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EDITORIAL

Genetic interactions and modifier genes in Hirschsprung's disease

Adam S Wallace, Richard B Anderson

Adam S Wallace, Richard B Anderson, Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Victoria 3010, Australia
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Correspondence to: Richard B Anderson, PhD, Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Victoria 3010, Australia. rba@unimelb.edu.au
Telephone: +61-03-83445783 Fax: +61-03-93475219
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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.

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[1]. During development, the ENS arises from a highly migratory population of cells, called the neural crest[2-4]. 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)[5,6]. 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)[7] and three weeks in humans (during 4th-7th wk of gestation)[8].

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 faecal material. In humans, this condition is known as Hirschsprung's disease (HSCR) and occurs in approximately 1:5000 live births[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[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)[11]. HSCR may present as an isolated condition (about 70% of cases) or as part of a syndrome, such as Mowatt-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[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[13,14].

In the majority of cases, the genetics of HSCR are complex and non-Mendelian in nature[15]. To date, more than a dozen genes have been identified as being associated with HSCR[9,16]. However, mutations in these genes account for only about 50% of all HSCR cases[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 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 *ZFHX1B*. 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[18]. GDNF binds to the glycosylphosphatidylinsoitol-linked receptor, GFRα1. The GDNF-GFRα1 complex then binds to and activates the transmembrane receptor tyrosine kinase, RET[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[17]. Non-coding mutations in RET have also been proposed to increase susceptibility to HSCR[20-23]. In mice, *Gdnf* is expressed by the gut mesenchyme prior to the entry of neural crest cells[24]. *Ret* is expressed exclusively by neural crest-derived cells and *Gfrα1* is expressed by both crest-derived cells and the gut mesenchyme[25]. *Gdnf*-, *Gfrα1*- 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[25-27]. $Gdnf^{+/-}$ and $Ret^{+/-}$ mice are viable and do not exhibit aganglionosis[28].

RET is subject to alternative splicing and translated into two functional isoforms, RET51 and RET9, which differ in the number of amino acids at their C terminal end[29]. These isoforms are highly conserved between human and mouse[30]. Mice lacking the Ret51 isoform ($Ret^{9/9}$ mice) have enteric neurons along the entire length of the gastrointestinal tract, while mice lacking the Ret9 isoform ($Ret^{51/51}$ mice) suffer colonic aganglionosis and kidney hypodysplasia[31]. The phenotype of the $Ret^{51/51}$ 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[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[32].

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 (Ret^{S697A}), results in aganglionosis of the distal colon[33]. Mutation of an intracellular docking site, which converts tyrosine to phenylalanine (Ret^{Y1062F}), induces total intestinal aganglionosis[34]. The mutation of cysteine to arginine (Ret^{C620R}), which is observed in some MEN2A/HSCR patients, has also been shown to result in total intestinal aganglionosis[35].

ENDOTHELIN SIGNALLING PATHWAY

Endothelin 3 (ET-3) is a secreted peptide, which is expressed by the gut mesenchyme[36]. ET-3 is initially expressed in an immature form before being processed to an active peptide by the enzyme, endothelin converting enzyme 1 (ECE1)[37,38]. ET-3 signals through the receptor Endothelin receptor B (EDNRB), which is expressed on migrating enteric neural crest cells[39].

Mutations in *ET3* and *EDNRB* account for around 5% of HSCR cases, whilst only a single case of *ECE1*-associated HSCR has been reported[40]. *ET3*- 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[37,41]. Although enteric neurons are absent only from the distal colon of *Et-3* and *Ednrb*-null mice, the migra-

