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

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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.

**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. 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. 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) 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. 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**.

**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. 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; 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 haplotypes and sex, age at diagnosis, or length of segment involved (short segment v long segment disease). However, some associations were found between specific haplotypes and 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).

**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.

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haptotypes 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 GRF1 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). Refined 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, 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 date, 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 possible 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.11

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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