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The Evolving Genetic Landscape of Hirschprung Disease: An Update and Review

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Abstract

Hirschsprung Disease (HD) is a developmental disorder characterized by the complete absence of ganglion cells in the distal gastrointestinal tract. It is the most common cause of functional intestinal obstruction in neonates and children. The aganglionosis is believed to be either due to failure of neural crest cells to migrate, proliferate, differentiate or survive during gut development in the embryonic stage. The incidence of HD is estimated at 1/5000 live births and shows a male predominance. It is usually sporadic, although it can be familial and may be inherited as autosomal dominant or autosomal recessive. In 70% of cases, HD occurs as an isolated trait and in the other 30% it is associated with other congenital malformation syndromes. HD has a complex multifactorial etiology, and genetic factors play a key role in its pathogenesis. Several gene loci appear to be involved. Many of these have been identified by conducting Genome Wide Association (GWAS) studies and recently by Next Generation Sequencing (NGS). These genes encode for receptors, ligands (especially those participating in the RET, EDNRB and Semaphorin signaling pathways), transcriptional factors (PHOX2B & SOX10). These genes are involved in the neural crest cell development and migration that give rise to ganglion cells. Overall, the RET proto-oncogene is considered the major disease causing gene in HD. A greater understanding of the genetic landscape of this disease might pave way for genetic counseling, prenatal and preimplantation diagnosis in the management of HD.

Keywords: Hirschprung disease; Genetics; RET; Genome wide association studies (GWAS); Next generation sequencing (NGS); Semaphorins

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Introduction

Hirschsprung Disease (HD), or congenital intestinal aganglionosis, is a pediatric disorder which is characterized by complete absence of ganglion cells from a part of the intestine. Its incidence is reported to be 1:5000 births [1]. The neuronal ganglion cells are of neural crest origin. Since during development the migration of these cells is from proximal to distal the aganglionic segment typically includes the distal rectum and proximal extent is variable. The absence of ganglion cells has been attributable to either failure of migration of these enteric neural crest cells or due to absence of survival, proliferation or differentiation of these cells [2].

When the aganglionosis is restricted to the rectosigmoid colon it is known as short-segment disease. These represent the vast majority (80%) of cases of HD. However, in approximately 15% to 20% patients the aganglionosis extends proximal to the sigmoid colon which is designated as long-segment disease. In about 5% cases the aganglionosis involves the entire colon called total colonic aganglionosis. In extremely rare situation the aganglionosis is seen extending into small intestine or even more proximally, the so called total intestinal aganglionosis [3]. This condition is invariably fatal [4].

The patients typically present with inability to pass meconium within first 48 hours of life. The other modes of presentation include constipation, vomiting along with painful and distended abdomen. The abdominal distension can be sometimes quite massive due to huge distension of the colon known as megacolon. However, sometimes it presents in older children or adult. Thus in cases presenting with chronic constipation in these age groups possibility of HD should be kept in mind.

The diagnosis of HD is established by histopathological examination to show absence of ganglion cells in submucosal plexus. Suction biopsy is the preferred method as it is safe and does not require general anesthesia. Other findings seen in biopsies include hypertrophic nerve fibers [5]. Acetylcholinesterase histochemistry shows abnormally increased nerve fibers in the mucosa

Table 1: Chromosomal abnormalities seen in association with HD and their underlying gene loci.

Chromosome abnormality	Clinical features	Chromosome locus (Gene)	% of individuals with HD
Down syndrome	Impaired learning ability, short stature, congenital heart disease, craniofacial features	Trisomy 21	0.6%-3%
Deletion 10q11	Impaired learning ability, hypotonia	del110q11.2(RET)	Unknown
Deletion 10q23	Mostly isolated HD; one with rectocutaneous fistula	del110q.23.1(NRG3)	Unknown
Deletion 13q	Impaired learning ability, growth failure, craniofacial features	del113q22(EDNRB)	Unknown
Deletion 2q22	Impaired learning ability, microcephaly, craniofacial features, seizures	del 2Qq22(ZEB2)	Unknown
Deletion 4p12-p13	Impaired learning ability, short stature, craniofacial features	del4p12(PHOX2B)	Unknown
Deletion/duplication 17q21	Impaired learning ability, multiple congenital anomalies	Del17q21/dup17q21-q23 (Unknown)	Unknown

Table 2: Syndromes associated with HD.

