

## ELECTRONIC LETTER

# Investigation of germline *GFRA4* mutations and evaluation of the involvement of *GFRA1*, *GFRA2*, *GFRA3*, and *GFRA4* sequence variants in Hirschsprung disease

S Borrego, R M Fernández, H Dziema, A Niess, M López-Alonso, G Antiñolo, C Eng

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The *RET* proto-oncogene on 10q11.2, which encodes a receptor tyrosine kinase expressed in neural crest and its derivatives, is the susceptibility gene for multiple endocrine neoplasia type 2 (MEN 2), characterised by medullary thyroid carcinoma, pheochromocytoma, and hyperparathyroidism, and one of several susceptibility genes for Hirschsprung disease (HSCR).<sup>1-4</sup> HSCR is characterised by aganglionosis of the gut resulting from inappropriate and premature apoptosis of the enteric ganglia. Initially, it was believed that approximately 50% of familial HSCR and 30% of isolated HSCR were the result of germline loss of function mutations in the *RET* proto-oncogene<sup>5,6</sup> (reviewed by Eng and Mulligan<sup>7</sup>). However, these data were obtained with highly selected series of families and patients with HSCR. A population based survey of HSCR cases showed that only 3% of isolated HSCR carried traditional germline *RET* mutations.<sup>8</sup> In the context of these data and the anecdotal observation that a *RET* codon 45 variant seemed to modify the expression of HSCR in a MEN 2/HSCR family with *RET* codon 618 mutation,<sup>9</sup> we began to examine the polymorphic alleles at codon 45 and the other coding variants as common low penetrance alleles for HSCR susceptibility. Indeed, we found that certain haplotypes or pairs of haplotypes ("genotypes") comprising specific combinations of *RET* polymorphic sequence variants were highly associated with isolated HSCR.<sup>9-11</sup> These observations were also noted among HSCR populations from elsewhere in the world.<sup>12-14</sup> These data implied that *RET* and/or loci in proximity to it could act as common low penetrance alleles which predisposed to isolated HSCR.

There are perhaps seven susceptibility genes for syndromic and non-syndromic HSCR.<sup>3,4,15-25</sup> Among these seven genes, *RET* is considered a major susceptibility gene for HSCR. *RET* is an unusual receptor tyrosine kinase in that it requires one of four co-receptors, *GFRα-1*, *GFRα-2*, *GFRα-3*, and *GFRα-4*, before it can bind one of four specific related ligands, GDNF, neurturin, persefin, and artemin.<sup>26-43</sup> While germline mutations in the genes encoding GDNF and neurturin, *GDNF* and *NTN*, have been rarely described in HSCR,<sup>18,21,44</sup> there has yet to be convincing data suggesting that *GFRA1* (10q26), *GFRA2* (8p21), or *GFRA3* (5q31) can act as "traditional" susceptibility genes for HSCR.<sup>45-48</sup> Thus, the genes which encode the co-receptors of *RET* are excellent candidates to serve as common low penetrance alleles for predisposition to isolated HSCR as well, and may interact with the *RET* sequence variants, and perhaps other variants, to modulate disease. To test our hypothesis, we examined a population based series of isolated HSCR cases for association with sequence variants within the genes which encode the four known co-receptors of *RET*. In addition, because *GFRA4* (20p12-p13), which encodes one of the four *RET* co-receptors and which is expressed in the derivatives of the neural crest including the enteric nervous system,<sup>49</sup> has not been evaluated as a traditional susceptibility

