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REVIEW ARTICLE

Genetic basis of Hirschsprung's disease

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Abstract Hirschsprung's disease (HSCR) is a developmental disorder characterized by the absence of ganglion cells in the lower digestive tract. Aganglionosis is attributed to a disorder of the enteric nervous system (ENS) whereby ganglion cells fail to innervate the lower gastrointestinal tract during embryonic development. HSCR is a complex disease that results from the interaction of several genes and manifests with low, sex-dependent penetrance and variability in the length of the aganglionic segment. The genetic complexity observed in HSCR can be conceptually understood in light of the molecular and cellular events that take place during the ENS development. DNA alterations in any of the genes involved in the ENS development may interfere with the colonization process, and represent a primary etiology for HSCR. This review will focus on the genes known to be involved in HSCR pathology, how they interact, and on how technology advances are being employed to uncover the pathological processes underlying this disease.

Keywords Hirschsprung's · RET · Genetics

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Introduction

Understanding how genes and other DNA sequences function together and interact with proteins and environmental factors is paramount to the discovery of the pathways involved in normal processes and in disease pathogenesis. In this respect, the study of the molecular basis of Hirschsprung's disease (HSCR) has made a marked contribution to the understanding of complex diseases. Complex diseases result from the interaction of two or more genes and/or gene–environment interactions [1] and therefore, have no identifiable pattern of inheritance.

Hirschsprung's disease (HSCR) is a developmental disorder characterized by the absence of ganglion cells in the lower digestive tract. Aganglionosis is attributed to a disorder of the enteric nervous system (ENS) whereby ganglion cells fail to innervate the lower gastrointestinal tract during embryonic development. There is a significant racial variation in the incidence of the disease, and it is most often found among Asians (2.8 per 10,000 life births) [2]. HSCR has a complex pattern of inheritance and manifests with low, sex-dependent penetrance (frequency of mutation carriers who develop disease as opposed to mutation carriers who remain healthy) and variability in the length of the aganglionic segment. HSCR patients are classified according to the length of the aganglionic segment into: short-segment HSCR (SS-HSCR) ($\sim$80% of HSCR cases) and long segment HSCR (LS-HSCR) ($\sim$20% of HSCR cases). The rectosigmoid region acts as the boundary between LS-HSCR and SS-HSCR. LS-HSCR is defined as aganglionic segment extending to or beyond the proximal sigmoid colon, whereas, the remaining cases are grouped as SS-HSCR.

The HSCR most commonly presents sporadically although it can be familial (up to 20% of the cases) and is

frequently associated with many other neurocristopathies and chromosome abnormalities [3, 4].

The genetic complexity observed in HSCR can be conceptually understood in light of the molecular and cellular events that take place during the ENS development in the embryonic stage. The ganglion cells of the fully developed ENS are derived from vagal neural crest cells (NCCs) of the neural tube [5]. During the colonization process, the NCCs have to adapt to a constantly changing intestinal environment that strongly influences their differentiation into enteric neurons [6]. The entire process is regulated by specific molecular signals from both within the neural crest and intestinal environment [7], and the success of the colonization of the gut depends on the synchronization and balance of the signaling network implicated. DNA alterations in any of the genes codifying for the signaling molecules may interfere with the colonization process, and consequently represent a primary etiology for HSCR. The HSCR phenotype may therefore result from (i) single severe DNA alterations (mutations) in a major gene encoding a crucial molecule; (ii) the summation of the effects of a series of less severe mutations in several genes; (iii) the former and the later combined. The existence of non-clinically affected individuals carrying mutations in major genes invokes a compensatory effect by other genes and reinforces the oligogenic nature of the disease. HSCR has become a model for oligogenic and polygenic disorders in which the phenotypes and mode of transmission result from interactions between different genes.

The genetic etiology of Hirschsprung's disease

In 1886, Danish pediatrician Harald Hirschsprung (1830–1916) described the disease as a cause of constipation in early infancy due to congenital dilatation of the colon. But it was not till the 1940s, when histological studies revealed absence of intramural ganglion cells of the myenteric and submucosa plexuses downstream the dilated colon (megacolon), that the primary pathology of the disease was discovered [8].

The development of a pull-through surgical procedure by Swenson and Bill in 1948 made a major contribution to our understanding of the disease [9]. This innovative surgical technique enabled a far higher survival rate among patients, which created the conditions for the discovery of the familial transmission of the disease and the determination of its genetic nature. Additional evidence for a role of genetic factors in the pathology of HSCR was indicated by (i) an increased risk of recurrence for sibs of affected individuals compared with the population at large; (ii) an unbalanced sex-ratio; (iii) the association of HSCR with other genetic diseases, including malformation syndromes and chromosomal anomalies; and (iv) the existence of several animal models of colonic aganglionosis showing specific Mendelian modes of inheritance.

In the late 1980s, the locus for multiple endocrine neoplasia (MEN) was assigned to chromosome 10q11.2 [10, 11]. The same locus was also identified as the cause of papillary thyroid carcinoma (PTC) [12–14] and familiar medullary thyroid carcinoma (FMTC) [15]. The co-occurrence of HSCR with multiple endocrine neoplasia type 2 (MEN2) together with the observation of a deletion comprising the MEN2 locus in some HSCR patients [16, 17], prompted the search for a HSCR gene in that chromosomal region. Linkage analysis using chromosome 10 markers in HSCR families revealed a HSCR locus in the pericentromeric region of chromosome 10 [18, 19]. The observation of a HSCR-phenotype in mice with disrupted *Ret* gene [20] and the detection of *RET* rare deleterious DNA alterations ("mutations") in HSCR families [21, 22] lead to the recognition of *RET* as the main gene implicated in HSCR, almost a century after Harald Hirschsprung first described the disease.