Syndrome	Features	Chromosome locus/gene	% with HD
Bardet-Biedel syndrome	Retinal dystrophy, obesity, impaired learning ability, polydactyly, hypogenitalism, renal abnormalities	Atleast 14 loci/genes	2%-10% [9]
Cartilage hair hypoplasia anauxetic dysplasia spectrum disorder	Short limbed dwarfism, sparse hair, immune defects	9p13.3/RMRP	7%-9% [10]
Congenital central hypoventilation syndrome (CCHS)	Hypoxia, reduced ventilator drive, neuroblastoma	4p13/PHOX2B 10q11.21/RET 5p13.2/GDNF 20q13.32/EDN3 11p14.1/BDNF	20% [11]
Familial dysutonomia (Riley-day syndrome)	Sensory and autonomic dysfunction(including abnormal tear, sweat and saliva production)	9p31.3/ELP1(IKBKAP)	Unknown [12]
Fryns syndrome	Distal digital hypoplasia, diaphragmatic hernia, CHD, craniofacial, impaired learning ability	Unknown	Unknown [13]
Goldberg-Shprintzen syndrome	Craniofacial anomaly, microcephaly, impaired learning ability, polymicrogyria	10q22.1/KIFIBP(KIAA1279) Others	Common [14]
Llsyndrome	Impaired learning ability, hydrocephalus, corpus callosum agenesis, adducted thumbs	Xq28 / L1 CAM	Rare [15]
MEN 2A/FMTC	Medullary thyroid carcinoma, pheochromocytoma, hyperparathyroidism	10q11.21 I RET	1% [16]
MEN 2B	Medullary thyroid carcinoma, pheochromocytoma, mucosal and intestinal neuromas, skeletal abnormalities, corneal changes	10q11.21 /RET	Rare [17]
Mowat-Wilson syndrome	Impaired learning ability, microcephaly, craniofacial abnormalities,Congenital heart disease, corpus callosum agenesis, epilepsy, short stature	2q22.3 /ZEB2	41% - 71% [18]
Waardenburg syndrome type 4 (Waardenburg-Shahsyndrome)	Pigmentary abnormalities, deafness	13q22.3/EDNRB	Common [19]
		20q13.32/EDN3	
		22q13.1/SOX10	Almost 100%
Neurofibromatosis 1	Café-au-lait macules, neurofibroma, Lisch nodules	17q11.2/NF1, 5p13.2/GDNF?	Unknown [20]
Pitt-Hopkins syndrome	Craniofacial abnormalities, impaired learning ability, seizures, hyperventilation, hypoventilation, constipation	18q21.2/TCF4	Unknown [21]
Smith-Lemli-Opitz syndrome	Impaired learning ability, hypospadias, 2/3 syndactyly, congenital heart disease, craniofacial abnormalities	11q13.4/DHCR7	Unknown [22]

[6]. Other supporting investigations include anorectal manometry, abdominal radiographs with barium enema. Intraoperative frozen sections help in planning an accurate surgery.

The phenotype of these patients is however highly variable. This can be explained on the basis of the genetic abnormalities and the interactions between the various genes in cases of HD. An overview of the current understanding of this highly complex genetic landscape is presented in this article.

Genetic Abnormalities in Hirschprung Disease

The genetic abnormalities can be broadly classified into chromosomal anomalies and single gene abnormalities.

Chromosomal anomalies

The overall frequency of chromosomal aberrations in HD is about 12% [7]. The strongest association of HD is with Down syndrome (Trisomy 21) which has been reported to vary from as low as 2% to as high as 10% [8].

