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Do RET and APC Crosstalk in Hirschsprungs Disease Pathogenesis?

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Abstract: Although a number of genes have been shown to be involved in the pathogenesis of Hirschsprung's disease (HSCR), the RET proto-oncogene and the Endothelin receptor B (EDNRB) genes remain the major susceptibility genes. The final phenotypic expression appears to depend on gene-gene interaction but crosstalk with the APC/Wnt system has not previously been described. We report a case of short segment HSCR where mutational analysis showed both RET (V202M) and adenomatous polyposis coli (APC) (E1317Q) mutations. Several additional RET (A45, L769) and EDNRB (831G/A) polymorphisms but no EDN3 sequence variants were identified. We report the first association of the APC gene as a possible modifier locus with HSCR and explore possible mechanisms of action.

Key words: Hirschsprung's disease, polymorphism, APC gene, endothelin-B receptor gene, RET proto-oncogene

INTRODUCTION

Hirschsprungs disease (HSCR) is currently recognized as a multigenic congenital malformation resulting from failure of normal ganglion development and leads to aganglionosis of the distal bowel(Passarge, 2002). The disorder is extremely complex and at least 11 different genes have been implicated in its pathogenesis (Lantieri *et al*., 2006) suggesting a multiplicative effect model (Doray *et al*., 1998; Passarge, 2002). This is hardly surprising as the signals governing cell migration and development are extraordinarily complicated and signaling molecules are well known for their crosstalk and redundancy, as well as having coordinate and dependent regulation of expression on occasion.

The RET signaling pathway [REarranged during Transfection (RET) proto-oncogene and its ligands glial cell line-derived neurotrophic factor (GDNF) gene and neurturin (NTN)] (Angrist *et al*., 1995; Doray *et al*., 1998; Gianino *et al*., 2003; Jing *et al*., 1996; Treanor *et al*., 1996) appear to all be significant in the pathogenesis of Hirschsprungs disease (HSCR). RET remains the most significant pathogenetic mechanism for HSCR identified to date and recent reports suggest that variation within the RET promotor region (Burzynski *et al*., 2004; Griseri *et al*., 2005; Plaza-Menacho *et al*., 2006) accounts for a number of short segment HSCR.

Notwithstanding the central importance of RET in its pathogenesis, the ultimate HSCR phenotype may also be influenced by other genetic factors which influence cellular proliferation, maturation or apoptosis in the Enteric Nervous System (ENS) of the developing fetus. Among those not yet reported is the Wnt/beta-catenin signaling pathway which plays an extremely important role in regulating cellular differentiation, proliferation and migration, especially the process of somatogenesis (Wawra *et al*., 2007). As this takes place at the same time that RET exerts its influence on Embryonal development, it is not hard to imagine that cross-talk between these (and other) critical pathways may influence the phenotypic expression of RET gene variations and the resultant HSCR.

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We present a patient with sporadic HSCR who on molecular investigation had an exon 3 (V202M) RET mutation and an additional mutation in the Mutation Cluster Region (MCR) of the APC gene (E1317Q). The potential implications of this association are explored.

Ethical Permission

The study was approved by the Ethics Review Committee of the University of Stellenbosch which subscribes to the Declaration of Helsinki.

Case Presentation

The patient was a 3.58 kg baby born at full term to a G2P2 mother following an uneventful pregnancy. No family history of colonic disease was present. Neonatal jaundice was noted shortly after birth but required no treatment. Abdominal distension and constipation were also noted but not investigated and persisted until the patient presented at 35 months with severe constipation and a markedly distended abdomen. An abdominal X-Ray was suggestive of a low intestinal obstruction and a contrast enema identified a transitional zone in the recto-sigmoid area. The diagnosis of HSCR was confirmed on rectal biopsy, which demonstrated the typical aganglionosis and proliferation of thickened acetylcholinesterase (ACHE) staining neurofibrils in the lamina propria and muscularis mucosa of the intestinal wall. A colostomy was performed followed by a Soave pullthrough procedure 7 months later. At follow-up the patient was noted to have normal weight gain and development but suffered repeated bouts of diarrhea and enterocolitis with abdominal distension necessitating a secondary myectomy 4 months later. This led to a resolution of the early obstructive symptoms and the patient was noted to have normal stools on follow-up at 60 months.

DNA Analysis

DNA extraction was performed on the colonic tissue samples using standard techniques. Polymerase chain reaction (PCR) amplification was performed on RET, EDNRB, EDN3 and the mutation cluster region (MCR) of the APC gene (Bidaud *et al*., 1997; Ceccherini *et al*., 1994; Miyoshi *et al*., 1992; Tanaka *et al*., 1998). The PCR products were subjected to heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis (Kotze *et al*., 1995) and resolved by polyacrylamide gel electrophoresis (PAGE) with the gel supplemented with 7.5% urea at 4°C (350 V) for 18 h. The DNA fragments were stained in ethidium bromide and visualized by ultraviolet light transillumination. Semi-automated DNA sequencing (ABI PRISM 3130XL Genetic Analyzer) was performed on PCR products demonstrating mobility or conformational variants on the polyacrylamide (PAA) gels.

