

# Specific polymorphisms in the *RET* proto-oncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression

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## Abstract

**Hirschsprung disease (HSCR) is a common genetic disorder presenting with functional intestinal obstruction secondary to enteric aganglionosis. HSCR can be familial or sporadic. Although five putative susceptibility genes have been identified, only germline mutations in the *RET* proto-oncogene account for a significant minority (up to 50%) of familial HSCR; 3% of sporadic HSCR in a population based series carry *RET* mutations. From 1998 to February 1999, we prospectively ascertained 64 cases of sporadic HSCR from the western Andalusia region. To determine if polymorphic sequence variants within *RET* could act as low penetrance predisposing alleles, we examined allelic frequencies at seven polymorphic loci in this population based series. Whether allele frequencies differed from those in the control population were determined by either chi-squared analysis or Fisher's exact test. For two sequence variants, A45A (c 135G→A) (exon 2) and L769L (c 2307T→G) (exon 13), the rarer polymorphic allele was over-represented among HSCR cases versus controls ( $p < 0.0006$ ). In contrast, two other polymorphisms, G691S (c 2071C→A) (exon 11) and S904S (c 2712C→G) (exon 15), were under-represented in the HSCR patients compared to controls ( $p = 0.02$ ). Polymorphisms in the *RET* proto-oncogene appear to predispose to HSCR in a complex, low penetrance fashion and may also modify phenotypic expression.**

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Hirschsprung disease (HSCR, MIM 142623), which occurs in 1 in 5000 live births, is characterised by the absence of the intramural ganglia of Meissner and Auerbach in the hindgut and results in functional intestinal obstruction.<sup>1</sup> Usually occurring as isolated cases, HSCR can be familial and may be inherited as an autosomal dominant or autosomal recessive trait with reduced penetrance and male predominance.<sup>1-2</sup> To date, at least five related genes are believed to play some aetiological role in the pathogenesis of hereditary HSCR.

In 1994, a major susceptibility gene for HSCR was identified as the *RET* proto-oncogene, which is located on 10q11.2.<sup>3-5</sup>

The *RET* proto-oncogene encodes a receptor tyrosine kinase expressed in tissues and tumours derived from the neural crest and neuroectoderm.<sup>6-7</sup> 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, pheochromocytoma, and hyperparathyroidism.<sup>8-9</sup> Interestingly, loss of function germline *RET* mutations were discovered in HSCR.<sup>4-5</sup> Depending on the series, up to 50% of familial HSCR cases and anywhere between 10 and 30% of sporadic cases were reported to be accounted for by loss of function germline *RET* mutations.<sup>10-12</sup> 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* mutations in 69 unselected HSCR cases to be 7%.<sup>13</sup> However, only 3% of isolated HSCR cases in this population based cohort had germline *RET* mutations. While *RET* may be viewed as one of the major susceptibility genes for HSCR, the precise roles of the other putative susceptibility genes are unclear. Germline mutations in these non-*RET* genes have rarely been found and often coincide with other known reasons for the presence of HSCR (for example, co-presence of germline *RET* mutation). Thus, the aetiology of HSCR is complex and low penetrance polygenic inheritance will be the rule.

The existence of modifier loci, including those at 10q11/*RET*, chromosome 21, and 9q have been suggested,<sup>14-15</sup> but none has been formally identified to date. Because a single family segregating both HSCR and MEN 2 was found to harbour a germline *RET* C620S mutation and the expression of the HSCR phenotype seemed to occur only in the presence of both the C620S mutation and the homozygous sequence polymorphism A45A (c 135G→A),<sup>16</sup> we decided to examine a population based series of cases with sporadic HSCR for the frequency of the A45A sequence variant as well as six other polymorphic variants within *RET* compared to race matched, normal controls.

