

# Investigation of germline $GFR\alpha$ -1 mutations in Hirschsprung disease

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## Abstract

**Inactivating mutations of the RET proto-oncogene and of one of its soluble ligand molecules, glial cell line derived neurotrophic factor (GDNF), have been found in a subset of patients with Hirschsprung disease (HSCR). However, the majority of HSCR mutations remain unidentified. As normal RET function requires a multi-component ligand complex for activation, other members of the RET ligand complex are primary candidates for these mutations. We investigated the presence of mutations in another member of the RET signalling complex, GDNF family receptor alpha-1 ( $GFR\alpha$ -1), in a panel of 269 independent cases of HSCR. We identified 10 polymorphisms at the  $GFR\alpha$ -1 locus. Surprisingly, however, we did not identify any sequence variants in our HSCR population that were not also present in a normal control population. Our data suggest that mutations of the  $GFR\alpha$ -1 gene are not a common aetiological event in HSCR.**

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The RET receptor tyrosine kinase is activated by binding of a multimeric complex comprising both a soluble and a cell surface bound component. Although there is some heterogeneity in the components forming specific complexes, members of both of these families are required for RET mediated signalling. In the absence of a member of either family, RET cannot be activated. RET's soluble ligands are members of the glial cell line derived neurotrophic factor (GDNF) family of target derived neurotrophic molecules.<sup>1</sup> To date, two GDNF family members, GDNF and neurturin,<sup>1,2</sup> have been shown to contribute to RET phosphorylation.<sup>3-7</sup> However, further family members with similar functions are predicted. GDNF family members do not bind directly to RET but require the presence of an adapter molecule, a member of the GDNF family receptor alpha ( $GFR\alpha$ ) family, in the formation of a multicomponent complex. The  $GFR\alpha$  family is a novel group of extracellular proteins attached to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage.<sup>3-8</sup> The first identified member of this family,  $GFR\alpha$ -1 (previously called GDNFR- $\alpha$ , TrnR1, or RETL1), has been shown to bind GDNF with high affinity and neurturin with lower affinity.<sup>3-6,9,10</sup> In the presence of these soluble

ligands,  $GFR\alpha$ -1 also binds RET with high affinity and stimulates RET activation. In the absence of  $GFR\alpha$  proteins RET is not activated.

RET and GDNF have been shown to play an important role in migration and maturation of neural crest derivatives and in induction of the kidney. During murine embryogenesis, RET, GDNF, and  $GFR\alpha$ -1 are all highly expressed in the developing digestive tract and kidney.<sup>4,5,11-14</sup> Consistent with this pattern of expression, mice null for GDNF or RET have dysgenic or agenetic kidneys and lack enteric ganglia,<sup>15-18</sup> suggesting a requirement for RET signalling in kidney induction and in development of the enteric nervous system. In humans, germline mutations of RET have been identified in patients with Hirschsprung disease (HSCR), a developmental anomaly characterised by the absence of the enteric ganglia from all or part of the lower gut.<sup>19</sup> Inactivating RET mutations including deletions, insertions, and point mutations have been detected in approximately 15-30% of sporadic and 50% of familial HSCR cases.<sup>20,21</sup> Several studies have suggested that mutations of one RET ligand, GDNF, may also occur infrequently in HSCR.<sup>22-24</sup> Taken together, mutations of RET, GDNF, and other known HSCR genes are found in less than 50% of HSCR cases. Thus, additional candidate loci for HSCR remain to be identified.  $GFR\alpha$ -1, which can mediate GDNF dependent activation of RET, represents an excellent candidate. Mutations of  $GFR\alpha$ -1 which abrogate its ability to interact with either GDNF or RET would be predicted to mimic the effect of mutations of the latter molecules and thereby cause the HSCR phenotype. In order to investigate this potential role, we have examined a panel of DNA from 269 patients with HSCR for mutations of the  $GFR\alpha$ -1 locus. Here we show that mutations of  $GFR\alpha$ -1 are not a common cause of HSCR.

