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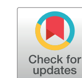
Review article

Literature review: enteric nervous system development, genetic and epigenetic regulation in the etiology of Hirschsprung's disease



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ABSTRACT

Hirschsprung's disease (HSCR) is a developmental disorder of the enteric nervous system (ENS) derived from neural crest cells (NCCs), which affects their migration, proliferation, differentiation, or preservation in the digestive tract, resulting in aganglionosis in the distal intestine. The regulation of both NCCs and the surrounding environment involves various genes, signaling pathways, transcription factors, and morphogens. Therefore, changes in gene expression during the development of the ENS may contribute to the pathogenesis of HSCR.

This review discusses several mechanisms involved in the development of ENS, confirming that deviant genetic and epigenetic patterns, such as DNA methylation, histone modification, and microRNA (miRNA) regulation, can contribute to the development of neurocristopathy. Specifically, the epigenetic regulation of miRNA expression and its relationship to cellular interactions and gene activation through various major pathways in Hirschsprung's disease will be discussed.

1. Introduction

Hirschsprung's disease (HSCR) is a developmental failure of the enteric nervous system (ENS) derived from neural crest cells (NCCs) which affects their migration, proliferation, differentiation, or preservation in the digestive tract. This alteration causes aganglionosis in the distal intestine, resulting in intestinal obstruction. Various genes and signaling pathways are tightly regulated during the development of the ENS by controlling both NCCs and their surrounding environment [1].

The development of HSCR is a complex process that is influenced by various factors, with 20% of HSCR cases are familial, and the rest are sporadic. At least 20 genes are known to have a role in the pathogenesis of HSCR including *rearranged during transfection (RET), glial cel-derived neurotrophic factor (GDNF), GDNF family receptor alpha 1 (GFRα1), neurturin (NRTN), endothelin receptor type B (EDNRB), endothelin 3 (ET3), zinc finger homeobox 1B (ZFHX1B)* or *ZEB2, paired-like homeobox 2b (PHOX2B), SRY-box 10 (SOX10), Indian Hedgehog (IHH)* and *Sonic Hedgehog (SHH)*. These genes encode receptors, ligands, and transcription factors. It has been established that *RET* is the major gene in the etiology of HSCR disease, with other known genes having a minor contribution accounting for about 30% of all HSCR cases [2, 3, 4, 5, 6].

These genes can interact and activate each other via several specific signaling pathways such as RET/GFRα1/GDNF, EDNRB/EDN3/endothelin converting enzyme 1 (ECE1), and Semaphorin, involving transcription factors and morphogens to guide complex molecular signals [7]. Another mechanism that causes changes in gene expression without changing the DNA sequence is known as epigenetics, which occurs through three mechanisms, namely DNA methylation on cytosine nitrogen bases, modification of histone molecules, and microRNA (miRNA) expression [8].

MicroRNA downregulates gene expression through post-transcription mechanisms, namely translation inhibition, and RNA breakdown. MicroRNA are associated with a variety of diseases by influencing cell migration, metastasis, proliferation, and apoptosis [9, 10, 11]. The roles of miRNA in NCCs migration and ENS development have been investigated, and this review will discuss further how the epigenetic regulation of miRNA plays a role in the pathogenesis of HSCR.

2. Hirschsprung's disease

2.1. Definition and incidence

Hirschsprung's disease (HSCR) is a congenital abnormality characterized by loss of ganglion cells in the wall of the distal intestine

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throughout various length. This condition causes the distal segment to lose its ability to perform peristalsis and stimulate the flow movement of feces to the distal intestine. Consequently, the proximal part of the intestine undergoes dilation (distension) and thickening of the wall due to muscle hypertrophy to encourage the movement of feces through the spastic part of the intestine [12, 13].

The incidence of HSCR is 1: 4400–7000 live births, and it is four times more common in male than female babies, with several case series showing that there are racial differences in HSCR incidence. The estimated worldwide incidence of HSCR is around 1 in 5000 live births [5]. Most HSCR cases are sporadic (80%) rather than familial. Mutation in the *RET* gene has been identified in 50% of familial cases and 15–35% of sporadic cases. However, the penetrance of the *RET* mutation is 72% in men and 51% in women [5]. There are no nationwide data regarding the incidence of HSCR in Indonesia. However, based on the database of Dr. Hasan Sadikin Hospital, the referral hospital for the West Java province in Indonesia, there have been 298 HSCR cases in last three years.

2.2. Signs and symptoms

Patients with HSCR present a history of constipation since the neonatal period [14]. The median age at presentation of the symptoms is two days old (age range 1–159 days old), and the median age at diagnosis is nine days old (age range 1–177 days old) with the median time from symptom presentation to diagnosis is five days (range 0–176 days) [12]. The clinical characteristics of HSCR are a distended abdomen, delayed meconium discharge over the first 24 h, and vomiting [13], however, only 26.2% of patients show this classic triad [12].

The first defecation occurs within 24 h after birth in 94% of full-term babies, and within 48 h in 98.5% of full-term babies [12]. A previous study shows that less than 10% of infants with HSCR release meconium within 24 h after birth [15]. Another study has found that 24% of HSCR patients excrete meconium within 24 h and 44% within 48 h [13]. The National Institute for Health and Care Excellence (NICE) guideline regarding constipation in children and adolescents now includes delays in meconium excretion more than 48 h in full-term infants as a red flag and should be referred immediately to rule out the possibility of HSCR [16].

Older children at diagnosis tend to show symptoms of chronic constipation, distended abdomen, vomiting, and failure to thrive. In 10% of cases, enterocolitis, fever, and septicemia can occur [12, 13, 15].

3. Enteric nervous system (ENS)

3.1. Development of the ENS

The mammalian gastrointestinal tract consists of cells derived from all three germ layers, with the epithelial lining of the gastrointestinal tract, liver parenchyma, and pancreatic cells all originating from the endoderm. The mesoderm layer forms mesenchymal elements, including smooth muscle and stromal cells. The juxtaposition of the ectoderm with the neural plate induces the neural crest which in turn generates NCCs, with the migrating pool of enteric neural crest-derived cells eventually giving rise to the ENS [17]. The fetal digestive tract is divided into three segments based on the supply of blood vessels. The foregut, which is supplied by the celiac arteries, consists of the esophagus, stomach, part of the duodenum, and biliary tract. The midgut, which is supplied by the superior mesenteric artery, includes the small intestine and large intestine to the level of the splenic flexure. The hindgut supplied by the inferior mesenteric artery consists of a portion of the remaining large intestine down to the superior anal canal [13].

The ENS is the largest part of the peripheral nervous system with autonomic function, but the regulation of ENS development is different from other peripheral nervous systems [13, 18]. Its organization also appears to be different from that of sympathetic or parasympathetic ganglia, more closely resembling the central nervous system (CNS) than any other region in the peripheral nervous system. There are structural, functional, and chemical similarities between the ENS and the CNS [19].

The ENS assesses the state of the lumen and intestinal wall and responds appropriately, activating intrinsic reflexes that cause peristaltic movements and fecal propulsion, control of blood flow, as well as water and electrolyte secretion to maintain physiological balance [13, 18]. Interdependent mechanisms between neural components such as enteric ganglia and non-neural components, like Cajal interstitial cells control intestinal motility. Cajal interstitial cells act as pacemaker cells that cause and disseminate slow waves, leading to intestine smooth muscle contraction [13, 18, 20].

