Genetic interactions and modifier genes in Hirschsprung's disease

Adam S Wallace, Richard B Anderson

Abstract

Hirschsprung's disease is a congenital disorder that occurs in 1:5000 live births. It is characterised by an absence of enteric neurons along a variable region of the gastrointestinal tract. Hirschsprung's disease is classified as a multigenic disorder, because the same phenotype is associated with mutations in multiple distinct genes. Furthermore, the genetics of Hirschsprung's disease are highly complex and not strictly Mendelian. The phenotypic variability and incomplete penetrance observed in Hirschsprung's disease also suggests the involvement of modifier genes. Here, we summarise the current knowledge of the genetics underlying Hirschsprung's disease based on human and animal studies, focusing on the principal causative genes, their interactions, and the role of modifier genes.

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Key words: Neural crest; Enteric nervous system; Hirschsprung's disease; Aganglionosis; Modifier genes

Peer reviewer: Nageshwar D Reddy, Professor, Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad-500 082, India

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INTRODUCTION

The enteric nervous system (ENS) comprises neurons and glial cells within the wall of the gastrointestinal tract. It is responsible for regulating intestinal motility, immune function, luminal secretions, and blood flow. During development, the ENS arises from a highly migratory population of cells, called the neural crest. The neural crest arises as a result of an epithelial to mesenchymal transition during the formation of the neural tube. Two separate populations of neural crest, arising from different axial levels, contribute to the ENS - the vagal level (defined as the post-otic hindbrain adjacent to somites 1-7) and the sacral level (caudal to somite 24 in mice and humans). Vagal neural crest cells migrate ventrally to the presumptive foregut, and then into and along the entire length of the gastrointestinal tract, a process that takes five days in mice (embryonic day E9.5-E14.5) and three weeks in humans (during 4th-7th wk of gestation). The formation of a functional ENS requires the coordination of many processes, including survival, migration, proliferation, and differentiation of precursor cells within the gastrointestinal tract. Failure of neural crest cells to fully colonise the entire length of the gastrointestinal tract results in a region of gut that lacks enteric neurons, called an “aganglionic zone”, which affects a variable length of the distal most bowel. As enteric neurons are essential for motility, the aganglionic zone remains...
tonically constricted, preventing the passage of fecal material. In this condition, this is known as Hirschsprung’s disease (HSCR) and occurs in approximately 1:5000 live births\(^\text{[9]}\). HSCR can either be familial or sporadic, and is subdivided into short or long segment HSCR (S-HSCR and L-HSCR), which refers to the extent of the aganglionic zone\(^\text{[10]}\). The less severe S-HSCR (about 80% of cases) is more common than L-HSCR (about 20% of cases] and displays a more pronounced gender bias (4:1 male:female in S-HSCR compared to 1.2:1 male:female in L-HSCR\(^\text{[11]}\). HSCR may present as an isolated condition (about 70% of cases) or as part of a syndrome, such as Moewat-Wilson or Waardenburg Shah type 4 (Table 1). Although HSCR is normally detected soon after birth, there have been reports of HSCR being identified in patients after childhood\(^\text{[12]}\). Failure to treat HSCR is often fatal because of malnutrition or sepsis following rupture of the bowel. Present treatment involves surgery to remove the affected portion of bowel and re-anastomosis of the remaining gut to the anus. Although refinements to surgical techniques have improved patient outcome, post-operative complications persist in a large number of patients\(^\text{[13,14]}\).

In the majority of cases, the genetics of HSCR are complex and non-Mendelian in nature\(^\text{[15]}\). To date, more than a dozen genes have been identified as being associated with HSCR\(^\text{[16]}\). However, mutations in these genes account for only about 50% of all HSCR cases\(^\text{[17]}\). The phenotypic variability and incomplete penetrance observed in HSCR also suggests the involvement of modifier genes. The aim of this review is to summarise the current knowledge of the genetics underlying HSCR. We first discuss the principal causative genes and detail the current knowledge of the genetics underlying HSCR. We then review the associated HSCR genes. The aim of this review is to summarise the current knowledge of the genetics underlying HSCR. We first discuss the principal causative genes and detail the interactions between these genes that alter the severity or incidence of HSCR. Finally, we discuss the accumulating evidence for the role of modifier genes in the development of HSCR.