## Key points

- Hirschsprung disease (HSCR) occurs in 1:5000 live births and is currently acknowledged as having a polygenic aetiology. Originally reported as the major susceptibility gene for both inherited and isolated HSCR, germline loss of function mutations in the *RET* proto-oncogene, encoding a receptor tyrosine kinase on 10q11.2, have only been implicated in 3% of a population based series of HSCR.
- Recently, we and others have shown that germline sequence variants within the *RET* coding sequence are significantly associated with isolated HSCR cases. In fact, when cases were compared to controls, HSCR cases were found to have specific *RET* haplotypes and pairs of haplotypes which were distinct from those of controls. Thus, the genes which encode the four known co-receptors of *RET*, *GFRA1-4*, are excellent candidates to play roles in the aetiology of HSCR.
- It has already been shown that traditional mutations in *GFRA1-3* do not play a prominent role in HSCR. Here, we have found that among 72 isolated HSCR cases from the population base of southern Spain, there were no traditional germline mutations in *GFRA4*. We have also investigated this cohort for associations with sequence variants spanning *GFRA1-4* to search for the possibility that these genes serve to harbour common low penetrance alleles for HSCR.
- None of the sequence variants within these four genes was associated with HSCR compared to controls. Further, no variants considered at single loci or haplotypes or haplotype combinations were associated with clinical parameters. We did find, however, a large germline deletion in *GFRA1* of unknown significance.
- In sum, our data suggest that *GFRA1-4* are not major contributors of high or low penetrance alleles for susceptibility to isolated HSCR. This may reflect their overlapping function.

gene for HSCR, we also performed mutation analysis of the entire gene in our HSCR cohort.

## MATERIALS AND METHODS

### Subjects

Seventy-two consenting cases of isolated HSCR originating from southern Spain were ascertained within a 16 month period, in accordance with the respective human subjects protection committees. Further, at least one of the two unaffected

parents of each of the 70 isolated HSCR cases also donated their blood for these studies. One hundred and fifteen race matched, geographically matched normal controls were also gathered.

### Single nucleotide polymorphism (SNP) and mutation analysis

Genomic DNA was extracted according to standard protocols.<sup>9-10,30</sup> Genotyping at each SNP locus within *GFRA1*, *GFRA2*, *GFRA3*, and *GFRA4* was performed as previously described, either by SSCP or FRET, and if necessary confirmed with differential restriction digestion or direct sequence analysis.<sup>10,11,51,52</sup> A total of 14 SNP loci within the four co-receptor genes were examined. Well documented biallelic SNPs at each locus belonging to *GFRA1* (seven loci), *GFRA2* (one locus), and *GFRA3* (two loci)<sup>46,47,51</sup> were analysed.

Because *GFRA4* had not been previously examined in HSCR, its exons and flanking intronic regions were analysed in their entirety using a combination of SSCP and semi-automated sequence analysis as previously described.<sup>53</sup> Four SNPs in *GFRA4* were uncovered using SSCP and confirmed with sequencing (see Results below).

For each subject, haplotypes comprising combinations of alleles at each SNP locus within each gene were formed for every HSCR case and control, where possible. Haplotype formation was made possible because phase could be determined by having available genotypes among HSCR cases and their unaffected parents in a similar manner as previously described for haplotype formation within *RET*.<sup>11</sup>

### Statistical analysis

The frequencies of each allele at each SNP locus was compared between cases and controls using chi-squared analysis with

