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Genetics of Syndromic and Non-Syndromic Hirschsprung Disease

Genetische aspecten van syndromale en niet-syndromale Morbus Hirschsprung

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“The brain is for philosophy, science and love, however, food is for the gut”

Michael D. Gershon,
Third International Meeting: Hirschsprung disease and related neurocristopathies,
February 5-8, 1998, Evian, France

Contents

In herinnering aan mijn oma en mijn moeder

List of abbreviations

BBS	Bardet-Biedl syndrome
BERA	brain stem evoked response audiometry
BGP	bilateral generalized polymicrogyria
CCHS	Congenital Central Hypoventilation syndrome
CGH	Comparative Genomic Hybridization
ECE1	Endothelin converting enzyme
EDN3	Endothelin-3
EDNRB	Endothelin receptor-B
ENS	Enteric nervous system
GDNF	glial cell line derived neurotrophic factor
GOSHS	Goldberg-Shprintzen syndrome
HSCR	Hirschsprung disease
ICD	inter canthal distance
L(S)-HSCR	Long-segment Hirschsprung disease
LD	Linkage disequilibrium
MEN2A	Multiple endocrine neoplasia type 2A
MKKS	McKusick-Kaufman syndrome
MMR syndrome	megalocornea-mental retardation syndrome
MRI	magnetic resonance imaging
MWS	Mowat-Wilson syndrome
OCD	outer canthal distance
OFC	occipitofrontal circumference
PCR	polymerase chain reaction
PCWH	peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease
PHOX2B	Paired-like homeobox 2
PMG	polymicrogyria
RET	Rearranged during Transfection
S(S)-HSCR	Short-segment Hirschsprung disease
SIP1	Smad interacting protein 1
SNP	single-nucleotide polymorphism
SOX10	sex-determining region Y-related HMG-box gene 10
TCA	Total colonic aganglionosis
TGFβ	Transforming growth factor beta
TPR	tetratrico peptide repeats
VEP	visual evoked potential
WS	Shah-Waardenburg syndrome
ZFHX1B	Zinc finger homeobox 1B

CHAPTER 1

General Introduction and Aims and Outline of the Thesis

Based on the article:
Studying the genetics of Hirschsprung's disease: unraveling an oligogenic disorder.

A.S. Brooks, B.A. Oostra, R.M.W. Hofstra.
Clin Genet 2005;67:6-14

1. General introduction

1.1 History, definition and frequency of Hirschsprung disease

Harald Hirschsprung (1830-1916) was the senior physician at Denmark's oldest children's hospital, Queen Louise Children's Hospital, Copenhagen. He was appointed as a professor in Pediatrics in 1877 [1]. He described 2 boys who died at 8 and 11 months of age respectively, having presented with a typical clinical history of severe constipation [2]. By 1904 Hirschsprung had seen 10 cases; 9 were boys. He focused on the dilated colon as the source of the patients' problems. However, the earliest observation of this clinical entity is probably attributed to the famous Frederik Ruysch (1638-1731). The city council of Amsterdam not only appointed him as praelector but also as professor of anatomy, surgery and botany and overseer of midwifery in Amsterdam [3]. He reported a girl with "enormis intestini colo dilatatio" [4].

What Hirschsprung was describing and what we now call Hirschsprung disease (HSCR) or congenital megacolon, is clinically characterized by failure to pass meconium (the first stool) within 48 hours after birth, a distended abdomen, vomiting or neonatal enterocolitis [5]. HSCR is the most common cause of bowel obstruction in childhood and occurs worldwide. Chronic constipation is the major presentation in childhood. The estimated prevalence in the population is 1:5000 live born children [6-11], which means approximately 40 children are born each year in the Netherlands with this congenital malformation (2003: 200.297 live born children, 2004: 194.007 live born children, CBS http://statline.cbs.nl).

1.2 Diagnosis and treatment of HSCR

Although the clinical condition became well recognized, the histopathological features of HSCR were not understood until 1948. Histopathology of the bowel wall in a full-thickness rectal biopsy is the golden standard in reaching the diagnosis [12]. It characterizes this congenital abnormality by the absence of ganglia (groups of neurons) in the myenteric (or Auerbach) plexus, between the circular and longitudinal muscle layers of the gut, and in the submucosal (or Meissner) plexus, internal to the circular muscle layer [13, 14]. More frequently, because it is less invasive and is associated with less complications, a suction rectal biopsy is performed, demonstrating an increase in acetyl cholinesterase (AChE) activity in the lamina propria and muscularis mucosae; which is pathognomonic for HSCR [15]. Usually two pieces are taken: one is frozen and processed for AChE histochemistry and counterstained with hematoxylin (fig 1), and the other is embedded in paraffin and the sections are stained with hematoxylin and eosin to examine for the presence of ganglion cells. It is important to note that on a water-soluble contrast or barium enema radiograph the grossly abnormal-looking, distended colon ("megacolon") contains the normal enteric nervous system (ENS). Often a transition zone between the normal and agaglionic bowel is seen (fig 2).
Anorectal manometry can also aid in the diagnosis of HSCR through the absence of the rectoanal inhibitory reflex after a distention bolus [16]. The department of Pediatric Surgery of the Erasmus MC/Sophia Children's Hospital advocates the diagnostic approach of a child clinically suspected of HSCR consisting of radiographic studies (in neonates a water-soluble contrast enema is preferred), histopathological studies (suction biopsy and/or a full thickness biopsy), and anorectal manometry according to the diagnostic guidelines of the Dutch Pediatric Surgery Association.

The treatment of HSCR is surgical and is based on the resection of the aganglionic

segment of intestine; the chief difficulty encountered by pediatric surgeons is precisely establishing the proximal extent of the functional abnormality. Swenson and Bill performed the first corrective procedure for HSCR-rectosigmoidectomy and primary colo-anal anastomosis [17].

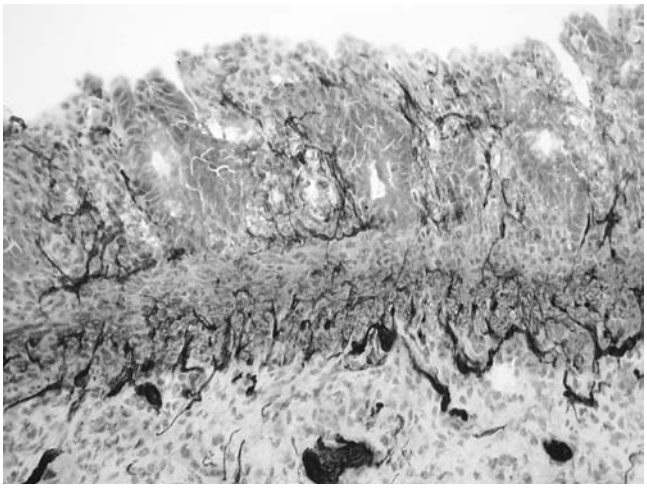

Figure 1

Histopathological findings in a patient with HSCR. Acetylcholinesterase histochemical staining in HSCR. Increased acetylcholinesterase staining with coarse, thick nerve twigs surrounding muscularis mucosa and extending into the lamina propria. *Courtesy Dr. J. den Hollander.*

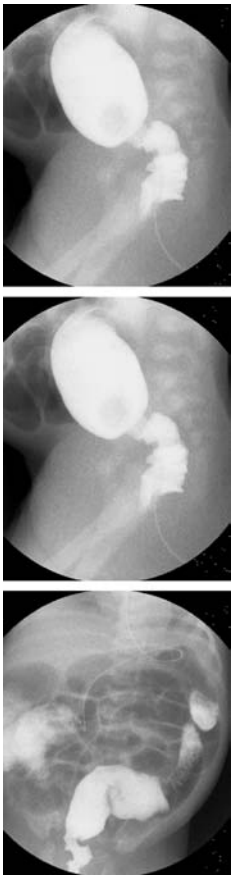

Figure 2

Water-soluble contrast enema of a patient showing the typical transition zone in the rectosigmoid region, which is the most common location. *Courtesy Dr. M. Lequin.*

Throughout the years, all kinds of variants to remove the aganglionic bowel and to reconstruct the intestinal tract by bringing the normally innervated bowel down to the anus while preserving normal sphincter function have been described. Many operations have been devised to accomplish these goals, but the most commonly performed at the present time are the Swenson, Duhamel, Soave and Rehbein procedures [1]. Current trends include primary neonatal and laparoscopic assisted pull-through, and transanal approaches [18-22].

When not treated correctly, HSCR may lead to death. However, the mortality rate has dropped from >75% in the 1940's to <2% in the 21st century, a trend which is associated with advancements in peri-operative treatment of children over the years [1, 23]. The most serious complication of HSCR is enterocolitis, in which necrosis and ulcerations affect the dilated proximal segment of the colon and may extend into the small bowel [23, 24]. It is characterized by fever, abdominal distention, and diarrhea, which may be severe and lead to life-threatening dehydration or sepsis.

1.3 Pathogenesis; normal and abnormal embryology of the enteric nervous system

HSCR results from a failure of neural crest cells to migrate, proliferate, differentiate or survive in the bowel wall to form both plexuses of the ENS [25, 26]. The ENS is a division of the autonomic nervous system and is defined as the system of neurons and their supporting cells (i.e. glial cells) that is present within the enteric wall. The ENS mediates motility reflexes and plays a major role in controlling water and electrolyte transport by the mucosal epithelium; it also regulates intestinal blood supply.

The development of the ENS is the focus of many different types of studies including e.g. studies of human congenital malformations of the ENS (i.e. the study of familial cases of HSCR, as discussed in paragraph 2.1), classic embryological ablation studies in chick embryo [27] and, transgenic experiments in rodents [28-30]. In addition, data from developmental studies of the ENS in fish and amphibians are accumulating [31-34], supporting the notion that the network of molecules and receptors that determine the development of the ENS is ancient since it is conserved in detail among many species (fish, amphibians, birds, and mammals).

Defects that interfere with cell migration could arise in the neural crest cells themselves, or in their local environment, either at the site of their origin, in their migration pathways towards the gut, in the gut in general, or in the normal ganglionic zone of the gut. Neural crest cells are produced from the entire length of the neural axis but only certain tightly defined regions of the neural crest give rise to the ENS, predominantly the vagal neural crest adjacent to the first seven somites-the primitive segments of the body- [26, 35].These precursor cells first migrate from the vagal neural crest into the rostral end of the intestinal tract early in embryonic life, and then migrate along the gut in a rostrocaudal manner to colonize the entire intestine. In the human fetus, neural crest cells migrate from the 5th to 12th week of gestation [25], in mice this process is completed by E13,5 [36-38]. The role of the sacral neural crest in the development of the distal part of the ENS is still disputed (for review see Burns) [39].

The neural crest cells also produce the cells of the sympathic and parasympathic nervous systems, dorsal root ganglia, and of several other cell types in specific locations (such as neuronal, endocrine and paraendocrine, craniofacial, conotruncal heart, and pigmentary tissues) [40, 41] . HSCR is therefore classified as a "neurocristopathy" [42] . Neurocristopathies encompass tumors, malformations, and single or multiple abnormalities of tissues mentioned above.

1.4 Classification of HSCR

In the majority of cases the lack of ganglion cells is restricted to the sigmoid and the rectum (fig 3). This form is called short-segment HSCR (S-HSCR). However, in 20% of cases the aganglionosis extends proximally [6, 9] which could be involving the complete colon and the terminal portion of the ileum (total colonic aganglionosis or TCA). In a small proportion of cases the aganglionosis extends to the whole bowel [43] .This is a lethal condition. These two latter forms are classified as long-segment or L-HSCR. However, not everybody is using the same classification; sometimes aganglionosis extending to the splenic flexure is called S-HSCR.

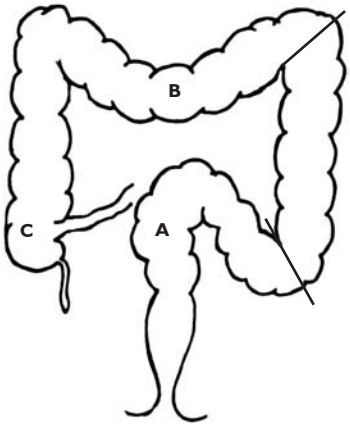

Figure 3

Classification of HSCR on the basis of the length of intestine with aganglionosis.

A=S-HSCR

B=L-HSCR

C=TCA

2. Studying the genetics of HSCR

What was the rationale for investigating a genetic basis for HSCR? Several lines of evidence suggested the involvement of genetic factors in the etiology of HSCR. First, familial occurrence ranges from approximately 5 -15% [6, 9, 44]. Badner and others performed complex segregation analysis of data on 487 probands and their families. They could show a male predominance of HSCR (3.9 males:1 female), consistent with other observations that HSCR is more common in boys [6-10, 44]. This unbalanced sex ratio suggests a genetic component [45]. Furthermore they demonstrated an elevated risk to sibs (4% or 0.04), as compared with the population frequency of 1 in 5000 (0.0002) (risk ratio λ=200) [45]. It was observed that the sex ratio decreased and the recurrence risk to sibs increased as the aganglionosis became more extensive. For the cases with aganglionosis beyond the sigmoid colon, the mode of inheritance was compatible with a dominant gene with incomplete penetrance, whereas for cases with S-HSCR, the inheritance pattern was equally likely to be multifactorial or recessive with very low penetrance. Another indication for involvement of genetic factors in HSCR comes from the association of HSCR with chromosomal aberrations such as trisomy 21 [6-11, 46] (it is estimated that 2-10% of HSCR cases have Down syndrome, whereas 5‰-1.4% of Down syndrome cases have HSCR [47, 48]) and structural anomalies of chromosome 2,7,10,13 and 20 [49-53]. The association of HSCR with a myriad of Mendelian inherited syndromes also indicated a possible genetic basis. Finally, the existence of several animal models of colonic aganglionosis showing specific Mendelian modes of inheritance was compatible with the notion that genetic factors were involved in HSCR as already mentioned in paragraph 1.3 [54, 55].

The search for genes involved in HSCR is a striking example of combining several strategies for the genetic dissection of a disorder with a complex etiology [56-58]. Furthermore this search illustrates the deeply interdependent relationship between clinically driven research and basic developmental studies. HSCR is mainly seen as a sporadic disorder and in most cases this is S-HSCR. In a minority of cases (5-15%) [6], the disease shows a clear familial occurrence mostly with a Mendelian autosomal dominant pattern with incomplete penetrance and variable expression [45]. One in three children has additional congenital anomalies. However, in only a minority of these children is a syndrome diagnosed [10, 11, 44, 59, 60]. The syndromes include chromosomal abnormalities (like trisomy 21) and Mendelian inherited syndromes, as extensively reviewed by Amiel and Lyonnet [61] (see fig 4 for examples of Mendelian syndromes associated with HSCR).

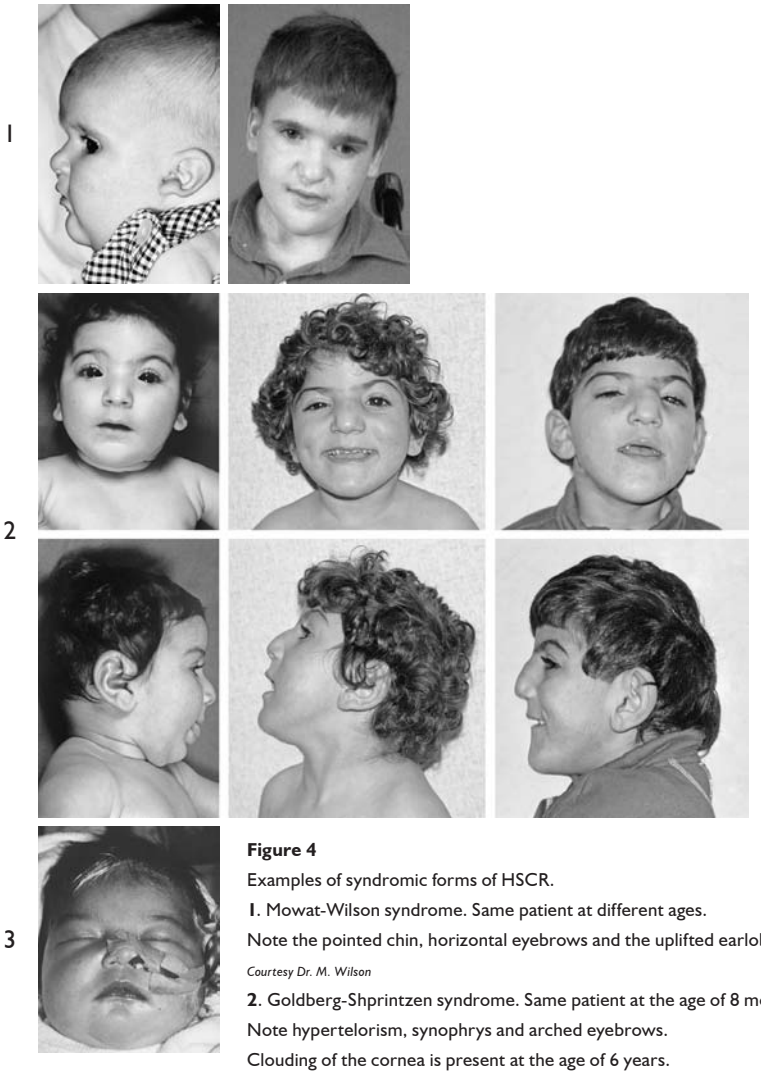

Figure 4

Examples of syndromic forms of HSCR.

1. Mowat-Wilson syndrome. Same patient at different ages.

Note the pointed chin, horizontal eyebrows and the uplifted earlobes.

Courtesy Dr. M. Wilson

2. Goldberg-Shprintzen syndrome. Same patient at the age of 8 months, 4 years and 6 years.

Note hypertelorism, synophrys and arched eyebrows.

Clouding of the cornea is present at the age of 6 years.

3. Shah-Waardenburg syndrome. Note the white forelock in a neonate.

For some of these Mendelian syndromes the genes have been identified. In the next paragraphs the genes and loci identified for non-syndromic (isolated) and syndromic forms of HSCR (table 1, 2 and 3) are reviewed. We will illustrate the overlap between syndromic and non-syndromic forms of HSCR, highlighting the fact that variable expression and reduced penetrance in those syndromes can be due to abnormalities in other non-syndromic HSCR susceptibility genes that act on the same or on interdependent developmental pathways. In contrast, we speculate that variants in new genes associated with rare syndromic forms might be discovered that may also confer a low risk in non-syndromic HSCR.

2.1 Non-syndromic HSCR: genes and loci involved

Multigenerational families with HSCR

Classical linkage mapping in large multigenerational families enriched for L-HSCR resulted in the mapping of a HSCR gene on 10q11.2 [62, 63]. Linkage was directed by information based on chromosomal aberrations in 2 HSCR patients [51, 64]. Both patients with total colonic aganglionosis showed an interstitial deletion of 10q11.2. *RET (*REarranged during Transfection), which encodes a receptor tyrosine kinase, and which was considered to be a good positional and functional candidate gene mainly based on expression studies. *RET* is predominantly expressed in neural crest derived tissues and tumors [65, 66]. The hypothesis of *RET* being a perfect candidate gene was strengthened by the finding of mutations in *RET* in a cancer syndrome, namely multiple endocrine neoplasia type 2A (MEN2A) [67, 68]. MEN2A is a cancer syndrome characterized by medullary thyroid carcinoma (MTC), phaechromacytoma, and hyperplasia of the parathyroid glands. The observation of the co-occurrence of MEN2A and HSCR in some families made a shared pathogenesis likely [63]. Shortly after the first *RET* mutations in HSCR patients were reported [69, 70].

All published families with HSCR, except one, are linked to *RET* [71]. In 50% of the multigenerational families a mutation in the coding sequence of *RET* was identified [72, 73]. The mutation yield of only 50% in HSCR families might be explained by the presence of mutations located outside the coding region of the *RET* gene in elements that control timing, location and /or level of gene expression. On the other hand the linkage to 10q in families without a *RET* coding sequence mutation, might also be explained by a susceptibility gene at a locus near *RET* and which is in linkage disequilibrium with *RET*, although so far no direct evidence points in that direction.

Failure to detect the biologically relevant *RET* mutations in half the reported HSCR families resulted in additional linkage studies using classical and non-parametric linkage methods. Linkage to *RET* was found in 11 out of 12 multigenerational families. In 6 of these families a pathogenic *RET* mutation was found. More interestingly a locus at 9q31 was found to segregate with the disease phenotype. This 9q31 locus was found in those families that did not have a mutation in the coding sequence of *RET*. It was suggested that the combination of these specific loci most likely explains the disease phenotype in these families [71].

Siblings with HSCR

As mentioned above, classical linkage mapping led to the identification of the *RET* gene as the major locus in families in which both S-HSCR and L-HSCR was segregating. However, these studies failed to explain the much more frequent occurrence of S-HSCR. Therefore series of siblings with S-HSCR were studied in a genomic wide screen for susceptibility factors other than *RET*. All 67 sib-pairs, excluding 2, showed haplotype sharing of 10q11.2. In 40% of these sib pairs a mutation in

the coding sequence of *RET* was reported, again underlining the central role of *RET* in HSCR. Two additional susceptibility loci, one at 3p21 and one at 19q12, were identified, and conferred a lower risk than *RET* [74]. This study showed that what seemed to be "simple" dominant inheritance proved not to be in the small HSCR families studied: heterozygous mutations at 3 different loci worked in concert and explained in a multiplicative model the genetics of S-HSCR. Moreover, multiplication of the estimated frequencies of these 3 susceptibility alleles is consistent with the frequency of HSCR in the population [6]. Furthermore, when only the *RET* negative siblings and the siblings with "mild" *RET* changes were analyzed, non-random allele sharing at 9q31 was confirmed. This again suggests a modifier locus at 9q31, as was already proven to exist in multigenerational families enriched for L-HSCR [71].

The sporadic HSCR patient

Direct mutation analysis revealed that mutations of the *RET* gene are also the most common genetic cause of HSCR in sporadic patients identified today although they are only found in 15-35% of the cases [72, 73]. For the genetic dissection of the sporadic form of HSCR a candidate-gene/association-analysis approach was chosen. As only 50% of the *RET* linked families had mutations, *RET* involvement without an obvious mutation was also considered in sporadic HSCR. Therefore case-control or trio-design studies evaluated haplotypes encompassing the *RET* locus. All studies (all performed on European HSCR patients originating from Spain, Italy, Germany, France and the Netherlands) showed the same alleles/haplotypes to be over represented in sporadic HSCR [75-80]. Up to 55% of the patients were carrier of the same haplotype and consequently the same unidentified variant whereas this variant was only present in 16% of the controls [79]. This same haplotype has a 66% frequency in Chinese sporadic HSCR patients [81]. In Asian American and European American control patients, the common non-coding *RET* variant (named RET+3) was identified in 45% and 25% of cases respectively; however, it is virtually absent in African Americans where it has a frequency of less than 1% [82]. The high frequency of this common non-coding *RET* variant in Asians probably correlates with an increased frequency of S-HSCR among Asian American newborns [83]. The data clearly show that the *RET* gene is also the major gene in sporadic HSCR cases, even when no clear mutation can be identified. Only one variant or one combination of ancestral variants in *RET*, specifically in the 5' end region (upstream the gene and in intron 1), contribute to the development of the majority of HSCR worldwide. Remarkably, a dose dependent effect seems present; homozygosity for certain *RET* haplotypes is associated with a higher odds ratio [79, 80]. The question remains: which of the identified variants is contributing to the disease development? Several candidates have already been put forward [78, 84, 85]. Both Fitze et al.[78] and Garcia Barcelo et al. [84] showed reduced expression associated with two risk-associated SNPs in the basal promoter. However, Griseri et al. could not confirm these findings [85]. Furthermore, Garcia-Barcelo and co-workers showed that these two basal promoter SNPs overlap a TTF-1 binding site and that TTF-1 activated RET transcription is also decreased by the HSCR-associated SNPs. In addition to these possible disease associated SNPs, two SNPs (RET3+, rs2435357 and IVS+9494, rs2506004) in this common risk haplotype are particular interesting as disease-associated *RET* variants, because of the homology and evolutionary conservation between rodents and primates, the differences in allele frequencies among patients and controls, and the fact that these SNPs show the highest association with the disease [82, 86]. Recent data show that RET3+ might lie within, and might compromise the activity of an enhancer-like sequence in *RET* intron 1 [82]. IVS+9494 is located in a region also conserved in nonmammalian species, and was therefore considered to be the most likely HSCR-associated variant [86].

Direct mutation analysis in *GDNF* (glial cell line derived neurotrophic factor) and *Neurturin*, both encoding ligands of the RET protein and therefore considered as plausible functional candidate genes, has rarely revealed mutations in sporadic HSCR patients. Single mutations in these genes are thought to contribute to the phenotype but are insufficient to cause HSCR [87] [88].

2.2 Syndromic HSCR: autosomal recessive forms

Waardenburg syndrome type 4 or Shah-Waardenburg syndrome (OMIM 277580) is characterized by sensorineural deafness, pigmentary abnormalities of the skin, eyes (heterochromia of the irises) and hair (poliosis or white forelock) and HSCR [89] [90]. Shah-Waardenburg syndrome is traditionally classified as a "neurocristopathy"; both melanocytes and ganglion cells that migrate into the gut are neural-crest-derived [42]. The first locus for this syndromic form of HSCR was mapped to 13q22 by an identity by descent approach in an inbred Mennonite population with considerable degrees of consanguinity and a ten-fold increased prevalence of HSCR indicating a high carrier frequency for a HSCR related mutation [91]. The affected cases shared a common ancestor 12 generations ago, and thus should be classified as a very young genetic isolate (<20 generations). The advantage of a young isolate is the fact that the genetic make-up of this isolated population more closely resembles that of the general population [92]. However in the case of the Mennonites it is important to realize that the founders were Swiss immigrants and therefore the resemblance to the general Caucasian population in the US might probably be limited [93]. Again as was the case with the identification of a HSCR locus on chromosome 10 (*RET*), the finding of aberrations (interstitial deletions) of chromosome 13 in HSCR cases helped to pinpoint this region as a HSCR related locus [52, 94]. The identification of the *EDNRB* (endothelin receptor-B) gene at 13q22 as the disease gene in this inbred population was further facilitated by knowledge based on phenotypic similarity to a mouse mutant [29, 95].