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Table 1 *RET* polymorphic alleles in HSCR and normal controls

	HSCR alleles	Control alleles
(A) Over-representation of the A45A polymorphic allele in HSCR versus controls		
Polymorphic A	75	32
Wild type G	53	168
Chi-squared, Yates's correction = 62.50, $p < 0.0001$		
(B) Over-representation of the L769L polymorphic allele in HSCR versus controls		
Polymorphic C	39	28
Wild type G	89	172
Chi-squared, Yates's correction = 12.03, $p = 0.0005$		
(C) Under-representation of the G691S or S904S polymorphic allele in HSCR versus controls		
Polymorphic allele	16	47
Wild type allele	112	153
Chi-squared, Yates's correction = 5.40, $p = 0.02$		
(D) Polymorphisms with similar frequencies in HSCR and controls		
V125V (exon 3), $p = 0.15$		
Polymorphic allele	9	6
Wild type allele	119	194
A432A (exon 7), $p = 0.14$		
Polymorphic allele	28	60
Wild type allele	100	140
S836S (exon 15), $p = 0.54$		
Polymorphic allele	3	8
Wild type allele	125	192

## Materials and methods

### PATIENTS

The University Hospital Virgen del Rocío in Seville is the major referral centre for the western Andalusia region of Spain, and therefore all HSCR cases seen at this institution can be considered representative of the population. The Seville HSCR work began only recently (January 1998). For this study, the first 64 consecutive cases of clinically sporadic HSCR seen at the University Hospital based office practice of three of us (SB, ML-A, and GA) were ascertained beginning in January 1998 and ending on 15 February 1999, in accordance with the ethical standards of the institutional review board. Parenthetically, during this period when the 64 isolated HSCR cases were ascertained, three unrelated families with HSCR were also found. Thus, the proportion of familial to isolated cases is concordant with that from another population based survey.<sup>13</sup>

HSCR was diagnosed based on histological examination of either biopsy or surgical resection material. Histopathological criteria for HSCR are (1) absence of enteric plexuses with histological evaluation of the aganglionic tract, and (2) increased acetylcholinesterase immunohistochemical staining in the nerve fibres. A family history of HSCR was recorded only when more than one confirmed HSCR case was noted.

Normal controls were unselected, unrelated, race matched subjects from Andalusia without a diagnosis of HSCR. The control samples were tested in an anonymised fashion.

### MUTATION ANALYSIS

Genomic DNA was extracted from peripheral blood leucocytes using standard techniques.<sup>17</sup> The appropriate *RET* amplicon for each of exons 2, 3, 7, 11, 13, 14, and 15 was generated as previously described.<sup>18</sup> The presence or absence of each polymorphism within each amplicon was assessed by differential restriction digestion with the appropriate enzymes, as previously described,<sup>19,20</sup> according to the manufacturers' recommendations (Boehringer-Mannheim, 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 transillumination after ethidium bromide staining. When the primers were 5' labelled with fluorescent dyes, the restricted amplicons were subjected to electrophoresis through an Alf-Express Automated DNA Sequencer (Pharmacia Biotech).

### STATISTICAL ANALYSIS

Allelic frequencies between HSCR cases and controls were compared using standard chi-squared analysis with Yates's correction or, where appropriate, the Fisher two tailed exact test. Statistical significance was taken as  $p < 0.05$ . Only allelic frequencies were compared because full haplotypes could not be consistently generated for the entire cohort because of the lack of availability of other family members.

## Results

We have analysed 64 unrelated cases of sporadic HSCR compared to race matched, normal controls for the frequency of polymorphic alleles at seven loci within the coding region of the *RET* proto-oncogene. Of these seven loci, over-representation of the respective polymorphic allele occurred at two loci in cases compared to controls (table 1). The two associations were noted at codons 45 (exon 2) and 769 (exon 13). Among a total of 128 HSCR chromosomes, 75 (59%) harboured the polymorphic variant A and 53 (41%) the wild type G at nucleotide 135 (codon 45). In contrast, among 200 control chromosomes, 32 (16%) had the variant A and 168 (84%) the wild type G. The difference in allelic distribution at this locus between HSCR cases and normal controls was statistically significant (chi-squared with Yates's correction = 62.50,  $p < 0.0001$ ). Similarly, for the exon 13 polymorphism, L769L (c 2307T→G), 39 of 128 (30%) HSCR alleles carried the polymorphic c 2307G compared to 28 of 200 (14%) control alleles (chi-squared with Yates's correction = 12.03,  $p = 0.0005$ ).

Whenever the two polymorphisms occurred in a single person ( $n = 27$ ), inspection showed that the two polymorphic alleles always occurred in synteny.

Two polymorphisms, G691S (c 2071C→A) in exon 11 and S904S (c 2712C→G) in exon 15, which are in linkage disequilibrium with each other, appear to be under-represented in cases compared to controls (chi-squared with Yates's correction = 5.40,  $p = 0.02$ , table 1C). Three of the seven tested loci, codons 125 (exon 3), 432 (exon 7), and 836 (exon 14), had allelic distributions which were similar in both HSCR cases and normal controls ( $p \geq 0.14$  for each, table 1D).

