

A Founding Locus within the *RET* Proto-Oncogene May Account for a Large Proportion of Apparently Sporadic Hirschsprung Disease and a Subset of Cases of Sporadic Medullary Thyroid Carcinoma

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Hirschsprung disease (HSCR) is a common congenital disorder characterized by aganglionosis of the gut. The seemingly unrelated multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominant disorder characterized by medullary thyroid carcinoma (MTC), pheochromocytoma, and hyperparathyroidism. Yet, germline mutations in the *RET* proto-oncogene are associated with both MEN 2 and HSCR. In the former, gain-of-function mutations in a limited set of codons is found, whereas, in the latter, loss-of-function mutations are found. However, germline *RET* mutation is associated with only 3% of a population-based series of isolated HSCR, and little is known about susceptibility to sporadic MTC. We have found previously that specific haplotypes comprising *RET* coding single-nucleotide polymorphisms (SNPs) comprising exon 2 SNP A45A were strongly associated with HSCR, whereas haplotypes associated with exon 14 SNP S836S were associated with MTC. In this study, we describe three novel intron 1 SNPs, and, together with the coding SNP haplotypes, the data suggest the presence of distinct ancestral haplotypes for HSCR and sporadic MTC in linkage disequilibrium with a putative founding susceptibility locus/loci. The data are consistent with the presence of a very ancient, low-penetrance founder locus ~20–30 kb upstream of SNP A45A, but the failure of the SNPs to span the locus presents challenges in modeling mode of transmission or ancestry. We postulate that this founding locus is germane to both isolated HSCR and MTC but also that different mutations in this locus would predispose to one or the other.

Introduction

Germline gain-of-function mutations of the *RET* proto-oncogene, encoding a receptor tyrosine kinase on 10q11.2, cause multiple endocrine neoplasia type 2 (MEN 2 [MIM 164761]) and loss-of-function mutations are associated with a small subset of Hirschsprung disease (HSCR [OMIM 142623]). Germline mutations in six different exons of the *RET* proto-oncogene (exons 10, 11, 13, 14, 15, and 16) account for at least 92% of cases of MEN 2, which is characterized by the triad of medullary thyroid carcinoma (MTC), pheochromocytoma, and hyperparathyroidism (Eng et al. 1996a). MEN 2, the heritable form of MTC, is believed to account for 25% of all MTC presentations. Thus, 75% are believed to be sporadic.

The etiology of sporadic MTC is not well understood

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(for review, see Eng 1999). Limited somatic genetic alterations—such as loss of heterozygosity of markers and somatic mutations in *RET*, principally M918T—have been described in sporadic MTC (Mulligan et al. 1993; Eng et al. 1994, 1995, 1996b; Hofstra et al. 1994) (reviewed in Eng 1999). Recently, we reported an overrepresentation of a germline *RET* sequence variant in exon 14, S836S (c.2439 C→T), among isolated patients from Germany affected with MTC, compared with a control population with the same geographic origin and ethnic and genetic backgrounds (Gimm et al. 1999). The association of S836S with MTC was confirmed, independently, in another series of European patients with MTC (Ruiz et al. 2001). These observations suggest that the phenomenon of association of S836S with MTC could be universal and that there is an ancestral low-penetrance susceptibility marker for MTC within the *RET* proto-oncogene (Ruiz et al. 2001). To date, S836S is the only germline susceptibility factor that has been identified for sporadic medullary thyroid cancer. Although various hypotheses have been suggested for its molecular mechanism, this remains unknown at this time (Gimm et al. 1999; Ruiz et al. 2001).

HSCR or aganglionic megacolon is a common disorder, occurring in 1 in 5,000 live births characterized by the absence of the intramural ganglia of Meissner and Auerbach in the hindgut and resulting in functional intestinal obstruction. HSCR most commonly presents in isolated cases, although it can be familial and may be inherited as autosomal dominant or autosomal recessive, with reduced penetrance and male predominance (Badner et al. 1990; Passarge 1967). The *RET* proto-oncogene is considered a major susceptibility gene for HSCR (Lyonnet et al. 1993; Edery et al. 1994; Romeo et al. 1994; Chakravarti 1996). Depending on the referral 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 (Angrist et al. 1995; Attié et al. 1995; Eng 1996). The only population-based series, however, estimates the frequency of germline *RET* mutation in 69 unselected patients with HSCR to be 7%, and only 3% of isolated patients with HSCR in this population-based series had traditional germline *RET* mutations (Svensson et al. 1998). Several other putative HSCR susceptibility genes have been proposed for syndromic and nonsyndromic HSCR (Puffenberger et al. 1995; Angrist et al. 1996; Amiel et al. 1996, 2001; Auricchio et al. 1996; Edery et al. 1996; Bidaud et al. 1997; Doray et al. 1998; Pingault et al. 1998; Southard-Smith et al. 1998; Wakamatsu et al. 2001).

The molecular mechanism responsible for the majority of isolated patients with HSCR remains unknown. It has been suggested that the sporadic cases of HSCR might be accounted for by a model of polygenic inheritance (Bolk et al. 2000). We have found a significant over-representation of the *RET* A45A (nt c135G→A, exon 2) and L769L (nt c2307T→G, exon 13) intragenic sequence variants in our cohort of patients with sporadic HSCR compared to the control group ($P < .0006$) (Borrego et al. 1999). These findings were independently confirmed in a HSCR cohort of German origin (Fitze et al. 1999). It was proposed that the A45A and L769L polymorphisms could act as low-penetrance alleles and/or as factors modifying the phenotypic expression or even be in linkage disequilibrium with an unknown functional variant (Borrego et al. 1998, 1999, 2000; Fitze et al. 1999). We subsequently studied the haplotypes on the basis of the combination of the seven polymorphic variants in the *RET* coding region. The over-represented haplotypes in HSCR were the so-called haplotype B (which includes only the A45A polymorphism) and haplotype C (which includes A45A and L769L). Analysis of phase-known genotypes (i.e., paired haplotypes) indicated an over-representation of genotypes BB, BC, and CC in the HSCR series versus the control group. On the basis of these data, it was proposed that the association of these genotypes with

HSCR susceptibility occurs in an autosomal recessive manner in an additive dose-dependent manner (Borrego et al. 2000).

