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Hirschsprung's disease and variants in genes that regulate enteric neural crest cell proliferation, migration and differentiation

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Abstract

Hirschsprung's disease (HSCR) results from failed colonization of the embryonic gut by enteric neural crest cells (ENCCs); colonization requires RET proto-oncogene (*RET*) signaling. We sequenced *RET* to identify coding and splice-site variants in a population-based case group and we tested for associations between HSCR and common variants in *RET* and candidate genes (*ASCL1*, *HOXB5*, *L1CAM*, *PHOX2B*, *PROK1*, *PROKR1*) chosen because they are involved in ENCC proliferation, migration, and differentiation in animal models. We conducted a nested case-control study of 304 HSCR cases and 1 215 controls. Among 38 (12.5%) cases with 34 *RET* coding and splice-site variants, 18 variants were previously unreported. We confirmed associations with common variants in *HOXB5* and *PHOX2B* but the associations with variants in *ASCL1*, *L1CAM*, and *PROK1* were not significant after multiple comparisons adjustment. *RET* variants were strongly associated with HSCR (*P* values between 10^{-3} and 10^{-31}) but this differed by race/ethnicity: associations were absent in African-Americans. Our population-based study not only identified novel *RET* variants in HSCR cases, it showed that common *RET* variants may not contribute to HSCR in all race/ethnic groups. The findings for *HOXB5* and *PHOX2B* provide

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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supportive evidence that genes regulating ENCC proliferation, migration, and differentiation could be risk factors for HSCR.

Keywords

congenital abnormalities; enteric nervous system; Hirschsprung disease; RET

INTRODUCTION

Hirschsprung's disease (HSCR; MIM# 142623) is the congenital absence of ganglion cells in the submucosal and myenteric plexi of the gut.[1] The length of the aganglionic segment is variable,[2] and in 70% of cases, HSCR is an isolated trait.[3] Overall prevalence of HSCR is estimated at 1/5 000 live births.[3] HSCR is a multifactorial disorder exhibiting non-Mendelian inheritance and low, sex-dependent penetrance with male preponderance.[4] The high recurrence among siblings and the occurrence of HSCR as part of the phenotype of various syndromes suggest the importance of genetic factors.[1,4]

RET proto-oncogene (*RET*), which encodes a receptor tyrosine kinase, is the main gene implicated in HSCR.[5,6] Approximately 50% of familial cases and 7–35% of non-familial cases have loss-of-function germline *RET* mutations.[7,8] Common variants in the *RET* promoter (rs10900296; rs10900297), at a *SOX10* binding site in intron 1 (rs2435357), and in exon 2 (rs1800858; c.135G>A; p.A45A) have also been associated with HSCR,[9,10] suggesting that common as well as rare variants might influence the occurrence of HSCR.

HSCR is attributed to impeded migration of enteric neural crest cells through the embryonic hindgut between weeks 5–12 of gestation.[11,12] Animal studies indicate that the GDNF-GFRA1-RET signaling pathway (in which RET forms a ligand/receptor complex with one of its ligands, GDNF, and its co-receptor, GFRA1) is important to the survival, proliferation, and migration of enteric neural crest cells in the developing gut.[11,13,14] Other genes may also be involved. Knockdown of the transcription factor achaetescute complex homolog 1 (Drosophila) (*Ascl1*) in mice embryos retards the differentiation of myenteric neurons in the intestine.[15] Disruption of the transcription factors, homeobox B5 (*Hoxb5*) and paired-like homeobox 2b (*Phox2b*), and the L1 cell adhesion molecule (*L1cam*), results in the delay or failure of migration of enteric neural crest cells to the distal intestine in mice embryos.[16–18] In cell culture, *Prok1*, which encodes the secreted protein prokineticin 1, induces enteric neural crest cell proliferation and differentiation; this effect on proliferation is eliminated by knockdown of its receptor, Prokr1.[19]

Given the potential importance of common genetic variants in HSCR, and the failure to identify disease-causing rare mutations in most non-familial HSCR cases, our objective was to examine associations between HSCR and single nucleotide polymorphisms (SNPs) in candidate genes (*ASCL1*, *HOXB5*, *L1CAM*, *PHOX2B*, *PROK1*, *PROKR1*) for which there is evidence of a role in the proliferation, migration, and differentiation of enteric neural crest cells. We also investigated differences in the associations between selected *RET* SNPs and HSCR by race/ethnicity because such differences might exist but have received little attention.

MATERIALS AND METHODS

Subjects

This was a population-based, nested case-control study that included HSCR cases born from 1998 through 2005 and identified from the New York State Congenital Malformations

Registry. Physicians and hospitals are mandated by law to report birth defect cases that come to their attention if the child is under two years of age and was born, or resides, in New York State. Cases had to have at least one British Pediatric Association code for HSCR (751300, 751310, 751320, 751330) in the registry records. There were 420 live-born HSCR cases among 2 023 083 resident live births (1 case per 4 817 live births) in New York State from 1998–2005. Thirty-two (7.6%) HSCR cases with chromosomal anomalies (all Down syndrome) and 81 (19.3%) cases with other major congenital malformations were excluded. The remaining 307 cases had HSCR as their only major congenital malformation (isolated HSCR cases); one HSCR case was subsequently excluded because of missing data on maternal race/ethnicity. A random sample of controls was frequency-matched to HSCR cases by race/ethnicity at a control:case ratio of 4:1, yielding 1 216 controls. Controls had no congenital malformations and were selected from the New York State Newborn Screening Program's records for the birth years 1998–2005.

New York State birth certificates were obtained for all study subjects and were linked to the records of the New York State Newborn Screening Program for retrieval of archived residual dried blood spots. One case could not be matched, and another case and one control were mismatched. After exclusion of these subjects, 304 cases and 1 215 controls remained.