Because only 87% of the affected individuals in the inbred Mennonite population carried a missense mutation in the *EDNRB* gene a genome-wide association study in 43 Mennonite family trios was performed leading to the identification of a susceptibility locus on 16q23 in this kindred [96]. Furthermore, a clear association with certain *RET* alleles was demonstrated in the Mennonite population. However, after extensive mutation analysis, no mutation was identified of the *RET* gene in a selected panel of Mennonite HSCR patients. Apart from *RET* and *EDNRB*, none of the other HSCR genes or loci (on 9q31, 3p21, 19q31) showed an association. So even in this inbred population, mutations and/or variants in multiple genes at 3 loci cause HSCR with, again, a role for the *RET* gene or for one or more HSCR-susceptibility variants in linkage disequilibrium with *RET*. These data showed that variable expression and incomplete penetrance in syndromic HSCR (i.e. WS) is not only associated with variants in a syndromic HSCR gene (i.e. *EDNRB*) but is most probably also due to an abnormality in a gene involved in non-syndromic HSCR disease (*RET*) that acts in another neural crest development-related pathway. Association with 16q23 has not been confirmed in a non-Mennonite dataset, so it is unclear whether this susceptibility locus is unique for this inbred population or may also confer a low risk in the general population. No evidence was found for haplotype sharing with 16q23 in the 67 siblings with non-syndromic S-HSCR [74]. However, the elucidation of the gene located at 16q23 may yield clues toward other key proteins involved in HSCR genesis that may play a role in the general HSCR population.

The finding of mutations in the gene encoding the ligand of *EDNRB*, that is *EDN3* (endothelin-3) in Shah-Waardenburg patients was not surprising. A well-informed functional candidate gene approach and knowledge based on a mouse model helped in its identification [28, 97, 98]. It shows that two different genes in the same developmental pathway can cause the same phenotype. Heterozygous

mutations in these two genes were found in less than 5% of the non-syndromic HSCR population. Illustrating again the fact that although rare, mutations in these genes indeed confer a risk in non-syndromic forms of HSCR [99-103].

2.3 Syndromic HSCR: autosomal dominant forms

To date four autosomal dominant forms with predominantly *de novo* mutations are known in syndromic HSCR patients. To decipher the molecular basis of three syndromes, knowledge derived from animal models was crucial.

In one patient with HSCR, a complex heart defect and autonomic dysfunction, a mutation in *ECE1* (endothelin converting enzyme) was reported [104]. *ECE1* was considered as a candidate gene because it was known that *Ece1*$^{+/-}$ mice are normal, whereas *Ece1*$^{-/-}$ mice exhibit neonatal lethality because of craniofacial and cardiac defects. In addition, *Ece1*$^{-/-}$ newborns lack enteric ganglia in the terminal colon [30].

A dominant form of Shah-Waardenburg syndrome, sometimes with additional neurological features such as ataxia, leukodystrophy, polyneuropathy, is caused by mutations in *SOX10* (sex-determining region Y-related HMG-box gene 10) [105], a gene that was suspected to be a candidate for HSCR because of the Dominant megacolon or Dom mouse, a spontaneous mouse mutant with aganglionosis [106]. These mice have aganglionosis of the colon, pigmentation defects, and a loss of neurons and glia in the peripheral nervous system, consistent with the human phenotype.

SOX10 mutations are associated with 3 phenotypes: classical Shah-Waardenburg syndrome, a complex neurocristopathy also named PCWH (OMIM 609136); peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, HSCR [107, 108] and Yemenite deaf-blind-hypopigmentation syndrome [109].

In 75% the classical Shah-Waardenburg cases the mutations are *de novo* dominant, as reviewed by Verheij and Hofstra [103]. However, in the 25% of cases with an inherited mutation, the parent has no clinical features of Shah-Waardenburg syndrome. The reason for existence of so many *de novo SOX10* mutations may be that the severity of most associated phenotypes precludes reproduction. To explain the inheritance of several *SOX10* mutations one must assume that these mutations have incomplete penetrance or variable expression with only mild features in the parent. This suggests the existence of modifying factors in the parent and/or the patient.

Congenital Central Hypoventilation syndrome (CCHS) or Ondine's curse (OMIM 209880) is a life-threatening disorder characterized by failure of the autonomic control of breathing, especially during sleep [110]. The observation of HSCR in an estimated 15-20% of CCHS cases suggests a developmental link and common disease pathogenesis. Also, approximately 1.5% of HSCR cases have CCHS [111]. This specific combination is named Haddad syndrome [112, 113]. Therefore several known HSCR genes (*RET, GDNF, EDN3*) were considered as candidate genes for CCHS [114 115, 116]. Recently the major CCHS gene, *PHOX2B* (the paired-like homeobox) gene or *PMX2b* on 4p12, has been identified [117]. *PHOX2B* was considered to be a candidate gene in CCHS because in mice, the development of the reflex circuits of the autonomic nervous system is dependent on *Phox2b* [118]. At present *PHOX2B* mutations have been reported in over 300 CCHS patients, a minority of whom also had histologically proven HSCR [116, 117, 119-123]. Among 66 CCHS cases with *PHOX2B* mutations, 4 parents of the 43 pairs of parents available for DNA analysis, were mosaic for the mutation. This suggests that not all mutations in CCHS probands with healthy parents are *de novo* mutations [119].

The accurate syndrome delineation of HSCR with microcephaly and mental retardation in

cases with deletions of chromosome 2q22, led Mowat *et al*, to the conclusion that this entity should be separated from the clinical entity originally described by Goldberg and Shprintzen as an autosomal recessive syndrome [124, 125]. *De novo* mutations in *ZFHX1B* (zinc finger homeo box 1B, also known as Smad interacting protein or *Sip1*) were described in HSCR patients with microcephaly. This gene was located in the deleted segment at 2q22 from HSCR patients with a *de novo* t (2;13)(q22;q22) and t (2;11)(q22.2;q21). Again, a chromosomal abnormality helped to identify the culprit gene by using a positional cloning approach [126 127]. At present reports of over 100 cases with a *ZFHX1B* mutation and variable clinical features-now named Mowat-Wilson syndrome- have been published [127-141]. HSCR was a variable feature in 40-70% of cases [133].

Goldberg-Shprintzen syndrome is another clinical entity that is unlinked to 2q22 (chapter 3). Specifically, the facial features in Goldberg-Shprintzen syndrome appear to be different from those in Mowat-Wilson syndrome. Most striking is the difference in configuration of the eyebrows. In Goldberg-Shprintzen syndrome the eyebrows are arched, and may cross the midline (synophrys), while in Mowat -Wilson syndrome the eyebrows are horizontal (see fig 4). Furthermore a substantial part of patients with Mowat-Wilson syndrome have epilepsy and agenesis of the corpus callosum, while these features are rarely reported in Goldberg-Shprintzen syndrome [124, 125, 127-130, 133].

2.4. Relationship between gene mutations and aganglionic phenotype

In summary, many of the genetic abnormalities seen in animal models are phenotypically similar or identical to human HSCR, and the human genes were predominantly discovered through studies of mice with similar defects who had either spontaneous or designed mutations [28, 29, 54, 55, 142]. The most important of these genes code for molecules in the GDNF/RET signaling pathway, or the EDN3/EDNRB signaling pathway. These two signaling pathways have been identified as critical players in enteric neurogenesis. However, the genotype-phenotype relationship for genes involved in HSCR is not simple, and can only be understood with knowledge of the development of the ENS (see paragraph 1.3).

GDNF/RET signaling pathway

This signaling pathway is of importance for subpopulations of both peripheral and central neurons, having been shown by in vitro and in vivo assays to promote survival of neurons, mitosis of neural progenitor cells, differentiation of neurons, and neurite extension. The main purpose of this signaling pathway is the migration of the ENS precursors into the foregut [143, 144]. Experimental work with mice has demonstrated a high expression of *Gdnf* in the stomach at embryonic day 9.0, when the precursors start to migrate into the foregut. This seems to attract the *Ret* positive precursor cells (i.e. the vagal neural crest cells). Through time the *Gdnf* expression increases in more posterior parts of the intestine, with highest expression in the cecum around E10.5 [145]. Using in vitro gut cultures, it was established that RET was essential for the GDNF-mediated migration. Disruption of this signaling pathway would thus influence the migrational behavior of the ENS precursors. Interestingly, only the distal part of the gut is aganglionic. This could indicate that as it is the last part to be reached, any disruption in migration would affect only the distal part of the intestine. In addition to its chemoattractive role in ENS development, GDNF has also been reported to be necessary for the proliferation and survival of ENS precursors [146, 147], processes that may be modulated by EDN3 [148, 149] .

EDN3/EDNRB signaling pathway

EDN3 and its G-protein coupled receptor EDNRB are other important components of the signaling process, particulary in the development of the distal colon. EDN3 is expressed mainly in the cecum around E10.5, whereas its receptor EDNRB is expressed in the migratory ENS precursors. An interaction between the GDNF and the EDN3 signaling pathways has been demonstrated in both human and mouse [96, 150] Mice lacking *Edn3* have an aganglionic gut, however reducing the signaling capacity of *Ret* results in an improved colonization of the hindgut, indicating a fine balance between the 2 signaling pathways [151]. EDN3 has a role in inhibiting crest cell differentiation to ensure that sufficient ENS precursors are available to completely colonize the gut [148, 149]. In spontaneous or targeted mutations affecting *Edn3*, the distal hindgut fails to be colonized by neural crest cells and remains aganglionic [28, 152], supporting the hypothesis that in the absence of Edn3, enteric precursors differentiate prematurely and therefore fail to colonize the entire length of the intestine. Furthermore it was shown that EDNRB modifies the migratory response of neural crest cells to GDNF [153].

ECE-1 plays a role in activating, amongst others, EDN3, thus enabling EDNRB signaling. ECE-1 is a protease that performs the second cleavage to generate functional EDN3. Impaired function of ECE1 may thus lead to impaired function of EDN3: similar abnormalities are found in mice lacking *Ece-1, End3* or *Ednrb* [30].

Transcription factors

Three transcription factors SOX10, PHOX2B and ZFHX1B are involved in syndromic HSCR (see previous paragraph). *Sox10* encodes a transcription factor that can regulate expression of *Ednrb* [154] and *Ret* [118]. EDNRB has an enhancer element with binding sites for SOX10. Partial deletion of this enhancer results in mice that die postnatally from megacolon suggesting that the corresponding region in human EDNRB may be relevant to HSCR. It is therefore likely that mutations in *SOX10* result in misregulation of *EDNRB*, which will have an effect on the migration of the ENS precursors as well. The severity of the aganglionosis phenotype could be dependent on such interactions [155].

Phox2b is also a transcription factor, and is expressed in several classes of differentiating neurons of both the peripheral nervous system (including the ENS) and the central nervous system. Homozygous disruption of the *Phox2b* gene in mice results in the absence of all enteric ganglia, a clinical picture reminiscent of HSCR. Furthermore, *Phox2b* mutants do not show *Ret* expression and it has then been postulated that the *PHOX2B* gene may play a regulatory role in *RET* expression and account for the lack of enteric nervous system development in HSCR [156 118].

Finally *ZFHX1B*, a transcription modulator (a repressor of transcription) in the TGFβ signaling cascade , is expressed in early neural development in xenopus [157]. Its exact role in HSCR pathogenesis is unknown, however a possible functional link between ZFHX1B and RET is suggested [127]. Studies in homozygous *Zfhx1b* mutant mice have shown a complete lack of vagal neural crest cells cell precursors reflecting the dependence of these precursors on Zfhx1b activity for their normal development [158].

3. Aims and outline of the thesis

The preceding paragraphs discussed the history, etiology, pathogenesis and genetics of HSCR. This thesis will address the different aspects of the genetics of HSCR and, in particular, of the syndromic forms of HSCR. As discussed, the genetic studies of HSCR as a complex disorder shifted from family-based studies towards sib-pair and population-based studies. Despite the improvement in

the genetic analysis of HSCR patients following the discovery of 9 susceptibility genes, there remains a group of patients with histological proven HSCR that do not have a mutation in the currently known genes. Although it is shown that HSCR is oligogenic and that *RET* is involved in almost all cases [71, 74], additional HSCR loci and genes are likely to be identified in the future. However, finding these HSCR susceptibility loci and genes might turn out to be difficult. A single approach might not suffice. Studying families with rare presumptive single-gene associations of HSCR with other congenital abnormalities might be worthwhile. The genes involved in these syndromes might shed further light on the pathways underlying HSCR in general and may be useful in the understanding of the non-syndromic form of HSCR as well.

From 1970 to1995, 239 consecutive children with HSCR were admitted at the Pediatric Surgery Department of the Erasmus MC/Sophia Children's Hospital [159]. A total of 71 children (30%) had additional anomalies, consistent with data from the literature [8, 10, 59]. Chromosomal and monogenic syndromes were reported in 15 and 4 patients respectively. Fourteen males and 2 females had trisomy 21. In one female patient a duplication of chromosome 11 was detected. Shah-Waardenburg syndrome occurred in only two patients, a brother and a sister [97]. Two sporadic cases had CCHS; one of them also had a neuroblastoma.

The aim of our studies was to identify new genes involved in HSCR susceptibility by studying both non-syndromic and syndromic families, treated at the Pediatric Surgery Department of the Erasmus MC/Sophia Children's Hospital. To this end we chose the traditional linkage study design. From the 239 consecutive HSCR patients, we selected 3 families to study in detail.

In part 1, we characterized families in which HSCR segregates with additional congenital malformations. We performed phenotypic studies and DNA analyses. Our objectives were 1). to delineate the clinical features of Goldberg-Shprintzen syndrome in a consanguineous Moroccan family (chapter 2); 2). to identify the genetic defect in this family by homozygosity mapping (chapter 3); 3). to prove that Goldberg-Shprintzen syndrome is not only clinically but also genetically different from Mowat-Wilson syndrome (chapter 3); 4). to perform clinical and molecular studies in additional families with Goldberg-Shprintzen syndrome (chapter 4); 5). to characterize a family in which HSCR segregates with postaxial polydactyly and to discuss the differential diagnostic considerations (chapter 5).

In part 2, we present a genome-wide linkage analysis of a non-syndromic HSCR family who did not have a *RET* mutation and identified suggestive linkage for a new HSCR susceptibility locus at 4q31.3-q32.3 (chapter 6).

Tables

Table 1 Current genes and loci involved in HSCR

Gene	OMIM	Map Position	Inheritance	Phenotype
RET	164761	10q11.2	Dominant, incomplete penetrance	Non-syndromic MEN2A
GDNF	600837	5p13	Non-Mendelian	Non-syndromic
Neurturin	602018	19p13	Non-Mendelian	Non-syndromic
EDNRB	131244	13q22	Recessive	Shah-Waardenburg ABCD
			Dominant (de novo in 80%)	Non-syndromic
EDN3	131242	20q13	Recessive	Shah-Waardenburg
			Dominant, incomplete penetrance	Non-syndromic
SOX10	602229	22q13	Dominant (de novo in 75%)	Shah-Waardenburg
ECE-1	600423	1p36	De novo dominant	Congenital heart malformation
ZFHX1B	605802	2q22	De novo dominant	Mowat-Wilson
PHOX2B	603851	4p12	Dominant (de novo in 90%)	CCHS
Unknown		3p21	Non-Mendelian	Non-syndromic, S-HSCR
Unknown		9q31	Dominant, incomplete penetrance	Non-Syndromic, L-HSCR
Unknown		19q12	Non-Mendelian	Non-syndromic, S-HSCR
Unknown		16q23	Non-Mendelian	Shah-Waardenburg

Table 2 Clinical features of syndromes with HSCR as a mandatory feature

Gene	OMIM	Syndrome	Clinical Features
EDNRB	277580	Shah-Waardenburg	Deafness, pigmentary abnormalities
	600501	ABCD	Albinsm, black lock, cell migration disorder of the neurocytes of the gut (i.e.HSCR), and deafness
EDN3	277580	Shah-Waardenburg	Deafness, pigmentary abnormalities
SOX10	277580	Shah-Waardenburg	Deafness, pigmentary abnormalities, polyneuropathy
	277580	Shah-Waardenburg and neurological features	Ataxia, leukodystrophy, demyelinating polyneuropathy, mental retardation
PHOX2B	209880	Haddad or Ondine-Hirschsprung	Congenital Central Hypoventilation Syndrome, neuroblastoma, autonomic dysfunction
ZFHX1B	235730	Mowat-Wilson	Mental retardation, microcephaly, epilepsy, corpus callosum agenesis

Table 3 Oligogenic inheritance in HSCR

Genes/loci	Phenotype	Study design	Ref
RET/ 3p21/ 19q12	Non-syndromic HSCR	Linkage in an affected sib-pairs design	[74]
EDNRB/ 16q23/ 10q11.2	Shah-Waardenburg	Association studies in trio-design in isolated population	[96]
RET/ 9q31	Non-syndromic HSCR	Classical linkage in multigenerational families	[71]

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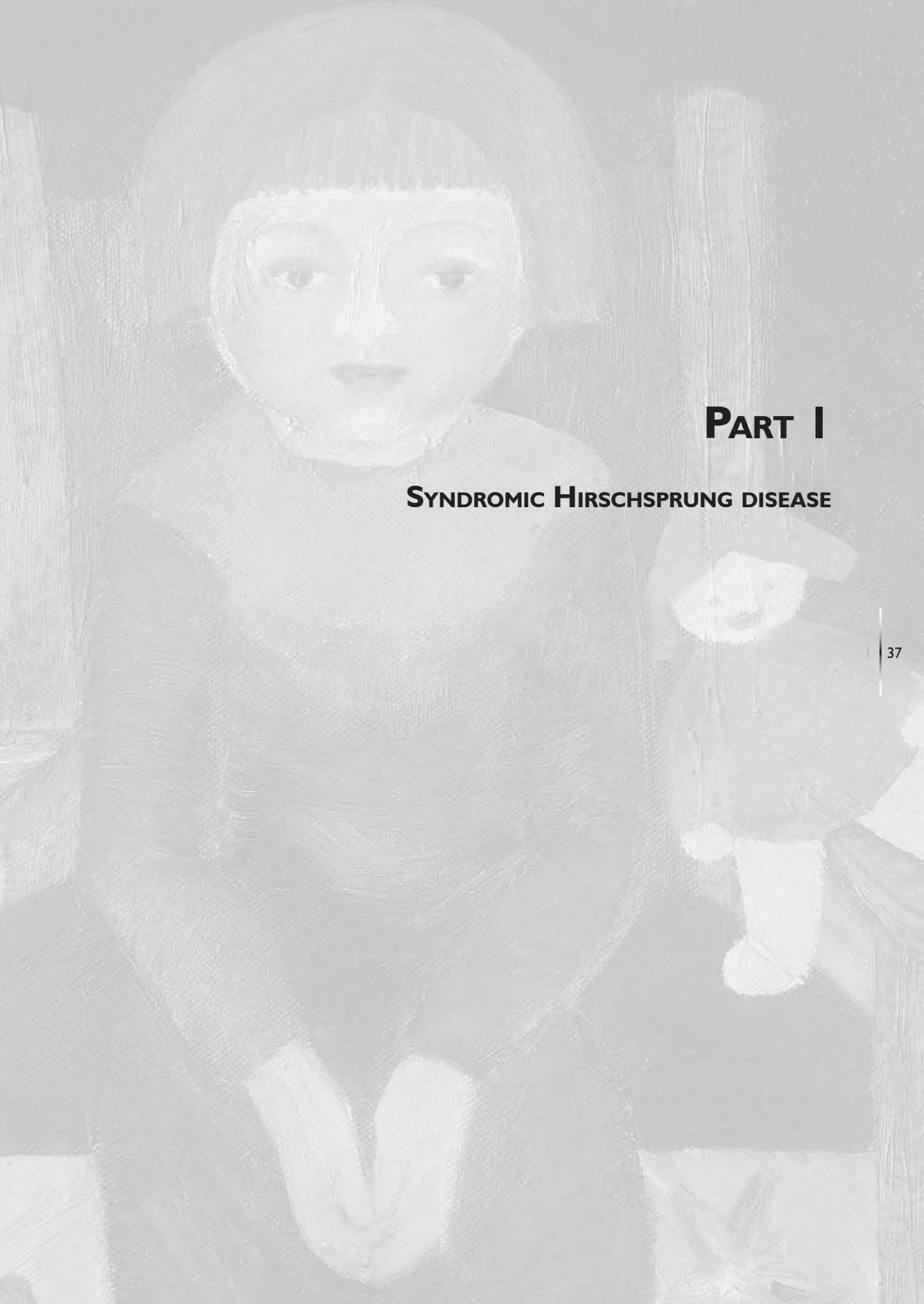

Part I

Syndromic Hirschsprung disease

CHAPTER 2

A Consanguineous Family with Hirschsprung Disease, Microcephaly, and Mental Retardation (Goldberg-Shprintzen syndrome)

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Abstract

Hirschsprung disease, mental retardation, microcephaly, and specific craniofacial dysmorphism were observed in three children from a large, consanguineous, Moroccan family. A fourth child showed similar clinical features, with the exception of Hirschsprung disease. The association of these abnormalities in these children represents the Goldberg-Shprintzen syndrome (OMIM 235730). Mutation scanning of genes potentially involved in Hirschsprung disease, *RET*, *GDNF*, *EDN3*, and *EDNRB*, showed a sequence variant, Ser305Asn, in exon 4 of the *EDNRB* gene in the index patient of this family. The Ser305Asn substitution present in two of the four patients and four healthy relatives and absent in one of the remaining two patients illustrates the difficulties in interpreting the presence of mutations in families with Hirschsprung disease. It is unlikely that the EDNRB variant contributes to the phenotype. This consanguineous family might be useful for the identification of a Goldberg-Shprintzen locus.

Introduction

Hirschsprung disease (HSCR) is a congenital disorder characterised by the absence of ganglion cells from the bowel wall. The estimated incidence is 1 in 5000 live born children. Boys are affected four times as often as girls. Additional anomalies, mostly of the heart and kidneys, occur in 10-30% [1, 2]. HSCR is a variable feature in a large number of monogenic syndromes, such as Waardenburg syndrome, cartilage-hair hypoplasia syndrome, Smith-Lemli-Opitz syndrome, Nager acrofacial dysostosis, Kaufman-McKusick syndrome, Bardet-Biedl syndrome, and primary hypoventilation syndrome (Ondine-Hirschsprung disease) [3-7]. This variety of syndromes associated with HSCR implies considerable genetic heterogeneity in the aetiology of HSCR, although some of these syndromal associations may be through chance.

Traditionally, HSCR as an isolated entity is viewed as having a complex inheritance and being transmitted as a sex modified multifactorial trait. However, clear autosomal dominant and autosomal recessive inheritance have been reported [8]. Mutations in five genes have been implicated in isolated or syndromic HSCR: *RET*,[9, 10] which encodes a receptor tyrosine kinase and its ligand, glial cell line derived neurotrophic factor (GDNF),[11, 12] *EDNRB*, the gene encoding the endothelin B receptor,[13] and endothelin 3 (*EDN3*), one of its ligands [14, 15]. Homozygous mutations in the latter two genes are associated with HSCR, pigmentary abnormalities, and deafness (Shah-Waardenburg syndrome). A similar phenotype is associated with heterozygous mutations in the *SOX10* gene.[16]. RET mutations have been observed in approximately 50% of familial cases and 10-30% of sporadic cases. Mutations of *EDNRB*, *EDN3*, and *GDNF* have been reported in only a few cases [17].

Here we describe a syndrome consisting of HSCR, mental retardation, microcephaly, and dysmorphic features in four patients of a large, consanguineous, Moroccan family. These phenotypic features are consistent with Goldberg- Shprintzen syndrome. A rare *EDNRB* variant was found, not cosegregating with the phenotype.

Case reports

Patient VI.1 is the first daughter of a consanguineous Moroccan couple; their maternal grandmothers were sisters (fig 1). After premature rupture of the membranes, there was spontaneously delivery at 40 weeks of gestation; her birth weight was 3280 g (50th centile) and occipitofrontal circumference

(OFC) 33 cm (3rd centile). She was admitted to our hospital because passing of meconium was delayed and she had feeding problems. Short segment HSCR was diagnosed. She had a temporary colostomy at 2 weeks of age and intestinal reconstruction at the age of 6.5 months.

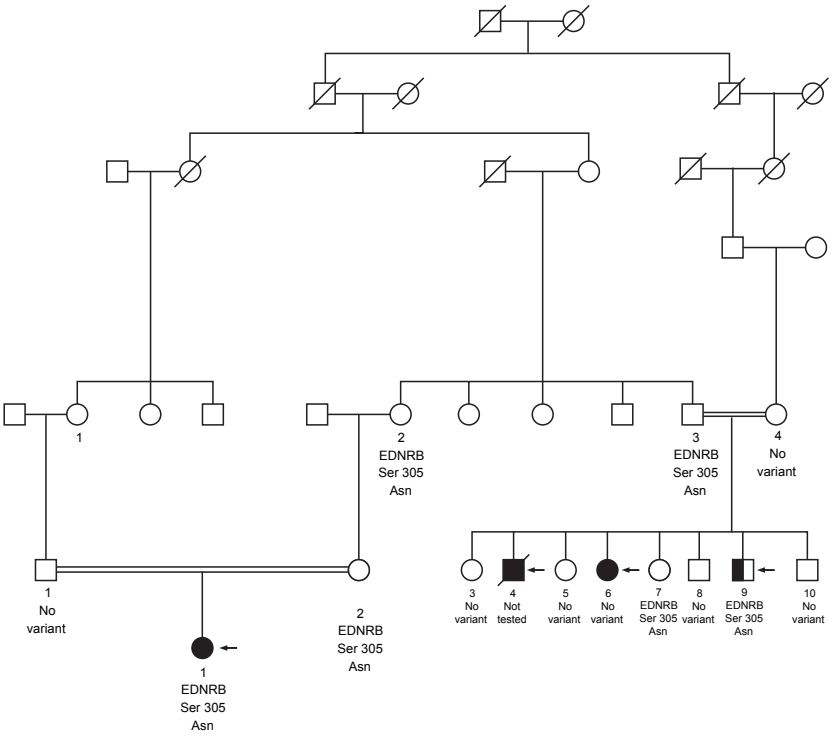

Figure 1

Pedigree of family. Subjects with the Goldberg-Shprintzen phenotype are indicated by blackened symbols. Patients VI.1, V.4, and V.6 have histologically proven Hirschsprung disease. V.9 has microcephaly, mental retardation, and characteristic facial abnormalities, but no Hirschsprung disease.

