Gut Bacteria in Hirschsprung-Associated Diseases

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Hirschsprung disease (HSCR) is a congenital malformation of the enteric nervous system, characterized by the absence of ganglion cells in the distal intestine, resulting in spastic contractions at the affected bowel, and functional obstruction of the intestine above the aganglionic levels. The worldwide incidence of HSCR is approximately 1 in 5000 live births with various spectrums of defective involvements, clinical manifestations, and outcomes of treatment. Up to now, the only treatment of choice for HSCR is surgery by resection of an aganglionic bowel segment and reconstruction of the normally innervated intestine to the anus.

Keywords: Hirschsprung ; aganglionosis ; gut microbiota

1. Introduction

Hirschsprung disease (HSCR) is a congenital malformation of the enteric nervous system, characterized by the absence of ganglion cells in the distal intestine, resulting in spastic contractions at the affected bowel, and functional obstruction of the intestine above the aganglionic levels [1]. The worldwide incidence of HSCR is approximately 1 in 5000 live births with various spectrums of defective involvements, clinical manifestations, and outcomes of treatment [1][2]. Up to now, the only treatment of choice for HSCR is surgery by resection of an aganglionic bowel segment and reconstruction of the normally innervated intestine to the anus [1][3]. Currently, the surgical management for HSCR significantly improves symptoms and prolongs patient life; however, the surgery does not always equate to curative therapy. About one-third of patients with HSCR still develop postoperative complications [4], including obstructive symptoms, fecal soiling, and Hirschsprung-associated enterocolitis (HAEC) [1]. HAEC is a common life-threatening complication with about 5% mortality in HSCR patients [3]. HAEC is a severe inflammation of the intestine, and patients with HAEC will present with abdominal distension, vomiting, fever, and the passage of foul-smelling or bloody stools. The overall incidence of HAEC is 25–35% [4], and 25–37% of HSCR patients can develop HAEC after definitive treatment [2]. The etiology of HAEC is unclear. Some studies proposed underlying mechanisms behind HAEC, including decreased intestinal blood flow from marked and prolonged bowel dilation, impaired epithelial barrier function, bacterial translocation, gut immune dysfunction, and dysbiosis of gut microbiota [2][3][4].

2. The Alterations of Taxa in Gut Microbiota between Subjects with Hirschsprung Disease and Healthy Controls: Evidence from Animal to Clinical Studies

The majority of HSCR studies in animals found a significant increase in *Proteobacteria* and *Bacteroidetes* with a decrease in *Firmicutes* at the phylum level [5][6][7][8]. Some studies reported the reduction in Actinobacteria and the TM7 phylum in HSCR mice [9]. These findings were consistent with a relative increase in *Proteobacteria* and *Bacteroidetes* with a decrease in *Firmicutes* in patients with bowel obstruction [10]. With regard to the identifiable microbes of HSCR at the genus, family, and species levels, most studies detected an increase in *Escherichia* , especially *Escherichia coli* under the phylum of *Proteobacteria* [9], an increase in *Bacteroides* [9][5] and *Tannerella* [5] under *Bacteroidetes phylum* , and a decrease in *Lactobacillus* [9][5] and *Staphylococcus* [5] under the *Firmicutes phylum* . Comparison of gut microbiota at different ages of mutant mice found a decrease in Lactobacillus over time and an absence of this bacterium at a late age [9][5]. In a study using piglets, the stools of HSCR piglets tended to show an increase in Proteobacteria at the phylum level and an increase in *Fusobacterium , Mogibacterium ,* and *Bilophilia* , these bacteria acting as proinflammatory bacteria [6].