We considered the possibility that monozygous twins discordant for HSCR might have genetic differences that result in one twin, but not the other, being affected with HSCR. Therefore, the unaffected siblings from the same gestation as HSCR cases (12 twin and 2 triplet sets) were also included to permit comparison of genetic data between monozygous twin pairs discordant for HSCR. Data from unaffected siblings were not used in statistical analyses.

After records were matched and biological specimens were processed, the specimens and associated data were made anonymous. This study was approved by the Institutional Review Board of the New York State Department of Health and reviewed by the Office of Human Subjects Research at the National Institutes of Health.

DNA extraction

DNA was extracted from 3 mm-diameter segments punched from the dried blood spots. Extraction involved the removal of cellular debris and DNA precipitation with sodium hydroxide.

Identity testing

Births from the same gestation were tested for zygosity by genotyping one sex marker and 13 short tandem repeat loci using the AmpFlSTR COfiler and Profiler plus polymerase chain reaction (PCR) amplification kits (Applied Biosystems, Foster City, CA, USA). Four pairs of monozygous twins (all male) discordant for HSCR were identified.

RET Sequencing

RET exons and flanking regions in introns were sequenced for all 304 cases and the four unaffected siblings of monozygous twin pairs discordant for HSCR (conditions and primers[20–23] described in Supplementary Information and Supplementary Table 1). Sequencing was also performed for 10 randomly selected controls to assess *RET* sequence diversity among unaffected individuals and to check that there were no systematic sequencing errors among cases. In addition, exon 1 of *RET* was sequenced for all controls to obtain genotypes for the rs10900296 and rs10900297 promoter SNPs. We used GenBank reference sequence NG_007489.1 for genomic DNA and NM_020975.4 for cDNA. Nucleotides were numbered with +1 representing the A of the ATG translation initiation

codon (codon 1) of the reference cDNA sequence. The bioinformatic tools, PolyPhen-2 and SIFT, were used to predict the effects of novel *RET* missense variants.[24,25] Human Splicing Finder was used to predict the effects of novel variants on mRNA splicing.[26]

Genotyping

Thirty-seven haplotype-tagging SNPs in the six candidate genes were genotyped (listed in Supplementary Table 2). SNPs with a minor allele frequency of ≥0.1 and r^2<0.8 were selected based on the HapMap European, Chinese, Japanese and Yoruban populations to permit representation of genetic variation in the race/ethnic groups that make up the study population. In addition to the two exon 1 SNPs, five SNPs in *RET* were genotyped (listed in Supplementary Table 2). The seven *RET* SNPs were chosen because they had been reported to be associated with HSCR.[9,27,28] Whole-genome amplification and genotyping of DNA was performed by KBiosciences (Herts, UK) (conditions described in Supplementary Information).

Tests for deviation from Hardy-Weinberg equilibrium (HWE) were performed for all 44 SNPs, separately for cases and controls and stratified by race/ethnicity within each group, considering adjustment for multiple comparisons using the Bonferroni method (352 tests: P<0.00014). In non-Hispanic white cases, *PROKR1* rs6722313 and *RET* rs10900296, rs1864410, rs2435357, and rs1800858 were not in HWE. In non-Hispanic white and Hispanic controls, *PROKR1* rs6722313 was not in HWE and was excluded from further analyses. No deviations from HWE were observed for other race/ethnic groups. The lack of HWE for selected *RET* SNPs in cases has been described in other reports that have examined their association with HSCR,[28,29] and is expected because of the strong relationship between *RET* and HSCR.

For each race/ethnic group, linkage disequilibrium (LD) measures were estimated using Haploview based on the genotypes of controls.[30]

Statistical analysis

The main statistical analysis included 1 215 controls and 301 unrelated, isolated cases. The case group comprised the older sibling from each of three case sibling pairs (from different gestations) and 298 unrelated cases. Data on maternal and infant characteristics were obtained from the birth certificates and compared between case and control groups using Fisher's exact test. Characteristics that could be biologically relevant to birth defects and that had P values <0.1 in bivariate analyses were included as covariates in regression models; because infant sex was not considered to be a cause of birth defects, it was not included as a covariate in the models. Logistic regression was used to compare genotype distributions between cases and controls and to estimate odds ratios (OR) and 95% confidence intervals (CI). Homozygosity for the major allele was the reference group with which being heterozygous and being homozygous for the minor allele were compared. Analyses were performed for the overall group of study subjects, and separately by race/ethnic group. Analyses involving all case and control infants were adjusted for race/ethnicity. Subjects whose race/ethnicity was categorized as 'other' were not analyzed separately because of small numbers.

Additional analyses included the younger case sibling from each of the three sibling case pairs; generalized estimating equations were used to account for the relatedness between siblings. Statistical analyses were performed using SAS software, version 9.2 (SAS Institute, Cary, NC, USA).

Haplotype analyses were performed using HPlus software (http://cdsweb01.fhcrc.org/HPlus/); these analyses involved only unrelated individuals and included the same covariates

as the genotype analyses. The most frequent haplotype among controls was used as the reference for calculating odds ratios and 95% CI. Only haplotypes with a frequency >0.01 among cases or controls were considered in the analyses. Genotype and haplotype analyses involving SNPs in *L1CAM*, a gene on the X chromosome, were performed for males and females separately.

All analyses were repeated excluding subjects with rare *RET* variants and restricting to singleton births to determine whether these factors influenced the results. The Bonferroni method was used to adjust for multiple testing (43 tests; $P<0.0012$).

RESULTS

Case mothers were more likely than control mothers to be multiparous (Table 1). The two groups did not differ significantly by maternal age, race/ethnicity, education, maternal diabetes, use of *in vitro* fertilization or other assisted reproductive techniques, plurality, or birth year. There were more males among cases than among controls; the sex ratios were 2.46 and 1.07 for the case and control groups, respectively.

