations in EDNRB occur in a significant minority of non-syndromic HSCR.

When HSCR and MEN 2 occur together, the great majority are found to have C620R and C618R mutations. A single case family segregating both HSCR and MEN 2 was found to harbour a germline RET C620S mutation, and at the time of original ascertainment, the only subject to have both HSCR and MEN 2 carried a C620S RET mutation and the homozygous sequence polymorphism in exon 2, A45A (c.135G→A). Extending this observation in a population based series of isolated HSCR cases from western Andalucía, Spain, we found that the A45A (c.135G→A) sequence variant and L769L (c.2307T→G) (exon 13) were significantly over-represented (p<0.0006) when compared to region matched, race matched normal controls. In contrast, two other polymorphisms, G691S (c.2071C→G, exon 11) and S904S (c.2712C→G, exon 15), were under-represented in the HSCR patients compared to controls (p=0.02). Interestingly, similar findings were independently obtained in a series of HSCR cases from different population bases, Germany and the UK. Using the same 64 isolated HSCR patients from our previous population based study and newly accrued normal parents of these cases, we therefore sought to determine whether distinct germline RET sequence variant haplotypes could be directly associated with predisposition to HSCR.

**Materials and methods**

**ISOLATED HSCR CASES**

The western Andalucía region of Spain is serviced by the University Hospital “Virgen del Rocío” in Seville and is the major referral centre for HSCR, and so all HSCR cases seen at this institution may be considered representative of the population. This study used the first 64 consecutive cases of clinically sporadic HSCR seen at this institution in the first 13.5 months of study, described in detail previously. In contrast, two other polymorphisms, G691S (c.2071C→G, exon 11) and S904S (c.2712C→G, exon 15), were under-represented in the HSCR patients compared to controls (p=0.02). draw from the HSCR patients compared to controls (p=0.02). Interestingly, similar findings were independently obtained in a series of HSCR cases from different population bases, Germany and the UK. Using the same 64 isolated HSCR patients from our previous population based study and newly accrued normal parents of these cases, we therefore sought to determine whether distinct germline RET sequence variant haplotypes could be directly associated with predisposition to HSCR.

**CASES AND NORMAL CONTROLS**

Near the end of the study period, the unaffected parents of the isolated HSCR cases were recontacted to participate in the research protocol (in accordance with local institutional human protection committee rules). Forty-nine parental couples and eight single parents agreed to participate.

Normal controls were unselected, unrelated, race matched subjects from Andalucía without a diagnosis of HSCR and who did not attend the medical genetic, paediatric surgical, or gastroenterological clinics.

**MUTATION ANALYSIS**

Genomic DNA was extracted from peripheral blood leukocytes from HSCR cases, their clinically unaffected parents or relatives, and normal controls using standard techniques. To examine variant status at each of the polymorphic loci within RET, the appropriate RET amplicon for each of exons 2, 3, 7, 11, 13, 14, and 15 was generated as previously described. The presence or absence of each polymorphism within each amplicon was assessed by differential restriction digestion with the appropriate enzymes as described, according to the manufacturers’ recommendations (Roche Life Technologies and Pharmacia Biotech). The products of restriction digestion were fractionated by electrophoresis through 2% agarose or 6% polyacrylamide gels and visualised under UV transilumination after ethidium bromide staining. When the primers were 5’ labelled with fluorescent dyes, then the restricted amplicons were subjected to electrophoresis through an Alf-Express Automated DNA Sequencer (Pharmacia Biotech).

**STATISTICAL ANALYSIS**

Allelic frequencies at all seven RET polymorphic loci were determined and haplotypes formed (table 1). The frequencies of each haplotype were compared between the cases and race matched, region matched, normal controls who were unrelated to the HSCR subjects. Comparisons were performed using χ² contingency table tests with Yates’s correction or (when computationally feasible) Fisher’s exact test for tables with small expected cell counts. The criterion for statistical significance was set at α=0.05. Odds ratios with Cornfield 95% confidence intervals were also generated at each haplotype. The asymptotic p values from the transmission disequilibrium tests were supported in each case by exact p values computed via direct evaluation or 10 000 simulations.