The existing data for HSCR suggest one of two hypotheses. Either the sequence variation representing each of the “at risk” haplotypes is itself functional and leads to low-penetrance loss of function, or the “at risk” haplotypes represent linkage disequilibrium with the actual low-penetrance susceptibility locus. There also appear to be consistent haplotypes containing S836S in patients with MTC, and the same two hypotheses hold as with HSCR: either the variant is functional, or these haplotypes are in linkage disequilibrium with a low-penetrance susceptibility locus. Because the haplotypes containing S836S in sporadic MTC never coincide with the HSCR “risk” haplotypes, we might postulate the existence of a single modifying/regulating locus of *RET* function where different specific alterations lead to opposing functional effects, conferring specific susceptibility to either MTC or HSCR. To begin testing these hypotheses, we sought to identify and characterize novel SNPs upstream of the earlier examined haplotypes and to use all of the data in haplotype analyses.

Material and Methods

Patients and Control Subjects

In the present work, we have included 103 patients affected with clinically sporadic HSCR, their unaffected parents, 51 patients affected with sporadic MTC, and 100 normal control subjects. Traditional germline MEN 2-defining *RET* mutations are absent in the 51 patients with sporadic MTC. HSCR and MTC cohorts have been described in our previous publications (Borrego et al. 1999, 2000; Ruiz et al. 2001). Normal control subjects were unselected, unrelated race-, age-, and gender-matched individuals from Spain. Informed consent was obtained in accordance with the approved protocols of the respective institutional review boards for the protection of human subjects.

Sequence Analysis of Intron 1 of RET

Genomic DNA was obtained from peripheral blood leukocytes using standard protocols (Dracopoli et al. 1994). To meticulously search for new SNPs within intron 1 of the *RET* proto-oncogene, we performed enzymatic mutation detection (EMD), beginning from 3' of the intron, on overlapping fragments of 300 bp to 1 kb in length, according to the manufacturer's recommendations (del Tito et al. 1998) (fig. 1A and table 1). When any putative DNA variant was detected, we proceeded to direct sequencing of the fragments, by use of conditions published elsewhere (Marcos et al. 2000), to characterize the changes observed.