Table 1 Genes associated with Hirschsprung's disease

Locus	Gene	Associated syndrome	Incidence	Penetrance	Inheritance	Ref.
10q11	*RET*	Non-syndromic HSCR	50% familial 30% sporadic	70% male 50% female	Dominant	82-84
5p13	*GDNF*	Non-syndromic HSCR	5 cases	Low	Dominant	85-89
13q22	*EDNRB*	Shah-Waardenburg Non-syndromic HSCR	5%	Low	Dominant or recessive	44,90
20q13	*ET3*	Shah-Waardenburg 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 Non-syndromic HSCR	> 5%	~80%	Dominant	47,49,50,92
2q22	*ZFHX1B*	Mowat-Wilson	< 5%	60%	Dominant	62,93-95
4p12	*PHOX2B*	CCHS–Ondines Curse	< 5%	20%	Dominant	96
19p13	*NTN*	Non-syndromic HSCR	1 case		Dominant	97
18q21	*TCF4*	Epileptic encephalopathy	1 case		Dominant	98
10q21.1	*KIAA1279*	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	+/- 9/9 Y162F/+	51/51 S697A/S697A	-/- Y1062F/Y1062F C620R/C620R	C620R/+
Ednb	sl/+	sl/sl $Ednrb^{tm1Ywa}/Ednrb^{tm1Ywa}$		
Et3	ls/+	ls/ls $Et3^{tm1Ywa}/Et3^{tm1Ywa}$		
Sox10	Dom/+ (~80%) LacZ/+ (~80%)	Dom/+ (~20%) LacZ/+ (~20%)	Dom/Dom LacZ/LacZ	
Interactions		$Ret^{+/-}$; $Ednrb^{s/s}$ $Ret^{51/+}$; $Et3l^{s/ls}$	$Ret^{51/51}$; $Et3l^{s/ls}$ $Sox10^{Dom/+}$; $Et3l^{s/ls}$ $Sox10^{Dom/+}$; $Edrnb^{sl/sl}$	
Other genotypes		$Sall4^{-/-}$ B1Intergrin$^{-/-}$ $Ece1^{-/-}$	$Gdnf^{-/-}$ $Gfra1^{-/-}$ $Phox2b^{-/-}$ $Pax3^{-/-}$	$Gdnf^{+/-}$ $Hlx1^{-/-}$

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

SRY (*Sex determining region Y*)-*box 10* (*SOX10*) is a high mobility group transcription factor of the SRY (sex determining factor) family. Mutations in *SOX10* account for around 5% of HSCR cases[46-48], and comprise both syndromic [Waardenburg-Shah types 4 (WS4)] and non-syndromic forms[49]. Some WS4 patients with *SOX10* mutations also suffer dysmyelination of the central and peripheral nervous systems[47]. *Sox10* is expressed by migrating enteric neural crest cells[50]. *Dom* is a naturally occurring mouse mutant of *Sox10*, which carries a single base insertion in the *Sox10* locus that prematurely truncates the transcription factor downstream of the DNA binding domain, producing a dominant negative form of the protein[50]. Mice lacking *Sox10* are devoid of enteric neurons throughout the entire gastrointestinal tract[50,51]. Around 20% of $Sox10^{+/-}$ mice suffer colonic aganglionо-

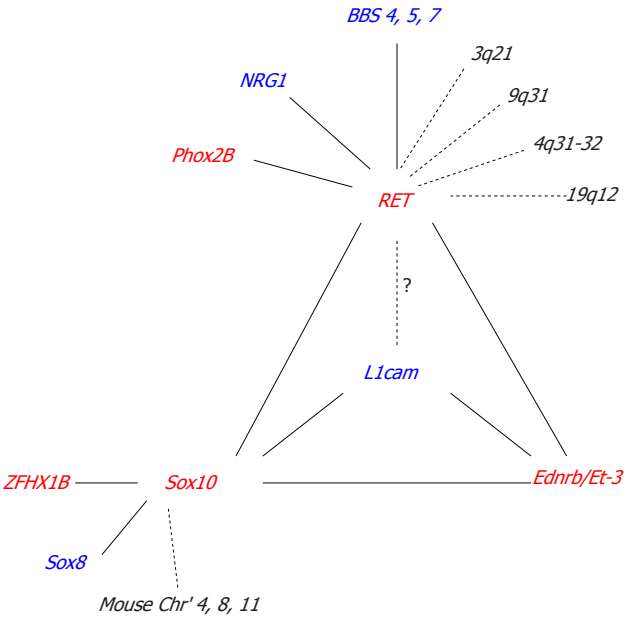

Figure 1 Interactions between known Hirschsprung's disease associated genes and their modifiers. Confirmed and putative interactions are shown by the solid and dotted lines, respectively. Red: HSCR associated gene; Blue: Known HSCR modifier gene; Black: Modifier loci with unknown gene. The question mark represents a disparity in the existing data obtained from human clinical and animal studies. HSCR: Hirschsprung's disease; BBS: Bardet-Biedel syndrome.

sis, although the incidence varies depending on the background strain[45,50,52,53]. These mice also exhibit coat colour defects analogous to the pigmentation defects seen in WS4 patients. *Sox10* has been shown to regulate the expression of both *Ret* and *Ednrb*[54-56].

ADDITIONAL TRANSCRIPTION FACTORS ASSOCIATED WITH HSCR

PHOX2B

Paired-like homeobox 2b (*PHOX2B*) is a transcription factor that is expressed by enteric neural crest cells[57]. Human studies have linked mutations in *PHOX2B* with HSCR associated with congenital central hypoventilation syndrome (CCHS)/Ondines curse in two thirds of patients[58]. The causative mutations in these patients are most commonly polyalanine expansions[59]. *Phox2b* null mice lack enteric neurons along the entire length of the gastrointestinal tract[60]. *Phox2b* has been shown to regulate the expression of *Ret*[60-61].

