

Review

Hirschsprung's disease as a model of complex genetic etiology

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Summary. Hirschsprung disease (HSCR), or aganglionic megacolon, is a developmental disorder characterised by the absence of ganglion cells along variable length of the distal gastrointestinal tract, leading to the most common form of functional intestinal obstruction in neonates and children. Aganglionosis is attributed to a failure of neural crest cells to migrate, proliferate, differentiate or survive during enteric nervous system (ENS) development in the embryonic stage. The incidence of HSCR is estimated at 1/5000 live births and most commonly presents sporadically with reduced penetrance and male predominance, although it can be familial and may be inherited as autosomal dominant or autosomal recessive. In 70% of cases, HSCR occurs as an isolated trait and in the other 30% HSCR is associated with other congenital malformation syndromes. HSCR has a complex genetic etiology with several genes and loci being described as associated with either isolated or syndromic forms. These genes encode for receptors, ligands (especially those participating in the RET and EDNRB signaling transduction pathways), transcriptional factors or other cell elements that are usually involved in the neural crest cell development and migration that give rise to ENS. Nevertheless, the *RET* proto-oncogene is considered the major disease causing gene in HSCR. A common *RET* variant within the conserved transcriptional enhancer sequence in intron 1 has been shown to be associated with a great proportion of sporadic cases and could act as a modifier by modulating the penetrance of mutations in other genes and possibly of those mutations in the *RET* proto-oncogene itself.

Key words: Hirschsprung's disease, Enteric nervous system, RET proto-oncogen, Molecular genetics

Introduction

Hirschsprung disease (HSCR, OMIM: 142623), or aganglionic megacolon, is a developmental disorder characterised by the absence of intramural ganglion cells in the submucosal and myenteric plexuses along variable length of the distal gastrointestinal tract, leading to the most common form of functional intestinal obstruction in neonates and children (Hirschsprung, 1888; Whitehouse and Kernohan, 1948). Such aganglionosis is attributed to a failure of neural crest cells to migrate, proliferate, differentiate or survive during enteric nervous system (ENS) development in the embryonic stage (Okamoto and Ueda, 1967).

The incidence of HSCR is estimated at 1/5000 live births (Bodian and Carter, 1963). There is a sex bias with a preponderance of affected males and a sex ratio of 4/1 (Badner et al., 1990). Interestingly, the male:female ratio is significantly higher for S-HSCR (4.2–4.4) than for L-HSCR (1.2–1.9) (Badner et al., 1990; Torfs et al., 1998). HSCR most commonly presents sporadically, although it can be familial and may be inherited as autosomal dominant or autosomal recessive, with reduced penetrance and male predominance (Passarge, 1967; Badner et al., 1990). In 70% of cases, HSCR occurs as an isolated trait and in the other 30% HSCR is associated with other congenital malformation syndromes (Amiel et al., 2008).

The classification of HSCR is according to the length of the aganglionic segment (Chakravarti and Lyonnet, 2001). While the internal anal sphincter is the constant inferior limit, patients could be classified as short-segment HSCR (S-HSCR: 80% of cases) when the aganglionic segment does not extend beyond the upper sigmoid, and long-segment HSCR (L-HSCR: 20% of cases) when aganglionosis extends proximal to the sigmoid. A less common HSCR variety included within the L-HSCR forms is total colonic aganglionosis (TCA, 3–8% of cases) in which the entire colon and the terminal portion of the ileum is involved (Nihoul-Fékété

et al., 1986).

Molecular genetics in HSCR

HSCR has a complex genetic etiology with several genes being described as associated with either isolated or syndromic forms. These genes encode for receptors, ligands (especially those participating in the RET and EDNRB signaling transduction pathways), transcriptional factors or other cell elements that are usually involved in the neural crest cell development and migration that give rise to ENS (Table 1, Fig. 1). Nevertheless, the *RET* proto-oncogene is considered the major disease causing gene in HSCR.

The *RET* signalling pathway

The *RET* (REarranged during Transfection) proto-oncogene (OMIM +164761), localized at 10q11.2 (Ceccherini et al., 1993), encodes a transmembrane receptor with a cadherin-like extracellular domain, a cysteine-rich region and an intracellular tyrosine kinase domain (Schneider, 1992). *RET* is activated by the glial cell line-derived neurotrophic factor family ligands (GDNF family ligands, GFLs), a neurotrophic factor family comprising four members with approximately 40% aminoacid identity with each other: GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) (Lin et al., 1993, Kotzbauer et al., 1996; Baloh et al., 1998; Milbrandt et al., 1998). The GFLs function as homodimers and activate *RET* through four different

glycosyl phosphatidylinositol-linked co-receptors (GFR·1-4). The formation of such multisubunit complexes promotes the transient dimerization of *RET*, leading to the autophosphorylation of specific tyrosine residues located in the intracellular domain and the subsequent activation of a wide spectrum of signalling pathways (Takahashi, 2001; Airaksinen and Saarma, 2002). *RET* activation is crucial in ENS development, and both GDNF and NRTN have been demonstrated to promote the survival, proliferation, and differentiation of enteric neurons (Taraviras et al., 1999; Natarajan et al., 2002). Mice lacking *Ret*, *Gdnf*, or *Gfr·1* share a similar phenotype, showing total intestinal aganglionosis caused by impaired migration of immature enteric neural crest-derived cells, whereas NRTN or GFR·2 knockout mice show a middle phenotype with moderate deficit of enteric neurons (Schuchardt et al., 1994; Moore et al., 1996; Cacalano et al., 1998; Heuckeroth et al., 1999; Rossi et al., 1999).

RET

Over 100 mutations have been identified in HSCR patients, including large deletions encompassing the *RET* gene, microdeletions and insertions, nonsense, missense and splicing mutations (Edery et al., 1994; Romeo et al., 1994; Yin et al., 1994; Attie et al., 1995a; Angrist et al., 1995; Seri et al., 1997; Svensson et al., 1998; Fitze et al., 2002; Garcia-Barceló et al., 2004; Ruiz-Ferrer et al., 2006; Núñez-Torres et al., 2011), The Human Gene Mutation Database: <http://www.hgmd>.

Table 1. Genes associated with isolated or syndromic HSCR.