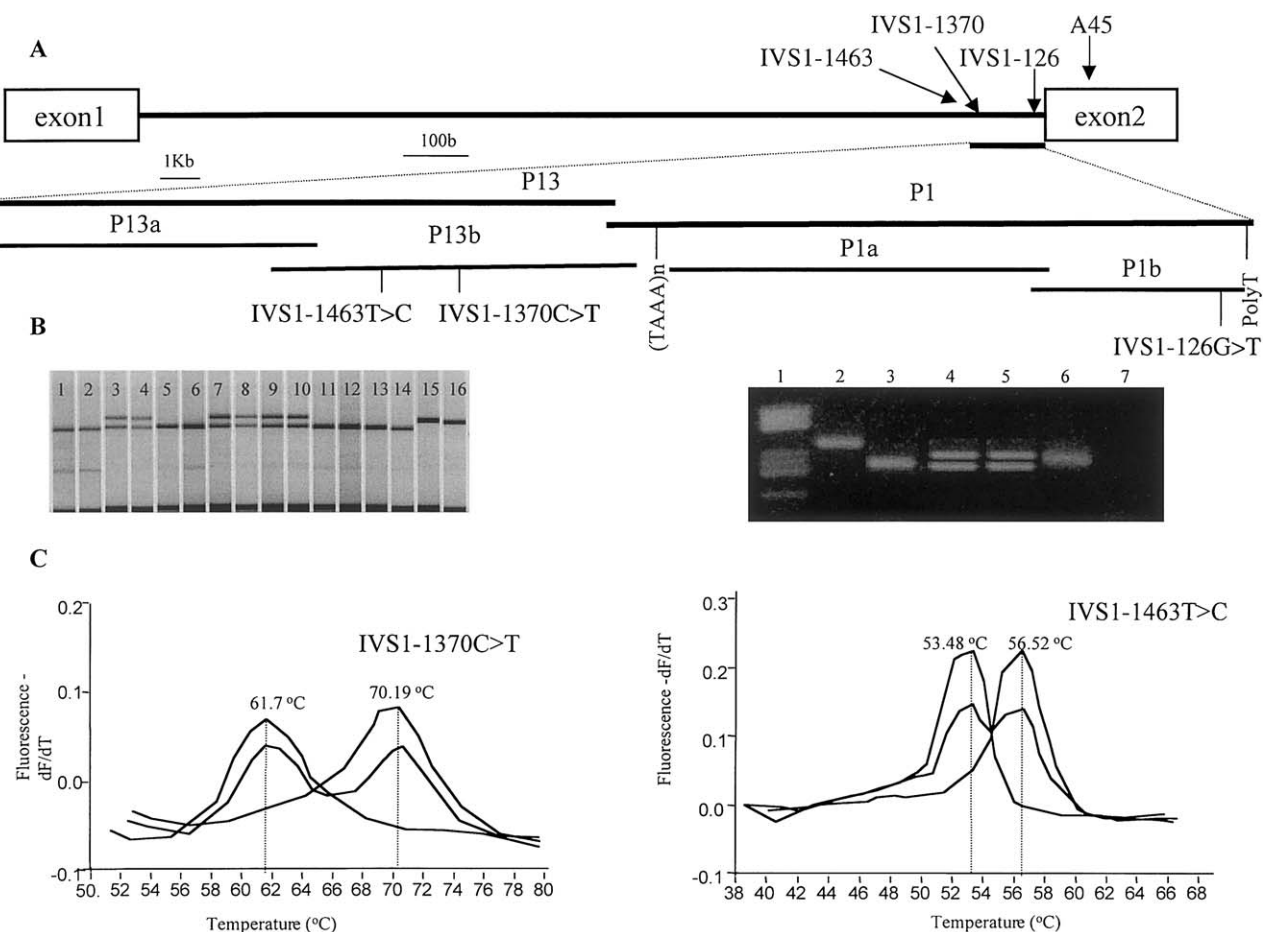


Figure 1 Characterization of novel SNPs in *RET* intron 1. *A*, Schematic representation on 3' end of intron 1 of the *RET* proto-oncogene. Codon 45 and the 3 IVS1 SNPs are indicated. *B*, *left panel*, genotyping of IVS1-126G→T by fluorescent SSCP. *Lanes 1, 2, 5, 6, and 11–14*, genotype *-/-*. *Lanes 3, 4, and 7–10*, genotype *+/-*. *Lanes 15 and 16*, genotype *+/+*. *Right panel*, genotyping of IVS1-126G→T by differential restriction with *Nla*III. *Lane 2*, a nondigested sample. *Lane 3*, genotype *-/-*. *Lanes 4 and 5*, genotype *+/-*. *Lane 6*, genotype *+/+*. *C*, FRET analysis of IVS1-1370C→T and IVS1-1463T→C. In each case, amplicons containing the polymorphisms have a melting point (MP) which is >9°C lower (in the case of IVS1-1370C→T) and 3°C higher (in the case of IVS1-1463T→C) than the amplicons with the wild-type sequence. Genotype *-/-*, *+/-*, and *+/+* are represented in red, blue, and green, respectively.

Genotyping of SNPs

The variant IVS1-126G→T was genotyped using fluorescent SSCP analysis, after digestion of the PCR product with *Msp*I (Roche), under conditions described elsewhere (Marcos et al. 2000). In addition, the results were confirmed by differential restriction digestion with *Nla*III (New England Biolabs), since this enzyme loses a restriction site in the presence of the variant (fig. 1B).

To detect IVS1-1370C→T and IVS1-1463T→C, we have developed an automated method using fluorescence resonance energy transfer (FRET) technology. Internal probes were designed according to the sequences of interest and were purchased from TIB Molbiol. The whole procedure, including real-time PCR and a melting curve, was performed in a LightCycler machine (Roche) (fig. 1C and table 2).

Phase of haplotypes was determined by typing of the corresponding parents of patients with HSCR and control subjects where available (86% of patients with HSCR and 65% of control subjects had parents available for typing [data not shown]). Haplotypes (i.e., phase) were similar in patients and control subjects, and, thus, haplotypes from patients with MTC were inferred from the haplotypes available in patients with HSCR and control subjects. The median age of sporadic MTC diagnosis is 55–60 years, and, hence, the parents of patients with MTC were, in general, deceased.

Statistical Analysis

Allelic frequencies of the three new *RET* polymorphic loci were determined, and haplotypes were constructed (tables 3 and 4). The frequencies of each haplotype were

Table 1**PCR Conditions of Individual Fragments within Intron 1 of *RET* Proto-Oncogene**

FRAGMENT	PRIMERS (5'→3')		SIZE (bp)	ANNEALING TEMPERATURE (°C)	CYCLES
	Forward	Reverse			
P1	M13F*-GTGGAAGTTGTGGTGAGCCAAG	M13R*-GTGGGAAGTAGGGAAGTGAGTGAG	1,057	67	35
P13	M13F-TGGCACAATCTCGGCTCACTAC	M13R-CTTGGCTCACCACAACCTCCAC	1,051	68	35
P1b	M13F-ATGACTTTCCTGTAAAGTGC	M13R-GGAGTTTTTCATCTCTGTTC	338	54	35
P1a	M13F-AGATAAGATGCACGGACCTTAG	M13R-GCAACAGTTGCCAAAAAATG	596	60	35
P13b	M13F-CCAGAAGTGGGATTGGTAG	M13R-TCACCACAACCTCCACCTC	597	59	35
P13a	M13F-GTGAATGGCACAATCTC	M13R-GAAAAACAAGAAGGTAGTCCC	552	58	35

NOTE.—Primers were designed using the PRIME command of GCG-Wisconsin software, and all of them had universal M13 primers attached at the 5' ends. M13F*: CGCCAGGTTTTCCAGTCACGAC; M13R*: TTTACACAGGAAACAGCTATGAC. Fragments P1 and P13 were each divided into two smaller fragments (a and b), to obtain better results in the performance of EMD technology. Fragments were overlapping, except for P1a and P13b, which were separated by the STR (TAAAn).

compared between the patients with MTC and control subjects, as well as between patients with HSCR and control subjects and between patients with MTC and patients with HSCR. Comparisons were performed using either χ^2 analysis with Yates's correction or, when appropriate, Fisher's two-tailed exact test. Nominal statistical significance was considered when $P < .05$.

Haplotype analyses were performed by reconstructing and comparing the transmitted versus nontransmitted haplotypes to patients with HSCR, with the understanding that meiotic recombination is negligible for a single generation across such a short region. This approach is consistent with the haplotype relative risk approach of Falk and Rubinstein (1987) and is largely robust to population stratification (described by Ott [1999], p. 293).

Full multipoint model-based analyses of a founding locus were performed using DMLE+ (Reeve and Rannala 2002), which uses Monte Carlo integration to approximate Bayesian posteriors for locus position and age. Calculations were performed on a Pentium 4 workstation, using recommended burn-in and sampling intervals and a variety of modeling assumptions and parameter ranges, including population growth rates. The composite likelihood ratio of DISMULT (Terwilliger 1995) was also computed, using a Unix workstation. Numerical difficulties in evaluating the likelihood over such short intervals was resolved by artificially inflating the map distances, while interpreting the mutation age as increased by the same proportion.

In addition to these approaches, simple haplotype methods were used to test against the null hypothesis of equal transmission probabilities. These included specification of putative ancestral haplotypes and permutation testing by drawing 10,000 random permutations of the transmission status of all haplotypes. For each permutation, the maximum nominal χ^2 statistic was recorded in the association of each haplotype (vs. all others combined) with transmission status. A somewhat more structured approach is described below.

The degree of linkage disequilibrium between disease

and a marker locus is often described as p_{excess} , which measures the proportion of alleles at a locus caused by the putative founding mutation (de la Chapelle and Wright 1998). If an allele is associated with disease (e.g., the polymorphic allele A at codon 45 (Borrego et al. 1999, 2000)), then we can compare the proportion of transmitted chromosomes which have the allele (p_{affected}) with the proportion in nontransmitted chromosomes (p_{normal}). From these, we can compute $p_{\text{excess}} = (p_{\text{affected}} - p_{\text{normal}})/(1 - p_{\text{normal}})$.

