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Tissue specific somatic mutations and aganglionosis in Hirschsprung's disease

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ABSTRACT

Background: RET proto-oncogene intron 1 variations [e.g. SNP1 (rs2506004) and SNP2 (rs 2435357)] have been shown to be etiologically important in the pathogenesis of Hirschsprung's disease (HSCR). Although activating somatic RET rearrangements have been identified in certain tumours, this is the first study to confirm somatic gene variation in HSCR.

Methods: DNA was extracted from 53 paraffin embedded tissue samples (HSCR patients n = 33, multiple levels n = 17), and controls (n = 3). Patients were grouped into aganglionic (Group 1), ganglionated (group 2), and transitional (group 3). PCR products of *RET* intron 1 were screened for genetic variation by semi-automated bi-directional sequencing analysis and matched to unaffected controls from the general population. Comparison was by Fishers exact test. $P < 0.05$ was regarded as significant.

Results: HSCR patients included short segment (n = 26), long segment colonic [(n = 4 (24%)], and total colonic aganglionosis (n = 3). RET intronic variations [SNP1 (rs2506004) or SNP2 (rs 2435357)] showed somatic homozygous in affected tissue in 9/12 (75%) Group 1 (aganglionic tissue) compared with 2/5 (40%) and 1/10 (10%) of groups 2 and 3 ($P < 0.001$). Homozygous SNP2 variation was observed in all long segment versus 4/10 short segment. 50% of the short segment cases showing homozygous SNP 1 variation.

Conclusion: We report somatic mutations in the RET intron 1 region of affected HSCR tissue, confirming for the first time that somatic mutations are present in aganglionic tissue and may promote local aganglionosis through deregulated receptor activity. Detailed understanding of the somatic genetic events that drive congenital aganglionosis may have bearing on diagnosis and therapy.

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Hirschsprung's disease (HSCR) has a complex pathogenesis as shown by the number of genes implicated — at least 11 genes and 5 gene loci in the germline have been reported to be associated with HSCR [1]. The reported genetic associations with HSCR are significant because they give insights into the abnormal genetic signalling during ENS development, thus giving clues as to the genetic mechanisms involved in pathogenesis.

The RET gene encodes a receptor tyrosine kinase essential for development of the enteric nervous system and kidney and appears to be the major gene involved in Hirschsprung's disease (HSCR) development. The centrality of the RET proto-oncogene in the pathogenesis of both activating and inhibiting conditions is now well established with RET playing a pivotal role in at least 4 clinical syndromes (HSCR, MEN2 A and B and Familial Medullary thyroid carcinoma (FMTC)) in a unique "switch on", "switch off" manner [2]. Somatic RET mutations have been described in hereditary tumours, particularly those occurring within the thyroid and parathyroid and multiple endocrine neoplasia type 2 [3]. Furthermore, the presence of somatic RET proto-oncogene mutations appear to correlate with a worse outcome in these tumours [4].

Current knowledge includes both infrequent RET gene coding sequence variations occurring throughout the gene as well variants located in an enhancer section which appears to be important predisposing factors in HSCR [5,6]. In particular, the RET intron 1 variations [e.g. SNP1 (rs2506004) and SNP2 (rs 2435357)] have been shown to be etiologically important.

Much of the genetic research to date has concentrated on germ-line variations of the involved genes. This, however, does not fully explain the variable nature of the disease especially in terms of the variability of the length of the aganglionic zone (particularly the extended forms).

The aim was to investigate tissue sample DNA in HSCR and compare histological findings with local genetic variations in order to provide further clues as to its pathogenesis.

1. Methods

DNA was extracted from paraffin embedded tissue samples from patients with histologically proven HSCR. Tissue samples were histologically grouped into aganglionic (Group 1); ganglionated (group 2) and transitional histological groups (group 3). DNA was extracted by standard techniques from cut 10×5 μ sections which were deparaffinated according to the method of Min et al. [7] and the DNA

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Table 1
Tissue SNP1 variation in HSCR tissue at ganglionated, transitional and aganglionic levels.

	Short segment (S-HSCR)	S-HSCR (Hom)	Long segment (L-HSCR)	L-HSCR (Hom)	TCA	TCA (Hom)	TOTAL	Total (Hom)
Aganglionic	10	5 (50%)	1	0	2	0	18	5 (42%) P < 0.05[a]
Transitional	4	1 (25%)	1	0			5	1 (20%)
Ganglionic	10						10	0
Total pts	24	6	2	0			33	6 (19%) N.S.
Control							3	0

Notes.
HSCR = Hirschsprung's disease, TCA = total colonic aganglionosis, Hom = homozygous.
[a] Fisher's Exact test.

extracted using an established extraction buffer [10 mM Tris (ph 8.3), 1 mM EDTA, 0.5% Triton X-100, 0.001% SDS (w/v), 500 μg/ml Proteinase K] [8]. The enzyme was then inactivated and the DNA stored at 4 °C.

Following DNA extraction, PCR products of *RET* intron 1 [i,e. SNP1 (rs2506004) and SNP2 (rs 2435357)] were screened for genetic variation by semi-automated bi-directional sequencing analysis and matched to unaffected controls from the general population. Statistical comparison between the groups was by Fishers exact test. A P value of < 0.05 was regarded as satisfactory.