At 2 weeks of age, she showed telecanthus (her ICD was 2.7 cm (>97th centile) and OCD 7 cm (75th-97th centile)), a prominent nasal bridge, tapering fingers, and widely spaced nipples. Involuntary movements of the head started from 3 months of age. She was able to sit without support at the age of 7 months, but showed titubation of the head when seated or vertically suspended. An MRI scan of the brain at 9 months of age showed a slight general brain atrophy with delayed myelinisation. At 2 years, she manifested the described dysmorphism, progressive microcephaly, mildly downward slanting palpebral fissures, a flat occiput, short philtrum, and large ears. At the age of 26 months, her development is retarded and is equivalent to a chronological age of 14 months.

Patient V.4 was the second child of a consanguineous couple. The boy's father (IV.3) is the brother of the maternal grandmother of the case described above. Pregnancy and delivery (at 38 weeks) were uneventful. Birth weight was not recorded and OFC was 33.5 cm (3rd-10th centile). A broad nasal bridge, low set ears, and a short neck were reported. Failure to pass meconium and vomiting were indications for referral to the Department of Paediatric Surgery. Long segment HSCR was diagnosed and an ileostomy was made. He died from complications of sepsis at the age of 3 weeks. Necropsy was not permitted. He had a normal 46,XY karyotype.

Patient V.6, a 12 year old girl, is the fourth child of this couple. She was born at term after an

uneventful pregnancy. Her birth weight was 4000 g (50th-97th centile) and OFC was 33.5 cm (3rd-50th centile). A lesion of the right inferior part of the brachial plexus or Erb-Duchenne palsy was noted. From 2 months of age, she developed progressive constipation, which prompted admission to our department. Her abdomen was distended. A barium enema was suggestive of HSCR. Absence of ganglion cells and increased acetylcholinesterase staining in nerve fibres were found at biopsy. A low anterior resection with a primary anastomosis was performed. The postoperative course was uncomplicated.

At the age of 5 months, the child neurologist saw her for suspected psychomotor retardation, microcephaly (OFC 39 cm, 3rd centile), and a mild convergent strabismus. BERA (brain stem evoked response audiometry) and VEP (visual evoked potential) showed no overt abnormalities. Brain CT at the age of 5 years showed a slight asymmetry of the lateral ventricles and a hypoplastic septum pellucidum. The karyotype was 46,XX. Dysmorphological evaluation at 5 years (fig 2) showed high arched eyebrows, dense curled eyelashes, a broad nasal bridge, blue sclerae, large corneae (corneal diameter 13.5 mm), a mild thoracic scoliosis, mild syndactyly of digits II and III, and a broad hallux.

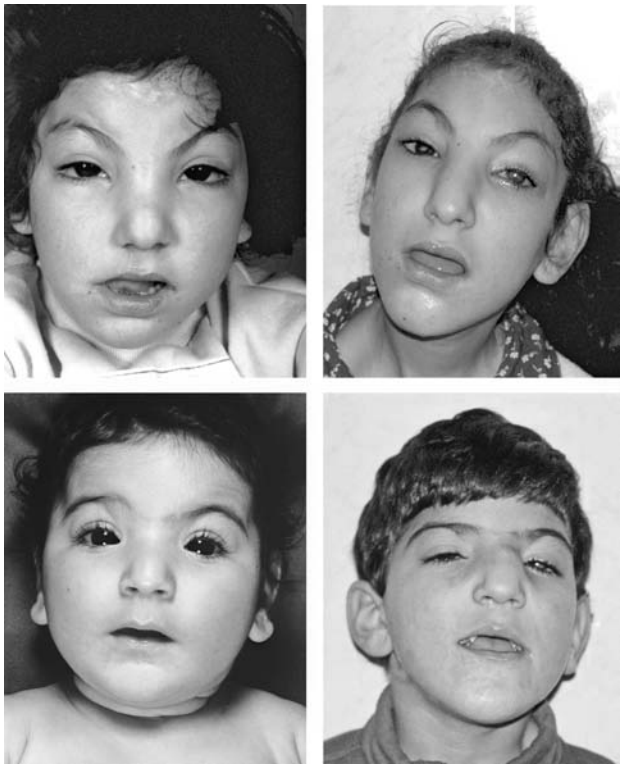

Figure 2

(Above) Patient V.6 at 5 years and 12 years. Note hypertelorism and curled eyelashes and clouding of the cornea at the age of 12 years. (Below) Patient V.9 at 8 months and 7 years. Note hypertelorism, synophrys, and curled eyelashes. Clouding of the cornea is present.

She has mental retardation and a severe conductive hearing loss, related to therapy resistant middle ear infections. Follow up at 6, 8, and 11 years (fig 2) showed height following the 3rd centile and head circumference paralleling the 3rd centile. Progressive scoliosis and proximal muscle weakness were noted from 10 years of age. Lung function showed a strongly reduced vital capacity, not proportional to the severity of the kyphoscoliosis. Recurrent corneal infections suggested a primary corneal hypoaesthesia.

The seventh child (V.9) was born after a healthy girl and boy. Caesarean section was indicated because of a transverse lie. His birth weight was 4115 g (90th centile). When he was 8 months, the parents noticed developmental delay and remarkable physical resemblance to their older daughter (V.6) (fig 2). His OFC was 42 cm (<3rd centile). He had dense eyebrows with mild synophrys, a broad nasal bridge, low set ears, and large corneae (corneal diameter 14.5 mm) with blue sclerae. Hypotonia and slipping through on vertical suspension were present. At the age of 13 months his height was 75 cm (10th-25th centile), OFC 43 cm (2 cm <3rd centile), ICD 3.2 cm (>97th centile), and OCD 8.8 cm (>97th centile). He had telecanthus, downward slanting palpebral fissures, broad nasal bridge with a bulbous nose, a high arched palate with a bifid uvula, and large anteverted ears. His thumbs were short and broad and his toes overlapped. He had corneal hypoaesthesia and recurrent corneal infections (fig 2). Defecation was reportedly normal. Brain imaging was not performed. Table 1 lists the phenotypic features of all four patients.

Table 1 Phenotypic features in four Goldberg-Shprintzen syndrome patients

	Case 1 VI.1	Case 2 V.4	Case 3 V.6	Case 4 V.9
HSCR	SS-HSCR	TCA	SS-HSCR	No HSCR
Mental retardation	+	?	+	+
Hypotonia	+	?	-	-
Microcephaly	+	+	+	+
Brain scan	Abnormal	?	Abnormal	?
Arched,dense eyebrows	+	?	+	+
Curled eyelashes	+	?	+	+
Synophrys	+	?	+	+
Hypertelorism	+	?	+	+
Corneal ulcer	-	?	+	+
Megalocornea	?	?	+	+
Wide nasal brigde	+	+	+	+
Bulbous nasal tip	+	?	+	+
Anteverted ears	+	+	+	+
Bifid uvula	-	?	?	+
Toes			Broad hallux	Overlapping
Skeletal abnormalities	-	?	Scoliosis	-
Ataxia of the head	+	?	-	-
Karyotype	46, XX	46, XY	46, XX	46, XY
DNA analysis	S305N	?	-	S305N

TCA=total colonic aganglionosis. SS-HSCR=short segment Hirschsprung disease. ?=not evaluated/described. +=present.-=absent.

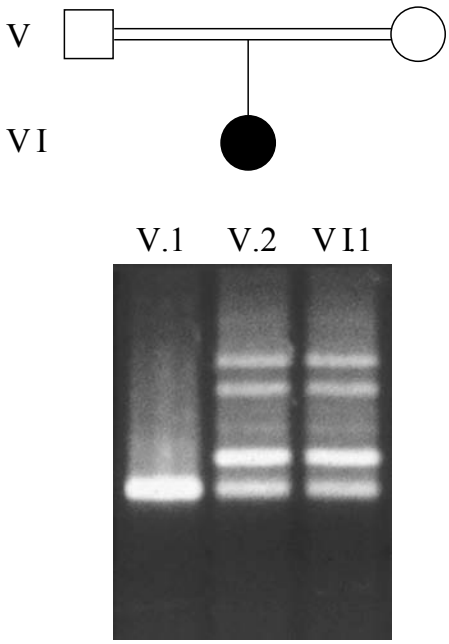

Figure 3

DGGE analysis of EDNRB exon 4. PCR products were amplified from genomic DNA from patient VI.1 (lane 3), her father V.1 (lane 1), and mother V.2 (lane 2). A mobility shift was found in the patient and her mother. Sequencing of the PCR products showed the Ser305Asn substitution. Both mother and patient are heterozygous.

Laboratory studies

All four patients appeared to have a normal karyotype.. DNA was isolated from leucocytes prepared according to standard methods. DGGE analysis of all exons of *RET*, *GDNF, EDNRB*, and *EDN3* was performed on the index patient VI.1 as described before (RMW Hofstra and J Osinga)[14, 18]. Apart from known neutral variants, a similar mobility shift of exon 4 of *EDNRB* was detected in index patient VI.1 (fig 3). Sequencing showed a G to A transition that at the amino acid level results in the replacement of a serine by an asparagine (Ser305Asn). On screening 12 available family members (IV.2, IV.3, IV.4, V.1, V.2, V.3, V.5, V.6, V.7, V.8, V.9, and V.10), five (IV. 2, IV.3, V.2, V.7, and V.9) were found to be carriers of the EDNRB variant. The Ser305Asn substitution was not identified in 100 (unselected) HSCR patients or 70 healthy Dutch controls (140 chromosomes). However, we did not genotype unrelated Moroccan subjects for this substitution.

Discussion

The dysmorphic features in these four patients closely resemble the syndrome described by Goldberg and Shprintzen [19] in 1981 and later by others [20-24]. Common features in reported cases were microcephaly, mental retardation, distinctive face, and HSCR. Goldberg and Shprintzen [19] only reported submucous cleft palate. Coloboma of the iris was not always present [19, 20]. An autosomal recessive mode of inheritance was suggested, based upon two sibs in one pedigree with unaffected parents and one consanguineous pedigree. This mode of inheritance is also most likely in our

consanguineous pedigree. This syndrome should not be confused with the Shprintzen-Goldberg syndrome (OMIM 182212, craniosynostosis, marfanoid habitus).

The neurological abnormalities seen in our patients have not been reported before. They might either belong to the clinical spectrum of Goldberg-Shprintzen syndrome, occurring at a later stage, or be caused by another autosomal recessive trait segregating in this family from a common ancestor. This second trait could cause proximal muscle weakness, hypotonia, and the eye abnormalities. Kim *et al* [25] recently reported a similar phenotype in a 15 year old boy with microcephaly, mental retardation, a characteristic face, HSCR, proximal muscle weakness, cerebellar dysfunction, and scoliosis. The parents were not consanguineous. His brother had mild developmental delay and a paternal cousin had isolated HSCR.

The eye abnormalities (megalocorneae and corneal hypoaesthesia) in two out of four patients have not been reported previously in Goldberg-Shprintzen syndrome. Their differential diagnosis includes megalocornea-mental retardation or MMR syndrome (OMIM 249310) [26]. HSCR and mild microcephaly was described once in combination with familial dysautonomia (OMIM 223900) [27]. Both syndromes can be excluded in our patients. Thus we propose that the Goldberg-Shprintzen syndrome can be associated with neurological and ophthalmic symptoms at a later age.

A causative role for the Ser305Asn EDNRB variant?

Among causative mutations involved in HSCR, 10 were reported to be in the *EDNRB* gene. They occurred predominantly in sporadic cases and were located in the transmembrane spanning domains [28-30] . The Ser305Asn substitution was previously reported by Auricchio *et al* [31] as a causative mutation in a sporadic Italian male patient with isolated HSCR. Biochemical data showed that in cows Serine305 is involved in post-translational modification (that is, phosphorylation) of the Ednrb protein [32]. Therefore, substitutions of this amino acid might have functional consequences, resulting in HSCR. The Ser305Asn substitution cannot explain the Goldberg-Shprintzen phenotype by itself since it does not cosegregate with the phenotype (for example, while V.6 is affected with Goldberg-Shprintzen syndrome, but does not have the EDNRB variant, the reverse applies to V.7). The most likely explanation for the phenotype in this consanguineous family seems to be homozygosity for a mutation in a presumed Goldberg-Shprintzen gene; none of the patients in this family was found to be homozygous for the Ser305Asn substitution. Although we do not know the frequency of Ser305Asn substitutions in the Moroccan population, it is unlikely that the EDNRB variant contributes to the phenotype in our family. This family might be instrumental in identifying a Goldberg-Shprintzen gene. The presence of a mutation in the *EDNRB* gene in this family illustrates the difficulties in interpreting the presence of mutations in families with HSCR [33].

Although the function and interactions of the multiple HSCR susceptibilty genes await further clarification, mutation analysis in syndromic HSCR patients might offer insight into the pleiotropic effects of these genes. Detailed dysmorphological evaluation of syndromic HSCR patients may give clues as to additional susceptibility genes for HSCR.

Acknowledgements

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HOMOZYGOUS NONSENSE MUTATIONS IN *KIAA1279* ARE ASSOCIATED WITH MALFORMATIONS OF THE CENTRAL AND ENTERIC NERVOUS SYSTEMS

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Summary

We identified, by homozygosity mapping, a novel locus on 10q21.3-q22.1 for Goldberg-Shprintzen syndrome (GOSHS) in a consanguineous Moroccan family. Phenotypic features of GOSHS in this inbred family included microcephaly and mental retardation, which are both central nervous system defects, as well as Hirschsprung disease, an enteric nervous system defect. Furthermore, since bilateral generalized polymicrogyria was diagnosed in all patients in this family, this feature might also be considered a key feature of the syndrome. We demonstrate that homozygous nonsense mutations in *KIAA1279* at 10q22.1, encoding a protein with two tetratrico peptide repeats, underlie this syndromic form of Hirschsprung disease and generalized polymicrogyria, establishing the importance of *KIAA1279* in both enteric and central nervous system development.

Goldberg-Shprintzen syndrome (GOSHS)[1, 2] is a disorder characterized by microcephaly, mental retardation, facial dysmorphism, and Hirschsprung disease (HSCR). GOSHS shares the same accession number (MIM 235730) in the Online Mendelian Inheritance in Man (OMIM) database as the comparable disorder named Mowat-Wilson syndrome (MWS) [2]. Genetically, however, they are different. MWS occurs as a *de novo* dominant syndrome; whereas GOSHS is most likely inherited as an autosomal recessive trait, a hypothesis based on the occurrence of the disease in consanguineous pedigrees and in affected sibs with unaffected parents [3, 4]. MWS is associated with *de novo* mutations in *ZFHX1B* located at 2q22 [5-7], whereas, for GOSHS, no causative gene has yet been identified. Also, some clinical differences exist. In patients with MWS, neurological abnormalities such as epilepsy (in a high percentage), agenesis of the corpus callosum (in 35% of patients), and cortical malformations (in a minority of patients) have been reported [5, 7, 8]. In the few patients with GOSHS discussed in published reports, neurological symptoms have not been studied in detail, and the cause of the microcephaly and mental retardation remains obscure [1, 3].

Here, we describe a family with HSCR (an anomaly of the enteric nervous system that is of neural crest origin and is histologically characterized by the absence of ganglion cells in the myenteric and submucosal plexuses) [9] as a variable feature and bilateral generalized polymicrogyria (PMG) (a developmental malformation of the cerebral cortex, characterized by an enlarged number of smaller convolutions or gyri and disruption of the normal six-layered cerebral cortical structure) [10] as a constant feature. The established diagnosis for this family was GOSHS, although bilateral generalized polymicrogyria (BGP) has not been reported as part of the syndrome before.

To unravel the genetic basis of GOSHS, we performed a complete genome scan and homozygosity mapping in a large consanguineous Moroccan family reported elsewhere [4]. The segregation of the disease in the pedigree is consistent with an autosomal recessive mode of inheritance, with several consanguineous loops (fig 1), and the family therefore was considered suitable for homozygosity mapping [11].

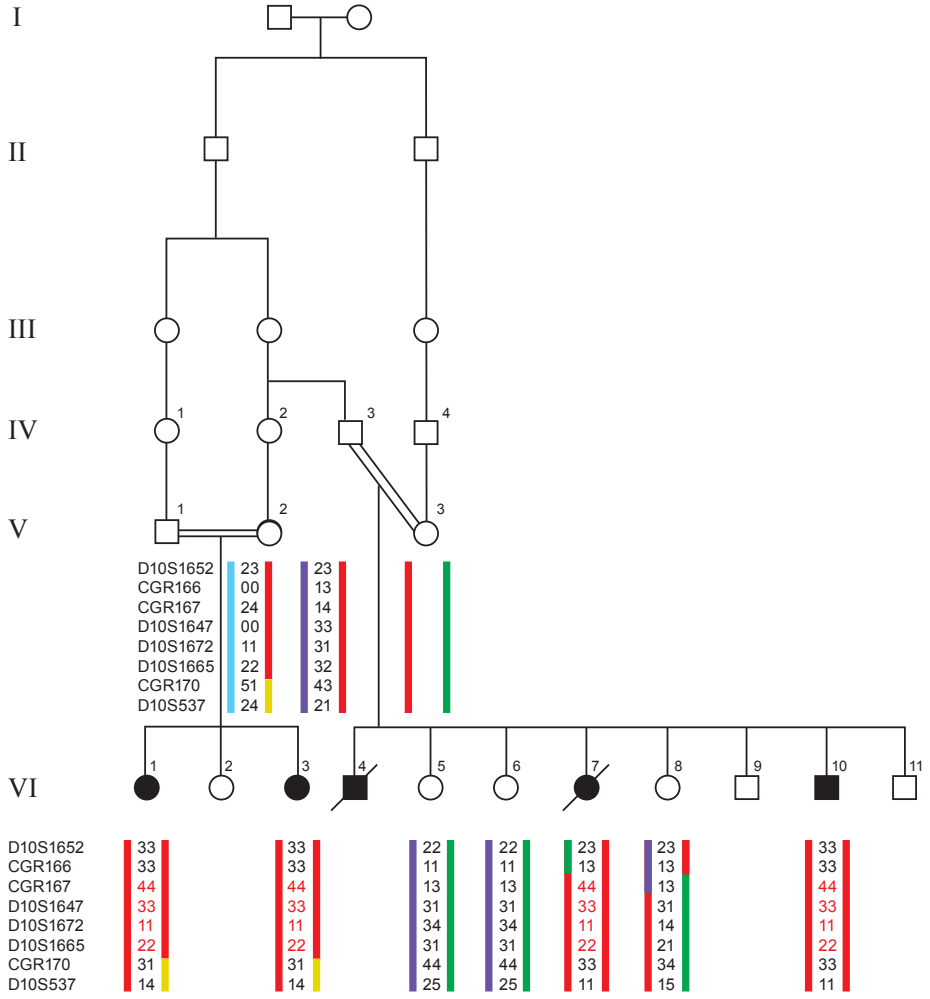

Figure 1 Pedigree structure and haplotypes of the Moroccan family with GOSHS. Patients are represented as blackened symbols. The shared region of homozygosity (*red*) in all affected cases is delimited by markers *CGR166* and *GCR170*. Six of the 11 markers typed for refining the candidate region between marker *D10S1652* and *D10S537* are shown.

Informed consent was obtained from the parents of all subjects. In this family, five patients had the cardinal signs characterizing GOSHS (one boy lacked HSCR). One child with long-segment HSCR died in the neonatal period; his DNA was unavailable for our study. When the youngest patient was diagnosed, brain magnetic resonance imaging (MRI) revealed BGP. MRI scans of all living patients also revealed BGP (fig 2). Gait disturbance, speech defect, excessive drooling, and pseudobulbar signs indicate diffuse cortical dysfunction including the perisylvian areas.

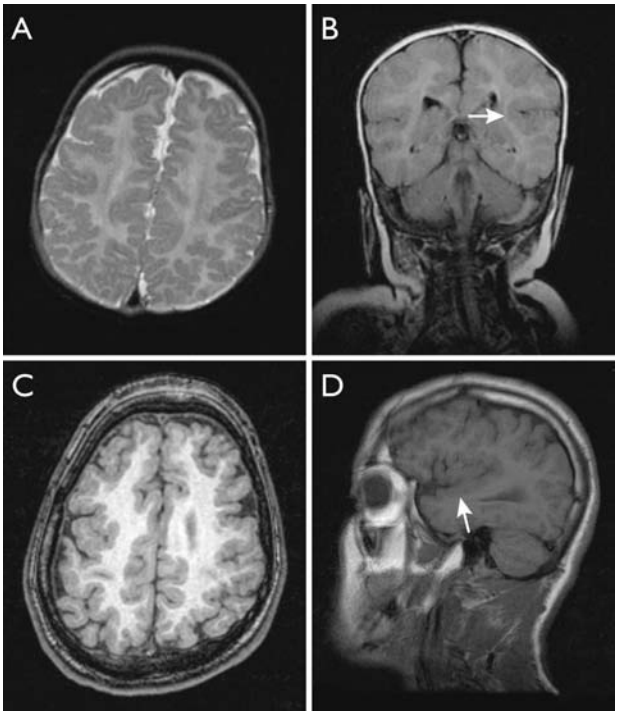

Figure 2

Representative MRIs of patient VI-1 (*panels* A and B) and patient VI-10 (*panels* C and D) showing BGP. *A*, Axial T2- weighted MRI of patient VI-1 at age 5 mo, showing frontoparietal and occipital bilateral PMG. *B*, Coronal FLAIR image of the same patient showing perisylvian PMG (*arrow*). *C*, T1-weighted axial image of patient VI-10 at age 14 years, showing BGP with distribution similar to that seen in patient VI-1. *D*, Parasagittal T1-weighted image of patient VI-10, showing PMG of the right peri-insular temporal lobe.

Genomic DNA was isolated from peripheral leukocytes by use of the method described by Miller et al. [12]. By haplotyping, FISH, and sequence analyses, we excluded the *ZFHX1B* gene as a cause of GOSHS in this family (data not shown). For the systematic genome scan, 381 markers (STRPs) from the ABI Prism Linkage Mapping Set MD-10 (version 2.5), with an average spacing of 10cM, were tested. DNA amplification was performed using 25 ng genomic DNA in 7.5-μl PCRs containing 1xPCR Gold Buffer, 2.5 mM $MgCl_2$, 10 μM primer pair mix, and 0.4 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems). Amplification conditions were 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min 30 s at 72°C; amplification was ended by a final extension for 5 min at 72°C. PCR products were pooled in panels and loaded on an ABI 3100 automated sequencer. Data were analyzed using GeneMapper software (version 2.1) (Applied Biosystems).

After this initial screening a region on 10q21.3-q22.1 was further investigated, since one of the four affected individuals was homozygous by descent for two consecutive markers, *D10S1652* and *D10S537*, whereas the other three patients were homozygous for one of these two markers. No other regions of homozygosity for consecutive markers were identified. We saturated the region between markers *D10S1652* and *D10S537* (12.3 cM) with 11 additional markers; they either were obtained from the Marshfield genetic map (Center for Medical Genetics Website) or were newly developed. Marker order and genetic distances were obtained from the Marshfield genetic map (Center for Medical Genetics Website) and the Celera physical map. All patients were homozygous

for 7 of these 11 markers. A recombination event between loci *CGR166* and *CGR167* in patient VI-7 defined the centromeric boundary of the genetic interval. The telomeric border was defined by recombination events between markers *D10S1665* and *CGR170* in patients VI-1 and VI-3 (fig 1). These recombination events reduced the candidate region to a minimum of 2.8 Mb and a maximum of 3.8 Mb.

Parametric two-point and multipoint linkage analyses were performed using MLINK program from the LINKAGE (version 5.1) software package [13] and SimWalk2 program (version 2.9) [14]. LOD scores were calculated, under the assumption that the disease phenotype is a rare autosomal recessive disorder with 100% penetrance, with a gene frequency of 1:5000. No phenocopies were allowed, and equal allele frequencies were used because of the limited number of available independent family members. A significant two-point LOD score was obtained (maxLOD score 3.29 and $\theta=0$ for marker *CGR167*) (table 1); which increased to a maximum multipoint LOD score of 5.9 between markers *CGR167* and *D10S1647* (fig 3).

Table 1

Results of two-point LOD score analysis, performed for markers used in fine mapping

	LOD at $\theta=0$						
Marker[a]	0	.01	.05	.1	.2	.3	.4
D10S196	-13.81	-3.96	-1.52	-.65	-.08	.02	0
D10S1652	-6.96	-2.39	-1.85	-1.36	-.68	-.35	-.15
D10S1743	-4.97	1	1.39	1.31	.9	.46	.14
CGR166	-3.7	.58	1.03	1.02	.73	.4	.13
CGR167	3.29[b]	3.21	2.9	2.51	1.73	1	.4
D10S210	2.59	2.53	2.26	1.92	1.26	.67	.24
D10S1678	1.49	1.45	1.29	1.09	.72	.4	.16
D10S1647	1.84	1.79	1.58	1.33	.86	.47	.19
D10S1672	2.88	2.81	2.52	2.15	1.42	.76	.25
D10S1665	2.77	2.7	2.43	2.08	1.39	.75	.25
CGR172	1.36	1.32	1.17	.97	.62	.32	.11
CGR170	-.28	1.59	1.97	1.88	1.4	.83	.33
D10S676	-.28	1.25	1.64	1.56	1.1	.59	.18
D10S537	-.11	1.45	1.82	1.71	1.2	.65	.21

[a] Marker order follows that of the Celera physical map.