The fact that not all HSCR patients bore mutations in *RET*, implied that mutations in other genes accounted for the rest of the HSCR patients or/and that mutations occurred in regions of the gene which had not been investigated, namely regulatory non-coding regions. Subsequent studies lead to the identification of other genes in which DNA alterations or mutations lead to the HSCR phenotype. To date, mutations in the coding regions of nine different genes (*RET*, *GDNF*, *NRTN*, *PHOX2B*, *EDNRB*, *EDN3*, *ECE1*, *SOX10*, *ZFHX1B*, *KIAA1279*, and *NRG1*; Garcia-Barceló and Tam et al. unpublished data) [8, 23] have been detected in individuals affected with either isolated or syndromic HSCR (Table 1). Most of these genes encode protein members of important inter-related signaling pathways that are critical for the development of enteric ganglia: the GDNF/RET receptor tyrosine kinase, the endothelin type B receptor, and the SOX10-mediated transcription. *RET* is, however, the major gene involved in HSCR, and the proper expression of the RET protein expression is critical for the normal development of the ENS [24–27]. There is evidence that the manifestation of the HSCR phenotype may result from the interaction and/or accumulation of DNA variants in genes of these signaling pathways as illustrated by the identification of patients bearing mutations in *RET* in combination with mutations in other genes namely *GDNF*, Neurturin (*NTN*), or *EDNRB* [28] (see below).

Common features to the mutations found in the HSCR genes are low and sex-dependent penetrance and variable expression of the HSCR phenotype for a given mutation.

Table 1 Genes in which coding sequence mutations are associated with isolated or syndromic HSCR

Gene	Phenotype	Inheritance	Frequency[a]
RET	Isolated HSCR	Dominant, incomplete penetrance	50% Familial/7–35% sporadic
	HSCR-MEN2/FMTC	Dominant	–
GDNF	Isolated HSCR	Non-Mendelian	Very rare
NRTN	Isolated HSCR	Non-Mendelian	Very rare
EDNRB	Isolated HSCR	Dominant	3–7%
	Waardenburg-Shah syndrome	Recessive	–
EDN3	Isolated HSCR	Dominant, incomplete penetrance	<5%
	Waardenburg-Shah syndrome	Recessive	–
ECE-1	HSCR with cardiac defects, craniofacial abnormalities and autonomic dysfunction	Dominant	–
ZFHX1B	Mowat–Wilson syndrome + HSCR	Dominant	–
SOX10	Waardenburg-Shah syndrome	Dominant	–
PHOX2B	Haddad Syndrome (CCHS + HSCR)	Dominant	–
	Neuroblastoma + HSCR	Dominant	–
KIAA1279	Goldberg–Shprintzen syndrome + HSCR	Recessive	–
NRG1[b]	Isolated	Dominant, incomplete penetrance	6%

[a] % Of isolated HSCR patients with coding region mutations in these genes

[b] Unpublished data

These characteristics are consistent with the expression of a HSCR gene subject to modification by other loci [29, 30], perhaps as a result of cross-talk between signaling pathways (see below).

RET receptor tyrosine kinase pathway

The RET protein, functions as a receptor for the Glial cell line-Derived Neurotrophic Factor (GDNF) family. Receptor–ligand interactions are mediated by RET co-receptors (GDNF-Family Receptors-α; GFRα) [31–33]. Activation of the RET receptor by these neurotrophic factors is essential for the migration and differentiation of NCCs into enteric neurons [20, 34, 35]. The correct development of the ENS therefore depends on the ability of these neurotrophic factors to activate RET, on the ability of the RET receptor to transduce the signal, and on the competence of the intracellular machinery to elaborate a response (Figs. 1 and 2).

RET proto-oncogene and HSCR

The *RET*, also known as *RET* proto-oncogene, is composed of 21 exons that encode the RET receptor tyrosine kinase protein, a cell-surface molecule that transduces signals. Germline mutations in *RET* are responsible for four unrelated disorders in humans, HSCR, multiple endocrine neoplasia; type IIB (MEN2B), multiple endocrine neoplasia; type IIA (MEN2A), and medullary thyroid carcinoma (MTC) [36]. In PTC, somatic *RET* rearrangements are the most commonly detected genetic alterations. No germline mutations are found in PTC patients.

The RET is a single-pass membrane protein with two main domains: extracellular (EC) and intracellular (IC).

Ligand and co-receptor complexes interact with the EC domain of two RET molecules inducing their interaction with various intracellular signaling proteins [37]. Activating mutations in the *RET* gene cause MTC, MEN2A, and MEN2B, whereas inactivating mutations lead to HSCR [38]. A large number of *RET* mutations have been reported in HSCR patients. In general, *RET* gene mutations affecting the RET EC domain alter the folding of the protein impairing its maturation [39, 40]. *RET* mutations affecting the RET IC interfere with the binding to intracellular signaling proteins [40–42]. Mutations in any intron/exon boundary affecting the splicing consensus sequences can alter mRNA processing. Remarkably, silent mutations (i.e., the mutation does not result in an amino-acid change in the protein) in any of the *RET* exons can also affect mRNA processing [28]. Unlike in MEN 2, *RET* mutations causing HSCR are, with a very few exceptions [29], scattered throughout the gene, affecting indiscriminately any part of the RET protein. However, many *RET* mutations have not been functionally tested and it is very difficult to discern between truly deleterious substitutions and non-deleterious ones. A recent study based on the alignment of the human