It is however interesting to note that chromosome 21 does not contain any gene which is known to be associated with HD. Therefore, in addition to trisomy 21 there are other chromosomal abnormalities which specifically involve HD associated genes like EDNRB, RET, NRG3, ZEB2 and PHOX2B. In case of all of these genes both the alleles need to be functional for normal status. In case even a single allele gets inactivated due to mutation there will be clinical manifestation. This loss of single allele is known as haploinsufficiency. The details of the chromosomal abnormalities leading to HD are presented in Table 1. It was observed that only some of the patients of HD possessed some of these chromosomal anomalies that are every patient of HD does not carry all of these anomalies. On further investigation the causative genes were also identified. However, as it is quite evident from Table 1 that still there are chromosomal abnormalities in which the underlying genes have not been identified.

Table 3: Genes involved in cases of HD that are not associated with any syndromes.

Gene/OMIM	Protein	Chromosome locus	Frequency	Type of HD
RET/164761 [23]	Proto-oncogene tyrosine protein kinase receptor ret	10q11.21	17%-38%	Short segment
			70%-80%	Long segment
			50%	Familial
			3%-7%	Simplex
GDNF/600837 [24]	Giant cell derived neurotrophic factor	5p13.2	1%	Variable
NRTN/602018	Neurturin	19p13.3	1%	Variable
EDNRB/131244 [25]	Endothelin B receptor	13q22.3	3%-7%	Variable
EDN3/131242 [25]	Endothelin 3	20q13.32	5%	Variable
ECE1/600423	Endothelin-converting enzyme	1p36.12	1%	Variable
NRG1/142445 [25]	Neuregulin 1	8p12	1%	Variable
NRG3/605533 [27]	Neuregulin 3	10q23.1	1%	Short segment
SEMA3C/602645 [28]	Semaphorin 3C	7q21.11	5%	Short segment
SEMA3D/609907 [29]	Semaphorin 3D	7q21.11	5%	Short segment

Table 4: HD associated with congenital anomalies of an unknown etiology.

Anomaly	Features	Mode of inheritance	Genetic locus/gene	% in HD
Central nervous system	Impaired learning ability, Dandy-Walker malformation, microcephaly	Unknown	Unknown	3.6%-3.9%
Congenital heart disease	Atrial septal defect, Ventricular septal defect , Patent ductusarteriosus, Tetralogy of fallot	Unknown	Unknown	2.3%-4.8%
Gastrointestinal	Malrotation, imperforate anus, Meckel diverticulum, sacral-rectal fistula	Unknown	Unknown	3.3.%-3.9%
Genitourinary	Cryptorchidism, inguinal hernia, hypospadias, Kidney malformation, urethral fistula	Unknown	Unknown	5.6%-.7.3%

Single gene mutations

Besides the chromosomal abnormalities as described above a certain group of HD patients show mutations involving single gene. The various modes of inheritance that have been reported in these cases include autosomal dominant, autosomal recessive, or X-linked. Further these cases may or may not show coexisting syndromes.

HD associated with syndromes

There are large numbers of syndromes that may have an association with HD. These cases usually present clinically as long segment HD. The details of these syndromes are summarily presented in Table 2.

HD not associated with syndromes

In these cases of HD there is no definite association with any congenital anomaly. Broadly describing these cases show mutation in either one of these four genetic pathways –

1. RET along with its ligands GDNF and NRTN
2. EDNRB and its associated genes EDN3 and ECE1
3. NRG pathway and the genes involved being NRG1 and NRG3
4. SEMA signaling pathway and the related genes SEMA3C and SEMA3D

The details of the genes involved in these cases of HD are shown in Table 3.

HD associated with congenital abnormalities of unknown etiology

There are certain cases of HD which have at least one congenital abnormality associated with them. However, it is not possible to place these cases into definite syndromes. The currently available information about these cases of HD is summarized in Table 4.