Intestinal motility is a complex process mediated by interactions between intestinal smooth muscle, Cajal interstitial pacemaker cells, and the ENS. Cajal interstitial cells are responsible for the slow-wave activity of muscles that can spread to surrounding muscles. Although these waves produce contractile activity and propagation of enteric contents craniocaudally, the ENS is important for broader coordination coupled with amplitude modulation and frequency of smooth muscle contraction to produce two types of contractions, namely segmentation and peristaltic. Both of which occur without the existence of extrinsic innervation [20]. A complex circuit consists of enteric neuron structures that regulate various intestinal functions such as motility, secretion, vascular tone, and hormone release. Although there is usually an interplay between the ENS and CNS, in most regions of gastrointestinal tracts, the ENS can function independently. In mammals, enteric neurons aggregate in myenteric ganglia and submucosal ganglia [16]. At the end of the digestive tract is an internal sphincter, a special thickening of the circular smooth muscle in the distal rectum to maintain the status of tonic contractions for continence function. Rectal distension stimulates the sphincter relaxation reflex (rectoanal inhibitory reflex) which depends on the ENS.

Formation of the ENS requires extensive cell migration, controlled cell proliferation, regulated differentiation, directed neurite growth, and interconnected neuron networks [1]. The congenital absence of ganglion in the distal intestine in HSCR is a consequence of either migration disruption, proliferation, differentiation, survival, or apoptosis of normal NCCs [13, 18]. Three important aspects of NCCs to develop ENS are migration, proliferation and differentiation.

3.1.1. Migration

Previous studies on NCCs ablation in animals have shown that neurons and glia of the ENS originated from the vagal and sacral segments of NCCs [21, 22]. The vagal crest (somite level 1–7) provides most of the precursors of enteric neurons and glia and colonization of the entire intestine. These cells are known as enteric neural crest derived cells (ENCCs), the progenitors of the enteric nervous system. Vagally derived NCCs migrate and enter the foregut mesenchyme at human embryonic age 4–5 weeks [23, 24]. The cells migrate rostrocaudally along the gastrointestinal tract to reach the distal ileum at the 7th week, mid-colon at the 8th week, and distal rectum at the 12th week of gestation [13, 18, 25]. By the 10th week, ENCCs reach the distal intestine and intertwine into the myenteric plexus [26]. The ENS in the intestinal segment below the umbilicus is derived from the sacral crest cells (caudal to somite 28) [27, 28]. Vagal and sacral ENCCs appear to be two different distinct cell populations, regarding not only their origin but also their migration behavior. The direction of sacral ENCCs migration is opposite of that of the vagal ENCCs pool; while vagal derived ENCCs migrate rostrocaudally, the sacral derived ENCCs migrate cudorostrally. Vagal ENCCs are more invasive as they colonize almost the entire gut but sacral ENCCs only colonize a small end-part of gastrointestinal tract [20]. When Vagal NCCs are grafted into sacral region, their intrinsic entities are still the same and they are still able to colonize further than do sacral NCC [29].

The enteric ganglia are interconnected to form two plexuses that extend along the intestine with the myenteric plexus (Auerbach) in the outer layer and the submucosal plexus (Meissner) in the inner layer. The myenteric plexus that develops early on lies between the layers of the

longitudinal and circular muscles and plays a role in intestinal motility. The submucosal plexus that develops afterward plays an important role in motility, regulation of blood flow, and ion transport through the intestinal epithelium [13].

Migration of ENCCs is not only a mere movement toward the anus but a dynamic process that involves complex interactions between migrating ENCCs and developing intestines [26]. Different populations of ENCCs exhibit different migration behavior pattern, depending on their position along the migration trail. Cells in the migration wavefront show a significant caudal expansion, while cells in the trailing wave tend to be limited, therefore, the ENCCs in the wavefront are responsible for the colonization of the hindgut [13].

Previous studies have found many molecular signaling mechanisms involved in regulating migration, proliferation, and differentiation of ENCCs, such as RET/GDNF, EDNRB/EDN3 and several morphogens such as Netrin, SHH [26, 30, 31]. It has also been shown that RET/GDNF signals are crucial for the survival and proliferation of ENCCs (Figure 1) [18].

3.1.2. Proliferation

The size of the ENCC population determine the normal speed of cells and completion of cell invasion in the gut [32]. Reduction of vagal NCCs by partial ablation *in vivo* before migration in avians leads to Hirschsprung-like aganglionosis· [21, 29, 33] It is presumed that if the remaining ENCCs are given sufficient time to colonize the gut, eventually complete cell colonization in the gut will occur. However, if complete cell colonization in the gut is required in a particular time frame, then the reduction of initial ENCCs will lead to failure of complete cell colonization· [21, 34].

Simpson, et al. in 2007 developed a mathematical model to predict cell invasion in the gut by incorporating two basic cell functions as parameters, cell motility and proliferation. Their study showed that cell proliferation in the wavefront is critical in driving the invasive process and this mathematical model was validated by experimental data using gut organ culture with chick-quail grafting [35]. However, Newgreen et al. (in 1979) demonstrated that ENS density was independent of the initial number of ENCCs in avian organ culture, implying that ENCC density increases through proliferation to reach the final number of cells desired for proper function [36].

In an animal model, the HSCR-like phenotype occurs when ENCCs failed to reach the caudal part of the gut; the same situation is likely to occur in human leading to HSCR phenotype. Landman et al. (in 2007) developed a mathematical model to predict cell invasion in growing tissue by incorporating cell motility and proliferation as well as gut growth [37]. The cell invasion simulation using this mathematical model generates result similar to those from an *in vitro* experiment using donor-host grafting method. Those *in silico* and lab experimental results showed that in a non-growing gut condition, ENCC invasion waves are organized so that wavefront cells act as a proliferation source generating ENCCs that colonize the reminder of the gut. However, in the growing gut, ENCCs behind the migrating wavefront will actively proliferate to expand and increase the number of ENCCs to fill the developing gut, while the wavefront provides progenitor cells for distal colon colonization [37]. Taken together, these results provide an understanding of why some genes implicated in HSCR affect the ENCCs proliferation.

3.1.3. Differentiation

The ENS in human consist of 200–600 millions neurons of 20 functional classes, comprising a wide range of neurotransmitter, projection and electrical properties [38]. Appropriate differentiation into many neuronal classes and glial is critical for ENS function. Many genes involved in ENCC differentiation during ENS development have been identified, mostly from animal studies. In the large and small intestines, neurogenesis occurs before gliogenesis. Upon neurogenesis, neuron-fated progenitors maintain expression of *Ret* and *Phox2b* but also express neuron markers such as *Tubb3*, *PGP9.5*, *Nf*, and *TH*. As they develop into mature neurons, they become negative for glial markers and do not further proliferate [39]. Different neuronal-progenitor cells exit the cell cycle at different time points, with cells expressing early neuron markers such as Tuj1 and HuC/HuD appearing in the ENS soon after colonization begins, although most ENCCs, particularly in the migrating wavefront, remain undifferentiated. The earliest formed neurons are serotonin (5-HT) neurons arising at day 8–14 (peak at day 10), followed by cholinergic neurons which produce choline acetyltransferase (peak at day 12), and enkephalin (peak at day 14) [40].

