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The *RET* gene and its associated diseases

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The *RET* gene and its associated diseases

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Scope and outline of the thesis

The *RET* proto-oncogene encodes a receptor tyrosine kinase involved in the normal development and the neoplastic growth of neural crest lineages. The ligand of the receptor is as yet unidentified. During embryogenesis *RET* expression is high in neuroectodermal tissues, suggesting a function of RET in the proliferation, the migration and the differentiation of these cell types. In adult tissues the gene is hardly expressed. Expression is high in several tumor types derived from neural crest cells.

Transfection studies with DNA from different tumors revealed focal proliferation due to the presence of different DNA sequences that, however, shared a common part called *RET*. The original *RET* gene turned out to be rearranged in such a way that the sequences coding for the extracellular part of its protein product were replaced by sequences from elsewhere, resulting in a rearranged protein with a constitutive tyrosine kinase activity. The same rearrangement occurs in papillary thyroid carcinoma (PTC).

After the genes involved in multiple endocrine neoplasia types 2A (MEN 2A) and 2B (MEN 2B) and in Hirschsprung disease (HSCR) had been mapped to the centromeric region of chromosome 10 by linkage analysis, mutations of *RET*, a gene which lies in this very region, appeared responsible for the development of these diseases. For MEN 2A and MEN 2B the mutations were activating the protein translated, for HSCR the mutations resulted in a functional loss of the protein translated.

Much is known about the *RET* gene, its protein product and its involvement in at least four different diseases (PTC, MEN 2A, MEN 2B, HSCR) as briefly summarized above. In this thesis on the *RET* gene and its associated diseases, an overview of the relevant *RET* literature will be given and our own data as well as those obtained in collaborative efforts with other groups is presented in the appendices. Appendix 1 displays the *RET* sequence of both the long and the short isoforms. Appendix 2 shows the genomic structure, i.e. the intron-exon junctions of all 20 exons of the *RET* gene. It is followed by a paper giving SSCP conditions for mutation detection of the *RET* gene [Appendix 3]. Our finding that a single mutation is uniquely associated with MEN 2B is presented in appendix 4. Appendix 5 shows that sporadic medullary thyroid carcinoma and sporadic pheochromocytoma can only partly be explained by *RET* mutations. A paper on the possible involvement of *RET* in neuroblastoma and a paper concerning the involvement of *RET* in families with MEN 2A and cutaneous lichen amyloidosis (CLA) and in families with hereditary "CLA only" are presented in appendices 6 and 7, respectively.

The *RET* gene and its associated diseases

Protein kinases

The protein kinases are a large family of enzymes, many of which mediate external stimuli of eukaryotic cells, that are involved in cell growth, proliferation and differentiation. The enzymes respond to stimuli by becoming activated (see 1.3) and thereby able to phosphorylate target proteins (substrate molecules) in the cytoplasm.

1.1 Classification of protein kinases

Some protein kinases add phosphate to serine or threonine residues (serine/threonine kinases), whereas others only phosphorylate tyrosine (tyrosine kinases). Although the serine/threonine kinases and the tyrosine kinases phosphorylate different amino acids their catalytic domain is similar. Of the two classes, yeasts have only the serine/threonine kinases, whereas tyrosine kinases are observed in multicellular organisms that also have serine/threonine kinases. This difference and the observation that many tyrosine kinases are growth factor receptors suggest that tyrosine kinases play a role in the cell to cell communication in multicellular organisms.

The kinase family can also be classified in two groups dependent on their location in the cell. Some of the protein kinases, both serine/threonine and tyrosine kinases, are located entirely within the cytoplasm, although often in association with other proteins integrated in the cell membrane that act as an intermediate between external stimuli and the protein kinase. The other group consists of the receptor protein kinases, again containing both serine/threonine and tyrosine kinases. The receptor proteins extend their amino terminus through the cell membrane, the carboxyl terminus being intracellular. Their external domain recognizes the presence of extracellular factors and transmit external signals to the cytoplasmic domain which contains the kinase domain.

Within the two classes, serine/threonine kinases and tyrosine kinases, many subfamilies can be distinguished (Table 1) according to similarities in primary structure and to the deduced catalytic domain phylogeny (Hanks *et al*., 1988; Hunter *et al*., 1992).

1.2 Structure of protein kinases

As mentioned, protein kinases can be subdivided into families according to similarities

within their respective defined catalytic domains. However, the amino acid sequences outside the catalytic domain also show striking similarities within the different subfamilies. Structural motifs and cysteine residues present in the external domain are usually conserved within the different subfamilies and the same holds for the spacing between the transmembrane domain and the catalytic domain and between the catalytic domain and the carboxyl-terminal tail.

The kinase catalytic domain is usually located near the carboxyl terminus of the protein and ranges from 250 to 300 amino acids, the boundaries being determined through an analysis of conserved sequences (see, Hanks *et al.*, 1988). Eleven major conserved subdomains are present in the catalytic domain. They are separated by less conserved regions which in some cases contain relative large inserts or gaps. These conserved subdomains are part of the active domain and contribute to the necessary tertiary structure. Certain short amino acid stretches in subdomains VI and VIII indicate whether the protein is a putative tyrosine or serine/threonine kinase (Hanks, 1988). Probable functions of the different subdomains are reviewed in Hanks *et al.* (1988).

Table 1. Subfamilies of protein serine/threonine and tyrosine kinase (Hanks et al. 1988; Hunter et al., 1992).

Protein-serine/threonine kinase subfamilies	Protein-tyrosine kinase subfamilies
1. Cyclic nucleotide-dependent subfamily	1. SCR subfamily
2. Calcium-phospholipase-dependent subfamily	2. ABL subfamily
3. Calcium-calmodulin-dependent subfamily	3. Epidermal Growth Factor Receptor subfamily
4. SNF1 subfamily	4. Insulin Receptor subfamily
5. CDC28-CDC2^{+} subfamily	5. Platelet Derived Growth Factor subfamily
6. Casein kinase subfamily	6. Fibroblast Growth Factor Receptors
7. RAF-MOS proto-oncogene subfamily	7. Nerve Growth Factor Receptors
8. STE7 subfamily	8. ECK protein kinase like Receptors
9. Others	9. ARK protein kinase like Receptors
	10. Others

1.3 Activation of receptor protein kinases

Receptor protein kinases are activated by ligands which bind to the external domain of the receptor. The most plausible explanation for this activation is that ligand binding either stabilizes or induces receptor dimerization. Dimerization juxtaposes the two catalytic domains, which then leads to a conformational change allowing phosphorylation of specific tyrosine or serine/threonine residues in the cytoplasmic domain irrespective of their location within or outside the catalytic domain. Only when the ligand-bound receptor dimer becomes autophosphorylated it is able to phosphorylate substrate proteins. The ligands and substrates involved in this process have not yet been found for all protein kinases. The signal pathways activated by protein kinases may be common in most cases, specificity being provided by the ligand-binding domain and the availability of the ligand. The spectrum of substrates that bind to the protein kinase and subsequently are phoshorylated can differ based upon the binding properties and expression of the substrates in each cell type. Protein kinases are not involved in one specific process, as they are found in all kinds of tissue and at different stages of development and differentiation during embryogenesis (for review see Hunter *et al*., 1992).

1.4 Protein kinase genes as oncogenes

Since the growth of malignant cells in contrast to normal cells seems to have escaped normal control mechanisms, it was hypothesized that such cells could produce "abnormal" growth factors or respond to growth factors in an "abnormal" way. They would miss certain feedback mechanisms or simply be irreversibly committed to growth.

Among the oncogenes known to date, the genes coding for the family of protein kinases play a prominent role. Most of them were identified following transfection of NIH3T3 mouse cells with human or rodent tumour DNA. Analysis of the transfected genes showed that activation of the protein kinases is due to different mechanisms. One type of alteration often found is a DNA rearrangement resulting in a fusion gene whose product shows an exchange of amino terminal sequences between the protein kinase and the product of another gene. Examples of such rearrangements involve the tyrosine kinases genes *RET* (Takahashi & Cooper, 1985), *MET* (Chan *et al.*, 1987), *TRK* (Martin-Zanca *et al.* 1986), and *ROS* (Birchmeier *et al.*, 1986), and the serine/threonine kinase *RAF* (Shimizu *et al.*, 1985). These findings suggest the presence of regulatory domains in the lost amino terminal region, which, when abnormally replaced, could result in abnormal catalytic activity, eventually leading to tumor formation.

In tumors, amplification of genes coding for protein kinases has also been found, usually associated with tumor progression. Thus, amplification of *C-ERB-2* has prognostic implications in mammary and ovarian (Slamon *et al.*, 1989; Tsuda *et al.*, 1989), and in gastric tumors (David *et al.*, 1992). Although the gene products are not mutated, amplification of the gene causes a dosage effect, resulting in tumor progression.

C-ERB-2 also illustrated that somatic point mutations can be found associated with neoplasia as well. Point mutations in this gene have been found in human brain tumors (Kamitani *et al.*, 1992). Another example of a protein kinase gene in which point mutations can lead to neoplasms is *RET*, which will be more extensively discussed in the next chapter.

1.5 Protein kinase genes involved in hereditary disease

Protein kinases can also be involved in the development of hereditary diseases. The way in which they are genetically altered varies. In myotonic dystrophy (DM), one of the most prevalent dominant hereditary diseases in adults (1/8000), a "dynamic mutation" has been found in the *DM-PK* gene (Aslanidis *et al.*, 1992; Brook *et al.*, 1992; Buxton *et al.*, 1992; Fu *et al.*, 1992; Harley *et al.*, 1992; Jansen *et al.*, 1992; Mahadevan *et al.*, 1992). This dynamic mutation affects the length of a trinucleotide repeat (CTG) in the 3’ non-coding region of this putative serine-threonine protein kinase (Brook *et al.*, 1992; Fu *et al.*, 1992; Jansen *et al.*, 1992). It remains to be clarified how this repeat expansion alters the transcription or splicing of this and/or neighbouring genes or affects the functioning of the protein (for review see Wieringa, 1994).

