The Molecular Genetics of Hirschsprung's Disease

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5.1 Epidemiology and Genetics of HSCR

Hirschsprung's disease (HSCR), or aganglionic megacolon, is a classic example of a complex genetic disease, characterized by the lack of enteric ganglia in the submucosal and myenteric plexuses, along variable portions of the distal gut. Since it is caused by a premature arrest of the migration of neural crest cells along the hindgut, it is defined also as a neurocristopathy. The variable extent of aganglionosis correlates with severity of the disease, leading to a classification of HSCR into short- and long-segment phenotypes [1, 2]. S-forms include aganglionosis confined below the rectosigmoid junction (80% of patients), while L-forms (20% of patients) can extend below the splenic flexure (colonic forms, 9%), to the whole colon (total colonic aganglionosis, TCA, 5–10%), or up to the whole bowel (total intestinal). The disease is a congenital malformation occurring in 1 in 5,000 live births, with the highest incidence in Asian populations (2.8 in 10,000), intermediate in Afro-Americans (2.1 in 10,000) and Caucasians (1.5 in 10,000) and lowest in Hispanics (1 in 10,000). The male to female ratio is 4:1, and the sex imbalance is particularly evident for S-forms (ranging from 4.2 to 5.5 in S-form and from 1.2 to 1.9 in L-form aganglionosis) [1–3] (Table 5.1). A proportion of cases are familial (20%), but HSCR most commonly presents with a sporadic occurrence. Approximately 30% of patients show an association with other disorders such as chromosomal abnormalities (12%) or different neurocristopathies, and with a variety of additional isolated or syndromic anomalies [4]. In this respect, patients with Down's syndrome are at higher risk of HSCR (5%, vs 1/5,000 in the general population), suggesting that dosage-sensitive susceptibility gene(s) are located on chromosome 21. Interestingly, the sex ratio is balanced among patients with Down's syndrome [4].

While possible environmental effects on disease pathogenesis have not yet been demonstrated, the genetic component has been recognized since the 1960s on the basis of both increased recurrence risk for sibs of affected individuals as compared to the general population, the association with other genetic diseases and the existence of several animal models of colonic aganglionosis showing Mendelian inheritance.

Notwithstanding the clear hereditability, segregation analyses suggest a complex mode of inheritance. In particular, an autosomal dominant and an autosomal recessive or multifactorial models of segregation have been suggested for L- and S-forms, respectively (Table 5.1). The recurrence risk for siblings varies from 1% to 33% depending on gender and length of aganglionosis of the probands and the gender of the sibs. In particular, it is estimated to be 3% and 17% for S- and L-forms, respectively, and to be higher for female rather than male probands and for males rather than female sibs, attesting to the fact that HSCR is a sex-modified multifactorial disorder [2]. Finally, it is higher in multiplex families (Table 5.2).

Table 5.1 Genetic and epidemiological features of different HSCR phenotypes

	Short-segment	Colonic-segment	Long-segment
Affected (%)	79	11	10
Sex ratio (M:F)	4.9:1	1.6:1	1.9:1
Genetic model	Multifactorial or recessive	Dominant or additive	Dominant or additive
Heritability (%)[a]	87	100	100
Penetrance (%)[a]			
Males	17	37	66
Females	4	29	51
Sporadics (%)[a]			
Males	4	39	41
Females	0	21	13
New mutations (%)[a]	0	0	15

[a]Based on segregation models (dominant or additive for long- and colonic-segment and recessive or multifactorial for short-segment), according to Badner et al. [2]

Table 5.2 Risk of recurrence based on segregation models (dominant or additive for long- and colonic-segment and recessive or multifactorial for short-segment, according to Badner et al. [2])

Risk Recurrence	Short-segment		Colonic-segment		Long-segment	
	Male	Female	Male	Female	Male	Female
Risk to sib (%)						
male proband	5	1	10	7	11	8
female proband	6	2	13	10	23	18
Offspring (%)						
of affected male	0	0	11	9	18	13
of affected female	0	0	15	11	28	22
Risk to second-degree relatives (%)	0	0	5	4	4–9	3–7
Risk in multiplex families (%)	5–10	1–4	19	14	33	25

In summary, the most relevant factors providing evidence that underlines the complex genetics of the disease are:

1. High proportion of sporadic cases
2. Variable expressivity, depending on the length of gut involved
3. Incomplete and sex-dependent penetrance
4. Risk to siblings varying by gender, segment length and co-occurrence of nonenteric phenotypes

5.2 The *RET* Protooncogene

5.2.1 Identification of *RET* as the Major Disease Locus in Isolated HSCR

The protooncogene *RET* is the major gene responsible for HSCR, with *RET* mutations implied also in different pathologies: multiple endocrine neoplasia of type 2A (MEN2A) and 2B (MEN2B) and medullary thyroid carcinoma, both sporadic (MTC) and familial (FMTC). The starting point for the identification of *RET* mutations in HSCR was the observation in 1992 of a patient with total colonic aganglionosis carrying a de novo interstitial deletion of chromosome 10 (46, XX, del10q11.21) [5]. The presence of a gene responsible for HSCR located on chromosome 10 was confirmed by two independent linkage studies [6, 7]. Moreover, the co-occurrence of HSCR with MEN2 syndromes, which had already been mapped to 10q and found in association with *RET* gain-of-function mutations, proved *RET* as a good candidate for HSCR as well [8–10]. Finally, the description of two other interstitial deletions allowed the smallest region of overlap (sro) among the deleted chromosomes to be narrowed to an interval of less than 250 kb, where *RET* was the only

known and already cloned gene [11, 12].

The exon–intron organization of *RET* was therefore reconstructed, starting from the published cDNA sequence [13, 14] and by using a PCR-based approach [15]. This allowed DNA fragments flanking both sides of each exon to be sequenced, thus making possible the mutation screening of the whole coding region of the *RET* gene.