Approaches Taken to Study the Genetic Landscape of Hirschprung Disease

Genome wide association studies (GWAS)

Genome wide association studies are those studies in which the DNA sample is analyzed for the presence of various single nucleotide pleomorphisms (SNPs) in the genome. Those SNPs which are found to be more frequent in patients are said to be associated with that particular disease. Thus to find additional loci that contribute to the development of HD, a GWAS was carried out in Chinese cases with sporadic HD [30]. As expected, most frequent association was found with the RET gene. However, two additional SNPs located in intron 1 of Neuregulin-1 gene (NRG1) were also found to be strongly associated, pointing to NRG1 as a plausible candidate gene. This was further corroborated by the identification of coding sequence mutations in NRG1 [30-32]. Copy number variation analysis on this data revealed another gene to be associated with HD, namely NRG3, a paralog of NRGI. Further validation on Chinese HD patients identified nine deletions and two de nono duplications in NRG3, suggesting a role of this gene in HD [33].

A novel pathway based analysis approach was used by Fernandez et al. [34] to initially prioritize candidate genes in a Spanish cohort of 53 cases of short-segment Hirschsprung disease. Candidate genes were further validated in an independent population of 106 cases. Their study revealed a strong association of 11 Gene Ontology (GO) modules which were related to signal transduction and its regulation, Enteric Nervous System (ENS) formation and other HD-related processes. Among the preselected genes, a total of 4 loci, RASGEF1A, IQGAP2, DLC1 and CHRNA7, related to signal transduction and

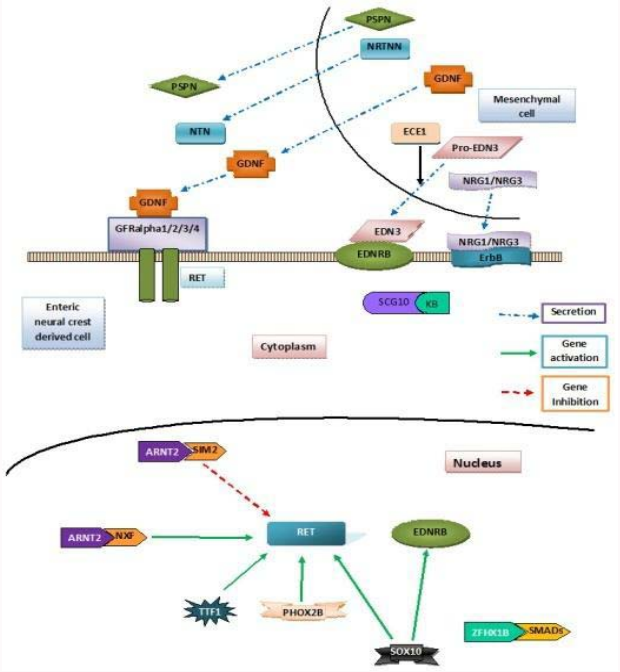

Figure 1: Role of RET, EDNRB and NRG pathways in HD: Giant cell derived neurotrophic factor (GDNF) secreted by the mesenchymal cells (Blue dashed arrow) binds to its receptor RET on the enteric neural crest derived cell. This causes proliferation of these cells by further downstream mechanisms. Endothelin-3 (EDN3) also secreted by the mesenchymal cells goes and binds to its receptor Endothelin receptor B (EDNRB) on the enteric neural crest derived cells. This causes their proliferation and survival. PHOX2B and SOX-10 increase the expression (green solid arrow) of RET and EDNRB genes. SIM2 inhibits the expression of RET (red dashed arrow).

In order to discover additional genetic loci, Kim et al. [35] performed a GWAS of 123 sporadic HD patients and 432 unaffected controls using a large-scale platform. They also found mutation in the RET-CSGALNACT2-RASGEF1A genomic region and NRG1 as susceptibility loci. In addition, they identified SLC6A20 and ABCC9 as new potential susceptibility loci. Although none of the SNPs in these genes passed the Bonferroni correction. In further subgroup analysis it was observed that the RET-CSGALNACT2-RASGEF1A genomic region had a differential significance pattern amongst the various types of HD. This suggests that other genomic loci or mechanisms may affect the length of aganglionosis in HD subgroups during Enteric Nervous System (ENS) development.