## Materials and methods

### PATIENTS AND CONTROL SAMPLES

DNA isolated from a panel of 269 unrelated HSCR patients, including 117 independent familial cases and 152 sporadic cases, was analysed in this study. All patients had been previously tested for mutations of RET and GDNF. As previous studies have shown that the presence of RET mutations does not preclude mutation of the RET ligand GDNF in the same person,  $GFR\alpha$ -1 was investigated irrespective of the presence of a mutation at one of these loci. In addition, DNA from normal controls was obtained from blood samples from

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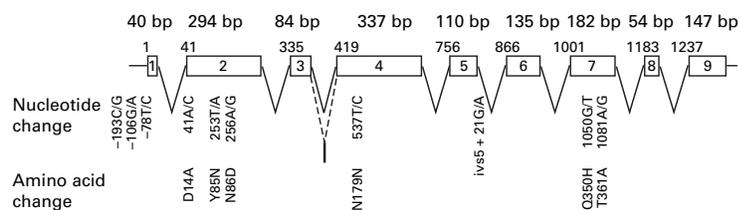
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**Figure 1** Schematic representation of the *GFRα-1* gene showing the location of polymorphisms. Nucleotide positions are given with reference to the *GFRα-1* sequence of Samicola *et al.*<sup>26</sup> Nucleotide changes for each site are given. Where polymorphisms occurred within the gene coding sequence, any changes in the coded amino acid are also indicated. The position of the alternatively spliced exon is indicated by dotted lines and a black box.

the DNA Diagnostic Laboratory at Kingston General Hospital. DNA was extracted from peripheral blood samples using standard protocols.

#### DNA ANALYSES

##### PCR analyses

PCR was carried out using a sense and antisense primer pair in 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.75–1.75 mmol/l MgCl<sub>2</sub>, 0.01% gelatin, 1 μmol/l of each primer, 200 μmol/l dNTPs, and 1.5 units of *Taq* DNA polymerase (Life Technologies). Reactions were subjected to 40 cycles of 95°C for one minute, 55°C or 53°C for one minute, and 72°C for one minute followed by 10 minutes at 72°C, or to 30 cycles of 40 seconds at each of 94°C, 53, 55, or 57°C, and 72°C, followed by one cycle of seven minutes at 72°C. PCR for single strand conformation polymorphism (SSCP) analyses were as described above except that in most cases only 100 μmol/l dNTPs were used and 0.5 μCi of [ $\alpha$ -<sup>32</sup>P]dCTP was added. Reactions were denatured at 95°C for 5–10 minutes and electrophoresed at 6 W overnight at 15°C on 8% acrylamide or 6% acrylamide with glycerol varied between 2.5 and 7.5% in 0.5 × TBE buffer or electrophoresed on Hydrolink MDE gels (Bioprobe) for 18 hours at 4 W. A minimum of two electrophoretic conditions were used in analysis of each DNA sample for each exon in order to increase the efficiency of SSCP variant detection.

##### Sequencing

When an abnormal SSCP pattern was observed, direct DNA sequencing was performed using the DyeDeoxyTerminator Cycle Sequencing Kit (Applied Biosystems). Alternatively, amplified products were purified on 2% low melting temperature agarose and eluted through Wizard PCR Prep columns (Promega). Products were directly sequenced using the  $\Delta$ *Taq* cycle sequencing kit (Amersham-Life Sciences) by incorporation of [ $\alpha$ -<sup>35</sup>S]dATP or [ $\alpha$ -<sup>32</sup>P]dCTP.

##### Primers

Primers used to amplify *GFRα-1* for SSCP are shown. Forward (f) and reverse (r) strand primers are indicated. Intronic primer sequences are shown in lower case.