5.2.2 *RET* Gene Mutations

A variety of mutations of the *RET* protooncogene have been detected in HSCR patients including microdeletions, insertions, variants affecting the correct RNA splicing, nonsense mutations, and, above all, missense mutations (Fig. 5.1). To date, more than 100 different missense mutations have been described, with a recurrent mutation described in the Chinese population (R114H) [16]. De novo mutations can be found in approximately 16–65% of patients with a *RET* mutation, are associated with L-forms [17–22], and have been demonstrated in a limited number of patients to arise equally on both paternal and maternal chromosomes [21]. Mutations found in HSCR patients are scattered throughout the gene while in MEN2 syndromes and in MTC mutations occur in specific codons, among which the cysteine residues of the cys-rich extracellular domain are the most frequently affected [23–26]. Moreover, *RET* mutations in HSCR generally result in the loss-of-function of the protein due to misfolding, failure in transportation to the cell surface or suppression of its biological activity, and the identification of deletions also supports the haploinsufficiency effect in disease pathogenesis [26–29]. As well as the localization, this loss-of-function mechanism contrasts with the MEN2 pathogenesis in which gain-of-function, due to constitutive dimerization of the RET receptor or dysregulated activation of the tyrosine kinase activity, has been demonstrated [30–32]. Surprisingly, some MEN2A-typical *RET* mutations seem to result in both gain- and loss-of-function, since they have been detected in families presenting a certain degree of co-occurrence of MEN2A and HSCR [18–35].

Despite the central role played by *RET* in HSCR and the extensive mutation screenings performed by many groups in the last 10 years, the mutation rate remains quite low, and only about 50% of familial and 7–35% of sporadic cases (15–20% in most of the series) present with *RET* mutations [17–19, 22, 36]. Moreover, *RET* mutation frequency has been shown to be higher in TCA and the L-form than in the S-form, overall suggesting the effects of multiple genes which would work particularly in the least severely affected, and providing an explanation for the still very poor genotype–phenotype correlation in HSCR. It was initially supposed that such a limited mutation detection rate might have derived from pitfalls in the screening procedure. More recently it has become clear that this is not the case and in the majority of HSCR cases still await a clarification of the underlying pathogenetic mechanism. Several hypotheses have been advanced to explain these cases, such as:

1. The possible effect of neutral gene variants acting as low penetrant alleles
2. The presence of still undetected mutations in RET non-coding regions involved in either regulatory functions or transcript processing and maturation
3. The existence of another gene in the vicinity of the *RET* locus

5.3 Other Genes Involved in HSCR Pathogenesis

To date, molecular and genetic analyses have allowed eight other different HSCR susceptibility genes to be identified (*GDNF, NRTN, ECE1, EDN3, EDNRB, SOX10, ZFHX1B, PHOX2B*), and found to be related to both the *RET*-mediated signaling pathway and other cellular programs crucial for the normal development of the enteric nervous system (ENS). A role for the *KIAA1279* gene in HSCR etiology has also recently been hypothesized.

5.3.1 The *RET* Signaling Pathway

In 1996, *GDNF*, which is known to be a major survival factor for many types of neurons, was shown to be the *RET* ligand by both phenotypic similarities between *Ret*–/– and *Gdnf*–/– knockout mice [37–39], and Xenopus embryo bioassays [40]. *GDNF* is a TGF-β-related protein of 211 residues, proteolytically cleaved to a 134-residue mature protein that homodimerizes. To activate *RET*, *GDNF* needs the presence of the coreceptor *GFRA1* linked to glycosylphosphatidylinositol (GPI) [41, 42]. Four related GPI-linked coreceptors, *GFRA1–4* [43], and four related soluble growth factor ligands of *RET* have been identified, namely: *GDNF*, *NTN* [44], persephin (*PSPN*) [45] and artemin [46]. Specific combinations of these proteins are necessary for development and maintenance of both central and peripheral neurons, and all can signal through *RET*. Based on its crucial role in *RET* activation and the presence of aganglionosis in *Gdnf*–/– mice, extensive mutation screening has been performed, but only seven mutations have been described so far [47–53]. Absence of genotype–phenotype correlation and cosegregation of *GDNF* variants with *RET* mutations and with trisomy 21 have suggested a weak role for *GDNF* in HSCR pathogenesis [49]. Moreover, none of the five *GDNF* mutations tested in vitro can interfere with *RET* activation and consequently none is causative of HSCR per se [54, 55]. Among other *RET* ligands, only *NTN* has been found mutated in a patient with familial HSCR, where a *RET* mutation also cosegregates [56].

Finally, although *Gfra1* homozygous knockout mice are phenotypically very similar to *Ret*–/– and *Gdnf*–/– mice, no *GFRA1* mutations have been identified in HSCR