RESULTS

Mutation analysis of the RET proto-oncogene in the patient revealed a potentially disease-related mutation in exon 3 (V202M) (Julies *et al*., 2001) in addition to polymorphisms in exons 2 (A45) and 13 (L769). HEX-SSCP analysis showed no aberrant banding patterns in the remaining exons. The variants identified in exons 3 and 13 were in the heterozygous state whereas the variant in exon 2 was homozygous. Analysis of the EDNRB gene revealed variation only in exon 4 (831G/A). Polymorphism 831G/A was present in a homozygous state. No aberrant banding patterns were observed for the EDN3 gene. Mutation analysis of the coding region of the APC gene by HEX-SSCP analysis revealed a variant in exon 15 (E1317Q) of the gene. The APC mutation caused a G to C transition at nucleotide 3949, changing glutamic acid to glutamine and was present in the heterozygous state.

DISCUSSION

In the patient presented in this study, sporadic short segment HSCR was associated with a novel exon 3 (V202M) RET mutation together [as well as additional polymorphic RET variants (eg A45)] coupled with an APC mutation (E1317Q) in the MCR (codons 1286-1513) of the gene. As such, it describes the first association between RET and APC mutations in HSCR.

Chromosome 5 has become an important area for investigating possible genetic basis of disease as it contains several disease loci, [viz: growth factor and growth factor receptor genes (including the APC gene and the Wnt signaling pathway)(Masure *et al*., 1998). It also contains the genes for the RET-cofactors (GDNF and GFR) in neighbouring areas of the gene. It would also appear that the APC gene has a potential activating or up-regulatory role on RET and RET associated conditions and possible associations have been demonstrated between altered function of the APC gene and RET in thyroid carcinoma (Cetta *et al*., 2001). There are also a few clinical indications suggesting an association of RET activation with the APC gene on 5q21 [normally associated with familial adenomatous polyposis (FAP)](Cetta *et al*., 2001; Marchesi *et al*., 2001; Scopsi *et al*., 1998). It is therefore possible that concomitant gene variations in APC may modulate RET activation through gene interaction.

The Wnt genetic pathway is understood to be critical in controlling cell proliferation and body patterning during normal development. The APC gene has been identified as a multifunctional gene within this system which plays an important role in epithelial differentiation, brain development as well as other neuronal functions, being present in developing astrocytes (Senda *et al*., 2005). The large APC protein (2843 amino acids) binds to beta-catenin (as well as Axin) in order to downregulate the Wnt signaling pathway via one of 2 possible pathways, depending on the required function (Katoh and Katoh, 2007). The canonical pathway appears to function via receptors in the Frizzled family and the beta-catenin signaling cascade, whereas the non-canonical pathway is involved in cell movement and tissue polarity(Schlessinger *et al*., 2007). Aberrant APC proteins lack the necessary beta-catenin phosphorylation repeats thereby failing in Wnt signaling down regulation. As a result, prevention of further cellular differentiation or uncontrolled cellular proliferation may well result.

It is now well recognized that whereas major RET mutations may give rise to HSCR by haploinsufficiency, lesser mutations require the multiplicative effects of other critical genes that control the mechanisms of cell proliferation, differentiation and maturation (Amiel and Lyonnet, 2001; McCallion *et al*., 2003). The RET mutation in this patient appears to be significant in terms of HSCR and is in the extracellular domain of the gene, encompassing exon 3 (V202M) which partly encodes the cadherin-like domain in the promotor area of the gene (Lantieri *et al*., 2006). Mutations in this area can potentially result in RET loss-of-function by a dominant-negative mechanism (Overduin *et al*., 1995), especially if associated with a haplotype with A45 (as in this case). The question as to whether this variation is sufficient to produce HSCR remains unclear.

Gene interaction between Ret and Wnt signaling systems have recently been demonstrated in a number of situations including the interaction of the epithelial WNT11 on the largely mesenchymal RET/GDNF signalling to control ureteric branching and development during kidney development (Majumdar *et al*., 2003; Michos *et al*., 2007). In addition, a subset of ovarian carcinomas show interaction of the PI3K (downstream from RET) and the wnt/betacatenin signalling system in both animals (Wu *et al*., 2007) and humans (Sarrio *et al*., 2006).

The association of RET and APC mutations in this patient suggest functionality of these lesions and warrants further investigation. The potential link between the inter-related functions of APC, β-catenin, E-Cadherin and RET and may indicate how the multiplicative effect of gene-gene interaction may influence the phenotypic expression. A reasonable case can therefore be made to support the

novel hypothesis of the APC gene as a putative modifier gene in HSCR. Whether the corollary is true that RET is related to FAP/colonic cancer or thyroid cancer is less clear and should remain the subject of future studies. The risks of FAP/colonic carcinoma or even thyroid carcinoma in our particular patient are undetermined and warrant careful follow-up.

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