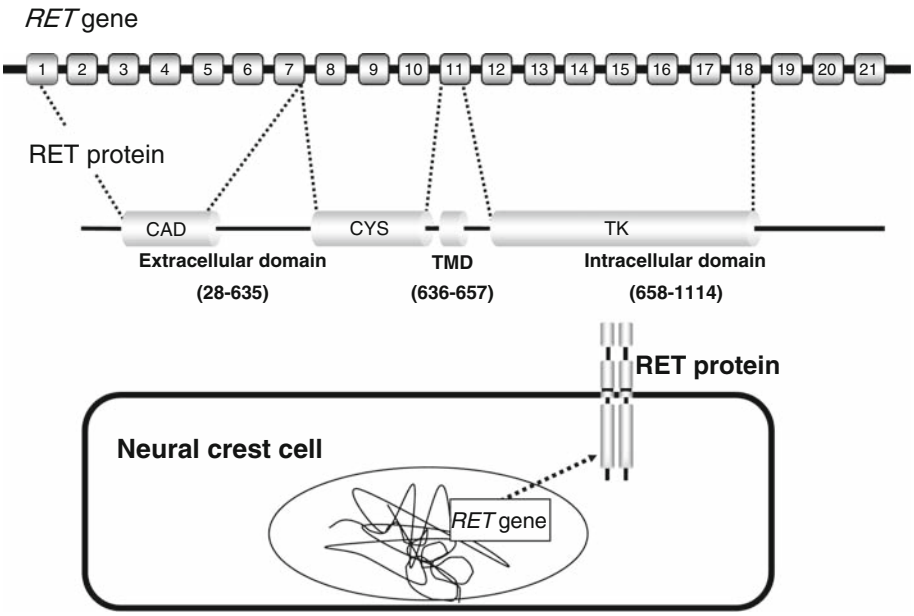

Fig. 1 Schematic drawing showing the *RET* gene and the RET protein receptor (adapted from [1]) and its localization on the neural crest cells. Exons of *RET* gene are indicated with *numbered boxes* and *doted lines* indicate the domains of the protein they encode. Different domains of RET receptor are indicated with cylinders. The extracellular part of RET contains cadherin domains (CAD) and the intracellular part of RET contains two tyrosine kinase domains (TK). TMD, transmembrane domain. In brackets, amino-acid residues

RET protein with 12 orthologous sequences provides an insight on the prediction of the effect of the *RET* mutations [43].

Mutations in the coding regions of *RET* and other HSCR genes (see below) only account for over 50% of the familial cases, and between 7% and 35% of the sporadic cases [44–59]. Intriguingly, genetic-linkage analyses in HSCR families keep implicating the 10q11 chromosomal region (*RET* locus) even though no *RET* coding regions can be found [29, 30]. This, besides confirming the central role of *RET* in HSCR, also indicates that mutations in non-coding (regulatory) regions *RET* remains to be found. Mutations in regulatory regions can alter the DNA binding sites of transcriptional regulators and reduce or abolish the expression of *RET*.

In addition to *RET* mutations, a possible role in HSCR has been attributed to several single nucleotide polymorphisms (SNPs; variations in the DNA present in >1% of the population) of the *RET* gene since they have been found associated with the disease (the frequency of the DNA variation varies significantly between patients and controls) [60–63], indicating that may indeed play a role, although subtle, in the disease. The current data indicates that these *RET*-SNPs could act as modifiers by modulating the penetrance of mutations in other HSCR genes and possibly of those mutations in the *RET* gene itself [64–68]. It has been shown that HSCR-associated *RET*-SNPs in the promoter region [69–71] and in intron 1 [72–74] quantitatively affect the expression *RET*. In particular, the *RET* promoter SNPs associated with HSCR disrupt the binding site of the transcription factor NKX2.1 (previously TTF-1; thyroid transcription factor 1).

It is worth noting that the overall frequencies of the HSCR-associated *RET*-SNPs and haplotypes are significantly higher in Chinese, not only in patients but also in the general population [63, 74], possibly explaining the higher incidence of HSCR in Asians when compared to Caucasians [2].

GDNF-family ligands and their receptors (GDNF-family receptors-α; GFRα)

The relevance of RET signaling pathway in the ENS development and the phenotypic similarities between *Ret*, *Gdnf*, and *Gfrα*-1 knock-out mice [20, 75–77], prompted an extensive mutation screening of the genes encoding the GDNF family of ligands—*GDNF*, neurturin (*NTN*), artemin (*ARTN*), and persepin (*PSPN*)—and of the genes encoding their co-receptors (*GFR*-α1–4) and adaptors. However, mutations in those genes have been identified in a very small number of patients and generally co-segregate with mutations in *RET* [78–82]. Screening of patients affected with idiopathic slow transit constipation and megacolon revealed no mutations associated with the phenotype [83]. Also, none of the of mutations identified in the *GDNF* gene is likely "per se" to cause HSCR, although it is thought they can contribute to disease via interaction with other susceptibility loci [33, 84–86]. Surprisingly, *RET* is virtually the only target of HSCR mutations in the RET signaling pathway. It is possible that homozygous mutations in the GDNF family of ligands were lethal, and that these ligands, particularly GDNF, may have other functions not mediated by RET, and therefore come under selective pressure against mutations.