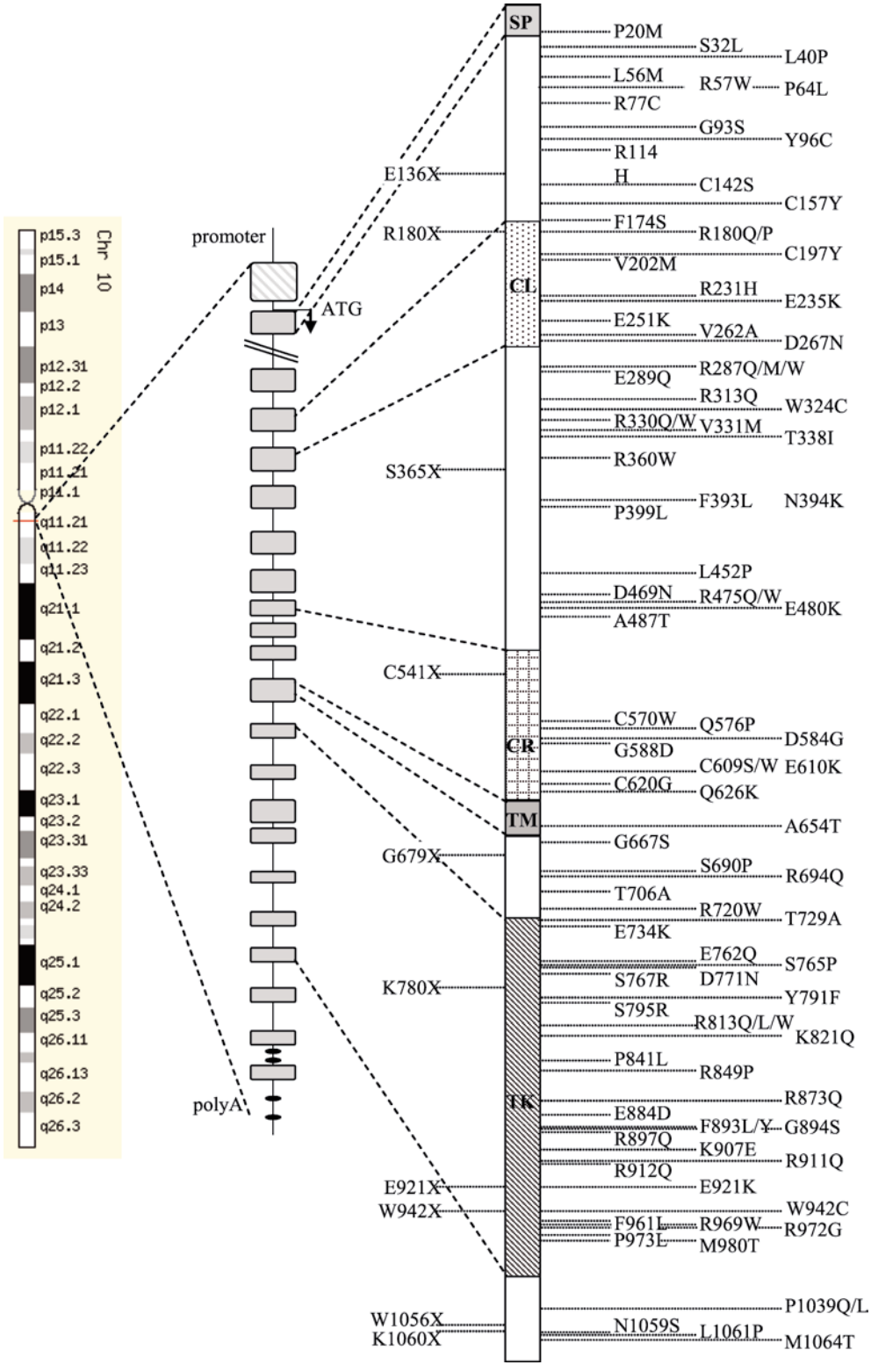

Fig. 5.1 Representation of chromosomal location, gene structure, protein domains and HSCR associated single nucleotide mutations of the *RET* protooncogene (*SP* signal peptide, *CL* cadherin-like, *CR* cysteine rich, *TM* transmembrane, *TK* tyrosine kinase)

patients in spite of extensive screenings performed to this end [57–60].

5.3.1.1 RET and GDNF Proteins in Normal and HSCR Gut

The early studies on Ret protein expression in mammalian tissues showed that this receptor tyrosine kinase (RTK) might be a receptor normally functioning in particular differentiation stages or restricted tissue lineages [61]. As expected from knockout mice, Ret mRNA is highly expressed in both the developing peripheral nervous system and the excretory system during mouse embryogenesis [62]. Successive studies, using immunohistochemical techniques on embryonic, infant and adult normal tissues from rats, showed Ret protein expression not on ly in the nervous system but also in acinal cells of the salivary glands, epithelial cells of the thymus, and follicular dendritic cells of the spleen and lymph nodes [63].

The first localization of the RET protein in the ENS was achieved in 1995 [64] through immunohistochemical studies using three different anti-RET protein antibodies, namely anti-RET R5, anti-RET C and anti-RET K [65]. In normal ganglia of control subjects, diffuse granular red-staining cells and some strongly positive ganglion cells were found, while in HSCR patients with complete deletion or stop codon mutations of the *RET* gene [64, 66] a markedly reduced staining was clearly observed (Figs. 5.2 and 5.3). This finding may support the hypothesis of loss of function due to reduced amounts of RET protein.

A possible deficit in the expression of GDNF protein in the ENS of HSCR patients with no *GDNF* mutations was searched for: the immunohistochemistry assay was performed in 30 HSCR patients and 10 control subjects with GDNF D-20, an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 186–205 mapping within the carboxyl terminal domain of human *GDNF* [51]. GDNF immunoreactivity was localized in the ganglia of the myenteric and submucous plexuses. In normal colon and in the ganglionic segment of HSCR, a strong granular red staining was obtained in the satellite elements and on the cellular membranes of the ganglion cells. In ganglionic intestine, GDNF-positive nerve fibers were not observed. The small ganglia of the hypoganglionic segment showed a reduced GDNF immunoreactivity when compared with the proximal normoganglionic segment. The muscular interstitium showed trunks of nerve fibers and persistence of some small cellular elements of glial origin that showed GFAP and S-100 protein immunoreactivity. GDNF immunoreactivity was absent in the aganglionic segment of HSCR. A deficit in the expression of *GDNF* in the distal aganglionic segment could be a cofactor in HSCR pathogenesis. The absence of GDNF in the distal hindgut could result in a missed or reduced autophosphorylation (activation) of the RET receptor in the absence of *RET* protooncogene mutations, causing enteric neuroblast migration arrest and HSCR.