The most common alterations of protein kinases associated with hereditary disease, however, are missense mutations, nonsense mutations, or frameshifts, affecting the function or expression of the protein in different ways. Germline *RET* mutations, as will be discussed in chapter 3, are not only found associated with the neoplastic syndromes MEN 2A and MEN 2B, but also in the non-neoplastic congenital disorder called Hirschsprung disease (for more details see chapter 3). Further examples are insulin resistance, caused by point mutations in the insulin receptor gene (for review see Taylor *et al.*, 1992), piebaldism, caused by mutations in *c-KIT* (Giebel & Spritz 1991; Spritz *et al.*, 1992), and X-linked agammaglobulinaemia caused by alterations in *ATK* (Vetrie *et al.*, 1993). As most of the mutations found give rise to truncated proteins, a non-functioning of gene-products is likely causing the development of the hereditary diseases mentioned.

The human protein kinase RET

Transfection studies using DNA from human T cell lymphoma led to the isolation of a transforming gene, designated *RET* (REarranged during Transfection), which consisted of two sequences linked *in vitro*, due to cointegration during transfection (Takahashi & Cooper, 1985). Similar results were obtained when DNA from human colon cancer (*RET*-II) (Ishizaka *et al.*, 1988) and stomach cancer (Kuneida *et al.*, 1991) was used. The 3' half of these transforming genes were identical, whereas the 5' parts were different.

2.1 *RET* sequence and gene structure

Using the 3' part of the rearranged *RET* genes as a probe, cDNAs of the *RET* gene could be isolated and their sequences could be determined (Takahashi *et al.*, 1988; Takahashi *et al.*, 1989; Itoh *et al.*, 1992). As the reports published show only parts of the sequence, confusion about the correct numbering of nucleotides and amino acids has occurred. In Appendix 1 the respective sequences have been combined to represent the full length cDNA sequence of both isoforms. The derived amino acid sequence of RET, the *RET* gene protein product, is also given. The indicated numbering is used throughout this thesis.

Several approaches have been applied to determine the genomic structure. Kwok *et al.* (1993) used exon trapping. Ceccherini *et al.* (1993) sequenced cloned PCR products and cosmid subclones. The cDNA sequences turned out to be spread over 20 exons (Figure 1). Appendix 2 shows the intron-exon junctions found. Expression studies, however, showed that the gene is expressed in at least two different isoforms, coding for proteins of 1072 and 1114 amino acids, respectively. They differ in their last exon, which in the short form codes for 9 amino acids, in the long form for 51 amino acids. These isoforms are the result of alternative splicing involving the last two exons (Tahira *et al.*, 1990). A recent report (Xing *et al.*, 1994) showed that alternative splicing can also occur in intron 4, as demonstrated by the detection of two different splice forms, one with an insertion of 62 base pairs, the other with an insertion of 69 base pairs between the exons 4 and 5. These two isoform transcripts are present in lower amounts than the transcript without an insertion between exons 4 and 5. Whether these isoforms are also translated remains to be determined.

The *RET* gene was localized on chromosome 10 (Donghi *et al.*, 1989; Ishizaka *et al.*, 1989). Further genetic and physical mapping refined the location of the *RET* gene and its linked markers (Norum *et al.*, 1990; Brook-Wilson *et al.*, 1993; Gardner *et al.*, 1993;

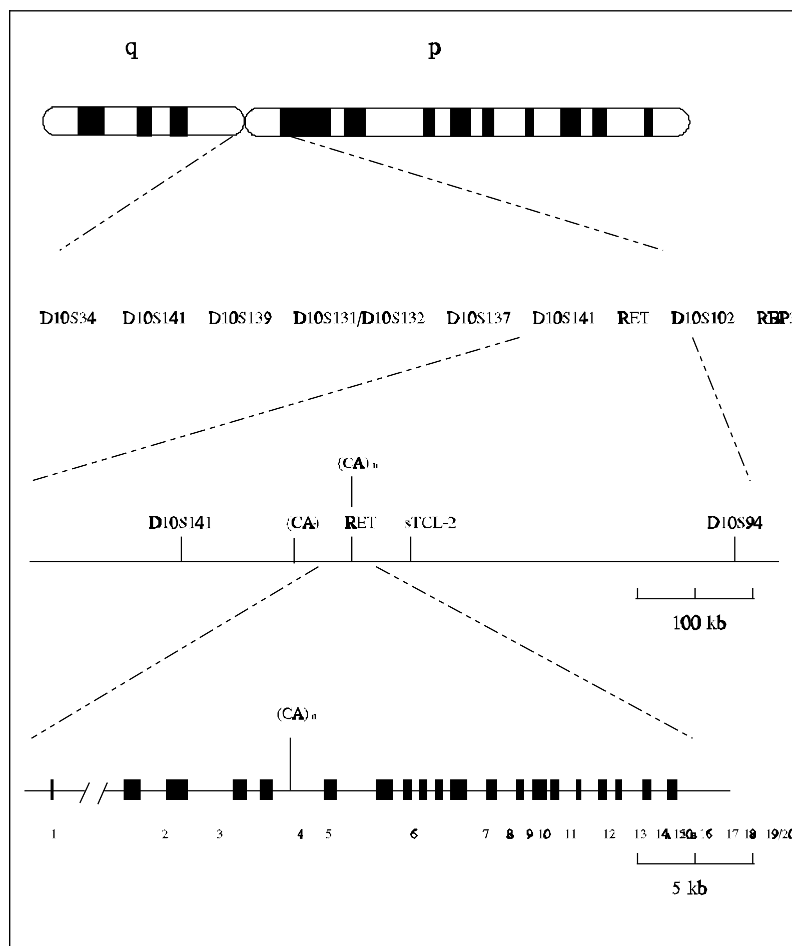


Figure 1. *Map of the centromeric region of chromosome 10 giving the order of a number of loci including RET (Hofstra et al., in press), the subregion where the RET gene is located and a diagram of the intron-exon structure of RET (Ceccherini et al., 1993, Pasini et al., submitted).*

Mole *et al.*, 1993, Lairmore *et al.*, 1993; Hofstra *et al.*, in press). In a collaborative effort we cloned and physically characterized a 150 kb region around *RET* (Pasini *et al.*, submitted). It could be demonstrated that the gene is spread over a minimum distance of 55 kb, in *Eco*RI fragments of 68 kb. The gene contains a putative CA repeat in intron 5 and is flanked by two other CA repeats (Pasini *et al.*, submitted) (Figure 1).

2.2 RET protein structure

From the cDNA sequence it could be inferred that the *RET* gene product, RET, is a cell surface protein belonging to the family of protein kinases, more specifically to the receptor tyrosine kinases (Takahashi *et al.*, 1985; Takahashi & Cooper, 1987). The extracellular domain of RET has no homology with other receptor tyrosine kinases (Takahashi *et al.*, 1988; Takahashi *et al.*, 1989).

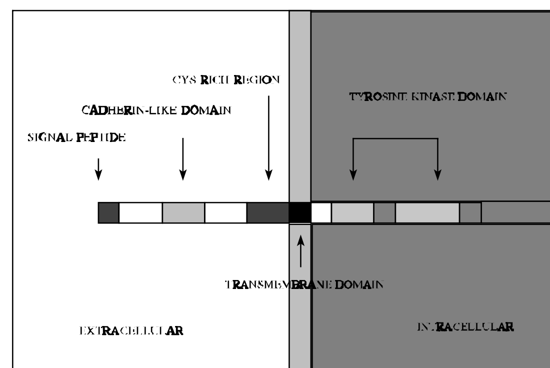


Figure 2. Schematic representation of the RET protein.

It contains a cleavable signal sequence of 28 amino acids, as well as a conserved cysteine-rich region close to the cell membrane and a cadherin-like region more toward the amino terminus (Schneider, 1992; Iwamoto *et al.*, 1993; Kuma *et al*, 1993). A single transmembrane domain is followed by an evolutionary conserved tyrosine kinase domain (Takahashi *et al.*, 1988) interrupted by an inter-tyrosine kinase region of 27 amino acids. Similarities have been found between the tyrosine kinase domains of *RET* and those of the subfamily of platelet-derived growth factor receptors (Hanks, 1988).

2.3 Expression of the *RET* gene

As already mentioned, the RET protein is expressed in two isoforms of 1072 and 1114 amino acids, differing from each other in their 9 and 51 carboxy-terminal amino acids, respectively, due to alternative splicing involving the last two exons of *RET* (Tahira *et al.*, 1990). Upon Northern blot analysis, this causes five different bands representing transcript sizes of 7.0, 6.0, 4.6, 4.5 and 3.9 kb (Tahira *et al.*, 1990).

Expression studies of *RET* in normal adult rat tissue showed very low levels of expression in lung, heart, spleen, and small intestine, whereas high levels of *RET* were observed in brain, thymus, and testis (Tahira *et al.*, 1988). In developing mice it was shown that *RET* is expressed during specific phases and in specific tissues. In the early stages of embryonic development *RET* was found expressed in the excretory system, and in the peripheral and central nervous systems (Pachnis *et al.*, 1993; Avantaggiato *et al.*, 1994; Schuchardt *et al.*, 1994). In agreement with this analysis, homozygous knock-out mouse showed intestinal aganglionosis and renal agenesis (Schuchardt *et al.*, 1994). Until now little is known about the expression of the *RET* gene in adult human tissues. Only in the thyroid a low expression of the *RET* gene was detected (Santoro *et al.*, 1990), due to expression in some but not all C cells (Fabien *et al.*, 1994). Studies of human neoplasia showed that *RET* expression is mainly limited to some solid tumor types which derive from migrating neural crest cells, such as neuroblastoma (Ikeda *et al.*, 1990; Nagao *et al.*, 1990; Tahira *et al.*, 1991; Takahashi *et al.*, 1991; Hofstra *et al.*, submitted [Appendix 6]), medullary thyroid carcinoma and pheochromocytoma (Santoro *et al.*, 1990; Itoh *et al.*, 1992; Miya *et al.*, 1992).

2.4 Function of the RET protein

The RET protein, being a tyrosine kinase receptor for a yet unidentified ligand, is thought to be involved in the signal transduction required for proliferation, migration, differentiation, and survival of neural crest cells as well as for kidney organogenesis (Pachnis *et al.*, 1994; Schuchardt *et al.*, 1994). It is not clear whether the RET protein can also function as an adhesion protein (Takahashi *et al.*, 1993).

Preliminary studies on the *RET* signal transduction pathway revealed that the RET intracellular domain is able to bind and phosphorylate SHC adaptor, PLC-gamma, and possibly RAS-GAP associated proteins, and suggest the existence of a RET-specific mitogenic pathway (Borrello *et al.*, 1994; Santoro *et al.*, 1994a).