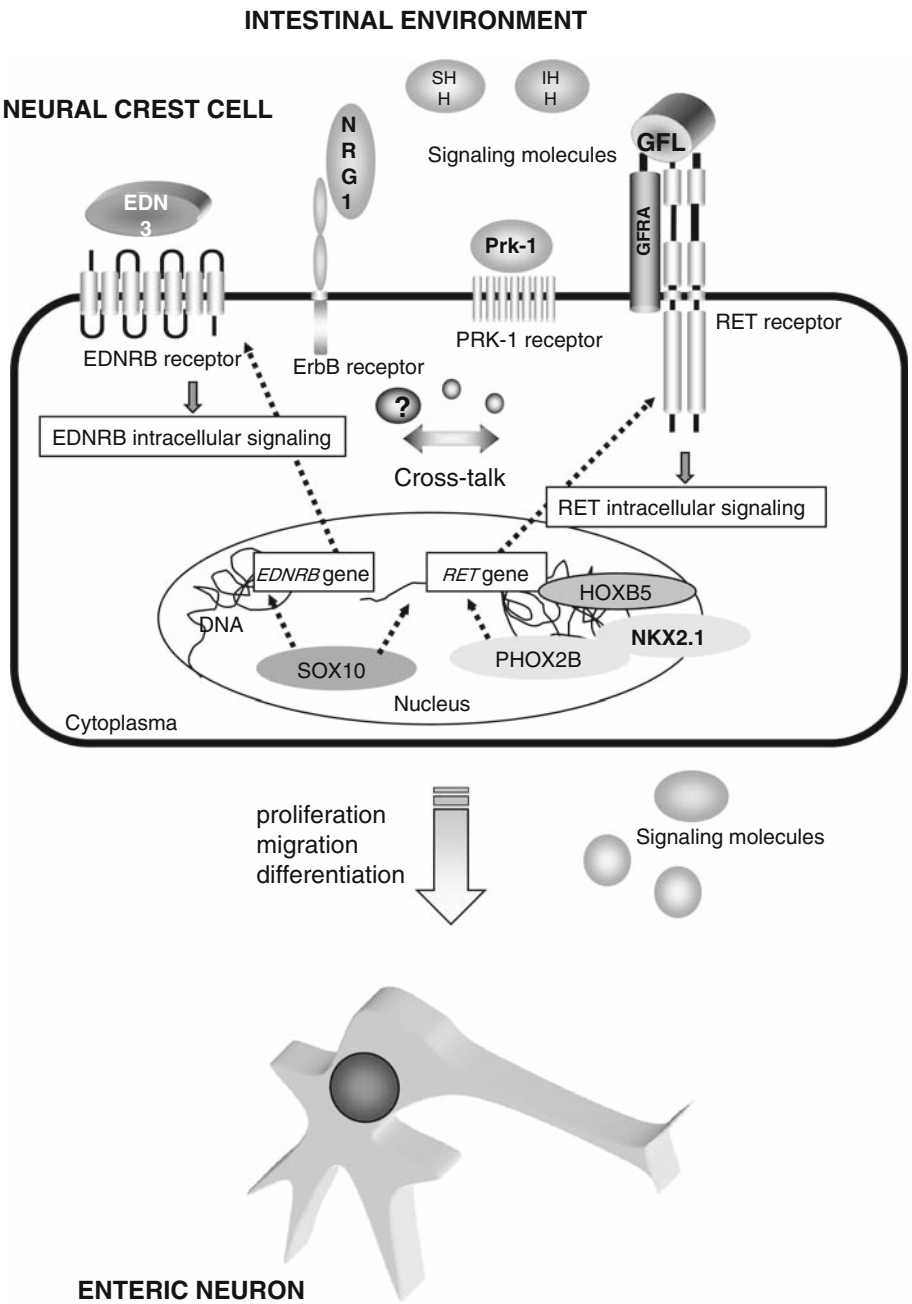

Fig. 2 Schematic representation of the network of interactions that govern the development of the enteric nervous system (adapted from [1]). Expression of the *RET* gene (regulated by transcription factors) leads to the formation of the RET protein which functions as a receptor for the Glial cell line-Derived Neurotrophic Factor family (GFL) through co-receptors (GFRA). Activation of the RET receptor by these signaling molecules of the gut environment activates the intracellular machinery necessary for the migration, proliferation, and differentiation of neural crest cells into enteric neurons. Similarly, the EDNRB receptor is activated by EDN3 and initiates a series of events that will also regulate the development of the enteric nervous system in conjunction with the RET pathway. The Prok-1 and NRG1 pathways, thought to be involved in the ENS development, are also represented. *Double head arrows* indicate interrelation between pathways. Transcription factors involved in *RET* regulation are pictured in the nucleus. *Question marks* represent yet unidentified molecule members of the network (see section "Modifying genes and interaction between signaling pathways" in the text)

HSCR genes involved in the transcriptional regulation of *RET*

Because of the key role played by *RET* in HSCR, mutations in genes known to be involved in the regulation of *RET* are likely to give rise to phenotypes involving aganglionosis. Not all the genes involved in the regulation process are known and the precise mechanisms of regulation remain unclear. Thus, further investigation on the molecules involved in *RET* regulation is likely to shed more light to the mechanisms underlying HSCR. In these respect, several *RET* regulators have been scrutinized.

SOX10

The *SOX10* gene encodes a transcriptional regulator, the expression of which is essential for the development of cells in the NCC lineage, including melanocites and enteric neurons. During the development of the ENS, the SOX10 protein physically interacts and cooperates with another transcriptional regulator (PAX3) to activate transcription of *RET* [35, 87, 88]. Importantly, *Sox10* also interacts with *Ednrb* in mice [89, 90].