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[62]. In mice, *Zfhx1b* is expressed by vagal neural crest cells, which are absent in *Zfhx1b* null mutant mice[63].

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[64]. 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 $Ret^{+/-}$; $Ednrb^{s/s}$ mice resulted in colonic aganglionosis; a phenotype not observed in $Ret^{+/-}$ or $Ednrb^{s/s}$ mice alone[64,65]. A similar genetic interaction was also reported using Ret^{51} and $Et\text{-}3^{ls}$ mice[42]. A significant increase in aganglionosis, extending all the way to the stomach, was observed in $Ret^{51/51}$; $Et\text{-}3^{ls/ls}$ mice compared to the colonic aganglionosis normally observed in $Ret^{51/51}$ or $Et\text{-}3^{ls/ls}$ mice alone[42]. The mechanism underlying these interactions is not yet known; however, it has been proposed that *Ret* and *Ednrb* may interact by activating common downstream signalling molecules, such as PKA[42].

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[66], *Sox10* has been shown to form a transcriptional complex with the transcription factor, *Pax3*, to directly regulate the expression of RET[67]. In addition to *Sox10*, *Phox2B* has also been shown to bind the *RET* promoter and regulate transcription[61]. Although no genetic interactions were observed in double heterozygotic mice ($Ret^{+/-}$; $Phox2b^{+/-}$ mice), human clinical studies have reported interactions between *RET* and *PHOX2B* in CCHS patients[68].

Sox10 and Zfhx1b

Sox10 has been shown to interact with the transcription factor, *Zfhx1b*, in mice[69]. The generation of double heterozygotic progeny ($Sox10^{LacZ/+}$; $Zfhx1b^{\lrcorner/+}$ mice) resulted in a significant increase in the severity of aganglionosis compared to a mutation in $Sox10^{LacZ/+}$ or $Zfhx1b^{\lrcorner/+}$ mice alone[69]. The mechanism underlying this interaction is not known, but is likely to be mediated by the modulation of *Bmp* expression[69].

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[45,52]. Using a two-locus complementation approach, mice carrying mutations in *Sox10* and *Et-3* ($Sox10^{Dom/+}$; $Et\text{-}3^{ls/ls}$) or *Ednrb* ($Sox10^{Dom/+}$;$Ednrb^{s/s}$ and *Sox-*

10$^{Dom/+}$; *Ednrb*$^{sl/sl}$) exhibited a significant increase in the severity of intestinal aganglionosis compared to mutations in *Sox10*, *Et-3*, or *Ednrb* alone[45,52]. In addition, the expression of Ednrb has been shown to be significantly reduced in *Sox10*$^{Dom/+}$ mice[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[56].

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[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[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*)[72]. Association studies have shown that individuals that possess a specific *NRG1* haplotype have an increased risk of HSCR conferred by *RET*[72]. *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*[73].

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[74]. In zebrafish, suppression of *Ret* in conjunction with a loss of either *Bbs 4*, *5*, or *7* has been shown to significantly increase the severity of ENS defects compared to loss of these genes independently[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[76]. However, animal model studies using a two-locus complementation approach failed to detect any genetic interaction between *L1cam* and *Ret*[70]. One reason for this discrepancy could be that humans are more sensitive to a reduction in RET levels than mice[28,32]. It is not yet known whether interactions with *L1cam* can be detected in *Ret*$^{51/51}$ and *Ret*$^{S697A/S697A}$ mice that exhibit colonic aganglionosis and more closely resemble human HSCR[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[66]. Two of these loci have been identified as Ednrb and Phox2b[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*[53]. *Sox8* is a transcription factor that is closely related to *Sox10*, and is expressed by all enteric neural crest cells[53]. *Sox8*$^{-/-}$ mice are viable and fertile and show no ENS phenotype[53]. Using a two-locus complementation approach, double heterozygotic progeny (*Sox8*$^{+/-}$; *Sox10*$^{+/-}$ mice) were shown to have a significant increase in the incidence and severity of aganglionosis compared to a mutation in *Sox8* or *Sox10* alone[53]. 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[77-80] and is capable of activating Sox10 target genes[78].

The X-linked gene, *L1cam*, can also act as a modifier gene for *Sox10* in mice[70]. 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 *Sox10* alone[70]. *Sox10* has been shown to directly regulate the expression of endogenous *L1cam*[70].

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 *Et-3* or *Ednrb* significantly increases the severity of intestinal aganglionosis compared to a loss of *Et-3* or *Ednrb* alone[81]. 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[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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