RET coding and splice-site variants

A *RET* coding or splice-site variant was present in 38 (12.5%) of 304 cases; the variants were heterozygous in 37 of the 38 cases. Thirty-four cases had one variant each and four cases had two variants each. In all, 32 different coding and two different splice-site variants were observed (Table 2). We searched for these variants in databases of genetic variants and in previous reports[7,31–36] to determine whether any were novel. The databases included the Human Gene Mutation Database (www.hgmd.cf.ac.uk) , the Multiple Endocrine Neoplasia type 2 RET proto-oncogene database,[37] dbSNP (www.ncbi.nlm.nih.gov/projects/SNP), 1000 Genomes (www.1000genomes.org), and the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project database (http://evs.gs.washington.edu/EVS/).[38] There were 17 coding variants and one splice-site disruption variant that have not been previously reported; each was observed in only one individual. Twenty of the 27 missense variants are predicted by PolyPhen-2 or SIFT or both to disrupt protein function. The nonsense and frameshift variants are potentially damaging, as well as the c.1759+1G>A and c.1879+1G>A variants located at the first base pair of introns and predicted by Human Splicing Finder to disrupt a splice site. Of the 16 previously reported variants, nine (p.L56M, p.A386V, p.G446R, p.L452I, p.Y791F, p.V804M, p.P841L, p.R886Q, p.R982C) were present in the NHLBI Exome Sequencing Project database. Variants in this database were identified by sequencing exomes in 5 379 DNA samples obtained from European-American and African-American individuals that had participated in large epidemiological studies.[38] In the database, the minor allele frequency was 1.7% for p.R982C but was less than 1% for the other eight variants. This indicates that the minor alleles of these nine variants are likely to be rare in the general population.

RET variants in controls, non-twin siblings, and monozygous twins

Of the 10 controls sequenced for *RET*, only one had a coding variant and none had a splice-site variant. The coding variant (c.1465G>A; p.D489N) has been reported previously (dbSNP rs9282834) and is predicted to be benign by PolyPhen-2 and SIFT. This variant was not observed in any of the Hirschsprung's disease cases.

RET missense, nonsense, frameshift, and splice-site variants were not observed among the three pairs of case siblings (from different gestations). However, the siblings from one pair were both heterozygous for the previously unreported c.654G>A (p.P218P) variant which is

predicted by Human Splicing Finder to generate a cryptic splice site. This variant was also observed in 10 other cases.

There were no differences in either *RET* coding sequences or genotypes for the common variants in *RET* and the candidate genes between monozygous twins (N = 4 pairs) discordant for HSCR. One pair had the p.Y146H variant which has been reported previously and is predicted to be benign by PolyPhen-2 and SIFT. This pair also had the c.654G>A (p.P218P) variant.

Case characteristics according to presence of *RET* coding and splice-site variants

Race/ethnicity, sex, and other characteristics for HSCR cases with (N=38) and without (N=263) *RET* coding and splice-site variants are shown in Table 1. There were no statistically significant differences between controls and the cases with *RET* coding and splice-site variants. Cases in whom these *RET* variants were absent were more likely than controls to have mothers who were multiparous and smoked during pregnancy. Both groups of cases had more males than females but the comparison with controls was only statistically significant in the group without *RET* variants. We also calculated minor allele frequencies for the 43 SNPs in *RET* and the six candidate genes, and compared them between the two groups of cases (Supplementary Table 3). We found no comparisons that remained statistically significant after adjustment for multiple testing.

Associations with *RET* SNPs by race/ethnicity

Table 3 presents odds ratios and 95% CI for the associations between HSCR and *RET* SNPs. Having at least one copy of the minor allele of six of the seven *RET* SNPs was associated with HSCR in study subjects overall, and in non-Hispanic white, Hispanic, and Asian subgroups (Table 3). The strongest associations were observed for having two copies of the minor allele of rs10900296, rs1864410, rs2435357, and rs1800858: all odds ratio point estimates were >10 and *P* values ranged between 10^{-3} for the smallest subgroup (Asians) to 10^{-31} for study subjects overall. These associations in study subjects overall, non-Hispanic whites, and Hispanics, and the association with rs1800858 in Asians, remained statistically significant after adjustment for multiple testing. There was variation in the magnitude of odds ratios by race/ethnicity. Although some odds ratios were elevated for African-Americans, there were no statistically significant associations between any of the seven *RET* SNPs and HSCR in this subgroup. For six of the seven SNPs there was a low frequency of individuals homozygous for the minor allele among African-Americans (Supplementary Table4).

SNPs rs1864410, rs2435357, and rs1800858 were in strong LD with each other in all race/ethnic groups (all $r^2 \geq 0.80$). They were also in strong LD with rs10900296 in the non-Hispanic white, Hispanic, and Asian subgroups (all $r^2 > 0.70$) but not in African-Americans (all $r^2 < 0.40$).

Genotype-phenotype associations for other candidate genes

Table 4 shows *P* values, calculated from two degree-of-freedom tests in logistic regression, comparing SNP genotypes between cases and controls. Based on a nominal *P* value <0.05, some of the SNPs in the candidate genes involved in enteric neural crest cell proliferation and migration were associated with HSCR and these associations varied by race/ethnicity (number of subjects with each genotype is shown in Supplementary Table 5). *ASCL1* SNPs were associated with HSCR in non-Hispanic whites (rs1874875; *P*=0.015) and African-Americans (rs17450122; *P*=0.029). In addition, *PROK1* rs7513898 was associated with HSCR in African-Americans (*P*=0.044).

Because we wanted to determine whether the SNPs were associated with HSCR among cases that did not have *RET* variants that might cause HSCR, we repeated the logistic regression analyses excluding the 38 cases with *RET* coding and splice-site variants. In addition to the findings already noted for *ASCL1* and *PROK1* SNPs, *L1CAM* rs4646265 was associated with HSCR in females among study subjects overall (P=0.0094) and among non-Hispanic whites (P=0.020). Also, *HOXB5* rs4793943 (P=0.034), rs4793589 (P=0.033), rs872760 (P=0.034), and rs1529334 (P=0.036), and *PHOX2B* rs6811325 (P=0.049) were associated with HSCR in Hispanics.