The significance of the *SOX10* gene in HSCR was revealed through the study of a mouse model of HSCR

(*Dom*) fortuitously generated at the Jackson Laboratory [91]. The molecular defect in *Dom* mice was a mutation in the *Sox10* gene [92–95]. Heterozygous *Dom* mice presented with distal colonic aganglionosis and localized hypomelanosis of the skin and hair (features similar to those in Waardenburg's syndrome), which indicated that neural crest-derived melanocytes and enteric neurons were affected in *Sox10* mutants. *Dom* homozygous mice were embryonic lethal.

In humans, heterozygous *SOX10* mutations have been identified in patients with Waardenburg-Shah syndrome (WS4; HSCR type 2). Patients with WS4 display a combination of the features of Waardenburg syndrome (wide bridge of the nose, pigmentary abnormalities, and cochlear deafness) and HSCR [92, 96–102]. *SOX10* mutations also lead to Yemenite deaf-blind hypopigmentation syndrome and other myelin deficiencies [103]. To date, no *SOX10* mutations have been found in patients presenting aganglionosis as an isolated trait. *SOX10* is therefore unlikely to be major gene in isolated HSCR. Interestingly, and despite the absence of genetic mutations, abnormal *SOX10* gene expression can be observed in aganglionic intestine of isolated HSCR patients [102, 104].

PHOX2B

The paired mesoderm homeobox 2b gene (*PHOX2*) encodes a transcription factor which is involved in the development of several noradrenergic neuron populations. In mice homozygous disruption of the *Phox2b* gene results in absence of enteric ganglia, a feature reminiscent of HSCR. Furthermore, there is no *Ret* expression in *Phox2b* mutant embryos indicating that regulation of *Ret* by *Phox2b* could account for the failure of the ENS to develop [105, 106]. All these observations make the *PHOX2B* a candidate gene for HSCR. Interestingly, a chromosomal alteration involving the deletion of the *PHOX2B* locus has been described in a patient with syndromic HSCR (developmental delay, severe hypotonia, facial dysmorphism, and short-segment aganglionosis) [107]. This suggests that *PHOX2B* haploinsufficiency may predispose to HSCR. A SNP of the PHOX2B gene has been found to be associated with HSCR and importantly, the interaction between *PHOX2B* and *RET* HSCR-associated SNPs increases susceptibility to HSCR [108, 109].

PHOX2B represents the first gene for which germline mutations predispose to neuroblastoma [110–112] and is the major locus for congenital central hypoventilation syndrome (CCHS, Ondine's curse) [113, 114]. In particular, 25–30% of the CCHS patients are affected with aganglionosis (Haddad syndrome) [115]. Intriguingly, both neuroblastoma and CCHS are frequently associated with HSCR [8, 116, 117]. Taken together these data support the contribution of *PHOX2B* to HSCR.

HOXB5

Recently, using transgenic mouse technology, it has been shown that the transcription factor Hoxb5 may contribute to HSCR by interfering with the regulation of the *Ret* gene. A fraction of Hoxb5 mutant mice presented with reduction of ganglia (hypoganglionosis) and slow peristalsis and *Ret* expression was markedly reduced or absent. In addition, *HOXB5* SNPs were found associated to HSCR in Chinese patients. All these data suggest that DNA alterations in *Hoxb5* contribute to the etiology of HSCR [118, 119].

As all *HOX* genes have a major role in gut development (the enteric *Hox* code), the implications of alterations in other gene members of the human *HOX* clusters was also investigated in Chinese HSCR patients. Genetic interaction was found among SNPs in two *HOX* loci (5′-*HOXA13* and 3′UTR-*HOXB7*) and the most HSCR-associated *RET*-SNP, suggesting that the interacting *HOX* loci may affect the penetrance of the *RET*-risk locus [120].

NKX2.1

It was shown later that *RET* transcription was decreased due to alteration of the NKX2-1 transcription factor binding site by two HSCR-associated *RET* promoter SNPs (see above), the *NKX2-1* gene was investigated for mutations in HSCR patients. Direct sequencing of 188 Chinese and 70 Caucasian patients revealed the presence of a Met3Leu mutation in two Caucasian patients. This mutation reduced the activity of the *RET* promoter and it is thought to contribute to HSCR by affecting the *RET* expression through defective interactions with other transcription factors [71, 121].

Endothelin type B receptor pathway

The endothelins are a family of three signaling peptides (EDN1, EDN2, and EDN3) that can act on two subtypes of G protein-coupled receptors, termed endothelin-A and endothelin-B receptors (EDNRA and EDNRB) (Fig. 2). The endothelins are synthesized as much larger proteins which are cleaved by the endothelin converting enzyme (ECE-1) to produce an active peptide. The human genes coding for these proteins are *EDN1*, *EDN2*, *EDN3*, *EDNRA*, and *EDNRB*, respectively.

The involvement of the endothelin type B receptor pathway in the pathology of HSCR was demonstrated when a targeted disruption of the mouse endothelin-B receptor gene (*Ednrb*) resulted in an autosomal recessive phenotype

with aganglionic megacolon and white spotting of the coat [122]. A targeted disruption of the mouse endothelin-3 ligand (*Edn*3) gene produced a similar recessive phenotype of megacolon and white coat spotting [123]. These data indicated an essential role for the members of the EDNRB pathway in the development of two neural crest-derived cell lineages, enteric neurons and epidermal melanocytes. EDNRB signaling not only regulates migration of the ENS progenitors but can also modulate the response of NCCs to GDNF [6, 124]. This provides evidence of interaction between two different pathways implicated in the pathogenesis of HSCR.