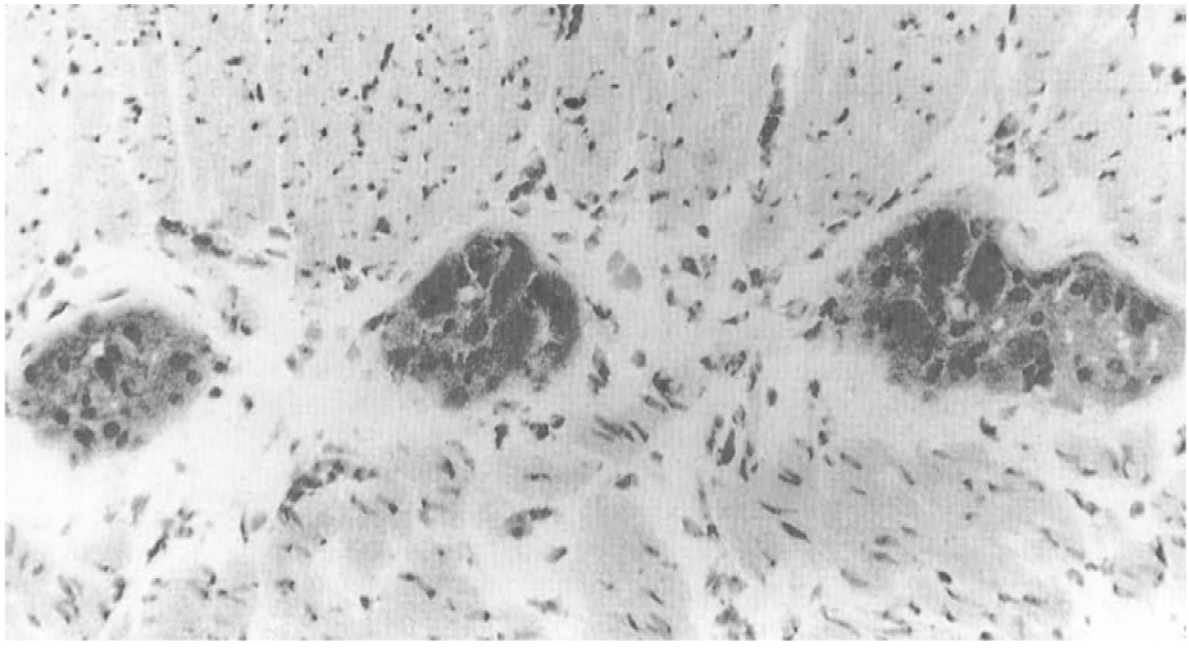

Fig. 5.2 Normoganglionic myenteric plexus. The ganglia of Auerbach plexus show a diffuse granular staining with anti-Ret K polyclonal antibody. Some strongly stained ganglion cells are present

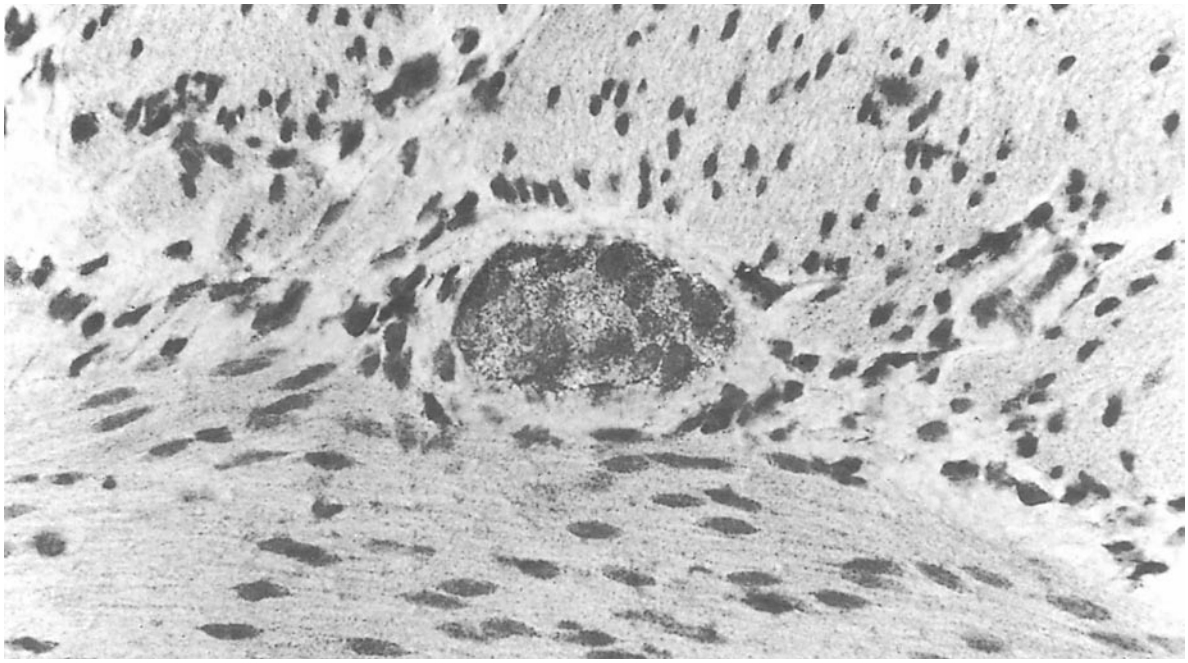

Fig. 5.3 Ultralong Hirschsprung's disease with complete deletion of the RET protooncogene. In the hypoganglionic segment, a ganglion shows a reduced content of Ret protein

5.3.2 The Endothelin Signaling Pathway

The critical role of the endothelin pathway in HSCR was demonstrated with the finding that *piebald-lethal* (s^l), a murine model of aganglionosis, is allelic to the endothelin receptor b (*Ednrb)* knockout mouse and harbors an *Ednrb* mutation (Table 5.3) [67]. Subsequently, an *EDNRB* missense mutation (W276C) was identified in a large inbred Old Order Mennonite community with multiple patients with HSCR [68–70].