Whether the different isoforms differ in function is presently unclear, although preliminary data suggests that the expression of the two isoforms could be tissue-specific (Pachnis *et al.*, 1993). Furthermore, they differ in their ability to bind certain factors (e.g the *GRB2* adaptor) of the signal transduction pathway (Borrello *et al.*, 1994).

The *RET* gene and its associated diseases

3.1 Papillary thyroid carcinoma

Although the first *RET* rearrangements were found *in vitro* (Takahashi *et al.*, 1985; Takahashi & Cooper, 1987), reports on *in vivo* rearrangements of this gene soon followed (Fusco *et al.*, 1987). They were named PTC, as they were found in papillary thyroid carcinoma.

Three different rearranged forms of *RET* have been identified so far, *RET-PTC*1 (Fusco *et al.*, 1987), *RET-PTC*2 (Bongarzone *et al.*, 1993), and *RET-PTC*3 (Bongarzone *et al.*, 1994; Santoro *et al.*, 1994). In all three situations genomic rearrangements between *RET* and another gene were found, resulting in the exchange of amino terminal sequences between the protein kinase and another protein. *RET-PTC*1 is composed of *H4* (D10S170) and *RET*, *RET-PTC*2 of *RI*α and *RET* and *RET-PTC*3 of *ELE* 1 and *RET*. The rearrangements have only been observed in tumors. Together they account for not more than 35% of PTC (Bongarzone *et al.*, 1994). The rearranged proteins (PTCs) share some common features: (1) the genes to which RET is translocated are all expressed in the thyrocytes. These cells do not express wildtype *RET*. As a consequence of the translocation, however, the tyrosine kinase domain of RET becomes expressed. (2) the rearrangements always take place in intron 11 of the *RET* gene; (3) the genes with which *RET* is rearranged confer to the RET intracellular domain a novel amino-terminal portion which enables the chimeric proteins to dimerize in the cytoplasm. This results in a constitutive catalytic activity of the RET-PTC protein which is ligand-independent (Bongarzone *et al.*, 1989; Ishizaka *et al.*, 1992; Lanzi *et al.*, 1992).

3.2 Multiple endocrine neoplasia Type 2A - Familial medullary thyroid carcinoma

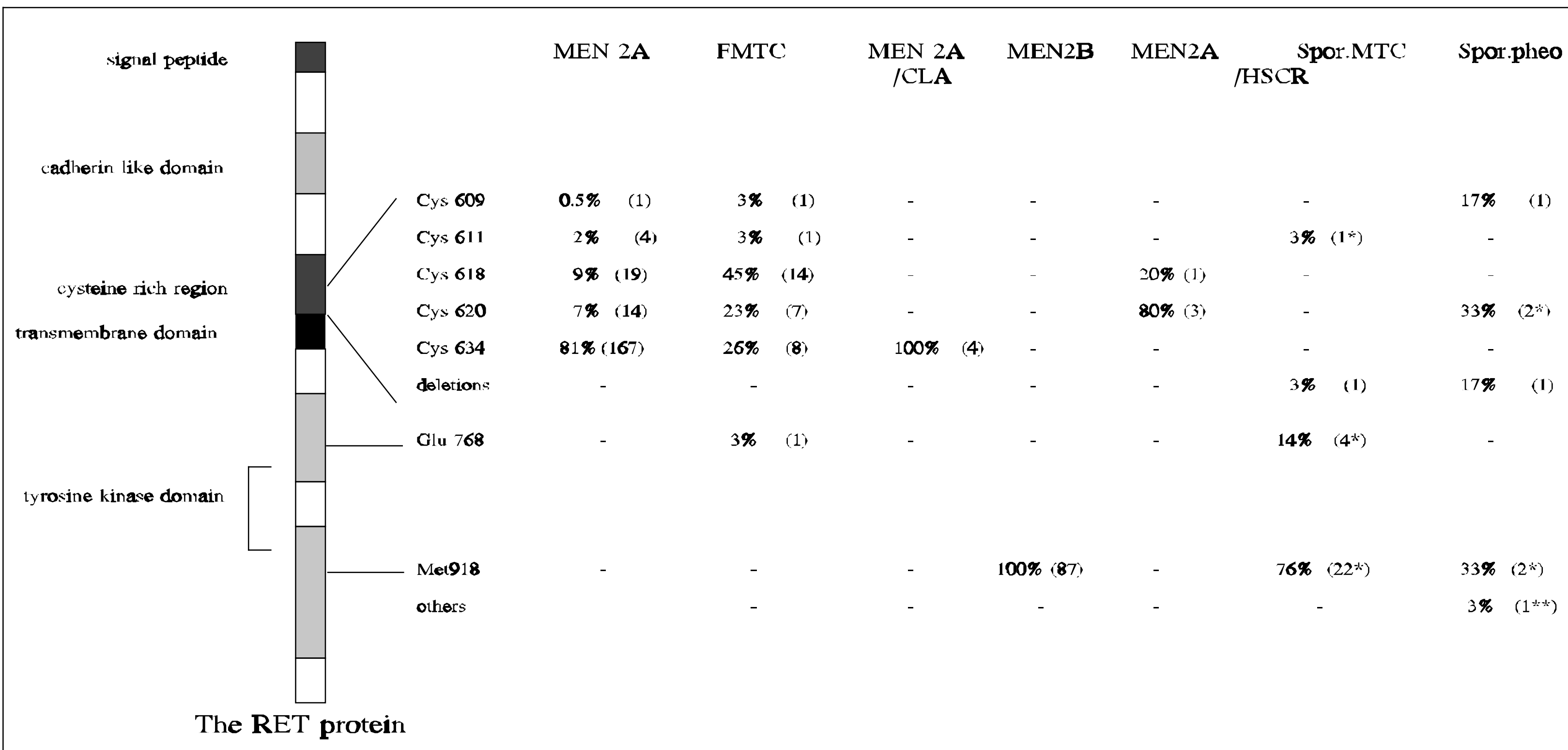
Multiple endocrine neoplasia type 2 (MEN 2) comprises at least two clinically distinct dominantly inherited cancer syndromes. MEN 2A patients develop medullary thyroid carcinoma (MTC), pheochromocytoma and parathyroid hyperplasia. MEN 2B patients do not have hyperplasia of the parathyroid, but in addition to MTC and pheochromocytoma show ganglioneuromas of the gastro-intestinal tract, and skeletal abnormalities. Familial

MTC (FMTC), usually considered a distinct third type of the MEN 2 syndromes, is characterized by MTC only. Most cases of MTC are sporadic. Approximately 25% of cases appear in the context of inherited disease, *i.e.* MEN 2 and FMTC (Saad *et al.*, 1984; Raue *et al.*, 1993). For pheochromocytoma, the percentage of cases belonging to an inherited neoplastic syndrome is similar to that found in MTC (Neumann *et al.*, 1993). The remaining pheochromocytoma cases are sporadic.

Linkage analysis suggested that either one gene or a few closely linked genes in the pericentromeric region of chromosome 10 could be involved in all three hereditary diseases (Mathew *et al.*, 1987; Simpson *et al.*, 1987; Norum *et al.*, 1990; Lairmore *et al.*, 1991; Mole *et al.*, 1993). Involvement of a single gene was confirmed by the finding of germline *RET* mutations in patients of all three inherited diseases (Donis-Keller *et al.*, 1993; Mulligan *et al.*, 1993; Carlson *et al.*, 1994; Eng *et al.*, 1994; Hofstra *et al.*, 1994) (see Table 2 for an overview).

All *RET* mutations found in MEN 2A occur in one of five codons specifying cysteine residues in the transition of the RET extracellular and transmembrane domains. They are present in a conserved region containing a total of 20 cysteine residues. FMTC is found associated with mutations in the same codons, with one exception where a mutation was found in codon 768 (Glu768→Asp) (Eng *et al.*, 1995), which has not been detected, sofar, in MEN 2A patients.

*Table 2. RET mutations found in MEN 2A, MEN 2B, FMTC, MEN 2A associated with CLA, MEN 2A associated with HSCR and sporadic MTC and pheochromocytoma. MEN 2A and FMTC data come from Donis-Keller et al., 1993; Eng et al., 1995; Komminoth et al., 1994; Marsh et al 1994; Maruyama et al., 1994; McMahon et al., 1994; Mulligan et al., 1994; Schuffenecker et al., 1994; Tsai et al., 1994; Xue et al., 1994; Zedenius et al., 1994; Takiguchi et al., 1995; Landsvater et al., in press. Data on MEN 2B from Blaugrund et al., 1994; Carlson et al., 1994; Eng et al., 1994; Hofstra et al., 1994 [Appendix 5]; Maruyama et al., 1994. MEN 2A associated with CLA from Ceccherini et al, 1994; Hofstra et al., submitted [Appendix 7]. MEN 2A associated with HSCR from Hofstra et al., 1994a; Mulligan et al., 1994a. Sporadic MTC and pheochromocytoma from Blaugrund et al., 1994; Eng et al., 1994, 1995 & 1995a; Hofstra et al., 1994 & submitted [Appendices 4 & 5]; Lindor et al., 1994; Zedenius et al., 1994. Figures between brackets are the absolute numbers of cases. * The somatic nature of the mutation found was proven in some or all cases. ** The case described by Lindor et al., (1994) having two exon 16 mutations in one tumor sample (see chapter 3.5).*



	MEN 2A	FMTC	MEN 2A /CLA	MEN2B	MEN2A /HSCR	Spor.MTC	Spor.pheo
Cys 609	0.5% (1)	3% (1)	-	-	-	-	17% (1)
Cys 611	2% (4)	3% (1)	-	-	-	3% (1*)	-
Cys 618	9% (19)	45% (14)	-	-	20% (1)	-	-
Cys 620	7% (14)	23% (7)	-	-	80% (3)	-	33% (2*)
Cys 634	81% (167)	26% (8)	100% (4)	-	-	-	-
deletions	-	-	-	-	-	3% (1)	17% (1)
Glu 768	-	3% (1)	-	-	-	14% (4*)	-
Met918	-	-	-	100% (87)	-	76% (22*)	33% (2*)
others		-	-	-	-	-	3% (1**)

The RET protein

Using site-directed mutagenesis it was shown that *RET* constructs having mutations leading to (RET/MEN2A)proteins with Cys634→Tyr, Cys634→Arg or Cys634→Trp amino acid changes, act as dominant transforming genes in NIH3T3 cells as a result of a constitutive activation of the RET protein (Santoro *et al.*, 1994). This constitutive activation was caused by a ligand-independent dimerization of the protein. Furthermore, Santoro *et al*. (1994) suggested that the mutant RET-MEN2A protein interacts with the same substrates as the wildtype protein, suggesting a change of only the catalytic properties of the RET-MEN2A protein.