Gene	Map location	Phenotype	Inheritance	Mouse models
<i>RET</i>	10q11.2	HSCR/HSCR-MEN2/FMTC	Dominant, incomplete penetrance Dominant	TIA, renal agenesis
<i>GDNF</i>	5p13.2	HSCR	Dominant, low penetrance	TIA, renal agenesis
<i>NRTN</i>	19p13.3	HSCR	Dominant, low penetrance	Moderate deficit of enteric neurons
<i>PSPN</i>	19p13.3	HSCR	Dominant, low penetrance	-
<i>GFR1</i>	10q25.3	HSCR	Dominant, low penetrance	TIA, renal agenesis
<i>EDNRB</i>	13q22.3	WS4/HSCR	Recessive/ Dominant	Aganglionosis, coat spotting
<i>EDN3</i>	20q13.32	WS4/HSCR	Recessive/ Dominant	Aganglionosis, coat spotting
<i>ECE1</i>	1p36.12	HSCR with cardiac defects, craniofacial abnormalitie and autonomic dysfunction	Dominant	Aganglionosis, coat spotting, craniofacial defects
<i>SOX10</i>	22q13.1	WS4/HSCR	Dominant	Aganglionosis, coat spotting
<i>PHOX2B</i>	4p12	CCHS/Neuroblastoma+HSCR	Dominant	TIA, no autonomic nervous system, ventilatory anomalies
<i>NTF3</i>	12p13.31	HSCR	Dominant, low penetrance	Reduced enteric neurons
<i>NTRK3</i>	15q25.3	HSCR	Dominant, low penetrance	Reduced enteric neurons
<i>PROKR1</i>	2p14-p13.3	HSCR	Dominant, low penetrance	-
<i>PROKR2</i>	20p12.3	HSCR	Dominant, low penetrance	Hypoplasia of the olfactory bulb and reproductive system
<i>PROK1</i>	1p13.3	HSCR	Dominant, low penetrance	-
<i>SEMA3A</i>	7p12.1	HSCR	Dominant, low penetrance	Deficit of cardiac sympathetic innervation and stellate ganglia malformation
<i>SEMA3D</i>	7q21.11	HSCR	Dominant, low penetrance	-
<i>NRG1</i>	8p12	HSCR	Dominant, low penetrance	Letal from cardiac defect
<i>NRG3</i>	10q23.1	HSCR	Dominant, low penetrance	-
<i>ZFHX1B</i>	2q22.3	MWS	Dominant	Letal at gastrulation
<i>KIAA1279</i>	10q22.1	GSS	Recessive	-
<i>L1CAM</i>	Xq28	HSAS/MASA spectrum+HSCR	X-linked	Hydrocephalus

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org/)]. However, *RET* Copy Number Variations (CNVs) are not a common molecular cause of Hirschsprung disease (Núñez-Torres et al., 2009). In contrast to multiple endocrine neoplasia type 2 (MEN 2), a cancer syndrome caused by specific germline mutations of *RET* (Eng, 1999), there is no mutational hot spot and *RET* mutations identified in HSCR occur throughout the gene. Biochemical studies demonstrated variable functional consequences: mutations affecting coding sequences in the extracellular domain of *RET* result in disturbed transport of *RET* to the plasma membrane; mutations affecting the cystein-rich domain result in covalent dimerization of the protein and reduced localization at the plasma membrane; mutations targeting the kinase domain cause the disruption or alteration of the catalytic activity of the receptor and; mutations located in the C-terminal tail cause alteration of binding proteins and hence disruption of signaling (Kashuk et al., 2005). While *in vitro* MEN 2 mutations have been shown to be activating mutations leading to constitutive dimerisation of the receptor and to transformation (Santoro et al.,

1995), haploinsufficiency is the most likely mechanism for HSCR mutations (Pasini et al., 1995; Carlomagno et al., 1996; Iwashita et al., 1996, 2001; Pelet et al., 1998).

Despite extensive screening, mutations in the *RET* coding sequence (CDS) account for only up to 50% of familial and 7-20% of sporadic cases (Amiel et al., 2008). However, the involvement of *RET* in the pathogenesis of HSCR has been further supported by the existence of a specific haplotype, constituted by common *RET* polymorphisms, which seems to have a key role in the majority of sporadic forms. The starting point to the identification of such haplotype was the finding of a family segregating both HSCR and MEN2, where the homozygous silent variant A45A (c.135G>A, rs1800858, exon 2) was present in the only member with HSCR phenotype (Borrego et al., 1998; Fernández et al., 2003). Further analysis of this common variant together with another 6 common polymorphisms, in the context of sporadic HSCR, revealed a significant over-representation of this variant and the *RET* haplotypes carrying it (Borrego et al., 1999, 2000). These findings

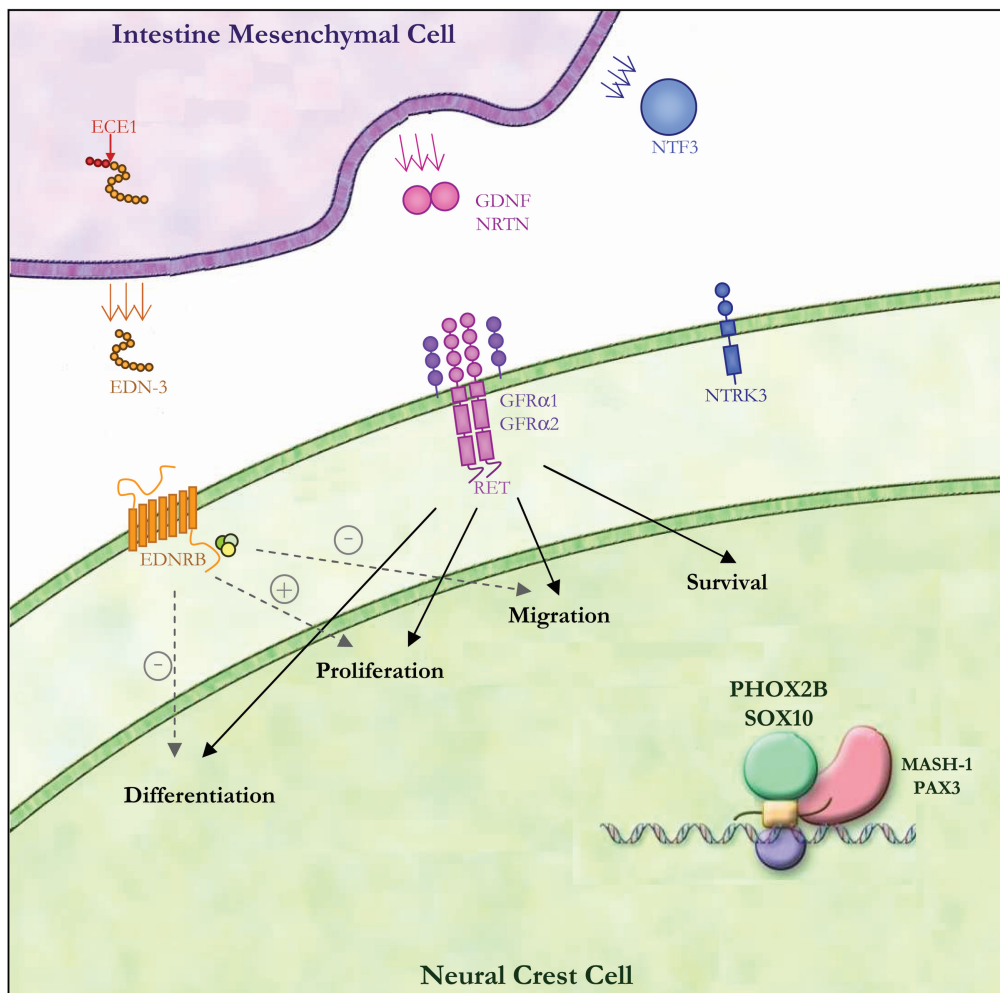


Fig. 1. The genetic complexity observed in HSCR could be explained by the complex nature of ENS development, which is regulated by an ever-increasing range of molecules and signalling pathways involving both the NCCs and intestinal environment.

were independently confirmed in additional HSCR series of different origins (Fitze et al., 1999; García-Barceló et al., 2003a,b). Subsequently, it was demonstrated that A45A anchors ancestral haplotypes in linkage disequilibrium, with a putative common founding susceptibility locus estimated to be 22 to 50 kb upstream (Borrego et al., 2003a; Sancandi et al., 2003). Systematic screening of the region upstream of the A45A SNP, comprising *RET* intron 1, exon 1, and promoter, revealed that the ancestral haplotype associated with HSCR was characterised by the presence of two specific SNPs located at -5 and -1 from the transcription start site (-5 A>G, rs10900296 and -1 C>A, rs10900297) (Fernández et al., 2005; Burzynski et al., 2005; Garcia-Barceló et al., 2005; Griseri et al., 2005; Pelet et al., 2005). It was shown that the AC-haplotype decreased the *RET* promoter activity in luciferase assays (Fitze et al., 2003; Fernández et al., 2005).