Where available, parental haplotypes were examined in the context of the affected children’s haplotypes. Transmitted and non-transmitted haplotypes were noted and compared. Statistical analyses and simulations were performed using S-Plus 4.5 (Mathsoft Inc.). Stepwise logistic regression was performed using SAS v6.09 (SAS Institute), with entry and retention significance level criteria of 0.05. Although the transmitted haplotypes are matched with non-transmitted haplotypes,
unconditional logistic regression was appropriate (chapter 6 of Breslow and Day) because marginal distributions of the predictors are assumed not to contain information about relative risk.

Results

Haplotypes, comprised of variants across seven polymorphic loci in the coding region of \textit{RET}, could be generated for 62 of the 64 HSCR cases and for 65 of the 104 race matched, region matched controls. Phase was successfully determined for 62 of 64 HSCR cases, 54 parents, and 65 controls. Among the 62 HSCR cases, seven with both parents lost to follow up had their haplotypes inferred from the haplotype compositions of the other subjects. This inference has no effect on the transmission disequilibrium test results, which use only families with informative parents. There were 12 unique haplotypes, labelled A-L, among cases and controls combined (tables 1 and 2A).

Since recombination events among loci will be extremely rare, the haplotypes are viewed as individual alleles of a single locus. Apart from the haplotype comprised of the wild type allele at each of the polymorphic loci (haplotype A), there were 11 haplotypes (B-L) made up of various combinations and permutations of sequence variant and wild type sequence at each locus (table 1).

The three most common haplotypes represented among controls occurring in more than 15% of chromosomes were A (36.2%), D (A432A only, rest wild type; 20%), and G (G691S (c.2071C→A) and S904S (c.2712C→G; 17.7%) (table 2A, fig 1). In contrast, the most common haplotypes occurring in 15% or more of HSCR chromosomes were haplotypes B (A45A (c.135G→A) only; 33% of chromosomes) and C (A45A

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\textbf{Haplotype} & \textbf{HSCR patients} & \textbf{Normal controls} & \textbf{Statistic} & \textbf{p value} \\
\hline
& No chrom & % & No chrom & % & $\chi^2$ \\
\hline
A & 10 & 8.1 & 47 & 36.2 & 27.2 <0.0001 \\
B & 38 & 30.7 & 8 & 6.2 & 24.0 <0.0001 \\
C & 25 & 20.2 & 4 & 3.1 & 16.7 <0.0001 \\
D & 15 & 12.1 & 26 & 20 & 2.4 0.12 \\
E & 8 & 2.4 & 8 & 6.2 & 1.3 0.25 \\
F & 6 & 4.8 & 0 & 0 & Fisher 0.01 \\
G & 9 & 7.3 & 23 & 17.7 & 5.4 0.02 \\
H & 9 & 7.3 & 6 & 4.6 & 0.4 0.53 \\
I & 5 & 4.0 & 7 & 5.4 & 0.04 0.83 \\
J & 1 & 0.8 & 0 & 0 & Fisher 0.49 \\
K & 2 & 1.6 & 0 & 0 & Fisher 0.24 \\
L & 1 & 0.8 & 0 & 0 & Fisher 1.0 \\
\hline
\textbf{Totals} & 124 & 100 & 130 & 100 & \\
\hline
\end{tabular}
\caption{Comparison of haplotype frequencies among HSCR cases and population normal controls.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Haplotype} & \textbf{HSCR patients} (n=62 patients and 124 chromosomes) & \textbf{Non-transmitted chromosomes (107 chromosomes)} & \textbf{Control population (n=65 unrelated subjects and 130 chromosomes)} & \\
\hline
A & 10 & 27 & 47 & \\
B & 38 & 12 & 8 & \\
C & 25 & 5 & 4 & \\
D & 15 & 26 & 26 & \\
E & 3 & 5 & 8 & \\
F & 7 & 1 & 0 & \\
G & 9 & 17 & 23 & \\
H & 9 & 2 & 6 & \\
I & 4 & 2 & 7 & \\
J & 1 & 2 & 0 & \\
K & 2 & 0 & 0 & \\
L & 1 & 8 & 1 & \\
\hline
\textbf{Total} & 124 & 107 & 130 & \\
\hline
\end{tabular}
\caption{Comparison of haplotype frequencies between HSCR patients, non-transmitted chromosomes of their parents, and control population. (Both homozygous and heterozygous parents are included).}
\end{table}

Comparison of transmitted vs non-transmitted: $\chi^2 = 53.1$, p<0.0001.
Comparison of non-transmitted v control: $\chi^2 = 18.1$, p=0.039.

