

THE ROLE OF THE *ret* PROTO-ONCOGENE IN HUMAN DISEASE

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ABSTRACT

The *ret* proto-oncogene encodes a receptor tyrosine kinase with a cadherin-like motif in the extracellular domain. Recently, it turned out that *ret* is the causative gene for the development of multiple endocrine neoplasia (MEN) type 2A and type 2B and Hirschsprung's disease. MEN 2A and MEN 2B mutations represent activating changes of *ret* whereas Hirschsprung mutations inactivate *ret*. In addition, another activating change of *ret* was found in papillary thyroid carcinoma, particularly in those cancers which developed in children from areas contaminated by the Chernobyl accident. This review summarizes the role of *ret* in the development of human disease.

Key Words: *ret*, multiple endocrine neoplasia, Hirschsprung's disease, papillary thyroid carcinoma, glial cell line-derived neurotrophic factor

INTRODUCTION

Receptor tyrosine kinases are involved in the control of cell growth and differentiation. Some of them are known to be activated as oncogenes in human tumors. The *ret* proto-oncogene is the most interesting gene because its mutations were found in several hereditary and non-hereditary human diseases such as multiple endocrine neoplasia (MEN) 2A and 2B, Hirschsprung's disease and papillary thyroid carcinoma.¹⁻³⁾

The *ret* proto-oncogene was first identified by transfection of NIH 3T3 cells with human T cell lymphoma DNA.^{4,5)} Its oncogenic version was reported by an Italian group, in a proportion of papillary thyroid carcinoma in which rearrangements of *ret* with unrelated gene sequences occurred.⁶⁾ The rearrangements were generated mostly by a paracentric inversion of chromosome 10.⁷⁾ Subsequently, germline mutations of *ret* were identified in four different neural crest disorders including MEN 2A, MEN 2B, familial medullary thyroid carcinoma (FMTC) and Hirschsprung's disease.⁸⁻¹³⁾

ret is highly expressed in the enteric and sympathetic nervous system during mouse and rat embryogenesis.^{14,15)} In addition, the high expression was observed in the developing excretory system such as the branching ureteric bud. Consistent with this expression pattern, homozygous *ret* knock-out mice showed lack of enteric neurons and severe renal abnormality,¹⁶⁾ suggesting its importance in renal organogenesis and enteric neurogenesis. Recently, since several reports showed that glial cell line-derived neurotrophic factor (GDNF) represents a Ret ligand,¹⁷⁻²¹⁾ the intracellular signalling pathway through Ret is expected to be extensively studied from now on.

MULTIPLE ENDOCRINE NEOPLASIA TYPE 2 SYNDROME

MEN 2A and MEN 2B are autosomal dominant cancer syndromes involving medullary thyroid carcinoma and pheochromocytoma. In addition to these tumors, 10–20% of MEN 2A patients develop parathyroid hyperplasia whereas the clinical features of MEN 2B are characterized by development of mucosal neuroma, intestinal ganglioneuromatosis and marfanoid habitus. It is well known that medullary thyroid carcinoma associated with MEN 2B is more aggressive than that of MEN 2A.

The MEN 2A mutations always involve one of six cysteines (codons 609, 611, 618, 620, 630 and 634) in the Ret extracellular domain and were found in 98% of the MEN 2A family investigated (Fig. 1).^{1–3)} Mulligan *et al.*²²⁾ reported that the mutations at codon 634 were present in more than 80% of the MEN 2A family and have a strong association with the development of pheochromocytoma. In the case of MEN 2B, a single point mutation in the tyrosine kinase domain was identified in 95% of the patients.^{1–3)} Methionine at codon 918, normally present in the catalytic core region, was replaced by threonine (Fig. 1). Interestingly, the somatic MEN 2B-type mutation was also found in 30–40% of sporadic medullary thyroid carcinomas, although the MEN 2A mutations were rarely present.