Based on association studies and comparative genomics focused on conserved non-coding sequences, one of the HSCR-associated *RET* SNPs (c.73+9277C>T, rs2435357), located within a highly conserved enhancer-like sequence in intron 1 (MCS+9.7) (Emison et al., 2005, 2010), was identified to make a 20-fold greater contribution to risk than coding mutations (Emison et al., 2005). These data led the authors to postulate that the SNP rs2435357 is most likely a low-penetrance disease-causing variant. They supported this idea with reporter (luciferase) assays and showed that, indeed, the MCS+9.7 region, containing the disease-associated variant (T-allele), reduced promoter activity when compared to the wild-type sequence (C-allele). Additional functional studies demonstrated that the T variant disrupts the SOX10 binding site within MCS+9.7 and compromises *RET* transactivation (Emison et al., 2010). Interestingly, the frequency of the predisposing T allele varies according to HSCR prevalence in various ethnic backgrounds and the penetrance is both dose-dependent, greater in males than in females and also increased in patients with rare *RET* coding mutations (Emison et al., 2010; Núñez-Torres et al., 2011). Recently, one SNP located close to rs2435357 and in complete linkage disequilibrium with it (c.73+9494A>C, rs2506004) has been identified as a binding site for NXF/ARNT2 and SIM2-ARNT2 that modifies *RET* expression, demonstrating that more than one SNP can influence gene expression and ultimately HSCR phenotype (Sribudiani et al., 2011). In summary, the combination of common variants, such as the so-called enhancer variants, and rare variants, such as *RET* CDS mutations, contributes to the risk of HSCR and seems to explain in part the complexity of the disease. In addition, a gender effect exists on both the transmission and distribution of rare coding and common HSCR causing mutations. In this sense, an asymmetrical parental origin is observed for *RET* CDS mutations with a higher maternal inheritance due to a differential reproductive rate between male and female carriers (Jannot et al., 2012).

GDNF-family ligands and co-receptors

The relevance of *RET* signaling pathway during ENS development and the prominent role of the *RET* receptor in the pathogenesis of HSCR suggested that the genes encoding the GFLs and their co-receptors could be excellent candidates to be involved in the disease. With regard to the genes encoding the co-receptors (*GFRA1-4*), no mutations have been identified in HSCR patients (Angrist et al., 1998; Myers et al., 1999; Onochie et al., 2000; Vanhorne et al., 2001) except for a deletion at the *GFRA1* (OMIM *601496) locus with incomplete penetrance in two non-related families (Borrego et al., 2003b; Sánchez-Mejías et al., 2010a). In contrast, regarding the genes encoding the ligands, different mutational screenings in HSCR patients have identified several heterozygous germline mutations in *GDNF* (OMIM *600837), although often in combination with *RET* mutations or other genetic alterations (Angrist et al., 1996; Ivanchuk et al., 1996; Salomon et al., 1996; Hofstra et al., 2000; Martucciello et al., 2000; Ruiz-Ferrer et al., 2011a), indicating that those mutations could be neither necessary nor sufficient to cause HSCR. Subsequent analysis of the functional role of mutations located in the mature region of *GDNF* demonstrated no effect on *RET* phosphorylation, even when a reduction in the binding affinity to GFR α 1 was observed (Eketjall et al., 1999; Borghini et al., 2002). Taking these findings into account, such mutations could be regarded as genetic changes with a modulatory effect that could contribute to the disease via interaction with other susceptibility loci.

On the other hand, three mutations have been reported in *NRTN* (OMIM *602018) (Doray et al., 1998; Ruiz-Ferrer et al., 2011a), only one in *ARTN* (OMIM *603886) (Ruiz-Ferrer et al., 2011a) and another in *PSPN* (OMIM *602921) (Ruiz-Ferrer et al., 2011a). Interestingly, *in vitro* analyses revealed a decreased secretion level of correctly processed peptide for the variant R91C in *PSPN* and altered *RET* activation by a significant reduction of autophosphorylation also for this variant and F127L in *NRTN*, supporting their effects on HSCR phenotype (Ruiz-Ferrer et al., 2011a). Generally, the variants in the GFL genes behave as incompletely penetrant, since they were carried by other healthy members of the family. This would completely fit with the additive model of inheritance proposed for HSCR, in which the expression of the disease seems to depend on the contribution of different combinations of gene alleles acting in an additive or multiplicative fashion. Following this model, those genetic variants could modulate the penetrance of mutations located in other genes or modify expressivity of the disease in affected individuals. These variants may be contributing to the final phenotype acting in combination with additional mutational events in other genes, such as the common *RET* variant within the transcriptional enhancer in intron 1, since most of the patients have inherited these variants from their healthy parents (Ruiz-Ferrer et al., 2011a). Presumably, none of

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these mutations are likely to cause HSCR independently, but the co-occurrence of both different mutational events in the same patient may have contributed to the manifestation of the phenotype.

The endothelin signalling pathway

A second pathway involved in HSCR is the Endothelin Receptor Type B (EDNRB) pathway. EDNRB and EDNRA are G-protein-coupled heptahelical receptors that transduce signals through the endothelins (EDN1, 2, 3) (Inoue et al., 1989; Sakurai et al., 1992). The endothelins are synthesized as much larger proteins which are cleaved by two related membrane-bound metalloproteases, the endothelin converting enzymes (ECE1, 2), to produce an active peptide. In mouse embryonic gut, *Ednrb* is expressed in the migrating enteric neural crest cells and *Edn3* is mainly expressed in the midgut and the hindgut mesenchyma during the early phases of enteric neural crest cell migration, and at high levels in the caecum and the proximal colon when the enteric neural crest cells colonize the terminal gut region (Leibl et al., 1999; Barlow et al., 2003). Furthermore, *Edn3* mutant mouse has fewer neural crest stem cells compared to the wild-type mouse (Barlow et al., 2003), and it was demonstrated that cell differentiation is inhibited by EDN3-EDNRB signalling (Bondurand et al., 2006). These studies together showed that EDN3-EDNRB signalling is important for enteric neural crest cell migration and proliferation, and for maintaining enteric neural crest cells in their progenitor state during ENS development. In addition, *Edn3*, *Ednrb* and *Ece1* knockout mice showed aganglionosis and pigmentary abnormalities (Baynash et al., 1994; Hosoda et al., 1994; Yanagisawa et al., 1998), similar to the phenotypic abnormalities seen in human patients with Shah-Waardenburg syndrome (Waardenburg syndrome type 4, WS4, OMIM 277580). Waardenburg syndrome (WS, OMIM 193500, OMIM 148820, OMIM 193510), a clinically and genetically heterogeneous autosomal dominant condition, is by far the most frequent condition combining sensorineural deafness and pigmentary anomalies due to an abnormal proliferation, survival, migration, or differentiation of neural-crest-derived melanocytes, and in combination with HSCR defines the WS4 type.