If there is a predominant ancestral haplotype, then the value of p_{excess} will tend to reach a maximum at markers very near the mutation and to descend as one moves away from the mutation. Moreover, the maximum value of p_{excess} (attained at the mutation locus) represents the proportion of transmitted chromosomes that contain the ancestral mutation. For a rare recessive disease, this can be, at most, 1, and for a rare dominant disease, it can be, at most, 0.5. The estimate of p_{excess} at A45A is ~ 0.45 (derived from the frequency estimates of Borrego et al. [1999]), which suggests that, if the mutation exists, it is responsible for a large portion of sporadic HSCR in this population. Often, p_{excess} will not achieve the theoretical maximum value because of the presence of additional founding mutations that account for some of the sporadic cases.

To generalize this approach to multiple markers, we used a method (Gao and Wright 1999) that examines haplotypes in varying window widths of successive SNPs to find the window in which haplotypes (all treated separately) are most strikingly associated with disease, as judged by a χ^2 statistic. The null distribution of this maximal statistic can be generated by recomputing it over permutation samples of transmission status. Also, we recorded, for each SNP, the single haplotype (over all window widths) most associated with transmission, which was then treated as if it were a single allele in a p_{excess} plot. The result is a plot of so-called h_{excess} (similar to p_{excess} , but with less error variation) for the excess of the haplotype among transmitted chromosomes as a

Table 2**FRET Conditions for Detection of the Polymorphisms within Intron 1 of *RET* Proto-Oncogene**

POLYMORPHISM GENOTYPED	PROBES ^a	MELTING CONDITIONS AFTER AMPLIFICATION		
		Temperature Transition (°C)	Transition Rate (°C/s)	Gains (F1:F2:F3)
IVS1-1370C→T	Anchor: 5'- YgCTCACTCAgCCACAgCCgAggC p Sensor: 5'- TgCACCgTgCCCCTgCTTg X	From 50 to 95	.40	1:45:30
IVS1-1463T→C	Anchor: 5'- TTTTgTgggAACTACCTTCTTgTTTTTCAT Sensor: 5'- Y-ACTgTATATTATTTTCCACTCACCgA p	From 40 to 95	.20	

NOTE.—We used 50 cycles of PCR at an annealing temperature of 66°C.

^a Boldface italics denote the nucleotides that are sensitive for the wild-type or the polymorphic allele. “X” represents fluorescein, and “Y” represents LC-Red 640. Some probes have modifications (represented by “p”) in their 3' that disable extension of *Taq* polymerase.

function of physical position, and it is similar to the multiple-SNP haplotype-based disequilibrium measure described by Daly et al. (2001). Under the assumption that the ancestral haplotype has been correctly identified, h_{excess} at the mutation also represents the proportion of transmitted chromosomes containing the mutation.

Finally, noting that expected linkage disequilibrium decays exponentially with increasing recombination fraction (see, e.g. Hastbacka et al. 1992), simple linear regression of $\log(h_{\text{excess}})$ on genomic position was performed, under the assumption that all transmitted chromosomes contain the ancestral mutation. This simple moment-based approach is based on our method for testing against a null hypothesis, but with no specification of the complicated dependencies arising under the alternative. Thus, it does not naturally give rise to CIs or precise inferential statements about the mutation age or location.

Results

Identification of Three Novel *RET* Intron 1 SNPs

Using a series of bioinformatics manipulations within GenBank and the National Center for Biotechnology Information (NCBI), we uncovered a 505-kb genomic contig on 10q23 that contained the entire *RET* sequence (GenBank accession number AC010864 [subfile of NT_033987]). Using the Blast 2 sequences tool at the NCBI Web site, we aligned the AC010864 sequence with those of exons 1 and 2 of the *RET* proto-oncogene described earlier. The result was a segment of contig AC010864 that contained the complete sequence of intron 1 of *RET* (23,127 bp). We were able to identify three novel SNPs within intron 1: IVS1-126 G→T, IVS1-1370 C→T, and IVS1-1463 T→C (fig. 1).

Studies of Association Between the Three Intron 1 SNPs and HSCR or MTC

Patients with HSCR versus control subjects.—The three newly identified intron 1 SNPs were found to be

under-represented in the HSCR cohort compared to the control population (table 3). For example, of a total of 206 HSCR chromosomes, there were 44 chromosomes (21%) with the T variant at the IVS1-126G→T locus, and 162 (79%) the wild-type G allele. The T variant was significantly under-represented when compared to the control series (χ^2 with Yates's correction 8.69, $P = .0032$). Similarly, the IVS1-1370C→T and IVS1-1463T→C polymorphic alleles (T and C, respectively) were also under-represented in the HSCR cohort compared to those observed in the control group (table 3; $P \ll .000001$).

Patients with MTC versus control subjects.—In contrast to the HSCR cohort, the IVS1-126G→T polymorphic allele was over-represented in patients with sporadic MTC. Of 102 MTC chromosomes studied, 49 (48%) carried the polymorphic T allele, and 53 (52%) carried the wild-type G ($\chi^2 = 4.28$; $P = .038$ with Yates's correction) (table 3). We did not find any statistically significant differences when comparing the allelic frequencies of the IVS1-1370C→T and IVS1-1463T→C loci between MTC and control subjects (table 3).

Patients with HSCR versus patients with MTC.—One highlight of the results obtained in the analysis of allele frequencies of the newly identified loci was the inversion in the frequency of IVS1-126G→T when comparing the series of patients with MTC to the HSCR series. The frequency observed for this marker in the control population appeared in a range intermediate between those observed in patients of both pathologies (T: 70 [35%]; G: 130 [65%]). In comparison of the allele distribution of the SNPs between cohorts (HSCR vs. MTC), it was remarkable to note an inversion of the allele frequencies for each genotyped marker (tables 3 and 4).