Figure 1 Frequency of RET genotypes in HSCR cases (white bars) versus normal controls (striped bars). X axis, genotypes; Y axis, absolute number of cases.
Table 3  Transmission disequilibrium testing comparing each haplotype to the group of remaining haplotypes

<table>
<thead>
<tr>
<th>Haplo</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>χ²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>14.2</td>
<td>10.5</td>
<td>10.7</td>
<td>4.5</td>
<td>0.5</td>
<td>4.0</td>
<td>3.9</td>
<td>4.5</td>
<td>1.3</td>
<td>0.3</td>
<td>2.0</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>0.0002</td>
<td>0.0012</td>
<td>0.0011</td>
<td>0.0339</td>
<td>0.4795</td>
<td>0.0455</td>
<td>0.0495</td>
<td>0.0339</td>
<td>0.2568</td>
<td>0.5637</td>
<td>0.1573</td>
<td>0.0196</td>
</tr>
</tbody>
</table>

(c.135G→A) and L769L (c.2307T→G); 19%). Overall, the distribution of HSCR and control alleles across these 12 haplotypes was significantly different (χ² = 81.4, p << 0.0001, table 2A, B).

Three haplotypes, B, C, and F, were over-represented among HSCR cases compared to normal controls (table 2A). Haplotype B, having only the polymorphic A45A (c.135G→A) and the rest of the loci wild type, was found in 38 (30.7%) HSCR chromosomes and only eight (6.2%) control chromosomes (OR=6.74 (2.84-16.53), χ² = 24.0, p << 0.0001). Haplotype C, harbouring A45A (c.135G→A) and L769L (c.2307T→G), with the rest of the loci wild type, was represented among 25 (20.2%) HSCR alleles compared to four (3.1%) control alleles (OR=7.95 (2.61-32.25), χ² = 16.7, p << 0.0001). Haplotype F, comprising A45A (c.135G→A), A432A, and L769L (c.2307T→G), comprised seven (5.6%) HSCR alleles compared to no control chromosomes (p = 0.006, Fisher two tailed exact test).

Two RET haplotypes, A and G, appeared under-represented among HSCR cases compared to matched normal controls (table 2A). Haplotype A (all loci wild type) occurred among 10 (8.1%) HSCR chromosomes compared to 47 (36.2%) control chromosomes (OR=0.15 (0.07-0.34), χ² = 27.2, p << 0.0001). Haplotype G, with G691S (c.2071C→G), comprised among 25 (20.2%) HSCR alleles compared to 47 (36.2%) control chromosomes (p = 0.012), B (p = 0.0017), C (p = 0.0027), D (p = 0.046), H (p = 0.034), and L (p = 0.0196).

The transmission disequilibrium test tests association in a manner that acknowledges the matching of observed alleles, or in this instance haplotypes, in the parents of affected subjects. Only heterozygous parents are informative when performing this test. Although originally designed for biallelic markers, we applied multiple allelic/haplotype extensions here. Table 3 presents the results comparing each haplotype to the group of remaining haplotypes. The highest frequencies of non-transmission were observed with haplotypes A, D, and G, and this was more pronounced when compared to the frequency of these haplotypes among the affected offspring. In contrast, haplotypes B, C, F, and H had the lowest rates of parental non-transmission, that is, these haplotypes were the most frequently transmitted from parent to affected offspring. As an overall test of association, Spielman and Ewens propose a summation of the individual contributions. The corresponding statistic is χ² = 56.7, p << 0.0001. Another overall test uses the Bonferroni corrected p value to the maximum χ² statistic of the individual haplotypes, which yields p = 0.002. Simulation based methods conditioned on the parental genotypes produced p values of 0.0001 and 0.0004, respectively.