**GENES INVOLVED IN ENS DEVELOPMENT**

Many of the genes associated with HSCR encode members of the Glial cell line-derived neurotrophic factor (GDNF)/RET- and ET-3/EDNRB-signalling pathways or transcription factors, such as SOX10, PHOX2B or ZF-HX1B. Mutations in these genes have been shown to result in Hirschsprung’s disease in humans (Table 1) or aganglionosis in mice (Table 2).

**GDNF/RET-GFRα1**

GDNF is a secreted protein and a distant member of the TGF-β superfamily\(^\text{[18]}\). GDNF binds to the glycosylphosphatidylinositol-linked receptor, GFRα1. The GDNF-GFRα1 complex then binds to and activates the transmembrane receptor tyrosine kinase, RET\(^\text{[19]}\). Mutations in genes encoding members of the GDNF/RET-GFRα1 signalling pathway account for about 50% of familial cases and around 30% of sporadic cases of HSCR\(^\text{[17]}\).

Non-coding mutations in RET have also been proposed to increase susceptibility to HSCR\(^\text{[20-23]}\). In mice, *Gdf1* is expressed by the gut mesenchyme prior to the entry of neural crest cells\(^\text{[24]}\). *Ret* is expressed exclusively by neural crest-derived cells and *Gfra1* is expressed by both crest-derived cells and the gut mesenchyme\(^\text{[25]}\). *Gdf1*, *Gfra1* or RET-null mice die within 24 hours of birth, and lack enteric neurons along the entire length of the gastrointestinal tract caudal to the stomach\(^\text{[21,22]}\). *Gdf1* and *Ret* mice are viable and do not exhibit aganglionosis\(^\text{[26]}\).

*RET* is subject to alternative splicing and translated into two functional isoforms, RET151 and RET9, which differ in the number of amino acids at their C terminal end\(^\text{[26]}\). These isoforms are highly conserved between human and mouse\(^\text{[15]}\). Mice lacking the Ret51 isoform (Ref\(^\text{[12]}\) mice) have enteric neurons along the entire length of the gastrointestinal tract, while mice lacking the Ret9 isoform (Ret\(^\text{[15]}\) mice) suffer colonic aganglionosis and kidney hypoplasia\(^\text{[15]}\). The phenotype of the Ret\(^\text{[15]}\) mice is highly reminiscent of the colonic aganglionosis observed in patients with HSCR. Interestingly, the developing ENS in humans appears to be more sensitive to reduced RET signalling than that of the ENS in mice. RET mutations in humans act dominantly to give rise to HSCR, whereas ENS development is normal in Ret heterozygous mice\(^\text{[28]}\). In fact, it has recently been shown that a loss of around 60%-70% of Ret expression in mice is required to mimic the aganglionic phenotype observed in humans\(^\text{[22]}\).

Targeted mutations in RET have identified signalling sites that are required for ENS development. Mutation of a putative protein kinase A phosphorylation site, which changes serine to alanine (Ref\(^\text{[29]}\) ), results in aganglionosis of the distal colon\(^\text{[30]}\). Mutation of an intracellular docking site, which converts tyrosine to phenyalanine (Ref\(^\text{[30]}\) ), induces total intestinal aganglionosis\(^\text{[31]}\). The mutation of cysteine to arginine (Ref\(^\text{[32]}\) ), which is observed in some MEN2A/HSCR patients, has also been shown to result in total intestinal aganglionosis\(^\text{[33]}\).