Among Hispanics, three of the four *HOXB5* SNPs (rs4793943, rs4793589, rs872760) were in strong LD (r^2>0.9) with each other and were in moderately strong LD with *HOXB5* rs1529334 (r^2=0.77–0.79).

Except for *RET*, none of the associations in the candidate genes were statistically significant after adjustment for multiple comparisons using the Bonferroni method. Similar results were obtained after including the three younger case siblings, and after restricting the analyses to singleton births.

Haplotype-phenotype associations

Haplotypes with the *RET* rs10900296 minor A allele (in non-Hispanic whites), and the rs10900296-rs10900297-rs1864410 A-C-A alleles (in Hispanics and Asians) were associated with HSCR (Supplementary Table6). *RET* haplotypes were not associated with HSCR in African-Americans. The *HOXB5* rs4793943 minor G allele and *ASCL1* rs2291854 minor T allele also differentiated risk haplotypes in Hispanics. In African-Americans, *ASCL1* haplotypes associated with HSCR had the major A allele for rs9782; the haplotype with the strongest association (P=0.005) also had the minor G allele for rs17450122.

DISCUSSION

Most previous studies of HSCR have focused on *RET* because of the crucial importance of *RET* signaling in enteric nervous system development. However, attention must be given to other genes for several reasons: our data and previous studies show that only a small proportion of HSCR cases have known *RET* coding sequence mutations,[7,8,39] penetrance differs by sex,[4] and the correlation between specific *RET* mutations and HSCR severity varies.[40] Genes that regulate enteric neural crest cell proliferation, migration, and differentiation, are strong candidates because their disruption in animals leads to phenotypes that resemble HSCR in humans.[15–19] We confirmed associations between HSCR and common variants in *HOXB5* and *PHOX2B*, and observed that associations with *RET* SNPs varied by race/ethnicity. After adjustment for multiple comparisons, many associations with *RET* SNPs remained statistically significant but our findings for variants in other candidate genes did not. Others have reported associations between HSCR and SNPs in *HOXB5* and *PHOX2B*,[16,41,42] evidence which suggests that common variants in these genes could be involved in HSCR. We have extended the investigations of previous studies by using a large population-based sample of HSCR cases, examining SNPs in additional candidate genes, and exploring associations in multiple race/ethnic groups. We have also provided precise estimates of the prevalence of HSCR among live births and the proportion of cases with other birth defects, based on a consecutive case group born over an 8-year period. These estimates are in the range reported by others using data collected from smaller cohorts.[3,43,44]

Animal studies suggest that there are interrelationships between the candidate genes we studied and *RET* expression. In cultures of rat neural crest stem cells, *Ascl1* induces *Ret* expression and promotes neurogenesis.[45] *Hoxb5* disruption in mouse neural crest cells leads

to reduced *Ret* expression and impaired migration of the cells through the embryonic gut.[16] *Phox2b* inactivation results in down-regulated expression of *Ascl1* and *Ret* in mouse embryonic enteric neural crest cells.[17] In humans, a genome-wide association study conducted in a Chinese population also found an interaction between another gene (*NRG1* which encodes neuregulin 1) and *RET*.[46] Two SNPs in *NRG1* were associated with HSCR if subjects were also homozygous for the minor T allele of *RET* rs2435357. These interrelationships suggest that variants in the selected candidate genes could influence *RET* signaling in humans and affect HSCR risk. Therefore, a more comprehensive examination of both the rare and common variants in these genes would be worth further investigation.

In our population-based sample of HSCR cases, 34 *RET* coding and splice-site variants were identified, 18 (52.9%) of which were novel. Most of the 34 variants were heterozygous, and therefore dominant, in contrast to the recessive effects we observed for common variants in *RET* and the other candidate genes. Notably, there were no differences between members of monozygous twin pairs discordant for HSCR with regard to coding, splice-site, and common variants in *RET* and common variants in the candidate genes. Possible reasons for HSCR discordance include de novo mutations in other genes involved in enteric nervous system development, the influence of epigenetic factors, and differences in intrauterine insults experienced by each twin.

Emison *et al.*[10] observed differences by race/ethnicity in the association between *RET* rs2435357, which disrupts an enhancer site in intron 1, and HSCR. The minor allele was twice as frequent in haplotypes transmitted to Chinese than European cases and this correlated with the 2-fold higher minor allele frequency in chromosomes from Chinese than European individuals. We added to these findings by including other race/ethnic groups in our analysis of *RET* SNPs. We found that *RET* SNPs were associated with HSCR among all race/ethnic groups except African-Americans. For six of the seven SNPs tested, the minor allele was least frequent in African-Americans. Therefore, the small number of African-American individuals that were homozygous for the minor allele could have contributed to the lack of association between these SNPs and HSCR in this group.

A major strength of this study was the large, population-based sample of cases and controls. The case group is a consecutive sample from all live births in New York State. In a previous report, the New York State Congenital Malformations Registry ascertained at least 86.4% of cases when all types of major malformations were considered.[47] Furthermore, our study included subjects of different race/ethnic groups to test for associations in each of these groups. The limitations of the study included the lack of medical record data; consequently, the extent of aganglionosis in cases could not be determined. Because of small sample sizes, there was low power to examine associations in some race/ethnic groups. In addition, we were unable to perform functional assessments of the genetic variants that we analyzed. As a result, we could not determine whether the *RET* coding and splice-site variants identified directly affected gene function.