## Discussion

Classically, polymorphisms represent sequence variations, which are present in the general population, and confer no deleterious effects. However, as the human genome project evolved and molecular epidemiological studies

were performed, it became clear that some "polymorphisms" were not entirely harmless. For example, a glutamine to arginine (Q192R) polymorphism in the paraoxonase gene, which encodes an enzyme capable of hydrolysing lipid peroxides, has been shown to be associated with coronary heart disease in the setting of type 2 diabetes mellitus.<sup>21</sup> Although the variant occurs in the general population, it is still plausible that a change from a neutral amino acid to a charged amino acid could have some effect. However, the germline I1307K sequence variant in the *APC* gene, the susceptibility gene for familial adenomatous polyposis syndrome, has recently been shown to be associated with an increased risk of non-syndromic familial and apparently sporadic colorectal cancer in the Ashkenazim.<sup>22</sup> Initially this was surprising, as an isoleucine to leucine change is considered neutral. However, such a sequence variant was shown to create a small hypermutable region in the gene, thus predisposing to somatic mutations in that area, that is, it does not directly cause an alteration in the function of the encoded protein.<sup>22</sup> The converse observation of that seen in the present study is the two to threefold over-representation of the *RET* S836S sequence variant, which does not alter the amino acid, among subjects with sporadic medullary thyroid carcinoma compared to race matched, normal controls.<sup>20</sup>

Polymorphic alleles at two loci within *RET*, at codons 45 and 769, appear to be over-represented in a cohort of 64 unselected, isolated HSCR cases compared to race matched controls. The A45A polymorphic allele and the L769L allele were each strongly associated with the occurrence of HSCR. It is more likely than not that these associations are true given the high statistical probability with which the null hypothesis was rejected in each case, and that the allele frequencies at five of the seven loci obtained in the Spanish controls were almost identical to those obtained in other studies.<sup>19, 20</sup> The frequency of the polymorphic alleles at two loci, A45 and L769, appeared to be lower than that found in French and German populations. Given that 200 chromosomes were examined, it is highly unlikely that this is artefactual. Indeed, it probably reflects the documented north-south gradient (highest frequencies in the north, lowest in the south) of some polymorphism frequencies.<sup>23</sup> More importantly, the S836S polymorphic allele, which was found to be over-represented in sporadic MTC patients, was not over-represented among HSCR cases. Indeed, one could postulate that the S836S polymorphic allele might be under-represented in HSCR cases versus controls if numbers were larger (0.02 and 0.04, respectively).

If this current molecular epidemiological observation is valid, then several mechanisms are possible. First, a new cryptic splice donor, acceptor, or enhancer site could be created. This would certainly be plausible for the A45A-HSCR association; the G to A substitution could result in the creation of a new alternative splice acceptor site 4 bp downstream of nucleotide 135.<sup>16</sup> This could result in a truncated protein or in a receptor that did not

bind ligand well. Unfortunately, RNA from a proper tissue source is not available to test this hypothesis and *RET* is not expressed in peripheral lymphocytes. Second, the sequence variant(s) may predispose to decreased expression of the variant bearing allele, thus leading to low level functional haploinsufficiency. Third, these loci, whether over- or under-represented in cases compared to controls, may lie in linkage disequilibrium with other sequences that may directly confer low level predisposition to or protection against HSCR. Fourth, preferential usage of tRNA molecules may be invoked, although this has yet to be shown in humans, but is well described among prokaryotes. If this were true, the wild type would be the favoured sequence with the variant(s) less favoured, thus resulting in slightly decreased efficiency of *RET* translation in the latter. Finally, when an amino acid is altered, for example, G691S, one may postulate that such an apparently conservative change could subtly alter structure or function or both if located in a critical region. These postulated mechanisms are not mutually exclusive, and may account for the *RET* sequence variants acting as common low penetrance alleles in HSCR predisposition.

These observations, taken together, argue for the validity of the association of A45A and L769L variants with the development of HSCR, perhaps in a low penetrance fashion. In addition, the data might also suggest that other polymorphic alleles, for example, G691S and S904S, might protect against the development of HSCR in a low penetrance manner.

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