**ENDOTHELIN SIGNALLING PATHWAY**

Endothelin 3 (ET-3) is a secreted peptide, which is expressed by the gut mesenchyme\(^\text{[34]}\). ET-3 is initially expressed in an immature form before being processed to an active peptide by the enzyme, endothelin converting enzyme 1 (ECE1)\(^\text{[35-37]}\). ET-3 signals through the receptor Endothelin receptor B (EDNRB), which is expressed on migrating enteric neural crest cells\(^\text{[38]}\). ET-3 is initially expressed in an immature form before being processed to an active peptide by the enzyme, endothelin converting enzyme 1 (ECE1)\(^\text{[35-37]}\). ET-3 signals through the receptor Endothelin receptor B (EDNRB), which is expressed on migrating enteric neural crest cells\(^\text{[38]}\). Mutations in ECE1 and EDNRB account for around 5% of HSCR cases, whilst only a single case of ECE1-associated HSCR has been reported\(^\text{[39]}\). ET-3 and EDNRB-associated HSCR can present as both syndromic (such as Wardenburg-Shah syndrome) and non-syndromic forms of HSCR. In mice, lethal spotted (ls) and piebald lethal (sl) are naturally occurring mutants of Et-3 and Ednrb respectively, and lack enteric neurons in the distal bowel\(^\text{[40-42]}\). Although enteric neurons are absent only from the distal colon of Et-3 and Ednrb-null mice, the migra-
tion of neural crest cells through the small intestine is also delayed[42,43]. As with RET, the human ENS appears to be more sensitive to reduced EDNRB signalling than that in mice. Around 21% of patients heterozygous for the W276C mutation in EDNRB develop HSCR[44], while heterozygous piebald lethal (sl) mice do not develop any form of aganglionosis[45].

SOX10

SOX10

SOX10

Table 1  Genes associated with Hirschsprung's disease

| Locus | Gene | Associated syndrome | Incidence | Penetrance | Inheritance | Ref.
|-------|------|---------------------|-----------|------------|-------------|------|
| 10q11 | RET  | Non-syndromic HSCR  | 50% familial | 70% male | Dominant | 82-84 |
| 5p13  | GDNF | Non-syndromic HSCR  | 30% sporadic | 50% female | Dominant | 85-89 |
| 13q22 | EDNRB| Shah-Waardenburg    | 5 cases    | Low      | Dominant or recessive | 44,90 |
| 20q13 | ET3  | Non-syndromic HSCR  | 1 case     | N/A      | Dominant or recessive | 91   |
| 1p36  | ECE1 | Cardiac and autonomic nervous system defects with HSCR | 1 case | N/A | Dominant | 40 |
| 22q13 | SOX10| Shah-Waardenburg    | > 5%       | ~80%     | Dominant | 47,49,50,92 |
| 2q12  | ZFHX1B| Mowat-Wilson        | < 5%       | 60%      | Dominant | 62,93-95 |
| 4p12  | PHEX | CCHS-Ondines Curse  | < 5%       | 20%      | Dominant | 96   |
| 19p13 | NTN  | Non-syndromic HSCR  | 1 case     | Dominant | 97     |
| 18q21 | TCFC4| Epileptic encephalopathy | 1 case | Dominant | 98 |
| 10q21.1| KOAA1279| Goldberg-Shprintzen | Rare | Recessive | 21 |

HSCR: Hirschsprung's disease; CCHS: Congenital central hypoventilation syndrome.

Table 2  Phenotypes of mouse models of enteric nervous system defects

| Wild-type | Colonic aganglionosis | Total intestinal aganglionosis | Hypoganglionosis |
|-----------|-----------------------|-------------------------------|------------------|
| Ret +/+   | +/-                   | 1/1                           | +/-              |
| Ednrb     | +/-                   | +/-                           | +/-              |
| Etn       | +/-                   | +/-                           | +/-              |
| Sox10     | +/-                   | +/-                           | +/-              |

Interactions

| Ret / Ednrb | +/- | +/- | +/- |
|------------|-----|-----|-----|
| Ednrb       | +/- | +/- | +/- |
| Sox10       | +/- | +/- | +/- |

Other genotypes

| Sal14 | BlIntegrin | Ecr1 |
|-------|------------|------|
| +/- | +/- | +/- |

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Interactions between known HSCR associated genes and their modifiers. Confirmed and putative interactions are shown by the solid and dotted lines, respectively. Red: HSCR associated gene; Blue: Known HSCR modifier gene; Black: Modifier loci with unknown gene. The question mark represents a disparity in the existing data obtained from human clinical and animal studies. HSCR: Hirschsprung’s disease; BBS: Bardet-Biedel syndrome.

ADDITIONAL TRANSCRIPTION FACTORS ASSOCIATED WITH HSCR

PHOX2B

Paired-like homeobox 2b (PHOX2B) is a transcription factor that is expressed by enteric neural crest cells. Human studies have linked mutations in PHOX2B with HSCR associated with congenital central hypoventilation syndrome (CCHS)/Ondines curse in two thirds of patients. Sox10 has been shown to regulate the expression of both Ret and Ednrb.