[b] Max LOD score.

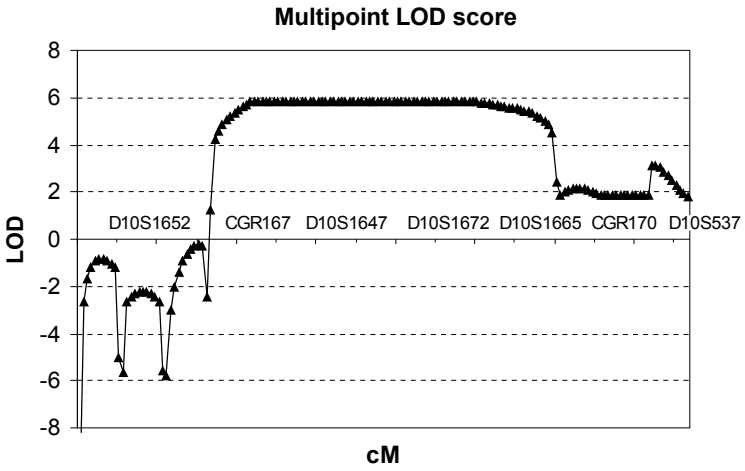

Figure 3

Multipoint LOD score analysis across and beyond the region of homozygosity, flanked by markers *D10S1652* and *CGR170*, with a peak LOD score of 5.9.

The maximum 3.8-Mb linked region of shared homozygosity (defined by *CGR166* and *CGR170*) contained in total 25 known genes and 5 genes encoding hypothetical proteins, in accordance with the National Center for Biotechnology Information (NCBI) build 34 of the human genome and the Ensemble Genome Browser. We first sequenced seven functional candidate genes on the basis of their putative involvement in neurogenesis (*CTNNA3, LRRTM3, ATOH7, DDX50, DDX21, NET-7 and NEUROG3*), either using cDNA obtained by RT-PCR from a lymphoblastoid cell line of patient VI-10 and from a control or using genomic DNA from both. Mutations were not identified. We then performed a systematic mutational screening of all transcripts from the candidate region. PCR products from either genomic DNA (exons including flanking intronic sequences) or reverse-transcribed products (ORFs) were amplified and purified (ExoSapit [USB]). PCR primers, which were designed using standard software (Primer3), and conditions are available on request. Bidirectional sequencing was performed using BigDye Terminator chemistry on an ABI 3100 sequencer (Applied Biosystems). With the use of the software package SeqScape (version 2.1) (Applied Biosystems), sequences were aligned and compared with consensus sequences.

Analysis of 98% of all coding sequences from the region revealed one likely disease-causing homozygous nucleotide substitution, 303C→T, in exon 1 of the *KIAA1279* gene (fig 4*A*). This transversion leads, at the amino acid level, to a replacement of an arginine by a stop codon (R90X), resulting in a shortened protein of 89 amino acids. The 303C→T mutation showed complete segregation with the disease in the family. One hundred ethnic matched control chromosomes (of North African origin) were screened for the R90X mutation, and none of the controls carried this mutation. Subsequently, one additional family of British Pakistani ancestry, with a similar phenotype to that found in the Moroccan family, was screened for mutations in this gene. This family had four affected individuals with multiple inbreeding loops. Clinical characteristics were consistent with GOSHS, and CT brain scans of this family were described in a previous study and were thought to represent an abnormality of neuronal migration [3]. In the affected individuals, a homozygous G→T transversion was identified at nt 285, resulting, at the amino acid level, in the replacement of a glutamic acid with a stop codon (E84X)(fig 4*B*). Two different homozygous nonsense mutations in *KIAA1279* in two independent families with GOSHS strongly indicate that *KIAA1279* is the gene responsible for GOSHS.

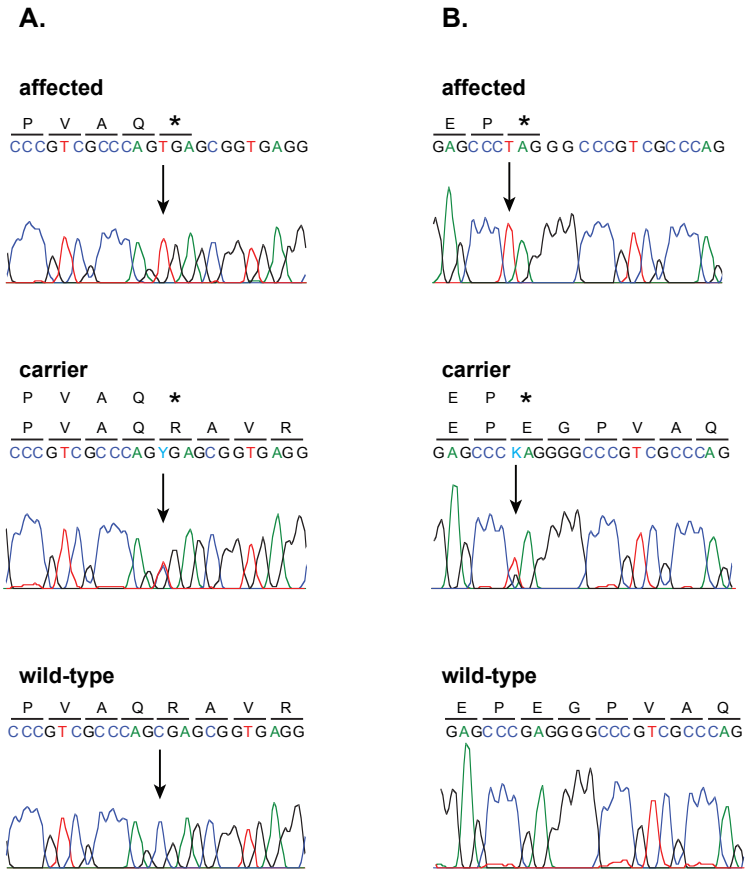

Figure 4

Mutations of *KIAA1279* associated with GOSHS. *A*, Electropherogram of the mutation identified in the Morrocan family. The 303C→T mutation causes the replacement of an arginine with a stop codon (R90X) in exon 1. *B*, Electropherogram of the mutation identified in Pakistani family. A homozygous G→T transversion at nt 285, results in the replacement of a glutamic acid with a stop codon (E84X) in the patients.

KIAA1279 consists of seven exons spanning 28 kb of genomic DNA. The transcript encodes a protein of 621 aa. We identified orthologs of human KIAA1279 in fruit fly, frog, rat, mouse, bee, chicken, and Japanese puffer fish, using BLAST comparison (NCBI Web site). There is strong sequence conservation during evolution; the protein sequence of human KIAA1279 shares 89% amino acid identity with mouse product, 88% with rat product and 29% with fruit fly product. Multitissue northern blots (both adult and fetal) (fig 5) showed ubiquitous expression of the 2.4-kb *KIAA1279* mRNA without a specific pattern, with expression lacking only in blood leukocytes (fig 5*A*). In addition, northern blot hybridization identified *KIAA1279* mRNA molecules in different parts of the adult CNS,-namely cerebellum, cortex, medulla, spinal cord, occipital lobe, frontal lobe, temporal lobe, and putamen (fig 5B).

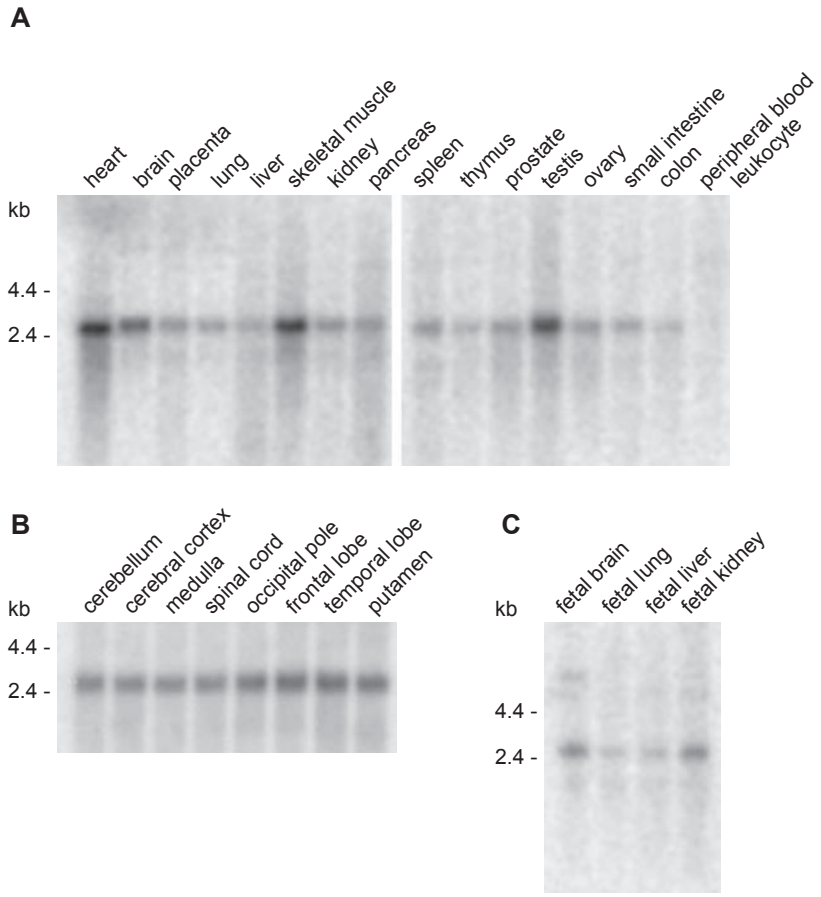

Figure 5

Expression of *KIAA1279* in human adult and fetal tissues. *A*, Multi-tissue northern blots, incubated with a *KIAA1279* probe, showing ubiquitous expression without a specific pattern (note the lack of expression in blood leukocytes). *B*, Northern blots from adult brain tissues, incubated with a *KIAA1279* probe. *C*, Human fetal multi-tissue northern blot, showing widespread expression.

This widespread *KIAA1279* expression is consistent with data available in gene expression databases such as the Gene Expression Omnibus, the human Unidentified Gene-Encoded (HUGE) Protein Database, and the University of California-Santa Cruz (USCS) Genome Browser. The function of KIAA1279 is unknown. Neither the gene nor its derived protein shows any significant sequence similarity to known human cDNA or protein sequences. Possible aspects of the molecular function of KIAA1279 may be gleaned from its primary sequence. We predicted protein motifs and domains using the program SMART (Simple Modular Architecture Research Tool). The predicted protein contains two tetratrico peptide repeats (TPRs), which are structural motif consisting of 34 amino acid residues, in exon 1/2 and exon 4, respectively. Because of these repeats KIAA1279, may be considered a member of the TPR protein family [15]. The most basic function of TPR motifs is to mediate protein-protein interactions. Proteins with TPR motifs are involved in a variety of biological processes, such as cell-cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding. Several diseases have been described as being caused by mutations in genes encoding proteins containing TPR domains- for example,

Leber congenital amaurosis (*AIPL1*) [16] and Charcot-Marie-Tooth type 4C neuropathy (*KIAA1985*) [17].

Since mutations in *KIAA1279* are associated with HSCR and PMG, its protein product may play a pivotal role in both peripheral and central nervous system development. PMG is traditionally classified as a result of a disruption such as fetal hypoxic-ischemic damage, infection or exposure to toxic drugs in the second trimester (13-21 weeks) [18, 19]. However, the identification of *KIAA1279* as the GOSHS gene underlines the importance of genetic factors in the etiology of this neuronal migration disorder of the cortex, as was already proven [20 21] or suggested [22 23] for other forms of PMG. Under the assumption that the main function of KIAA1279 is to bind other proteins, these target peptides might be (known) HSCR- or PMG- associated susceptibility factors. More likely, however, these targets might play a more general role in neuronal development, since a malformation of cortical organization is a constant feature in the Moroccan family, whereas HSCR is a variable feature. Elucidation of KIAA1279 function, and more important, studies of *KIAA1279* expression and protein interactions may provide new insight into the molecular basis of PMG and HSCR.

Acknowledgments

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Web resources

Accession numbers and URLs for data presented herein are as follows:

Celera, http://www.celeradiscoverysystem.com/
Center for Medical Genetics, Marshfield Clinic Research Foundation, http://research.marshfieldclinic.org/genetics/
Ensembl Genome Browser, http://www.ensembl.org/
GenBank, http://www.ncbi.nlm.nih.gov/GenBank/ (for *CGR170* [accession number BV212295], *CGR167*[accession number BV 212296], *CGR166* [accession number BV212297], D10S196 [accession number Z16598], D10S1652 [accession number Z52339], D10S1743[accession number Z53951], D10S210 [accession number Z16813], D10S1678 [accession number Z52660], D10S1647 [accession number Z52188]
Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/
Human Unidentified Gene-Encoded Proteins database, http://www.kazusa.or.jp/huge/
NCBI, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/OMIM/(for GOSHS and MWS)
Primer3,http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi
SMART,http://smart.embl-heidelberg.de/.
UCSC Genome Browser, http://genome.ucsc.edu/.

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CLINICAL AND MOLECULAR STUDIES IN GOLDBERG-SHPRINTZEN SYNDROME

Clinical and molecular studies in Goldberg-Shprintzen syndrome

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Manuscript in preparation

Abstract

In order to establish the phenotypic spectrum of Goldberg-Shprintzen syndrome (GOSHS), we studied 3 new GOSHS families including the original siblings described by Goldberg and Shprintzen. GOSHS is characterized by Hirschsprung disease, mental retardation, microcephaly, and a typical facial dysmorphism. MRI studies showed polymicrogyria in the proband of one of these families, and a simplified gyral pattern in one of the original GOSHS patients, indicating that malformations of cortical development are part of the clinical spectrum of GOSHS.

In all three families novel truncating recessive *KIAA1279* mutations were identified. To study the involvement of *KIAA1279* in phenotypes related to GOSHS, we also screened 14 individuals (12 sporadic cases, and 2 sibs) that were initially considered as possible Mowat-Wilson syndrome cases, a related syndrome with Hirschsprung disease-mental retardation-microcephaly, in whom mutations of the Mowat-Wilson syndrome gene *ZFHX1B* had been excluded. Furthermore, we studied *KIAA1279* in a group of 26 sporadic, non-syndromic Hirschsprung disease cases, and 6 cases with isolated diffuse polymicrogyria. In none of these 3 groups, however, *KIAA1279* mutations could be identified, indicating that *KIAA1279* mutations specifically lead to GOSHS. Finally, additional genes for the combination of Hirschsprung disease with mental retardation and microcephaly are likely to exist, since not all cases can be attributed to mutations in either *KIAA1279* or *ZFHX1B*.

Introduction

Goldberg-Shprintzen syndrome or Goldberg-Shprintzen megacolon syndrome (GOSHS) (MIM 609460), is a rare autosomal recessive syndrome with 6 families described in the literature [1-6]. GOSHS presents with Hirschsprung disease (HSCR), microcephaly and moderate mental retardation [1, 2]. Distinctive associated facial features include high-arched eyebrows, synophrys, hypertelorism, cleft palate/bifid uvula, and clouding of the cornea related to a primary hypoesthesia of the cornea [1, 6]. Furthermore, an occasional patient with GOSHS is diagnosed with a congenital heart defect [5], or develops a progressive scoliosis starting in late childhood or early adolescence [1, 6]. The eldest patients described in the literature are the 2 original cases reported by Goldberg and Shprintzen (respectively 13.5 years old and 10 years old at the time of publication), and case V.6 (age 12 years) previously reported by us in a Moroccan family.[1, 6] HSCR proved to be a variable feature in GOSHS patients [5, 6]. Both short segment HSCR and long segment HSCR have been described in GOSHS [6]. Most patients with documented brain MRI were diagnosed with polymicrogyria (PMG) [7], and this clinical feature might be a key feature of GOSHS. Recently we identified the gene causing GOSHS as *KIAA1279*, a gene located at 10q22.1 and encoding a hypothetical protein with 2 tetratrico peptide repeats. GOSHS patients were shown to have inactivating homozygous *KIAA1279* mutations [7].

GOSHS shows some clinical overlap with Mowat-Wilson syndrome (MWS) (OMIM 235730) [8], which is characterized by uplifted ear lobes, hypertelorism, a pointed chin and is associated with *de novo* mutations in *ZFHX1B* located at 2q22 [9] [10-13]. Recently, however, sib recurrence is described in MWS consistent with a germline mosaicism in one of the parents [13, 14]. The clinical distinction between these two entities might be difficult in the young sporadic HSCR patient with microcephaly and mental retardation; several MWS cases have been initially misdiagnosed as GOSHS [15-18]. Most typically the shape and position of the eyebrows differ; i.e. horizontal, wedge-shaped eyebrows in MWS [11] and arched eyebrows that may cross the midline (synophrys) in GOSHS [1 2, 6].

To further delineate the phenotypic spectrum of GOSHS and to study which phenotypic features are associated with mutations in *KIAA1279*, we analysed 5 patients (from 3 families) with

clinical features of GOSHS, including the 2 original sibs described by Goldberg and Shprintzen in 1981[1]. Furthermore, we studied a group of 14 cases with presumptive MWS, 26 sporadic HSCR patients, and 6 cases with bilateral diffuse polymicrogyria, in order to assess the involvement of *KIAA1279* in these cases.

Patients and Methods

GOSHS patients

Three families were considered as having GOSHS. The first family was the original family with GOSHS consisting of 2 affected sibs (US1 and US2) reported by Goldberg and Shprintzen [1]. Two additional GOSHS families were selected that were not described previously. As major inclusion criteria for GOSHS in our study the following features were used: i) HSCR of variable length, ii) facial dysmorphism (high arched eyebrows, synophrys, a high nasal bridge, hypertelorism), iii) small head circumference or microcephaly (defined as an occipito-frontal circumference or OFC< 3rd percentile), and iv) psychomotor retardation (Table 1).

DNA was available from 5 patients; from family 1 (US1 and US2), family 2 (CYP1), and family 3 (UK1 and UK2). Informed consent was obtained from the parents of the patients.

Table 1. Most prominent features of the three new GOSHS families, and the two previously described families with documented *KIAA1279* mutations

Family	Patient	Sex	MR	Micro Cephaly	Seizures	HSCR	Facial dysmorphism	Other	Ref
1	US1	M	+	+	-	+	+	Scoliosis, simplified gyral pattern	[1],This study
1	US2	F	+	+	-	+	+	Scoliosis, corneal transplants, cleft palate	[1],This study
2	CYP1	M	ND	+	-	+	+	diffuse PMG	This study
2	CYP2	F	+	ND	-	-	+	Scoliosis, leg muscle weakness	This study
3	UK1	M	+	+	+	+	+		This study
3	UK2	M	+	+	-	+	+	ASD, submucous cleft palate	This study
4	Case 1	M	+	+	-	+	+	Scoliosis	[2]
4	Case 2	M	+	+	-	+	+	Iris coloboma	[2]
4	BP1	F	+	+	-	+	+	VUR	This study
4	BP2	F	+	+	-	-	+		This study
5	V-4	M	ND	-	-	+	+		[6]
5	V-6	F	+	+	-	+	+	Scoliosis, corneal transplants, diffuse PMG	[6]
5	V-9	M	+	+	-	-	+	Scoliosis, corneal transplants, bifid uvula, diffuse PMG	[6]
5	VI-1	F	+	+	-	+	+	Diffuse PMG	[6]
5	VI-3	F	+	+	-	+	+	Difuse PMG	[7]

ND: not documented. MR: psychomotor retardation; HSCR: Hirschsprung disease; PMG: polymicrogyria; VUR: vesicoureteral reflux

MWS-like patients

We also sequenced the *KIAA1279* gene in 14 cases (8 males, 6 females) which were considered as presumptive/possible MWS cases. This included 4 patients previously described by Zweier et al. as belonging to either group 2 having an ambiguous facial Gestalt of Mowat-Wilson syndrome (patients Erl1and Erl7), or to group 3 displaying a non-specific facial phenotype (patients Erl2 and Erl4) [13]. The remaining 10 MWS-like cases are unpublished, but could be classified in either of these 2 groups. All were mentally retarded, and 9 out of 14 had HSCR (Table 2). These 14 MWS-like patients were found to be negative for whole gene deletions or intragenic mutations in *ZFHX1B*.

Table 2. Overview of the *ZFHX1B*-negative Mowat-Wilson-like syndrome and polymicrogyria cases analysed in this study

MWS like patients	Sex	MR	Micro cephaly	Seizures	HSCR	PMG	Other
Erl1	M	+	+	+	+		congenital heart defect, cerebral atrophy, fetal finger pads
Erl2	F	+	-	-	+		cleft palate
Erl3	M	+	+	ND	+		polydactyly
Erl4	M	+	+	-	+		
Erl5	M	+	+	+	ND		sibs, consanguineous parents, agenesis of the corpus callosum, optical and cortical atrophy
Erl6	F	+	+	+	ND		
Erl7	M	+	-	+	+		3 further affected sibs; 1 with HSCR
Erl8	M	+	ND	ND	+		abnormal genitals
Erl9	F	+	-	ND	+		renal anomaly
Erl10	F	+	+	+	-		agenesis of the corpus callosum
SB	M	+	+	-	+		CHD, broad gyri of the frontal cortex
SF	F	+	+	+	-		constipation
MS	F	+	+	-	-		agenesis of the corpus callosum
KAL	M	+	ND	-	+		unilateral cleft lip/palate
PMG1	F	+	-	+	-	fronto-parietal	spasticity, affected brother
PMG2	F	+	-	+	-	biparietal	hypotonia, consanguineous parents
PMG3	M	+	-	+	-	bilateral parieto-occipital	spasticity, consanguineous parents
PMG4	M	+	-	-	-	closed lip schizencephaly	spasticity
PMG5	F	+	-	+	-	bilateral diffuse	nystagmus, spasticity, kyfoscoliosis
PMG6	M	+	+	-	-	biparietal	strabismus

ND: not documented.

MR: psychomotor retardation; HSCR: Hirschsprung disease; PMG: polymicrogyria; CHD: congenital heart defect

Table 3. PCR primers and conditions for amplification of *KIAA1279* exons

Primer Sequences		bp	Size(bp)	Tm °C	Exon	seq. primer
KIAA1AF	CAACGTGTCGAGAGCCGTAA	20	424	58	1	1AF
KIAA1AR	CTCCGTGTCGATGTGGTTCA	20				
KIAA1BF	TCCGGAGAAGGAACCATACA	20	446	58	1	1BF
KIAA1BR	GAATTGCGCCTTACAACGTC	20				
KIAA2F	CTGCACTACATAAGGTCAAT	20	355	58	2	2F
KIAA2R	AGTGAAGAGTCACACCAATA	20				
KIAA3F	TTGGCTTGTAGAAGTTGA	18	277	52	3	3F
KIAA3R	GAATCCACATTCTATAACC	19				
KIAA4F1	GCCTGACTATTATGGTGAA	19	479	58	4	4R1
KIAA4R1	CTCACTATTGGTACTCTGTA	21				
KIAA5F	TTCTGAATGATCTGCAACTC	20	328	58	5	5F
KIAA5R	CTCTTCTGTTCTTGCAACTT	20				
KIAA6F	ACAGTTGTGACCATTAACTT	20	281	58	6	6R
KIAA6R	ACATTCTACACACCTTTCAT	20				
KIAA7AF	GGAGGTGAGTGTCCTACTTA	20	423	58	7	7AR
KIAA7AR	GCTCTAGCATGGCTATTCTG	20				
KIAA7BF	ACCACAGTGCTCTGTTTAAGG	21	427	58	7	7BF
KIAA7BR	TCCAGCTCTTTCTTGGGATCT	21				
KIAA7CF	AGACCCAAATAAATGTATTCC	20	390	58	7	7CF
KIAA7CR	CTGGCTATAAAGGTAAACAT	20				

F= forward, R=reverse

Sporadic HSCR patients and PMG cases

Twenty-six sporadic HSCR patients (23 with short-segment aganglionosis and three with unknown length of aganglionosis) and six cases (PMG1-PMG6) with bilateral polymicrogyria and suggestive autosomal recessive inheritance (Table 2) were also screened for mutations in *KIAA1279*. In the latter PMG group microdeletions of 22q11 [19] were excluded.

Molecular analysis of KIAA1279

Genomic DNA was isolated from peripheral leukocytes by the use of standard methods. Sequencing of exons 1-7 of *KIAA1279* was performed using 9 sets of primers (Table 3). DNA amplification was performed using 25-100ng genomic DNA in 30 µl PCRs containing 10X reaction Buffer, 8 µM primer pair mix, and 1 U Taq Polymerase (Amersham). PCR products were amplified and purified (Multiscreen PCR u96 Filter Plate -Millipore-). Unidirectional sequencing was performed using BigDye Terminator chemistry on an ABI 3100-16 capillary sequencer (Applied Biosystems). Using the software package SeqMan II (Version 5.07, DNASTAR) DNA sequences were aligned and compared with consensus sequences.

Results

Clinical data

Family 1

Patients US1 and US2 were previously reported by Goldberg and Shprintzen as the first patients with GOSHS [1]. These sibs manifested an unusual facies (microcephaly, fine scalp hair, apparent hypertelorism, synophrys, thick curly eyelashes, prominent nose, and maxillary hypoplasia), neonatal hypotonia, short stature, HSCR, and a submucous cleft palate.

Both siblings are now adults: US1 is 40 and US2 is 37 years old. They both had surgery for severe scoliosis. Furthermore, US 2 had corneal transplants because she suffered from recurrent corneal infections. Revision of MRI of the brain of US1, performed at the age of 37 years because of abnormal gait and lack of balance, showed a simplified gyral pattern and diffuse white matter loss. Unfortunately, the quality of the MRI was insufficient to rule out PMG.

