REVIEW

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The advances of genetics research on Hirschsprung's disease

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ABSTRACT
Hirschsprung's disease (HSCR) is a rare and complex congenital disorder characterized by the absence of the enteric neurons in lower digestive tract with an incidence of 1/5 000. Affected infant usually suffer from severe constipation with megacolon and distended abdomen, and face long-term complications even after surgery. In the last 2 decades, great efforts and progresses have been made in understanding the genetics and molecular biological mechanisms that underlie HSCR. However, only a small fraction of the genetic risk can be explained by the identified mutations in the previously established genes. To search novel genetic alterations, new study designs with advanced technologies such as genome/exome-wide association studies (GWASs/EWASs) and next generation sequencing (NGS) on target genes or whole genome/exome, were applied to HSCR. In this review, we summaries the current development of the genetics researches on HSCR based on GWASs/EWASs and NGS, focusing on the newly discovered variants and genes, and their potential roles in HSCR pathogenesis.

KEYWORDS
Genetics, Genome/Exome-wide association study, Hirschsprung's disease, Next generation sequencing

Introduction

Hirschsprung's disease (HSCR, or congenital intestinal aganglionosis), is a complex developmental disorder characterized by the absence of parasympathetic intrinsic ganglion cells in submucosal and myenteric plexuses of the hindgut.[1,2] It is attributed to the failure of enteric neural crest cells to migrate, proliferate or differentiate in the bowel wall during embryogenesis, leading to the aganglionosis in lower gastrointestinal tract. Severity of the disease is classified into short-segment HSCR (S-HSCR: 80% of cases) when the aganglionic segment is limited in the rectosigmoid colon, long-segment HSCR (L-HSCR: 15% of cases) when aganglionosis extends to the sigmoid, and total colonic aganglionosis (TCA: 5% of cases) when the entire small and large intestines are aganglionic.[2-4] Disappearance of propulsive motility in the aganglionic bowel would result in chronic constipation, abdominal distension, growth failure and bilious vomiting,[5,6] with a series of complications such as bowel perforation and enterocolitis. Even with surgical treatments removing and bypassing aganglionic bowel, about one-third of affected children still suffer from constipation, faecal incontinence or long-term enterocolitis.[7-9]

As a potentially fatal birth defect, the incidence of HSCR is about 1/5 000 live births, but varies across different ethnic groups, with the highest reported rate in Asians (2.8/10 000 live births).[2,10] There is a strong male gender bias with a ratio of about 4:1.35, which is much higher in

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S-HSCR [(4.2–4.4):1] than L-HSCR [(1.2–1.9):1].[11] HSCR has been considered to be a sex modified multifactorial disorder, the effect of environmental factors (like vitamin A deficiency)[12] just playing a minor role as compared to genetic factors with a relative risk of about 1/200.[13] And the genetics of HSCR is complex. Syndromic HSCR, such as Mowatt-Wilson or Waardenburg Shah type 4, presents a Mendelian mode of inheritance, while isolated HSCR (>70% of cases) appears to be of non-Mendelian inheritance with low penetrance.[9] For cases with L-HSCR or TCA, the inheritance mode is much likely due to a dominant gene with incomplete penetrance, while for cases with S-HSCR, the inheritance pattern is multifactorial or compatible with a recessive gene with low penetrance.[11]

Since 1994, positional cloning and candidate gene studies have identified a number of genes with mutations in HSCR patients, including *RET*, *GDNF*, *GFRA1*, *NRTN*, *PHOX2B*, *NKX2.1*, *SOX10*, *EDNRB*, *EDN3*, *ECE-1*, *KIAA1279*, *ZFXH1B*, *NTRK3*, *L1CAM*, *TCF4*, *and HOXB5*.[10,14-23] Most of them encode proteins that are members of three important inter-related signaling pathways: the *GDNF/RET* receptor tyrosine kinase, the endothelin type B receptor, and the *SOX10*-mediated transcription. And there have been much evidence that interactions existed between genes in those different signaling pathways.[2,10,24] RET is considered to be the most important gene involved in HSCR, and its sufficient expression is essential for the development of enteric nervous system (ENS).[23,25,26] However, coding or splice junction mutations at these genes account for only about 50% of familial cases and 20% of sporadic cases, and explain just 0.1% of the heritability.[4] Hence, there must be additional genetic defects responsible for HSCR.

As effective strategies with new technologies emerged, genetics researchers started to apply chip-based genome/exome-wide association study (GWAS/EWAS) and next generation sequencing (NGS) on target genes or whole genome/exome, to search novel genes and corresponding variants or mutations associated with different diseases, including HSCR. In this review, we focus on the advances of HSCR's genetic etiology revealed by GWAS/EWAS and NGS.