Sox10 and Edn3 are both important in the maintenance of ENCCs in mammals, however, *Sox10* is not sufficient to maintain ENCCs in an undifferentiated state as glial cells also express this gene [41]. Notch in

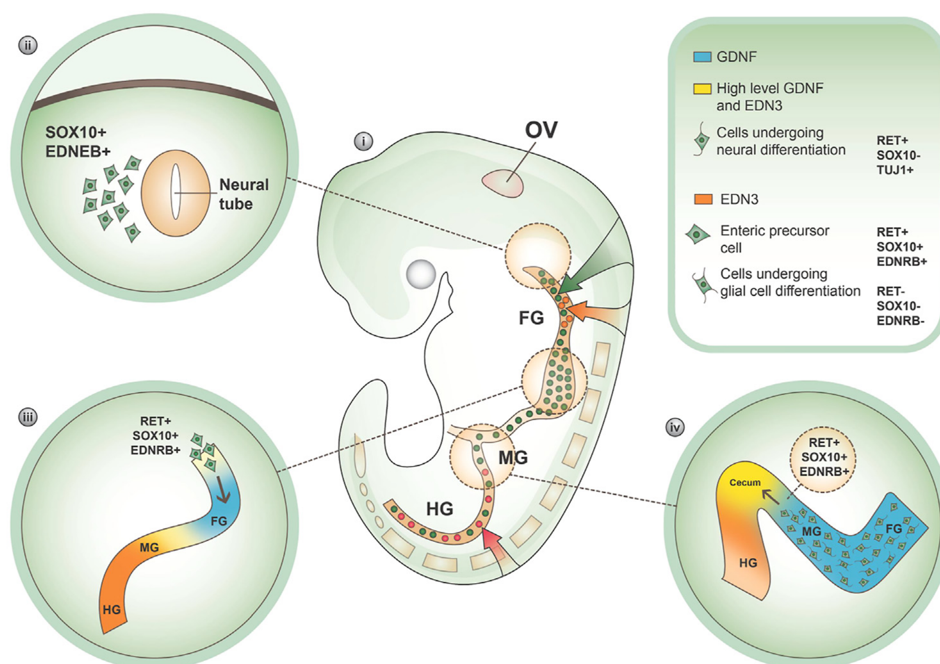

Figure 1. (i) ENCC migration routes. FG: foregut, MG: midgut, HG: hindgut, OV: otic vesicle. Orange arrows: vagal NCCs, green arrows: anterior truncal NCCs, red arrows: sacral NCCs.(ii) ENCCs migrating from the neural tube express *SOX10* and *EDNRB*. (iii) ENCCs migrate along the gastrointestinal tract in a rostral to caudal direction following the expression of *GDNF*, which is highly expressed in the foregut (FG) and midgut and *EDN3* which is highly expressed in the hindgut (HG). This enteric neural crest cells (ENCCs) population expresses *RET, SOX10*, and *EDNRB*. ENCCs behind the migration wavefront are starting to differentiate into neurons and glial cells at the same time.

mice is required to prevent premature differentiation and depletion of undifferentiated ENCCs. Transcriptomics studies on gut tissue and animal study performed by Nishino et al. (2010) showed that Lgi4 is required for the differentiation of glial cells [42]. BMP signaling involved in the cell cycle exit of the ENCCs and induces glial differentiation in the ENS, subsequently, developing glial cells are dependent on glial growth factors such as GGF2 and NRG1. Enteric glial cells start to form from day 12 onward in rat and continue to mature to 4 weeks postnatal [40, 43].

The neurons are grouped into ganglia, with each ganglion containing glial cells and many different types of neurons such as motor neurons, sensory neurons, interneurons, secretomotor neurons, and vasomotor neurons [38]. Many signaling pathways, including BMP2 and BMP4, NOTCH, SHH, SEMA3A, NRG1, and Neurturin play a role in plexus patterning [31]. The balance between proliferation and differentiation is necessary to maintain sufficient progenitor cell collections to ensure total colonization of the ENS [13, 19].

3.2. Genetic aspects of the development of the ENS

NCCs are transient and multipotent progenitors that produce a variety of cell types, including neural, endocrine, pigment, craniofacial, and conotruncal cardiac cells. Neurocristopathy is a diverse class of pathologies that may arise from developmental defects of NCCs [13, 25]. Conditions that cause interference with the migration of enteric neuroblasts can also affect migration, differentiation, or preservation of other cells that are also derived from NCCs. Disruption of genetic sequences that produce changes in the function of any gene responsible for migration, proliferation, differentiation, preservation, or changing the permissive environment for migration of NCCs has the potential to cause the failure of the development of the ENS [20].

Mutations in target genes alter the molecules involved in intestinal colonization by ENCC, including factors secreted by intestinal mesenchyme acting on receptors expressed by ENCCs, transcription factors, morphogens, proteins that send signals from the cells membranes to the nucleus, and adhesion molecules. Mutations in genes that encode many of these components have been linked to the occurrence of HSCR in humans and most of these factors are known to affect many cellular processes during development [44].

Many genes contribute to the normal migration, proliferation and differentiation of ENCCs which eventually form a functional ENS, including *RET, GDNF, NRTN, EDNRB, EDN3, ECE1, PHOX2B, SOX10, PAX3*, *SHH, IHH, GLI,* and *ZEB2* (*SIP1, ZBFX1B*). Mutations can be in the form of nonsense mutations, missense mutations, small deletions, and insertions. Three major contributing genes to the pathogenesis of HSCR are *RET, EDNRB,* and *GDNF* [12, 45, 46]. Each of these complex cellular events must be guided by certain molecular signaling pathways such as RET/GDNF and EDNRB/EDN3, transcription factors such as SOX10, PAX3, PHOX2B, ZFHX1B, TITF-1 or even among several morphogens like Hedgehog (HH), Netrins, or Semaphorins [7, 26, 47, 48, 49].

3.2.1. RET/GDNF pathways

The *RET* proto-oncogene is a major gene that causes HSCR and *RET* mutations have been identified in 50% of familial cases and 15–35% of sporadic cases of HSCR. In total, at least 20% of all HSCR cases are caused by the *RET* mutation, hence this gene is the main gene involved in HSCR etiology [2, 13, 18, 50].

The *RET* proto-oncogene is located on chromosome 10 band q11.2 and encodes a tyrosine kinase transmembrane receptor. Its expression in ENCCs requires SOX10 and PHOX2B transcription factors, with the loss of either factor leading to intestinal aganglionosis. The RET protein has a large extracellular domain, a transmembrane region, and an intracellular kinase domain. It functions as a signaling receptor and has four ligands, namely GDNF, NRTN (neurturin), ARTN (artemin), and PSPN (persephin) [51, 52, 53, 54].

These ligands activate RET by binding to their respective GPI (glycosylphosphatidylinositol*)*-linked GDNF family of receptors (GFRα1-4) to form a ligand-co-receptor complex, which binds to RET and induces its dimerization and autophosphorylation of the tyrosine residue in the intracellular domain [55]. These tyrosine residues act as docking sites for adapters and signaling proteins to stimulate multiple downstream pathways [26]. These activated downstream effector molecule are respectively RAS/mitogen-activated protein kinase (MAPK) pathway, Jun-associated N-terminal kinase (JNK), and phosphatidylinositol-3 kinase (PI3K-AKT), JAK-STAT, ERK, and PKC which support cell growth, proliferation, preservation, and differentiation of cells [56].

The *GDNF* gene consists of two exons (151 and 485 base pairs) located on chromosome 5. The GDNF protein consists of 134 amino acids with a molecular weight of 32–42 kDa (kD) in the form of dimers, which act as ligands for the multi-subunit glycosylphosphatidylinositol (GPI)-anchored co-receptor GFR-α [57]. This complex binds to RET providing signaling components that are involved in and support migration, proliferation, differentiation, and survival of neurons in the ENS [58, 59, 60].

GDNF is a chemoattractant signal for ENCCs and when the wavefront of ENCCs reaches the esophagus, *GDNF* is expressed in the stomach, then increases again in the cecum as ENCCs approach the distal small intestine. This suggests that GDNF attracts ENCCs that express *RET* and *GFRα* to the right location [60]. Thus, aganglionosis can occur by the absence of the RET-GFRα1-GDNF complex caused by a mutation in either one of those encoded genes [6, 45, 61, 62, 63, 64, 65]. Functional tests show that the absence of either GDNF or GFR-α decreases or eliminates RET/GDNF signaling [19].

Pathology examination revealed that GDNF immunoreactivity is found in the ganglion in the myenteric and submucosal plexus. In the hypoganglionic segment, GDNF immunoreactivity was found to decrease and GDNF immunoreactivity was absent in the aganglionic segment [25]. The *GDNF* mutation has been identified in limited cases, therefore, *GDNF* is considered a rare susceptibility gene for HSCR [6, 64, 66]. Recently, Sribudiani et al (2018) identified multiple mutations in known HSCR genes in a large family with HSCR, one of which was an in-frame deletion of *GDNF.* Functional studies *in vitro* showed that this mutation prevented secretion of its product reducing RET activation [6].