3. The Diversity of Gut Microbiota in Hirschsprung Disease with and without Enterocolitis: Evidence from Animal to Clinical Studies

Owing to its importance in postoperative complications and clinical outcomes, several researchers studied and reported the alterations in gut microbiota in HAEC episodes. Cheng Z et al. reported clinical enterocolitis or HAEC in HSCR mice after microsurgical resection of the aganglionic segment [11]. The alpha diversity of HAEC mice significantly reduced when compared with wild-type mice, and tended to reduce when compared with HSCR mice without HAEC [11]. Several pieces of evidence reported alterations in gut microbiota in HAEC patients. Most of this evidence was cross-sectional in nature, comparing gut microbiota between HSCR patients with clinical enterocolitis and those without enterocolitis, by measuring gut microbiota at the time of the surgery and in the postoperative period [12][7][13][14]. Two continuous studies showed a significant difference in the beta diversity of gut microbiota between patients presenting with and without HAEC during the operative time [13][14]. Both studies also detected a distinct microbiome between the proximal ganglionic and distal

aganglionic parts of the intestine in Hirschsprung disease [13][14]. Yan Z and his colleagues found a greater alpha diversity of gut microbiota in the distal aganglionic than that of the proximal intestine. They also found a significant increase in the alpha diversity in HAEC when compared with HSCR patients without HAEC at the time of surgery [14]. In a subgroup analysis of Li and his colleagues, the authors detected a similarity in beta diversity of gut microbiota in both proximal and distal parts of HAEC specimens [13]. The comparison of gut microbiota between HAEC patients and patients who had a history of HAEC, called “HAEC-remission”, found a similarity in the beta diversity in both groups. These findings concluded that gut microbiota in an HAEC episode could lose the site-specific microbiome presented in Hirschsprung disease. Interestingly, the disturbance in gut microbiota persisted even after the symptoms of enterocolitis were resolved [13]. There were two studies into the gut microbiota in patients who had a history of postoperative HAEC compared with HSCR patients without a history of HAEC after definitive surgery, which implied that all the patients would not have the symptoms of an intestinal obstruction. Frykman PK et al. showed that the bacterial microbiome significantly increased in the enterocolitis group, while the diversity of the fungal mycobiome significantly reduced with an expansion of mycotic pathogens such as Candida albicans , especially in severe HAEC [12]. Moreover, the authors observed the depletion of Malassezia and Saccharomyces species in the patients who had a history of HAEC after surgery [12]. There was less evidence to determine the mechanism of the overabundance of Candida due to limited information regarding long-term antibiotic treatment which usually caused the fungal expansion. However, it was suggested there was a role of antifungal therapy in selected HAEC patients [12]. In addition, several clinicians found the co-incidence of prenatal cytomegalovirus infection with HSCR patients. Some studies proposed the controversial association of prenatal cytomegalovirus infection and the development of HSCR symptoms [15][16], but no study found a direct association between this virus and the development of HAEC. All these of findings suggest that the contribution to enterocolitis by disturbance of the gut microbiota may extend beyond bacteria [12]. On the other hand, another study found a decrease in the richness of the microbiota in HAEC patients after definitive surgery [7]. The possible explanation for this inconsistency between studies may be due to the difference in the ages of participants in each study and antibiotic usage. Regarding ages of participants, the median age of patients was 2.7 years old in the HAEC group in a study by Frykman PK and colleagues, while the median age in the HAEC group in a study by Neuvonen et al. was 12 years old. Regarding antibiotic usage, only three patients out of nine in Frykman’s study received antibiotics for 2 months prior to the stool collection, while nearly all patients in Neuvonen’s study used prophylactic oral antibiotics.

There were two longitudinal studies into intestinal tissue microbiota at the time of surgery and the development of postoperative HAEC. Tang W et al. studied mucosal gut microbiota in Chinese HCSR patients at the time of surgery, then followed up those patients for the potential development of clinical enterocolitis. The results found a decrease in alpha diversity of patients who developed HAEC, known as “HAEC potential patients” [17]. However, the study by Arbizu RA in American HSCR patients showed an increase in the alpha diversity of aganglionic colonic tissue microbiota at the time of surgery in the patients who developed postoperative HAEC [18]. Although the purpose of the two studies was to identify the characteristic of gut microbiota in the patients who would develop postoperative enterocolitis or HAEC potential patients, there were differences in specimen collection for the investigation of gut microbiota between the studies. Tang’s study used fresh mucosal specimens from the dilated colon to represent normal innervated tissue, while Arbizu’ study used formalin- and paraffin-fixed tissue from the aganglionic segment to study gut microbiota. Therefore, the differences in results of gut microbiota between the studies [17][18] may be due to the differences in collection and preparation of the specimens.