Haplotype Analysis Using Intron 1 SNPs: Evidence of Association of Haplotype 0 and HSCR, and Haplotype 2 with MTC

Using the information on the segregation of the SNPs

Table 3

Comparative Studies between the Groups, using χ^2 Analysis with Yates's Correction

	RESULTS OF COMPARISON					
	HSCR vs. Control		sMTC vs. Control		sMTC vs. HSCR	
	HSCR Alleles	Control Alleles	sMTC Alleles	Control Alleles	sMTC Alleles	HSCR Alleles
IVS1-126G→T:						
No. of polymorphic T alleles	44	70	49	70	49	44
No. of wild-type G alleles	162	130	53	130	53	162
χ^2 with Yates's correction (<i>P</i> value)	8.69 (<i>P</i> = .0032053)		.28 (<i>P</i> = .0385776)		21.79 (<i>P</i> = .000003)	
IVS1-1370C→T:						
No. of polymorphic T alleles	39	94	40	94	40	39
No. of wild-type C alleles	167	106	62	106	62	167
χ^2 with Yates's correction (<i>P</i> value)	35.03 (<i>P</i> < .000001)		1.36 (<i>P</i> = .2438972)		13.67 (<i>P</i> = .0002175)	
IVS1-1463T→C:						
No. of polymorphic C alleles	84	163	89	163	89	84
No. of wild-type T alleles	122	37	13	37	13	122
χ^2 with Yates's correction (<i>P</i> value)	68.94 (<i>P</i> < .000001)		1.23 (<i>P</i> = .2674883)		57.99 (<i>P</i> < .000001)	

identified in HSCR patients and their parents, we performed a construction of haplotypes comprising different combinations of the three SNPs at the 3' end of *RET* intron 1 (table 5). The method and the group of patients used for the haplotype construction was similar to that described elsewhere (Borrego et al. 2000). We found a total of five haplotypes in the patients with HSCR and their parents (haplotypes 0–4). As the recombination events between the markers under study were predicted to be extremely rare (distance between SNPs <1,200 bp), we considered each haplotype as an individual allele within the same locus (3' intron 1, *RET* proto-oncogene). Parents of MTC or control subjects were not available, so the haplotype construction in both groups was inferred from the haplotype of the HSCR patients. The haplotype distribution observed in the MTC, control, and HSCR groups was different. Thus, haplotype distribution of each cohort compared to the control group (MTC vs. control subjects or HSCR vs. control subjects), was found to be statistically significant (*P* < .0023) (fig. 3).

We verified that haplotype 0 was the most common in the HSCR group (59%) (fig. 3). It is important to note that haplotype 0 is in complete linkage disequilibrium with all the risk haplotypes described so far (Borrego et al. 1999, 2000). That is, all the risk haplotypes share the same sequence of SNPs in this genomic segment of intron 1 (haplotype 0). This is consistent with our studies of linkage disequilibrium mapping and would place the locus of susceptibility to HSCR at ~20 kb from the A45A marker (see below as well).

Haplotype 2 is the most frequent in patients with sporadic MTC (48%) (haplotype that contains IVS1-126G→T and IVS1-1463T→C in disequilibrium), whereas the frequency of haplotype 0 is notably low (12.7%). The MTC patients that carry S836S also carry intron 1 haplotype 2, which suggests that S836S and haplotype 2 are in linkage disequilibrium. These data have been confirmed

in three patients with MTC who were homozygous for haplotype 2 and heterozygous for S836S.

The haplotypes observed in the control series show a distribution intermediate to that observed in the MTC and HSCR cohorts. Haplotype 1 was the most common in the control subjects (43%). Furthermore, haplotype 4, which is not represented in any of the patient series being studied, appears with a certain frequency (4%). Haplotype 0 has a frequency of 18.5% in control subjects, compared with 59% in patients with HSCR and 12.7% in patients with MTC. The frequency of haplotype 2 in control subjects was 31%, compared with 21.3% in individuals with HSCR and 48% in individuals with MTC. These observations suggest that haplotype 0 could be a haplotype associated with HSCR, and haplotype 2, a haplotype associated with MTC (fig. 3; also see below).

Analysis of the IVS1 Genotypes in HSCR and MTC

We studied the genotype composition (pairs of hap-

Table 4
Polymorphic Frequencies in Patients with sMTC, Control Subjects, and Patients with HSCR

ALLELE	FREQUENCY IN		
	Patients with sMTC	Control Subjects	Patients with HSCR
IVS1-126G→T:			
Polymorphic T	.48	.35	.21
Wild-type G	.52	.65	.79
IVS1-1370C→T:			
Polymorphic T	.39	.47	.21
Wild-type C	.61	.53	.79
IVS1-1463T→C:			
Polymorphic C	.9	.82	.41
Wild-type T	.1	.18	.59

Table 5**Haplotypes Based in the Combination of Three SNPs Located within Intron 1 of *RET***

HAPLOTYPE	PRESENCE/ABSENCE OF		
	IVS1-126G→T	IVS1-1370C→T	IVS1-1463T→C
0	–	–	–
1	–	+	+
2	+	–	+
3	–	–	+
4	+	+	+

NOTE.—+ = present; – = absent.

lotypes) in each study group (fig. 3). Genotypes 00 and 02 predominate in HSCR (70%), whereas, in MTC, genotypes 12 and 22 are more predominant (67%). In the control group, the most common genotype was 12, followed by 11 and 22. Analysis of the genotypes observed/expected in the study groups shows that the results are in Hardy-Weinberg equilibrium. If we compare the distribution of genotypes between both study groups (MTC and HSCR), the deviation is rather striking (fig. 3). As a whole, these findings corroborate the observations made in individual allele as well as haplotype frequency studies.

*Haplotype Analysis Using All 10 *RET* SNPs: Evidence for a Novel Founding Locus for HSCR*

Using the data from the same consecutive incident cases of isolated HSCR from the Western Andalusia region described in Borrego et al. (1999, 2000), we have analyzed the observed *RET* haplotypes and their association with HSCR for evidence of a mutation or variant contributing to sporadic cases of disease. This offers a potentially more parsimonious explanation for the linkage disequilibrium than the hypothesis that the polymorphisms in *RET* have subtle functional effects.

We analyzed the transmitted versus nontransmitted haplotypes using DMLE+ (see the “Material and Methods” section), which is a fully model-based approach for linkage disequilibrium mapping. The results were difficult to interpret, as the posterior for mutation location was extremely short (<1% of allowed range) and always placed at the extreme 5' end of the allowed range. Mutation age estimates were very highly sensitive to starting values, despite long burn-in times for DMLE+ and ranged from 200 to 1,500 generations, under the assumption of an average correspondence of 1 cM = 1 Mb. We speculate that the difficulty in obtaining estimates stems from the failure of the SNPs to span the mutation, in contrast to classic examples used to evaluate the approach (e.g., diastrophic dysplasia [Reeve and Rannala 2002]). The program DISMULT achieved maximum-likelihood values at or just upstream of the most

5' SNP IVS1-1463, with estimated mutation age (see the “Material and Methods” section) sharply changing from 4,100 to 8,000 generations in a short interval having high likelihood, and with estimated proportion of the mutation, among transmitted chromosomes, of ~0.45.