Familial medullary thyroid carcinoma (FMTC) is a disease related to MEN 2 syndromes and is characterized by the development of medullary thyroid carcinoma alone. Most FMTC mutations were found in cysteine residues in the extracellular domain as observed with MEN 2A mutations.²²⁾ In addition, two FMTC-specific mutations were identified at codons 768 and 804 in the tyrosine kinase domain (Fig. 1) and their somatic mutations were reported to be present in fewer than 10% of sporadic medullary thyroid carcinomas.²³⁾

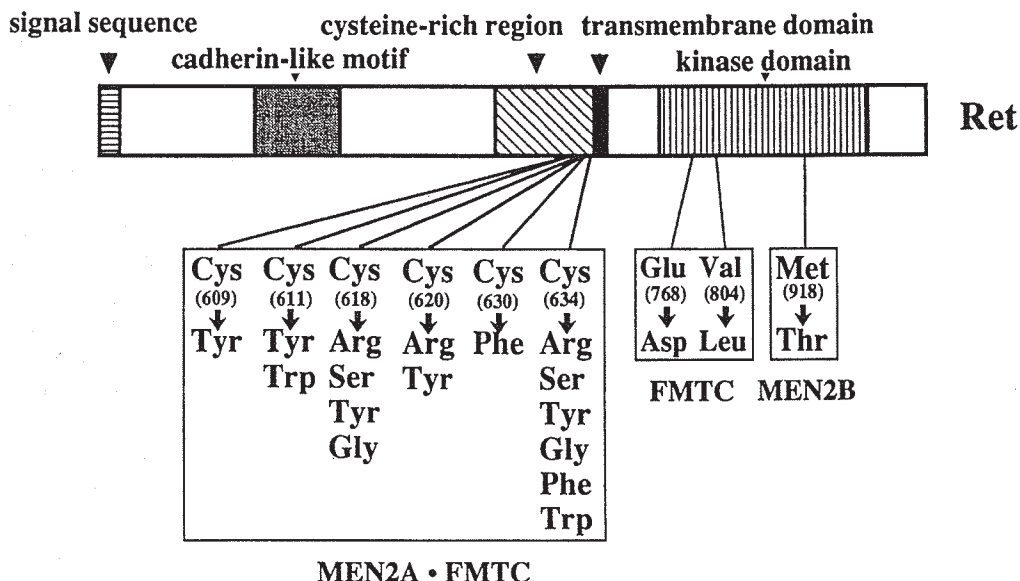


Fig. 1. *ret* mutations in MEN 2A, MEN 2B and FMTC. Germline mutations of *ret* identified in MEN 2A and FMTC involve one of six cysteine residues present in the cysteine-rich region of the extracellular domain. A single point mutation that substitutes threonine for methionine at codon 918 was detected in MEN 2B. Two FMTC-specific mutations were reported at codons 768 and 804 in the kinase domain.

MEN 2 SYNDROME DEVELOPMENT MECHANISM BY *ret* MUTATIONS

To investigate whether MEN 2A and MEN 2B mutations represent gain of function mutations of *ret*, we introduced the mutations into *ret* cDNA and transfected mutant *ret* cDNAs into NIH 3T3 cells. As a result, we found that all mutations which were found in MEN 2 patients have high transforming activity, confirming that the mutations can activate Ret.

To investigate further the molecular development mechanisms of MEN 2 syndromes, NIH 3T3 cells expressing normal c-Ret, Ret with the MEN 2A mutation (MEN 2A-Ret) or Ret with the MEN 2B mutation (MEN 2B-Ret) were metabolically labeled with ³⁵S-methionine, and the nature of the mutant Ret proteins was analyzed. When the cell lysates were immunoprecipitated with anti-Ret antibody and separated on SDS-polyacrylamide gels under reducing conditions, the 150 kDa and 170 kDa Ret proteins were detected in each sample. However, it is interesting to note that when the immunoprecipitates were separated under non-reducing conditions, the 340 kDa band was detected only in the lysate from the cells expressing MEN 2A-Ret. To see the nature of this 340 kDa band, the band was cut out from the gel and separated under reducing conditions again. Consequently, the 340 kDa band became a single 170 kDa band, suggesting that it represents homodimers of the 170 kDa Ret protein. Since the 170 kDa Ret protein is the mature glycosylated form present on the cell surface and the 150 kDa Ret protein is the immature glycosylated form present in the endoplasmic reticulum, this result means that Ret dimerization by MEN 2A mutations occurred on the cell surface in a ligand-independent manner.²⁴⁻²⁶⁾

In the case of the MEN 2B mutation, we found that Ret is activated in the monomeric form. As mentioned above, the MEN 2B mutation is present in the catalytic core region of the tyrosine kinase domain. Thus, it is conceivable that the mutation induces a conformational change in this region and activates Ret by an intramolecular mechanism, resulting in an enhancement of its intrinsic tyrosine kinase and/or a change in its substrate specificity.²⁵⁾ In this respect, we found that the tyrosine residues essential for any transforming activity are different in MEN 2A-Ret and MEN 2B-Ret.²⁶⁾ When tyrosine 905 was changed to phenylalanine, the transforming activity of MEN 2A-Ret was completely abolished while MEN 2B-Ret was not affected. On the other hand, the transforming activity of MEN 2B-Ret significantly decreased when tyrosine 864 or 952 was changed to phenylalanine, and the double mutation of these two tyrosines abolished the activity of MEN 2B-Ret. MEN 2A-Ret was not affected. These results also supported the view that a conformational change in the Ret catalytic core region is important for Ret activation by the MEN 2B mutation.