However, the W276C mutation was neither necessary (since the presence of affected wild-type homozygotes in the pedigree) nor sufficient (nonaffected mutant homozygotes were found) to cause HSCR, and penetrance showed sex-dependence (more in males than in females) [68]. *Piebald-lethal* can be considered a mouse model for Shah-Waardenburg type 4 (WS4) in humans and, in agreement with this notion, some of the affected Mennonite individuals have pigmentary anomalies and sensorineural deafness in addition to HSCR [69]. This prompted a study of the *EDNRB* gene in WS4, and homozygous mutations in some families were found [71]. At the same time, an *Edn3* mutation was identified in the *lethal spotting* (ls) mouse, another natural murine model of human WS4 [72], and *EDN3* homozygous mutations were accordingly identified in patients (Table 5.3) [73, 74].

Both *EDNRB* and *EDN3* were screened in large a series of isolated HSCR patients. While *EDN3* mutations were seldom found [75], *EDNRB* mutations could be identified in approximately 5% of the patients [20, 76–78]. It is worth mentioning that these studies were able to demonstrate that penetrance of *EDN3* and *EDNRB* heterozygous mutations is incomplete in HSCR patients as de novo mutations are not observed and that S-HSCR is largely predominant. The observation of interstitial 13q22 deletions, encompassing the *EDNRB* gene, in HSCR patients makes haploinsufficiency the most likely mechanism for *EDNRB*-mediated HSCR development (Table 5.3).

Although *EDNRB* binds all three known endothelins (*EDN1*, *2*, *3*), the similarity of the phenotypes associated with both *Ednrb* knockout mice and *Edn3* knockout mice [67, 72] suggests that *EDN3* is the major ligand of *EDNRB*. Pre-proendothelins are proteolytically cleaved by two related membrane-bound metalloproteases giving rise to the mature 21-residue endothelins. Ece1 processes Edn1 and Edn3 and *Ece1* knockout mice show colonic aganglionosis in addition to craniofacial defects and cardiac abnormalities [79]. Accordingly, a heterozygous *ECE1* mutation has been identified in a patient with HSCR and associated craniofacial and cardiac defects (R742C) [80].

5.3.3 *SOX10*

Dominant megacolon (*Dom*) is a mouse model of human WS4, the homozygous *Dom* mutation being embryonically lethal [81]. The *Dom* gene is *Sox10*, a member of the

Table 5.3 Genes involved in Hirschsprung's disease (*CCHS* congenital central hypoventilation syndrome; *GOSHS* Goldberg-Shprintzen syndrome; *MEN2* multiple endocrine neoplasia type 2; *PCWH* peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome and Hirschsprung's disease; *WS4* Waardenburg syndrome type 4)

Gene	Map locus	Related syndromes	HSCR reported mutations	Animal model
RET	10q11	MEN2	Heterozygotes	Knockout
GDNF	5p13	–	Heterozygotes	Knockout, s[l]
EDNRB	13q22	WS4	Hetero/homozygotes	Knockout, s
EDN3	20q13	WS4	Hetero/homozygotes	Knockout
SOX10	22q13	WS4/PCWH	Heterozygotes	Dom
NTN	19p13	–	Heterozygotes	Knockout
ECE1	1p36	–	Heterozygotes	Knockout
SIP1	2q21–23	Mowat-Wilson	Heterozygotes	Knockout
PHOX2B	4p12	CCHS	Heterozygous deletion[a]	Knockout
KIAA1279	10q21.3–22.1	GOSHS	Homozygotes	–

[a] One patient reported [100]

SRY (sex-determining factor)-like, high-mobility group (HMG) DNA-binding proteins [82]. Both inherited and de novo heterozygous *SOX10* mutations have been identified in familial and isolated HSCR patients with WS4 [83–85] and, more recently, in a severe phenotype designated PCWH (*P*eripheral demyelinating neuropathy, *C*entral dysmyelinating leukodystrophy, *W*aardenburg syndrome and *H*irschsprung's disease) [86]. These two phenotypes, PCWH and WS4, are caused by two distinct molecular mechanisms. While all mutations have enhanced DNA-binding affinity, and potent dominant-negative activity, only the WS4 mutation activates the nonsense-mediated decay (NMD) while PCWH escapes it as the stop codon lies in the last exon [86].

5.3.4 *ZFHX1B*

The SMAD-interacting protein1 gene (SIP1), better named as *ZFHX1B*, located in 2q22, encodes a transcriptional corepressor of Smad target genes. In 2001, the gene was found to be mutated in Mowat-Wilson syndrome (MWS) by the cloning of two de novo translocation break points [87, 88]. MWS is a multiple congenital anomaly syndrome characterized by dysmorphic features, severe intellectual disability and microcephaly, and is commonly associated with congenital anomalies, including HSCR, heart defects, hypospadias, genitourinary anomalies, postnatal microcephaly, agenesis of the corpus callosum, severe mental retardation, short stature and facial dysmorphic features. The facial gestalt is so distinctive that the diagnosis can be suspected in patients with no congenital malformation [89, 90]. HSCR, which at first was considered a mandatory feature to suspect the diagnosis of MWS, is now described in about 60% of the patients only (see reference [91] for a review). The MWS phenotype is the result of de novo heterozygous deletions or truncating mutations of the *ZFHX1B* gene, suggesting that haploinsufficiency for *ZFHX1B* is sufficient to cause the disease phenotype. The study of the expression pattern of the gene in early embryonic and fetal stages in humans argues for a pleiotropic role of the gene [92]. *Zfhx1b* knockout mice do not develop postotic vagal neural crest cells, the precursors of the ENS that are affected in patients with HSCR, and they display a delamination arrest of cranial neural crest cells, which form the skeletomuscular elements of the vertebrate head [93]. This suggests that *Zfhx1b* is essential for the development of vagal neural crest precursors and the migratory behavior of the cranial neural crest in the mouse.