There was one self-control study of fecal microbiota between HAEC and normal episodes in same patient after surgery [19]. The authors found a significant difference in the beta diversity of fecal microbiota between the time of HAEC and in normal episodes [19]. Due to the limited number of studies and different design of each study, a direct comparison of the diversity of gut microbiota from each study has been impossible. Therefore, standardization of technique needs to be adhered to in any future studies which compare the changes in gut microbiota at different time points, such as preoperative, peri-operative, and postoperative periods, to discover the association between gut microbiota and the development of HAEC. All these findings are illustrated in **Table 1** .

Table 1. The diversity in gut microbiota in Hirschsprung disease with and without enterocolitis: evidence from animal to clinical studies.

HSCR with EC Model/Age (N)	HSCR without EC Model/Age (N)	Specimens/Time of Collection	Methods	Diversity			Other Findings	Interpretation	Re
				Alpha	Type of Analysis	Beta			
HAEC patients/2, 6 mo. (*n* = 2)	HSCR patients/7, 12 mo. (*n* = 2)	Intestinal content from different sections/during surgery	16S rDNA sequencing (V1–3)	↑	OTUs	Difference	HSCR had greater diversity in distal than proximal samples. HAEC had greater diversity in proximal than distal samples.	HAEC samples increased the alpha diversity, while both HSCR-HAEC had a difference of microbiome between proximal (ganglionic) and distal (aganglionic) parts of the intestine.	[14
HAEC/HAEC-R patients/10 d.–2 yrs. (*n* = 5/3)	HSCR patients/10 d.–2 yrs. (*n* = 5)	Intestinal contents from difference sites/during surgery	16S rRNA sequencing (V4)	N/A		Difference (HSCR-HAEC) Similarity (HAEC-HAEC-R)	HSCR showed distinct microbiomes between the proximal-distal intestine. Both HAEC and HAEC-R specimens showed no different microbiota in sites.	HAEC specimens found a loss of a site-specific microbiome of HSCR and HAEC-R had persistent disturbance similar to HAEC even when symptoms of EC were resolved.	[13
HAEC patients/5 mo.–8 yrs. (*n* = 9)	HSCR patients/5 mo.–8 yrs. (*n* = 9)	Stools/after definitive surgery	16S rRNA sequencing (V1–4)	↑ ↓ (mycobiome)	Shannon OTUs	N/A		The stools of HSCR patients had an increased alpha diversity in the microbiome but a decreased alpha diversity in the mycobiome.	[12
HAEC patients/3–25 yrs. (*n* = 26)	HSCR patients/3–25 yrs. (*n* = 8)	Stools/post definitive surgery	16S rDNA sequencing (V3–4)	↓ richness		N/A		The loss of richness in the microbiota led to an increase in vulnerability to colonizing pathogens in HAEC.	[7
HAEC episodes/3 yrs. (*n* = 3)	Non-HAEC episodes/3 yrs. (*n* = 3)	Self-comparisons of stools	16S rRNA sequencing	↔	Chao1	Difference		There was a difference in Beta diversity between HAEC and non-HAEC periods.	[19
post-op HAEC patients/mostly <3 mo. (*n* = 25)	HSCR patients/mostly <3 mo. (*n* = 50)	Mucosa at edge of dilated segment close to normal/at time of surgery	16S rRNA sequencing (V4)	↓	OTUs Chao1 Shannon Simpson PD	Difference		In postoperative HAEC patients, a decrease in the alpha diversity of microbiota in a mucosal specimen at the time of surgery was found.	[17