The critical role of the endothelin pathway in HSCR was demonstrated with the study of an inbred Old Order Mennonite community. The occurrence of multiple cases of both isolated HSCR and WS4 in the community facilitated the mapping of another major HSCR susceptibility gene to the chromosomal region 13q22 (Puffenberger et al., 1994a), and an *EDNRB* (OMIM *131244) missense mutation (W276C, c.828G>T) was identified (Puffenberger et al., 1994b). However, this mutation was neither fully dominant nor fully recessive. Subsequent *EDNRB* mutation analyses conducted on both isolated HSCR and WS4 patients

revealed that homozygous *EDNRB* mutations were associated with WS4 (Attie et al., 1995b; Hofstra et al., 1996; Edery et al., 1996; Pingault et al., 2001; Verheij et al., 2002) and heterozygous mutations with isolated HSCR (Amiel et al., 1996; Auricchio et al., 1996; Chakravarti, 1996; Kusafuka et al., 1996; Kusafuka and Puri, 1997; Tanaka et al., 1998; Gath et al., 2001; Garcia-Barceló et al., 2004; Sánchez-Mejías et al., 2010b). Functional analyses of *EDNRB* missense mutations showed impairment of the intracellular signalling (Kusafuka et al., 1996; Abe et al., 2000; Fuchs et al., 2001). Overall, *EDNRB* mutations account for 5% of the isolated HSCR phenotype.

HSCR patients have also been screened for mutations in the human *EDN3* (OMIM *131242) and *ECE-1* (OMIM *600423). Only one heterozygous *ECE-1* mutation has been identified in a single patient combining HSCR with craniofacial and cardiac defects (R742C, c.2224C>T) (Hofstra et al., 1999). Very few *EDN3* mutations have been characterized in HSCR patients and, with exceptions, similar genetic behaviour to *EDNRB* mutations was observed. In this sense, 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 signalling (McCallion and Chakravarti, 2001). The evaluation of *EDN3* as a susceptibility gene for HSCR using common polymorphisms revealed the association of a SNP (rs6064764) with the disease (Sánchez-Mejías et al., 2010b). It is plausible the existence of linkage disequilibrium with some functional, still unidentified allele of these genes, or this allele might be acting as a functional variant *per se* leading to a higher predisposition for HSCR, although its precise molecular mechanism remains to be elucidated. In any case, those results suggest that this gene might be considered as a common susceptibility gene for sporadic HSCR in a low-penetrance fashion, more than a minor gene for this disease as it is currently considered (Fuchs et al., 2001).

NTF-3/TRKC signalling pathway

Neurotrophin 3 (*NTF-3*, OMIM *162660) encodes a 257 amino acid protein member of the highly homologous neurotrophin family (Levi-Montalcini et al., 1987). The signal transduction pathway of NTF-3 is initiated by high-affinity binding to the extracellular domain of the tyrosine kinase receptor TRKC (encoded by *NTRK3*, OMIM *191316) (Klein et al., 1989; Lamballe et al., 1991). Several studies on mice have provided evidence that NTF-3 is secreted by the non-crest-derived enteric mesenchyme and promotes the development and/or survival of neurons and glia from enteric neural crest-derived cells expressing the receptor TrkC (Chalazonitis et al., 1994; Chalazonitis, 2004). Mice lacking NTF-3 or its receptor TrkC have reduced numbers of both myenteric and submucosal neurons, and mice overexpressing NTF-3 have increased numbers of

myenteric neurons. Moreover, analysis of the localization of neurotrophins and their receptors in developing and postnatal human intestine, both in normal individuals and in patients with HSCR, revealed that NTF-3 is absent in the aganglionic colon and reduced in transitional intestine (Hoehner et al., 1996). Therefore, the requirement of the NTF-3/TrkC signalling for the proper development of the ENS, together with the evidence presented by the murine models, has prompted the investigation of the possible involvement of the human NTF-3 and TRKC in HSCR.

In this sense, mutational screening of *NTF-3* as well as *NTRK3* have been performed (Ruiz-Ferrer et al., 2008; Fernández et al., 2009). The most relevant findings were the identification of a novel sequence variant in *NTF-3*, *G76R* (c.226G>A), present in 2 unrelated patients and a novel variant within the tyrosine kinase domain of *NTRK3*, *R645C* (c.1933 C>T), in a family with two affected siblings also carrying a *RET* splicing mutation. It suggests that both *RET* and *NTRK3* mutations may act together, being necessary and sufficient for the appearance of the disease. Interestingly, the detection of a novel *EDN3* mutation in the patient with aganglionosis extending to the ileum, and not in his affected sibling with a milder phenotype, strongly suggests that the *EDN3* gene is acting as a phenotype-modifier factor in this particular family and that the accumulation of gene variants predisposing to HSCR in the genetic background of the patient has a greater impact on expression of the disease (Sánchez-Mejías et al., 2009). These results assess the importance of the NTF-3/NTRK3 signalling pathway in ENS disorders and support the complex additive model of inheritance proposed for HSCR disease.

Prokineticins signalling pathway

Prokineticins (PROK1 and PROK2) belong to the AVIT protein family, a recently identified family of cysteine-rich secreted protein that share an identical amino terminal sequence crucial for their biological activities (Li et al., 2001; Kaser et al., 2003). These proteins are known to bind and activate two closely related G protein-coupled receptors, PROKR1 and PROKR2, leading to the mobilization of calcium, the stimulation of phosphoinositide-3-kinase turnover, and the activation of the mitogen activated protein kinase (MAPK) signalling pathway (Lin et al., 2002; Soga et al., 2002). PROKR1 expression was demonstrated in mouse enteric neural crest derived cells and Prok-1 was shown to work coordinately with GDNF in the development of the ENS (Ngan et al., 2007a, 2008). Firstly, both GDNF and Prok-1 share common downstream elements, prominently the MAPK and Akt pathways, which provide multiple points of insertions between these two factors and lead them to exhibit similar biological functions (Ngan et al., 2007a). In addition, GDNF potentiate the proliferative and differentiation effects of Prok-1 by up-regulating

PROKR1 expression in enteric NCCs (Ngan et al., 2008). This functional redundancy of PROKR1/Prok-1 and RET/GFRa1/GDNF signalling supports the idea that Prok-1/PROKR1 provides a compensatory pathway to ensure the proper development of ENS.

Recently, using neurosphere cultures obtained from human ENS derived cells, it has been demonstrated that not only PROKR1 was present in neural stem cells and neuronal precursors, but the PROKR2 receptor was also observed (Ruiz-Ferrer et al., 2011b). These results suggest that PROKR2 would have a relevant role by inhibiting apoptosis of enteric neuronal precursors, as it was previously described in neural crest-derived neuroblastoma cells (Ngan et al., 2007b). Therefore, PROKR2 could mediate neuronal protection or survival not only in the central nervous system (Melchiorri et al., 2001), but also during ENS development. Accordingly, *PROKR1*, *PROK1*, *PROKR2* and *PROK2* were evaluated as susceptibility genes for HSCR, based on the etiopathogenesis of the disease (Ruiz-Ferrer et al., 2011b). Several missense variants in *PROKR1*, *PROK1* and *PROKR2* genes were detected, most of them affecting highly conserved amino acid residues of the protein and located in functional domains of both receptors, which suggests a possible deleterious effect in their biological function. Interestingly, it has been observed that the presence of sequence variants in these genes in HSCR patients are frequently associated to mutations in *RET* proto-oncogene or *GDNF*, contributing to the manifestation of the more severe phenotypes. These results provide the first evidence to consider them as susceptibility genes for HSCR.