5.3.5 *PHOX2B*

The paired-like homeobox gene *PHOX2B*, located in 4p12, encodes a transcription factor (homeodomain protein) that has been regarded as a candidate gene in the

association of HSCR and congenital central hypoventilation syndrome (CCHS; Haddad syndrome). Indeed, the neuronal circuits of the autonomic nervous system that control vegetative functions have been shown to depend on the expression of the *Phox2b* homeodomain transcription factor as the neurons either fail to form or degenerate in mouse mutants null for *Phox2b* (paired-like homeobox 2B) [94, 95]. Heterozygous *PHOX2B* mutations, clustered in the C terminus of the gene, have recently been detected in 50–98% of patients affected by CCHS [96, 97], a very rare neonatal disorder characterized by an abnormal ventilatory response to hypoxia and hypercapnia owing to failure of autonomic respiratory control [98]. Subsequently, *PHOX2B* was reported to be the first susceptibility gene in TSNS (tumors of the sympathetic nervous system). Therefore, HSCR, CCHS and TSNS can be found in various combinations and can be ascribed to various *PHOX2B* gene mutations with some genotype/phenotype correlation. Although a deletion encompassing the *PHOX2B* gene has been described in a patient with HSCR, mental retardation and failure to thrive, there is only weak evidence to support a role of *PHOX2B* in the development of isolated HSCR [99, 100].

5.3.6 *KIAA1279*

The *KIAA1279* gene has been found to carry homozygous nonsense mutations in two different families diagnosed with Goldberg-Shprintzen syndrome (GOSHS) [101], a disorder characterized by microcephaly, mental retardation, facial dysmorphisms, and HSCR [102], clinically but not genetically similar to MWS.

The gene, mapped in 10q21.3-q22.1, encodes a protein with a still-unknown function, predicted to contain two tetratrico peptide repeats (TPRs) and likely involved in a variety of biological processes. In the two families, HSCR is present as a variable feature, while bilateral generalized polymicrogyria (PMG, a neuronal migration disorder resulting in malformation of the cerebral cortex) is constantly associated with the affected status. This has led to the hypothesis that the protein plays a central role in the development of both the peripheral and central nervous systems, possibly through binding HSCR- or PMG-associated susceptibility factors [101].

5.3.7 Interactions Between Pathways

Formation of the mammalian ENS requires a coordinated and balanced interaction of signaling molecules and transcription factors which play a critical role in the formation of normal enteric ganglia (Fig. 5.4). Failure of this integration leads to the absence of enteric ganglia and therefore to the HSCR phenotype. Two signaling systems mediated by *RET* and *EDNRB* have been identified as critical players in enteric neurogenesis.

RET and EDNRB signaling pathways were considered as biochemically independent until 1999 when the first genetic evidence was reported that some interaction was going on between the two signaling pathways, namely the description of an HSCR patient, heterozygote for weak hypomorphic mutations of both *RET* and *EDNRB* genes each inherited from one of the healthy parents [103]. Subsequently, two systematic genetic studies have sustained this hypothesis. First, a genome-wide association study in 43 Mennonite family trios and noncomplementation of aganglionosis in mouse intercrosses between *Ret* null and the *Ednrb* hypomorphic piebald alleles suggested the presence of epistasis between *EDNRB* and *RET* [104]. Second, by using two-locus noncomplementation of known mouse *Ret* and *Ednrb* mutations, it was demonstrated that compound genotypes of the two major HSCR genes *Ret* and *Ednrb*, which independently fail to yield intestinal aganglionosis, can result in an enteric defect in mice [105].

Moreover, developmental studies have shown that activation of *EDNRB* specifically enhances the effect of *RET* signaling on the proliferation of uncommitted ENS progenitors and that protein kinase A is a key component of the molecular mechanisms that integrate signaling by the two receptors [106].

SOX10 is involved in cell lineage determination and is capable of transactivating both *RET* and *MITF* synergistically with *PAX3* [107, 108] Moreover, the *Ednrb* transcript is absent or drastically reduced in *Dom*−/− and *Dom*+/− mice, respectively [109], due to either a direct effect of Sox10 or an indirect effect due to the common fate of the NC cell progenitors.

On the basis of the above discussion, a few conclusions can be drawn:

1. *RET* is the major HSCR gene with heterozygous mutations found in 50% of familial cases and 15–20% of isolated cases.
2. Penetrance of *RET* mutations is incomplete and sex-dependent.
3. Genotype–phenotype correlation is poor in isolated HSCR.
4. HSCR is genetically heterogeneous and can arise from mutations in distinct pathways.
5. Some patients with mutations in more than one HSCR susceptibility gene (*RET* + *GDNF, RET* + *NTN, RET* + *EDNRB*) are known.

These and other observations have confirmed the complex inheritance of HSCR disease. In this respect, some data have already been collected and used to reconstruct a preliminary picture of the different genetic components involved, as shown in the following paragraphs.