A positive correlation between a specific mutation (Cys634→Arg) and the presence of parathyroid disease in MEN 2A families has been suggested (Mulligan *et al.*, 1994). Schuffenecker *et al.* (1994) found a correlation between the presence of parathyroid disease and codon 634 mutations rather than a specific amino acid substitution. This finding of Schuffenecker *et al*. (1994), was confirmed by the data of the International *RET* Mutation Consortium (Mulligan *et al*., in press). Furthermore, a positive correlation between the presence of pheochromocytoma and codon 634 mutations was detected (Mulligan *et al.*, 1994; Schuffenecker *et al.*, 1994; Mulligan *et al*., in press). Again, no correlation with a specific amino acid substitution of this codon could be shown. Codon 634 mutations occur in approximately 87% of all MEN 2A kindreds screened to date (Mulligan *et al*., in press). Based on the above correlations, it cannot be excluded that codon 634 mutations are associated with a higher risk for pheochromocytoma and parathyroid disease. Despite such a possible genotype-phenotype correlation, different germline mutations do exist that lead to similar disease phenotypes and different disease phenotypes exist that are associated with a single specific mutation (for review see Goodfellow, 1994). Conceivably, some mutations result in a higher or lower catalytic function as compared with others. This, in combination with a different *RET* sensitivity of progenitor cells of the different cancers, may account for the differences found.

Not all MEN 2A and FMTC cases exhibit detectable *RET* mutations. These are found in over 90% and in 87% of cases, respectively (Mulligan *et al*., in press). A full scale mutation scanning of *RET* in those families which did not show any of the known *RET* mutations sofar, might, however, reveal additional MEN 2A or FMTC mutations.

3.3 MEN 2A associated with cutaneous lichen amyloidosis

In some families MEN 2A has also been found associated with cutaneous lichen amyloidosis (CLA). (Gagel *et al.*, 1989; Nunziata *et al.,* 1989; Ferrer *et al.*, 1991; Kousseff *et al.*, 1991; Chabre *et al.*, 1992; Robinson *et al.*, 1992; Pacini *et al.,* 1993).

CLA is a rare skin disorder, characterized by deposits of amyloid in the papillary dermis. We screened 2 families in which both MEN 2A and CLA occurred. A mutation was detected in codon 634, namely Cys634→Arg (Hofstra *et al.*, submitted [Appendix 7]). The mutations were present in both MEN 2A and MEN 2A/CLA patients. *RET* mutation screening has been reported for another MEN 2A family with CLA-like lesions (Ceccherini *et al.*, 1994a) in which a Cys634->Tyr mutation was found.

3.4 MEN 2B

For MEN 2B, a single mutation in the *RET* proto-oncogene has been found uniquely associated (Carlson *et al.*, 1994; Eng *et al.*, 1994; Hofstra *et al.*, 1994 [Appendix 4]; see Table 2). This is a T→C transition in codon 918 of the *RET* gene, resulting in the substitution of a threonine for a methionine. Two cases have been reported that do not harbour this codon 918 mutation (Eng *et al.*, 1994). In those cases no other *RET* mutations have been detected.

It has been shown that the MEN 2B mutation, present in the catalytic domain of the protein, gives rise to a constitutively activated protein, in this case, however, with an alteration of both the catalytic function and the substrate specificity of the protein (Santoro *et al.*, 1994). Another study arrived at the same conclusion by using a degenerated peptide library to show that the *RET* mutation causing MEN 2B results in a shift in the peptide substrate specificity of the translated RET protein (Songyang *et al.*, 1995). In contrast to EGFR-RET and RET-MEN2A proteins, RET-MEN2B proteins did not form dimers (Santoro *et al.*, 1994).

In 25 out of 25 *de novo* MEN 2B cases analyzed the new mutation was of paternal origin (Carlson *et al.*, 1994). There was no indication of an imprinting phenomenon. Possibly, spermatogenesis may be more susceptible to mutations than oogenesis. In both *de novo* MEN 2B patients and in the affected offspring of MEN 2B transmitting males also a distortion of the sex ratio was observed (Carlson *et al.*, 1994).

3.5 Sporadic MTC and pheochromocytoma

RET mutations have also been reported to occur in sporadic MTC and pheochromocytoma. The mutation which occurs constitutively in MEN 2B (Met918→Thr) is also found somatically in one third of sporadic MTC (Blaugrund *et al.*, 1994; Eng *et al.*,

1994; Eng *et al.*, 1995a; Hofstra *et al.*, 1994 & submitted [Appendices 4 & 5]; Zedenius *et al.*, 1994). Three other *RET* mutations have been described in sporadic MTC, namely a 6 base pair deletion in exon 11 encompassing codon 630 (Donis-Keller *et al.*, 1993), a mutation affecting codon 768 (Glu768→Asp) of exon 13 in four sporadic MTC (Eng *et al.*, 1995), and a somatic mutation in exon 15 in several cases (Eng *et al.*, 1995a).

In sporadic pheochromocytoma, *RET* mutations have been described to occur in three exons. In exon 16, a mutation like the one found in MEN 2B was detected in two cases (Eng *et al.*, 1994; Lindor *et al.*, 1995). In one of these, an additional second exon 16 mutation (a G→C transversion affecting codon 925) was found (Lindor *et al.*, 1995). In exon 11, a 6 base pair deletion encompassing codons 632 and 633 was detected in the tumor only (Lindor *et al.*, 1995). For the mutations that are different from those found in MEN 2A and MEN 2B, it can only be speculated that they also lead to a constitutive activation of the protein product. In exon 10, a mutation affecting codon 609 was found somatically in one pheochromocytoma (Lindor *et al.*, 1995). This mutation has previously been reported in MEN 2A. In two sporadic pheochromocytoma reported by Eng *et al.* (1994) mutations were found in codon 620 of exon 10, a codon also known to be mutated in MEN 2A. A somatic nature could not be proven, since constitutional DNA was not available. Although we did find somatic *RET* mutations in some sporadic MTC, we failed to detect them in the majority of the sporadic cases (Hofstra *et al.* submitted [Appendix 5]).

The mutation data from the sporadic tumors might shed some light on the genetic basis of phenotype diversity. We and others never found somatic mutations such as those described for MEN 2A (Blaugrund *et al.*, 1994; Eng *et al.*, 1994; Eng *et al.*, 1995a; Hofstra *et al.*, 1994 & submitted [Appendices 4 & 5]; Zedenius *et al.*, 1994). The likely absence in MTC of somatic mutations identical to the constitutional mutations observed in MEN 2A patients, suggests that MEN 2A mutations *per se* cannot cause MTC, implying that constitutional *RET* mutations are probably a necessary but not sufficient condition for the development of this tumor. In pheochromocytoma, the situation seems to be somewhat different. As mentioned above, a codon 609 mutation found by Lindor *et al.* (1995) as a somatic event, may also rarely occur in MEN 2A (Mulligan *et al.*, 1994). There is, however, a notable difference in tumor behaviour between pheochromocytoma and MTC. Whereas the latter may metastasize to the lungs, liver and bones, pheochromocytoma frequently remains unnoticed, since many individuals with pheochromocytoma are asymptomatic. These arguments suggest that pheochromocytoma and parathyroid disease might be a direct result of some specific *RET* mutations, whereas MTC might be the result of a multiple step process.

3.6 Hirschsprung disease

Besides the MEN 2 syndromes also Hirschsprung disease (HSCR) was found to be linked to the centromeric region of chromosome 10 (Angrist *et al.*, 1993; Lyonett *et al.*, 1993). HSCR is a congenital disorder characterized by the absence of parasympathic intrinsic ganglion cells in the submucosal and myenteric plexuses of the hindgut, resulting in intestinal obstruction in neonates and in severe obstipation in infants. HSCR is regarded to be the consequence of a premature arrest of the craniocaudal migration of neural crest cells toward the anal end of the rectum during early embryonic development. A further reduction of the region for the HSCR gene strongly indicated *RET* as a candidate for HSCR (Yin *et al.*, 1994). A mutation analysis of *RET* was carried out and proved that *RET* was indeed involved in HSCR (Edery *et al.*, 1994; Romeo *et al.*, 1994). The *RET* mutations observed do not seem to be restricted to certain codons and are scattered all over the gene. They can be divided in three groups, namely those leading to truncated proteins (nonsense mutations, deletions and insertions), those consisting of missense mutations, and those including deletions of the entire *RET* gene, thereby causing haploinsufficiency. Table 3 summarizes the mutations reported by Yin *et al.* (1994a), Angrist *et al.* (in press) and Attie *et al.* (submitted).

Mutations leading to truncated proteins obviously have a major deleterious effect on the protein and its function. The effects of missense mutations are more difficult to predict. Some seem to inactivate the protein, as demonstrated by site-directed mutagenesis carried out on PTC2 constructs (Pasini *et al.*, 1995). Introduction of HSCR missense mutations in the *RET* part of PTC2 causes a complete loss of transforming capacity of the mutated PTC2 proteins. This suggests that loss of function of the RET protein translated from the mutant *RET* allele could be the cause of the aganglionosis found in HSCR patients. This idea is supported by the finding that mice homozygous for a null mutant at the *RET* locus, have total intestinal aganglionosis (Schuchardt *et al.*, 1994). Although heterozygous mice did not show an abnormal phenotype, in man HSCR cases have been described with heterozygous deletions of 10q11.2, implying that, in man, haploinsufficiency for *RET* is critical for the development of the disease.

Mutations are found in both short and long segment HSCR, as well as in cases with total colonic aganglionosis, suggesting that if there is a difference between these forms of HSCR it should be allelic (Edery *et al.*, 1994a; Angrist *et al.*, in press). No specific mutation pattern could, however, be distinguished.