NRGs signalling pathway

Through a Genome Wide Association Study (GWAS), the *NRG1* gene (OMIM *142445) was successfully identified as a new candidate gene for HSCR (Garcia-Barcelo et al., 2009). *NRG1* is a trophic factor that contains an epidermal growth factor (EGF)-like domain that signals by stimulating ErbB receptor tyrosine kinases and activates some cellular processes such as proliferation, differentiation, migration, apoptosis and cellular survival (Riese et al., 1995; Tzahar et al., 1996). It has been described that *NRG1* receptors ErbB2/ErbB3 are expressed in mouse vagal neural crest cells entering the developing gut and in adult intestinal epithelia of both humans and mice (Prigent et al., 1992; Britsch et al., 2001; Britsch, 2007; Paratore et al., 2002). In addition, *NRG1* is also expressed in mice and human intestinal mucosa and enteric ganglia (Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994). Moreover, a recent study demonstrated that the mRNA expression levels of *NRG1* were significantly higher in tissues of HSCR than those in controls, and the increased *NRG1* protein levels in HSCR were consistent with the mRNA levels, which suggests that the aberrant expression of *NRG1* may play an important role in the pathology of HSCR (Tang et al.,

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2011). To refine the locus on 8p12 linked to the disease (Garcia-Barcelo et al., 2009), a wide spectrum of SNPs were genotyped in ethnic Chinese HSCR patients and controls (Tang et al., 2012a). Genotype analysis narrowed down the HSCR-associated region to six of the most associated SNPs mapping to the *NRG1* promoter. Of note, significant differences in *NRG1* expression levels between patients and controls bearing the same rs10088313 risk genotype were detected (Tang et al., 2011). This seems to indicate that the effects of *NRG1* common variants are likely to depend on other alleles or epigenetic factors present in patients and would account among other factors for the variability in the genetic predisposition to HSCR. Finally, the implication of *NRG1* in HSCR has also been demonstrated through the identification of coding mutations whose pathogenic role was demonstrated by different functional approaches (Luzón-Toro et al., 2012; Tang et al., 2012).

On the other hand, with the data generated from the GWAS the contribution of CNVs to HSCR was also assessed, leading to the identification of many rare genetic CNVs exclusive to patients. In this regard, a deletion affecting the *NRG3* gene (OMIM *605533), a paralog of *NRG1*, was detected. This fact prompted a subsequent follow-up on *NRG3*, revealing the existence of both deletions and duplications within this gene, associated to HSCR. Stratification of patients by presence/absence of HSCR-associated syndromes showed that while syndromic-HSCR patients carried significantly longer CNVs than the non-syndromic or controls, non-syndromic patients were enriched in CNV number when compared to controls or the syndromic counterpart. These results suggested a role for *NRG3* in HSCR etiology and provide insights into the relative contribution of structural variants in both syndromic and non-syndromic HSCR (Tang et al., 2012b).

SEMA3 signalling pathway

Another GWAS led to the identification of a significant cluster of SNPs in a region on chromosome 7, containing significant association to HSCR with allelic effects independent of *RET*, which fall downstream from the protein *SEMA3D* (7q21.11; OMIM 609907) and upstream from *SEMA3A* (7p12.1; OMIM 603961), *SEMA3E* (7q21.11; OMIM 608166) and *SEMA3C* (7q21-q31; OMIM 602645). In this study, S-HSCR trios were analyzed to strengthen the significance of the *SEMA* SNPs cluster, and refined the location of its peak. The four *SEMA* family III members demonstrated very similar temporo-spatial patterns of expression throughout the gut. They were co-expressed with *RET* in these tissues, supporting the possibility that one or more might modify *RET* function in the developing ENS. In addition, next-generation sequencing technologies have allowed the detection of different missense mutations in the *SEMA* genes potentially involved in HSCR (Jiang et al., 2012; Luzón-Toro et al., 2013).

Different studies had suggested a role for members

of the *SEMA* family in NCC development defects, the proliferation, migration, and/or differentiation of which might be a cause of HSCR (Yu and Moens, 2005; Berndt and Halloran, 2006; Anderson et al., 2007; Lwigale and Bronner-Fraser, 2009). Recently, it has been proposed that increased *SEMA3A* expression may be a risk factor for HSCR pathology in a subset of HSCR patients, based on the upregulation in the aganglionic smooth muscle layer of the colon (Shepherd and Raper, 1999). In addition, the association between two *SEMA3A* common polymorphisms and the risk of HSCR in the Northeastern Chinese and Thai populations has been validated, as was previously demonstrated in Caucasian population (Wang et al., 2011; Phusantisampan et al., 2012).

Transcriptional Factors and other cell elements

SOX10

SOX10 (OMIM *602229) encodes a transcription factor belonging to an evolutionary conserved protein family, which contains a central high mobility group (HMG) DNA binding/DNA-bending domain and a C-terminal transactivation domain (Pevny and Lovell-Badge, 1997; Wegner, 1999). In a manner similar to all members of this family, *SOX10* exerts its function through binding to the promoters or enhancer sequences of its target genes, alone or in association with other transcription factors (Kuhlbrodt et al., 1998; Kamachi et al., 1999). *SOX10* is a key transcription factor during neural-crest derived cell migration and differentiation, and *RET* and *EDNRB* are known to be target genes for *SOX10* regulation (Kuhlbrodt et al., 1998; Kamachi et al., 1999; Bondurand et al., 2000, 2001; Peirano and Wegner, 2000; Lang and Epstein, 2003; Ludwig et al., 2004; Zhu et al., 2004; Murisier et al., 2007). *SOX10* modulates gene expression of pluripotent neural-crest cells that migrate from the neural tube throughout the embryo along several pathways during embryogenesis. Those precursors give rise to enteric neurons and glia, some of the craniofacial skeletal tissue, melanocytes of the skin and inner ear, in addition to other cell types (Le Douarin and Kalcheim, 1999).

The significance of *SOX10* in HSCR was revealed through the study of a mouse model for WS4 in human (*dominant megacolon, Dom*) (Lane and Liu, 1984). The molecular defect in *Dom* mice was a mutation in the *Sox10* gene (Pingault et al., 1997; Southard-Smith et al., 1998, 1999; Herbarth et al., 1998). Heterozygous *Dom* mice presented with distal colonic aganglionosis and localized hypomelanosis of the skin and hair (features similar to those in WS), which indicated that neural crest-derived melanocytes and enteric neurons were affected in *Sox10* mutants. *Dom* homozygous mice were embryonic lethal.

WS4 phenotype has been reported to be caused by heterozygous *SOX10* point mutations as well as gross deletions, presented in 45–55% of patients (Bondurand

et al., 2007; Pingault et al., 1998, 2002; Inoue et al., 1999; Touraine et al., 2000; Sham et al., 2001; Shimotake et al., 2007). Moreover, a study searching for deletions within *SOX10* regulatory sequences has described the first characterization of a WS4 patient presenting with a large deletion encompassing three of these enhancers (Bondurand et al., 2012). However, 15–35% of WS4 remains unexplained at the molecular level, suggesting that other genes could be involved and/or that mutations within known genes may have been missed in previous screenings. On the other hand, the role of the *RET* common hypomorphic allele has been studied in the context of WS patients leading to the conclusion that it is not significant for the manifestation of the HSCR phenotype in WS4 patients (de Pontual et al., 2007a).