EDNRB gene and HSCR

The occurrence of multiple cases of both isolated HSCR and WS4 in an inbreed Old Order Mennonite community facilitated the mapping of another major HSCR susceptibility gene to the chromosomal region 13q22 [125, 126]. The gene identified on 13q22 was the endothelin type B receptor (*EDNRB*) which had a mutation that resulted in the W276C amino-acid change in the protein. In contrast to the recessive, fully penetrant defects in the rodent model, this human mutation was neither fully dominant nor fully recessive. The homozygous W276C mutation (CC) was more penetrant than the heterozygous (WC), and that penetrance was sex dependent. Individuals homozygous (CC) presented with WS4 features, while those with the heterozygous mutation (WC) presented with isolated HSCR. Furthermore, some family members with no W279C mutation were clinically affected. This implied the presence of additional predisposing genes among this closely-related group. In fact, a genetic modifier of HSCR found among the group was mapped to chromosome 21q21 [125]. Subsequent *EDNRB* mutation analyses conducted on both isolated HSCR and WS4 patients revealed other *EDNRB* mutations with similar genetic behavior to W279C. Homozygous *EDNRB* mutations were associated with WS4 [127, 128] and heterozygous mutations with isolated HSCR [58, 59, 129–134]. Overall, *EDNRB* mutations account for 5% of the isolated HSCR phenotype. Functional analyses of *EDNRB* missense mutations showed impairment of the intracellular signaling [132, 135, 136]. *EDNRB* mutations found this far, are mainly inherited from unaffected parents, and associated with short-segment aganglionosis.

EDN3 and *ECE-1* genes

HSCR patients are being screened for mutations in the human *EDN3* because of the HSCR-like phenotype presented by mice with disruption of the *Edn*3 gene. To the best of our knowledge, very few *EDN3* mutations have been characterized in HSCR patients. With very few exceptions [94], heterozygous *EDN3* mutations are associated with isolated HSCR [137, 138] and homozygous mutations with WS4 [139, 140].

Again a knock-out mouse model provided evidence of the involvement of another member of the EDNRB pathway, *ECE-1*, in HSCR [141]. The gene encodes the endothelin converting enzyme (ECE-1), which cleaves the large inactive endothelins into smaller 21 amino-acids active endothelins. Mice carrying an *Ece-1* null mutation (no protein is synthesized) present with craniofacial and cardiac abnormalities, absence of melanocytes, and absence of the enteric neurons in the distal gut. This phenotype is similar to that presented by mice with mutations in the genes encoding other members of the EDNRB pathway (*EDN3* and *EDNRB*). To date, only one mutation in the *ECE-1* gene has been found and the patient was affected with syndromic HSCR [142].

As a general rule, severe homozygous mutations in genes involved in the EDNRB pathway are mainly associated with WS4 (absence of enteric ganglia and epidermal melanocytes, both neural crest cell derivatives). The association of homozygous mutations with WS4 and heterozygous mutations with isolated HSCR may indicate that melanocytes and enteric ganglia differ in sensitivity to the varying levels of EDNRB signaling [143].

The restriction of aganglionosis to the distal colon in mice with EBNRB and EDN3 deficiency and the fact that HSCR patients with mutations in these genes mainly present with short-segment aganglionosis suggested that the EDNRB signaling pathway is only required during the later stages of the colonization of the colon [144, 145]. However, it has recently been shown that the EDNRB signaling pathway is required for the colonization of both colon and small bowel [124]. The colonization process is subject to distinct spatial and temporal signaling requirements, and at some stage EDNRB signaling enhances the ability of the NCCs to migrate into the distal bowel [6].

ZFHX1B (previously SIP1)

The observation of a translocation involving the *ZFHX1B* locus (2q22) in a patient with HSCR disease, microcephaly, mental retardation, epilepsy and characteristic facial appearance, led to the first documentation of *ZFHX1B* involvement in this syndromic form of HSCR [146]. Other chromosomal abnormalities involving 2q22 and mutations in the *ZFHX1B* gene are being described in patients with similar clinical features (recently named Mowat–Wilson syndrome) although not all of them present with aganglionosis.

ZFHX1B encodes a transcriptional repressor (SIP1, Smad interacting protein 1), which interacts with several

members of the Smad family. Some Smad proteins act as tranducers in signaling cascades critical to embryogenesis. It is not yet known how mutations in *ZFHX1B* may result in defects of the ENS, although it is tempting to speculate on a possible functional link between SIP1 and the signaling pathways currently known to be essential for the ENS development. As in *SOX10*, no mutations have been found in patients with isolated HSCR, and therefore *ZFHX1B* is unlikely to be major gene in non-syndromic HSCR [147–149].

KIAA1279

By homozygosity mapping, a novel locus on 10q21.3-q22.1 for Goldberg–Shprintzen syndrome (GOSHS) was identified in an inbreed family. Phenotypic features of GOSHS in this inbred family included microcephaly, polymicogyria, and mental retardation, as well as HSCR. Homozygous missense mutations were identified in the KIAA1279 gene at 10q22.1 [27]. This finding established the importance of *KIAA1279* in both enteric and central nervous system development although the role of *KIAA1279* is not yet understood. No mutations in this gene have been found in isolated HSCR patients.

Modifying genes and interaction between signaling pathways

As indicated above, the successful colonization of the gut by the ENS precursors depends on a coordinated and balanced network of interacting molecules (Fig. 2). Conceivably, there should be a functional and genetic link among these molecules for them to interact. The mechanisms underlying these interactions may help to explain the complexity of the HSCR phenotype and resolve puzzling genetic observations, e.g., that in some cases more than one mutated gene is needed to produce the phenotype, while (conversely) healthy individuals exist with mutations in HSCR genes. Interaction between pathways requires not only coordination among the pathway members but also with those molecules that mediate their interaction.