Furthermore, it turned out that tyrosine 1062 regulates the activity of both MEN 2A-Ret and MEN 2B-Ret.²⁷⁾ When tyrosine 1062 was changed to phenylalanine, the transforming activity of MEN 2A-Ret and MEN 2B-Ret drastically decreased (less than 20%). Since the Shc adaptor protein is known to have phosphotyrosine binding sites named SH2 and PTB domains which are bound to the tyrosine 1062 of MEN 2A-Ret and MEN 2B-Ret, it seemed likely that tyrosine phosphorylation of Shc is associated with the transforming activity of the MEN 2A-Ret and MEN 2B-Ret proteins. We are currently investigating whether Shc phosphorylation induced by the mutant Ret proteins leads to activation of the Ras-mitogen activated protein kinase (MAPK) signalling pathway.

HIRSCHSPRUNG'S DISEASE

Hirschsprung's disease is a congenital disorder associated with the absence of enteric neurons. Based on the length of the aganglionic segment, it is classified into short segment Hirschsprung's

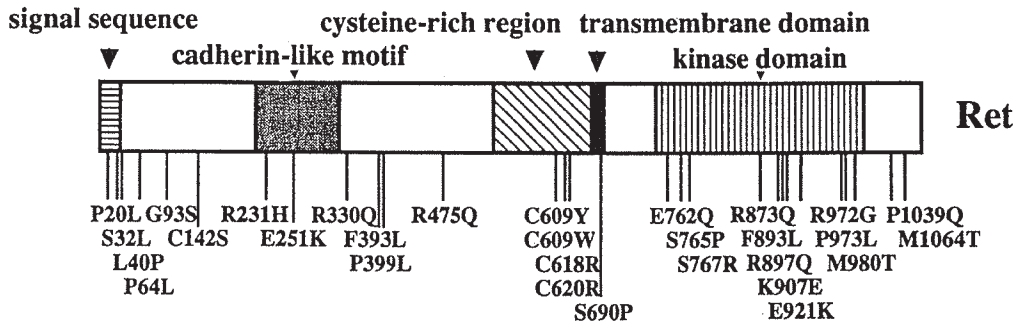


Fig. 2. *ret* mutations in Hirschsprung's disease. Missense mutations identified in Hirschsprung patients are shown.

disease and long segment Hirschsprung's disease. Approximately 10% of the cases are familial and inherited in an autosomal dominant manner with incomplete penetrance and variable expressivity. Many missense, nonsense and frameshift mutations as well as splice-site deletions of *ret* were identified in Hirschsprung's patients. *ret* mutations are heterozygous and account for 50% of familial and 15–20% of sporadic cases of Hirschsprung's disease.²⁸⁾ These mutations appear to be associated with the development of long segment Hirschsprung's disease rather than short segment Hirschsprung's disease. Unlike the MEN 2 mutations, Hirschsprung mutations are scattered along the extracellular and intracellular domains of Ret (Fig. 2).

HIRSCHSPRUNG'S DISEASE DEVELOPMENT MECHANISM BY *ret* MUTATIONS

So far, we have analyzed the biological and biochemical effects of six Hirschsprung mutations by introducing them into *ret* cDNA. Of these, five mutations were present in the extracellular domain and one in the tyrosine kinase domain. Five Hirschsprung's mutations in the extracellular domain resulted in a marked decrease in the expression of the 170 kDa cell surface form of Ret.²⁹⁾ Since the expression of the 150 kDa Ret protein present in the endoplasmic reticulum was not affected, this suggested that these mutations impair the transport of Ret from the endoplasmic reticulum to the plasma membrane.^{29,30)} Interestingly, our results also revealed that four long segment Hirschsprung mutations more severely impair Ret transport than a short segment Hirschsprung mutation, suggesting that the level of its cell surface expression might correlate to the phenotypes of Hirschsprung's disease.²⁹⁾ On the other hand, the mutations detected in the tyrosine kinase domain did not affect the expression of the 170 kDa Ret protein at all. These mutations impaired the kinase activity of the Ret protein.³¹⁾