Another potentially significant locus SLC6A20 was studied by Lee and coworkers [36]. SLC6A20 stands for solute carrier family 6, proline IMINO transporter, member 20 (SLC6A20). A total of 40 single nucleotide polymorphisms (SNPs) of SLC6A20 were genotyped in 187 HD subjects composed of 121 short-segment HD, 45 long-segment HD, 21 total colonic aganglionosis and 283 controls. The data analysis revealed that 13 SLC6A20 SNPs were significantly associated with HD. In further subgroup analysis, SLC6A20 polymorphisms appeared to have increased association with L-HSCR. Thus the results of their study suggest that SLC6A20 may have a role in aetiopathogenesis of HD and it may also contribute in determining the length of aganglionic segment.

The probability that a variant (rs6509940) of interleukin-11 (IL-11) may act as a potential locus for HD was studied by Kim et al. [37]. They further studied associations with HD of nine common SNPs on IL-11. A total of nine SNPs on IL-11 were genotyped in 187 HD patients and 283 controls using TaqMan genotyping assay. The data analysis revealed that several SNPs showed a statistically significant association with HD. Moreover, the most common haplotype was strongly associated with HD. In further analysis among three HD subtypes (short segment, long segment, total colonic aganglionosis), the results showed a different association pattern depending on the subtype. This suggests that genetic variations of IL-11 may be associated with the risk of HD.

Bae et al. [38] attempted to identify new HD genetic factors related to Copy Number Variation (CNV) and loss of heterozygosity (LOH) in Korean patients. They performed genome-wide genotyping, using Illumina's HumanOmni1-Quad BeadChip (1,140,419 markers), on 123 HD patients and 432 controls. A total of 8,188 CNVs (1 kb 1 mb) were identified by CNV partition. As a result, 16 CNV regions and 13 LOH regions were identified as associated with HD. Two top CNV regions (deletions at chr6:32675155-32680480 and chr22:20733495-21607293) were successfully validated by additional real-time quantitative polymerase chain reaction analysis. In addition, 2 CNV regions (6p21.32 and 22q11.21) and 2 LOH regions (3p22.2 and 14q23.3) were discovered to be unique to the HD patients group. Large-scale chromosomal aberrations (>1 mb) were identified in 11 HD patients.

A trans-ethnic meta-analysis of 507 HD cases and 1191 controls was carried out by Tang et al. [38]. They combined all published GWAS results on HD. It was seen that the effects of RET and NRG1 are universal across European and Asian ancestries. In contrast, a European-specific association was observed with a low-frequency variant, rs80227144, in SEMA3 gene. Further conditional analyses on the lead SNPs showed a secondary association signal, which corresponded to an Asian-specific, low-frequency missense variant encoding RET p.Asp489Asn (rs9282834). When in trans with the RET intron 1 enhancer risk allele, rs9282834 increases the risk of HD from 1.1 to 26.7. This is the largest meta-analysis study on HD and provides great insights into the genetic architecture of HD.

Next generation sequencing

A major advance in molecular biology in the last decade has been the availability of next generation sequencing as an investigative tool. While the speed has increased manifold the cost has dramatically come down. In HD this tool has been applied by some authors leading to discovery of newer genetic mutations.

In order to evolve an efficient approach for identifying rare mutations which could be possibly related to Hirschsprung disease (HSCR), Gui et al. [40] carried out a pilot study using a newly developed protocol for next generation targeted resequencing. A total of 20 HD patients and 20 sex-matched individuals with no HD as controls were included. In these patients coding sequences (CDS) of 62 genes known to be involved in signaling pathways related to enteric nervous system development were selected for capture and sequencing. The blood samples of these 40 cases were pooled to make a total of 8 pools. Each pool comprising of 5 patients. The pooled DNAs was enriched by PCR¬based Rain Dance technology (RDT) and then sequenced on a 454 FLX platform. For technical validation, five patients from Pool-3 were also independently enriched by RDT, indexed with barcode and sequenced with sufficient coverage. The evaluation of single nucleotide variants showed that DNA pooling performed well (specificity/sensitivity at 98.4%/83.7%) for the common variants. But in case of rare variants it did relatively worse (specificity/sensitivity at 65.5%/61.3%). Sanger sequencing only validated five out of 12 rare

mutations which were reported. Thus the authors suggest that more technical improvement is required in sequencing technology for variant detection for large-scale resequencing of pooled DNA.