Another role of RET is the regulation of apoptosis. When there are ligands, RET produces a positive control signal (stimulation) to maintain cell development and survival, whereas, in the absence of ligands, RET produces a negative control signal (inhibition) and apoptosis occurs. This apoptotic effect can be inhibited in the presence of GDNF [67].

3.2.2. EDNRB/EDN3 pathways

Endothelin receptor type B (*EDNRB)* gene is located on chromosome 13q22 and encodes heptahelical proteins, known as G-protein-coupled receptors that contain seven transmembrane domains expressed by the neural crest derivatives. The extracellular and transmembrane regions are involved in ligand binding, whereas the intracellular domains are involved in intracellular signaling pathways that are mediated by G proteins [67]. Endothelin 3 (EDN3) is a ligand of EDNRB and Endothelin converting enzyme 1 (ECE1) converts the inactive EDN3 precursor to the active form [30, 66, 67].

HSCR patients appear to have mutations in *EDNRB, EDN3,* and *ECE1* in 5% of cases. A study in a mouse model found that EDNRB/EDN3 signals are involved in ENCC migration as the *EDNRB* is expressed by ENCCs that are migrating while *EDN3* is expressed in the midgut and hindgut mesoderm. Other studies have found a role for EDNRB/EDN3 signals in the development of the ENS, EDN3 activation of EDNRB induces ENCCs to proliferate, maintains precursor status, and prevents premature differentiation [30, 31].

Mutation in the *EDNRB/EDN3* gene tends to cause short-segment HSCR (S-HSCR), whereas *RET* mutations play a role in both short- and long segment-HSCR (L-HSCR). This may be due to the dysregulation of the *EDNRB/EDN3* and *RET/GDNF* signaling impacting the NCCs at different times and location during ENCCs migration in the gut [65, 67, 68].

3.2.3. Semaphorin pathways

Semaphorin is a transmembrane protein or GPI-linked protein involved in neuronal migration, proliferation, survival, and axonal guidance. These proteins are grouped into various classes and subgroups [67]. Their main receptors are the Plexin family receptors. Plexin causes activation of Plexin-associated tyrosine kinase and inhibits integrin-mediated cell adhesion and events downstream of its activation pathway. Semaphorin was originally known as a protein that plays a role in the formation of neural circuits and mediates inhibition of cell motility, thereby interfering with ENCC migration [7, 69].

In the developing cecum and colon, *Semaphorin3A (SEMA3A)* is expressed by the mesenchyme. In mice, *Sema3a, Sema3c*, and *Sema3d* are expressed by the ENS. *sema3c* and *sema3d* are also expressed in zebrafish and their knockdown resulted in decreased migration of ENS precursors. [69] In the aganglionic colon segment of HSCR patients, *SEMA3A* was found to be expressed at a higher level than in the ganglionic segment of HSCR or of control patients, therefore, increased *SEMA3A* expression could be a risk factor for HSCR [70]. Recently, the signaling pathway of *Semaphorin3C/3D* has been considered a regulator in the development of the ENS [49]. Deviant expression of *SEMA3D* has been found in HSCR patients [48].

3.2.4. Transcription factors

Mutation in the transcription factor of *RET* such as the *SOX10* gene has been reported to cause a high percentage of Wardenburg syndrome type IV (WS4), a congenital disorder characterized by hearing loss, pigmentary abnormalities, and HSCR [71]. In 2010, Sanchez Mejias et al. identified for the first time the *SOX10* mutation in isolated HSCR cases. Mutation in *PHOX2B* has been identified in both isolated and syndromic HSCR cases, such as congenital central hypoventilation syndrome (CCHS) [72, 73]. An *in vitro* study showed that poly-alanine contraction at the 3′UTR of *PHOX2B* reduces the transcription activity, hence, it is likely to down-regulate *RET* expression and lead to HSCR [72]. Garcia-Barcello et al. (2005) reported that the expression of thyroid transcription factor 1 (TITF-1) overlapped with $p75^{NTR+}$, a marker for enteric neural crest progenitor cells in humans. TITF-1 trans-regulates *RET* expression by binding to the HSCR-associated locus region in the *RET* promoter [74]. The same group also identified a mutation in the *TITF-1* gene in Chinese and Caucasian cohorts, with the frequency of the TITF-1 mutation in Caucasians ~2.85% [75].

3.2.5. Hedgehog (HH) signaling

Glioma-associated oncogene homolog 1 (*GLI*), a zinc finger protein is a downstream effector of SHH and IHH in the Hedgehog signaling pathway. A mutation in *GLI1* was first identified in a Chinese HSCR patient (~25%) in 2015. Subsequently, a functional study *in vitro* showed that this mutation leads to an increase in *SOX10* expression which is known to play a role in regulating neuronal-glial differentiation and migration. The high expression of *GLI* due to loss of *Sufu* leads to defective axonal fasciculation and delayed cell colonization in the mouse gut, resulting in hypoganglionosis [76]. Sribudiani et al. (2018) identified a missense mutation in exon 1 of *IHH* (p.Gln51Lys) and a missense mutation in exon1 4 of *GLI3* (p.Pro707Ser) in a large family with HSCR. A functional study on the *IHH* mutation *in vitro* showed that the mutation leads to reduced expression of GLI1 protein. Injection of morpholinos which target *ihh* in zebrafish reduce the number of neurons by 13% compared to control [6].

About 30% of HSCR patients show mutations in genes organized into four categories: (a) genes that affect the RET/GDNF pathway; (b) genes involved in the EDNRB/EDN3 pathway; (c) transcription factors; and (d) other genes or cell elements involved in the development of ENS. Although *RET* mutation is known to be a major risk factor for HSCR, mutations in other additional genes have also been linked to HSCR (Figure 2).

Table 1 summarizes some of the known HSCR genes, their function, and their role in ENS development. These genes do not act independently but also interact with each other. Initially, no connection was found between the RET/GDNF and EDNRB/EDN3 signaling pathway, so these pathways were assumed to operate separately. However, there is an interaction between the RET and EDNRB signaling pathways that regulates the development of the ENS.

EDNRB activation specifically enhances the effect of RET signaling for ENS progenitor proliferation, as well as modifies the migration response of NCCs. EDN3 and GDNF appear to have a synergistic effect on the proliferation of undifferentiated progenitors and an antagonistic effect on the migration of ENCCs. The interaction between the RET/GDNF and EDNRB/EDN3 signaling pathways has been found to regulate ENS development throughout the gut, providing evidence that there is a coordinated interaction between these signaling pathways [67].

3.3. Epigenetic aspects of ENS development

The term epigenetics was first introduced by Conrad Waddington in 1975 as a branch of biology that studies the causal interactions between genes and their products that form phenotypes. Epigenetic features are phenotypes that result from chromosome changes without changes in the order of deoxyribonucleic acid (DNA) [8, 128].