In conclusion, we found that associations between common *RET* variants and HSCR varied by race/ethnicity: no association was present in African-Americans. We also confirmed previously reported associations with *HOXB5* and *PHOX2B* suggesting that interactions between *RET* and genes that regulate proliferation, migration and differentiation of enteric neural crest cells may be important in HSCR. From a population-based perspective, the minor alleles of the *RET* SNPs we studied are probably important to HSCR susceptibility in non-Hispanic whites, Hispanics, and Asians but are unlikely to contribute to most cases in African-Americans, because the percentage of individuals homozygous for the minor alleles is very low. Additionally, our results for monozygotic twins discordant for HSCR suggest that coding and non-coding regions of other genes, epigenetic changes, and variation in the

intrauterine environment need to be investigated as determinants of HSCR. Our findings for variants in *HOXB5* and *PHOX2B* provide further evidence that genes regulating enteric neural crest cell activity during gut development are key elements in the mechanism of HSCR. It is possible that SNPs in these genes could alter the penetrance of *RET* risk alleles; therefore future work should explore the potential functional effects of SNPs in these genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Comparison of characteristics between Hirschsprung's disease cases and controls

Characteristic	Controls (N = 1 215)	All cases (N = 301)	P value[1]	Cases without *RET* coding and splice-site variants (N = 263)	P value[1]	Cases with *RET* coding and splice-site variants (N = 38)	P value[1]
	N (%)	N (%)		N (%)		N (%)	
Maternal age (years)			0.95		0.95		0.25
<20	99 (8.15)	26 (8.64)		21 (7.98)		5 (13.16)	
20–34	881 (72.51)	218 (72.43)		189 (71.86)		29 (76.32)	
≥35	235 (19.34)	57 (18.94)		53 (20.15)		4 (10.53)	
Maternal race/ethnicity			0.99		0.91		0.37
White, non-Hispanic	667 (54.90)	166 (55.15)		149 (56.65)		17 (44.74)	
African-American	250 (20.58)	61 (20.27)		48 (18.25)		13 (34.21)	
Hispanic	198 (16.30)	50 (16.61)		45 (17.11)		5 (13.16)	
Asian	92 (7.57)	22 (7.31)		19 (7.22)		3 (7.89)	
Other	8 (0.66)	2 (0.66)		2 (0.76)		0 (0.00)	
Maternal education (years)			0.12		0.25		0.053
<12	192 (15.80)	59 (19.60)		47 (17.87)		12 (31.58)	
12	355 (29.22)	95 (31.56)		86 (32.70)		9 (23.68)	
>12	658 (54.16)	145 (48.17)		128 (48.67)		17 (44.74)	
Missing	10 (0.82)	2 (0.66)		2 (0.76)		0 (0.00)	
Parity			0.030		0.0066		0.32
Nulliparous	499 (41.07)	103 (34.22)		84 (31.94)		19 (50.00)	
Multiparous	716 (58.93)	198 (65.78)		179 (68.06)		19 (50.00)	
Maternal smoking during pregnancy			0.099		0.049		0.77
Yes	107 (8.81)	36 (11.96)		34 (12.93)		2 (5.26)	
No	1106 (91.03)	265 (88.04)		229 (87.07)		36 (94.74)	
Missing	2 (0.16)	0 (0.00)		0 (0.00)			
Maternal pre-pregnancy diabetes			0.73		1.00		0.29
Yes	10 (0.82)	3 (1.00)		2 (0.76)		1 (2.63)	
No	1205 (99.18)	298 (99.00)		261 (99.24)		37 (97.37)	

Characteristic	Controls (N = 1 215)	All cases (N = 301)	P value[1]	Cases without *RET* coding and splice-site variants (N = 263)	P value[1]	Cases with *RET* coding and splice-site variants (N = 38)	P value[1]
	N (%)	N (%)		N (%)		N (%)	
Gestational diabetes			0.87		0.62		0.40
Yes	51 (4.20)	13 (4.32)		13 (4.94)		0 (0.00)	
No	1164 (95.80)	288 (95.68)		250 (95.06)		38 (100.00)	
In vitro fertilization or other assisted reproductive technique			0.19		0.16		1.00
Yes	16 (1.32)	7 (2.33)		7 (2.66)		0 (0.00)	
No	1199 (98.68)	294 (97.67)		256 (97.34)		38 (100.00)	
Infant sex			<0.0001		<0.0001		0.32
Male	628 (51.69)	214 (71.10)		191 (72.62)		23 (60.53)	
Female	587 (48.31)	87 (28.90)		72 (27.38)		15 (39.47)	
Plurality			0.15		0.13		1.00
Singleton	1179 (97.04)	287 (95.35)		250 (95.06)		37 (97.37)	
Multiple birth	36 (2.96)	14 (4.65)		13 (4.94)		1 (2.63)	
Birth year			0.32		0.52		0.32
1998	143 (11.77)	34 (11.30)		29 (11.03)		5 (13.16)	
1999	158 (13.00)	42 (13.95)		40 (15.21)		2 (5.26)	
2000	168 (13.83)	28 (9.30)		25 (9.51)		3 (7.89)	
2001	152 (12.51)	37 (12.29)		34 (12.93)		3 (7.89)	
2002	152 (12.51)	44 (14.62)		36 (13.69)		8 (21.05)	
2003	156 (12.84)	32 (10.63)		28 (10.65)		4 (10.53)	
2004	146 (12.02)	45 (14.95)		37 (14.07)		8 (21.05)	
2005	140 (11.52)	39 (12.96)		34 (12.93)		5 (13.16)	

[1]Fisher's exact test used to compare characteristics between cases and controls