Family 2

Family 2 is originally from Cyprus. The family resides in Australia, and the parents, although not known to be consanguineous; both originate from the same village in Cyprus. Their first child, a girl is healthy. The male proband (CYP1) in this family, is their second child. The pregnancy of the index patient was complicated by hyperemesis, and was induced at term resulting in a normal vaginal delivery. This infant's birth weight was 3100 g, OFC 33 cm (< 10th percentile). The finding of delayed passage of meconium led to the suspected diagnosis of HSCR. A rectal biopsy done on day 4 was consistent with the diagnosis of HSCR. On examination he was hypotonic, with severe progressive microcephaly with an OFC of 33.1 cm at 4 months (<< 3rd percentile). He had dysmorphic features including: a low anterior hairline, overriding sutures, open mouthed posture, arched and prominent eyebrows, long, thick and curled eyelashes, prominent nasal bridge, a pointed chin, and long, slender fingers and toes (fig 1).

Microcephaly and irregularity of the cortical grey/white matter junction were seen on cerebral MRI consistent with polymicrogyria, involving the entire hemispheres with sparing of the inferior frontal lobes, inferior portion of the Sylvian fissure and the medial lobes. A skeletal survey was normal. A mild pelvicalyceal dilatation was reported on ultrasound. Conventional karyotyping, subtelomeric FISH, and FISH for the MWS region, were normal. Mutation analysis of the *ZFHX1B* gene by direct sequencing was negative. Given the child's poor prognosis and his expected severe neurodevelopmental delay, a joint medical and parental decision was made for conservative care of HSCR. He died at 6 months of age. The family history is of particular relevance. A female second cousin (CYP2) of the mother of the proband suffers from chronic constipation, mental retardation, scoliosis, and leg muscle weakness. Moreover, a brother of CYP2 died at 2 months of age after complicated surgery for HSCR. Further clinical details of this boy are lacking. Review of the photographs of CYP2 confirmed that this girl has GOSHS. The medical history of her brother is also suggestive for GOSHS.

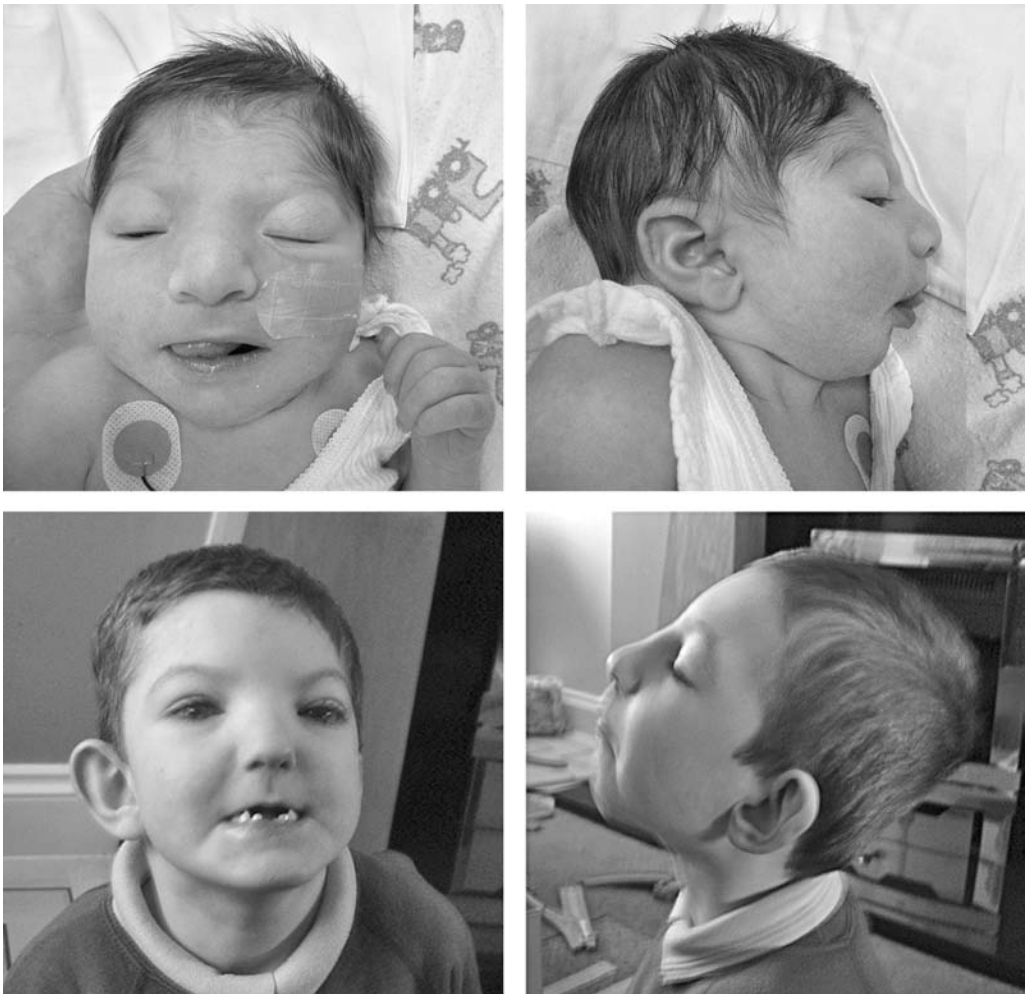

Figure 1

CYP1 as a neonate**.** Note the high-arched eyebrows, and microcephaly.

UK2 at age 5 years. Note the high- arched eyebrows.

UK1 at age 7 years. Note the high-arched eyebrows, and the high nasal bridge.

Family 3

In the third Caucasian family, 2 sons (UK1 and UK2) of a non-consanguineous couple of British ancestry were diagnosed with HSCR. For an extensive clinical description see Murphy et al. (submitted). UK1 is now 8 years of age (fig 1). Passing of meconium was delayed and he developed a lower bowel obstruction within 48 hours after birth, which was suggestive of HSCR. Colonic biopsies were consistent with long-segment HSCR. His OFC at birth was < 3rd percentile. He currently is microcephalic (OFC < 3rd percentile), whereas his height and weight are normal (25th-50th percentile). On examination he was noted to have high-arched eyebrows, down slanting of the palpebral fissures, bilateral ptosis, prominent ears, sloping forehead, a prominent nose, and a

short philtrum. At age of 12 months, his psychomotor development was delayed and equivalent to a chronological age of 6 months. His motor milestones were delayed: he sat at 13 months, walked independently at 4 years, and began to talk at 2 years and 10 months of age. At age of 71/2 years, his development is moderately retarded. Brain MRI at the age of 2 years, performed after he had an absence seizure, was reported as normal. UK2, a 6-year-old boy (fig 1), is the second child of this British couple. He was born after an uneventful pregnancy. His birth weight was 3120 g (10^{th}-25^{th} percentile); OFC was 31 cm (< 3^{rd} percentile). Failure to feed and vomiting were indications for transfer to the pediatric surgical unit. Short segment HSCR was diagnosed; he had a temporary colostomy at 2 days of age, and intestinal reconstruction at the age of 2 months. He was noted to have an ASD and a submucous cleft palate. At age 7 months, he manifested a flat occiput and a remarkable physical resemblance to his elder brother; he has high-arched eyebrows, curled eyelashes, large anteverted ears and a prominent nose. His psychomotor development is markedly delayed; he sat at 12 months, and walked at 22 months of age. At 51/2 years of age, he is only able to follow simple verbal instructions. Brain MRI at age 7 months, showed asymmetrical lateral ventricles, but was not conclusive for a malformation of cortical development. Neither corneal hypoesthesia nor recurrent corneal infections were reported. Both boys have a normal 46, XY, male karyotype. FISH analysis of the *ZFHX1B* region showed no submicroscopic deletions.

Family 4

Previously we identified a homozygous mutation in the *KIAA1279* gene [7], in the family described by Hurst and others [2]. Clinical follow-up of this British-Pakistani family revealed that 2 additional affected children were born in this family. A sister (BP1) of case 1 has typical facial characteristics (ptosis, a prominent nasal bridge, a high arched palate), microcephaly (OFC< 3^{rd} centile) and was diagnosed with HSCR. A sister of case 2 (BP2) has the typical GOSHS Gestalt (prominent nasal bridge, high arched eyebrows), is microcepalic and also exhibits developmental delay, but she did not have HSCR. Case 1 was noted to have a clumsy, slightly broad base gait and he appeared slightly ataxic. He died at the age of 20 years because he had respiratory failure as a consequence of a progressive scoliosis.

Molecular analysis of KIAA1279

Table 4 summarizes the *KIAA1279* mutations found in this and in our previous study.

In family 1, a homozygous insertion of an A at nucleotide 1551 in exon 7 of *KIAA1279* leading to a frameshift and a premature stop codon was found in US1 and US2. The parents were both heterozygous carriers of this mutation.

In family 2, a G→T substitution at nt 753 in exon 4 was identified that results at the protein level in the replacement of a glutamic acid with a stop codon (E240X). This nonsense mutation was found homozygous in CYP1, whereas the parents of CYP1 were both heterozygotes.

In family 3, the boys (UK1 and UK2) and their father are heterozygote for an insertion of an A at nt 1117, leading to a frame shift with a premature stop. We have not identified a second mutation so far but analysis is on-going.

In family 4, the British-Pakistani family described by Hurst et al. [2], we previously identified a homozygous G→T transversion at nt 285, resulting, at the amino acid level , in the replacement of a glutamic acid with a stop codon (E84X).

In family 5 (the Moroccan family) a homozygous nucleotide substitution 303C→T, leading to the

replacement of an arginine with a stop codon at the amino acid level (R90X) was identified [7]. All mutations identified so far are inactivating

No *KIAA1279* mutations were identified in 26 HSCR cases, in 6 cases with PMG and in the group of possible MWS-like cases.

Table 4. *KIAA1279* mutations in GOSHS patients from this study and the literature

Mutation	Type	Exon	Family	Reference
c.1551-1552insA	Frameshift	7	Family 1(US) with 2 affected sibs	This study
c.753G>T	Nonsense	4	Family 2 (CYP) with 3 affected probands	This study
c.1117-1118insA	Frameshift	7	Family 3 (UK) with 2 affected sibs	This study
c.285G>T	Nonsense	1	Family 4 (British-Pakistani family) with 4 affected patients	[2, 7]
c.303C>T	Nonsense	1	Family 5 (Moroccan family) with 5 affected patients	[6, 7]

Discussion

Clinical spectrum

GOSHS is a clearly defined clinical entity, and although rare, it can be recognized in children from different geographic regions and ethnic backgrounds. Moderate mental retardation and microcephaly (which can be progressive as in case CYP1 of family 2 and patient V.6 in family 5 (Moroccan family [6]) are obligate (Table 1). The facial Gestalt is discrete and consists of high arched eyebrows, curled eyelashes, and a prominent nose. HSCR of variable length occurred in all 5 families, but not every GOSHS case had HSCR: CYP2 in family 2 suffered from constipation, but was never diagnosed as having intestinal aganglionosis. In total 13/16 (81%) affected cases in these 5 families had HSCR (Table 4). Therefore, HSCR is a variable and not a mandatory feature in GOSHS. This is consistent with earlier observations [5]. Possibly, like in MWS, also in GOSHS the actual frequency of HSCR is lower than presented here, as a selection bias in recognizing families with HSCR might have occurred [13].

Diffuse PMG could be part of the core phenotype, but this feature is likely to be underdiagnosed because of the lack of detailed MRI studies in many cases. So far in 4/5 families with mutations in *KIAA1279*, brain MRI studies were performed. In 2 families (family 2 and family 5) PMG was diagnosed [7] , whereas in the other 2 families (family 1 and family 3) PMG was clinically suspected, however revision of the available MRIs was inconclusive. Importantly, the MRI studies of the eldest GOSHS patient, US1 of family 1, showed a simplified gyral pattern and a diffuse white matter loss. Although a simplified gyral pattern is actually classified as a defect of cortical proliferation, distinct from PMG (often classified as a postmigratory defect) [20] , its presence suggests that malformations of cortical development represent core features of GOSHS, and underlie the obligate microcephaly.

We, therefore, recommend that every child with HSCR and mental retardation and/or microcephaly is referred to a pediatric neurologist and that a MRI of the brain is performed. Furthermore, MRI studies in adolescent and adult GOSHS cases are needed to study whether progressive white matter loss is part of the GOSHS phenotype.

Additional features present in some but not all of the patients include corneal infections requiring corneal transplants (present in 2 cases from family 5 -the Moroccan family- and in US2 from family 1 at an older age), and progressive scoliosis (present in all children who reach adolescence). This progressive scoliosis was associated with pulmonary failure and early death in 2 cases (case V.6 of family 5 died at 15 years, and case 1 of family 4 -the British-Pakistani family- died at age 20 years, respectively) [2, 6]. We underlined earlier that neurological and ophthalmologic complications may develop at an elder age in GOSHS. Follow-up of the original 2 cases reported by Goldberg and Shprintzen and the cases described by Hurst confirmed this observation [1, 2, 6]. These 2 features warrant clinical follow-up by a neurologist and an ophthalmologist. Well-timed surgery to correct the scoliosis might improve the prognosis.

Mutational spectrum

We have identified 12 patients from a total of 5 unrelated pedigrees with *KIAA1279* mutations to date. The pattern of transmission observed is consistent with an autosomal recessive trait, with occurrence in both sexes and consanguinity in 2/5 families (families 4 and 5, and possibly in family 2 originating from Cyprus). We identified in total 5 different mutations in the *KIAA1279* gene, 4 of which were homozygous. These include 3 nonsense (family 2, 4 and 5), and 2 frame shift mutations (family 1 and 3). In family 3 (the UK family) we did not identify a second mutation; one possible reason is that a large deletion (>100 bp) could have been overlooked using a mutational scanning method with PCR as a first step. Comprehensive mutation analysis should not only include screening of the coding sequences, intron-exon boundaries, and promoter sequences, but also scanning for large deletions. Therefore, quantitative PCR studies will be performed in family 3.

All mutations are inactivating indicating that loss of function of *KIAA1279* leads to GOSHS. Non-syndromic HSCR and PMG unrelated to GOSHS are not caused by mutations in this gene.

Finally, since not all HSCR cases associated with microcephaly and mental retardation can be attributed to mutations in either *KIAA1279* or *ZFHX1B,* there must exist further genetic heterogeneity [11].

Acknowledgments

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CHAPTER 5

TWO SIBS WITH HIRSCHSPRUNG DISEASE AND OVERLAPPING PHENOTYPES OF MCKUSICK-KAUFMAN SYNDROME AND BARDET-BIEDL SYNDROME

Two sibs with Hirschsprung disease and overlapping phenotypes of McKusick-Kaufman syndrome and Bardet-Biedl syndrome

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Submitted

Abstract

We describe a patient with hydrometrocolpos, postaxial polydactyly of the feet, subvalvular pulmonary artery stenosis, dysplastic kidneys, and short-segment Hirschsprung disease (HSCR), in which the presumptive diagnosis of McKusick-Kaufman syndrome was made. Her brother was diagnosed with Bardet-Biedl syndrome based on the combination of four limb postaxial polydactyly, hypogenitalism, cystic dysplastic kidney changes and long-segment HSCR.

Sequencing 3 of the 9 *BBS genes* (*BBS1, BBS2,* and *MKKS*/*BBS6*) did not reveal any mutations. Mutation scanning of *RET*, the major gene in HSCR, revealed a sequence variant Lys716Asn in exon 12 in the affected girl, but not in the affected brother. It is uncertain whether this variant contributes to HSCR in this family. The existence of a gene causing the combination of BBS/MKKS and HSCR can however not be excluded.

Introduction

McKusick-Kaufman syndrome (MKKS, OMIM 236700) and Bardet-Biedl syndrome (BBS, OMIM 209900) are rare autosomal recessive syndromes with overlapping clinical and molecular spectrum. Hydrometrocolpos is regarded as a primary sign in MKKS. Other hallmarks of MKKS are kidney abnormalities (hydronephrosis secondary to hydrometrocolpos, primary cystic kidney disease) postaxial polydactyly and congenital heart disease (predominantly septal defects) [1].

BBS is characterised by rod-cone dystrophy, postaxial polydactyly, central obesity, mental retardation, hypogonadism, and renal dysfunction [2].

Since hydrometrocolpos may occur as a neonatal manifestation of BBS syndrome [3 4], while the main clinical features such as retinitis pigmentosa and obesity may not be apparent in infancy and early childhood [5], no definite distinction between BBS and MKKS can be made on clinical grounds in a young child. Phenotypic features that allow discrimination between the two syndromes in the neonatal period do not exist, although it was suggested that upper female genital tract malformations (uterine, ovarian, and fallopian tube abnormalities) are rare in MKKS, but common in BBS [6]. Clinical follow-up of these patients include ophthalmologic studies, monitoring of weight and development, and will eventually permit discrimination between MKKS and BBS [7, 8]. As MKKS was shown to be caused by one of the *BBS* genes (*BBS6*/*MKKS*) the distinction between both syndromes is no longer clear [9, 10 11].

Hirschsprung disease (HSCR), a congenital disorder of the enteric nervous system, characterized by the absence of ganglion cells from the bowel wall [12], can be a feature of both MKKS and BBS. HSCR is a also a variable feature in a large number of uncommon autosomal recessive inherited syndromes, such as Shah-Waardenburg syndrome, Smith-Lemli-Opitz syndrome, Goldberg-Shprintzen syndrome [13], Mowat-Wilson syndrome and Congenital Central Hypoventilation syndrome [14].

The elucidation of the genetic background of both MKKS and BBS, as well as HSCR has been fruitful with the identification of 9 *BBS* genes [15 16] and 10 genes and 4 loci for HSCR [17]. Functional studies show emerging evidence that some of the BBS proteins are involved in multiprotein complexes that localize to centrosomes [15]. Centrosomal dysfunction is not only associated with abnormalities in cell division and chromosomal segregation, but can also lead to abnormal neuronal migration and assembly of cilia [18]. The main purpose of the RET signalling pathway, the major pathway involved in HSCR development, is the migration of the enteric nervous system precursors into the foregut [19].

The aim of this paper is to present a clinical description and molecular studies in a family

with 2 sibs affected with the combination of HSCR and a syndrome representing McKusick-Kaufman syndrome or Bardet-Biedl syndrome.

Patients

Case 1

The proband, a girl, is the first child of parents of native Dutch origin. Their family history was unremarkable and in particular there was no prior history of bowel dysfunction in either parent. Consanguinity between the parents or grandparents was unknown, however four of the eight grandparents originated from a small Dutch island in the south of the Netherlands.

At prenatal fetal ultrasonography in the third trimester mild hydrops fetalis, mild ventriculomegaly of the brain, cystic kidneys and a large abdominal cyst were noted. Polydactyly of the feet was suspected. Amniocentesis at 32.5 weeks was performed and showed a 46, XX normal female karyotype and an elevated alpha-fetoprotein level (28.5 mg/L) (normal < 5mg/l). Birth was preterm at 33 4/7 weeks. Birth weight was 3,155 g (>p97). Her abdomen was extremely distended and a mass was palpable. She had generalized edema (fig 1a). Abdominal ultrasonography showed that the abdominal mass was due to hydrometrocolpos, which was surgically treated at day 2. X-rays showed fusion of metacarpals III and IV of the left hand, and postaxial polydactyly of both feet (fig 1b). Cardiological evaluation revealed an asymptomatic subvalvular pulmonary artery stenosis. Ophthalmologic studies were normal in the neonatal period, however she gradually developed mild myopia (-3/-3) during childhood. Since intestinal obstruction persisted after surgical correction of the hydrometrocolpos, HSCR was suspected. Suction biopsy showed an aganglionosis of the recto-sigmoid colon (short-segment HSCR). A low anterior resection (Rehbein) with a primary anastomosis was performed at 14 months. Dysmorphologic evaluation at 2 months also showed frontal bossing and deep-set eyes. She had dysplastic, malfunctioning kidneys with a glomerular filtration rate 35ml/min/1.73m^2 at the age of 7 (normal value 100ml/min/1.73m^2).

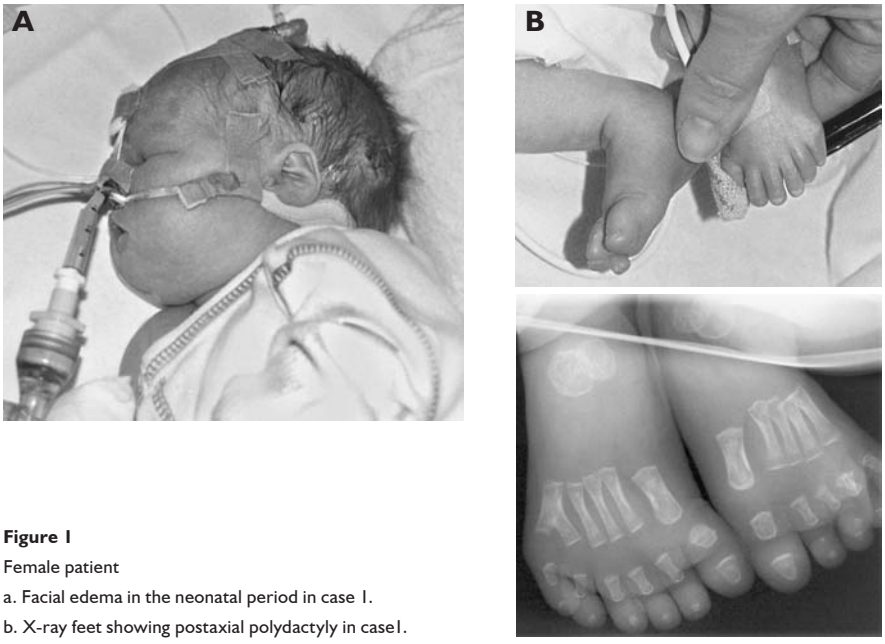

Figure 1

Female patient

a. Facial edema in the neonatal period in case 1.

b. X-ray feet showing postaxial polydactyly in case1.

Case 2

In the second pregnancy prenatal ultrasound studies were performed. A bichorionic-biamniotic twin-pregnancy was diagnosed. One male fetus had mild ventriculomegaly of the brain, large kidneys and polydactyly of the feet. The other male child had no congenital malformations. Delivery was at 37 4/7 weeks. The weight of the first-born twin was 2975 g; the second child's weight was 3420 g. The latter twin brother had four limb postaxial polydactyly, with partial digital syndactyly IV-V of the left hand. X-rays of the feet showed 6 metatarsal bones, and postaxial hexadactyly (fig 2). He was admitted to the pediatric surgical ward because of delayed passing of meconium in addition to feeding problems. At day 2, HSCR was suspected; biopsies were taken at laparotomy, showing a total colonic aganglionosis, consistent with long-segment HSCR. A perforation of the ileum was observed. After ileocecal resection, a temporary ileostomy was fashioned. A final re-anastomosis was completed at the age of 5 months. Ultrasound studies of the kidneys showed large dysplastic kidneys with some cysts. The glomerular filtration rate was 45ml/min/1.73m^2 (normal 100ml/min/1.73m^2). He had a micropenis and a mild phimosis. Ophthalmological studies at the age of 4 years showed mild myopia.

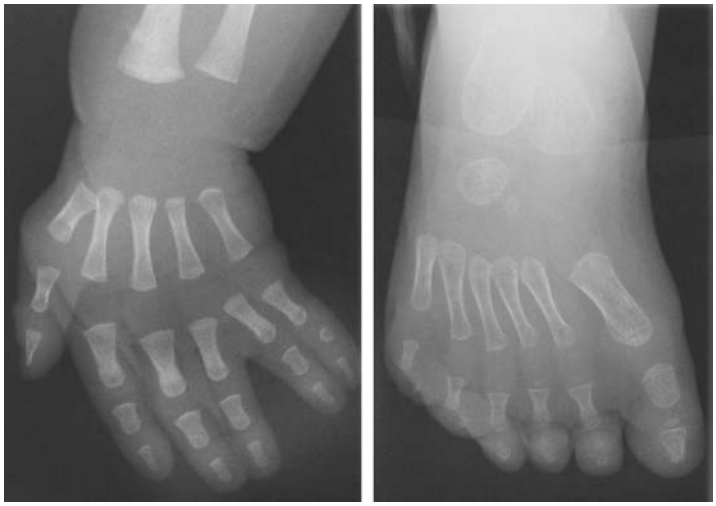

Figure 2
Male patient
Postaxial polydactyly of hands and feet in case 2

Methods

Molecular studies of BBS1, BBS2, BBS6 and *RET.*

DNA was isolated from leukocytes prepared according to standard methods.

BBS1 and *BBS2* of the patients were analyzed using capillary heteroduplex analysis.

The coding exons of *MKKS*/*BBS6* of the patients and parents were sequenced.

DGGE analysis of all exons of *RET* was performed on the patients and their parents as described before [20].

Results

Both *BBS1* and *BBS2* were analyzed using capillary heteroduplex analysis and proved to be normal.

Sequencing the coding exons of *MKKS*/*BBS6,* revealed two single-base substitutions believed to be polymorphisms (Arg517Cys and Gly532Val).

A mobility shift of exon 12 of *RET* was detected in both the index-patient and her father. Sequencing demonstrated a G to A transition resulting in the amino acid change Lys716Asn. This missense mutation was not identified in 70 healthy Dutch controls and in 200 (unselected) HSCR patients. This variant was, however, absent in the affected brother with long-segment HSCR.

Discussion

Clinical findings

At prenatal ultrasound Meckel syndrome (OMIM 249000) was considered in the first-born child of this family, based on the combination of postaxial polydactyly and cystic kidneys. Although an elevated alpha-fetoprotein level (28.5 mg/L) was found, no encephalocele, neural tube defects or abdominal wall defects were present. Recently it was shown that antenatal presentation of BBS might mimic Meckel syndrome. In 6/13 cases of prenatally diagnosed Meckel-like cases presenting with cystic kidneys and polydactyly without an encephalocele, mutations in 3 Bardet-Biedl syndrome genes (*BBS2*, *BBS4* and *BBS6*) were identified underlining the clinical overlap between these 2 syndromes [21].