HSCR with EC Model/Age (N)	HSCR without EC Model/Age (N)	Specimens/Time of Collection	Methods	Diversity			Other Findings	Interpretation	Re
				Alpha	Type of Analysis	Beta			
post-op HAEC patients/mostly <1 mo. (*n* = 4)	HSCR patients/mostly <1 mo. (*n* = 4)	Aganglionic colonic tissue formalin and paraffin fixation/at time of surgery	16S rDNA sequencing (V3,4)	↑	observed alpha diversity	No significant difference		In postoperative HAEC patients or HAEC potential patients, an increase in the alpha diversity of microbiota in aganglionic colon specimens at the time of surgery was found without significant difference in the beta diversity between HSCR and potential HAEC groups.	[16]
Ednrb-/- mice with HAEC/3–6 wks. (*n* = 6)	WT mice/3–6 wks. (*n* = 4)	Stool/PO d0,14,28	16S rRNA sequencing (V4–5)	↓	OTUs Chao1 Shannon Simpson PD	N/A		In a potential HAEC model, a decrease in the alpha diversity of microbiota in stools at the time of surgery was found.	[11]

Abbreviations: EC: enterocolitis; HAEC: Hirschsprung-associated enterocolitis; HSCR: Hirschsprung disease; rDNA: ribosomal deoxyribonucleic acid; OTUs: Operational taxonomic units; HAEC-R: Hirschsprung-associated enterocolitis remission; rRNA: ribosomal ribonucleic acid; EdnrB: Endothelin receptor type B; PO: postoperative day. ↑: increase or higher in diversity, ↓: decrease or lower in diversity, ↔: no change or equal in diversity.

Due to the variation in design in the studies, it could not directly compare the concordant or discordant results regarding the microbiota in HAEC patients. However, those previous studies were divided into three groups with regard to timing of specimen collection. The first group was the studies that collected specimens at the time of surgery and compared the microbiota in patients with and without a history of HAEC [13][14]. The authors did not always clearly identify that the patients in the HAEC group were in an acute phase of HAEC at the time of surgery. Both studies of this group proposed that the differences in beta diversity between patients with and without enterocolitis were observed [13][14]. The second group were in the postoperative condition, which compared the microbiota in patients with and without a history of HAEC, without information regarding acute symptoms [12][7]. This second group showed discordant diversity of postoperative gut microbiota between patients with and without history of HAEC [12][7]. The third group involved studies investigating gut microbiota at the time of surgery without clinical HAEC to predict postoperative HAEC [17][18]. Information regarding the history and duration of anti-biotic treatment, which could greatly affect the alteration of gut microbiome, was not clearly reported in many studies, making it impossible to determine the confounding effect of antibiotics. Until now, no study has investigated the changes in gut microbiota at different time points of treatment in HSCR patients. In addition, no direct comparison of gut microbiota in patients at an acute phase of enterocolitis and patients with a history of HAEC has been carried out.

4. The Alterations in Gut Microbiota Taxa in Hirschsprung Disease with and without Enterocolitis: Evidence from Animal to Clinical Studies

HSCR mice with enterocolitis after microsurgery showed a significant increase in *Akkermansia* in the phylum Verrucomicrobia with a decrease in *Bacteroides* (phylum: Bacteroidetes) and Clostridium XIVa (phylum: Firmicutes) [11]. It was [11][20][21] demonstrated that *Akkermansia* , a mucin-degrading bacteria found in rodents and humans, plays an important role in intestinal barrier function as well as having an anti-inflammatory effect on the host. However, recent studies found a reduction in *Akkermansia* in IBD patients [20][21]. Some studies also showed a significant increase in *Akkermansia* in rodents with colitis [22]. Therefore, the role of *Akkermansia* is still elusive, particularly as to whether it acts as a pathogen predisposing factor to instigate HAEC, or has a compensatory preventive role.