Table 2

RET coding and splice-site variants in patients with Hirschsprung's disease

DNA change[1]	Protein change	Number of affected subjects[2]	Variant type	Exon/ intron	Location on chromosome 10[3]	PolyPhen-2 Prediction[4]	SIFT prediction[4]
c.166C>A	p.L56M	1	missense	exon 2	43595999	benign	tolerated
c.286T>G	p.Y96D	1	missense	exon 2	43596119	probably damaging	affect protein function
c.418C>T[5]	p.P140S	1	missense	exon 3	43597870	possibly damaging	tolerated
c.436T>C	p.Y146H	1	missense	exon 3	43597888	benign	tolerated
c.523C>T[5]	p.R175C	1	missense	exon 3	43597975	probably damaging	affect protein function
c.628G>A[5]	p.E210K	1	missense	exon 4	43600402	benign	tolerated
c.898G>A[5]	p.D300N	1	missense	exon 5	43601854	probably damaging	affect protein function
c.1157C>T	p.A386V	1	missense	exon 6	43604572	benign	tolerated
c.1187C>T[5]	p.S396L	1	missense	exon 6	43604602	benign	affect protein function
c.1193T>C[5]	p.L398P	1	missense	exon 6	43604608	probably damaging	affect protein function
c.1336G>C	p.G446R	2	missense	exon 7	43606727	benign	tolerated
c.1354C>A	p.L452I	1	missense	exon 7	43606745	benign	tolerated
c.1387G>A[5]	p.G463R	1	missense	exon 7	43606778	probably damaging	affect protein function
c.1400T>C[5]	p.V467A	1	missense	exon 7	43606791	probably damaging	affect protein function
c.1701C>A[5]	p.D567E	1	missense	exon 9	43608353	possibly damaging	affect protein function
c.1894G>A	p.E632K	1	missense	exon 11	43609942	benign	tolerated
c.2372A>T	p.Y791F	2	missense	exon 13	43613908	possibly damaging	tolerated
c.2390A>T[5]	p.D797V	1	missense	exon 13	43613926	probably damaging	tolerated
c.2410G>A	p.V804M	1	missense	exon 14	43614996	probably damaging	affect protein function
c.2522C>T	p.P841L	1	missense	exon 14	43615108	probably damaging	affect protein function
c.2550C>G[5]	p.D850E	1	missense	exon 14	43615136	probably damaging	tolerated
c.2590T>C[5]	p.Y864H	1	missense	exon 14	43615176	probably damaging	affect protein function
c.2657G>A	p.R886Q	1	missense	exon 15	43615578	probably damaging	affect protein function
c.2680G>A	p.G894S	1	missense	exon 15	43615601	probably damaging	affect protein function

DNA change[1]	Protein change	Number of affected subjects[2]	Variant type	Exon/ intron	Location on chromosome 10[3]	PolyPhen-2 Prediction[4]	SIFT prediction[4]
c.2944C>T	p.R982C	6	missense	exon 18	43620335	probably damaging	affect protein function
c.3142C>G[5]	p.L1048V	1	missense	exon 19	43622125	probably damaging	tolerated
c.3278A>G[5]	p.D1093G	1	missense	exon 20	43623650	probably damaging	affect protein function
c.750_751delCG	p.E251G frameshift X102	1	frameshift	exon 4	43600524 - 43600525	-	-
c.1261C>T	p. Q421X	1	nonsense	exon 6	43604676	-	-
c.1370C>A[5]	p.S457X	1	nonsense	exon 7	43606761	-	-
c.1914delC[5]	p.I638M frameshift X37	1	frameshift	exon 11	43609962	-	-
c.2943C>G[5]	p.Y981X	1	nonsense	exon 18	43620334	-	-
c.1759+1G>A[5]	-	1	splice-site disruption	intron 9	43608412	-	-
c.1879+1G>A	-	2	splice-site disruption	intron 10	43609124	-	-

[1] Nucleotides numbered with +1 representing the A of the ATG translation initiation codon of the GenBank reference sequence for *RET* cDNA (NM_020975.4)

[2] Thirty-four cases had one variant each and four cases had two variants each. Among the four cases with two variants each, all variants except for p.I638M frameshiftX37 have been reported previously: one case had p.L452I and p.P841L, another had p.L56M and p.A386V, the third had p.V804M and p.R982C, and the fourth had p.I638M frameshiftX37 and p.R982C.

[3] Based on GenBank human reference sequence for chromosome 10 (NC_000010.10), Genome Reference Consortium Human Build 37 (GRCh37.p5), February 2009

[4] PolyPhen-2 and SIFT used to predict the effect of missense variants on protein function based on sequence comparisons and/or the physical characteristics of amino acids

[5] Previously unreported variant, based on a search for these variants in previous reports and in the Human Gene Mutation Database, the Multiple Endocrine Neoplasia type 2 RET proto-oncogene database, dbSNP, 1000 Genomes (www.1000genomes.org), and the National Heart, Lung, and Blood Institute Exome Sequencing Project database (http://evs.gs.washington.edu/EVS/)

Table 3

Odds ratios and 95% confidence intervals for associations between *RET* SNPs and Hirschsprung's disease, by race/ethnicity[1,2]