After birth the presumptive diagnosis of the first-born changed into MKKS based on postaxial polydactyly of the feet, hydrometrocolpos and congenital heart defect. The phenotype of the affected brother with four limb polydactyly, hypogenitalism, enlarged cystic kidneys with renal dysfunction, and myopia, most likely fits BBS (the first 3 being major features) rather than MKKS [5]. This makes the older sister the one who is difficult to classify and illustrates that overlapping phenotypes of MKKS and BBS can be present within the same family, which is consistent with the idea that MKKS might represent a hypomorphic variant of BBS [6] , and that MKKS and BBS can be caused by mutations in the same gene [10, 11]. Although there is significant overlap in clinical features between MKKS and BBS, MKKS patients are not obese and do not develop retinopathy. So far both sibs have normal weight gains and regular ophthalmology consultations revealed mild myopia but no retinopathy.

The occurrence of HSCR in MKKS and BBS is well documented [22]; the simplest explanation for this combination is that they co-occur by chance. This seems highly unlikely since the frequency of HSCR in MKKS (12-14% or 1:7) [23 6] and in BBS (2-12%) [5, 6] is much higher than the prevalence of HSCR in the general population (1:5000) [24]. Based on a literature review, Slavotinek and Biesecker concluded that a subtype of BBS might exist in which hydrometrocolpos, structural abnormalities of the female genital tract, cardiac malformations, gastrointestinal malformations (imperforate anus and HSCR), and metacarpal involvement are more common [6]. Also male cases with HSCR and BBS are reported [25].

As far as we know no specific *BBS* gene has been implicated in the combination of HSCR and BBS. We analysed 3 (*BBS6, BBS1, BBS2*) of the 9 known *BBS* genes. We selected these genes because *BBS1* and *BBS2* are the genes most frequently mutated in Caucasians [26], and because of the fact that *BBS6* mutations can either be associated with MKKS or BBS [10, 11]. No mutations in these *BBS* genes were found. Given the occurrence of HSCR in both patients, we decided to screen *RET*, the major gene involved in HSCR susceptibility. A *RET* missense mutation Lys716Asn was identified

in the index patient and her father, but was not present in the second affected patient. Therefore, the Lys716Asn mutation cannot be the sole determinant of the extended phenotype in this family. Whether it contributes to the HSCR phenotype is hard to say as functional data of this variant are lacking. In favour of a causative role are the observations that i) this substitution involves amino acids that differ in polarity and size, ii) the Lys716Asn substitution was not identified in 70 healthy Dutch control individuals, and iii) Lysine716 is located just outside the tyrosine kinase domain, a region which is highly conserved between different species. Given the prior evidence for oligogenic inheritance of S-HSCR, involving loci at 3p21 and 19q12 as RET-dependent modifiers [27], we hypothesise that the RET gene/protein might interact with one or more BBS genes/proteins, to explain the frequent occurrence of HSCR in both syndromes. However, the existence of a gene causing the combination of BBS/MKKS and HSCR can not be excluded.

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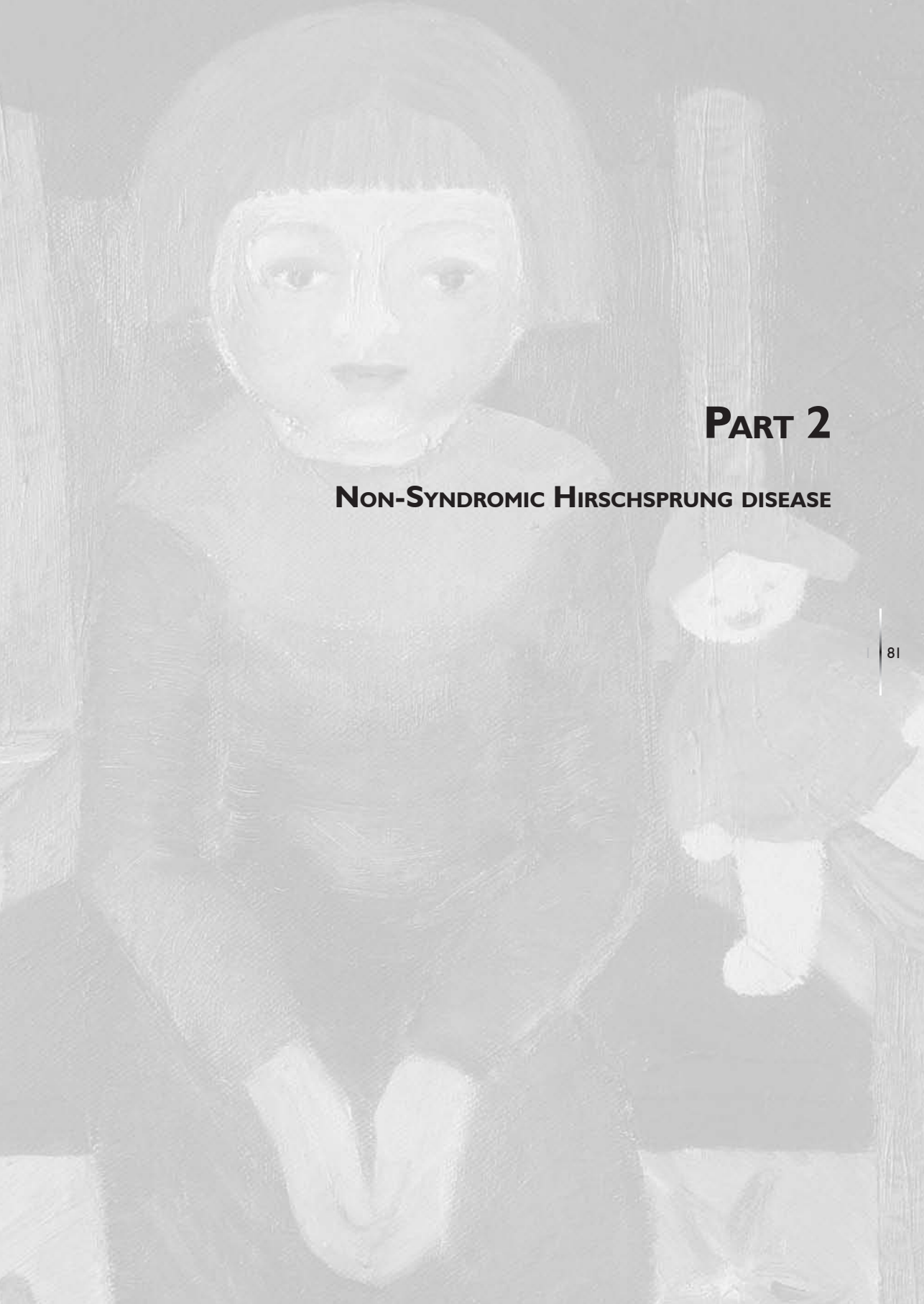

PART 2

NON-SYNDROMIC HIRSCHSPRUNG DISEASE

CHAPTER 6

A Novel Susceptibility Locus for Hirschsprung Disease Maps to 4q31.3-q32.3

A novel susceptibility locus for Hirschsprung disease maps to 4q31.3-q32.3

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Submitted

Abstract

We report on a multigenerational family with isolated Hirschsprung disease (HSCR). Five patients were affected by either short-segment or long-segment HSCR. The family consists of two main branches, one with 4 patients (3 sibs and 1 maternal uncle) and one with 1 patient, respectively. Analysis of the *RET* gene, the major gene involved in HSCR susceptibility, revealed neither linkage nor mutations. A genome-wide linkage analysis was performed, revealing suggestive linkage to a region on 4q31-q32 with a maximum parametric multipoint LOD score of 2.7. Furthermore, non-parametric analysis of the genome-wide scan data revealed a NPL score of 2.54 (p= 0.003) for the same region on chromosome 4q (*D4S413-D4S3351*). The minimum linkage interval spans a region of 11.7 cM (12.2 Mb). No genes within this chromosomal interval have previously been implicated in HSCR. Considering the low penetrant-disease in this family, the 4q locus may be necessary but not sufficient to cause HSCR in the absence of modifying loci elsewhere in the genome. Our results are suggesting the existence of a new susceptibility locus for HSCR at 4q31.3-q32.3.

Introduction

Hirschsprung disease (HSCR, OMIM 143623) is a congenital disorder characterized by the absence of enteric neurons, which are neural-crest derived, in the digestive tract. Delayed passage of meconium is the cardinal symptom in neonates with HSCR. If untreated, bowel hypomotility leads to severe constipation often associated with obstruction, gross distention of the bowel and vomiting. The prevalence of HSCR is approximately 1 in every 5000 liveborns. In the majority of cases (75-80%) the aganglionosis typically involves the rectum and the sigmoid (short segment Hirschsprung disease: S-HSCR). In 20-25% of the patients the aganglionosis extends proximally of the rectosigmoid, and the disease is called long segment HSCR (L-HSCR) [1]. HSCR mostly presents as an isolated congenital malformation (non-syndromic HSCR), but can be found in association with other congenital abnormalities (syndromic HSCR) [2].

So far mutations in 10 genes (*RET*, *GDNF*, *EDNRB*, *EDN3*, *ECE1*, *SOX10*, *ZFHX1B, NTN, PHOX2B,* and *KIAA1279*) have been implicated in HSCR [3 4 5 6 7, 8 9 10 11 12, 13 14 15 16].

The *RET* gene located at 10q11.2 is the major susceptibility locus in HSCR: 15-35% of the sporadic patients have inactivating mutations in the coding sequence of *RET* [17 18 19 20], whereas linkage analysis showed that all, but one, autosomal dominant families are linked to *RET* [21]. High-penetrance mutations in the coding sequence of the *RET* gene, however, are found in only 50% of the *RET*-linked families. *RET*-linked multigenerational families without a coding sequence mutation, might have a mutation in the non-coding sequence of the *RET* gene, including alterations in intronic and promoter sequences or harbor (frequent) variants that do change the function of the RET protein slightly [21]. Furthermore, similar haplotypes are found in the 5' region of the *RET* locus in HSCR populations patients from all over the world indicating the segregation of identical ancestral variant(s) [22 23 24 25 26]. Evidence is accumulating that specific (common) non-coding low-penetrance variants just before the gene and within intron 1 of *RET* are associated with HSCR susceptibility [22 25 27 28 29 30].

Single mutations leading to either isolated or syndromic HSCR have been found in the above-mentioned 10 genes, although evidence is building up that HSCR is a multigenic congenital malformation in the majority of HSCR patients. A "multiplicative model" has been suggested which assumes that additional loci are involved apart from the *RET* locus, and their individual effects can be multiplied.[31]

Furthermore, 4 HSCR susceptibility loci (9q31, 3p21, 19q12, and 16q23) have been identified, harboring unidentified genes [21 31 32]. Linkage at 9q31 was reported in 5 families that also showed linkage with *RET*, however no causative *RET* mutation could be identified. A sixth family that was *RET*-unlinked, however, was linked to the locus at 9q31 [21]. Furthermore susceptibility loci at 3p21 and 19q12 have been identified in affected nuclear families, suggesting that these 2 loci probably function as *RET*- dependent modifiers. Non-random allele sharing was also found at 9q31 in those nuclear families in which no *RET* mutation was identified, confirming the segregation of the 9q31 locus in multiplex families [31 21].

In an inbred Mennonite population, *RET* not only interacts with *EDNRB*, but also with an unknown gene on chromosome 16q23. This locus is probably only of importance in this genetic isolated population [32] in which HSCR can be associated with symptoms also found in Waardenburg-Shah syndrome [7]. Conversely, no linkage with the other susceptibility loci at 3p21, 9q31 and 19q12 was found in this Mennonite kindred.

Clearly, HSCR is a heterogeneous congenital malformation. It is estimated that only 30% of cases can be attributed to mutations of the known genes [33]. Thus, a considerable number of additional genes involved in enteric nervous system development might be identified in the future [28]. Here, we describe a five-generation family with non-syndromic HSCR with suggestive evidence for a new susceptibility locus on chromosome 4.

Methods

Patients

The family we describe here is a five-generation pedigree of native Dutch origin with 5 cases presenting HSCR. The segregation pattern is compatible with an autosomal dominant mode of inheritance with incomplete penetrance. One branch of the family consists of a sibship with 3 affected children and an affected uncle. Two sisters (V-1 and V-3) have S-HSCR. Their brother (V-2) was diagnosed with total intestinal aganglionosis, both large and small intestines were aganglionic. Given the boy's poor prognosis, a joint medical and parental decision was made for conservative care of HSCR; he died at the age of 1 month. Delayed passage of meconium led to the suspected diagnosis of HSCR in all 3 children. Suction and/or full thickness biopsies were consistent with this diagnosis. Their maternal uncle (IV-3) was operated during childhood because of S-HSCR. The other branch of the family contains one affected female with S-HSCR (V-4). Her paternal grandfather (III-3) is a cousin of the maternal grandmother (III-2) of the 3 sibs (fig 1). Congenital malformations indicative of syndromic HSCR were lacking in all 5 patients as confirmed by two dysmorphologists (ASB and IVL). Karyotyping was normal in patients V-1 and V-2. Brainstem evoked response audiometry, which we performed because of his expected early death and to exclude hearing loss consistent with Shah-Waardenburg syndrome, showed no abnormalities in patient V-2. Chronic severe constipation was not reported in II-2, III-2, IV-4, although IV-2 as well as her father III-1 suffered from severe constipation in childhood. Informed consent was given by the parents and the adult patient (IV-3). Genomic DNA was isolated from peripheral blood obtained from II-2, III-2, IV-1, IV-2, IV-3, IV-4, IV-5, V-1, V-2, V-3 and V-4 using standard protocols [34].

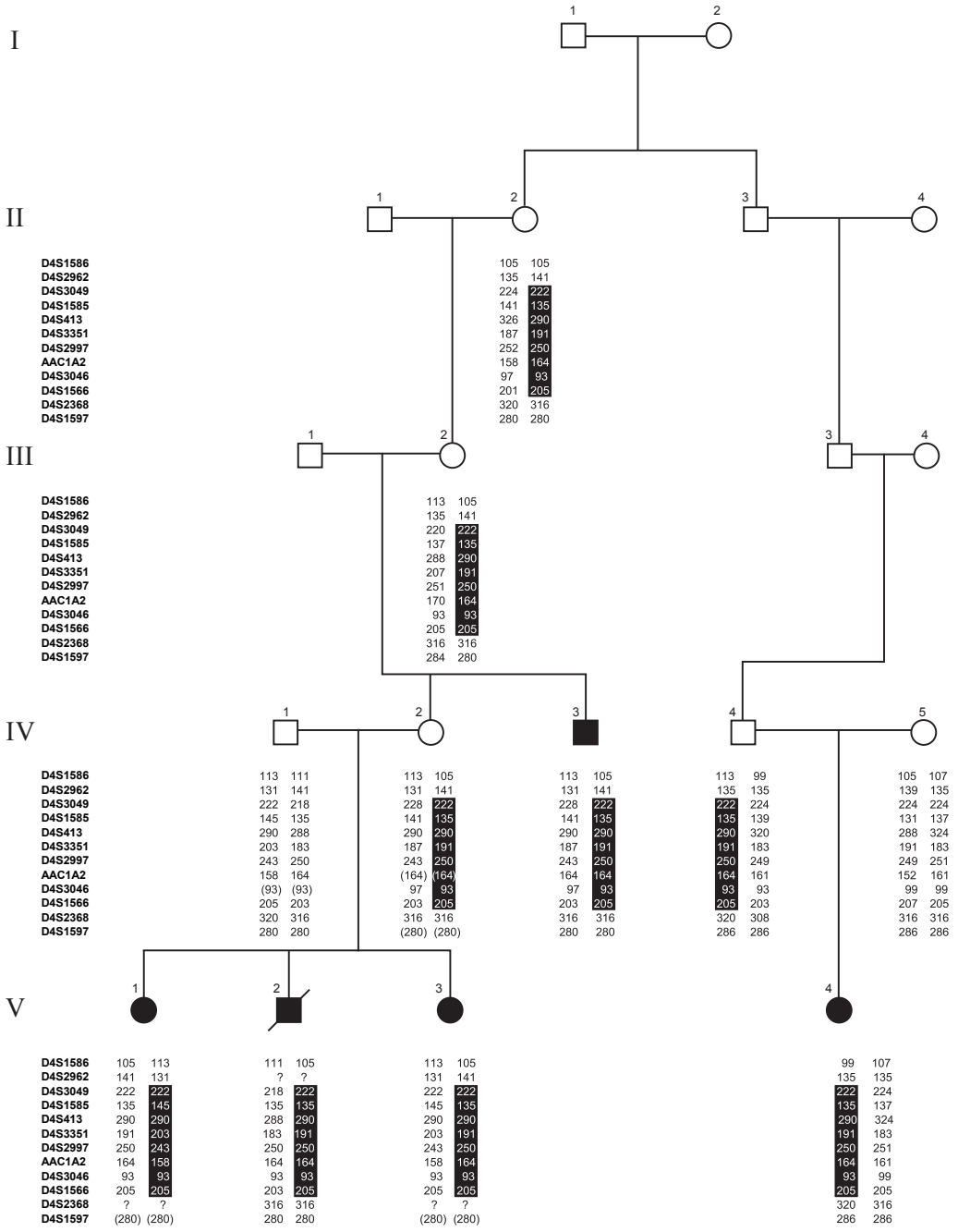

Figure 1

Pedigree structure and haplotypes. Patients are represented as blackened symbols. The segregating 4q31-q32 haplotype is depicted. The minimum critical region at chromosome 4 spans 11.7 cM between *D4S3049* and *D4S1566*.

Analysis of markers encompassing the *RET* locus

The following markers: *D10S141* (- 1 Mb to *RET*), *RET*int5, *D10S1099* (1.4 Mb downstream to *RET*) and 5 SNPs (rs741763, rs2435362, rs2565206, rs2506004) from the 5'*RET* region were analyzed as reported by us elsewhere [26] [27]and rs2435357 reported by others [28].

Mutational analysis of *RET*

Mutation analysis of the 21 exons of *RET* was performed in probands V-1 and V-4, as described by us elsewhere [20].

Genome wide linkage analysis

We performed a systematic genome scan using the ABI Prism MD-10 set (Applied Biosystems) consisting of 382 markers (STRPs), with an average spacing of 10 cM. Additional markers for further characterization of candidate regions were selected from the sex-average Marshfield genetic map or newly designed. Genomic DNA (20 ng) was used as a template in 7.5 µl PCR reactions, with 5 pmol of oligonucleotides, 0.3 units Amplitaq Gold polymerase in Gold buffer (Applied Biosystems) and 2.5 mM MgCl2. The thermal cycling consisted of an initial incubation at 95 °C for 5 minutes, followed by 10 cycles of 95 °C for 30 seconds, 55 °C for 15 seconds and 72 °C for 30 seconds and 25 cycles of 92 °C for 30 seconds, 55 °C for 15 seconds and 72 °C for 30 seconds. PCR products were pooled and loaded on an ABI3100 automated sequencer (Applied Biosystems). Data were analyzed using GeneMapper software (v 2.0). Mega2 [35] was used to process the genetic data into the appropriate format and perform data validation checks. Simulation analysis to estimate the probability of detecting genetic linkage given the pedigree structure (statistical power) was performed with the SLINK program [36]. Due to uncertainties related to the correct genetic model in this pedigree, parametric and non-parametric linkage analyses were performed using SimWalk2 (version 2.9) [37].

For the parametric analysis we specified an autosomal dominant mode of inheritance, a mutant allele frequency of 0.01% with a penetrance of 40% and equal marker allele frequencies. Pedigree's Location Scores were calculated; these location scores are directly comparable to multipoint LOD scores. For the non-parametric analysis, the max-tree statistic (the largest number of affecteds inheriting an allele from one founder-allele) is reported. This statistic was designed for traits best modeled by dominant inheritance and was formerly known as STAT B. The NPL_ALL (STAT E) statistics, a measure of whether a few founder-alleles are overly presented in affecteds (suitable for an additive model) is reported as well. A large value of the statistic indicates a high degree of identity by descent (IBD) allele sharing among the patients, usually a result above 2 can be considered significant. Empirical p-values (10,000 simulations) are also reported. Namely, this p-value is the probability of obtaining a value for that statistic equal to, or more extreme than, the observed value, if the trait were unlinked to the markers.

Results

Linkage analysis to the *RET* locus and sequence analysis of the *RET* gene

The family we investigated is a five-generation pedigree of native Dutch origin with 5 cases presenting HSCR (fig 1). Since the majority of the multigenerational families with HSCR show linkage to the *RET* gene [21], we investigated the *RET* locus by haplotype analysis and mutational analysis. We observed that not all 5 patients (IV-3, V-1, V-2, V-3 and V-4) shared the same haplotype at the *RET* locus (Table 1), excluding linkage with the *RET* locus in both branches of this family. However, IV-2, IV-3, V-1, V-2, and V-3 from branch 1 did share the same haplotype encompassing the *RET* locus, which must be inherited from III-1. We also performed sequence analysis of the entire coding region of the *RET* gene. Direct sequencing revealed no mutations in patients V-1 (branch 1) and V-4 (branch 2).

Table 1. *RET* haplotypes. Affected family members are in bold.

	Family members										
	II-2	**III-2**	**IV-1**	**IV-2**	**IV-3**	**IV-4**	**IV-5**	**V-1**	**V-2**	**V-3**	**V-4**
D10S141	3 1	3 2	2 2	4 3	**4 2**	3 4	2 2	**2 4**	**2 4**	**2 4**	**3 2**
rs741763	C G	C G	G G	C C	**C G**	C C	G G	**G C**	**G C**	**G C**	**C G**
rs2435362	C C	C C	A A	C C	**C C**	C C	C A	**A C**	**A C**	**A C**	**C C**
rs2435357	C C	C C	T T	C C	**C C**	C C	C T	**T C**	**T C**	**T C**	**C C**
rs2506004	C C	C C	A A	C C	**C C**	C C	C A	**A C**	**A C**	**A C**	**C C**
rs2565206	G T	G T	G G	G G	**G T**	G G	T G	**G G**	**G G**	**G G**	**G T**
Retint5	3 2	3 2	1 3	2 3	**2 2**	3 1	2 1	**1 2**	**1 2**	**3 2**	**3 2**
D10S1099	5 2	5 5	5 4	5 5	**5 5**	2 3	5 1	**5 5**	**5 5**	**4 5**	**2 5**

Genome search

Simulation analysis (SLINK) yielded an average LOD score of 1.54 and a maximum of 2.15. We performed a genome wide search using 382 STRPs. Results from the parametric linkage analysis excluded most of the genome (data not shown). Only two genomic regions displayed LOD scores above 1, a region on chromosome 17 (*D17S798* mLOD score=1.15) and on chromosome 4 (mLOD=1.84). We tested additional markers and performed haplotype analysis. The region on chromosome 17 was rapidly excluded since one patient (IV-3) was not sharing the haplotype observed in patients from branch 1.

The highest multipoint LOD score (mLOD=1.84) was obtained for chromosome 4 between markers *D4S424* and *D4S413*. This region fully segregated with the disease phenotype (fig 1). When we saturated the chromosome 4 region with additional markers, a maximum mLOD of 2.7 (fig 2) was reached between markers *D4S1585* and *D4S3351* which is consistent with suggestive linkage according to the Lander-Kruglyak guidelines for significance thresholds [38].

We also performed non-parametric analysis for our genome wide scan data. Only for this region on chromosome 4q, we found non-parametric scores (NPL) above 1. The maximum NPL was found for marker *D4S413* (158.0 Mb, NCBI build 35.1) located between markers *D4S1585* and *D4S3351* (NPL=2.55, p=0.003), under both dominant and additive mode of inheritance (table 2).

Table 2. Results from the non-parametric analysis on chromosome 4q after fine mapping

Marker Name	Genetic Position cM*	Physical Position Mb†	Max-tree‡	NPL_All§	Empirical p-value
D4S1575	132.1	135.1	0.35	0.38	0.418
D4S1644	143.3	142.1	0.80	0.83	0.148
D4S424	144.6	142.6	0.82	0.84	0.143
D4S1625	146.0	143.9	0.84	0.85	0.141
D4S1586	147.1	147.1	0.87	0.86	0.136
EDNRA	--	148.8	0.86	0.86	0.137
D4S2962	153.0	150.7	0.87	0.86	0.137
D4S3049	155.2	155.1	1.60	1.60	0.025
D4S1585	158.0	157.9	2.42	2.42	0.004
D4S413	158.0	158.7	2.54	2.55	0.003
D4S3351	158.7	159.9	2.54	2.54	0.003
D4S2997	158.7	160.0	2.52	2.51	0.003
AAC1A2	158.0	160.0	2.51	2.51	0.003
D4S3046	162.5	163.7	1.55	1.57	0.030
D4S1566	166.9	167.3	0.99	0.99	0.102
D4S2368	167.6	169.1	0.83	0.84	0.144
D4S1597	169.4	170.2	0.74	0.79	0.163
D4S2431	176.2	175.2	0.51	0.63	0.234
D4S415	181.4	179.1	0.10	0.11	0.776

*According to Marshfield sex average genetic map

†According to NCBI physical map, build 35.1

‡Max-tree is the allele-sharing statistic for traits best modeled as dominant inheritance.

§NPL-All is the statistics for traits following an additive inheritance.

Recombination events can be identified in individual IV-4 (and V-4) and show that marker *D4S2962* at the centromeric site, and *D4S2368* at the telomeric site limits the critical region. The maximum critical region between *D4S2962* and *D4S2368* spans approximately 16.4 cM (19.7 Mb according to NCBI physical map, build 35.1), the minimum shared region extend from marker *D4S3049* until *D4S1566* (11.7 cM or 12.2 Mb).