In this sense, non-syndromic HSCR disease was initially thought not to be associated to mutations at this particular locus. However, abnormal *SOX10* gene expression could be observed in aganglionic intestine of isolated HSCR patients suggesting a role in the pathogenesis of HSCR (Pingault et al., 1998; Chan et al., 2003). Recently, a screening of both point mutations and gene-dosage anomalies of its coding sequence was performed in a series of 196 HSCR patients, the largest patient series evaluated so far (Sánchez-Mejías et al., 2010c). Interestingly, a truncating mutation in *SOX10* has been found in a patient presenting aganglionosis as an isolated trait (Sánchez-Mejías et al., 2010c). This was the first time that a *SOX10* mutation was detected in an isolated HSCR patient, which points out the association of this gene with the pathogenesis of HSCR *per se*, not only as a part of a syndromic trait.

PHOX2B

The paired-like homeobox 2b gene (*PHOX2B*, OMIM *603851) encodes a transcription factor involved in the development of several noradrenergic neurone populations in mice. In the murine model, *Phox2b* expression starts as soon as enteroblasts invade the foregut mesenchyme and is maintained throughout development into enteric neurons, so that homozygous disruption of *Phox2b* in mice leads to an absence of enteric ganglia (Pattyn et al., 1999). 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 (Pattyn et al., 1999; Dubreuil et al., 2000). The *PHOX2B* homology observed between humans and mice, as well as the compelling evidence of its key function in the development of neural crest derivatives, have made it an attractive target for study as a potential gene involved in human neurocristopathies. In this sense, the major role of the gene is well established in the pathogenesis of Congenital Central Hypoventilation Syndrome (CCHS or Ondine's curse, OMIM 209880), a rare disorder which presents in newborns as apparent hypoventilation with monotonous respiratory rates and shallow breathing either during sleep only or while awake as well as

asleep; autonomic nervous system dysregulation (ANSD); or in some individuals, as altered development of neural crest-derived structures (i.e., HSCR) and/or tumors of neural crest origin (neuroblastoma, ganglioneuroma, and ganglioneuroblastoma) (Roshkow et al., 1988; Levard et al., 1989; Weese-Mayer et al., 1993). The association of HSCR and CCHS, known as Haddad syndrome (MIM 209880), is found in around 20% of CCHS patients (Haddad et al., 1978; Verloes et al., 1993). In contrast with isolated HSCR, in the majority of Haddad cases the length of colonic aganglionosis is long or even total, and the sex ratio male:female is around one (Croaker et al., 1998). Two types of *PHOX2B* mutations are observed in CCHS: (1) Polyalanine repeat expansion mutations (PARMs) between 24 and 33 repeats (Weese-Mayer et al., 2003; Repetto et al., 2009); and (2) Sequence alterations outside of the polyalanine repeat and frameshift mutations affecting the region encoding the polyalanine repeat (NPARMs), which are typically small out-of-frame deletions or duplications of approximately 1 to 38 nucleotides (Berry-Kravis et al., 2006; Weese-Mayer et al., 2010). As one of the targets of *PHOX2B* is the *PHOX2B* gene itself, the transcriptional activity of wild-type and mutant proteins on the *PHOX2B* gene promoter has been tested, and the transactivation ability of proteins with polyalanine expansions has been found to be decreased as a function of the length of the expansion, whereas DNA binding is severely affected only in the case of the mutant with the longest polyalanine tract (+13 alanine) (Di Lascio et al., 2012). Interestingly, genotype-phenotype studies have shown that individuals with the 20/27 genotype or longer PARMs are at greatest risk for HSCR, and almost all individuals with NPARMs have HSCR (Trochet et al., 2005a; Berry-Kravis et al., 2006). Most CCHS patients are heterozygous for a de novo mutation in *PHOX2B*. Nevertheless, parents of patients with molecularly proven CCHS must be tested for accurate genetic counselling, as about 10% carry a germline or somatic mosaic and some parents may develop late onset CHS (Weese-Mayer et al., 2003; Trochet et al., 2005a; Berry-Kravis, 2006). Mutations in genes other than *PHOX2B* have been identified in patients with CCHS, such as *RET*, *GDNF*, *EDN3*, *BDNF*, *HASH1*, *PHOX2A*, *GFRA1*, *BMP2* or *ECE1*, although their significance and real involvement in the disease is not known (Weese-Mayer et al., 2011). However, it has been verified that the frequent, low penetrant, predisposing allele of the *RET* gene can be regarded as a risk factor for the HSCR phenotype in CCHS (de Pontual et al., 2007a).

Interestingly, also in some cases of syndromic neuroblastoma (NB, OMIM 256700) combined with CCHS or HSCR, heterozygous mutations of the *PHOX2B* gene have been identified while they remain rare in sporadic, isolated NB (Rohrer et al., 2002; Mosse et al., 2004; Trochet et al., 2004, 2005b; van Limpt et al., 2004; Perri et al., 2005; McConville et al., 2006; de Pontual et al., 2007b).

HSCR as a model of complex genetic etiology

PHOX2B haploinsufficiency has also been suggested to predispose to HSCR. The c.429+100A>G 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 (Garcia-Barceló et al., 2003a,b; Miao et al., 2007). Taking all these data together, *PHOX2B* can be regarded as an interesting candidate gene for HSCR as well. Moreover, a recent study has led to the identification of a de novo and novel deletion (c.393_411del18) in a patient with HSCR and no other sign of CCHS or NB. Results of in silico and functional assays support its pathogenic effect related to HSCR, supporting that *PHOX2B* loss-of-function is a rare cause of HSCR phenotype (Fernández et al., 2013).

ZFHX1B

ZFHX1B encodes Smad-interacting protein-1 (SMADIP1 or SIP1), a transcriptional co-repressor involved in the transforming growth factor-beta signaling pathway. It is a highly evolutionarily conserved gene, widely expressed and with key functions in early embryological development, as demonstrated with animal models (Papin et al., 2002; Van de Putte et al., 2003, 2007). Mowat-Wilson syndrome (MWS, MIM 235730) is a condition associated with microcephaly, epilepsy, a facial gestalt and severe mental retardation (MR). The spectrum of possible associated malformations is wide and encompasses hypospadias, renal anomalies, congenital cardiac defect, agenesis/hypoplasia of the corpus callosum and HSCR (Mowat et al., 1998; Zweier et al., 2005; de Pontual et al., 2007a). Over 100 mutations have been described in patients with clinically typical MWS, who almost always have whole gene deletions or truncating mutations (nonsense or frameshift) of *ZFHX1B*, suggesting that haploinsufficiency is the basis of MWS pathology. No obvious genotype-phenotype correlation has been identified so far, but atypical phenotypes have been reported with missense or splice mutations in the *ZFHX1B* gene (Wakamatsu et al., 2001; Cacheux et al., 2001; Amiel et al., 2001; Dastot-Le Moal et al., 2007). In addition, the analysis of the distribution of the *RET* intronic mutation in the context of MWS patients revealed no association with the manifestation of the HSCR phenotype in these patients (de Pontual et al., 2007a).