The RET and EDNRB signaling pathways were initially thought to be biochemically independent. However, the identification of a HSCR patient carrying both a *RET* mutation inherited from the healthy mother and a *EDNRB* mutation transmitted by the healthy father suggested that these two pathways were related, and indeed, that more than one mutation was needed for the manifestation of the phenotype. The latter is especially true when the effect of the mutation on the protein function is not severe [28]. The genetic interaction between mutations in *RET* and *EDNRB* in HSCR was verified in 2002 in an association study conducted on 43 Mennonite family trios segregating the W276C mutation in the *EDNRB* described above [64]. The study demonstrated the joint transmission of both the W276C mutation in *EDNRB* and HSCR-associated *RET*-SNPs in affected individuals. The combination of these two genotypes increased the penetrance of the W276C mutation and therefore the risk to disease. Genetic interaction between *RET* and *EDNRB* pathways has also been demonstrated in mice [64, 124, 150]. The fact that two mutated genes are needed for the manifestation of the disease also implies functional interaction. EDN3 and GDNF seem to have a synergistic effect on the proliferation of the undifferentiated ENS progenitors and an antagonistic effect on the migration of differentiated enteric ganglia. It appears that EDN3 has a variable distribution along the developing gut with differential effects on processes regulated by the RET. It has been firmly established that the interaction between these signaling pathways controls the ENS development throughout the intestine [6, 124]. However, both pathways have to be integrated by additional molecules or "mediators". Protein kinase A has been suggested as a key component of the molecular mechanisms that mediate and link the RET and EDNRB signaling pathways [124]. Also, in mice, *Sox10* has been shown to act on both *Ret* and *EdnrB* genes [26, 87–90] further linking these two signaling pathways. All the molecule members of these pathways/networks are not known. This means that genes involved in the development of the ENS still await discovery, and that DNA alterations in multiple genes have the potential to combine and hinder a phenotype. Obviously, defective functioning of these still unknown "mediators" could modify the outcome of the development of the ENS. RET and EDNRB are central to the genesis of HSCR but little is known about the influence of the genetic background. Unknown loci (that could encode protein members of the RET and EDNRB pathways) can modify *RET* expression and act as a disease promoting or suppressing genes (modifiers). A linkage study conducted on 12 HSCR families enriched for L-HSCR form, demonstrated linkage to *RET* in all but one family. However, 6 out of the 11 families linked to *RET*, also showed linkage to the 9q31 locus. Interestingly, no severe *RET* mutations were detected in those 6 families. The effect of a mutated gene in the 9q31 region is thought to be required to produce the HSCR phenotype in those individuals bearing weak *RET* mutations [30]. A genome-wide scan on 49 S-HSCR families detected linkage to three chromosomal regions, 10q12, 3p21, and 19q12. Since S-HSCR did not segregate in the absence of *RET,* the authors suggested that 3q21 and 19q12 loci are *RET*-dependent, therefore, modifiers of the *RET* expression [29]. A recent study on the 3p21 chromosomal region narrowed down a HSCR-associated region

comprising three genes involved in neurological phenotypes [151]. Other examples of interaction include those reported between RET and genes of the NTF3/NTRK3 signaling pathway, which also plays an essential role in the ENS development [152].

These studies emphasize the central role of *RET* in all forms of HSCR (short and long) and explained the non-Mendelian inheritance of the disease. Also, they suggest that the genetics of long- and short-segment HSCR may depend on the effects of the different *RET* modifiers.

Other HSCR candidate genes

Mouse models and syndromes associated with HSCR provide insights into the genes that may share a common signaling pathway involved in the development of the ENS and therefore in the pathogenesis of HSCR. It is therefore logical to study the possible contribution of these genes to HSCR. This can be illustrated by the example posed by the Indian hedgehog gene (*IHH*). The hedgehog gene family encodes a group of secreted signaling molecules that are essential for growth and patterning of many different body parts of vertebrate and invertebrate embryos [153]. In particular, Ihh signaling controls growth of bones and is required for the proper development of the ENS and the intestinal stem cell proliferation and differentiation [154]. *Ihh* mutant mice present with dilated colon with abnormally thin wall, and enteric neurons missing from parts of the small intestine and from the dilated regions of the colon. This HSCR-like phenotype is observed with an incomplete penetrance of 50%, which suggest that interaction with other factors will be required for full expression of the phenotype. These features are strikingly similar to those observed in HSCR patients. In humans, mutations in the *IHH* gene (2q33-35) are associated with congenital limb malformations [155] and other skeletal dysplasias [156] some of which are also seen in some HSCR patients [3, 4]. Yet, *IHH* mutation analysis in over 60 HSCR patients with no mutations in the HSCR genes described so far, reveal no coding region mutations that could account for the disease [157].

Similar results were obtained for the human *L1CAM* gene. This gene encodes the L1 cell adhesion molecule involved in the development of the nervous system [158]. Mutations in *L1CAM* are linked to a recessive form of congenital hydrocephalus [159]. The detection of *L1CAM* mutations in individuals with congenital hydrocephalus and HSCR [160, 161] and the fact that *L1CAM* maps to chromosome Xq28, (which could account for the higher penetrance of HSCR in males) encouraged the mutational screening of this gene in isolated HSCR patients [162]. Although no coding region mutations were identified, it was hypothesized that L1CAM-mediated cell adhesion may be important for the ability of ganglion cell precursors to populate the gut, and that the *L1CAM* gene could modify the effects of a Hirschsprung disease-associated gene to cause intestinal aganglionosis.