Genome-wide association study and HSCR

To date, there are four well-design GWASs, one meta-analysis and one EWAS for HSCR in different populations (Table 1). Published in 2009, the first GWAS of HSCR not only ascertained the role of *RET* in Chinese patients, but also identified a new susceptibility gene neuregulin-1 (*NRG1*) that played an important role in survival and differentiation of the neural crest cells through binding and interaction with ErbB tyrosine kinase receptors.[27] The involvement of *RET* and *NRG1* in HSCR was also discovered in another GWAS in Korean population.[28] As for Caucasians, a family-based GWAS further reported the susceptibility of individuals with variants of *RET* and *NRG1*, and located a new risk locus containing class 3 Semaphorin gene cluster (*SEMA3A*, *SEMA3C*, *SEMA3D*). Analysis in *Ret* wild-type and *Ret*-null mice showed specific expression of *Sema3a*, *Sema3c*, and *Sema3d* in ENS, while the knockdown of *Sema3c* or *Sema3d* in zebrafish embryos demonstrated the loss of migratory ENS precursors.[29] To aggregate the data of three above-mentioned GWAS on HSCR, Tang et al[30] conduct a trans-ethnic meta-analysis containing totally 507 cases and 1 191 controls. They not only confirmed the associations of *RET*, *NRG1*, *SEMA3*, and one previously well-established locus 4p13 (*PHOX2B*) in syndromic HSCR, but also found one novel disease-susceptibility locus 2p16.1 (*VRK2/FANCL*). Encoding a serine/threonine protein kinase, VRK2 was strongly implicated in central nervous system and neurodevelopmental disorders, and might interacted with receptor ErbB2, which is the co-receptor of NRG1. More recently, another HSCR GWAS of Caucasians was published. It confirmed *RET* and *SMEA3* as being associated with HSCR in a Danish cohort, and additionally reported a novel low-frequency variant (rs144432435) of *RET*.[31]

Exome-wide association study and HSCR

Most of the susceptibility variants discovered by GWASs are common variants with minor allele frequency (MAF) > 0.05, conferring relatively small effect sizes with odds ratios (OR) from 1.1 to 1.5. These variants could explain only a small fraction of genetic risk of investigated diseases. Therefore, rare variants and loci that are undetected by GWAS-used chips may have a stronger effect and contribute to the missing heritability.[32] Exome-chip platforms have been developed to capture low-frequency variants in protein-coding regions and have been proved to be an effective complementary approach for genetic researches on complex diseases. An exome-wide association study was applied to scan the exonic variants for HSCR.[33] In this study, Tang et al identified ten variants and ten novel genes associated with HSCR at $P < 10^{-4}$ in a Chinese population. Among these SNPs, the missense variants in catechol-O-methyltransferase (*COMT*) and armadillo repeat gene deleted in velocardiofacial syndrome (ARVCF) indicated an ectopic expression in HSCR colons. Specially, the variant Ala72Ser in *COMT* decreased proliferation activity of neural cell via NOTCH signal pathway, while the mutant ARVCF suppressed cell migration by downregulating *RHOA* and *ROC* (Table 1).

Deep-targeted sequencing and HSCR

As NGS technologies emerged, some researchers started to apply deep-target sequencing on candidate genes or loci that have been implied in HSCR (Table 2). An early study in 2012 sequenced all 16 exons of the HSCR-associated gene NRG1 in 358 cases and 333 controls, and reported 13 different heterozygous variants.[34] *RET*, as the most well-

TABLE 1 Genes associated with HSCR identified by GWAS or EWAS.

Gene	Locus	tagSNP	Study Design	Population	Journal	Year
NRG1	8p12	rs16879552 /rs7835688	GWAS + Replication	Discovery: Chinese (181 cases/346 controls) Replication: Chinese (190 cases/510 controls)	PNAS	2009
RET	10p11.21	rs2742234				
RET	10p11.21	rs1864400	GWAS	Korean (123 cases/432 controls)	PLoS One	2014
NRG1	8p12	rs16879552				
SLC6A20	3p21.31	rs4299518/rs2159272				
RORA	15q22.2	rs1351544/rs8025324/ rs9920560/rs7183955				
ABCC9	12p12.1	rs704192/rs704191/ rs4148669/rs704190				
STIM2	4p15.2	rs11725593				
DEFB129	20p13	rs6074578				
LOC100509398	3q26.2	rs12639288				
RET	10p11.21	rs2506030/rs2435357	GWAS + Replication	Discovery : Caucasian (220 trios) / Replication: Caucasian (429 trios)	AJHG	2015
NRG1	8p12	rs4541858/rs7835688				
SEMA3A/ SEMA3C /SEMA3D	7q21.11	rs12707682/rs11766001				
RET	10q11.21	rs9282834/rs2505998/ rs2505998	Meta-analysis of GWAS	European (212 cases/202 controls) / Chinese (173 cases/615 controls) / Korean (122 cases/374 controls)	HMG	2016
SEMA3C/3D	7q21.11	rs80227144				
NRG1	8p12	rs7005606				
PHOX2B	4p13	rs6826373				
VRK2/FANCL	2p16.1	rs4672229				
SSPO	7q36.1	rs10250401	EWAS	Chinese (167 cases/900 controls)	Mol Neurobio	2017
EEF1D	8q24.3	rs10282929				
SLC34A3	9q34.3	rs35699762				
ABO	9q34.2	rs1053878				
BOC4L	12q24.33	rs78871841				
CACNA1H	16p13.3	rs36117280				
TELO2	16p13.3	exm1202536				
CARD14	17q25.3	rs11652075				
COMT	22q11.21	rs6267				
ARVCF	22q11.21	rs80068543				
SEMA3	7q21.11	rs62472985/rs117617821	GWAS	Discovery: Danish (170 cases/4717 controls) Replication: European (416 cases/903 controls)	EJHG	2018
MOB1AP1/ DDX6P2	13q31.1	rs12428625				
RET	10p11.21	rs17653445/rs2505994/ rs4519046/rs144432435				