Heterozygous deletion of *ret* was found in sporadic Hirschsprung patients, implying that haploinsufficiency of *ret* is critical for the development of Hirschsprung's disease. The fact that the *ret* mutations affecting its extracellular domain severely impair the transport of Ret to the plasma membrane also suggests that the haploinsufficiency effects of these mutations might be a possible mechanism in the development of Hirschsprung disease. As mentioned below, it was recently reported that GDNF can activate Ret after its binding to GDNFR- α .^{18,19)} Thus, sufficient levels of Ret expression on the cell surface for GDNF binding appear to be required for migration of enteric neuroblasts towards the distal portion of the colon or their full differentiation.

ROLE OF *ret* IN HUMAN DISEASETable 1. Frequency of Rearrangement of the *ret* Proto-Oncogene in Papillary Thyroid Carcinoma

Japan	Italy	France	U.S.A.
< 3%	33%	11%	17%

PAPILLARY THYROID CARCINOMA

Papillary thyroid carcinoma is the most common malignant tumor developed in the thyroid gland. In 1990, Grieco *et al.*⁶⁾ reported that rearrangement of *ret* was detected in approximately 30% of human papillary thyroid carcinomas in which *ret* was fused to unrelated gene sequences just before its tyrosine kinase domain. So far, three genes involved in the fusion, including H4, *ele1* and cyclic AMP-dependent protein kinase A regulatory subunit *RI α* genes, have been described in papillary carcinoma.³²⁻³⁶⁾ Among these, the H4 and *ele1* genes were more frequently involved in the rearrangement than the *RI α* gene in adult papillary carcinomas. The mechanism of *ret* activation in papillary carcinoma is reminiscent of the mode of several other tyrosine kinase receptors such as the EGF receptor, *met* and *trk* which were also activated by deletion of their ligand-binding domains. It is interesting to note that the frequency of rearrangement is different among races. It is high (11-33%) in papillary carcinomas from European and U.S. patients³⁵⁾ while it is very low (<3%) in those from Japanese and Saudi Arabian patients (Table 1).^{37,38)} Although the reason for this difference is unknown, it is possible that this could be due to racial differences in the genetic background.

The incidence of papillary carcinoma dramatically increased in children from areas contaminated by the Chernobyl reactor accident. Surprisingly, two thirds of the papillary carcinomas in children showed a *ret* rearrangement,³⁹⁾ suggesting that *ret* is very sensitive to radiation exposure. In this case, the fusion of *ret* with the *ele1* gene was the prevailing type of rearrangement.⁴⁰⁾ It was reported that papillary carcinoma developed in connection with this accident is relatively aggressive, showing direct invasion to the peripheral tissues and distant metastasis.

IDENTIFICATION OF Ret LIGAND

GDNF was isolated as a potent survival factor for midbrain dopaminergic neurons.⁴¹⁾ Extensive studies revealed that GDNF plays a crucial role in the survival and/or differentiation of various neurons in both the central and peripheral nervous systems.⁴²⁻⁴⁵⁾ GDNF belongs to members of TGF- β superfamily that bind and activate serine/threonine kinase receptors. Unexpectedly, GDNF-knock out mice showed phenotypes similar to those of *ret*-knock out mice,⁴⁶⁻⁴⁸⁾ implying that Ret receptor tyrosine kinase may be a functional receptor for GDNF.

Jing *et al.*¹⁸⁾ and Treanor *et al.*¹⁹⁾ cloned a novel glycosylphosphatidylinositol-linked cell surface receptor (designated GDNFR- α) for GDNF. Biochemical analyses demonstrated that GDNF induces the formation of a physical complex between GDNFR- α and Ret, leading to Ret activation. Thus, this complex appears to represent a unique ligand-receptor system similar to that of a ciliary neurotrophic factor that also requires an α component as a ligand-binding molecule.⁴⁹⁾ Identification of the Ret ligand will promote the study of intracellular signalling via Ret in neurons.

CONCLUDING REMARKS

The *ret* proto-oncogene is involved in the development of four different neural crest disorders: MEN 2A, MEN 2B, FMTC and Hirschsprung's disease. Thus, *ret* offers a unique opportunity for studying phenotypic diversity caused by gene mutations. In particular, it is of special interest to investigate the molecular mechanism that induces the different clinical phenotypes observed in MEN 2A and MEN 2B. Furthermore, identification of GDNF as a Ret ligand will provide an interesting basis for studying the differentiation mechanisms of neuronal cells.

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