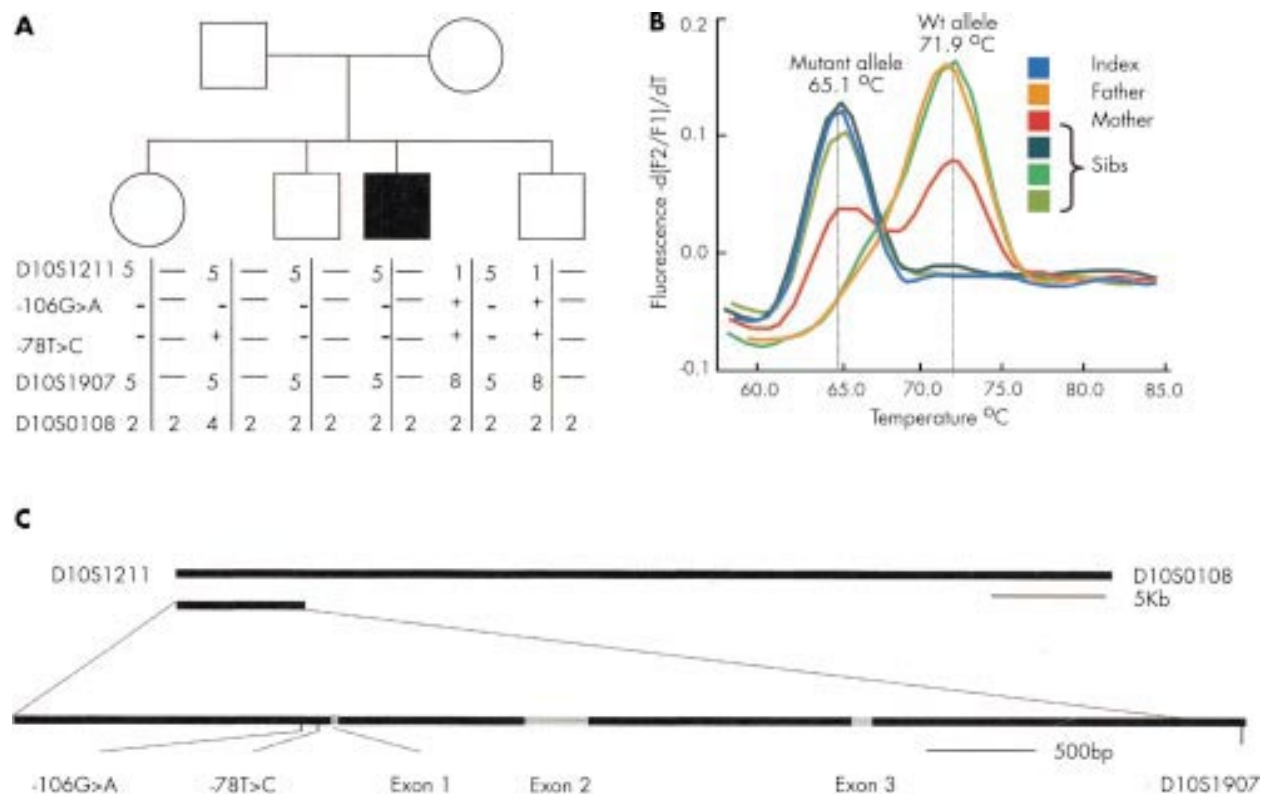
Yates' correction as previously described.<sup>10</sup> Frequencies of haplotypes across each of the four genes were then compared between cases and controls also using chi-squared analysis with Yates' correction as previously described<sup>11</sup>;  $p < 0.05$  was considered significant. In addition, haplotype frequencies were compared against various demographic and clinical features among all HSCR cases, such as sex, age at diagnosis, and length of segment involved (short segment v long segment disease) using either chi-squared analysis with Yates' correction or Fisher's exact test (two tailed).

### RESULTS

Because *GFRA4* had not been previously analysed as a susceptibility gene for HSCR, the exons, respective exon-intron boundaries, and flanking intronic regions were examined in their entirety. Of note, no germline mutations were found among 72 unrelated isolated HSCR cases. We did, however, find four polymorphic sequence variants within the exonic sequences of this gene, c.102G>A (T34T, exon 2), c.711G>A (P237P, exon 5), c.847C>T, and c.867G>A (exon 6, 3'UTR).

The frequencies of the alleles at each of the SNPs at every locus for each of the four co-receptor genes were not significantly different between HSCR cases and controls. Haplotypes could be formed for 63 of the 72 HSCR cases and 77 of the 115 controls. Nonetheless, no particular haplotype was observed to be particularly prevalent among HSCR cases and normal controls. Pairs of haplotype combinations (genotypes) across all four genes were noted and none was found to be particularly associated with cases compared to controls.

Haplotypes across each of the genes were formed and compared to various demographic and clinical parameters in all HSCR cases. No associations were found between specific



**Figure 1** (A) Segregation of the polymorphisms of the *GFRA1* gene in a family carrying a deletion in the 5' end of this gene. (B) FRET analysis of -78T>C (5'UTR of *GFRA1*) in the family carrying the *GFRA1* deletion. The homozygous genotype for the wild type allele has a higher melting temperature than the homozygous genotype for the mutant allele (71.9°C v 65.1°C); heterozygotes for this polymorphism show both peaks. (C) Schematic representation of the 5' end of the *GFRA1* gene.

haplotypes across all four genes and sex, age at diagnosis, and length of segment involved.