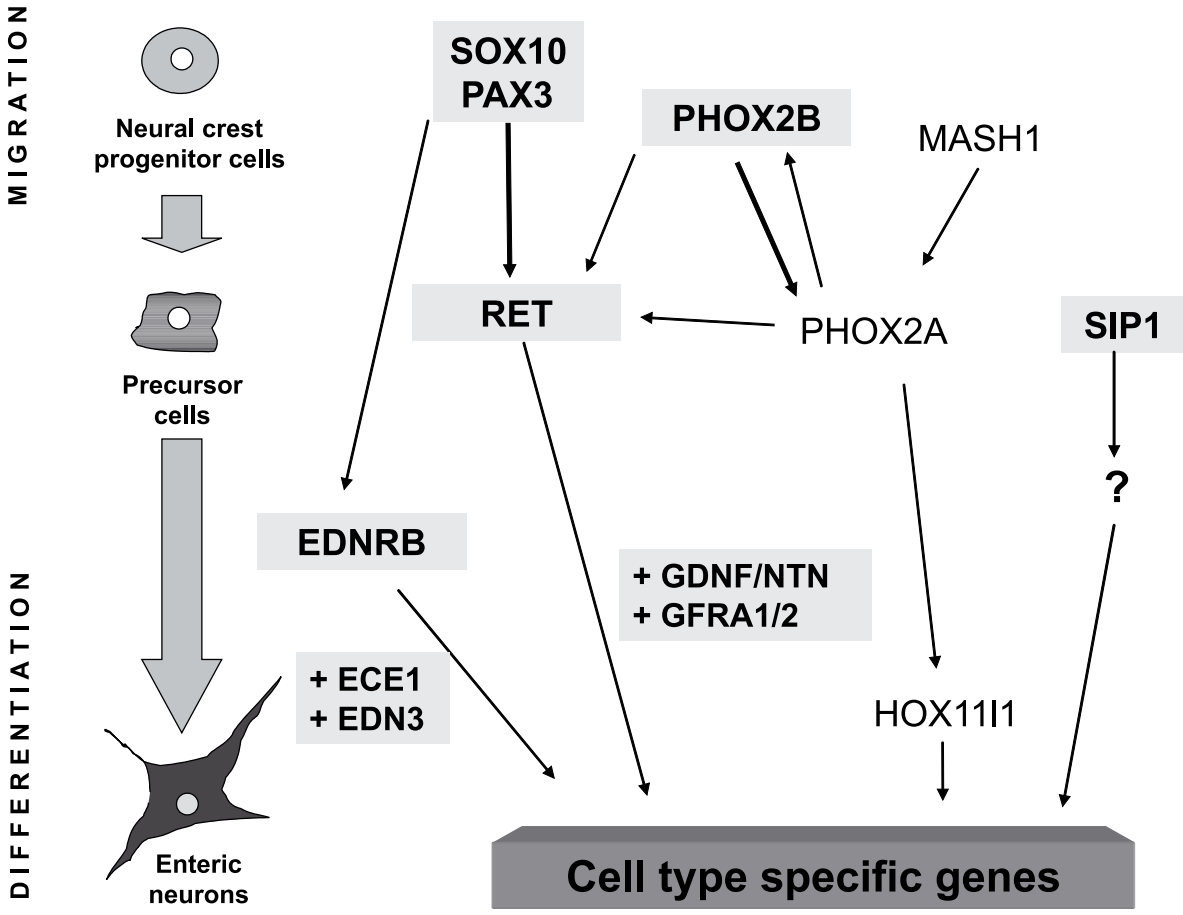

Fig. 5.4 Cascade of transcriptional activation in precursor cells of the ENS, as suggested by in vivo expression studies. *Bold arrows* indicate direct interaction demonstrated by specific functional assays

5.4 Genetic Analysis to Identify Other HSCR Loci

Linkage analysis

Linkage analysis in 12 vertical HSCR families with a large predominance of L-HSCR has shown linkage to the *RET* locus in all but one family [110]. Mutational analysis identified nonsense or missense mutations at highly conserved residues in six families, splice mutations in two families and noncoding sequence variations in three families. Linkage to a novel locus in 9q31 was identified only in families with no or hypomorphic *RET* gene mutations. Therefore, a severe *RET* mutation may lead per se to phenotypic expression by haploinsufficiency, while hypomorphic *RET* mutations would require the action of other mutations, probably located in an undiscovered gene in 9q31.

Sib-pair analysis

A sib-pair analysis in 49 families with S-HSCR probands [111] has shown that three loci, located on chromosomes 3p21, 10q11 and 19q12, are both necessary and sufficient to explain the incidence and sib recurrence risk in HSCR. A multiplicative risk across loci, with most affected individuals being heterozygotes for all three corresponding genes, seems the best genetic model. The HSCR susceptibility gene at the 10q11 locus is *RET* and the two other genes in 3p21 and 19q12 remain to be identified. Interestingly, marker analysis showed a significant parent-of-origin effect at, and only at, the *RET* locus, 78% of shared *RET* alleles being maternally derived, which could explain the sex difference in HSCR expression.

5.5 Additional Contribution of the *RET* Gene: SNPs and Haplotypes

The *RET* protooncogene is mutated in 50% of familial cases, 7–35% of sporadic cases, and up to 75% of L-HSCR, while other genes account for less than 5% of cases, either in syndromic patients or in combination with *RET* mutations in a few isolated HSCR cases. Moreover, as already anticipated, several genetic linkage analyses have shown that in over 90% of families HSCR is linked to the *RET* gene, even in the absence of clearly functional mutations of the coding region of the gene [110, 111]. This and other observations opened the new perspective of a different additional role of *RET* in HSCR onset, sustained by a still-unknown mechanism.

Several hypotheses have been proposed, such as an epistatic regulation of *RET,* requiring the interaction of several genes to produce the phenotype [20, 104], and/or specific *RET* SNPs (single nucleotide polymorphisms) or *RET* haplotypes, acting as either low penetrant alleles themselves or in linkage disequilibrium (LD) with an unknown susceptibility locus [112–116].