In a subsequent study Luzón-Toro and coworkers carried out a study with the aim of designing of a panel of HD associated genes which could be used to carry out genetic screening. They performed NGS-based targeted sequencing (454-GS Junior) using a panel containing 26 associated or candidate genes in 11 patients of HD. The most notable finding in their study was that they found a total of 13 new coding variants and 11 new variants within the regulatory regions.

In another study Luzón-Toro et al. [42] performed whole exome sequencing in familial HD cases (n=16). They used the SOLID platform for their study. They looked for genes that were recurrently mutated. They found that variations in the FAT3 gene were significantly seen. Within-family analysis showed compound heterozygotes for ANNAK and several genes (n=23) with heterozygous variants that co-segregated with the phenotype. Network and pathway analyses led to the discovery of polygenic inheritance involving FAT3, HD associated genes and their gene partners. Their approach led to the detection of more than one damaging variants in genes that could together contribute to the overall phenotype. They concluded that these findings further elucidate the complex interactions that occur during enteric nervous system development and the etiopathogenesis of familial HD.

Understanding the genetic pathways

The important genes involved in HD have been mentioned above and shown in Table 3. Based on current understanding majority of these genes involved in HD can be divided into four groups based on the pathways in which they are involved. These are: RET activation pathway (RET, GDNF, PSPN), EDNRB pathway (EDNRB, EDN3, ECE1), transcription factors involved in both pathways (SOX 10, PHOX2B) and semaphorin pathway (SEMA3C, SEMA3D). Out of these RET and EDNRB pathways are relatively well known. As shown in Figure 1 giant cell derived neurotrophic factor (GDNF) which is secreted by the mesenchymal cells binds to its receptor RET on the enteric neural crest derived cell. This causes proliferation of these cells by further downstream mechanisms. Endothelin-3 (EDN3) also secreted by the mesenchymal cells goes and binds to its receptor Endothelin receptor B (EDNRB) on the enteric neural crest derived cells. This causes their proliferation and survival.

Semaphorin pathway is however a more recently discovered pathway. Semaphorins are proteins which were originally discovered for their role in assembly of neural circuitry [43,44]. Their role in HD has been investigated by several authors. Wang et al. [45] examined expression of semaphorin 3A in different colonic segments of HD patients. They studied expression levels of SEMA3A in both ganglionic and aganglionic colon tissues of 32 patients with HD and in colon tissue of 5 normal newborns. The tissues were examined by real-time RT-PCR, Western-blot, and immune histology. Comparison of SEMA3A expression levels between ganglionic and aganglionic tissues in HD revealed up regulation of SEMA3A expression in 43.75% (14/32) of the aganglionic colons. SEMA3A was expressed in the ganglion cells of the myenteric plexus, submucosa, as well as in the longitudinal and circular muscle layer of the normal colon of both unaffected newborns and patients with HSCR. In the aganglionic segment of patients with HD, SEMA3A was highly expressed in the circular muscle layer and was also detected in the submucosa