Cross-sectional studies in children with and without a history of HAEC found an increase in Proteobacteria [12][17][13][14][19], especially *Escherichia* [7][13] and *Enterobacteriaceae* [17][14], with a decrease in the *phylum Firmicutes* [12][13][14] and Bifidobacterium [23][19] from the phylum Actinobacteria in the patients with a history of HAEC. A predominance of the *phylum Proteobacteria* was still detected in patients with a history of HAEC, when compared with patients without enterocolitis episodes. This finding emphasized that the expansion of Proteobacteria was mainly associated with gut dysbiosis, which can lead to many diseases. In addition, several studies reported a correlation between the susceptibility to colitis and the overabundance of *Proteobacteria* [24][25]. For example: (1) The genetically susceptible colitis mice, lacking Toll-like receptor (TRL)-5, exhibited a disturbance of colonic mucous layers and delayed clearance of infectious bacteria, leading to a dominance of *Proteobacteria* , especially from the family *Enterobacteriaceae* , resulting in a predisposition to chronic colitis [25]; (2) IBD, chronic inflammation of the intestine, which has been proved to be associated with innate and adaptive immune defects [26]. Several IBD studies also demonstrated an increase in *Proteobacteria* with a depletion of Firmicutes , when compared with normal controls [27][28][29]; (3) Tash-T mutant mice or HSCR mice showed a dysregulated activity of Toll-like receptors (TLRs) at the surface of enteric neurons in the mutant intestine. Those mice also showed an increase in *Proteobacteria* with a depletion of Firmicutes in HSCR mice, when compared with wild type [8]. All these findings in animal studies and clinical studies suggest that defects of both the mucosal barrier and immune function in the HSCR intestine lead to increased susceptibility for colonization and invasion by infectious pathogens, resulting in a predisposition to enterocolitis.

The alterations in the bacteria in the phylum Bacteroidetes were diverse. Some studies found an increase in bacteria in the *Bacteroidetes phylum* [12][14], while others reported a decrease in *Bacteroidetes* [13] and *Prevotella* [7] in patients with a history of HAEC. However, the Bacteroides bacteria were reduced in IBD patients [30]. Therefore, an increase in Bacteroides might play a protective role in HAEC, or might be associated with intestinal inflammation, rather than be specific to HAEC.

Prospective studies into colonic microbiota at the time of surgery in postoperative HSCR participants showed an increase in *Enterobacteriaceae* and *Escherichia* from the phylum *Proteobacteria* [17][18] and an in phylum *Firmicutes* [18] in HAEC potential patients. Tang and colleagues also detected a total of 131-OTUs of microbiomes that had differences in potential postoperative HAEC specimens in comparison with specimens of non-HAEC patients. It also identified 21-OTUs bacteria, including nine OTUs in the *Enterobacteriaceae* family, to predict postoperative HAEC with 85% accuracy [17]. This finding is potentially useful in predicting the risk of postoperative HAEC in the patients in whom these colonic microbiomes were detected at the time of surgery. The findings of the study by Arbizu differed from the study mentioned previously that reported a tendency for *Firmicutes* bacteria to decrease in patients with a history of HAEC. The possible explanation may be that the analysis of gut microbiota in the later study was carried out from the aganglionic segment of the colon at the time of surgery which was free from enterocolitis [18]. All these findings are illustrated in **Table 2** .

Table 2. The alterations of gut microbiota taxa in Hirschsprung's disease with and without enterocolitis: evidence from animal to clinical studies.