KIAA1279

This gene has been identified as disease causing in a large consanguineous family with Goldberg-Shprintzen syndrome (GSS, MIM 609460), an autosomal recessive multiple congenital anomaly syndrome that combines HSCR, moderate MR, microcephaly, polymicrogyria, facial dysmorphic features (hypertelorism, prominent nose, synophrys, sparse hair), cleft palate and iris coloboma (Goldberg and Shprintzen, 1981; Brooks et

al., 1999, 2005). Through animal model studies, it has been proposed that such protein is an important regulator of axonal development and that axonal cytoskeletal defects underlie the nervous system defects in GSS (Lyons et al., 2008). Another hypothesis is that KBP is involved in neuronal differentiation and the central and enteric nervous system defects seen in GSS are likely caused by microtubule-related defects (Alves et al., 2010).

LICAM

There exist some clinical presentations of HSCR with central nervous system anomalies, including the HSAS spectrum (Hydrocephalus due to Stenosis of the Aqueduct of Sylvius, OMIM 307000) ascribed to mutations in the X-linked *LICAM* gene (Okamoto et al., 2004). *LICAM* encodes a neuronal cell adhesion molecule with key functions in the development of the nervous system. Indeed, until now *LICAM* pathogenic mutations have been found in at least 11 patients reported to show association of X-linked hydrocephalus (XLH) or acrocallosal syndrome (ACS) and HSCR (Fernández et al., 2012; Takenouchi et al., 2012). It has been hypothesized that in those cases in which XLH presents together with HSCR, either *RET* or another HSCR gene contributes to aganglionosis under the influence of a defective *LICAM* gene, and *LICAM* may act as an X linked modifier gene for the development of HSCR (Parisi et al., 2012). Animal model studies have shown that *L1cam* is required for neural crest migration, but loss of *L1cam* on its own is not sufficient to produce aganglionosis, supporting that *L1cam* may act only as a modifier gene (Anderson et al., 2006). Moreover, it has been shown that an interaction between *L1cam* and *Sox10* significantly perturbs neural crest migration within the developing gut (Wallace et al., 2010). Thus, *L1cam* may act as a modifier gene for *Sox10*, which is one of the HSCR associated genes. In addition, *L1cam* has also proven to act as a modifier gene for members of the endothelin signalling pathway during ENS development (Wallace et al., 2011), so that all these genes should also be investigated in the context of XLH-HSC (Fernández et al., 2012).

Other susceptibility loci for HSCR

Several studies have been searching for additional susceptibility loci in HSCR. First, a linkage analysis was performed in 12 multiplex HSCR families with three or more affected individuals in two or more generations where L-HSCR is largely predominant (Bolk et al., 2000). A new locus at 9q31 was identified in conjunction with *RET* and it was hypothesized that 9q31 was probably a modifier locus for development of HSCR disease. Recently, fine mapping of the locus firstly revealed an association with the gene *SVEPI*, but this result was not replicated in 107 independent HSCR Dutch patients (Tang et al., 2010). However, in a

Chinese HSCR population it was found to be associated with the gene *IKBKAP*, confirmed in a different cohort and suggesting that the association with chromosome 9 is population-specific (Tang et al., 2010). In another study, Gabriel et al. performed a genome-wide scan in 49 families with S-HSCR. They carried out a sib-pair analysis and found significant allele sharing with markers on 10q11, where *RET* is located, 19q12 and 3p21 (Gabriel et al., 2002). To validate these data, they checked the families' mutation status for *RET*. Surprisingly, they only identified *RET* CDS mutations in 40% of *RET*-linked families, suggesting the importance of non-coding variants. They therefore hypothesized that all three loci were necessary and possibly sufficient for the observed occurrence of S-HSCR. A multiplicative risk across loci with most affected individuals being heterozygotes at all three loci seems to be the best genetic model.

A fourth locus was identified by performing a genome-wide scan on 43 Mennonite trios, all belonging to the same large kindred (Carrasquillo et al., 2002). They identified three loci, two of which were known loci: 10q11.21 where the *RET* gene is located and 13q22.3-q31.1 containing the *EDNRB* gene, previously described as the primary susceptibility factor for the disease phenotype in this kindred (Puffenberger et al., 1994a). The new locus identified was located on 16q23.3 and the authors suggested two candidate genes in this region involved in ENS development (*CDH13* and *PLCG2*). However, no association study have been reported so far regarding these genes and HSCR. Later, a linkage analysis was performed through genotyping of 4,244 SNPs in 35 HSCR Mennonite families (Lin et al., 2004). The loci 10q11 and 13q22 were again associated to the disease but the locus 21q21 was identified for the first time, postulated as an interesting region given the association between HSCR and Down Syndrome. Finally, studying a large multi-generational Dutch family with an isolated HSCR phenotype resulted in the identification of a new susceptibility locus on 4q31-32 (Brooks et al., 2006). The low penetrance of the locus in this family suggests that this mutation is necessary but not sufficient for disease development.

On the other hand, a pilot study utilized a custom-designed array CGH to detect gene-sized or smaller CNVs within 67 proven and candidate HSCR genes in 18 heterogeneous HSCR patients. Using stringent criteria, they identified CNVs at three loci (*MAPK10*, *ZFHX1B*, *SOX2*) that are novel, involve regulatory and coding sequences of neuro-developmental genes, and show association with HSCR in combination with other congenital anomalies. Additional CNVs were observed under relaxed criteria. This kind of research again suggests a role for CNVs in HSCR and, importantly, emphasizes the role of variation in regulatory sequences, although much larger studies will be necessary both for replication and for identifying the full spectrum of small CNV effects (Jiang et al., 2011).

The way to identification of novel susceptibility

genes for HSCR is nowadays based on different approaches such as Genome-wide associations studies (GWAS), Genome Wide Expression Studies (GWES) and Next Generation Sequencing (NGS).

Syndromic HSCR

HSCR presents as a syndromic form in approximately 30% of cases, of which 18% are associated with congenital anomalies, such as gastrointestinal malformation, cleft palate, polydactyly, cardiac septal defects or craniofacial anomalies, among others (Spouge and Baird, 1985; Brooks et al., 1998) (Summarized in Table 2). The higher rate of associated anomalies in familial cases than in isolated cases (39% vs 21%) supports a Mendelian inheritance for these syndromic cases (Amiel et al., 2008; Brooks et al., 1998). Some associations are well characterised with a penetrance of HSCR ranging from 5% to >80%. However, for most rare disorders it is not easy to discriminate if HSCR is really connected to the presentation of the disease.

Up to 12% of HSCR cases have been described associated with a large number chromosomal abnormalities. Among this group of syndromic HSCR patients, Down syndrome (DS) due to free trisomy 21 is by far the most frequent (90%), involving 2-10% of the ascertained HSCR cases (Bodian and Carter, 1963; Spouge and Baird, 1985; Torfs, 1998; Brooks et al., 1998; Jiang et al., 2011). In those cases, both the unbalanced sex ratio (5.5–10.5:1 male:female) and the predominance of S-HSCR are greater than in isolated HSCR. Moreover, the risk-ratio of HSCR in DS is known to be greater than the risk conferred by any of the single gene mutations for HSCR (Gabriel et al., 2002). Several hypotheses have been suggested, although they could not be subsequently confirmed (Yamakawa et al., 1998; Korbelt et al., 2009). In addition, while *RET* coding mutations have rarely been found in DS+HSCR patients, the common HSCR predisposing *RET* hypomorphic allele is over-represented in DS+HSCR patients when compared to DS patients without HSCR (de Pontual et al., 2007a). Moreover, an association and interaction between *RET* and chromosome 21 gene dosage has been proposed, since the *RET*+9.7 T allele frequency is significantly different between individuals with DS alone, HSCR alone, and DS+HSCR patients (Arnold et al., 2009). Finally, although predominance of some *EDNRB* variants in DS+HSCR versus HSCR patients have been reported (Zaahl et al., 2003), those findings could not be subsequently confirmed in other populations (Sánchez-Mejías et al., 2010b).