Our haplotype-based linkage-disequilibrium analysis is in rough agreement with the DISMULT results, except that we believe the data do not preclude the possibility that a mutation may exist further upstream of the describe *RET* polymorphisms (5' direction, centromeric).

The h_{excess} plot in figure 2 (see the “Materials and Methods” section) is maximized at ~0.45, at the most 5' SNPs, and reveals that the linkage disequilibrium descends toward the 3' end of *RET*. The permutation-based empirical *P* value for the association of ancestral haplotype with transmission status was <.0001. The fact that the linkage disequilibrium levels off for a few markers at a time is expected—this reflects that historical crossovers occurred at only a few points across *RET*.

Most compelling is the fact that the “most significant” short haplotypes at each marker position (i.e., in χ^2 tests of association with disease) are consistent with a single, longer ancestral haplotype. The ancestral haplotype for the 10 SNPs appears to be (5'→3') WWMWW-WMWW, where M signifies mutant and W wild type. This is haplotype C from the Borrego et al. (2000) study, with the addition of the three new SNPs from intron 1. Haplotype MWMWMMMM is associated with disease (9 transmitted vs. 0 nontransmitted) but may be part of a different minor founding mutation.

The maximum value that the linkage-disequilibrium measure attains is not known and awaits further determination of SNPs in intron 1 and upstream of *RET*. An exponential fit to the decaying linkage-disequilibrium measure (which cannot exceed 1.0) in figure 2 (*dashed line*) suggests that the major mutation is likely to reside in intron 1 or just upstream of *RET*.

A recent refined genetic map indicates that the rate of recombination per unit of physical distance in the vicinity of *RET* is ~0.8 cM/Mb (Kong et al. 2002). Combining this information with the fitted rate of decay results in an estimated time from the founding mutation of 3,800 generations. Such a surprisingly ancient estimate must be interpreted with caution, as the recombination rate and mutation age are confounded, and the resolution of such recombination rate estimates remains low. Moreover, although linkage disequilibrium is clearly present, the current data do not support the construction of plausible CIs. Nonetheless, the data are consistent with a founder mutation old enough to be widespread in European populations. This is further supported by the report from Fitze et al. (1999), in which genotypes of the same seven polymorphisms studied earlier by us (Borrego et al. 2000) display a very similar

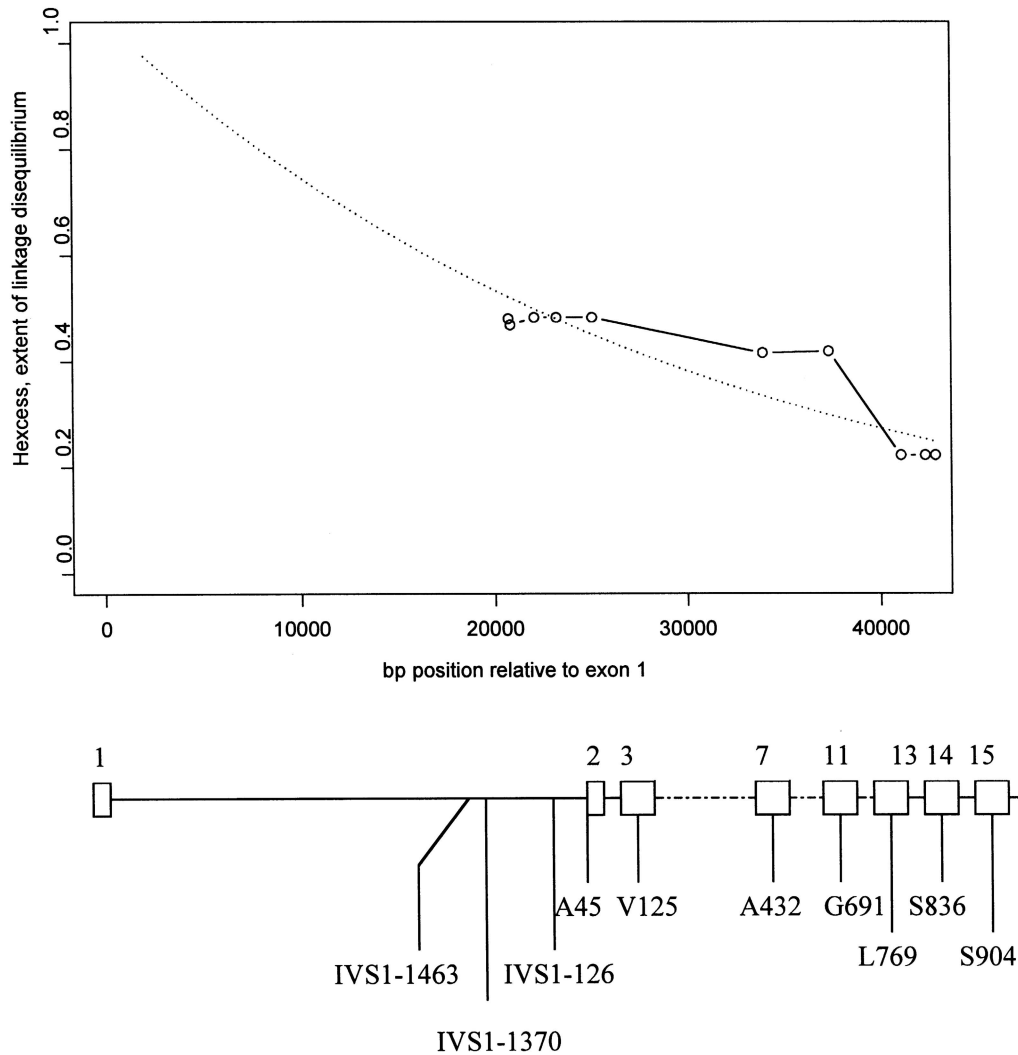


Figure 2 Excess proportion of ancestral haplotypes among transmitted chromosomes to HSCR patients (see text). The fitted line of expected linkage disequilibrium suggests that the ancestral mutation lies in or near the 5' region of *RET*—that is, within intron 1. A schematic of partial *RET* gene structure on which the positions of the 10 SNPs are placed lies below the plot. Numbers on top of the gene schematic are exon numbers, and the codes below represent the nature and position of the SNPs.

pattern of association with sporadic HSCR in a German population.