GWAS, genome-wide association study; EWAS, exome-wide association study; PNAS, Proceedings of the National Academy of Sciences of the United States of America; AJHG, The American Journal of Human Genetics; HMG, Human Molecular Genetics; EJHG, European Journal of Human Genetics.

TABLE 2 Genes associated with HSCR identified by NGS.

Gene	Locus	Mutation	Study Design	Population	Journal	Year
NRG1	8p12	A28G / E134K / V266L / H347Y / P356L / V486M /P24P / T169T / L483L / E239fsX10 / c.503-4insT	TES	Chinese (358 cases and 333 controls)	Hum Genet	2012
GLI1	12q13.3	R557C	TES	Chinese (20 cases and 20 controls)	Gastroenterology	2015
GLI1	12q13.3	P763S				
GLI2	2q14.2	G191R				
GLI3	7p14.1	H1200D				
RET	10p11.21	c.254G > A / c.754G > T / c.789C > G / c.2308C > T / c.2333delT / c.2578C > T / c.2802-2A > G / c.229C > T / c.200insTCC	TES & RET single gene screening	Chinese (152 patients)	Genet Med	2017
NRG3	10q23.1	chr10:84118524	WES	Chinese (2 affected familial patients)	Mol Neurobio	2013
TMPRSS11E	4q13.2	chr4:69342021				
SPRY1	4q28.1	chr4:124323240				
OR8J3	11q12.1	chr11:55905101				
PRSS1	7q34	chr7:142460335				
LAMA3	18q11.2	chr18:21453118				
RNF10	12q24.31	chr12:121004700				
VARS2	6p21.33	chr6:30884719				
KRT6A	12q13.13	chr12:52885316				
PLA2G4C	19q13.33	chr19:48558271				
JARID2	6p22.3	chr6:15520402				
PRB4	12p13.2	chr12:11461427				
BRIP1	17q23.2	chr17:59878736				
GSTM4	1p13.3	chr1:110200278				
NBPF16	1q21.2	chr1:148591281				
NRG3	10q23.1	chr10:84733588	TES	Chinese (96 cases and 110 controls)		
NRG3	10q23.1	chr10:84733624				
NRG3	10q23.1	chr10:84118499				
RET	10p11.21	3splicing9 + 1 / c.2511_2519 delCCCTGGACC:p.S837fs / c.1818_1819insGGCAC:p.Y606fs / c.1761delG :p.G588fs / c.1858 T > C:p.C620R / c.409 T > G:p.C137G / c.1710C > A:p.C570X / c.526_528delGCA:p.R175del	WES	Chinese (5 trios) + Caucasian (19 trios)	Genome Biol	2017
NCLN	19p13.3	c.496C > T:p.Q166Xb				
NUP98	11p15.4	c.5207A > G:p.N1736S				
DENND3	8q24.3	c.1921delT:p.K640fs				
TBATA	10q22.1	c.157C > T:p.R53C				
LRBA	4q31.1	rs140666848	TES	Dutch (A multi-generational family: 5 patients and 2 functional constipation)	Gastroenterology	2018
RET	10p11.21	c.1196C>T:p.P399L	WES			
NRP2	2q33.3	rs114144673				
PGRMC2	4q28.2	rs41298555				
OR1F1	16p13.3	rs142486394				
CLUH	17p13.3	rs201361018				
PELP1	17p13.2	rs199636910				
PELP1	17p13.2	rs200062536				