SNP[3]	Genotype	All subjects	Non-Hispanic white	African- American	Hispanic	Asian
rs10900296: G>A	GA	1.68 (1.24, 2.27) $P=8.1\times10^{-4}$	1.85 (1.22, 2.82) $P=4.0\times10^{-3}$	1.15 (0.61, 2.17) $P=6.7\times10^{-1}$	1.97 (0.95, 4.09) $P=7.0\times10^{-2}$	2.95 (0.56, 15.69) $P=2.0\times10^{-1}$
	AA	10.64 (7.14, 15.85) $P=3.1\times10^{-31}$	11.59 (7.08, 18.99) $P=2.2\times10^{-22}$	2.59 (0.41, 16.27) $P=3.1\times10^{-1}$	17.82 (5.83, 54.49) $P=4.4\times10^{-7}$	12.73 (2.60, 62.35) $P=1.7\times10^{-3}$
rs10900297: C>A	CA	1.01 (0.67, 1.53) $P=9.5\times10^{-1}$	1.05 (0.54, 2.04) $P=8.8\times10^{-1}$	1.02 (0.52, 2.01) $P=9.5\times10^{-1}$	0.88 (0.35, 2.21) $P=7.8\times10^{-1}$	-
	CC	2.30 (1.54, 3.44) $P=4.4\times10^{-5}$	3.03 (1.63, 5.65) $P=4.9\times10^{-4}$	0.81 (0.36, 1.83) $P=6.1\times10^{-1}$	1.97 (0.80, 4.85) $P=1.4\times10^{-1}$	-
rs1864410: C>A	CA	1.83 (1.33, 2.51) $P=2.2\times10^{-4}$	1.78 (1.17, 2.71) $P=6.9\times10^{-3}$	1.47 (0.64, 3.39) $P=3.7\times10^{-1}$	2.37 (1.14, 4.94) $P=2.1\times10^{-2}$	3.37 (0.63, 18.01) $P=1.6\times10^{-1}$
	AA	11.49 (7.55, 17.47) $P=4.2\times10^{-30}$	11.64 (7.02, 19.28) $P=1.6\times10^{-21}$	-	25.81 (7.31, 91.06) $P=4.4\times10^{-7}$	12.79 (2.61, 62.73) $P=1.7\times10^{-3}$
rs2435357: C>T	CT	1.89 (1.37, 2.61) $P=1.1\times10^{-4}$	1.87 (1.22, 2.86) $P=3.9\times10^{-3}$	1.37 (0.57, 3.28) $P=4.8\times10^{-1}$	2.54 (1.23, 5.26) $P=1.2\times10^{-2}$	2.98 (0.56, 15.85) $P=2.0\times10^{-1}$
	TT	11.43 (7.59, 17.22) $P=2.1\times10^{-31}$	11.51 (7.05, 18.80) $P=1.7\times10^{-22}$	4.16 (0.57, 30.55) $P=1.6\times10^{-1}$	21.38 (5.92, 77.23) $P=3.0\times10^{-6}$	12.57 (2.57, 61.43) $P=1.8\times10^{-3}$
rs1800858: G>A	GA	1.88 (1.37, 2.58) $P=1.0\times10^{-4}$	1.89 (1.24, 2.88) $P=3.0\times10^{-3}$	1.14 (0.48, 2.70) $P=7.7\times10^{-1}$	2.83 (1.36, 5.91) $P=5.6\times10^{-3}$	2.92 (0.55, 15.51) $P=2.1\times10^{-1}$
	AA	10.82 (7.22, 16.21) $P=7.6\times10^{-31}$	10.99 (6.73, 17.94) $P=9.2\times10^{-22}$	4.55 (0.62, 33.52) $P=1.4\times10^{-1}$	16.55 (5.24, 52.26) $P=1.7\times10^{-6}$	14.25 (2.90, 69.98) $P=1.1\times10^{-3}$
rs1800861: T>G	TG	1.67 (1.26, 2.21) $P=3.5\times10^{-4}$	1.64 (1.14, 2.37) $P=8.0\times10^{-3}$	1.05 (0.53, 2.07) $P=8.9\times10^{-1}$	1.62 (1.32, 5.20) $P=6.0\times10^{-3}$	2.70 (0.51, 14.44) $P=2.4\times10^{-1}$
	GG	2.94 (1.90, 4.55) $P=1.3\times10^{-6}$	2.51 (1.42, 4.43) $P=1.5\times10^{-3}$	1.20 (0.12, 11.92) $P=8.8\times10^{-1}$	3.95 (1.28, 12.17) $P=1.7\times10^{-2}$	6.55 (1.33, 32.15) $P=2.1\times10^{-2}$
rs2075912: C>T	CT	1.83 (1.38, 2.43) $P=2.9\times10^{-5}$	1.94 (1.35, 2.79) $P=3.5\times10^{-4}$	0.81 (0.35, 1.86) $P=6.1\times10^{-1}$	2.56 (1.31, 5.01) $P=6.1\times10^{-3}$	3.40 (0.67, 17.26) $P=1.4\times10^{-1}$
	TT	4.69 (2.81, 7.83) $P=3.3\times10^{-9}$	4.81 (2.39, 9.69) $P=1.1\times10^{-5}$	1.66 (0.15, 18.88) $P=6.8\times10^{-1}$	3.89 (1.03, 14.66) $P=4.4\times10^{-2}$	9.87 (1.97, 49.95) $P=5.3\times10^{-3}$

[1] Analyses performed using logistic regression with adjustment for maternal smoking and parity (analyses that include all subjects are also adjusted for race/ethnicity)

[2] Reference group is homozygous for major allele; effect estimates are for being heterozygous and homozygous for minor allele; effect estimates were not available when there were no cases or controls in a genotype group

[3] Major allele is listed first

Table 4

P values for associations between Hirschsprung's disease and SNPs in candidate genes for enteric nervous system development, including (+) and excluding (-) cases with *RET* coding and splice-site variants[1]