Genetic counselling

Genetic counselling remains a challenge in HSCR because of its multifactorial etiology. Essentially, the genetic counselor should at least be able to describe both inherited and acquired risk factors to affected families,

*HSCR as a model of complex genetic etiology***Table 2.** Syndromes associated with HSCR

Syndromes	MIM	Key features
<i>Syndromic NCC disorders</i>		
WS4 (Shah-Waardenburg)	277580	Pigmentary anomalies (white forelock, iris hypoplasia, patchy hypopigmentation)
Yemenite deaf-blind hypopigmentation	601706	Hearing loss, eye anomalies (microcornea, coloboma, nystagmus), pigmentary anomalies
BADS	227010	Hearing loss, hypopigmentation of the skin and retina
Piebaldism	172800	Patchy hypopigmentation of the skin
Haddad	209880	Congenital central hypoventilation
MEN2	171400	Medullary thyroid carcinoma, pheochromocytoma, hyperplasia of the parathyroid
Riley-Day	223900	Autonomic nervous system anomalies
HSCR mandatory		
Goldberg-Shprintzen	235730	MR, polymicrogyria, microcephaly, CF, coloboma, facial dysmorphic features
HD with limb anomalies	235740 235750 235760 604211 306980	Polydactyly, unilateral renal agenesis, hypertelorism, deafness Postaxial polydactyly, ventricular septal defect Hypoplasia of distal phalanges and nails, dysmorphic features Preaxial polydactyly, heart defect, laryngeal anomalies Brachydactyly type D
BRESHEK	-	Brain abnormalities , Retardation, Ectodermal dysplasia, Skeletal malformation, Hirschsprung disease,
Ear/eye anomalies, Kidney dysplasia		
Mowat-Wilson	235730	MR, microcephaly, epilepsy, facial gestalt, hypospadias, renal anomalies...
HSCR occasionally associated		
Bardet-Biedl syndrome	209900	Pigmentary retinopathy, obesity, hypogenitalism, mild mental retardation, postaxial polydactyly
Kauffman-McKusick	236700	Hydrometrocolpos, postaxial polydactyly, congenital heart defect
Smith-Lemli-Opitz	270400	Growth retardation, microcephaly, mental retardation, hypospadias, 2–3 toes syndactyly
Cartilage-hair hypoplasia	250250	Shortlimb dwarfism, metaphyseal dysplasia immunodeficiency
HSAS/MASA	307000	Hydrocephalus, aqueductal stenosis, spasticity adducted thumbs, ACC, mental retardation
HSCR rarely associated		
Fukuyama congenital muscular dystrophy	253800	Muscular dystrophy, polymicrogyria, hydrocephalus, MR, seizures
Clayton-Smith	258840	Dysmorphic features, hypoplastic toes and nails, ichthyosis
Kaplan	304100	Agenesis of corpus callosum, adducted thumbs, ptosis, muscle weakness
Okamoto	308840	Hydrocephalus, cleft palate corpus callosum agenesis
Werner mesomelic dysplasia	188770	Hypoplasia of tibia with polydactyly
Pitt-Hopkins	610954	Epileptic encephalopathy, facial dysmorphic features, bouts of hyperventilation, dysautonomia
Jeune	208500	Severely constricted thoracic cage, short-limbed short stature, and polydactyly
Pierre Robin	261800	Glossoptosis, micrognathia, and cleft palate
Miscellaneous associations		
Pallister-Hall (CAVE)	146510	Hypothalamic hamartoma, pituitary dysfunction, central polydactyly and visceral malformations
Fryns	229850	Diaphragmatic hernia, abnormal face, and distal limb anomalies
Aarskog	100050	Short stature, hypertelorism, and shawl scrotum
Fronto-nasal dysplasia	136760	True ocular hypertelorism, broadening of the nasal root, median facial cleft palate...
Osteopetrosis	Various	Macrocephaly, progressive deafness and blindness, hepatosplenomegaly, and severe anemia
Goldenhar	164210	Craniofacial anomalies, and eventually cardiac, vertebral, and central nervous system defects
Lesch-Nyhan	300322	Mental retardation, spastic cerebral palsy, choreoathetosis, uric acid urinary stones...
Rubinstein-Taybi	180849	Broad thumbs and great toes, characteristic facies, and mental retardation
Toriello-Carey	217980	Agenesis of corpus callosum, with facial anomalies and robin sequence
SEMDJL	271640	Vertebral abnormalities and ligamentous laxity resulting in early death
OSCS	300373	Osteopathia striata with cranial sclerosis

Adapted from: Scriver CM et al. The metabolic and molecular bases of inherited diseases. 8th ed. McGraw-Hill, pp 6231-6255. Updated from Amiel et al., 2008

Table 3. Epidemiology and recurrence risk figures in HSCR (12).

	L-HSCR	S-HSCR
% probands	19	81
Sex ratio (male:female)	1.75	5.5
Genetic model	Dominant	Multifactorial or recessive
Penetrance (%) (male:female)	52:40	17:4
Recurrence risk to sibs* (%)		
Male proband	17/13	5/1
Female proband	33/9	5/3

Relative risk=200 *Recurrence risk is given for male/female siblings, respectively.

investigate, and interpret personal and family histories to assess HSCR-recurrence risk, as well as to discuss the potential advantages and disadvantages of genetic testing. They should also be able to assist in evaluating the psychosocial aspects and to identify educational and support resources for both patients and families.

HSCR has been assumed to be a sex modified multifactorial disorder, the effect of genes playing a major role as compared to environmental factors (relative risk of 200). In these terms, the overall recurrence risk in siblings of a HSCR proband has been estimated at around 4%. In isolated HSCR, adequate relative risk figures need to be provided by taking into account the sex and length of the aganglionic segment in the proband and the gender of the sibling (2–33%). Therefore, a relatively precise recurrence risk tailored to individual families could be estimated based on the estimates provided by Badner (Badner et al., 1990). According to Carter's paradox, the highest recurrence risk is for a male sibling of a female proband with L-HSCR (Table 3). Nonetheless, the reduced penetrance of the HSCR mutations makes it difficult to rationally predict and assess the actual recurrence risk for HSCR. According to poor genotype–phenotype correlation thus far, the benefit of mutation screening for HSCR patients appears low, except for systematic testing of mutational hot-spots within RET proto-oncogene related to MEN2 syndrome. This, however, is not a routine practice in most countries.

Finally, many HSCR cases are associated with other congenital anomalies. In these cases, the long term prognosis is highly dependent on the severity of the associated anomalies. Several known syndromes have straight Mendelian inheritance. This emphasises the importance of careful assessment by a clinician trained in syndromology of all newborns diagnosed with HSCR.

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