REVIEW

The Hirschsprung's–multiple endocrine neoplasia connection

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The risk of patients with Hirschsprung's disease later developing multiple endocrine neoplasia remains a matter of concern. The multiple endocrine neoplasia 2–Hirschsprung's disease association has been shown to cosegregate in Hirschsprung's disease patients with both short- and long-segment aganglionosis, although patients with long-segment aganglionosis a to carry the greatest risk. The Hirschsprung's disease–medullary thyroid carcinoma relationship also appears to be bi-directional, and activation or suppression of the *rearranged during transfection* gene appeared to vary over succeeding generations within the same family. *Rearranged during transfection* gene variations are associated with both conditions. The cosegregation of Hirschsprung's disease and multiple endocrine neoplasia 2 is particularly interesting as it involves both ''switch off'' and ''switch on'' of the *rearranged during transfection* proto-oncogene in the same patient. This cosegregation mostly relates to the cysteine-rich area on *RET-620* (the ''Janus gene''). The mechanism whereby *rearranged during transfection* influences gene activation in multiple endocrine neoplasia 2 is complex, but genetic variations impair the *rearranged during transfection* tyrosine kinase response to tyrosine kinase activation, thus appearing to dictate downstream signaling cascade responses. Better understanding of the *RET*-620 relationship allows for a more cost-effective method of identifying those at risk by focusing *rearranged during transfection* gene testing to this specific area as a ''hot spot''. The clinical awareness of possible medullary thyroid carcinoma has led to timely intervention and early treatment of this chemo- and radioresistant tumor with poor prognosis. Establishment of ''risk'' by genetic testing has become a classic model of molecular medicine being integrated into patient care and offering *rearranged during transfection*-directed prophylactic surgical management. In addition, novel approaches to treatment based on this genetic knowledge have already shown early promise in randomized clinical trials.

KEYWORDS: Hirschsprung's disease; Genetics; Novel mutations; *RET*; *Endothelin receptor B gene*.

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INTRODUCTION

Hirschsprung's disease (HSCR), or aganglionic megacolon, occurs due to the congenital absence of parasympathetic neuronal ganglia in a segment of the hindgut, resulting in malfunction of the affected segment. The condition presents as a common cause of intestinal obstruction in neonates, with an incidence of 1 in 5,000 (0.02%) live births and an overall risk to siblings of 4% (1–3). A study of HSCR indicates several clear-cut associations that are known or are suspected to be related to an increased risk of HSCR. These include its recurrence in families and the association with the major susceptibility genes points convincingly to genetic factors in its multifactorial pathogenesis. The main susceptibility gene for Hirschsprung's disease is the *RET* (rearranged during transfection) proto-oncogene tyrosine kinase which is situated at chromosome 10q11.2.

There are early reports of associations between HSCR and a number of associated anomalies related to neural cell development. These include neuroblastoma (1), phaeochromocytoma (1,4,5) and multiple endocrine neoplasia (MEN) type 2 A and B syndromes (5–7), among others (1). Reports on this relatively uncommon cosegregation of HSCR and MEN2 in the same patient (6,8–20) exist because of the common factor of the *RET* gene being associated with both conditions (HSCR, MEN type 2 and medullary thyroid carcinoma (MTC)). This is an extremely interesting observation, as it involves both gain and loss of function of the same gene in the same patient.

Which patients are at risk?

The MEN–HSCR association has been shown to cosegregate in HSCR patients with both short- and long-segment aganglionosis (21). It seems to be particularly associated with long-segment aganglionic segments, and Decker et al. (21) reported a long aganglionic segment (L-HSCR) in seven out of 13 patients (54%). Our experience is of total colonic aganglionosis (TCA) in the index patient in all three families

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identified in our series (22). It therefore appears that patients with long-segment HSCR carry the highest risk of developing MTC and should have a detailed family history taken: the presence of a long-segment HSCR should be an important selection criterion for gene testing in HSCR.