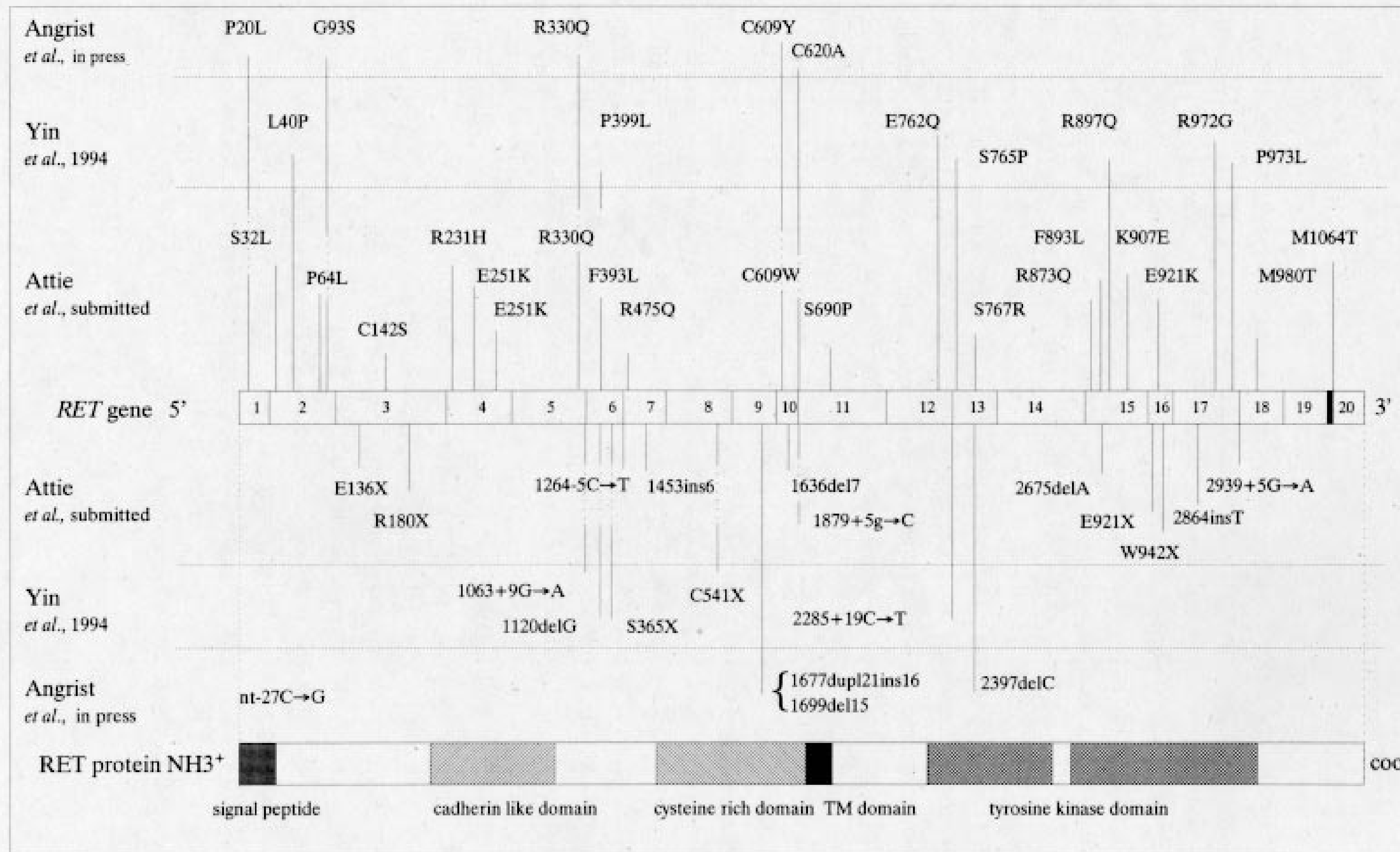

Angrist
et al., in press
P20L
G93S
R330Q
C609Y
C620A
Yin
et al., 1994
L40P
P399L
E762Q
R897Q
R972G
S765P
P973L
S32L
R231H
R330Q
F893L
K907E
M1064T
Attie
et al., submitted
P64L
E251K
F393L
C609W
R873Q
E921K
M980T
E251K
R475Q
S690P
S767R
C142S
RET gene 5'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
3'
Attie
et al., submitted
E136X
1264-5C→T
1453ins6
1636del7
2675delA
2939+5G→A
R180X
1879+5g→C
E921X
2864insT
W942X
Yin
et al., 1994
1063+9G→A
C541X
1120delG
S365X
2285+19C→T
Angrist
et al., in press
nt-27C→G
1677dupl21ins16
1699del15
2397delC
RET protein NH3+
coo-
signal peptide
cadherin like domain
cysteine rich domain
TM domain
tyrosine kinase domain

Table 3. RET mutations found in HSCR patients. Data from Yin et al., 1994, Angrist et al., in press and Attie et al., submitted.

3.7 Hirschsprung disease associated with MEN 2A

The rare occurrence of both MEN 2A and HSCR in some families, has been described. To our knowledge mutation analysis of *RET* has been reported in 5 families, one described by us (Hofstra *et al.*, 1994a) and four reported by Mulligan *et al.* (1994a). In our family and in three of Mulligan's families a Cys620→Arg mutation was found. The remaining family had a Cys618→Arg mutation. In all cases a arginine is substituted for a cysteine in either codon 618 or 620. These codons account for only 16 % of all MEN 2A mutations found (Table 2). It might well be that only the specific mutations mentioned account for the combined HSCR/MEN 2A phenotype. It should be noted that genetically HSCR is very heterogeneous. Puffenberger *et al*. (1994) showed linkage to chromosome 13q22 in a large consanguineous Mennonite family. Mutations associated with HSCR occurred in a gene, the endothelin B receptor gene, present in this region (Puffenberger *et al*., 1994a). They further found evidence for a modifier gene on chromosome 21 and for a possible involvement of *RET* on the expression of the HSCR phenotype in this family (Puffenberger *et al*., 1994). In mice four loci are known for congenital megacolon based on aganglionosis, namely lethal spotted (*ls*), piebald lethal (s^l), and dominant spotting (*DOM*) (for overview see Kapur, 1993), and *RET* (Schuchardt *et al*., 1994). In families showing both HSCR and MEN 2A a modifier gene might well modulate the expression of the HSCR phenotype. On the other hand, since MEN 2/HSCR patients occur in different branches of the families described, it is unlikely that a rare unlinked modifying locus would co-segregate with a *RET* mutation through multiple sibships in the same kindred (Mulligan *et al.*, 1994a). Consequently, the mutations found would indeed be responsible for the combined phenotype.

3.8 Possible involvement of *RET* in other neurocristopathies

Based on the involvement of *RET* in the development of neural-crest derived tissues and on the association of *RET* mutations with neurocristopathies such as the MEN 2 syndromes and HSCR, a search for *RET* mutations in other neurocristopathies seems justified. Neuroblastoma occasionally occurs in diseases associated with abnormal

neurocrest differentiation, e.g. Hirschsprung disease. Furthermore, neuroblastomas express *RET*. We therefore scanned the entire *RET* gene in a neuroblastoma patient belonging to a family in which different neurocrestopathies occurred, including Hirschsprung disease and ganglioneuroma, as well as in 16 neuroblastoma cell lines. No *RET* mutations were found. Therefore expression of *RET* in neuroblastoma might just reflect the differentiation status of the tumor cells, rather than indicating an involvement in the tumorigenesis of neuroblastoma (Hofstra *et al.* submitted [Appendix 6]) .

Summary

Protein kinases can be classified in two main classes serine/threonine and tyrosine kinases. They show auto-phosphorylation in response to stimuli (ligands) and can thereby phosphorylate substrate proteins. For many protein kinases the signalling pathways and also the ligands or stimuli which activate them, are still unknown.

The *RET* proto-oncogene encodes a receptor tyrosine kinase involved in the normal development and the neoplastic growth of neural crest cell lineages. The ligand of the receptor is as yet unidentified. During embryogenesis *RET* expression is high in neuroectodermal tissues, suggesting a function of RET in the proliferation, the migration and the differentiation of these cell types. In adult tissues the gene is hardly expressed. Expression is high in several tumor types derived from neural crest cells.

Transfection studies with DNA from different tumors revealed focal proliferation due to the presence of different DNA sequences that, however, shared a common part called *RET*. The original *RET* gene turned out to be rearranged in such a way that the sequences coding for the extracellular part of its protein product were replaced by sequences from elsewhere, resulting in a rearranged protein with a constitutive tyrosine kinase activity. The same rearrangement occurs in papillary thyroid carcinoma (PTC).

Protein kinases can be involved in various ways in neoplastic syndromes and tumors, and in non-neoplastic hereditary diseases. This also holds true for *RET*. After the genes involved in both MEN 2A and MEN 2B and in HSCR had been mapped to the centromeric region of chromosome 10 by linkage analysis, mutations of *RET*, a gene present in this very region, were found responsible for the development of these diseases. MEN 2A and MEN 2B are associated with specific mutations in the *RET* gene resulting in an activation of the protein translated, whereas HSCR is associated with mutations resulting in a functional loss of the translated protein.

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Appendix 1

The *RET* sequence

Sequence of the long and short isoforms of the RET gene (according to, Takahashi et al., 1988, Takahashi et al., 1989, Itoh et al., 1989). Base pair numbering starts from the first amino acid.