Discussion

Traditional germline mutations identified in the *RET* proto-oncogene have been associated with two different neurocristopathies (MEN 2 and HSCR). The phenotype observed in one is completely different from that in the other (for a review, see MIM 142623 and MIM 164761), and the molecular mechanism proposed for the two cases seem to be opposites, according to the available functional data. Mutations causing MEN 2 produce gain-of-function alterations in the cascade of RET signals,

either because of constitutive activation or alteration of substrate specificity (Santoro et al. 1990; Songyang et al. 1995). In patients with HSCR, traditional *RET* mutations typically result in loss of function, including structural and functional haploinsufficiency and a deficit in the cascade of RET signals in the target tissues (Pasini et al. 1995; Pelet et al. 1998). It has recently been proposed that the mechanism of some HSCR mutations could consist of an activation of cryptic proapoptotic functional motifs in the altered RET protein that lead to premature apoptosis (Bordeaux et al. 2000). The observation that the RET receptor is involved both in tumorigenesis and in the development of the nervous system is reminiscent of that observed for the p75^{NTR} and

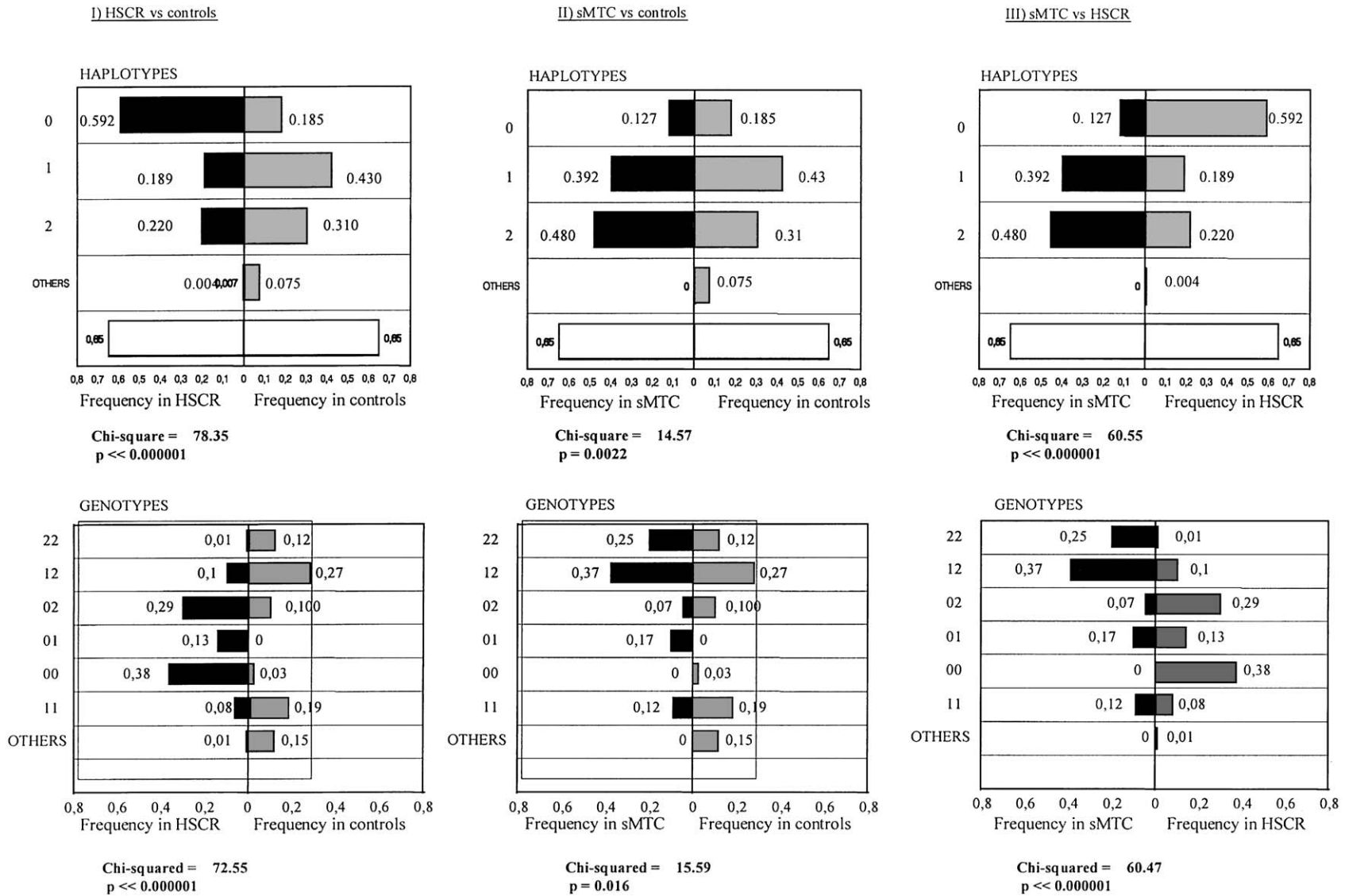


Figure 3 Comparison of the frequencies and distribution of *RET* IVS1 haplotypes and genotypes between HSCR or patients with MTC and control subjects. χ^2 (Yates's correction) and *P* values are denoted below each comparison.

DCC receptors, in which the proapoptosis mechanism seems similar to that observed in both cases (Rabizdeh et al. 1993; Mehlen et al. 1998).