2. Results

DNA was extracted from 53 tissue sections, 50 of which were from 33 HSCR patients (17 >1 level/patient) and 3 controls (specimens of normal colon taken at colonic interposition). Of the HSCR patients, 4 (24%) had long segment HSCR, and a further 3 had total colonic aganglionosis (TCA). DNA investigation for RET intronic variations [SNP1 (rs2506004) and SNP2 (rs 2435357)] showed mainly heterozygous SNP variation in ganglionated and transitional tissue whereas homozygous variation was observed in many aganglionic tissue specimens. Whereas control specimens showed no variation for either SNP, SNP 1 was noted to lose an allele in 50% of short segment cases changing from heterozygous in ganglionated and transitional tissue to homozygous in aganglionic tissue ($P < 0.05$). (Table 1). Homozygous intronic SNP2 *RET* variations were similarly identified in 9/12 (75%) of Group 1 (aganglionic tissue; 3 colonic aganglionosis; 2 TCA) whereas only 2/5 (40%) and 1/10 (10%) of groups 2 and 3 had the homozygous loss of allele ($P < 0.001$) (Table 2). All 7 patients with long segment aganglionic showed homozygous SNP2 variation as opposed to 4 out of 10 in the short segment aganglionic patients. In 3 individuals where sections of all 3 levels were available from the individual patients, the ganglionated and transitional bowel was heterozygous whereas aganglionic tissue showed homozygous SNP2 variation.

3. Discussion

The genetic origin of HSCR is complex and is attributed to loss of function germline mutations in a number of genes involved in the embryonic development of the enteric nervous system (ENS). Although these neuro-developmental genes number at least 11, 9 of them are related to two major susceptibility gene signalling pathways [viz: the REarranged during Transfection [RET (RET; GDNF; GFRα; NTN) signalling cascade and the Endothelin B receptor related pathways (EDNRB; EDN-3; ECE-1; PHOX2B and SOX10)]. The importance of the 2 described SNP's in Intron 1 (SNP1 (rs2506004) and SNP2 (rs 2435357) and HSCR pathogenesis, has been emphasized.

The genetic influence appears to vary in terms of the length of the affected segment, long segment Hirschsprung's disease and total colonic aganglionosis. As a result, the pathogenetic connection of a number of identified genes to HSCR is not as yet fully understood and low-penetrance polymorphisms have also been identified in other genes. These include polymorphisms (SNPs) within RET, NRG1 and possibly TCF4 genes which are thought to possibly act as genetic modifiers. Potential gene effects in the pathogenesis of HSCR within these pathways, include loss of function, apoptosis, aberrant splicing and decreased gene expression [9]. Although activating somatic RET rearrangements have been identified in certain tumours this is the first study to confirm somatic gene variation in Hirschsprung's disease by comparison of tissue sample DNA with germline observations.

It is now well established that in the developing ENS, the RET gene product and the allied GDNF family neurotrophic factors [mainly GDNF and GDNF receptor alpha1 (GFRalpha1)] stimulate the proliferation of enteric neural crest cells by activating numerous signaling pathways to determine ENS development [10,11]. Variations of the *RET* proto-oncogene, appear the most significant genetic association with hypomorphic deficits, being reported in up to 70% of patients [1,12–14]. This genetic variation occurs throughout the coding region of the gene, but more recently, strong associations have been reported for six markers in the 5' region of the RET proto-oncogene [15] and as a result, certain haplotypes and variations within intron 1 and promoter region of the *RET* proto-oncogene have been proposed as a highly significant causative associations [5,15,16]. This suggests an increased risk of HSCR in homozygotes for this haplotype (Odds ratio > 20) [15,17], which was found in one study to be present in 55.6% of HSCR patients versus 16.2% of controls [18]. Two common functional *RET* variants [viz: SNP1 (rs2506004) and SNP2: RET + 9.7 (rs2435357: C > T); 10q11.2] that lie within Intron 1 of this region have been shown to be associated HSCR, which have been reported in association with Down syndrome (DS) [6,19] where there is a well established Hirschsprung association (DS-HSCR) [20–23].

Table 2
Table showing tissue SNP2 (rs 2435357) variation in HSCR tissue at ganglionated, transitional and aganglionic levels.

	Short seg (SHSCR)	S-HSCR (Hom)	Long seg (L-HSCR)	L-HSCR (Hom)	TCA	TCA (Hom)	TOTAL	Total (Hom)
GROUP 1 (Aganglionic)	10	4 (40%)	4	4 (100%)	3	3 (100%)	17	11 (65%) p < 0.01[a]
GROUP 2 (Transitional)	4	1	1	1 (100%)			5	2 (40%)
GROUP 3 (Ganglionated)	10	1	1				11	1 (9%)
Total pts	24	5	6		3	3	33	13 (39%) NS[a]
CONTROLS							3	0

Notes.
HSCR = Hirschsprung's disease, TCA = total colonic aganglionosis, Hom = homozygous.
[a] Fisher's Exact test.