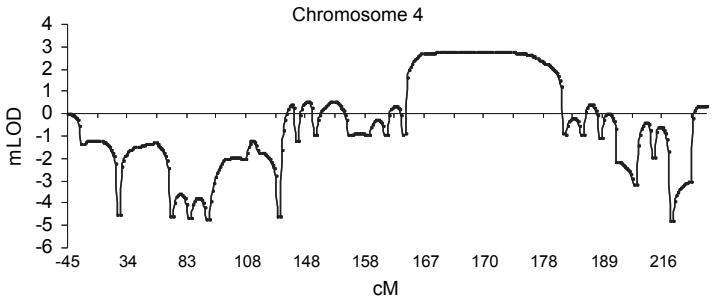

Figure 2

Multipoint LOD-score analysis with several markers on chromosome 4. The X- axis represents the chromosomal position of the markers. The Y- axis indicates the multipoint LOD score, with a peak LOD score of 2.7.

5' RET common risk haplotype analysis

To test whether non-coding low-penetrance variants just before or within intron 1 of *RET* were associated with HSCR susceptibility in a part of this family, we typed 5 SNPs all being part of an ancestral haplotype [27]. Spouse IV-1 (married-in individual) was homozygote for the GATAG haplotype that is part of the core risk SNP haplotype detected in European, Asian and European-American patients with sporadic HSCR [27 28, 39]. The 3 affected sibs (V-1, V-2 and V-3) were thus carriers of the core risk haplotype. However, the other 2 patients (IV-3 in branch 1 and V-4 in branch 2) were not carrying the 5' *RET* common risk haplotype (Table 1).

Discussion

We identified a five-generation family with 5 patients affected with HSCR. The patients were connected to a common ancestor within 3-4 generations. Two branches with HSCR patients within the family were identified. The inheritance pattern in the family is compatible with an autosomal dominant mode of inheritance with reduced penetrance of a single mutated gene.

Since *RET* is the major gene involved in HSCR susceptibility in multigenerational families [21], linkage to the *RET* locus and mutations in the coding sequence of *RET* were first excluded in both branches. However, under the assumption that HSCR is caused by two separate mutations in the two branches, an alternative hypothesis would be that the individuals of the main branch with 4 affected individuals carry a hitherto unidentified *RET* mutation (introduced in the family by III-1). Since an oligogenic model with the contribution of 2 or more loci could not be discarded, as was previously demonstrated for HSCR [21], we hypothesized that the possibility of linkage to *RET* in branch 1, did not mutually exclude the existence of an additional locus in this family. Subsequently, we performed a genome-wide scan to map additional disease gene(s) in this family. Model-free or non-parametric linkage analysis methods are more robust than parametric or model-dependent analysis, when the mode of inheritance or the genetic model is uncertain, such as the pedigree with isolated HSCR reported here. Consequently, we performed both parametric and non-parametric linkage analysis. Both methods highlighted the same region on chromosome 4q. No other known HSCR susceptibility loci such as the ones at chromosomes 3, 9, and 19 [21 31] showed positive LOD scores. When adjacent markers for chromosome 4 were tested, the evidence of linkage became stronger and we could observe a common haplotype extending at least 11.7 cM that was inherited by all the affected individuals from their common ancestor. Our results are suggesting the existence of a novel HSCR susceptibility locus on chromosome 4q.

Clearly, the chromosome 4q locus has incomplete penetrance. Is this 4q locus solely leading to HSCR or alternatively, is the phenotype only expressed in the presence of other susceptibility loci? Modifier loci either can increase susceptibility and severity of the phenotype or can act protectively to confer resistance to the disease in the face of a predisposing mutation [40]. Can variants within the *RET* gene explain the difference in penetrance observed in both branches? We investigated whether all patients shared a haplotype similar to the common risk haplotype defined by SNPs located in the 5' region of the *RET* locus reported in Dutch HSCR patients [26]. We identified non risk haplotypes in patients IV-3 and V-4. However, the spouse IV-1and the 3 affected children V-1, V-2, and V-3 were homozygote and heterozygotes, respectively, for the GATAG haplotype, which contains the core risk haplotype detected in European, and European-American patients with sporadic HSCR [27 28]. The third and fourth SNPs (RET3+, rs2435357 and IVS1+9494,

rs2506004) are particularly interesting as disease-associated *RET* variants, because of the homology and evolutionary conservation between rodents and primates and the differences in allele/genotype frequencies among patients and controls [27]. Recent data show that RET3+ might lie within, and might compromise the activity of an enhancer-like sequence in *RET* intron 1 [28]. These findings make us hypothesize that the 5' *RET* risk haplotype in combination with the identified chromosome 4 locus might play a role in penetrance and severity observed in the sibship with 3 affected children.

We looked for candidate genes in the minimum 12.2 Mb-linked region at 4q31.3-32.3 (between markers *D4S3049* and *D4S1566*) in the human genome sequence. This region contains at least 57 genes, in accordance with the National Center for Biotechnology Information (NCBI) build 35.1 of the human genome and the Ensemble Genome Browser. The maximum 20-Mb linked region between *D4S2962* and *D4S1597* encompasses 93 genes, including several interesting functional candidate genes that are proposed to be involved in neural crest development or neuronal development.

Unfortunately, the maximum genetic interval contains far too many candidates to begin functional evaluation of each gene individually; the best positional and functional candidate we could identify is *Mab21L2* (named after male abnormal 21 in C. Elegans). *Mab21L2* is expressed in the central nervous system and neural crest in midgestation embryogenesis in mice [41]. Furthermore, *Mab21L2* is linked to the TGFbeta signaling pathway to which also *ZFHX1B* belongs [13, 42]. *ZFHX1B*, the gene involved in Mowat-Wilson syndrome, a syndromic form of HSCR, is a transcription factor that functions as a transcriptional repressor [42]. For these reasons we sequenced the complete coding region of the *Mab21L2* gene for mutations; however we did not identify a sequence variant in *Mab21L2*. Besides *Mab21L2*, many other candidate genes are located in the region. Several proteins encoding neuropeptide Y receptors (NPY2R, NPY1R and NPY5R) are located in the minimum 12.2 Mb-linked region. In mammals NPY, the ligand, is mainly found in cells derived from the neural crest, and is widely distributed in the central and peripheral nervous system [43]. Furthermore, the gene for the secreted frizzled related protein 2 (*SFRP2*) is located in this region [44]. Wnt-frizzled signaling is involved in neural crest formation [45].

The 4q locus may be necessary but not sufficient to cause HSCR in the absence of modifying loci elsewhere in the genome. Linkage analysis in additional multigenerational HSCR families and association studies in sporadic patients with high-density marker sets covering the entire interval will be necessary to confirm this suggestive linkage to chromosome 4q. Eventually, identification of the causative gene defect will specify the susceptibility to HSCR conferred by this novel locus at 4q31.3-q32.3.

Electronic database information

Accession numbers and URLs for data in this article are as follows:
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for MIM 142623).

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CHAPTER 7

General Discussion and Future Considerations

1. Strategies for finding genes for Hirschsprung disease susceptibility

To date only 30% of HSCR cases can be attributed to mutations in the coding sequences of 9 known HSCR susceptibility genes [1]. The aim of our studies was to identify new genes involved in HSCR susceptibility by studying both syndromic and non-syndromic families segregating HSCR. To this end we have chosen the design of traditional linkage.

Additional HSCR susceptibility genes likely are bound to be identified in the future. However, finding these genes might turn out to be difficult, because the individual effects might be small [2 3].This problem is inherent in studies designed to identify susceptibility loci for complex diseases [4]. Yet one could think of plausible approaches to identify these HSCR susceptibility genes. Here strategies to identify genes involved in syndromic HSCR will be discussed, as well as possible approaches for the identification of non-syndromic HSCR susceptibility genes.

1.1 Syndromic Hirschsprung disease

1.1.1 Rare autosomal recessive syndromes

To identify genes involved in syndromic HSCR one could study rare presumptive Mendelian combinations of HSCR associated with additional major and/or minor congenital malformations. In general, mapping of genes involved in rare recessive syndromes is difficult because of the paucity of large families with enough affected progeny to provide sufficient statistical evidence for linkage. The alternative is using inbred affected families.

We have chosen this latter strategy to identify by homozygosity mapping the gene involved in Goldberg-Shprintzen syndrome, a rare autosomal recessive disorder [5, 6] (chapters 2 and 3). The basic concept of this method is to use the information provided by a genome-wide scan in inbred affected individuals in order to define a region of the genome where they are all homozygous [7]. The disease locus is then highly likely to map in this region. Identification of the gene defect causing this rare and relatively simple Mendelian disorder in only 4 affected children from one consanguineous family using this robust method, underlines the high power of this study design. The use of homozygosity mapping in a genome-wide scan requires a set of regularly spaced markers, which is of course also a condition sine qua non in classical linkage studies in multigenerational families from outbred populations. Spacing of the markers (typically around 400 CA repeat markers with an average spacing of 10 cM), should be based on the notion that the pedigree structure determines the expected length of the region of identity by descent around the disease locus. In our initial screen with 381 markers, only one of the four affected individuals was homozygous by descent for two flanking markers at chromosome 10q, whereas the other three were homozygous for one of these two markers. Excluding a region by the criterion that all affected individuals in the sample are not homozygous may increase the rate of false negatives and the locus of interest is likely to be missed [8]. More closely spaced STRPs (800 markers) or even high-density single-nucleotide polymorphism (SNP) genotyping arrays may be needed in extended pedigrees with low inbreeding coefficients [9]. For these reasons the choice of the distance between adjacent markers is not a trivial matter. Furthermore, the threshold criterion for exclusion of a region should be defined according to the specific family structure and the number of available affected family members.

1.1.2 De novo dominant syndromes

Gene finding in sporadic syndromic HSCR cases consistent with a *de novo* autosomal dominant trait requires other methods to identify the underlying defect. In the absence of patients with chromosomal

aberrations detectable by conventional G-banding (resolution 5-10 Mb), high-resolution techniques such as array Comparitive Genomic Hybridization (array-CGH) [10], oligo-microarray [11], or SNP arrays [12] might be instrumental to detect small deletions or duplications in those sporadic patients with a combination of HSCR, mental retardation and additional multiple congenital malformations. The most common technique uses a BAC array that covers the complete human genome with 1-Mb resolution [13]. Tiled (i.e. overlapping) BAC arrays have now become available with even higher resolution [14] and promising diagnostic yield [15]. The identification of a deletion by this technique might represent a promising starting point for further positional cloning experiments as was recently successfully demonstrated in patients with CHARGE syndrome (OMIM 214800) [16] and as was suggested in patients with syndromic congenital diaphragmatic hernia [17].

This approach is of course only effective if one assumes that not only intragenic mutations occur, but also microdeletions encompassing the underlying gene defect. This strategy is of particular interest for those sporadic syndromes that cannot be tackled by other mapping methods because of limited reproductive fitness often associated with mental retardation. In particular, one group of syndromic HSCR cases that might be studied with this approach is the group of HSCR-mental retardation-microcephaly cases unrelated to the known loci on 2q22 [18] or 10q22.1 (chapter 4).

Syndrome delineation and molecular studies are essential in this group of HSCR patients to further unravel the biological relationship between enteric nervous system and central nervous system development. This entity seems clearly to be heterogeneous. This genetic heterogeneity might reflect interactions at the protein level, such as ligand-receptor interactions, different subunits of a multiprotein complex or proteins that function at different steps of a pathway [19]. All three interactions are already known to exist in HSCR: GDNF-RET and EDN3-EDNRB are examples of ligand-receptor interactions, GDNF-GFRalfa functions as a multiprotein complex and EDN3-ECE1 represents the ligand and its cleavage enzyme [20-25]. Using expression arrays [26] to identify common affected pathways and integrating this knowledge with e.g. the data obtained with genomic array studies could lead more directly to the identification of the candidate gene(s).

1.2 Non-syndromic Hirschsprung disease

1.2.1 Linkage analysis

Linkage analysis is feasible in extended multigenerational families or multiple small nuclear families in which genetic homogeneity is assumed. If successful, linkage can identify a sometimes large genomic region, often containing many genes, in which the disease gene is sought. First of all one could collect familial cases with non-syndromic HSCR following a (presumptive) Mendelian inheritance pattern for gene mapping studies that might lead to the identification of putative HSCR susceptibility loci. Before a genome wide mapping procedure can be started, a thorough clinical diagnosis is required. In search of genes for HSCR susceptibility, only family members with histologically proven aganglionosis should be indicated as affected and not those who report constipation. Since *RET* is the major gene involved in HSCR susceptibility in multigenerational families [27], mutations in the coding sequence of *RET* and linkage to *RET* should be first excluded before starting gene mapping studies in suitable families. These linkage approaches might replicate or exclude the regions on 3p21, 9q31, 19q12 and 16q23 that were previously found to be implicated on the basis of genetic linkage and association studies [27-29]. Identification of additional large families might eventually confirm the localization of these putative HSCR susceptibility loci and hopefully delineate smaller regions of interest, or might identify new chromosomal regions associated with HSCR.

A genome-wide linkage analysis in a 5-generation pedigree with 5 family members presenting with isolated HSCR allowed us to identify a new locus with a lowered penetrance at 4q31.3-q32.3 (chapter 6). The result of this study underlines one of the problems of a traditional linkage design; the shared haplotype in the 5 affected family members extends around 12.2 Mb on the NCBI physical map and encompasses many genes. Unfortunately, the region is too large for mutational screening of every gene located in the region of interest. Importantly, the statistical analysis of such kind of families should allow for uncertainties related to the correct genetic model. Non-syndromic HSCR was proven to be an oligogenic inherited congenital malformation determined by a small number of genes acting together [27, 28]. Therefore, both parametric and non-parametric or model-free linkage analyses should preferably be performed.

1.2.2 Association analysis

As not many HSCR families with multiple affected individuals are available for linkage studies, a better approach seems screening of a dense set of SNPs (e.g. one SNP every 5 kb) confined to the regions implicated by initial low-resolution linkage studies [28-30], for regional association and transmission studies in siblings or sporadic patients in a trio design. This can be done by linkage disequilibrium (LD) mapping [3]; this implies a non-random association between a pair of markers. LD is common for markers that are located close to another. The presence of LD between SNP markers makes it possible to infer the location of a disease gene that is in LD with a genotyped SNP. The optimal SNP density for these studies is dependent on the extent of local LD [3]. The extent of LD determines the size of the shared ancestral haplotypes; the more time has elapsed since a mutation was introduced in the population, the more likely an ancestral haplotype has been disrupted by a recombination event, reducing the extent of LD and the size of the shared haplotype. This step is aimed at reducing as much as possible the size of the critical region, leaving a small number of candidate genes. This strategy has a higher resolution than linkage analysis in families; however, large patient cohorts are still needed.

1.2.3 Candidate genes

The identification of genes involved in non-syndromic HSCR might focus on candidate genes. Candidate genes could be selected on the basis of (1) expression within the tissue of interest, (2) functional criteria, such as membership of a known disease pathway, or (3) localization to an implicated chromosomal region, on the basis of previous linkage studies. For example, concerning the development of the enteric nervous system, genes involved in neural crest development might represent possible functional candidate genes. Of interest is the list of genes highly expressed in gut neural crest stem cells described by Iwashita and others [31]. Gene expression in gut neural crest stem cells from rat was compared with expression in the whole-fetus (E14.5). Genes that have been linked to HSCR are frequently expressed at higher levels in gut neural crest stem cells. Of the 10 known genes that are most highly expressed in gut neural crest stem cells relative to whole-fetus, mutations in 4 (*RET, SOX10, GFRA-1 and EDNRB*) have been implicated in HSCR. Consequently, the remaining genes that are more highly expressed in gut neural crest stem cells relative to whole–fetus, make up a list of candidate genes on the basis of expression within the tissue of interest.

2. The newly identified *KIAA1279* gene at chromosome 10 and the non-syndromic Hirschsprung disease locus at chromosome 4

2.1 KIAA1279

By identifying *KIAA1279* as the gene involved in GOSHS, we have proven that not only clinically but also genetically MWS and GOSHS are two separate conditions [32]. Apart from *RET*, *KIAA1279* is the second gene at the long arm of chromosome 10 involved in HSCR susceptibility. While *RET* is the major gene in non-syndromic HSCR, *KIAA1279* mutations are only found in a selected group of syndromic HSCR patients (chapters 3 and 4).

After fine mapping, a region of 2.8 Mb was identified at the long arm of chromosome 10 for which all patients were homozygous. All 30 transcripts located in this region were screened. In retrospect we wondered if we could have prioritized the order of most likely positional candidates by a tool other than searching the literature. The combination of gene mapping data with genetic expression profiling, which measures the expression levels of many genes at once [26], might provide a good option to select candidates [33]. This strategy might have been especially reasonable afterwards, since the mutations identified in *KIAA1279* were both nonsense mutations. This could mean that the mutant messenger RNAs trigger the nonsense mediated decay RNA surveillance pathway, which in turn degrades only transcripts containing nonsense codons that are followed by at least one intron [34]. What is more, one third of human disease genes are predicted to result from nonsense lesions or mutations that decrease transcript abundance [35 36]. Expression profiling studies in fibroblasts of 3 affected GOSHS individuals from the Moroccan family showed that *KIAA1279* was among a set of 20 genes with significantly decreased expression. Furthermore, *KIAA1279* was the only gene/transcript identified as having reduced expression in our region of interest (personal communication Van der Spek and Mancini). In this respect, integrating the expression microarray analysis and the linkage data would have led directly to the identification of the gene. Genetic expressing profiling may therefore represent a powerful tool in picking the right candidate genes and may dramatically speed up sequencing efforts and facilitate positional cloning approaches.

As patients with MWS and GOSHS have partly overlapping phenotypes (including malformations of cortical brain development in a set of patients) [32, 37], overlap at the molecular level is likely as well . The mechanisms underlying the phenotypic effects of a *ZFHX1B* mutation have not been fully determined. Interestingly, *ZFHX1B* mRNA is detected in nearly all human tissues (like is the case for *KIAA1279*) as well as mouse heart and brain. *ZFHX1B* expression is also detected in fetal human tissues including brain, kidney, liver, and spleen [38, 39]. Mouse *Zfhx1b* is expressed in many brain regions (hypothalamus, cerebellum, cortex, rhombencephalon, hippocampus, mid brain, and spinal cord) [40]. It would be of interest to see whether the expression patterns of mouse *Kiaa1279* and *Zfhx1b* overlap and to see whether both proteins could have critical functions in vertebrate neural crest and cortex development during embryogenesis. The function of *KIAA1279* is still unknown. Although this gene has probably no role in sporadic isolated HSCR or isolated polymicrogyria (chapter 4), the importance of this rare gene defect might lie in the identification of a pathogenic pathway. Further studies need to ascertain the function of *KIAA1279* and characterize the protein network in which KIAA1279 acts.

Besides the fact that KIAA1279 is highly conserved and expressed in specific tissues, we know that the protein contains two tetratrico peptide repeats (TPR), a structural motif consisting of 34 amino acid residues, in exon 1/2 and exon 4, respectively. Because of these repeats, KIAA1279 may be considered a member of the TPR protein family. The most basic function of TPR motifs

is to mediate protein-protein interactions [41]. Proteins with TPR motifs are involved in a variety of biological processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding.

A first possible indication of the function of KIAA1279 is derived from the investigation of the subcellular localization of the protein; antibody staining in HeLa cells showed that the KIAA1279 protein is localized in the cytoplasm (De Graaff; unpublished results). These preliminary data can be used as a starting point to identify the protein network in which KIAA1279 functions. The speculation would be that genes encoding these proteins are involved in related phenotypes, and that in this way clinical classification precedes molecular verification [19]. Therefore, functional studies of *KIAA1279* are hoped to provide more knowledge on the molecular basis of GOSHS, HSCR, and polymicrogyria- a disorder of cortical organization- as well, for which only a few genes and loci have been identified so far (for a review see Jansen and Andermann) [42, 43].

Study of these proteins will provide us with a window through which we can observe the development of both human cortex and enteric nervous system. Hopefully it will answer the questions why HSCR can be associated with microcephaly and/or malformations of cortical brain development and whether the underlying neurodevelopmental process is caused by either defective neuronal mitosis or defective neuron migration. The former was recently hypothesized for autosomal recessive inherited primary microcephaly, characterized by a substantial reduction in the size of the cerebral cortex, but with a normal architecture [44]; the latter for lissencephaly, a disorder of cortical brain development characterized by a smooth or agyric brain [45]. Since HSCR is generally regarded as a migration disorder of precursors derived from the vagal neural crest [46], an attractive hypothesis would be that HSCR and malformations of cortical brain development in GOSHS are the consequence of disturbed neuronal migration of vagal neural crest cells and of progenitor cells of the cerebral cortical neurons, respectively. A possible role of KIAA1279 in neuron proliferation (that is mitosis of the progenitor cells of the cerebral cortical neurons) during fetal life and/or neuronal migration will be object of further studies.

2.2. Chromosome 4 locus

We provided suggestive evidence for a fourth locus that could be involved in non-syndromic HSCR apart from the previously reported three loci at 3p21, 9q31 and 19q12 [27, 28]. Clinically this HSCR susceptibility locus seems to be representing a susceptibility locus with a lowered penetrance in the described family. In our genome-wide search no other regions were identified, however the low penetrance in this family is likely to be related to additional genes with low effects. We hypothesized that a 5'*RET* risk haplotype, similar to the common ancestral haplotype present in HSCR patients worldwide[47, 48], in combination with the identified chromosome 4 locus might be involved in the high disease penetrance and severity observed in one branch of this multigenerational family. Our suggestive evidence for linkage at chromosome 4 first needs to be confirmed in independent families, otherwise immense sequence efforts to identify the underlying gene might be without positive results.

3. The genetics of syndromic and non-syndromic Hirschsprung disease: Implications for Syndrome Diagnosis and Genetic Counseling

Since roughly 1 in 3 HSCR cases has additional congenital malformations, depending on the diligence with which they are sought [49-51], every child diagnosed with HSCR should at least once be seen by a geneticist-dysmorphologist to rule out recognizable patterns of malformations [52]. Sixty-six entries including HSCR as a clinical feature have been described in the London Dysmorphology database and 52 in the Australian POSSUM.

Lyonnet and Amiel divided HSCR-associated syndromes in 4 groups: (1) neurocristopathy syndromes, (2) syndromes with HSCR as a mandatory feature, (3) occasional or rare associations with recognizable syndromes, and (4) miscellaneous observations [49]. The clinical observation of specific entities with HSCR as a major feature in combination with a myriad of malformations related to the neural crest, suggested a molecular link between these neural crest derivatives hypothesized in 1974 by Bolande [53]. The identification of the genes *EDNRB* [23], *EDN3* [21, 54] and *SOX10* [55] involved in Shah-Waardenburg syndrome (OMIM 277580), proved that the hypothesized concept of neurocristopathies of Bolande indeed has a molecular basis. Thus, group 1 indeed represents a distinctive group of HSCR patients and should make the clinician aware of the possible presence of neural crest related features in every HSCR patient (e.g. neuroblastoma risk in HSCR cases with specific *PHOX2B* mutations) [56]. Group 2 (syndromes with HSCR as a mandatory feature) represents a much more diverse group of HSCR patients. GOSHS is categorized into this group. However, our studies have shown that HSCR is actually a major, albeit not a mandatory feature of GOSHS (chapters 2 and 4). It is barely understood why HSCR occasionally occurs in the syndromes grouped in category 3, such as the genetically heterogeneous Bardet-Biedl syndrome (OMIM 209900) (chapter 5) or the metabolic Smith-Lemli-Opitz syndrome (OMIM 270400) [57-60] One therefore could only speculate that cholesterol metabolism [61, 62] and cilia dysfunction [63] are somehow related to ENS development, which would explain the occurrence of HSCR in some, but not all cases with Smith-Lemli-Opitz syndrome or Bardet-Biedl syndrome, respectively. The variety of syndromes associated with HSCR implies considerable genetic heterogeneity in the etiology of HSCR although some of these syndromic associations may be due to chance, especially in group 4 of miscellaneous observations.

In anticipation of the unravelling of the genetic background of these syndromes, a classification system of syndromic forms of HSCR based on defined developmental pathways (i.e. grouping of clinical disorders by pathway), gene families, or biochemical processes might be preferable.

This classification system could be proposed for every multiple malformation syndrome [64]. In case of syndromic HSCR, patients might be categorized in disorders related to (1) the glial cell-derived neurotrophic factor signaling pathway (*RET* related HSCR/MEN2A), (2) the endothelin signaling pathway (*EDNRB, EDN3, ECE1* related cases), (3) the transforming growth factor β signaling pathway (*ZFHX1B*), and (4) the sonic hedgehog signaling pathway (HSCR related to Smith-Lemli-Opitz syndrome) (see table 1). The syndromes that cannot be placed within a specific pathway are grouped according to either (5) the gene families to which the responsible genes belong (e.g. GOSHS related to *KIAA1279* mutations might be categorized as a syndrome caused by a member of the TPR protein family) or (6) the processes or functions in which the genes are involved (e.g. microtubule motors in case of HSCR related to Bardet-Biedl syndrome or transcription factors in case of *SOX10* and *PHOX2B*). Finally (7) a remaining group should be composed of syndromic HSCR cases without a known gene defect so far. We realize that our attempt to classify syndromic HSCR

cases into these 7 groups is imperfect and also liable to changes in the future. For instance, as the function of KIAA1279 will be unravelled, GOSHS will most likely be categorized in another (new) group of syndromes. The grouping will benefit if a worldwide register will be set up for all the HSCR cases with or without a molecular diagnosis.