It is important to explore this concept further in families where HSCR and MTC coexist, as it will yield possible *RET* gene associations and insights into possible molecular reasons for the phenotypic expression. It is generally accepted that aberrant RET protein synthesis, due to inactivating genetic variations, lead to the congenital malformation of the enteric nervous system (ENS) which we call Hirschsprung's disease. *RET* gene activation occurs in MEN2A.

The HSCR–MTC relationship also appears to be bi-directional, and *RET* gene activation or suppression appeared to vary over succeeding generations within the same family (22). Butter et al. (23) reported a 50% incidence of HSCR in 20 patients undergoing a prophylactic thyroidectomy for *RET*-associated MTC risk (a *RET C620* mutation). In one further reported case of familial MTC (24) with a *C620S* point mutation, the MTC developed 12 years after surgical correction of HSCR in the child plus a maternal MTC 7 years after the child's birth. In our reported series (22), MTC was detected in the parent 5 years after the birth of the affected child.

Hirschsprung's, MEN and the *RET* proto-oncogene

The *RET* proto-oncogene [10q11.22] is the major gene involved in the pathogenesis of HSCR with causative loss-of-function mutations being identified in more than 70% of cases (25). In essence, the extracellular domain *RET* mutations alter the protein and possibly its processing in the endoplasmic reticulum (26). As a result, *RET* transport to and its expression at the cell surface is decreased.

Multiple endocrine neoplasia also results from autosomally dominant, inherited, highly penetrant germline *RET* mutations that predispose patients to the development of tumors in cells derived from neural crest origin. Of the more than 25 *RET* proto-oncogene gene mutations which have been described in association with MEN type 2 syndromes, the most important are associated with the six cysteine alleles of the extracellular portion of the *RET* proto-oncogene in MEN2A.

RET is a vital gene which directs the migration, proliferation and survival of the enteric neural crest-derived cells of the enteric nervous system (ENS) during embryogenesis. It is also responsible for the development of the autonomic nervous system as well as for controlling kidney development and spermatogenesis, among other functions. Genetic mutation and/or variation may result in *RET* malfunction, which has been associated with at least four clinical conditions (HSCR, MEN type 2 syndromes (A and B) and familial medullary thyroid carcinoma (FMTC)). In HSCR, hypomorphic *RET* alleles may cause delayed neuroblast migration and non-apoptotic cell death. This causes ENS maldevelopment, resulting in aganglionosis (HSCR) and other related conditions of the ENS.

The presence of a HSCR–MEN2 association raises the question of which HSCR patients are at greatest risk and should therefore be further investigated by *RET* gene analysis. Hereditary MTC can, with rare exceptions, be traced back in family trees (27) and the HSCR–MEN2 familial association may be missed because the MTC may appear later in life without the connection being made. As a result, the risk of HSCR patients later developing features of an MEN2 syndrome (with MTC) is thought to be under-evaluated. The risk of a future thyroid carcinoma remains a matter of general concern in long-term HSCR management. It is becoming clear that there is a need for screening in order to determine which patients require *RET* gene analysis as a means of determining risk.

The most frequently identified mutation responsible for MEN2A involves the substitution of the cysteine amino acid at position 634 (Cys634Arg or C634R) by the amino acid arginine. However, point mutations in the cysteine-rich *RET* area at the *620* position, along with some other *RET* gene areas (e.g. *C609, C611* and *C618*) have been reported as being mostly associated with the HSCR–MEN2 phenotype. By way of contrast, in >90% of MEN2B patients, the amino acid methionine is replaced by threonine at position 918 (M918T) in the intracellular portion of the gene. There are also a number of other *RET* variations that are associated with thyroid cancers and familial medullary thyroid carcinoma which may give rise to an overactive abnormal RET protein.

The cosegregation of HSCR and MEN2 is particularly interesting as it involves both a ''switch off'' and a ''switch on'' of the gene in the same patient. Some remarkable family lineages have been reported which demonstrate the occurrence of the two conditions in family lineages (17,22).