Exon 1: RAF5 (5'-CGACGTTGGGTC GGACCTG-3') (f), RAR21 (5'-CGATCC CCGACAGCTGTGCT-3') (r), RAF21 (5'-AGCACAGCTGTCCGGGATCG-3') (f),

RAR6 (5'-GCGCGAAGTACAGGGTCG CC-3') (r), ex1F (5'- CAGCGCGGCTCG GGATT-3') (f), ex1R (5'-taggaaagactcaga gggtaa-3') (r). Exon 2: RAF22 (5'-gc agACTTGCTCCTGTGCGGC-3') (f), RAR8 (5'-AGTTGGTCTCCTTGCCCCG-3') (r), RAF7 (5'-CGCACGCTAAGGCAGTG CGT-3') (f), HRAR2 (5'-CTGGTACATGCTCC AGTA-3') (r) ex2F (5'-cctgca ctcaggctccct cc-3') (f), ex2R (5'-CTCCAGTAAATGCGC AGGCAG-3') (r). Exon 3: ex3F (5'- cagcag-caaaaacctgcttg -3') (f), ex3R (5'-ccagaaac actgtgccattc-3') (r). Exon 4: ex4F (5'-gTGG AGCACATTCCCAAAGG-3') (f), RAR19 (5'-CATAGGAGCACACAGGCAG-3') (r). Exon 5: RAF5B (5'-TACCAACTGCCAGC CAGAGT-3') (f), RAR16 (5'-gaaagaacgcag gtatatgc-3') (r). Exon 6: ex6F (5'-cataca acaataaatgcct-3') (f), ex6R (5'-agctggagc tcggagaagaa-3') (r). Exon 7: ex7R (5'-GGCAAACATGAGTGGGAA-3') (r), ex7F (5'-gcttgactttgaaatgga-3') (f), Exon 8: RAF10 (5'-cagGCACAGAAGCTGAAATC-3') (f), RAR13 (5'-tctataaatgcagcaagcct-3') (r). Exon 9: ex9F (5'-agtggatgataatgaaccat-3') (f), ex9R (5'-TTTTTCATGTCCATATTGTATT-3') (r).

Primers for the five amino acid, alternatively spliced exon have been previously reported.<sup>25</sup> Primers used in sequencing *GFRα-1* included RAF19 in exon 4 (5'-ATCGTGCCTGTG TGCTCCTA-3') and previously published *GFRα-1* primers.<sup>26</sup>

#### Results

We have previously shown that the *GFRα-1* gene lies on chromosome 10q26.<sup>26</sup> *GFRα-1* expresses at least two alternatively spliced transcripts, both of which include nine common coding exons (fig 1).<sup>25–27</sup> An additional small exon, encoding five amino acids, is present in only one of these transcripts.<sup>25–27</sup> In this study, we analysed all 10 coding exons and the 5' untranslated region of *GFRα-1* for sequence variants in a panel of 269 HSCR cases using single strand conformation polymorphism analysis (SSCP). For larger exons, we analysed multiple overlapping PCR subfragments, which produced smaller amplification products, in order to increase the efficiency of SSCP variant detection. To confirm putative sequence variants, we also investigated a panel of 175 unrelated normal subjects.

We detected 10 electrophoretic variants at the *GFRα-1* locus (table 1, fig 1). All 10 variants were present in at least one subject in our control population. We did not detect any variants which were present in the HSCR population that were not also present in the controls. In order to confirm that frequent sequence variants were not present in *GFRα-1* but were undetected by our SSCP analyses, we performed direct genomic sequencing of >90% of the *GFRα-1* coding sequence in four HSCR samples. We did not detect any additional sequence variants in these analyses.

We used direct genomic sequencing analysis to confirm the nature of the sequence variants detected by SSCP. The positions of these variants are indicated relative to the *GFRα-1* sequence of Samicola *et al.*<sup>26</sup> (fig 1) (Genbank

Table 1 GFR $\alpha$ -1 polymorphisms in HSCR patient and control DNA samples

Amino acid change	Nucleotide	Frequency		
		HSCR	Control	
	-193	C	0.98	0.93
		G	0.02	0.07
	-106	G	0.96	0.86
		A	0.04	0.14
	-78	T	0.69	0.68
		C	0.31	0.32
D14A	41	A	>0.99	0.99
Y85N	253	C	<0.01	0.01
		T	0.98	0.96
N86D	256	A	0.02	0.04
		G	>0.99	0.99
N179N	537	T	<0.01	0.01
		C	0.6	0.59
Q350H	1050	G	0.4	0.41
		A	0.92	0.82
		A	0.08	0.18
T361A	1081	G	1.0	0.99
		T	0	0.01
		A	0.93	0.88
		G	0.07	0.12