Genome-wide association studies (GWASs) and HSCR

Current technology allows the search of the whole genome for common variants affecting the incidence of a disease. Recently, our group conducted the first GWAS on HSCR patients through which we identified the neuregulin1 gene (*NRG1*) as a new HSCR susceptibility gene. NRG1 (a signaling protein) and its receptors (the ErbB family of tyrosine kinase receptors) are among the molecular regulators of the NCCs' development by promoting neuronal survival amid other biological functions. In this study, which was conducted on individuals of Chinese origin, besides the *RET*-SNPs, the strongest overall associations were found for two SNPs located in intron 1 of *NRG1*. Importantly, *NRG1* SNPs increased risk to disease in the presence of the *RET* HSCR-associated SNPs [163] implying genetic and most likely, functional interaction between these two genes. Studies are underway to further identify the *NRG1* variants that disturb the function of the gene (Garcia-Barceló and Tam et al. unpublished data).

Genetic counseling

In isolated HSCR, a relatively precise recurrence risk tailored to individual families could be estimated based on the estimates provided by Badner [164]. The highest recurrence risk is for a male sib of a female proband with L-HSCR [3]. Nonetheless, the reduced penetrance of the HSCR mutations makes it difficult to rationally predict and assess the risk to disease. Genetic testing is only performed on a research basis, and due to the advances of the surgical management of HSCR, its utility is questionable.

As outlined earlier, mutations in the *RET* proto-oncogene are also the underlying cause of the inherited cancer syndromes MEN2A, MEN2B, and FMTC. In HSCR patients, *RET* mutations are dispersed throughout the gene, while in MEN2A and FMTC patients, mutations are clustered in the cysteine codons of the *RET* extracellular domain (exons 10 and 11). Although HSCR and MEN2A are two different entities, occasionally they co-segregate in some families [165–169], and affected individuals carry a single mutation in exons 10 or 11. Importantly, *RET* mutations identical to those found in MEN2A have been detected in HSCR patients with no clinical symptoms of MEN2A [170, 171]. This means that some HSCR patients may be exposed to a highly increased risk of tumors, where HSCR patients carry

these tumor-specific mutations, exploration of the family history of MEN2A and periodic screening for tumors is advisable. In families segregating both MEN2A and HSCR, *RET* gene testing, tumor screening, and prophylactic thyroidectomy is also warranted.

Future directions

Although modern surgical procedures have already achieved a high success rate of HSCR treatment, a better understanding of the mechanisms involved in the disease pathogenesis would enable improved diagnosis, prevention, and treatment.

The major breakthrough in the study of the pathogenesis of HSCR has been the demonstration of the genetic and functional interaction between the RET and EDNRB signaling pathways and their establishment as key players in the development of the ENS. These important findings should lead to the study of the complete gene network that makes the genesis of the ENS possible. Regarding HSCR gene-discovery, additional GWAS and whole-genome sequencing of DNA from HSCR patients are currently underway.

The GWAS studies are aimed at the discovery of genes whose common variants (SNPs) are associated with the disease, as per the common-disease-common variant hypothesis, whereby common variants (present in >1% of the population) might contribute to susceptibility to common diseases. These disease-associated common variants have a modest contribution to risk. Thus, more disease-associated functional common variants are to be identified when the disease results from their summative effect. Importantly, several lines of evidence indicate that in addition to common variants/SNPs, deleterious rare variants also contribute to the multifactorial inheritance of complex diseases although their effect and mode of inheritance is usually masked by the effect of other disease-contributing genes. The genes in which disease-associated common variants are found are to be considered as candidates for the search of deleterious rare variants (mutations) [172, 173]. Indeed, HSCR constitute a perfect example of the above. The *RET* gene contains high-frequency common variants with modest effects as well as deleterious rare variants (mutations). The latter may be transmitted in an autosomal dominant manner with reduced penetrance giving rise to familial HSCR. Because mutations are rare in the population, they must be identified by sequencing in cases and controls in each study. Yet, the contribution of common and rare variants to a disease or in particular, to HSCR, is not necessarily independent. Common variants may act as modifiers of the mutation effects as exemplified with the *EDNRB* W276C mutation, whose penetrance is increased by the presence of *RET* HSCR-associated SNPs in the same individual.

For obvious reasons, conducting gene or protein expression analyses for the study of HSCR and other human diseases that result from gene dysfunction during development is not feasible. Mice can provide access to the study of human genes and proteins that have an equivalent in mice. Scientists are now resorting to the isolation individual mouse cell types by making use of stem cell research. This has allowed the study the specific requirements of the gut neural crest stem cells (NCSCs) [124, 174, 175]. A recent study has shown that Prokineticin-1 (Prok-1) can induce both proliferation and expression of differentiation markers of *Ret* deficient mouse NCCs, suggesting that Prok-1 may provide a complementary pathway to GDNF/Ret signaling during the ENS development. This indicates that Prok-1 crosstalks with GDNF/Ret signaling and probably provides an additional layer of signaling refinement to maintain proliferation and differentiation of enteric NCCs. If the behavior of the gut NCSCs is better understood, it may be possible to treat HSCR by transplanting NCSCs directly into the aganglionic gut. It will be intriguing to determine whether these NCSCs are able to mature and engraft in the forming gut and enhance bowel motility, and further research of this kind should yield new therapeutic approaches.

The HSCR phenotype may therefore be a consequence of the interplay and/or accumulation of both common and rare deleterious DNA variants in gene members of the pathways involved in ENS development, and despite the importance of RET, additional HSCR susceptibility genes exist and are currently being uncovered through both genetic and cell functional studies.

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