Gene	SNP[2]	All subjects		Non-Hispanic white		African- American		Hispanic		Asian	
		+	−	+	−	+	−	+	−	+	−
ASCL1	rs9782:A>G	0.45	0.73	0.80	0.68	0.11	0.33	0.47	0.28	0.93	0.99
	rs1391682:G>A	0.70	0.71	0.67	0.58	0.98	0.98	0.75	0.66	0.88	0.75
	rs2291854:C>T	0.73	0.68	0.39	0.18	0.84	0.77	0.28	0.20	0.99	0.98
	rs17450122:A>G	0.97	0.88	0.86	0.60	0.029	0.0085	0.79	0.91	0.99	0.93
	rs1874875:G>C	0.10	0.12	0.015	0.024	0.88	0.98	0.91	0.91	0.26	0.36
HOXB5	rs4793943:C>G	0.86	0.93	0.80	0.67	0.19	0.25	0.052	0.034	0.95	0.85
	rs4793589:G>C	0.89	0.96	0.75	0.62	0.17	0.22	0.052	0.033	0.95	0.85
	rs872760:T>C	0.77	0.86	0.78	0.65	0.17	0.22	0.052	0.034	0.95	0.85
	rs9299:A>G	0.94	0.78	0.45	0.50	0.58	0.37	0.55	0.43	0.42	0.46
	rs7406798:C>T	0.91	0.80	0.54	0.63	0.98	0.71	0.96	0.83	0.99	0.99
	rs1529334:T>C	0.76	0.82	0.95	0.97	0.36	0.35	0.064	0.036	0.89	0.76
L1CAM	*Male*										
	rs4646266:C>A	0.34	0.37	0.98	0.88	0.37	0.48	0.30	0.26	0.98	0.98
	rs5987173:G>A	0.46	0.65	0.99	0.99	0.43	0.64	0.93	0.97	0.99	0.99
	rs4646265:T>C	0.75	0.75	0.99	0.75	0.75	0.70	0.23	0.15	0.98	0.98
	rs4646263:G>A	0.35	0.30	0.60	0.32	0.33	0.45	0.27	0.41	0.43	0.53
	Female										
	rs4646266:C>A	0.60	0.90	0.52	0.59	0.57	0.82	0.96	0.70	0.99	0.99
	rs5987173:G>A	0.69	0.89	0.99	0.99	0.80	0.98	0.97	0.97	0.99	0.99
	rs4646265:T>C	0.18	0.0094	0.051	0.020	0.40	0.34	0.31	0.60	0.89	0.58
	rs4646263:G>A	0.37	0.15	0.35	0.26	0.47	0.46	0.50	0.57	0.16	0.70
PHOX2B	rs11723860:G>A	0.48	0.67	0.15	0.11	0.96	0.91	0.42	0.38	0.92	0.99
	rs6826373:C>T	0.85	0.80	0.77	0.80	0.75	0.86	0.057	0.058	0.84	0.99
	rs2196822:A>C	0.99	0.70	0.79	0.80	0.34	0.13	0.28	0.12	0.81	0.98
	rs6811325:C>T	0.67	0.35	0.89	0.94	0.76	0.23	0.13	0.049	0.79	0.97

Gene	SNP[2]	All subjects		Non-Hispanic white		African- American		Hispanic		Asian	
		+	−	+	−	+	−	+	−	+	−
	rs4608840:C>T	0.67	0.39	0.71	0.76	0.47	0.12	0.20	0.084	0.81	0.98
PROK1	rs12405277:A>G	0.73	0.87	0.98	0.79	0.38	0.22	0.68	0.73	0.16	0.087
	rs1857512:G>A	0.21	0.61	0.34	0.55	0.55	0.76	0.46	0.40	0.91	0.75
	rs4839391:G>A	0.37	0.42	0.35	0.18	0.75	0.26	0.76	0.85	0.80	0.57
	rs884735:A>T	0.08	0.12	0.26	0.29	0.14	0.053	0.91	0.94	0.82	0.87
	rs3795828:C>T	0.38	0.59	0.75	0.86	0.73	0.61	0.44	0.52	0.99	0.99
	rs17628304:A>C	0.23	0.39	0.93	0.92	0.38	0.16	0.20	0.23	0.33	0.22
	rs7534330:C>T	0.78	0.74	0.56	0.50	0.86	0.77	0.31	0.36	0.99	0.99
	rs7513898:G>A	0.19	0.21	0.72	0.72	0.044	0.016	0.21	0.39	0.16	0.087
	rs7514102:G>A	0.18	0.49	0.84	0.84	0.078	0.058	0.14	0.22	0.20	0.14
	rs1044837:C>T	0.23	0.26	0.73	0.91	0.19	0.18	0.77	0.78	0.99	0.83
PROKR1	rs4854479:G>C	0.091	0.12	0.083	0.13	0.35	0.15	0.14	0.18	0.54	0.32
	rs7570797:A>G	0.51	0.44	0.69	0.99	0.11	0.090	0.42	0.35	0.30	0.75
	rs12713655:A>G	0.26	0.25	0.43	0.63	0.62	0.41	0.54	0.63	0.62	0.44
	rs4627609:T>C	0.25	0.24	0.43	0.65	0.67	0.43	0.67	0.76	0.62	0.44
	rs6731427:G>A	0.28	0.36	0.53	0.66	0.68	0.70	0.34	0.34	0.23	0.20
	rs4854436:G>A	0.26	0.20	0.18	0.21	0.88	0.90	0.38	0.59	0.86	0.91
RET	rs10900296:G>A	1.9×10^{-30}	1.2×10^{-30}	7.2×10^{-22}	7.0×10^{-22}	0.57	0.49	2.9×10^{-6}	4.0×10^{-6}	0.0015	0.0016
	rs10900297:C>A	4.9×10^{-9}	5.4×10^{-9}	3.1×10^{-8}	2.7×10^{-8}	0.81	0.61	0.062	0.11	0.11	0.11
	rs1864410:C>A	4.4×10^{-29}	6.8×10^{-29}	7.6×10^{-21}	9.8×10^{-21}	0.67	0.79	2.1×10^{-6}	3.7×10^{-6}	0.0022	0.0025
	rs2435357:C>T	1.8×10^{-30}	7.4×10^{-30}	4.9×10^{-22}	6.7×10^{-22}	0.31	0.59	9.5×10^{-6}	2.1×10^{-5}	0.0016	0.0018
	rs1800858:G>A	7.1×10^{-30}	1.7×10^{-29}	3.7×10^{-21}	3.7×10^{-21}	0.32	0.69	5.8×10^{-6}	1.2×10^{-5}	0.00075	0.00086
	rs1800861:T>G	9.4×10^{-7}	1.6×10^{-5}	0.0014	0.010	0.98	0.78	0.0070	0.0033	0.039	0.067
	rs2075912:C>T	6.8×10^{-10}	5.1×10^{-9}	2.0×10^{-6}	1.9×10^{-5}	0.80	0.50	0.0095	0.0057	0.011	0.010

[1]Logistic regression was used to calculate *P* values from two degree-of-freedom tests comparing SNP genotypes between cases and controls; models were adjusted for maternal smoking and parity (analyses that include all subjects are also adjusted for race/ethnicity)

[2]Major allele is listed first