HSCR with EC Model/Age (N)	HSCR without EC Model/Age (N)	Specimens/Time of Collection	Methods	Taxonomy			
				Phylum	Genus, Family, Species		
					Proteobacteria	*Firmicutes*	*Bacteroides*
HAEC patients/2, 6 mo. (*n* = 2)	HSCR patients/7, 12 mo. (*n* = 2)	Intestinal content from different sections/during surgery	16S rDNA sequencing (V1–3)	↑*Proteobacteria* ↓*Firmicutes*	↑*Enterobacteriaceae* ↓*Acinetobacter*	↑*Enterococcus*	↑*Bacteroide*
HAEC/HAEC-R patients/10 d.–2 yrs. (*n* = 5/3)	HSCR patients/10 d.–2 yrs. (*n* = 5)	Intestinal contents from different sites/during Surgery	16S rRNA sequencing (V4)	↑↑*Proteobacteria* ↓↓ *Bacteroidetes* ↓*Firmicutes*	↑↑*Escherichia* ↓*Acinetobacter*	↓*Veillonella*	↓*Bacteroide*
HAEC patients/5 mo.–8 yrs. (*n* = 9)	HSCR patients/5 mo.–8 yrs. (*n* = 9)	Stool/after complete definitive surgery	16S rRNA sequencing (V1–4)	↑*Proteobacteria* ↑*Bacteroidetes* ↓*Firmicutes* ↓*Verrucomicrobia*		No significant difference	
History of HAEC patients/3 mo–8 yrs. (*n* = 9)	HSCR patients/3 mo–8 yrs. (*n* = 9)	Stool/after definitive surgery	16S rRNA sequencing (V1–4)	Dominated by non-SCFA-producing bacteria			

HSCR with EC Model/Age (N)	HSCR without EC Model/Age (N)	Specimens/Time of Collection	Methods	Taxonomy			
				Phylum	Genus, Family, Species		
					Proteobacteria	*Firmicutes*	*Bacteroides*
HAEC patients/3–25 yrs. (*n* = 26)	HSCR patients/3–25 yrs. (*n* = 8)	Stool/post definitive surgery	16S rDNA sequencing (V3–4)		↑↑*Escherichia* ↑↑*Shigella* ↑*Proteobacteria*	↑*Lactococcus* ↑*Lactobacillus* ↓↓*Clostridia* ↓*Oscillospira* ↓*Holdemania*	↓↓*Prevotell*
HAEC patients/2 wks–2 yrs. (*n* = 10)	Non-HAEC patients/2 wks–2 yrs. (*n* = 20)	Stool	16S rRNA real time PCR				
HAEC episodes/3 yrs. (*n* = 3)	Non-HAEC episodes/3 yrs. (*n* = 3)	Self-comparison of stool	16S rRNA sequencing	↑*Proteobacteria* ↑Bacteroidetes ↑Cyanobacteria ↓Actinobacteria			
post-op HAEC patients/mostly <3 mo. (*n* = 25)	HSCR patients/mostly <3 mo. (*n* = 50)	Mucosa at dilated segment close to normal/at time of surgery	16S rRNA sequencing (V4)		↑*Enterobacteriaceae*		
post-op HAEC patients/mostly <1 mo. (*n* = 4)	HSCR patients/mostly <1 mo. (*n* = 4)	Aganglionic colonic tissue formalin and paraffin fixation/at time of surgery	16S rDNA sequencing (V3,4)	↑↑*Firmicutes* ↑Bacteroidetes ↑Cyanobacteria	↑*Escherichia*	↑*Dolosigranulum* ↑*Streptococcus* ↑*Roseburia* ↑*Enterococcus*	
Ednrb-/- mice with HAEC/3–6 wks. (*n* = 6)	Ednrb-/- mice without HAEC/3–6 wks. (*n* = 4)	Stool/PO d0,14,28	16S rRNA sequencing (V4–5)	↑↑Verrucomicrobia ↓Bacteroidetes ↔*Firmicutes*		↓*Clostridium XIVa*	↓*Bacteroide* ↓*Dysgonom*

Abbreviations: EC: enterocolitis; HAEC: Hirschsprung-associated enterocolitis; HSCR: Hirschsprung disease; rDNA: ribosomal deoxyribonucleic acid; HAEC-R: Hirschsprung-associated enterocolitis remission; rRNA: ribosomal ribonucleic acid; SCFA: short-chain fatty acid; PCR: polymerase chain reaction; EdnrB: Endothelin receptor type B; PO: postoperative day. ↑: increase or higher, ↓: decrease or lower, ↔: no change or equal.

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