This study shows that the SNPs 1 and 2 both lose an allele within the affected tissue to become homozygous in the affected HSCR tissue in a statistically significant fashion ($P < 0.001$). The heterozygous state is probably the germline predisposition which exists in the normally ganglionated and most of the transitional material, whereas the loss of an allele would tend to explain the localized nature of the pathology.

Thirdly, of the intronic SNPs tested, SNP2 appears to be more specific for long segment aganglionosis than SNP1 which appeared as homozygous in 50% of recto-sigmoid aganglionosis. As such, it may explain some of the increased genetic penetrance encountered in long segment aganglionosis.

Genetic variations such as single nucleotide polymorphisms (SNPs) are currently under study and are thought to be important in determining the biological basis of complex genetic conditions and diseases like HSCR. This study shows the significance of the intron 1-located RET SNPs (rs2506004; SNP1; AND C > T rs2435357 (SNP2)) in the hypomorphic RET expression and thus the maldevelopment of ENS neuroblasts in HSCR [18]. As opposed to germline gene variations (already established as being pathogenetic in HSCR), somatic gene mutations or variations represent acquired changes in DNA which take place after conception. Our findings in this study, suggest that these somatic mutations in the RET proto-oncogene may promote local aganglionosis through deregulated receptor activity. In hereditary RET-related tumours, somatic RET mutation has been shown to correlate with a worse outcome [4], suggesting increased gene penetrance. Recent analysis of genetic influences in phaeochromocytoma have shown that Loss of heterozygosity (LOH) of the known susceptibility genes (including RET) was the most frequent second allele inactivating event [24].

In tumour formation, germline gene variation which might predispose to the condition does not appear to lead to the tumour development unless a permissive microenvironment is also present [25]. Genes would thus require a permissive microenvironment to create local changes. A similar situation may be present in HSCR where local changes in microenvironment may result in or cause a somatic mutation thus “modifying” the germline genetic variation to achieve pathogenicity. In support of this, the somatic mutation theory now encompasses a wide spectrum of genes from suppressor genes, epigenetic inheritance to further mutations which are the basis of “modifier” genes and the multi-hit hypothesis of genetic disease.

Increased understanding of the microenvironment into which neuronal cells migrate shows that there are molecular cues which have established roles in controlling the fate of the migrating neuroblasts as well as controlling the migration of cells and the development of connecting axons within the tissue [26]. This suggests that micro-environmental changes may influence the inability of the colon to colonize neural crest derived cells. The “ground substance or local microenvironment” into which the developing neuroblasts migrate, includes the substrate or extracellular matrix, cell–cell interactions, mechanical forces, and soluble factors, as well as the local cellular architecture. These molecular cues have been shown to be disrupted in HSCR and may be the cause of premature neurite formation and inhibition of migration of enteric neuroblasts which has been shown to be promoted by the excessive secretion of laminin in the enteric mesenchyme of an ls/ls murine model [17,27].

The fact that these somatic mutations have been shown in critical areas of the RET proto-oncogene, strongly supports the argument that local changes in the ECM influence gene expression and neuroblast development. A more detailed understanding of the somatic genetic events that drive congenital aganglionosis is necessary as it may have bearing on diagnosis and therapy.

In summary, we present evidence of somatic mutation and the loss of a second allele of a known causative genetic variation of the RET proto-oncogene in the affected tissue in Hirschsprung's disease and suggest that it may be an important reflection of the pathophysiology of this condition. Our findings suggest that somatic mutations in the RET proto-oncogene may promote local aganglionosis through deregulated receptor activity. It also suggests that local micro-environmental factors may play a role in determining the phenotypic expression of the condition. A more detailed understanding of the somatic genetic events that drive congenital aganglionosis is necessary as it may have bearing on diagnosis and therapy.

Somatic variation of Intron 1 SNP in HSCR tissue appears important in the pathogenesis of HSCR. Both SNPs 1 and 2 somatically lose an allele and become homozygous in aganglionic HSCR tissue. SNP1 becomes homozygous in 50% of short segment HSCR ($P < 0.05$) whereas SNP2 loses an extra allele in 100% of long segment aganglionosis and total colonic aganglionosis cases investigated. This suggests a second “hit” within the affected tissue possibly on the basis of a localized micro-environmental influence. A more detailed understanding of the somatic genetic events that drive congenital aganglionosis is necessary as it may have bearing on diagnosis and therapy.

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Discussion

Discussant: Paul Tam (Hong Kong): Would you like to speculate as to when the somatic mutation occurred during development – was it at embryonic stage or nearer to term?

Response: Prof. Moore: Our feeling is it is something to do with the ground substance- laminin – this would be early on when the scaffolding for the gut is affected, but if it is a genetic one, and we haven't picked anything up yet at that level, well that would be early on too.

Paul Tam: Since you are postulating on the micro-environment, a factor that a lot of us are interested in, do you have data to differentiate between neural and non-neural tissue? That is do you find your somatic variation in the nerves or in the mesenchyme?

Response: Prof. Moore: We haven't done the immunochemistry yet but we will.