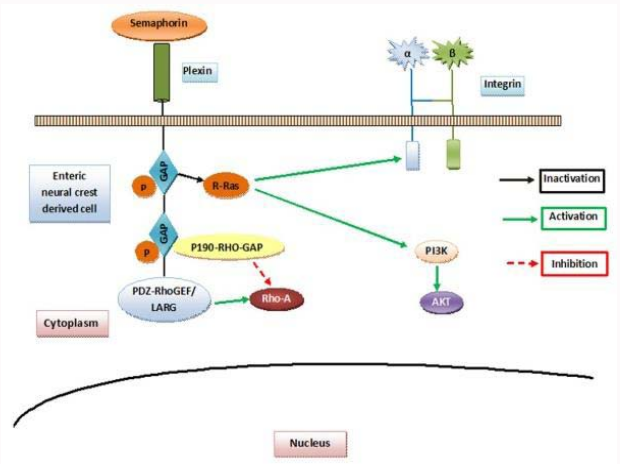

Figure 2: Role of semaphorins: Semaphorins bind to the Plexin family receptors. Plexins in turn can cause activation of Plexin associate tyrosine kinases. These plexins have intrinsic GTPase activating protein (GAP) activity which in turn leads to inactivation of R-Ras (black solid arrow). This leads to inhibition of integrin mediated activities and PI3K mediated AKT pathway. Another cytoplasmic domain of Plexins regulates the activation of a small GTPaseRhoA (red dashed arrow).

and in the longitudinal muscles layer. The fluorescence intensity of SEMA3A in the circular muscle layer in the aganglionic segment was much higher than that in ganglionic segment. Thus they concluded that SEMA3A expression was upregulated in the aganglionic smooth muscle layer of the colon in some patients with HD and increased SEMA3A expression may be a risk factor for HD pathology in a subset of patients.

In another subsequent studyLuzón-Toro et al. [46] carried out a comprehensive analysis of SEMA3A and SEMA3D in a series of 200 Spanish HD patients. RET mutations were also detected in some of those patients carrying SEMAs mutations. They evaluated the A131T-SEMA3A, S598G-SEMA3A and E198K-SEMA3D mutations using colon tissue sections by immunohistochemistry. All mutants showed increased protein expression in smooth muscle layer of ganglionic segments. Moreover, A131T-SEMA3A also maintained higher protein levels in the aganglionic muscle layers. These findings strongly suggest that these mutants have a pathogenic effect on the disease. Furthermore, coexistence with RET mutations, further substantiates the additive genetic model proposed for this rare disorder and further support the association of SEMAs genes with HD.

The major receptors for semaphorins are Plexin family receptors. Plexins in turn can cause activation of Plexin associate tyrosine kinases. Besides this plexins have intrinsic GAP activity which in turn leads to inactivation of an important G-protein R-Ras. This leads to inhibition of integrin mediated cell adhesion and the rest of downstream events. Another cytoplasmic domain of Plexins via signal transducers regulates the activation of a small GTPaseRhoA. In addition, GTPases are known to play important role in cell motility. Thus it is plausible that semaphorin mediated inhibition of cell motility may be responsible for migration failure of enteric neural crest derived cells in some cases of HD (Figure 2).

Role of genetic counseling

Since presently the understanding of the genetics of HD is not complete the role of genetic counseling is not clear. But it is likely that testing methodology and our understanding of genes, allelic variants,

and diseases will improve in the future. Thus, it is expected that genetic counseling will likely play an increasingly more important role in the management of these cases. DNA banking of affected patients may also be of value in the future.

Prenatal testing and preimplantation genetic diagnosis

Prenatal testing and preimplantation genetic diagnosis are presently not routinely performed in the cases of HD. However, since many of the pathogenic genetic variants are now well known this testing modality may be offered to some of the cases especially syndromic HD. However, one thing must be kept in mind that presence of a mutation does not necessarily mean that the child will develop clinical manifestations of HD. This fact must be discussed with the parents and they must be allowed to make an informed choice.

Conclusion

Thus to conclude HD is an extremely complex disease at genetic level. There is much more to be studied and learned before we are able to fully unravel this disease. The task at hand is proving to be extremely difficult. This is because a combinations of mutations together lead to the disease. Some of these are common and others are rare. But what is proving quite difficult is to associate a particular mutation to the disease. The present approach is to use NGS and GWAS. For any new variant discovered in these studies it is important to do functional analysis studies to prove their role in the pathogenesis of HD. In the future it is believed that a combination of these approaches and the development of an animal model will lead to further improvement in understanding of this disease.

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