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-648  CCG CGG GGT CGC ACC CCG AGC CAG TCG GCC AGA CCT GCA TCC CGC GTA GCA TCC CTG CCC   -589
-588  TCT CTG TGC AGC GGA AAG GGC AAA AGG CAG GGA CTG CAA GCG GGC GCG CAC CGG GTA GGA   -529
-528  AGA GCG GCT CTG CGT AGG TGC GCG GAC CCG GGC TCC TGG GTT CCA TCC CCG CCG CGC ACC   -469
-468  TCG GGG TCC GCA CCC GGC TCC TGC CGG GCC CTT TTC GGC CGC ACC CCG CTC CCG CAC CCC   -409
-408  GCT CCT CCC CAA GCC CCA CCC GGC CCA AGC CGC CGT CCC GCA CTG AGC TCC TAC ACG CGC   -349
-348  CGG CCC CGG CCG CAC CCC GCG CAC GCA GAG CAA GCA CTG GAG CCC CGC CCC TTC CCG CAC   -289
-288  CCC ACC CGC CTC CGG CCC CGC CTG GCC CAC CCC TGG ACC GCC CCC GCC CCG CCC CGC CCC   -229
-228  TAC CCG CTC CTC GGC GCA GCC GGC GCT TGC CTA GCT TCA GTC CCG CGA CCG AAG CAG GGC   -169
-168  GCG CAG CAG CGC TGA GTG CCC CGG AAC GTG CGT CGC GCC CCC AGT GTC CGT CGC GTC CGC   -109
-108  CGC GCC CCG GGC GGG GAT GGG GCG GCC AGA CTG AGC GCC GCA CCC GCC ATC CAG ACC CGC    -49

   1                                                              Met Ala Lys Ala      4
 -48  CGG CCC TAG CCG CAG TCC CTC CAG CCG TGG CCC CAG CGC GCA CGG GCG ATG GCG AAG GCG     12

   5  Thr Ser Gly Ala Ala Gly Leu Arg Leu Leu Leu Leu Leu Leu Leu Pro Leu Leu Gly Lys     24
  13  ACG TCC GGT GCC GCG GGG CTG CGT CTG CTG TTG CTG CTG CTG CTG CCG CTG CTA GGC AAA     72

  25  Val Ala Leu Gly Leu Tyr Phe Ser Arg Asp Ala Tyr Trp Glu Lys Leu Tyr Val Asp Gln     44
  73  GTG GCA TTG GGC CTC TAC TTC TCG AGG GAT GCT TAC TGG GAG AAG CTG TAT GTG GAC CAG    132

  45  Ala Ala Gly Thr Pro Leu Leu Tyr Val His Ala Leu Arg Asp Ala Pro Glu Glu Val Pro     64
 133  GCG GCC GGC ACG CCC TTG CTG TAC GTC CAT GCC CTG CGG GAC GCC CCT GAG GAG GTG CCC    192

  65  Ser Phe Arg Leu Gly Gln His Leu Tyr Gly Thr Tyr Arg Thr Arg Leu His Glu Asn Asn     84
 193  AGC TTC CGC CTG GGC CAG CAT CTC TAC GGC ACG TAC CGC ACA CGG CTG CAT GAG AAC AAC    252

  85  Trp Ile Cys Ile Gln Glu Asp Thr Gly Leu Leu Tyr Leu Asn Arg Ser Leu Asp His Ser    104
 253  TGG ATC TGC ATC CAG GAG GAC ACC GGC CTC CTC TAC CTT AAC CGG AGC CTG GAC CAT AGC    312

 105  Ser Trp Glu Lys Leu Ser Val Arg Asn Arg Gly Phe Pro Leu Leu Thr Val Tyr Leu Lys    124
 313  TCC TGG GAG AAG CTC AGT GTC CGC AAC CGC GGC TTT CCC CTG CTC ACC GTC TAC CTC AAG    372

 125  Val Phe Leu Ser Pro Thr Ser Leu Arg Glu Gly Glu Cys Gln Trp Pro Gly Cys Ala Arg    144
 373  GTC TTC CTG TCA CCC ACA TCC CTT CGT GAG GGC GAG TGC CAG TGG CCA GGC TGT GCC CGC    432

 145  Val Tyr Phe Ser Phe Phe Asn Thr Ser Phe Pro Ala Cys Ser Ser Leu Lys Pro Arg Glu    164
 433  GTA TAC TTC TCC TTC TTC AAC ACC TCC TTT CCA GCC TGC AGC TCC CTC AAG CCC CGG GAG    492

 165  Leu Cys Phe Pro Glu Thr Arg Pro Ser Phe Arg Ile Arg Glu Asn Arg Pro Pro Gly Thr    184
 493  CTC TGC TTC CCA GAG ACA AGG CCC TCC TTC CGC ATT CGG GAG AAC CGA CCC CCA GGC ACC    552

 185  Phe His Gln Phe Arg Leu Leu Pro Val Gln Phe Leu Cys Pro Asn Ile Ser Val Ala Tyr    204
 553  TTC CAC CAG TTC CGC CTG CTG CCT GTG CAG TTC TTG TGC CCC AAC ATC AGC GTG GCC TAC    612

 205  Arg Leu Leu Glu Gly Glu Gly Leu Pro Phe Arg Cys Ala Pro Asp Ser Leu Glu Val Ser    224
 613  AGG CTC CTG GAG GGT GAG GGT CTG CCC TTC CGC TGC GCC CCG GAC AGC CTG GAG GTG AGC    672

 225  Thr Arg Trp Ala Leu Asp Arg Glu Gln Arg Glu Lys Tyr Glu Leu Val Ala Val Cys Thr    244
 673  ACG CGC TGG GCC CTG GAC CGC GAG CAG CGG GAG AAG TAC GAG CTG GTG GCC GTG TGC ACC    732

 245  Val His Ala Gly Ala Arg Glu Glu Val Val Met Val Pro Phe Pro Val Thr Val Tyr Asp    264
 733  GTG CAC GCC GGC GCG CGC GAG GAG GTG GTG ATG GTG CCC TTC CCG GTG ACC GTG TAC GAC    792

 265  Glu Asp Asp Ser Ala Pro Thr Phe Pro Ala Gly Val Asp Thr Ala Ser Ala Val Val Glu    284
 793  GAG GAC GAC TCG GCG CCC ACC TTC CCC GCG GGC GTC GAC ACC GCC AGC GCC GTG GTG GAG    852

 285  Phe Lys Arg Lys Glu Asp Thr Val Val Ala Thr Leu Arg Val Phe Asp Ala Asp Val Val    304
 853  TTC AAG CGG AAG GAG GAC ACC GTG GTG GCC ACG CTG CGT GTC TTC GAT GCA GAC GTG GTA    912

 305  Pro Ala Ser Gly Glu Leu Val Arg Arg Tyr Thr Ser Thr Leu Leu Pro Gly Asp Thr Trp    324
 913  CCT GCA TCA GGG GAG CTG GTG AGG CGG TAC ACA AGC ACG CTG CTC CCC GGG GAC ACC TGG    972

 325  Ala Gln Gln Thr Phe Arg Val Glu His Trp Pro Asn Glu Thr Ser Val Gln Ala Asn Gly    344
 973  GCC CAG CAG ACC TTC CGG GTG GAA CAC TGG CCC AAC GAG ACC TCG GTC CAG GCC AAC GGC   1032
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345 Ser Phe Val Arg Ala Thr Val His Asp Tyr Arg Leu Val Leu Asn Arg Asn Leu Ser Ile 364
1033 AGC TTC GTG CGG GCG ACC GTA CAT GAC TAT AGG CTG GTT CTC AAC CGG AAC CTC TCC ATC 1092

365 Ser Glu Asn Arg Thr Met Gln Leu Ala Val Leu Val Asn Asp Ser Asp Phe Gln Gly Pro 384
1093 TCG GAG AAC CGC ACC ATG CAG CTG GCG GTG CTG GTC AAT GAC TCA GAC TTC CAG GGC CCA 1152

385 Gly Ala Gly Val Leu Leu Leu His Phe Asn Val Ser Val Leu Pro Val Ser Leu His Leu 404
1153 GGA GCG GGC GTC CTC TTG CTC CAC TTC AAC GTG TCG GTG CTG CCG GTC AGC CTG CAC CTG 1212

405 Pro Ser Thr Tyr Ser Leu Ser Val Ser Arg Arg Ala Arg Arg Phe Ala Gln Ile Gly Lys 424
1213 CCC AGT ACC TAC TCC CTC TCC GTG AGC AGG AGG GCT CGC CGA TTT GCC CAG ATC GGG AAA 1272

425 Val Cys Val Glu Asn Cys Gln Ala Phe Ser Gly Ile Asn Val Gln Tyr Lys Leu His Ser 444
1273 GTC TGT GTG GAA AAC TGC CAG GCG TTC AGT GGC ATC AAC GTC CAG TAC AAG CTG CAT TCC 1332

445 Ser Gly Ala Asn Cys Ser Thr Leu Gly Val Val Thr Ser Ala Glu Asp Thr Ser Gly Ile 464
1333 TCT GGT GCC AAC TGC AGC ACG CTA GGG GTG GTC ACC TCA GCC GAG GAC ACC TCG GGG ATC 1392

465 Leu Phe Val Asn Asp Thr Lys Ala Leu Arg Arg Pro Lys Cys Ala Glu Leu His Tyr Met 484
1393 CTG TTT GTG AAT GAC ACC AAG GCC CTG CGG CGG CCC AAG TGT GCC GAA CTT CAC TAC ATG 1452

485 Val Val Ala Thr Asp Gln Gln Thr Ser Arg Gln Ala Gln Ala Gln Leu Leu Val Thr Val 504
1453 GTG GTG GCC ACC GAC CAG CAG ACC TCT AGG CAG GCC CAG GCC CAG CTG CTT GTA ACA GTG 1512

505 Glu Gly Ser Tyr Val Ala Glu Glu Ala Gly Cys Pro Leu Ser Cys Ala Val Ser Lys Arg 524
1513 GAG GGG TCA TAT GTG GCC GAG GAG GCG GGC TGC CCC CTG TCC TGT GCA GTC AGC AAG AGA 1572

525 Arg Leu Glu Cys Glu Glu Cys Gly Gly Leu Gly Ser Pro Thr Gly Arg Cys Glu Trp Arg 544
1573 CGG CTG GAG TGT GAG GAG TGT GGC GGC CTG GGC TCC CCA ACA GGC AGG TGT GAG TGG AGG 1632

545 Gln Gly Asp Gly Lys Gly Ile Thr Arg Asn Phe Ser Thr Cys Ser Pro Ser Thr Lys Thr 564
1633 CAA GGA GAT GGC AAA GGG ATC ACC AGG AAC TTC TCC ACC TGC TCT CCC AGC ACC AAG ACC 1692

565 Cys Pro Asp Gly His Cys Asp Val Val Glu Thr Gln Asp Ile Asn Ile Cys Pro Gln Asp 584
1693 TGC CCC GAC GGC CAC TGC GAT GTT GTG GAG ACC CAA GAC ATC AAC ATT TGC CCT CAG GAC 1752

585 Cys Leu Arg Gly Ser Ile Val Gly Gly His Glu Pro Gly Glu Pro Arg Gly Ile Lys Ala 604
1753 TGC CTC CGG GGC AGC ATT GTT GGG GGA CAC GAG CCT GGG GAG CCC CGG GGG ATT AAA GCT 1812

605 Gly Tyr Gly Thr Cys Asn Cys Phe Pro Glu Glu Glu Lys Cys Phe Cys Glu Pro Glu Asp 624
1813 GGC TAT GGC ACC TGC AAC TGC TTC CCT GAG GAG GAG AAG TGC TTC TGC GAG CCC GAA GAC 1872