Which *RET* gene variations are important?

Like other receptor tyrosine kinases (RTKs), *RET* is made up of at least three functional areas (extracellular, transmembrane and intracellular regions) which appear to have specific roles in cellular function (28). It is interesting that, in contrast to other susceptibility areas, the *RET* mutations which have been identified as carrying both a risk for HSCR as well as MEN2 approximate the transmembrane domain of the gene (mostly *C620R* and occasionally *C620S* and rarely *C620W*) (21). Although the majority of reports connect these two conditions with point mutations in the cysteine-rich *RET*-area at the *620* position, other *RET* gene areas (e.g. *C609, C611* and *C618*) have been reported as alternative sites. The high frequency with which the C620 *RET* mutation occurs in HSCR–MEN patients suggests the concept of the so-called ''Janus gene'' mutation in this position, which, like the Roman god of doorways, can face in both directions (i.e. activation (MEN/MTC) and inactivation (HSCR)).

Our own studies (22) have shown the *RET*-620 cysteine variations to be present in 2.5% of HSCR families (three out of 118 families investigated), which is in keeping with other reports (21). There was, however, an uncertain prevalence of HSCR–MEN in related family members as not all could be tested.

Studies have also shown that the closer mutations are to the transmembrane domain, the higher their importance in terms of the development of cellular proliferation and tumor activation (29). A strong self-association has been shown in the transmembrane portion of the gene (*RET*-TM), which has been suggested as a possible explanation of the oncogenic activation due to *RET*–cysteine mutations in oncogenesis (29). This suggests an area that plays an important role in MTC risk evaluation and may identify a target area for novel therapeutic strategies.

How does *RET* gene variation result in cellular activation?

Because MEN2A relates mostly to the *RET* alleles at the six cysteine positions in the extracellular domain of the gene, it appears to induce a different set of downstream signaling genes from that carrying the MEN 2B mutation.

The multidocking intracellular portion of the *RET* gene where MEN2B *RET* variations are situated appears to be vital to both tyrosine kinase function and downstream signaling due to the number of signaling molecules that interact there (e.g. *Shc, Src, FRS2, IRS1, Gab1/2,* and *Enigma*) (30–32). It is therefore easier to understand how mutations within this tyrosine-kinase rich region (e.g. in 95% of MEN2B patients) alters the substrate specificity of RET tyrosine kinase and results in gene activation.

The mechanism whereby *RET* influences gene activation in MEN2A is more complex. Genetic variations impair the RET tyrosine kinase response to tyrosine kinase activation, thus appearing to dictate downstream signaling cascade response (33). The induction of a disulfide-linked homodimerization resulting from the cysteine-related mutations within the extracellular portion of the *RET* proto-oncogene promote dimer formation within the gene as covalent receptor dimers are linked by disulfide bonds between unpaired cysteines (34,35). It is known that the RET protein targets a number of intracellular signaling cascades, including the *RAS-RAF-ERK1/2, PIK3-AKT*, and *STAT* transcription pathways (36). The activation of the *MAPK* and *Ras/ERK* molecular signaling cascades involves *grb2/mSOS* recruitment (37). In addition, recent studies have suggested that the mammalian target of the rapamycin (mTOR) intracellular signaling pathway is functionally activated in MTC (especially in those with germline *RET* mutations) (38), and that the role of the other downstream RET tyrosine kinase signaling cascades (e.g. BRAF, RAS isoforms and PI3 kinase) is less certain.

How does the *RET*-620 relationship work?

Oncogenic *RET* mutations mostly occur *de novo* in sporadic MTC, and it is now well accepted that *RET* gene variations produce aberrant proteins which may bypass the normal linking to growth factors outside the cell, most probably triggering cells to grow and divide abnormally and, in turn, promoting tumor formation. Ret activation then promotes Ret-dimer formation and activation of the tyrosine kinase outside of the normal ligand binding system.