This latter possibility, in particular, has received increasing attention in the last 6 years, as attested by a new course of studies. Since 1999, several SNPs in the coding region of *RET* have been described as under- or over-represented in patients compared to controls [113, 114, 117–119], allowing the hypothesis to be advanced that common polymorphisms present in the general population and subsequently considered innocuous could be implicated in the pathogenesis of HSCR. Moreover, the involvement of *RET* polymorphisms has prompted the reconstruction of haplotypes and the study of their distribution within and among populations [104, 112, 116, 120–123]. In particular, a synonymous SNP in exon 2 (c.135G>A, A45A) and haplotypes comprising such a SNP, have repeatedly been shown in association with HSCR, and thus represent a sort of genetic marker of disease predisposition or increased recurrence risk [112, 116, 121, 122].

Recently, attention has focused on the 5' portion of the *RET* gene. Borrego et al., on the basis of the LD observed at several *RET* markers, have suggested the existence of a susceptibility variant in intron 1, in LD with an ancient low penetrant founder locus 20 to 30 kb upstream of SNP2 and related to the transcriptional activity of *RET* [115]. At the same time, Sancandi et al. described a three-locus haplotype named ACA, including the A variant allele of SNP2 and the A and C alleles of two novel SNPs identified in the *RET* promoter region, at −5 and −1 nucleotides from the transcription start codon, respectively. The ACA haplotype accounted, in the Italian population, for 62% of HSCR patients and only for 21.8% of healthy individuals [116] and, spanning from exon 2 up to the promoter, was defined by markers at the opposite end of the 23.5 kb long intron 1, and was thus consistent with the founding locus hypothesis just proposed. The association of this haplotype with the disease has been independently confirmed in other populations [121, 123], including the Chinese population, where the only haplotype found to be over-represented in sporadic HSCR patients included SNP2 [122] and the already known HSCR associated alleles of the promoter SNPs [124]. Notably, the SNP2 variant allele seems to display frequencies that correlate with the incidence of the disease in the different populations, being more frequent in Asiatics, lower in Caucasians and even lower in Hispanics, though no data are available yet to describe the distribution of the ACA haplotype in this latter population. The ACA haplotype probably extends from the 5'-UTR to at least intron 5 [115, 125], and has been suggested to act through an autosomal recessive or a dosage-dependent mechanism [123–127].

The direct role of the ACA haplotype, and especially of its single variants, is still disputed. Fitze et al., following in vitro experiments, hypothesized a direct role of the variant at −5 in determining a low level of *RET* expression [121]. In contrast, using a similar experimental approach, Griseri et al. showed that none of the promoter variants was functionally responsible for the association with HSCR and excluded a role of SNP2 in aberrant splicing. On the other hand, the same authors found that the whole ACA haplotype was associated with low in vivo *RET* gene expression, altogether denoting that the ACA haplotype could be in LD with a low-penetrance susceptibility locus, probably located in intron 1 [126]. Garcia-Barcelo et al. suggested that such discrepancies could be due to the different cell lines and lengths of promoter used in the transfection experiments, and, assuming that the promoter SNPs are located in a putative binding site for the transcription factor TTF-1, they found that the AC allele could decrease *RET* promoter expression by preventing TTF-1-induced trans-activation [124].

At present, the common belief that a frequent susceptibility variant could lie inside the long intron 1, in LD with the known HSCR predisposing *RET* haplotype is under investigation by several groups. To this purpose, the current approach involves a combination of comparative genomics between different species, to identify evolutionarily conserved regions likely relevant in gene expression, and genetic association studies [128]. Following such a strategy, Emison et al. have recently identified a common variant inside intron 1, named RET+3, which they propose as a major HSCR susceptibility mutation. RET+3 shows low penetrance, but still accounts for a 10–20-fold greater contribution to disease susceptibility than all the other known *RET* mutations, and exerts different genetic effects in males and females. The variant is located in a region, conserved among multiple vertebrate species, demonstrated to act as a cell-dependent expression enhancer, with the HSCR-associated allele significantly reducing the enhancer activity. RET+3, as well as the SNP2 discussed above, is more frequent in Asiatics,

and lower in Europeans. Moreover, it is almost absent in Africa. To explain such findings, the authors speculate about a selective advantage of the mutation in heterozygotes, and its possible protective role for another disease, as happens with malaria and microcytemia [129]. Such a hypothesis supports the "common variant–common disease" model of genetic disease which can also therefore be proposed for HSCR.

5.6 Genetic Counseling

HSCR is a sex-modified multifactorial congenital malformation with an overall recurrence risk in sibs of 4% (relative risk 200). In isolated HSCR, adequate relative risk figures can be provided by taking into account the sex and length of the aganglionic segment in the proband and the gender of the sib (2–33%), with the highest recurrence risk being for a male sib of a female proband affected with L-HSCR (Carter's paradox, see Table 5.1). In view of the poor genotype–phenotype correlation, and the low *RET* mutation rate in HSCR patients, thus far the benefit of mutation screening appears limited, except for systematic testing of exons 10 and 11. Special attention should be paid to patients who carry mutations of one of the critical cysteine residues of these exons, known to predispose to MEN2A [17, 22, 34]. In these patients, HSCR can be associated with development of neuroendocrine tumors such as MTC, for which a prophylactic thyroidectomy is advisable in the presence of a tumor causing *RET* mutation.

In particular patients HSCR is associated with other congenital anomalies. In these patients, the long-term prognosis is highly dependent on the severity of the associated anomalies. Several known syndromes have straight Mendelian inheritance. This emphasizes the importance of careful assessment by a clinician trained in syndromology of all newborns diagnosed with HSCR. The success in identifying specific genes for various syndromic and isolated forms of HSCR suggests that mutation detection in familial cases may be warranted. However, with few exceptions, the penetrance of single-gene mutations may be less than 100% so that genetic counseling in HSCR families is usually problematic and performing prenatal diagnosis cannot be advised. In addition, genetic counseling should take into account the great improvement of surgical management of HSCR achieved during the last decades.

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