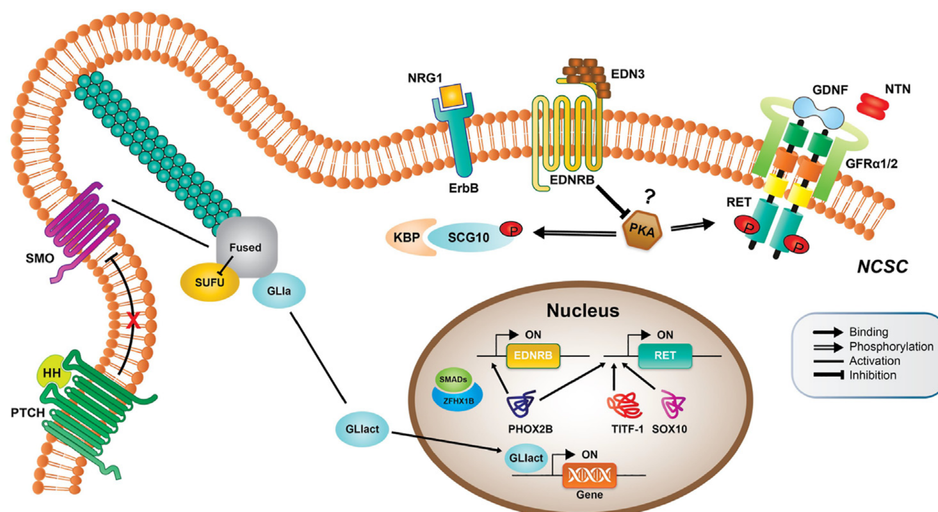

Figure 2. Genes and major signaling pathways (e.g RET/GDNF; EDNRB/EDN3) involved in the pathogenesis of Hirschsprung disease (modified from Alves, 2013 [2]). NCSC: neural crest stem cell.

Table 1. The genes involved in HSCR, the accompanying conditions, and their function during ENS development.

Gene	Abbreviation	Mutation	Underlying Condition	Function in ENS Development
Receptor tyrosine kinase	*RET*	Identified in 50% of familial cases [64, 77, 78] and 15–35% of sporadic cases [64, 79]	Isolated HSCR, Multiple endocrine neoplasia type IIA (MEN2A) syndrome, type IIB (MEN2B), and medullary thyroid carcinoma	Expressed by ENCCs [80] Supports proliferation, migration, survival and differentiation of ENCCs [81]
Neuregulin 1	*NRG1*	Mutation identified in familial and sporadic HSCR cases [77, 78].	Isolated HSCR cases	Suppress GDNF-induced neuronal differentiation. Survival of postnatal enteric neurons [82, 83]
Glial cell-line derived neurotrophic factor family receptor α	*GFRα*	-	-	Binding interaction with GDNF [57] Support survival of ENCCs [60, 84]
Ligands of RET: *glial cell-derived neurotrophic factor,* *Neurturin,* *Artemin,* *Persephin*	*GDNF,* *NRTN,* *ARTN,* *PSPN*	2.17% [85] <5% [66] 1.40% [85] 1.40% [85] 0.9% [85]	Isolated HSCR	*GDNF* family RET ligand produced by intestinal mesenchyma [59, 60, 86] Proliferation of undifferentiated ENCCs, promotes axon projections from excitatory motor neurons [87, 88, 89] Promote ENCCs proliferation, migration and differentiation [85, 90]
Endothelin B receptor	*EDNRB*	Identified in ~5% [91]	Waardenburg-Shah Syndrome (WS4)	Expressed by ENCCs, maintains *ENCCs* in an undifferentiated state; *EDNRB* expression is regulated by *SOX10* [92, 93, 94]
Endothelin 3	*EDN3*	Identified in <5% [95, 96, 97]	Waardenburg-Shah Syndrome (WS2), Isolated HSCR	*EDNRB* produced by intestinal mesenchymes especially the caecum, and its interaction with *EDNRB* allows distal bowel colonization [41, 92]
Endothelin-converting enzyme 1	*ECE-1*	1 case reported [98]		Proteolytic conversion of *endothelin-3* precursors into their active form [99]
SRY-related HMG-box 10	*SOX10*	Waardenburg-Shah Syndrome (WS4) [71, 99]	Hearing loss, HSCR, pigmentary abnormalilities	Expressed by ENCCs, maintains ENCCs in an undifferentiated state, cell fate, and glial cell differentiation; activates *RET* transcription and interacts with EDNRB [100, 101, 102, 103, 104]
Pairedlike homeobox 2B	*PHOX2B*	Mutation identified in syndromic and isolated HSCR cases [105, 106, 107]	Neuroblastoma congenital central hypoventilation syndrome (CCHS) and HSCR	Expressed by ENCCs; important for the development of derivatives of NCCs. PHOX2B is required for *RET* expression [108, 109]
Zinc finger homeobox 1B	*ZFHX1B (ZEB2)*	No mutations were found in isolated HSCR [110]	Mowat Wilson Syndrome	Expressed by ENCCs; important for the formation of vagal NCCs [111, 112]
Thyroid Transcription Factor-1	*TITF1*	Mutation identified in Chinese and in Caucasian (~2.85%) [74, 75]	Isolated HSCR cases	Express in myenteric and submucosal plexus; TITF-1 trans-activates *RET* gene expression [74].
Kinesin Binding Protein	*KBP*	Mutation identified in syndromic HSCR cases [113]	Goldberg-Shprintzen Megacolon syndrome (GOSH)	KBP binds to SCG10 to regulate axon growth and maintain neuronal development in the central and enteric nervous systems [114, 115].
L1 Cell Adhesion Molecule	*L1CAM*	No mutation was found in isolated HSCR [116, 117, 118]	X-linked hydrocephalus (XLH) and HSCR	Part of the neural cell adhesion molecule promotes ENCCs migration and differentiation [119, 120].
Semaphorins	*SEMA3A* *SEMA3C* *SEMA3D*	Mutation identified in HSCR cohorts [48, 69, 70, 121]		Delays entry of axons and sacral ENCCs into the hindgut. Control neurons connectivity and migration [122, 123, 124].
Hedgehogs Genes	*IHH, GLI*	Mutation identified in familial and sporadic HSCR cases [6, 76]	Isolated HSCR cases	Promote ENCC differentiation into neurons, proliferation and concentric patterning, as well as the survival of a subpopulation of [23], [125, 126, 127]

Epigenetics are involved in many normal cellular processes. Human cells have the same DNA but the body contains many different types of cells, such as neurons, liver cells, pancreatic cells, inflammatory cells, and others [129, 130]. Cells, tissues, and organs are different because

they have certain sets of genes that are "turned-on" or expressed, and other sets that are "turned-off" or inhibited. Epigenetic silencing is one way to turn off genes and can contribute to differential expression. Three systems can interact with each other in the process of gene silencing, DNA methylation, histone modification, and microRNA (miRNA) [129, 130, 131].

3.3.1. DNA methylation

DNA methylation is a chemical process that adds a methyl group to DNA. This is a very specific process and takes place in the area of the cytosine nucleotide located next to the guanine nucleotide and is connected by a phosphate called the *CpG* site. The *CpG* site is methylated by DNA methyltransferases (DNMTs) enzymes. Inserting a methyl group alters the appearance and structure of DNA and modifies gene interactions with other components in the cell nucleus required for transcription [8, 129]. This process is mediated by the family of DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B. Based on its function, DNMT1 represents the maintained methyltransferase and DNMT3A and 3B act as *de novo* methyltransferases [1].

Hypermethylation causes silencing of genes or decreases gene expression, while hypomethylation causes increased transcription activity thereby increasing gene expression (Figure 3) [130]. The *RET* promoter has a *5′CpG3* rich region that is easily influenced by methylation, so the degree of methylation regulates *RET* gene expression. Epigenetic inactivation through hypermethylation of *RET* promoters has been investigated [42]. The expression of *GDNF* (ligand of RET) is decreased in some HSCR patients due to hypermethylation of the promoter region, whereas *GDNF* demethylation increases cell proliferation and viability, cell cycle development, and cell invasion in *in vitro* studies of cells derived from HSCR tissue [130].

3.3.2. Histone modification

Histones are the main protein component of chromatin, a complex of DNA and proteins which can form nucleosomes. The histone acts as a spool where DNA can spin [129, 131]. Histones condense DNA strands into structural components called nucleosomes, with each nucleosome containing eight histones, two of each nucleus histones (H2A, H2B, H3 and H4), forming an octameric structure called the nucleosome nucleus, in which DNA is wrapped in an unstructured tail [130, 132].