However, little is known of the actual mechanisms whereby extracellular domain mutations (e.g. *C620S*) may result in C-cell proliferation (39). Current understanding of *RET* gene activation in MTC is that the gene is activated by alterations in autophosphorylation, or alternatively by the modification of the subcellular distribution of the active kinase or the induction of unusual interactions with other proteins (e.g. RET/PTC) (40).

It is possible that the MEN2A–HSCR connection represents a second alternative intracellular mechanism in the pathogenesis of MTC (41). The less common codons *C620, C618* and *C611* within exon 10 confer a weaker transforming activity *in vitro* than exon 11 (C634) *RET* mutations (42). It has been suggested that the MEN2A–HSCR connection represents a second alternative intracellular mechanism in the pathogenesis of MTC involving the endoplasmic reticulum which has been shown to decrease cell surface *RET* expression in HSCR (26). Changes in polarity in MEN2A–HSCR may result in the unfolding of the RET protein initially failing to achieve homeostasis via the unfolded protein response (UPR) of the endoplasmic reticulum, leading to apoptosis and HSCR. Subsequent upregulation of the UPR may then induce changes which allow gene activation, thus providing a growth advantage to tumor cells.

Oncogenic *RET* mutations affect protein synthesis and function, which then influence a number of downstream molecular signaling pathways (43). Studies have demonstrated that Ret influences the downstream phosphorylation of the insulin receptor substrate-2 (*IRS 2*) with subsequent activation of phosphatidylinositol 3 kinase (*PI 3-kinase*) and protein kinase B (*PKB/AKT*) signaling pathways (44). In addition, the induction of phosphorylation of SHC results in growth factor receptor binding protein 2 (*Grb-2*) binding and activation of the mitogen-activating protein (*MAP*) kinase pathway (45). The resulting *MAPK IRS-2, PI 3-kinase* and *PKB/AKT* activation could transduce the oncogenic Ret signal to promote gene transcription, possibly through activation of the jun/AP-1 transcription cascades (44).

Impact on treatment

This paper draws attention to the apparent genotype–phenotype correlation which exists between *RET* and the MEN2 syndromes (46,47). Clinical awareness of possible MTC has led to timely intervention and early treatment of this poor prognosis chemo- and radioresistant tumor. Establishment of ''risk'' by genetic testing has become a classic model of molecular medicine being integrated into patient care. It is therefore fairly clear that *RET* gene screening is of considerable value in familial screening, and offering *RET* testing is considered best practice for the clinical management of patients at risk of MEN2A and MEN2B.

Gene mutation in the *RET 620* position carries significant risk of developing HSCR and/or MTC. In the interests of cost-effectiveness, it may be part of a targeted investigation of high-risk areas of the gene in HSCR patients at risk, thus facilitating follow-up and future management. Genetic screening is an extremely sensitive method of identifying risk in MEN2 syndromes ($\pm$98%) and prophylactic surgical intervention may prevent the onset of the radio- and chemoresistant MTC (48).

Better understanding of the *RET*-620 relationship would also allow for a more cost-effective method of identifying those at risk by focusing *RET* gene testing to this specific area as a ''hot spot''. In addition, a number of attractive targets are being identified which may be suitable for novel therapeutic signaling pathway-specific drug design (49). Novel approaches to treatment based on this genetic knowledge have already resulted in randomized clinical trials into the effectiveness of multi-kinase inhibitors such as axitinib, sorafenib, motesanib, and XL-184. These have shown early promise in selected cases which may soon change the management of thyroid cancer (50).

AUTHOR CONTRIBUTIONS

Moore SW is an expert on the genetic connections and other aspects of Hirschsprung's disease and MEN2 syndromes. He wrote the review and was responsible for the clinical component and interpretation of results. Zaahl M is a scientist who collaborated for a long period on this project. She is the head of the Department of Genetics at Stellenbosch University

and runs the laboratory where the genetic work was carried out. She personally oversaw most of the laboratory work presented in this study.

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