While examining haplotypes in HSCR cases and their unaffected parents, we initially thought that we had made either technical or administrative errors in one family as the haplotypes of the parents and the proband were not compatible with Mendelian inheritance (fig 1). More specifically, the results of the genotyping of the  $-78T>C$  variant, located in the 5'UTR of the *GFRA1* gene, showed discordant results in the patient and his father (fig 1). Haplotyping at *GFRA2*, *GFRA3*, and *GFRA4* and genotyping at 10 other polymorphic microsatellite loci distant from *GFRA1* excluded non-paternity. Thus, we proceeded to perform further analysis of this family and the results showed a large deletion extending from within intron 3 at the 3' end to as 5' as the 5'UTR within *GFRA1*. Surprisingly, analysis of the unaffected father and three unaffected sibs showed this identical deletion (fig 1). Refinement of the *GFRA1* deleted region in this family was carried out by genotyping of three new informative STRs (D10S1211, D10S1907, and D10S0108) as well as by Southern blotting. The 3' boundary of the deletion is delimited by the D10S0108 microsatellite, which is located over 39 kb from the exon 3-intron 3 boundary. In addition, mutational screening in the *RET*, *GDNF*, *NTN*, *GFRA2*, *GFRA3*, *GFRA4*, and *EDNRB* genes was performed in the index patient, in order to find any mutation which explains why the rest of his relatives carrying the same rearrangement were healthy. However, no significant sequence changes were found in any of these genes. The precise functional significance of the *GFRA1* deletion is currently unknown.

## DISCUSSION

Like the genes encoding the first three co-receptors of RET, no traditional germline mutations in *GFRA4* were found in HSCR. Thus, none of the four co-receptor genes appears to contribute, to a significant degree, to the aetiology of HSCR, at least in the non-familial form. It is difficult to explain the lack of traditional germline mutations in the four co-receptor genes given the prominent involvement of *RET*, both in the traditional and low penetrance sense, in the pathogenesis of isolated and familial HSCR. One plausible explanation might be the overlapping functions of the co-receptors and cross reactivity of ligands, albeit with different affinities and with some tissue specific differences (see introduction). However, if this were a plausible explanation, it remains to be explained why germline variants in *GFRA1* appear to play low penetrance susceptibility roles in the aetiology of isolated medullary thyroid carcinoma (MTC), at least in Germany.<sup>31</sup>

Because traditional germline mutations in the four *GFRA* genes have not been shown to play pivotal roles in the pathogenesis of HSCR, we examined them for low penetrance alleles predisposing to isolated HSCR. We have comprehensively excluded this possibility by examining associations at the genotype level, haplotype level, and then combined haplotype level. Furthermore, none of the genotypes, single gene haplotypes, or combined haplotypes were associated with clinical presentation.

Each of the known traditional susceptibility genes for HSCR can also be considered candidates to contribute low penetrance alleles of susceptibility or phenotype modification. *RET* is one such example. Given the limitations of sample size and small effects, none of the genes which encode the four co-receptors of RET appears to play a prominent role as a traditional susceptibility gene or a gene which lends low penetrance susceptibility to isolated HSCR. Nonetheless, many other HSCR susceptibility genes exist as candidates, as well as those which encode molecules upstream and downstream of the proteins encoded by the known susceptibility genes for HSCR.

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## Authors' affiliations

**S Borrego, R M Fernández, G Antiñolo**, Unidad de Genética Médica y Diagnóstico Prenatal, Hospitales Universitarios Virgen del Rocío, Sevilla, Spain

**H Dziema, A Niess, C Eng**, Clinical Cancer Genetics and Human Cancer Genetics Programs, Comprehensive Cancer Center, and the Division of Human Genetics, Department of Internal Medicine, The Ohio State University, Columbus, OH, USA

**M López-Alonso**, Servicio de Cirugía Infantil, Hospitales Universitarios Virgen del Rocío, Sevilla, Spain

**C Eng**, Cancer Research UK Human Cancer Genetics Research Group, University of Cambridge, Cambridge, UK

Correspondence to: Dr S Borrego, Unidad de Genética Médica, Hospitales Universitarios Virgen del Rocío, Avda Manuel Siurot, Sevilla 41013, Spain; sborrego@hvr.sas.cica.es

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