Table 4  Transmission disequilibrium test by individual RET polymorphic locus

<table>
<thead>
<tr>
<th>Transmitted</th>
<th>Wild type</th>
<th>Variant</th>
<th>TDT</th>
<th>p value</th>
<th>Exact p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A45A</td>
<td>Wild type</td>
<td>37</td>
<td>9</td>
<td>28.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Variant</td>
<td>50</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V125V</td>
<td>Wild type</td>
<td>99</td>
<td>1</td>
<td>4.5</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>Variant</td>
<td>90</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A432A</td>
<td>Wild</td>
<td>70</td>
<td>23</td>
<td>6.2</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>Variant</td>
<td>72</td>
<td>19</td>
<td>1.6</td>
<td>0.209</td>
</tr>
<tr>
<td>G691S</td>
<td>Wild type</td>
<td>63</td>
<td>15</td>
<td>2.5</td>
<td>0.114</td>
</tr>
<tr>
<td></td>
<td>Variant</td>
<td>25</td>
<td>4</td>
<td>0.5</td>
<td>0.480</td>
</tr>
<tr>
<td>L769L</td>
<td>Wild type</td>
<td>99</td>
<td>5</td>
<td>0.5</td>
<td>0.480</td>
</tr>
<tr>
<td></td>
<td>Variant</td>
<td>73</td>
<td>19</td>
<td>1.6</td>
<td>0.209</td>
</tr>
<tr>
<td>S904S</td>
<td>Wild type</td>
<td>12</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ASSOCIATION OF RET HAPLOTYPE WITH HSCR BY THE TRANSMISSION DISEQUILIBRIUM TEST

Because the non-transmitted parental haplotypes are available, we have also used these for analyses of association with disease that are highly robust to population stratification. Table 2B shows the distribution of haplotypes that are transmitted to HSCR probands, those that are not transmitted from the parents, and those from the control group. The non-transmitted control haplotypes do not appear to differ greatly in distribution, although a comparison of these two groups is significant at the 0.05 level (χ² = 19.1, p = 0.039). A similar test of non-transmitted v transmitted haplotypes (chapter 4 of Lange) is highly significant (χ² = 33.1, p = 0.0001). The most significant contributions from individual haplotypes are A (p = 0.012), B (p = 0.0017), C (p = 0.0027), D (p = 0.046), H (p = 0.034), and L (p = 0.0196).

In order to analyse more fully the effect of each locus on the transmission of a haplotype...
Cases and controls was distinct (showed that the genotype distribution between Inspection of the genotypes in these two groups (six or 9.7%). Only two (3.1%) normal (seven or 11.3%), BC (six or 9.7%), and BH three most common HSCR genotypes were BB (three genotypes and all three were AD. The three (4.8%) HSCR cases carried one of these (12 or 18.5%), AG (seven or 10.8%), and AA parents, and controls. The three most common were generated for cases, their participating Genotypes comprising pairs of HAPLOTYPE PAIRS (GENOTYPE) IN HSCR CASES a haplotype is transmitted to the a multiple logistic regression (see Methods). Haplotype analysis in HSCR,27 was very rare by

<table>
<thead>
<tr>
<th>Table 5 Logistic regression data</th>
<th>Significance level (p)</th>
<th>Odds ratio</th>
<th>95% CI for odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A45A</td>
<td>0.0001</td>
<td>7.38</td>
<td>(3.93, 13.87)</td>
</tr>
<tr>
<td>V125V</td>
<td>0.006</td>
<td>9.11</td>
<td>(1.82, 45.49)</td>
</tr>
<tr>
<td>Regression on each locus</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A45A</td>
<td>0.0001</td>
<td>6.22</td>
<td>(3.36, 11.53)</td>
</tr>
<tr>
<td>V125V</td>
<td>0.075</td>
<td>4.11</td>
<td>(0.84, 20.08)</td>
</tr>
<tr>
<td>A432A</td>
<td>0.058</td>
<td>0.78</td>
<td>(0.43, 1.45)</td>
</tr>
<tr>
<td>G691S</td>
<td>0.081</td>
<td>0.52</td>
<td>(0.25, 1.10)</td>
</tr>
<tr>
<td>L769L</td>
<td>0.047</td>
<td>1.89</td>
<td>(1.0, 3.60)</td>
</tr>
<tr>
<td>S936S</td>
<td>0.069</td>
<td>0.51</td>
<td>(0.11, 2.23)</td>
</tr>
<tr>
<td>S904S</td>
<td>0.081</td>
<td>0.52</td>
<td>(0.25, 1.10)</td>
</tr>
</tbody>
</table>