Gene	Locus	Mutation	Study Design	Population	Journal	Year
IHH	2q35	c.151C>A:p.Q51K				
GLI3	7p14.1	rs121917716				
GDNF	5p13.2	c.676_681delGGATG:p.G226_C227del				
CCT2	12q15	g.69993654 G > A	WGS	Chineses (9 trios)	EJHG	2018
VASH1	14q24.3	g.77242233 A > G				
CYP26A1	10q23.33	g.7481 A > G				
PKD1L2	16q23.2	g.84039 G > A				
TMEM175	4p16.3	g.952275 C > T				
CSMD3	8q23.3	g.113841961 T > C				
CCDC82	11q21	g.96117858 A > T				
NRG1	8p12	g.667454 G > C, g.92222 G > T, g.146124 A > G				
ERBB4	2q34	g.835055_835059delAAACA				
SEMA3A	7q21.11	g.210732delT				
ZEB2	2q22.3	g.145137510 C > T				
DCC	18q21.2	g.651331 G > A				

TES, targeted exome sequencing; WES, whole exome sequencing; WGS, whole genome sequencing; EJHG, European Journal of Human Genetics.

established gene in HSCR, was also screened for somatic mutations through targeted exome sequencing and whole genome sequencing. Eight *de novo* mutations were found in 152 patients, of which six were pathogenic mosaic mutations.[35] These findings were in line with the evidence that genes containing common disease-associated variants were likely to harbor functional rare variants in coding exons. Considering that aberrant hedgehog signaling could disrupted neural crest cells (NCCs) differentiation and might cause Hirschsprung's disease, Li et al[36] performed targeted sequencing on *GLI1*, *GLI2*, *GLI3*, *SUFU*, and *SOX10* in 20 HSCR patients. Four rare heterozygous missense variants in the coding sequence of *GLI1*, *GLI2*, and *GLI3* were located for the first time, and aberrant Gli activity were found to perturb the Sox10-Sufu-Gli regulatory loop, leading to attenuated differentiation of enteric NCCs and delayed gut colonization.

Whole exome/genome sequencing and HSCR

In these years, whole exome sequencing (WES) and whole genome sequencing (WGS) have been more practical in genetics research on human diseases with technological development.[37-39] For HSCR, more risk genes were successfully identified via both two strategies (Table 2). In 2013, our group performed whole exome sequencing of two HSCR patients from a Han Chinese family, obtained a total of 15 novel nonsynonymous single nucleotide variants (SNVs) in 15 genes, and validated the involvement of *NRG3* mutations in 96 additional sporadic cases and 110 healthy controls by targeted sequencing of all nine exons.[40] Recently, Gui et al[41] reported another WES study in 24 HSCR trios and identified 28 *de novo* mutations in 21 different genes. They further showed that the orthologues of four genes (*DENND3*, *NCLN*, *NUP98*, and *TBATA*) are indispensable for ENS development in zebrafish, and these genes are also expressed in human and mouse gut and/or ENS progenitors. Lately, a targeted exome sequencing on a linkage interval 4q31.3–4q32.3 previously identified, coupled with a WES study identified several variants in *LRBA*, *RET*, *GDNF*, *IHH*, and *GLI3* in a multigenerational Dutch family with history of HSCR. Further functional experiments showed that these variants disrupted the function of their encoded proteins, and knockdown of *ihh* in zebrafish significantly reduced the number of enteric neurons in the gut.[42] In addition, a WGS study[43] was conducted on 9 trios where the sporadic probands had L-HSCR or TCA and harbored no rare coding variants affecting the function of *RET* and other known HSCR risk genes. The authors located de novo protein-altering variants in three genes *CCT2*, *VASH1*, and *CYP26A1*, and *de novo* SNV/indels in non-coding regions of *NRG1*, *ERBB4*, *SEMA3A*, *ZEB2*, and *DCC*. They further indicated that the shared genetic features of the patients were enriched in the extracellular matrix–receptor (ECM–receptor) pathway, which was involved in the migration of enteric neurons precursors.

Conclusions and perspectives

Unravelling the genetics of polygenic diseases is a major challenge in the field of human genetics. As HSCR is a representative example of complex multigenic disorders with limited treatments and poor prognosis, much effort has been made in the investigation on genetics and pathogenesis of the disease. With the applications of GWAS/EWAS and NGS, a sum of novel mutations and genes has been stated in these years as we discussed in this review. However, they could account for only a minority of the total genetic risk for HSCR. Additional pathogenic

mutations, causal variants and contributing genes are still to be found through more comprehensive genetics researches on subjects with larger sample size. Moreover, making use of GWAS/EWAS, NGS or both in combination with effective statistical analysis in silico, followed by the system biology approaches like high-throughput functional assays and appropriate models from animals or human induced pluripotent stem cell (HIPSC), should yielded huge advances in our understanding of the HSCR genetic basis. It may finally lead to precise prediction of HSCR risk and potentially to new therapies and improved outcomes.

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CONFLICT OF INTEREST

None of the authors declared any conflicts of interest.

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