625 Ile Gln Asp Pro Leu Cys Asp Glu Leu Cys Arg Thr Val Ile Ala Ala Ala Val Leu Phe 644
1873 ATC CAG GAT CCA CTG TGC GAC GAG CTG TGC CGC ACG GTG ATC GCA GCC GCT GTC CTC TTC 1932

645 Ser Phe Ile Val Ser Val Leu Leu Ser Ala Phe Cys Ile His Cys Tyr His Lys Phe Ala 664
1933 TCC TTC ATC GTC TCG GTG CTG CTG TCT GCC TTC TGC ATC CAC TGC TAC CAC AAG TTT GCC 1992

665 His Lys Pro Pro Ile Ser Ser Ala Glu Met Thr Phe Arg Arg Pro Ala Gln Ala Phe Pro 684
1993 CAC AAG CCA CCC ATC TCC TCA GCT GAG ATG ACC TTC CGG AGG CCC GCC CAG GCC TTC CCG 2052

685 Val Ser Tyr Ser Ser Ser Gly Ala Arg Arg Pro Ser Leu Asp Ser Met Glu Asn Gln Val 704
2053 GTC AGC TAC TCC TCT TCC GGT GCC CGC CGG CCC TCG CTG GAC TCC ATG GAG AAC CAG GTC 2112

705 Ser Val Asp Ala Phe Lys Ile Leu Glu Asp Pro Lys Trp Glu Phe Pro Arg Lys Asn Leu 724
2113 TCC GTG GAT GCC TTC AAG ATC CTG GAG GAT CCA AAG TGG GAA TTC CCT CGG AAG AAC TTG 2172

725 Val Leu Gly Lys Thr Leu Gly Glu Gly Glu Phe Gly Lys Val Val Lys Ala Thr Ala Phe 744
2173 GTT CTT GGA AAA ACT CTA GGA GAA GGC GAA TTT GGA AAA GTG GTC AAG GCA ACG GCC TTC 2232

745 His Leu Lys Gly Arg Ala Gly Tyr Thr Thr Val Ala Val Lys Met Leu Lys Glu Asn Ala 764
2233 CAT CTG AAA GGC AGA GCA GGG TAC ACC ACG GTG GCC GTG AAG ATG CTG AAA GAG AAC GCC 2292

765 Ser Pro Ser Glu Leu Arg Asp Leu Leu Ser Glu Phe Asn Val Leu Lys Gln Val Asn His 784
2293 TCC CCG AGT GAG CTT CGA GAC CTG CTG TCA GAG TTC AAC GTC CTG AAG CAG GTC AAC CAC 2352

785 Pro His Val Ile Lys Leu Tyr Gly Ala Cys Ser Gln Asp Gly Pro Leu Leu Leu Ile Val 804
2353 CCA CAT GTC ATC AAA TTG TAT GGG GCC TGC AGC CAG GAT GGC CCG CTC CTC CTC ATC GTG 2412

805 Glu Tyr Ala Lys Tyr Gly Ser Leu Arg Gly Phe Leu Arg Glu Ser Arg Lys Val Gly Pro 824
2413 GAG TAC GCC AAA TAC GGC TCC CTG CGG GGC TTC CTC CGC GAG AGC CGC AAA GTG GGG CCT 2472

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 825  Gly Tyr Leu Gly Ser Gly Gly Ser Arg Asn Ser Ser Ser Leu Asp His Pro Asp Glu Arg    844
2473  GGC TAC CTG GGC AGT GGA GGC AGC CGC AAC TCC AGC TCC CTG GAC CAC CCG GAT GAG CGG   2532

 845  Ala Leu Thr Met Gly Asp Leu Ile Ser Phe Ala Trp Gln Ile Ser Gln Gly Met Gln Tyr    864
2533  GCC CTC ACC ATG GGC GAC CTC ATC TCA TTT GCC TGG CAG ATC TCA CAG GGG ATG CAG TAT   2592

 865  Leu Ala Glu Met Lys Leu Val His Arg Asp Leu Ala Ala Arg Asn Ile Leu Val Ala Glu    884
2593  CTG GCC GAG ATG AAG CTC GTT CAT CGG GAC TTG GCA GCC AGA AAC ATC CTG GTA GCT GAG   2652

 885  Gly Arg Lys Met Lys Ile Ser Asp Phe Gly Leu Ser Arg Asp Val Tyr Glu Glu Asp Ser    904
2653  GGG CGG AAG ATG AAG ATT TCG GAT TTC GGC TTG TCC CGA GAT GTT TAT GAA GAG GAT TCC   2712

 905  Tyr Val Lys Arg Ser Gln Gly Arg Ile Pro Val Lys Trp Met Ala Ile Glu Ser Leu Phe    924
2713  TAC GTG AAG AGG AGC CAG GGT CGG ATT CCA GTT AAA TGG ATG GCA ATT GAA TCC CTT TTT   2772

 925  Asp His Ile Tyr Thr Thr Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu Trp Glu Ile    944
2773  GAT CAT ATC TAC ACC ACG CAA AGT GAT GTA TGG TCT TTT GGT GTC CTG CTG TGG GAG ATC   2832

 945  Val Thr Leu Gly Gly Asn Pro Tyr Pro Gly Ile Pro Pro Glu Arg Leu Phe Asn Leu Leu    964
2833  GTG ACC CTA GGG GGA AAC CCC TAT CCT GGG ATT CCT CCT GAG CGG CTC TTC AAC CTT CTG   2892

 965  Lys Thr Gly His Arg Met Glu Arg Pro Asp Asn Cys Ser Glu Glu Met Tyr Arg Leu Met    984
2893  AAG ACC GGC CAC CGG ATG GAG AGG CCA GAC AAC TGC AGC GAG GAG ATG TAC CGC CTG ATG   2952

 985  Leu Gln Cys Trp Lys Gln Glu Pro Asp Lys Arg Pro Val Phe Ala Asp Ile Ser Lys Asp   1004
2953  CTG CAA TGC TGG AAG CAG GAG CCG GAC AAA AGG CCG GTG TTT GCG GAC ATC AGC AAA GAC   3012

1005  Leu Glu Lys Met Met Val Lys Arg Arg Asp Tyr Leu Asp Leu Ala Ala Ser Thr Pro Ser   1024
3013  CTG GAG AAG ATG ATG GTT AAG AGG AGA GAC TAC TTG GAC CTT GCG GCG TCC ACT CCA TCT   3072

1025  Asp Ser Leu Ile Tyr Asp Asp Gly Leu Ser Glu Glu Glu Thr Pro Leu Val Asp Cys Asn   1044
3073  GAC TCC CTG ATT TAT GAC GAC GGC CTC TCA GAG GAG GAG ACA CCG CTG GTG GAC TGT AAT   3132

1045  Asn Ala Pro Leu Pro Arg Ala Leu Pro Ser Thr Trp Ile Glu Asn Lys Leu Tyr Gly Met   1064
3133  AAT GCC CCC CTC CCT CGA GCC CTC CCT TCC ACA TGG ATT GAA AAC AAA CTC TAT GGC ATG   3192
                                                                              ^
1065  Ser Asp Pro Asn Trp Pro Gly Glu Ser Pro Val Pro Leu Thr Arg Ala Asp Gly Thr Asn   1084
3193  TCA GAC CCG AAC TGG CCT GGA GAG AGT CCT GTA CCA CTC ACG AGA GCT GAT GGC ACT AAC   3252

1085  Thr Gly Phe Pro Arg Tyr Pro Asn Asp Ser Val Tyr Ala Asn Trp Met Leu Ser Pro Ser   1104
3253  ACT GGG TTT CCA AGA TAT CCA AAT GAT AGT GTA TAT GCT AAC TGG ATG CTT TCA CCC TCA   3312

1105  Ala Ala Lys Leu Met Asp Thr Phe Asp Ser ***                                       1114
3313  GCG GCA AAA TTA ATG GAC ACG TTT GAT AGT TAA CAT TTC TTT GTG AAA GGT AAT GGA CTC   3372

3373  ACA AGG GGA AGA AAC ATG CTG AGA ATG GAA AGT CTA CCG GCC CTT TCT TTG TGA ACG TCA   3432
3433  CAT TGG CCG AGC CGT GTT CAG TTC CCA GGT GGC AGA CTC GTT TTT GGT AGT TTG TTT TAA   3492
3493  CTT CCA AGG TGG TTT TAC TTC TGA TAG CCG GTG ATT TTC CCT CCT AGC AGA CAT GCC ACA   3552
3553  CCG GGT AAG AGC TCT GAG TCT TAG TGG TTA ACC ATT CCT TTC TCT TCA GTG CCC AGC AGC   3612
3613  ACC CAG TGT TGG TCT GTG TCC ATC AGT GAC CAC CAA CAT TCT GTG TTC ACA TGT GTG GGT   3672
3673  CCA ACA CTT ACT ACC TGG TGT ATG AAA TTG GAC CTG AAC TGT TGG ATT TTT CTA GTT GCC   3732
3733  GCC AAA CAA GGC AAA AAA ATT TAA ACA TGA AGC ACA CAC ACA AAA AAG GCA GTA GGA AAA   3792
3793  ATG CTG GCC CTG ATG ACC TGT CCT TAT TCA GAA TGA GAG ACT GCG GGG GGG GCC TGG GGG   3852
3853  TAG TGT CAA TGC CCC TCC AGG GCT GGA GGG GAA GAG GGG CCC CGA GGA TGG GCC TGG GCT   3912
3913  CAG CAT TCG AGA TCT TGA GAA TGA TTT TTT TTT AAT CAT GCA ACC TTT CCT TAG GAA GAC   3972
3973  ATT TGG TTT TCA TCA TGA TTA AGA TGA TTC CTA GAT TTA GCA CAA TGG AGA GAT TCC ATG   4032
4033  CCA TCT TTA CTA TGT GGA TGG TGG TAT CAG GGA AGA GGG CTC ACA AGA CAC ATT TGT CCC   4092
4093  CCG GGC CCA CCA CAT CAT CCT CAC GTG TTC GGT ACT GAG CAG CCA CTA CCC CTG ATG AGA   4152
4153  ACA GTG TGA AGA AAG GGG GCT GTT GGA GTC CCA GAA TTG CTG ACA GCA GAG GCT TTG CTG   4212
4213  CTG TGA ATC CCA CCT GCC ACC AGC CTG CAG CAC ACC CCA CAG CCA AGT AGA GGC GAA AGC   4272
4273  AGT GGC TCA TCC TAC CTG TTA GGA GCA GGT AGG GCT TGT ACT CAC TTT AAT TTG AAT CTT   4332
4333  ATC AAC TTA CTC ATA AAG GGA CAG GCT AGC TAG CTG TGT TAG AAG TAG CAA TGA CAA TGA   4392
4393  CCA AGG ACT GCT ACA CCT CTG ATT ACA ATT CTG ATG TGA AAA AGA TGG TGT TTG GCT CTT   4452
4453  ATA GAG CCT GTG TGA AAG GCC CAT GGA TCA GCT CTT CCT GTG TTT GTA ATT TAA TGC TGC   4512
4513  TAC AAG GTG TTT CTG TTT CTT AGA TTC TGA CCA TGA CTC ATA AGC TTC TTG TCA TTC TTC   4572
4573  ATT GC                                                                            4577