Haplotype PAIRS (Genotype) in HSCR Cases and Controls Genotypes comprising pairs of RET haplotypes were generated for cases, their participating parents, and controls. The three most common genotypes among the 65 controls include AD (12 or 18.5%), AG (seven or 10.8%), and AA (eight or 12.3%) (fig 1). Interestingly, only three (4.8%) HSCR cases carried one of these three genotypes and all three were AD. The three most common HSCR genotypes were BB (seven or 11.3%), BC (six or 9.7%), and BH (six or 9.7%). Only two (3.1%) normal controls carried any of these three genotypes and both were BC. In summary, there were 38 different genotypes among cases and controls combined. Inspection of the genotypes in these two groups showed that the genotype distribution between cases and controls was distinct (x2 = 93.8, p < 0.0001). For example, BB, BC, BD, and CD are prominently represented among HSCR cases, together accounting for >35% of the case genotypes, yet these four genotypes are not represented among the region matched, race matched normal controls. Conversely, AA, AG, DD, GG, and GJ are commonly represented in the controls, together accounting for 43% of the control genotypes, and yet they are never seen among the HSCR cases.

Discussion Among the western Andalucian HSCR cases, all RET haplotypes harbouring A45A (c.135G→A) appeared to be over-represented among HSCR cases compared to region matched, race matched, normal controls, thus confirming previous single site analyses and our hypotheses.27 Interestingly, the L769L (c.2307T→G) polymorphism, which was previously found to be over-represented in single locus analysis in HSCR,27 was very rare by itself. It almost always occurred with the A45A (c.135G→A) variant, suggesting perhaps that it could be in linkage disequilibrium with the A45A (c.135G→A) allele. Conversely, selected non-A45A (c.135G→A) bearing haplotypes such as A and G were over-represented among controls. Even more powerfully associated with HSCR are the genotypes comprising specific RET haplotype pairs, such that our data might suggest an autosomal recessive or dose dependent (additive) low penetrance mechanism for a large proportion of isolated HSCR.

The precise haplotype-HSCR and single locus-HSCR associations compared to unrelated controls differ slightly from those compared to the unaffected parents. In our earlier report,27 G691S (c.2071C→A)/S904S (c.2712C→G) were reported as conferring an apparent protective effect. Although the current results parallel an earlier report using unrelated control samples, the requirements of the transmission disequilibrium test reduce the effective sample size, so that the effect of G691S (c.2071C→A)/S904S (c.2712C→G) is not statistically significant here at the 0.05 level. Similarly, the stepwise multiple logistic regression shows that the effect of G691S (c.2071C→A)/S904S (c.2712C→G) is no longer suggestive in models which include A45A (c.135G→A). It can be seen by inspection of the haplotypes that variant A45A (c.135G→A) is associated with wild type sequence at codons 691 and 904 such that only one transmitted haplotype comprised variants A45A (c.135G→A) and G691S (c.2071C→A)/S904S (c.2712C→G). Thus, the apparent “HSCR protective” effect of variant G691S (c.2071C→A)/S904S (c.2712C→G) is largely confounded with the (stronger) effect at codon 45. Because of the association of the two loci, it is difficult to explore their precise joint effect on transmission status further. The observations using the transmission disequilibrium test have the advantage of being highly robust to population stratification, and there is evidence (table 2A, B) that the control haplotype distribution differs from that of the non-transmitted haplotypes.

Although this exploratory data set is relatively small, there are strong indications that haplotype pairs interact with each other to modulate HSCR phenotype. Under a simple additive (that is, dose dependent) or autosomal recessive model, therefore, we would expect the presence of two HSCR associated haplotypes would be overwhelmingly associated with HSCR and not controls. Conversely, the presence of two control associated haplotypes in a single subject should be strongly associated with normal controls and not with HSCR. In accordance with this model, the BB genotype was observed in seven (11.3%) HSCR cases but no controls. The AA, DD, and GG genotypes combined have been found in a total of 18 (27.7%) controls but no HSCR cases. Extrapolation of these observations would then lead to the hypothesis that heterozygous combinations of HSCR associated haplotypes would also be mainly associated with HSCR; similarly, heterozygous com-
RET genotypes predispose to Hirschsprung disease