accession U97144), which includes only the nine common coding exons present in all GFR $\alpha$ -1 transcripts.<sup>25-27</sup> The position of the additional, alternatively spliced exon is indicated in fig 1. No sequence variants were detected in this exon. Ten single base pair substitution polymorphisms in GFR $\alpha$ -1 were identified. Three of these lay in the 5' untranslated region at 193 bp (-193C/G), 106 bp (-106G/A), and 78 bp (-78T/C) upstream of the translation start site. A silent base pair substitution at codon 179 (537T/C) and a non-coding polymorphism within intron 5, 21 bp downstream of exon 5 (IVS5+21G/A) were also observed but were not predicted to affect the GFR $\alpha$ -1 protein. The remaining five polymorphisms were within the GFR $\alpha$ -1 coding sequences and resulted in amino acid substitutions. These coding polymorphisms were clustered in exons 2 and 7 (fig 1, table 1) at codons 14 (41A/C), 85 (253T/A), 86 (256A/G), 350 (1050G/T), and 361 (1081A/G). The allele frequency for each polymorphism was similar in the HSCR and control populations (table 1). Further, within our HSCR population, allele frequencies were also similar for both familial and sporadic cases.

## Discussion

We have detected 10 polymorphisms in the GFR $\alpha$ -1 locus, but no disease specific mutations of this gene in a large population of HSCR patients. Five of these resulted in amino acid substitutions, although none of these changes was found within predicted functional motifs based on homology searches. In a recent study, Angrist *et al*<sup>27</sup> identified two additional rare silent DNA sequence changes which lie within the coding portion of the GFR $\alpha$ -1 gene in 85 HSCR cases. Our data and those of Angrist *et al*<sup>27</sup> indicate that mutations of GFR $\alpha$ -1 are unlikely to be an aetiological event in HSCR. In humans, haploinsufficiency for functional RET is associated with the HSCR phenotype. Previous studies have suggested that development of the enteric ganglia is very sensitive to the amount of functional RET available and that even moderate reductions of

less than 50% in functional protein are sufficient to result in the HSCR phenotype.<sup>20 28 29</sup> The absence of disease specific mutations in GFR $\alpha$ -1 is surprising, given that this protein mediates the activation of RET by GDNF, an interaction required for enteric neurogenesis in mice.<sup>16-18</sup> Our data may suggest that GFR $\alpha$ -1 is not uniquely required for RET activation in development of the human enteric nervous system. It is possible that GFR $\alpha$ -2 or other members of this family may complement GFR $\alpha$ -1 loss or, in fact, may be required exclusively for RET signalling in development of the enteric nervous system. In situ hybridisation studies have shown that GFR $\alpha$ -1 is highly expressed in the developing mouse gut during the period in which enteric neurogenesis occurs.<sup>5</sup> While it is possible that GFR $\alpha$ -1 has a role in this process in murine but not human development, it is also possible that GFR $\alpha$ -1 plays a role unrelated to RET activation during this developmental period in the gut. The nature of such a role remains to be investigated.

Alternatively, mutations of GFR $\alpha$ -1 may occur in HSCR patients but may be limited to a very specific subset of cases. Mutations of the endothelin B receptor or endothelin 3 have been recognised in a specific subset of HSCR cases associated with a Waardenburg-like phenotype characterised by hypopigmentation and hearing loss,<sup>21</sup> although these are also found in non-syndromic HSCR.<sup>30 31</sup> Mutations of the SOX10 gene have also recently been recognised specifically in Waardenburg-Hirschsprung syndromes.<sup>32</sup> We did not detect GFR $\alpha$ -1 mutations in patients in our sample set with classical HSCR associated phenotypes, such as multiple endocrine neoplasia type 2 (n=4), Down syndrome (n=1), or the Waardenburg-like phenotype (n=5). However, we cannot exclude the possibility that such mutations are associated with a specific additional phenotype which was not present in our sample set.

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