In contrast to the traditional mutations described for a subset of HSCR and the majority of MEN 2, the isolated forms of both pathologies—namely, MTC and HSCR—appear to be attributable to low-penetrance alleles, the downstream functional mechanism of which is unknown. These alleles have been identified using common polymorphisms throughout the *RET* proto-oncogene sequence and studying genetic association or disequilibrium of transmission in families affected with HSCR with incomplete penetrance (Borrego et al. 1998, 1999, 2000; Fitze et al. 1999; Gimm et al. 1999; Bolk et al. 2000; Griseri et al. 2000). Initially, direct action mechanisms of the linked coding *RET* coding SNPs, such as the activation of cryptic splicing sites or altered expression of the allele with the variant (producing over- or underexpression of the *RET* proto-oncogene) were proposed. As an alternative, the mechanism was also suggested to be the preferential use of specific tRNAs that reduced the efficiency of the translation of the allele that carried the variant, and the alteration of functional motifs of the *RET* sequence which constituted the targets for DNA- or RNA-binding proteins. Although some functional studies have been performed on these polymorphisms, no alteration has yet been described that explains the direct effect of any of the SNPs associated with both pathologies (Griseri et al. 2000). Besides the hypothesis of direct action of the markers linked to each pathology, a mechanism related to the existence of linkage disequilibrium of some functional allele in noncoding sequence with the markers studied has also been considered (Borrego et al. 1998, 1999, 2000; Fitze et al. 1999; Gimm et al. 1999; Bolk et al. 2000; Griseri et al. 2000).

Together with our previous observations (Borrego et al. 1999, 2000), our current observations indicate that the A45A polymorphism, irrespective of the 3' haplotype, is in linkage disequilibrium with a group of markers within intron 1 of the *RET* proto-oncogene (haplotype 0-A45A). In other words, the under-representation of the variant alleles at all three new loci within intron 1 described in the HSCR cohort can be explained by the polymorphic variant at codon 45 (A45A, nt c135G→A) to be in complete linkage disequilibrium with the wild-type allele (G) at IVS1-126G→T, the wild-type allele (C) at IVS1-1370C→T and the wild-type allele (T) at IVS1-1463T→C. More importantly, our statistical analysis of all 10 *RET* SNPs in HSCR (see the last subsection under “Results”) strongly suggest the existence of a low-penetrance locus of susceptibility for HSCR at a distance of <20 kb from *RET* codon 45, perhaps still within intron 1.

In contrast to isolated HSCR, individuals with spo-

radic MTC, originating in Germany and Spain, were shown to have an over-representation of S836S (Gimm et al. 1999; Ruiz et al. 2001). Similarly, the same marker has been attributed with a protector effect against the appearance of the HSCR phenotype (Griseri et al. 2000, 2002). Whether the sequence variation in and of itself conferred a subtle effect on protein expression, splicing, or function remains to be elucidated. Like the association of specific *RET* haplotypes with isolated HSCR, the existence of a functional locus in linkage disequilibrium with S836S has also been suggested as an alternative to the hypothesis of the direct effect of S836S (Gimm et al. 1999; Griseri et al. 2000, 2002; Ruiz et al. 2001). This latter hypothesis and those that invoke expressional variation are not mutually exclusive, as the function of the linked upstream putative susceptibility locus has yet to be elucidated. Thus, our current observations shed some light to differentiate amongst these hypotheses. We have noted that there is a MTC-specific risk haplotype within intron 1: haplotype 2, which is always associated with S836S, and both haplotype 2 and S836S are concurrently over-represented among our isolated MTC series. Our current data, therefore, strongly suggest that the association of S836S with MTC can be most plausibly explained by linkage disequilibrium between S836S and haplotype 2. Further, preliminary germline intron 1 haplotyping data in the MTC cohort of German origin show a high frequency of homozygosity for haplotype 2 (data not shown). This suggests the existence of a low-penetrance susceptibility locus for MTC in close proximity to that for isolated HSCR, within intron 1 of the *RET* proto-oncogene.

It is tempting to speculate about the nature of the susceptibility loci that could be located within the immense intron 1 of the *RET* proto-oncogene. Perhaps the most plausible hypothesis would be the existence of two independent mutations within the same functional motif—unknown at present—that would control the transcriptional activity of the *RET* proto-oncogene. Little is known about the transcriptional factors that bind to the putative *RET* promoter. For example, there is in vitro evidence that RAF-1, PAX3, SOX10, AP1, and AP2 could act as transcriptional factors that bind upstream of exon 1 (Carson et al. 1995; Capes-Davis et al. 1999; Lang et al. 2000). Using the MatInspector program (Quandt et al. 1995), bioinformatic analysis of the region harboring the three novel SNPs predicts four motifs that could be binding sites for transcription factors BRN2, NFAT, IRF1, and IRF2 at IVS1-1463T (wild type; HSCR-associated). When IVS1-1463 is altered to the polymorphic C (which is MTC-associated), the BRN2 site is obliterated and a new site for OCT1 appears. Furthermore, the two IRF sites are strengthened, whereas the NFAT site is weakened, in the presence of the variant. Similarly, this program also predicts

that when the sequence is wild-type (G, HSCR-associated) at IVS1-126, there is the motif for a TCF1-binding site. When this sequence is variant, T (MTC-associated), an NFAT motif is created. It is interesting there is no difference at IVS1-1370 (both wild type) between haplotypes 0 (HSCR) and 2 (MTC), and no binding motifs are predicted at this site. Unfortunately, no in vitro or in vivo data exist regarding the plausibility of these binding motifs and actual binding sites within *RET* intron 1. To date, such bioinformatic mining for transcription binding sites has yet to yield functionally significant motifs for any gene in vivo. Therefore, further experimental work is required to prove the functional significance of these transcription factor binding sites predicted from bioinformatic analysis.

In summary, our data demonstrate that isolated MTC and HSCR are significantly associated with specific linked haplotypes (haplotypes 2 and 0, respectively) within *RET* intron 1. We believe that the data strongly suggest that each haplotype is likely to be in linkage disequilibrium with its respective putative low-penetrance susceptibility locus and that both loci may reside in a very small genomic segment (<30 kb). These loci might not only act in and of themselves as low-penetrance susceptibility loci for HSCR or MTC but would likely interact with other variants and with traditional germline HSCR-associated or MEN 2-associated mutations to modulate development of features, age at onset, and the like, as suggested by our prior, preliminary observations (Borrego et al. 1998).

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/index.html> (for *RET* sequence [accession number AC010864])
 NCBI, <http://www.ncbi.nlm.nih.gov/> (for Blast 2 sequences tool)
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for HSCR [MIM 142623] and MEN 2 [MIM 164761])

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