When histones are modified, this can affect the arrangement of chromatin determining whether the associated chromosomal DNA will be transcribed. If chromatin is less condense (euchromatin), it is in the active phase and the related DNA can be transcribed, whereas if chromatin is more condensed (creates a complex called heterochromatin), then chromatin is inactive and DNA transcription does not occur [129]. The histone core protein is highly conserved throughout evolution and the tail undergoes post-translational modifications such as methylation, acetylation, deacetylation, phosphorylation, ubiquitination, and sumoylation (Figure 4). So far, only methylation, acetylation and histone deacetylation have been described to play a role in the development of NCCs and neurochristopathic onset [130].

Various mechanisms of histone methylation and acetylation have been associated with the development of NCCs, although their implications for HSCR are unknown, hence their potential role in the pathogenesis of HSCR should be investigated. To date, the only histone modulator factor that has been associated with HSCR is *MECP2 (Methyl-CpG binding protein 2)*. The finding of decreased *MECP2* expression level in HSCR patients suggests that changes in *MECP2* expression level may be relevant in the etiology of HSCR through regulation of histone modification [133].

3.3.3. MicroRNA

MicroRNA (miRNA) is a non-coding RNA 19–25 (~22) nucleotides in length, which binds to the 3′ untranslated region (3′-UTR) messenger RNA (mRNA) target of the gene that encodes the protein [44, 134]. Biogenesis of miRNA starts from the transcription of the miRNA gene by RNA polymerase II which produces primary miRNAs (pri-miRNAs), followed by the breakdown by dsRNA-specific ribonuclease Drosha/-Pasha, which breaks down pri-miRNAs from hundreds to thousands of base pairs long into precursor miRNAs (pre-miRNAs) with ~70 base pairs [135]. This process is known as "cropping" [136]. Pre-miRNA, in the form of a hairpin, is transported from the nucleus to the cytoplasm, which is then broken down by RNAse III Dicer into a miRNA duplex of ~22 nucleotides consisting of 2 strands, the "guide" strand and "passenger" (Figure 5) [136]. The "passenger" strand is degraded, and the "guide" strand is incorporated into the RNA-induced silencing complex (RISC) and acts as a functional mature miRNA, working via different mechanisms based on complementarity with the target mRNA [137, 138].

This is achieved through a one-to-many or many-to-one pattern in regulating gene expression [139, 140, 141]. MiRNA guides the miRNA-induced silencing complex (miRISC) to recognize mRNA and decreases regulation of gene expression through post-transcriptional mechanisms, i.e. inhibition of translation and break down of mRNA [9]. If miRNA is almost completely complemented with the target mRNA, there will be degradation of mRNA but if the complementarity is only partial, translational inhibition will occur (Figure 6) [142].

Since miRNA was found as a potential target for HSCR treatment in the future, identification of miRNA and its target genes has become very important. Sergi et al. summarize the miRNA studies that have been conducted to date related to the involvement of miRNA in the pathogenesis of HSCR. Several miRNA target genes have been linked to HSCR, which are mostly involved in cell migration and proliferation. A previous study showed that *miR-34b, miR-146a, miR-196a2, miR-200a, miR141,*

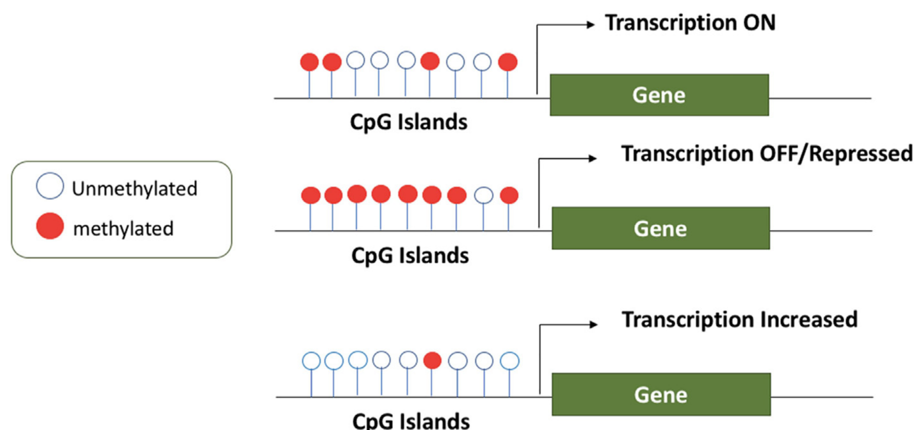

Figure 3. Illustration of transcription regulation by DNA methylation.

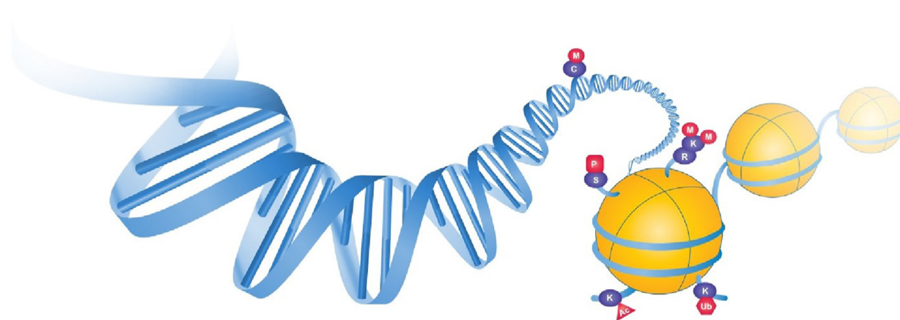

Figure 4. Illustration of histone modification. Ac: acetylation, M: methylation, P: phosphorylation, Ub: ubiquitin, C: cytosine, S: serine, R: arginine, K:lysine, Yellow spheres: histones.

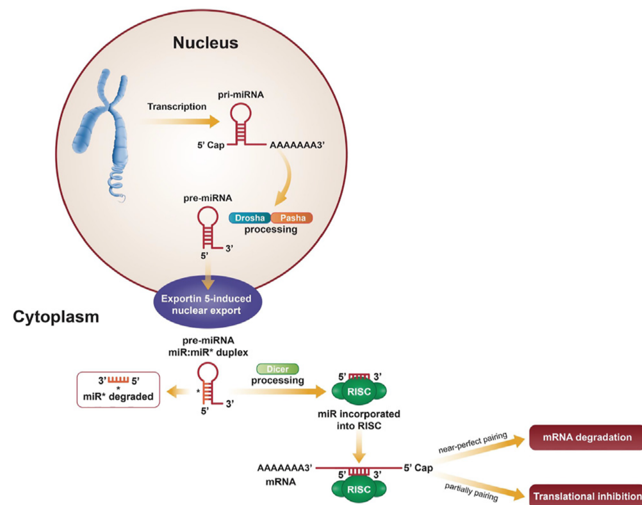

Figure 5. Illustration of miRNA Biogenesis.

and *miR-192* are downregulated, while *miR-195, miR206,* and *miR-218-1* are upregulated in HSCR; these miRNAs changes alter the expression of genes involved in the pathogenesis of HSCR [49].

One study in China analyzed the miRNA expression in a profile of colon tissue in HSCR patients, finding the increased expression of 73 miRNAs (*miR-141-3p, miR-200a-3p, miR-345-5p*, and *miR-194-5p)* and decreased expression of 89 miRNAs (*miR-1228-5p*, *miR-143-5p*, *miR-30a-3p*, and *miR3180)* [143].

Other studies describing miRNA profiles of colon tissue of HSCR patients found 168 expressed miRNAs (104 upregulated and 64 downregulated), including *miR-142-3p, miR-142-5p, miR-146b-5p, miR-369-3p,* and *miR-429* which represent the main targets of miRNA dysregulation; these miRNAs are significantly upregulated in the aganglionic colon segment of HSCR patients ($p < 0.05$) and their target genes encoded proteins are involved in the regulation of cell proliferation and migration through RET pathways and other related signaling pathways such as *MAPK* and *PI3K/Akt* [140].