ZFHX1B

ZFHX1B, also known as SMAD interacting protein 1 (SMADIP1/SIP1), is a zinc finger homeodomain transcription factor. Mutations in ZFHX1B are associated with Mowat-Wilson syndrome, and have been shown to result in HSCR with a varying degree of penetrance. In mice, Zfhx1b is expressed by vagal neural crest cells, which are absent in Zfhx1b null mutant mice.

INTERACTIONS BETWEEN KNOWN HSCR ASSOCIATED GENES

Interactions between known HSCR associated genes significantly influence the incidence and severity of intestinal aganglionosis (Table 2) (Figure 1).

Gdnf/Ret and Et-3/Ednrb signalling pathways

Genetic interactions were first proposed based on a human study of the genetically isolated Mennonite population, suggesting that the RET and EDNRB loci may interact to govern the susceptibility to Hirschsprung’s disease. Studies in mice, using a two-locus complementation approach, confirmed a genetic interaction between the Ret and Ednrb loci by showing that the generation of Ret+/l Ednrb+/ls mice resulted in colonic aganglionosis, a phenotype not observed in Ret+/l or Ednrb+/ls mice alone.

Gdnf/Ret signalling pathway and the transcription factors Sox10 and Phox2b

Although genome-wide linkage studies failed to detect any genetic interaction between the Sox10 and Ret loci, Sox10 has been shown to form a transcriptional complex with the transcription factor, Pax3, to directly regulate the expression of RET. In addition to Sox10, Phox2B has also been shown to bind the RET promoter and regulate transcription. Although no genetic interactions were observed in double heterozygotic mice (Ret+/l; Phox2B+/ls mice), human clinical studies have reported interactions between RET and PHOX2B in CCHS patients.

Sox10 and Zfhx1b

Sox10 has been shown to interact with the transcription factor, Zfhx1b, in mice. The generation of double heterozygotic progeny (Sox10+/l; Zfhx1b+/ls mice) resulted in a significant increase in the severity of aganglionosis compared to a mutation in Sox10+/l or Zfhx1b+/ls mice alone. The mechanism underlying this interaction is not known, but is likely to be mediated by the modulation of Bmp expression.

Et-3/Ednrb signalling pathway and Sox10

In mice, interactions between Sox10 and members of the endothelin signalling pathway (Et-3 and Ednrb) have been reported. Using a two-locus complementation approach, mice carrying mutations in Sox10 and Et-3 (Sox10+/l; Et-3+/ls) or Ednrb (Sox10+/l; Ednrb+/ls) and Sox-
10\textsuperscript{loxP/+}; Ednrb\textsuperscript{+/−} showed a significant increase in the severity of intestinal aganglionosis compared to mutations in Sox10, Et3-3, or Ednrb alone\textsuperscript{[45,50]}. In addition, the expression of Ednrb has been shown to be significantly reduced in Sox10\textsuperscript{loxP/+} mice\textsuperscript{[50]}. The mechanism underlying this interaction can be explained, at least in part, by the presence of SOX10 binding sites within a conserved enhancer region of the Ednrb promoter, which are required for the spatiotemporal expression of Ednrb in the ENS\textsuperscript{[90]}.

MODIFIER GENES

The incomplete penetrance and interfamilial variation commonly observed in HSCR strongly suggests the involvement of modifier genes. We define a modifier gene as a gene that, when mutated, is insufficient on its own to produce an effect, but, when coupled with another genetic mutation, it produces or enhances an effect\textsuperscript{[70]}. To date, only a handful of modifier genes have been identified for HSCR (Figure 1).

Modifiers for RET

Linkage studies and genome-wide screens have identified a number of putative modifying loci for RET, such as 3q21, 4q31-32, 8p12, 9q31, and 19q12\textsuperscript{[71,72]}. However, many of the genes responsible for interacting with RET at these loci are yet to be identified. One gene that has been identified is neuregulin 1 (NRG1)\textsuperscript{[72]}. Association studies have shown that individuals that possess a specific NRG1 haplotype have an increased risk of HSCR conferred by RET\textsuperscript{[73]}. NRG1 signals through ErbB2 and ErbB3 receptors to regulate neural crest cell development and in turn, ErbB3 is regulated by the HSCR associated gene Sox10\textsuperscript{[79]}.