Table 1. Proposed classification of syndromes associated with HSCR

Defined Pathways	Genes	Syndromes
1 GDNF signaling	*RET*	MEN2A-HSCR
2 Endothelin signaling	*EDNRB, EDN3*	Shah-Waardenburg syndrome
3 Sonic-hedgehog signaling	*DHCR7*	Smith-Lemli-Opitz syndrome
4 TGF-β signaling	*ZFHX1B*	Mowat-Wilson syndrome
5 Gene family		
TPR	*KIAA1279*	Goldberg-Shprintzen syndrome
6 Gene function		
Microtubule motors	*BBS* genes	Bardet-Biedl syndrome, Kaufman-McKusick syndrome
Transcription factors	*SOX10,*	Shah-Waardenburg syndrome PCWH syndrome
	PHOX2B	Haddad syndrome
7 Remaining group	?	

Although knowledge of the genes involved in HSCR susceptibility and the molecular processes involved in ENS development has grown rapidly over the past years, the impact of this knowledge is still relatively modest in clinical practice. One can question the benefit to the individual patient of testing for mutations predisposing to HSCR. In the Netherlands, genetic diagnostic testing is available for *RET*, *EDNRB, EDN3* and *SOX10*. To date, in our laboratory, 40 mutations of these 4 genes have been found among a total of approximately 250 HSCR cases (personal communication R.M.W. Hofstra). One reason for genetic testing of HSCR patients is to exclude the rare possibility of a MEN2A associated *RET* mutation which carries an elevated risk for MTC [65].

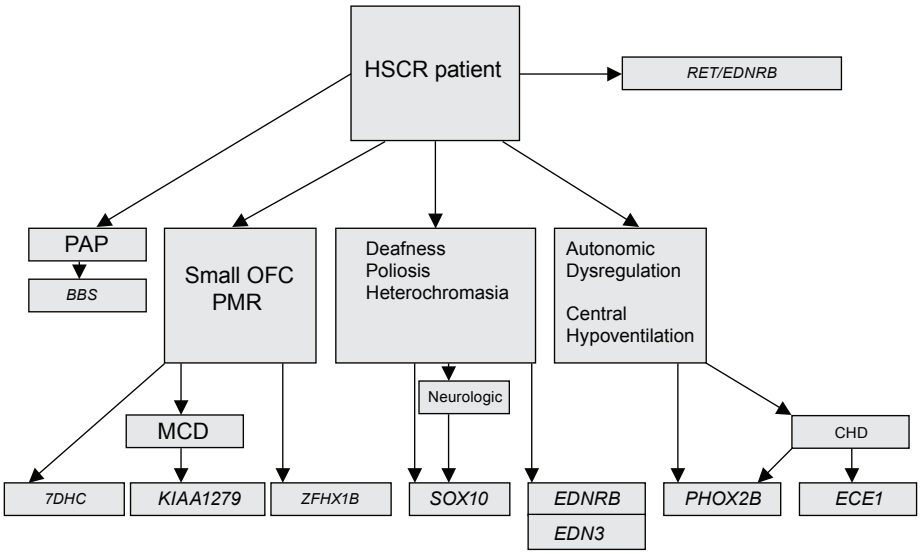
Another reason for genetic testing could be to give more accurate estimation for recurrence risks to parents of a HSCR patient. For example the finding of a pathogenic *RET* mutation in a male proband with L-HSCR and the exclusion of this mutation in the parents may lower the recurrence risk from13-17% to less than 1%, taking into account the theoretical possibility of a mosaic germ line mutation in one of the parents. The risk evaluation in this particular case is only justified by the assumption that *RET* is the major gene in HSCR development. We are not aware of requests for the prenatal diagnosis of a *RET* mutation with the aim of elective termination of the pregnancy, when a mutation was known to segregate in the family. This is probably related to the variable expression and incomplete penetrance of *RET* mutations and the good prognosis after surgical intervention in the majority of HSCR patients [66]. However, in our limited experience, parents who have lost a child with total intestinal aganglionosis or parents of syndromic HSCR cases (e.g. GOSHS) want to discuss options for prenatal diagnosis. In the absence of genetic testing and until all the genes involved in

HSCR genesis are elucidated, the risk estimation can be calculated from the segregation analysis by Badner [67]. At present the common risk *RET* haplotype [47] has no clinical implications; risk assessment by genetic risk profiling is not feasible because of unknown additional risk factors in HSCR.

Routine screening of the 10 known genes in every HSCR patient is time-consuming, expensive (the current cost for DNA diagnostics is 640 euro per gene in the Netherlands) and has a limited yield. We, therefore, propose the following guidelines for the practicing pediatrician, pediatric surgeon or clinical geneticist when dealing with a patient with HSCR. Both in non-syndromic sporadic (especially LS-HSCR) and familial HSCR cases, routine *RET* mutational screening is feasible [68]. However, before sampling and mutational analysis take place, genetic counseling should also address the issue of the co-occurrence of MEN2A and HSCR in occasional families [65] and how to deal with the follow-up when a MEN2A related *RET* mutation is identified [69]. Oncological and genetic counseling should always complement the management of such affected families. Routine screening of *GDNF* and *Neurturin*, both encoding ligands of the RET protein and therefore considered plausible functional candidates, is not indicated since these genes are mutated only rarely [70, 71]. Routine mutational screening of *EDNRB* in sporadic isolated HSCR cases should be considered, since it is the second most mutated gene in this group [72-74].

A scheme showing which genes to test for mutations in HSCR patients with and without additional malformations and dysmorphisms is proposed (fig 1).

Figure 1



PAP= postaxial polydactyly, OFC=occipito-frontal circumference,
PMR=psychomotor retardation; MCD=malformation of cortical development,
CHD= congenital heart defect
7DHC= 7-dehydrocholesterol (cholesterol precursor)

When a patient has additional abnormalities that can be classified as neural crest related (pigmentary abnormalities such as heterochromia of the irises, a white forelock, or sensorineural deafness) testing for *EDNRB* and *EDN3* mutations is justified. Shah-Waardenburg patients with an extended

neurological phenotype (e.g. a neuropathy) should be tested for mutations in *SOX10* [55, 75]. This latter phenotype is designated as PCWH (OMIM 609136); peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and HSCR [76]. When symptoms of autonomic nervous system dysregulation including congenital central hypoventilation are diagnosed [77], *PHOX2B* mutation analysis is advisable [78]. It is estimated that up to 30% of HSCR patients may have some form of dysautonomia [79] and approximately 1.5% of HSCR patients will have Congenital Central Hypoventilation Syndrome or Haddad syndrome (OMIM 209880). *PHOX2B* mutation analysis is also warranted in HSCR-neuroblastoma cases [56, 80]. Interestingly, analysis of genotype-phenotype interactions strongly supported the contention that patients with Congenital Central Hypoventilation Syndrome who develop malignant tumours of the sympathic nervous system will harbor either a missense or a frameshift heterozygous mutation instead of a polyalanine expansion mutation of the *PHOX2B* gene [56]. Pediatric surgeons should be aware of this specific association and may be alerted to perform strict follow-up protocols in this subgroup of HSCR patients (screen catecholamines in suspected neuroblastoma) [81].

Screening of *ZFHX1B* and *KIAA1279* is indicated in every HSCR patient with microcephaly and/or mental retardation. Without the help of pedigree information consistent with a clear-cut autosomal recessive inheritance discriminating between these 2 syndromes/genes might be difficult in a young child [82-84]. Clearly, additional minor facial features might help to decide which gene should be screened first. While high arched eyebrows are consistent with mutations in *KIAA1279* (chapters 2 and 4), the configuration of the eyebrows is horizontal and shows medial flaring in Mowat-Wilson syndrome [18, 85, 86]. Furthermore epilepsy is frequently reported in Mowat-Wilson cases[18], but so far it has been reported only once in our case series of patients with mutations in *KIAA1279* (chapter 4). We have proven that HSCR in GOSHS is a major, yet not a mandatory feature (chapter 4). Now the *KIAA1279* gene has been found to be associated with this syndrome, a clearer picture will emerge of what constitutes the core phenotype and its variants (chapter 3). We propose that a malformation of cortical development (diffuse polymicrogyria or a simplified gyral pattern) might be part of the core phenotype, but that this feature is probably underreported because of the lack of detailed MRI studies in all microcephalic or retarded HSCR cases (chapter 4). Therefore, the recommendation would be that every child with HSCR and mental retardation and/or microcephaly is referred to a pediatric neurologist and that an indication for MRI of the brain is established. Given the fact that Smith-Lemli-Opitz syndrome cases might also present with HSCR and microcephaly, plasma cholesterol levels should be measured in these cases. The facial Gestalt is striking; apart from microcephaly, Smith-Lemli-Opitz patients have bilateral ptosis, a broad and high forehead, a long philtrum and anteverted nares. These typical facial features together with structural defects of internal organs (e.g.HSCR) make that experienced dysmorphologists will recognize Smith-Lemli-Opitz patients easily.

The combination of postaxial polydactyly (PAP) and HSCR warrants a careful clinical follow-up during childhood; these children may develop retinopathy and represent Bardet-Biedl syndrome. Therefore, in a limited number of HSCR cases with PAP, screening of the *BBS* genes is advisable (chapter 5), especially in those cases with other features such as renal abnormalities and learning disabilities. However, because of the extensive genetic heterogeneity of Bardet-Biedl syndrome, mutation analysis will often be restricted to *BBS* genes involved in specific ethnic groups (e.g. *BBS1* screening in Caucasians) [63, 87].

Although an *ECE1* mutation was reported only once so far [22], one might consider *ECE1* mutational analysis in a HSCR patient with a complex heart defect after exclusion of a 22q11.2 deletion by FISH [88].

Provided that fruitful collaborations between departments of clinical genetics and pediatric surgery continue, HSCR patients in the coming years will benefit from further elucidation of the genes involved in HSCR susceptibility. Identification of these genes is of major importance for disease prognosis and accurate genetic counseling. Finally, a complete understanding of the genetic mechanisms that restrain the development of the ENS could have a major impact on clinical practice.

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Summary

Samenvatting

Summary

In *Chapter 1*, the genetic aspects of Hirschsprung disease are reviewed. With a prevalence of one in 5000 live born children, Hirschsprung disease is the most frequent cause of bowel obstruction in childhood, and it occurs worldwide. Neonates born with Hirschsprung disease are unable to pass their first stool, and as a consequence their bowels get distended ("congenital megacolon"). This congenital malformation is characterized by the absence of groups of neurons or nerve cells in the bowel wall. The role of inheritance in Hirschsprung disease was indicated by familial occurrence, male predominance (boys are affected four times as often as girls), the association with chromosomal aberrations such as trisomy 21, and a myriad of inherited syndromes caused by single gene defects. The search for genes involved in Hirschsprung disease has been successful; so far 9 genes (*RET, GDNF, Neurturin EDNRB, EDN3, ECE1, SOX10, ZFHX1B, and PHOX2B*) had been implicated in Hirschsprung disease. *RET* proved to be the major gene involved in Hirschsprung disease susceptibility. These 9 genes, however, do not explain the majority of patients with Hirschsprung disease yet.

The aim of our studies was to identify new genes or loci involved in Hirschsprung disease susceptibility by studying non-syndromic as well as syndromic Hirschsprung disease families, treated at the Pediatric Surgery Department of the Erasmus MC-Sophia Children's Hospital. Part 1 of this thesis (chapters 2-5) concerns the clinical and molecular characterization of families in which Hirschsprung disease is associated with additional congenital malformations. *Chapter 2* describes the study of a consanguineous Moroccan family with a rare autosomal recessive form of syndromic HSCR, Goldberg-Shprintzen syndrome. First described by Robert Shprintzen and Rosalie Goldberg in 1981 in a brother and a sister, this syndrome is characterized, apart from Hirschsprung disease, by mental retardation, a small head circumference (microcephaly) and typical facial features (e.g. high-arched eyebrows). We showed that Hirschsprung disease is a major, albeit not a mandatory feature of Goldberg-Shprintzen syndrome. We suggested that this inbred Moroccan family might be instrumental to identify the Goldberg-Shprintzen gene.

In *Chapter 3* we performed a genome-wide scan and homozygosity mapping in the consanguineous Moroccan family with Goldberg-Shprintzen syndrome. The family was considered suitable for homozygosity mapping. The basic concept of this method is to use the information provided by a genome-wide scan in inbred affected individuals in order to define a region of the genome where they are all homozygous. This homozygous region likely contains the mutated gene causing the disease. We demonstrated homozygosity of 10q22.1 and successively identified a homozygous truncating mutation in *KIAA1279* gene, one of the 30 genes located in this shared homozygous region. Identification of the gene defect causing this rare disorder in only 4 affected children from one family, underlined the high power of this study design. Since bilateral generalized polymicrogyria (a developmental disorder of the cerebral cortex or grey matter of the brains) was diagnosed in all patients in this family, we proposed that this feature might be considered a key feature of the syndrome.

Chapter 4 focused on the phenotypic diversity of *KIAA1279* mutations. We identified mutations in 3 new Goldberg-Shprintzen syndrome families. No *KIAA1279* mutations were identified in 26 sporadic, non-syndromic patients with Hirschsprung disease, in 14 individuals that were initially diagnosed as possible Mowat-Wilson syndrome cases or in 6 cases with polymicrogyria. Hirschsprung disease occurred in 81% of the affected cases with Goldberg-Shprintzen syndrome. Importantly, MRI studies of the eldest Goldberg-Shprintzen patient known to be alive to date showed a simplified gyral pattern and diffuse white matter loss. We concluded that malformations of cortical development represent core features of Goldberg-Shprintzen syndrome. Consequently,

we recommended that every child with Hirschsprung disease and mental retardation and/or microcephaly be referred to a pediatric neurologist and undergo brain MRI. Neurological and ophthalmologic complications might develop later; all older children showed progressive scoliosis and corneal infections. Follow-up of the original two cases reported by Goldberg and Shprintzen confirmed this observation. Since not all Hirschsprung disease cases associated with microcephaly and mental retardation can be attributed to mutations in *KIAA1279* or *ZFHX1B*, there must exist other genes whose mutations can lead to this specific combination of features.

Chapter 5 entailed clinical and molecular studies in a family with a sister and a brother affected with the combination of Hirschsprung disease and a syndrome representing McKusick-Kaufman syndrome or Bardet-Biedl syndrome. Both are rare autosomal recessive syndromes with overlapping clinical and molecular spectrum. Sequencing 3 of the 9 genes *BBS* genes did not reveal any mutations. Mutation scanning of *RET*, revealed a sequence variant Lys716Asn in the affected girl, but not in the affected brother. It is uncertain whether this variant contributes to Hirschsprung disease in this family.

Part 2 of the thesis (*Chapter 6*) is concerned with non-syndromic or isolated Hirschsprung disease. In *Chapter 6* the study of a multigenerational family with Hirschsprung disease segregating in two main branches, allowed us to provide suggestive evidence for the existence of a novel Hirschsprung susceptibility locus on chromosome 4q. We speculated that the penetrance in this family might be RET-dependent.

Chapter 7 is a general discussion on strategies for finding genes for Hirschsprung disease susceptibility and on our findings. Additional Hirschsprung disease susceptibility genes might be identified in the future, although the search for these genes might turn out to be difficult as the individual effects might be small.

Identification of the genes involved in Hirschsprung disease is of major importance for disease prognosis and accurate genetic counselling. As routine screening of the current genes known to be involved in Hirschsprung disease is not feasible, we propose guidelines for the practicing pediatrician, pediatric surgeon or clinical geneticist when dealing with a patient with Hirschsprung disease. Furthermore, a classification system based on defined developmental pathways for syndromes associated with Hirschsprung disease is proposed. Provided that fruitful collaborations between departments of clinical genetics and pediatric surgery continue, HSCR patients in the coming years are bound to benefit from further elucidation of the genes involved in Hirschsprung disease susceptibility.

Samenvatting

In *Hoofdstuk* 1 (Introduction) wordt een overzicht gegeven van de genetische aspecten van de ziekte van Hirschsprung. De ziekte van Hirschsprung is wereldwijd de meest frequente oorzaak van een darmobstructie op de kinderleeftijd; de frequentie wordt geschat op 1:5000 levendgeborenen. Kinderen die met de ziekte van Hirschsprung worden geboren, hebben vaak een vertraagde meconiumlozing, en dientengevolge ontstaat er een bolle buik met uitgezette darmen ("congenitaal megacolon"). Deze aangeboren aandoening wordt gekenmerkt door de afwezigheid van ganglioncellen (groepen van neuronen) in de darmwand. Vele factoren wezen erop dat erfelijkheid een rol speelt bij het ontstaan van de ziekte van Hirschsprung; het optreden van de ziekte van Hirschsprung bij meerdere leden van een familie, het feit dat jongens vier maal zo vaak aangedaan zijn dan meisjes, de associatie met chromosoom afwijkingen (bijvoorbeeld trisomie 21) en tal van monogene syndromen. De zoektocht naar genen betrokken bij de ziekte van Hirschsprung is zeer succesvol gebleken; tot nog toe kon worden aangetoond dat 9 genen (*RET, GDNF, Neurturin EDNRB, EDN3, ECE1, SOX10, ZFHX1B,* en *PHOX2B*) betrokken zijn. Van het *RET* gen is bewezen dat het de belangrijkste genetische aanlegfactor is voor de ziekte van Hirschsprung. Echter, mutaties in deze 9 genen, verklaren slechts een fractie van alle patiënten.

Ons onderzoek had als doel om nieuwe genen of loci betrokken bij de ziekte van Hirschsprung te identificeren, door zowel families met niet-syndromale als syndromale vormen van de ziekte van Hirschsprung te bestuderen. De kinderen met de ziekte van Hirschsprung uit deze families werden behandeld op de afdeling kinderchirurgie van het Sophia Kinderziekenhuis/ ErasmusMC te Rotterdam. Deel 1 van dit proefschrift (*Hoofdstukken 2-5*) omvat de klinische en moleculaire karakterisering van families waarin de ziekte van Hirschsprung voorkomt met andere aangeboren afwijkingen. In *Hoofdstuk 2,* wordt een consanguine Marokkaanse familie met het Goldberg-Shprintzen syndroom, een zeldzame autosomaal recessieve syndromale vorm van de ziekte van Hirschsprung, beschreven.Dit syndroom werd voor het eerst beschreven bij een broer en een zus door Robert Shprintzen en Rosalie Goldberg in 1981. Het Goldberg-Shprintzen syndroom wordt niet alleen gekenmerkt door de ziekte van Hirschsprung, maar ook door een ontwikkelingsachterstand, een kleine schedelomtrek (microcefalie) en opvallende gelaatskenmerken (waaronder hoog-gebogen wenkbrauwen). Wij toonden aan dat de ziekte van Hirschsprung een belangrijk, echter geen obligaat kenmerk is van het Goldberg-Shprintzen syndroom. Wij meenden dat genetisch onderzoek in deze bloedverwante familie zou kunnen leiden tot de identificatie van het genetische defect in het Goldberg-Shprintzen syndroom.

In *Hoofdstuk 3* verrichtten wij een scan van het genoom en homozygotie mapping in de consanguine Marokkaanse familie, beschreven in *Hoofdstuk 2*. Vanwege de bloedverwantschap tussen de ouders van de aangedane kinderen, meenden wij dat deze familie geschikt was voor homozygotie mapping. Het basisprincipe van deze methode is dat men gebruik maakt van de informatie verkregen uit de scan van het genoom bij aangedane kinderen van ouders die bloedverwant zijn, om zo een regio in het genoom af te bakenen waar alle patiënten homozygoot zijn. Deze overeenkomende homozygote regio bevat dan zeer waarschijnlijk het gen met de mutatie welke tot de ziekte heeft geleid. Wij toonden aan dat alle patiënten homozygoot waren voor een deel van chromosoom 10 (10q22.1). Vervolgens identificeerden wij een homozygote truncerende mutatie in *KIAA1279*, één van de 30 genen in de homozygote regio. Het feit dat het mogelijk was om met slechts 4 aangedane kinderen afkomstig uit 1 familie het genetische defect op te sporen, illustreerde de kracht van deze studie opzet.

Aangezien bilaterale gegeneraliseerde polymicrogyrie (een ontwikkelingsstoornis van de cerebrale cortex of grijze stof van de hersenen) in alle patiënten van deze familie werd gediagnosticeerd, meenden wij dat dit mogelijk een hoofdkenmerk van het syndroom betrof.

Hoofdstuk 4 is gericht op de fenotypische diversiteit van *KIAA1279* mutaties. Wij identificeerden mutaties in 3 nieuwe families met het Goldberg-Shprintzen syndroom. *KIAA1279* mutaties werden niet aangetoond bij 26 sporadische niet-syndromale patiënten met de ziekte van Hirschsprung, noch bij 14 kinderen bij wie eerder het Mowat-Wilson syndroom werd vermoed, noch bij 6 kinderen met polymicrogyrie. In de families met het Goldberg-Shprintzen syndroom en een *KIAA1279* mutatie, had 81% van de patiënten de ziekte van Hirschsprung. Van belang was dat MRI onderzoek van de hersenen bij de oudst levende patiënt met het Goldberg-Shprintzen syndroom, een "simplified gyral pattern" en een diffuus verlies van witte stof liet zien. Wij concludeerden dat malformaties van de ontwikkeling van de hersenschors hoofdkenmerken zijn van het Goldberg-Shprintzen syndroom. Om die reden, adviseren wij dat elk kind met de ziekte van Hirschsprung en een ontwikkelingsachterstand en/of microcefalie, verwezen wordt naar een kinderneuroloog en dat MRI onderzoek van de hersenen wordt verricht. Neurologische en oftalmologische complicaties ontstaan mogelijk op oudere leeftijd; alle oudere kinderen met het Goldberg-Shprintzen syndroom ontwikkelden een progressieve scoliose en infecties van de cornea. Follow-up van de 2 kinderen die gepubliceerd werden door Goldberg en Shprintzen bevestigde deze waarneming. Aangezien niet alle gevallen van de ziekte van Hirschsprung in combinatie met microcefalie en ontwikkelingsachterstand toegeschreven kunnen worden aan mutaties in *KIAA1279* of *ZFHX1B,* is het zeer waarschijnlijk dat er andere genen bestaan waarin mutaties kunnen leiden tot deze specifieke combinatie van kenmerken.

Hoofdstuk 5 betrof klinische en moleculaire studies in een familie met een zus en broer met de combinatie van de ziekte van Hirschsprung en een syndroom; het Kaufman-McKusick syndroom dan wel het Bardet-Biedl syndroom.Beide syndromen zijn zeldzame autosomaal recessieve syndromen met een overlappend klinisch en moleculair spectrum. Sequentie analyse van 3 van de 9 genen betrokken bij het Bardet-Biedl syndroom kon geen mutaties aantonen. Mutatie analyse van het *RET* gen toonde echter een verandering (Lys716Asn) aan bij het meisje, maar niet bij haar broer. Het is niet zeker dat deze variant een bijdrage levert aan het ontstaan van de ziekte van Hirschsprung in deze familie.

Deel 2 van dit proefschrift (*Hoofdstuk 6*) is gericht op de niet-syndromale vorm van de ziekte van Hirschsprung. *Hoofdstuk 6* beschrijft een studie van een familie waar in meerdere generaties (in 2 takken) de ziekte van Hirschsprung voorkomt. Deze familie stelde ons in staat om een nieuw gen voor de ziekte van Hirschsprung te lokaliseren op de lange arm van chromosoom 4. Het bewijs hiervoor was niet significant, maar wel suggestief. Wij speculeerden dat de penetrantie in deze familie mogelijk *RET*-afhankelijk is.

Hoofdstuk 7 (General discussion and future considerations) bevat een algemene discussie over de strategieën om genen betrokken bij de ziekte van Hirschsprung te identificeren en over onze bevindingen. Additionele genen betrokken bij de ziekte van Hirschsprung zullen zeer waarschijnlijk worden ontdekt in de toekomst, echter het vinden van deze genen zou wel eens moeilijk kunnen blijken te zijn, aangezien de individuele effecten klein zijn.

De identificatie van deze genen is van groot belang voor de prognose en accurate erfelijkheidsadvisering. Aangezien het routinematig screenen van de huidige set van genen betrokken bij de ziekte van Hirschsprung niet mogelijk is, stellen wij richtlijnen voor die in de praktijk gebruikt kunnen worden door de kinderarts, kinderchirurg of klinisch geneticus wanneer

zij een kind met de ziekte van Hirschsprung treffen. Bovendien trachten wij een voorstel te doen voor een classificatiesysteem voor syndromen geassocieerd met de ziekte van Hirschsprung. Dit classificatiesysteem is gebaseerd op wat bekend is over de functie van de betrokken eiwitten in de ontwikkeling van het embryo.

Mits vruchtbare samenwerkingsverbanden tussen kinderchirurgische en klinisch genetische afdelingen zullen voortbestaan, zullen kinderen met de ziekte van Hirschsprung ongetwijfeld hiervan profiteren in de komende jaren.

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Alice

Curriculum Vitae

Alice Brooks was born on December 3rd, 1968, in Apeldoorn, the Netherlands, as the second twin daughter of Yvonne Der Weduwen and Hugo Brooks. She completed her gymnasium β education at the "Waterlant College" in Amsterdam and started her medical training at the University of Amsterdam in 1988. As a student she took part in clinical research on the Fragile X syndrome in Hooge Burch, a center for the mentally disabled (supervisor Prof. H.M. Evenhuis). She obtained her medical degree (cum laude) in April 1996. She worked as a resident in Clinical Genetics (heads: Prof. H. Galjaard, Prof. J.W. Wladimiroff) at the department of Pediatric Surgery ErasmusMC- Sophia Children's Hospital, Rotterdam, where she started the work described in this thesis. January 2000, she started her training as a clinical geneticist and researcher (AGIKO) at the department of Clinical Genetics (instructors Prof. M.F. Niermeijer, Dr. E.J. Meijers-Heijboer). Her traineeship included a three-month clinical fellowship at the Center for Human Genetics, Leuven, Belgium (head: Prof. J.P. Fryns) and a five-month research fellowship at the department of Medical Genetics, University of Groningen (head: Prof. C.H.C.M. Buys, supervisor Prof. R.M.W. Hofstra). Alice Brooks will be registered as a clinical geneticist in December 2005. She is married to Jurgen Wegman.

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