Analysis of *miRNA-214* expression with the pleiomorphic adenoma gene-like zinc finger 2 (*PLAGL2*) gene target from the colon tissue of HSCR patients has also been investigated, showing increased levels of *miR-214* expression in the aganglionic colon segment, which inhibits cell migration and proliferation directly by deregulating the expression of the target gene *PLAGL2* [144]. In other studies, it has been found that the level of *miR-369-3p* expression in the aganglionic colon segment increased compared with the normal colon. The SRY-box 4 (*SOX4*) target gene is decreased in both mRNA and protein encoded by *SOX4*, significantly suppresses cell migration and proliferation [145].

Research on *miR-206* expression targeting the Fibronectin-1 (*FN1*) gene in an HSCR patient population found that *miR-206* expression levels were upregulated or two-fold higher in the ganglionic colon segment, and downregulated 0.5 times in the aganglionic colon segment [146]. This result is in contrast to other studies that found a downregulation or decreased level of *miR-206* expression results in overexpression of the target serum deprivation response (*SDPR*) gene. This causes deformation of neural crest cell caveolae, thus interfering with signal transduction in cell differentiation and migration [147]. A summary of studies relating to the involvement of miRNA in the pathogenesis of HSCR is provided in Table 2.

A summary of studies relating to the involvement of miRNA in the pathogenesis of HSCR is summarized in Table 2.

Previous studies have identified several miRNAs that are regulated and expressed differently in the stenotic colonic tissue of HSCR patients.

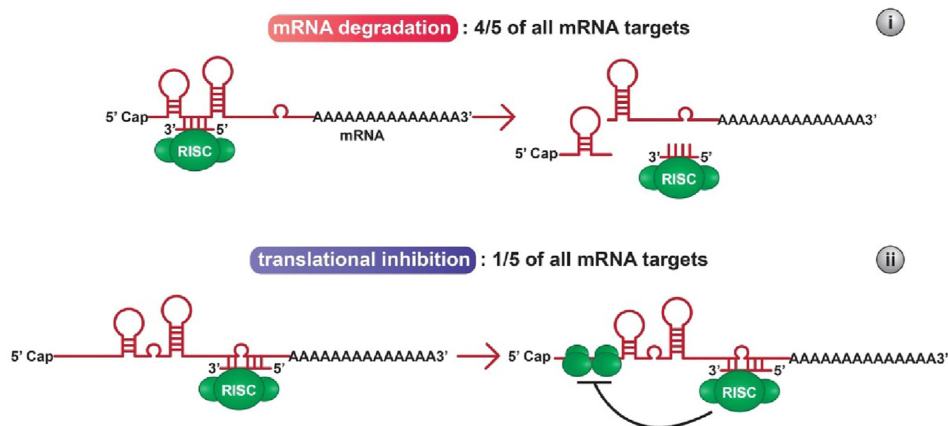

Figure 6. Mechanism of action of miRNA against mRNA targets based on complementarity between miRNA and 3′-UTR: (i). complete complementarity, (ii). partial complementarity.

Table 2. Research on miRNA and how it relates to HSCR.

No	Researcher	Years	Sample	Method	Result	Conclusion
1.	Tang et al. [148]	2013	70 HSCR colon and 60 controls	RT-PCR, western blot, MTT assay and flow cytometry	↓*miR-141*, ↑*CD47*, ↑*CUL3* in HSCR ($p < 0.05$)	An aberrant drop of *miR-141* plays a role in pathogenesis of HSCR with inhibits cell migration and cell proliferation
2.	Zhou et al. [133]	2013	73 pairs of colon/rectal tissue specimens, including HSCR stenotic, HSCR dilatation, and normal tissue	qRT-PCR, Western blot	↓*MeCP2* in a HSCR tissue. *miRNA-34b* expression not affected ($p < 0.05$)	*MeCP2* expression level may play an important role in the pathogenesis of HSCR by decreasing proliferation
3.	Tang et al. [141]	2014	95 HSCR serum and 104 controls	Taqman low density array, qRT-PCR	↑*miR-133a*, ↑*miR-218-1*, ↑*miR-92a*, ↑*miR-25*, ↑*miR-483-5p* ($p < 0.05$)	5 types of miRNAs in the serum as HSCR markers have the potential to be a non-invasive diagnostic tool for initial HSCR screening
4.	Mi et al. [149]	2014	50 HSCR patients		↑*miR-124* in aganglionic colon	*miR-124* and *SOX9* target gene is overexpressed in the aganglionic colon
5.	Zhu et al. [150]	2014	254 HSCR cases and 265 controls	SNP genotyping (rs2910164, rs11614913), qRT-PCR	The expression of miR-146a is higher for GG than CC genotype; *G allele of rs2910164 is* associated with HSCR ($p < 0.005$ OR, 1.54; 95% CI, 1.06–2.23).	G allele of rs2910164 is a risk factor for HSCR, increasing miR-146a expression and decreasing the expression of *ROB O 1* which might affect cell proliferation and migration of NCCs
6.	Li et al. [151]	2014	88 HSCR cases and 75 controls	qRT-PCR, western blot	↓*miR-200a* & *miR141* associated with ↑ *PTEN mRNA* and protein ($p < 0.05$)	Family of *miR*200 might play important roles in the pathogenesis of HSCR with coregulator *PTEN*.
7.	Lei et al. [152]	2014	78 HSCR colon samples and 66 controls	Cell counting kit 8(CCK-8)	↑*miR-195* in HSCR ($p < 0.05$)	Aberrant expression of *miR*-195 might be involved in the pathogenesis HSCR by ↓ *DIEXF* expression level.
8.	Sharan et al. [147]	2015	80 stenotic HSCR colon samples, 80 dilated HSCR colon samples, and 80 controls	qRT-PCR, western blot, transwell assay, CCK8 assay, flow cytometry, dual- luciferase reporter assays	↓*miR-206* in HSCR compared to control ($p < 0.05$), ↑*SDPR*	*miR-206* inhibitor suppresses cell migration and proliferation without affecting cell cycle and apoptosis; silencing of *SDPR* can reduce the extent of the suppressive effect of the *miR-206* inhibitor.
9.	Gao et al. [143]	2017	6 HSCR colon samples and 3 controls	Data set screening GSE77296, target gene prediction by miRWalk, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and gene ontology (GO) term analyses, DEM-target gene interaction network analysis	↑*miR-141-3p* ↓*miR-30a-3p*	Two miRNAs (*miR-141-3p* and *miR-30a-3p*), *MAPK* signaling pathway, and two major genes (*FZD3 and DOK6*) involved in the pathogenesis of HSCR
10.	Tang et al. [153]	2015	69 HSCR (42 S-HSCR, 27 L-HSCR) 49 controls	qRT-PCR, Western blot, cell proliferation, cell cycle, and apoptosis, transwell assay, dual luciferase reporter assays	↑*miR-218-1* ↑*SLIT2* ↓*RET, PLAG1* ($p < 0.05$)	Overexpression of *SLIT2* inhibits cell migration by binding to *ROB O 1* receptor
11.	Zhu et al. [154]	2015	80 HSCR colon samples and 77 controls	qRT-PRC western blot, a dual-luciferase reporter assay, transwell assay, CCK8 assay, flow cytometry	↓*miR-192/215* in HSCR ($p = 0.0001$) ↑*Nidogen1 (NID1)*	Reduced *mir-192/215* inhibits cell migration and proliferation; silencing of *NID1* can reduce the extent of the suppressive effect of *miR-192/215* inhibitor
12.	Li et al. [140]	2016	76 HSCR colon samples and 3 control	miRNA target prediction by miRWalk software, qRT-PCR	Significant ($p < 0,05$) *Upregulated* ↑: *miR-142-3p, miR-142-5p, miR-146b-5p, miR-369-3p*, dan *miR-429. Downregulated* ↓: *miR-885-3p.* ($p < 0.05$)	miRNA play important roles in the complex and multifactorial pathophysiology of HSCR
13.	Lei et al. [155]	2016	70 HSCR colon samples and 62 controls	qRT-PCR, western blot, dual-luciferase reporter assay, transwell assay, CCK8 assay, flow cytometry	↓*miR-215*, ↑*Sialic acid binding Ig-like lectin 8 (SIGLEC-8)* ($p < 0.05$)	*miR-215* causes *SIGLEC-8* inhibition by binding directly to the 3′-UTR region of *SIGLEC-8* Silencing of *SIGLEC-8* can reduce the extent of the effect of suppressing cell migration and proliferation due to the decline of *miR-215* *IARS2-miR-215-SIGLEC-8* pathway might play role in the pathogenesis of HSCR