Although not detected in any of the genome-wide screens, three further modifier genes for RET were identified through the Bardet-Biedel syndrome (BBS). Subsets of patients with BBS, a genetically heterogeneous disorder with 14 identified causative loci, also present with HSCR. BBS patients with HSCR are more frequent carriers of a common RET intronic hypomorphic allele than the general population\textsuperscript{[74]}. In zebrafish, suppression of Ret in conjunction with a loss of either Bbr 4, 5, or 7 has been shown to significantly increase the severity of ENS defects compared to loss of these genes independently\textsuperscript{[75]}.

Human clinical studies have also suggested that the X-linked gene L1CAM, may act as a modifier gene for RET. Some individuals with L1CAM mutations who have HSCR, also possess a common RET polymorphism that is over-represented in HSCR populations\textsuperscript{[76]}. However, animal model studies using a two-locus complementation approach failed to detect any genetic interaction between L1cam and Ret\textsuperscript{[74]}. One reason for this discrepancy could be that humans are more sensitive to a reduction in RET levels than mice\textsuperscript{[30,33]}. It is not yet known whether interactions with L1cam can be detected in Ret\textsuperscript{[47,13]} and Ret\textsuperscript{[50]/Sca/Sca} mice that exhibit colonic aganglionosis and more closely resemble human HSCR\textsuperscript{[31,33]}.

Modifiers for Sox10

A genome-wide screen in mice has identified five putative modifying loci for Sox10 on chromosomes 3, 5, 8, 11, and 14\textsuperscript{[66]}. Two of these loci have been identified as Ednrb and Phox2b\textsuperscript{[66]}, while the other three loci, on chromosomes 3, 8, and 11, are yet to be determined.

Although not identified in the Sox10 genome-wide screen, one modifier gene that has been shown to significantly increase the penetrance and extent of aganglionosis in Sox10 heterozygous mice, is Sox8\textsuperscript{[70]}. Sox8 is a transcription factor that is closely related to Sox10, and is expressed by all enteric neural crest cells\textsuperscript{[70]}. Sox8\textsuperscript{−/−} mice are viable and fertile and show no ENS phenotype\textsuperscript{[78]}. Using a two-locus complementation approach, double heterozygotic progeny (Sox8\textsuperscript{+/−}; Sox10\textsuperscript{+/−} mice) were shown to have a significant increase in the incidence and severity of aganglionosis compared to a mutation in Sox8 or Sox10 alone\textsuperscript{[66]}. The most likely mechanism underlying this interaction is genetic redundancy, as Sox8 has been shown to have DNA binding and subcellular redistribution properties similar to that of Sox10\textsuperscript{[77,80]} and is capable of activating Sox10 target genes\textsuperscript{[78]}.

The X-linked gene, L1cam, can also act as a modifier gene for Sox10 in mice\textsuperscript{[79]}. Loss or haploinsufficiency of L1cam in conjunction with a heterozygous loss of Sox10 significantly increases the incidence of intestinal aganglionosis compared to a mutation in Sox8 or Sox10 alone\textsuperscript{[79]}. Sox10 has been shown to directly regulate the expression of endogenous L1cam\textsuperscript{[81]}.

Modifiers of Et3/Ednrb

To date, only one modifier gene has been identified for members of the endothelin signalling pathway. Loss or haploinsufficiency of L1cam in conjunction with a null mutation in Et3-3 or Ednrb significantly increases the severity of intestinal aganglionosis compared to a loss of Et3-3 or Ednrb alone\textsuperscript{[80]}. Although the mechanism underlying these interactions is not yet known, it is most likely mediated through the activation of common downstream targets, such as PI3K\textsuperscript{[81]}.

CONCLUSION

HSCR research has now entered a second phase. Having identified many of the key genes capable of independently inducing HSCR, we are now undertaking the difficult task of identifying the interactions that modulate the severity and penetrance of this disease. By combining human genetic data from patients, family pedigrees, and genome wide association screens with animal studies, we are beginning to assemble the pieces of the HSCR puzzle into a coherent picture of multigenetic inheritance and interactions. To further aid this goal, as the cost of genome sequencing becomes more affordable, the potential to sequence the entire genome of individual HSCR patients becomes viable, which is likely to provide significant advances into our understanding of the genetic basis of HSCR.
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