(*continued on next page*)

Table 2 (*continued*)

No	Researcher	Years	Sample	Method	Result	Conclusion
14.	Pan et al. [145]	2017	60 HSCR colon samples and 47 controls	qRT-PCR, dual-luciferase reporter assay, transwell assay, CCK8 assay, flow cytometry	↑*miR-369-3p*, ↓*SOX4* ($p < 0.05$)	Aberrant expression of *miR-369-3p* may be involved in the pathogenesis of HSCR by regulating the expression of *SOX4*; dysregulation of *miR-369-3p* and *SOX4* suppresses cell migration and proliferation
15.	Wang et al. [156]	2017	20 HSCR colon samples and 20 controls	qRT-PCR, western blot, dual-luciferase reporter assay, transwell assay, CCK8 assay, flow cytometry	↑*miR-483-5p*, ↓*GFRA4* ($p < 0.05$)	*miR-483-5p* plays a role in the pathogenesis of HSCR with the target gene *GFRA4* Downregulation of *miR-483-5p* increases cell proliferation, cell cycle, cell invasion, and inhibit cell apoptosis
16.	Wu et al. [144]	2018	24 HSCR colon samples and 20 controls	qRT-PCR, western blot, dual-luciferase reporter assay, transwell assay, CCK8 assay, flow cytometry	↑*miR-214*, ↓*PLAGL2* ($p < 0.001$)	*miR-214* plays a role in the pathogenesis of HSCR by inhibiting cell migration and proliferation by directly decreasing *PLAGL2* expression
17.	Hu et al. [157]	2018	8 HSCR colon samples and 8 controls	qRT-PCR, western blotting, CCK-8 assay, luciferase reporter assay	↑*miR-431-5p*, ↓*LRSAM1*	Reduced *mir-431-5p* support ENCCs proliferation by targeting *LRSAM1*
18.	Gunadi, et al. [146]	2019	21 HSCR ganglionic (G), aganglionic (A), and 13 controls	qRT-PCR	↑*miRNA-206* (2-*fold*) in HSCR-G and↓(0.5-*fold*) in HSCR-A ($p = 0.48$ and $p = 0.46$). ↑*FN1* (38-*fold*) in HSCR-G and ↓(18-*fold*) in HSCR-A ($p = 0.001$ and $p = 0.038$).	Aberrant expression of *FN1* may play role in the pathogenesis of HSCR
19.	Lv et al. [158]	2020	48 HSCR blood plasma samples and 48 controls	qRT-PCR, western blotting	↑*miR-494-3p, miR-668-3p, miR-3231-3p,* and *miR-605-3p* ↓*miR-5701* ($p < 0.01$)	Those five miRNAs are significant markers for HSCR diagnostics

Another study attempted to identify the diagnostic marker for HSCR by analyzing miRNA that was expressed differently in the serum of HSCR patients and control subjects. However, the molecular mechanisms of those miRNAs involved in HSCR etiology have not been fully elucidated.

Li et al performed an analysis of the most relevant regulatory pathways associated with target genes from miRNA that were identified previously, revealing that many key pathways of cellular signaling can be disrupted by changes in miRNA expression. Fifty miRNA targets were experimentally validated for HSCR through identification via miRWalk software, GoGene database, and NCBI PubMed [140]. The study also identified signaling pathways that are related to cell proliferation and migration (RET, MAPK, phosphatase and tensin homolog [PTEN], PI3K/AKT, Hedgehog, p53, p21, and ZEB2), apoptosis (BCL-2, PTEN, PI3K/AKT, p53, p21, and c-Jun), and inflammation (TNFα, IL1β, IL-6, MAPK, and PI3K/AKT) [140].

The signaling pathway associated with *RET (MAPK* and *PI3/AKT)* contributes greatly to the pathogenesis of HSCR. [140] In this study, transcripts of 14 genes involved in those pathways *(RET, FGF, MAPK3, IL1B, JUN, MAPK1, MAP2K1, TGFB, TNF, TP53, AKT, FOS, p21,* and *PTEN)* are targets of miRNA that are expressed differently in HSCR. This study found a decreased expression of *RET* and molecules involved in RET-related signaling pathways. Six miRNAs *(miR-142-3p, miR-142-5p, miR-146b-5p, miR-369-3p,* and *miR-429)* were significantly upregulated in the aganglionic segments ($p < 0.05$), and one miRNA, namely *miR-885-3p,* was significantly downregulated ($p < 0.05$) [140].

The identification of miRNA profiles can identify *RET* dysregulation and RET-related signaling pathways, and miRNA can be individually or collectively influenced synergistically. Changes in the molecules related to the regulation of target genes and signaling pathways are involved in the pathogenesis of HSCR [140].

The regulation of *Semaphorins* expression by miRNA is found to vary, both in the physiological system and in pathological conditions such as immunity, cardiovascular system, nervous system, and cancer. However, the existing research data is still elementary, so further research is necessary. Semaphorin, Neuropilin 1 and 2, and Plexin are molecules affected by miRNA. Several miRNAs modulate Semaphorin signalling, namely *miR-9, miR-27a/b, miR-124, miR-141, miR-181b, miR-188, miR-214* and *miR-302a* [11].

To date, most miRNAs have been analyzed as segregated or individual components for specific target genes. However, these genes can interact with each other and they are activated by several specific signaling pathways, mainly *RET/GDNF, EDNRB/EDN3*, and *Semaphorin* pathways. The mechanism of action of miRNA can also be "one onto many" or "many onto one", therefore, a more thorough study of miRNAs and their effect, either as a single molecule or a group of molecules on gene(s) activation pathways would provide better insight into their role in the pathogenesis of HSCR.

4. Conclusion

HSCR is a developmental disorder of the ENS due to an abnormality in migration, proliferation, differentiation, and preservation of ENCCs. A comprehensive understanding of the complex pathogenesis of HSCR, involving genetic, epigenetic, cellular, and molecular events during the development of the ENS is essential. Epigenetics is known to be involved in the occurrence of HSCR and this could be a way of prevention and treatment in the future.

This review showed that there are many mechanisms involved in the development of NCCs and the ENS, which confirms that aberrant genetic and epigenetic patterns can contribute to the development of neurocristopathy. Some miRNA regulation appears to directly affect the expression of target genes and/or their allied receptors, also exerting an indirect effect by modulating the molecules that regulate the expression of target genes and signaling molecules, including transcription factors, influencing the exchange of receptors with certain cell subcompartments, or regulating the release and exposure of allied receptors at the right time and location. Apart from identifying the direct target of miRNA, it will be interesting to investigate how miRNAs participate in the molecular network and see how that network can ultimately modulate protein levels. Several studies show that the knockout of multiple single miRNAs

does not cause obvious phenotypic abnormalities or phenotypes with incomplete penetration. Hence, it is important to thoroughly analyze not only each miRNA separately but also groups of multiple miRNAs that target a single mRNA or are involved in the same signaling pathway.

Declarations

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Data availability statement

No data was used for the research described in the article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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