1063   ly Arg Ile Ser Tyr Ala Phe Thr Arg Phe ***                                       1072
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3188   GT AGA ATT TCC CAT GCA TTT ACT AGA TTC TAG CAC CGC TGT CCC CTC TGC ACT ATC CTT    3246

3247  CCT CTC TGT GAT GCT TTT TAA AAA TGT TTC TGG TCT GAA CAA AAC CAA AGT CTG TGC TCT    3306
3307  GAA CCT TTT TAT TTG TAA ATG TCT GAC TTT TGC ATC CAG TTT ACA TTT AGG CAT TAT TGC    3366
3367  AAC TAG TTT TCT AAA AGG T                                                         3385
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Appendix 2

The intron-exon junctions of *RET*

The table is showing all intron-exon junctions of the RET gene. Between parentheses the number of the first basepair of each exon is mentioned according to the sequence presented in appendix 1. The size of the exons, the approximate size of the introns and 10 flanking intronic basepairs are given according to Ceccherini et al., 1993, Pasini et al. submitted (for references see page 32)

Appendix 2

3' end intron	5' end exon (bp pos. RET	EXON number and size	3' end exon	5' end intron	Intron size
gcacgggcgATGGCGAAGG		exon 1	CTAGGCAAAGgtgagttctg		
	(1)	73 bp			24000 bp
cttcccacagTGGCATTGGG		exon 2	AGTGTCCGCAgtaagggagc		
	(74)	264 bp			1650 bp
ctctctgcagACCGCGGCTT		exon 3	CTCCTGGAGGgtgagtgccg		
	(338)	288 bp			2350 bp
tggtgcgcagGTGAGGGTCT		exon 4	AGCGGAAGGAgtgcttgtcc		
	(626)	242 bp			800 bp
catcctgcagGACACCGTGG		exon 5	CATGACTATAgtaagagggg		
	(868)	196 bp			2400 bp
ctacctgcagGGCTGGTTCT		exon 6	ATTTGCCCAGgtgagcccat		
	(1064)	200 bp			2000 bp
gccccctagATCGGGAAAG		exon 7	GAGGGGTCATgtgagtgcct		
	(1264)	259 bp			680 bp
ccacctgcagATGTGGCCGA		exon 8	GATGGCAAAGgtaagccctg		
	(1523)	126 bp			620 bp
tcctgtgcagGGATCACCAG		exon 9	GACTGCCTCCgtaagcaggg		
	(1649)	111 bp			610 bp
tctgcctcagGGGGCAGCAT		exon 10	GACATCCAGGgtgagtgggt		
	(1760)	120 bp			850 bp
ccacccacagATCCACTGTG		exon 11	CAAGATCCTGgtgagggtcc		
	(1880)	257 bp			1650 bp
tccaacatagGAGGATCCAA		exon 12	GATGCTGAAAgtacctgcca		
	(2137)	148 bp			1600 bp
tgcatttcagGAGAACGCCT		exon 13	AGCCAGGATGgtaaggccag		
	(2285)	108 bp			1100 bp
ccgcccccagGCCCGCTCCT		exon 14	CGAGATGAAGgtgcgtgcat		
	(2393)	215 bp			350 bp
ttcctcacagCTCGTTCATC		exon 15	GAGGAGCCAGgtgcccagtc		
	(2608)	123 bp			1700 bp
ttctctttagGGTCGGATTC		exon 16	AAAGTGATGTgtaagtgtgg		
	(2731)	71 bp			1650 bp
ctctctgcagATGGTCTTTT		exon 17	GCGAGGAGATgtgagcgggg		
	(2802)	138 bp			1100 bp
ttcccaccagGTACCGCCTG		exon 18	TAAGAGGAGAgtgagtgcct		
	(2940)	100 bp			1600 bp
tgtcttccagGACTACTTGG		exon 19	AAACTCTATATG		
	(3040)	148 bp			0 bp
GTAGAATTTCCCATGCATTTACTAGATTCtagcaccgct					
	(3188)	29 bp			1350 bp
tcatttttagGCATGTCAGA		exon 20b	CTTTGATAGTtaacatttct		
	(3188)	154 bp			

Appendix 3

DNA polymorphisms and conditions for SSCP analysis of the 20 exons of the *RET* proto-oncogene

Ceccherini, I.[1], Hofstra, R.M.W.[2], Yin, L.[1], Stulp, R.P.[2], Barone, V.[1], Stelwagen, T.[2], Boccardi, R.[1], Nijveen, H.[2], Bolino, A.[1], Seri, M.[1], Ronchetto, P.[1], Pasini, B.[1], Bozzano, M.[1], Buys, C.H.C.M.[2] & Romeo, G.[1]

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Abstract

Recently identified mutations affecting different domains of the RET proto-oncogene are associated with multiple endocrine neoplasia type 2A (MEN 2A) and type 2B (MEN 2B), familial and sporadic medullary thyroid carcinoma (MTC) and Hirschsprung disease (HSCR). In order to facilitate the screening for RET mutations and to study possible genotype-phenotype correlations we established exon-intron junctions and extended the intronic sequences flanking the 20 exons of this gene. This made it possible to design primers and develop PCR conditions useful for SSCP analysis of the whole RET coding sequence. Nine conformational variants were observed which after sequencing turned out to be 8 silent mutations and a conservative amino acid substitution. Restriction analysis performed on DNA samples from unrelated controls confirmed the polymorphic nature of six of these nucleotide changes and made it possible to estimate the frequency of the corresponding alleles.

The recent identification of mutations in different domains of the *RET* proto-oncogene in inherited human disease, namely multiple endocrine neoplasia type 2A (MEN 2A) (Donis-Keller *et al*., 1993; Mulligan *et al*., 1993) and type 2B (MEN 2B) (Carlson *et al*., 1994; Eng *et al*., 1994; Hofstra *et al*., 1994), familial and sporadic medullary thyroid carcinoma (FMTC or MTC) (Donis-Keller *et al*., 1993; Eng *et al*., 1994; Hofstra *et al*., 1994; Mulligan *et al*., 1994), MEN 2A with associated cutaneous lichen amyloidosis (CLA) (Ceccherini *et al*., 1994) and Hirschsprung disease (HSCR) (Edery *et al*., 1994; Romeo *et al*., 1994), suggests that *RET* plays a critical role in the differentiation of specific cell lineages of neural crest origin and in the maintenance of their differentiated state.

In order to facilitate the detection of point mutations responsible for these disorders we report in table 1 the intronic sequences flanking the 20 exons of the *RET* proto-oncogene. The intronic sequences flanking the 3' end of exon 19 which encodes the last 9 amino acids of the short form of the RET protein are also shown in the same Table. The latter amino acids are alternative to the 51 encoded by exon 20 in the long form of the RET protein (Tahira *et al*., 1990). An 'N' within a sequence indicates a nucleotide which could not be precisely identified.

One hundred and twenty three DNA samples from unrelated HSCR, MEN 2A, MEN 2B and MTC patients have been screened by PCR-SSCP analysis for the 20 exons of the *RET* proto-oncogene, during a study aimed at the detection of causative mutations which in part have already been reported (Ceccherini *et al*., 1994; Hofstra *et al*., 1994; Romeo *et al*., 1994), using oligonucleotide primers which are underlined in Table 1. Seven additional, equally efficient sets of primers, partially or totally located externally to the previous ones, are printed in capitals in the same Table for exons 5, 6, 12, 13, 15, 16 and
19, respectively. Forward and reverse primers specific for exons 6, 10, 11, 13 15, 16 and 17 were already reported (Ceccherini *et al*., 1994; Hofstra *et al*., 1994; Romeo *et al*., 1994). The forward primer used for exon 11 was previously described by Donis-Keller *et al*. 1993 (8AF primer).

The PCR conditions we used for the SSCP analysis (annealing temperature and magnesium concentration) are reported in Table 2 together with the expected size of the fragments. In order to improve the resolution of the SSCP analysis, restriction cleavage with the enzymes indicated was also carried out in all those cases where the amplification product was longer than 300 bp (Glavac & Dean, 1993; Hayashi & Yandell, 1993). Several gel compositions and running conditions were applied for each of the 20 exons in order to increase the probability of detecting SSCP variant bands (Glavac & Dean, 1993; Hayashi & Yandell, 1993). In particular 6-8 µl of either restricted or non-restricted PCR product were loaded on nondenaturating polyacrylamide gel containing 6% acrylamide prepared with a 49:1 ratio between acrylamide and bisacrylamide and alternatively without glycerol or with 5 and 10% glycerol, either in 1X TBE or 0.5X TBE buffer. Gels were run both at room temperature (overnight at 6W with 10% glycerol and in a cold room 1-6 h at 50 W without glycerol and with 5 and 10% glycerol). In a non radioactive PCR, DNA bands on the gel were visualized by silver staining according to a protocol already described (Budowle *et al*., 1991).

After SSCP analysis, every PCR product showing a conformation variant was sequenced as already reported (Hofstra *et al*., 1994; Romeo *et al*., 1994) and the corresponding nucleotide change thus assessed. Table 3 reports the nucleotide change changes and the corresponding amino acid changes associated with each of 9 mutations found after PCR-SSCP analysis and direct sequencing. Eight of these are silent mutations while one represents a conservative amino acid change, namely in exon11 where in our control sample we could detect either a glycine residue (79%) or a serine residue (21%) at codon 691 of the RET protein.

In order to make possible the screening of a large set of unrelated individuals for those variants which were observed by SSCP analysis in more than one sample (namely in exons 2, 3, 7, 11, 13 and 15), the DNA sequence surrounding each nucleotide change was analyzed and restriction sites generated or destroyed by the mutation were identified. The restriction sites are reported in Table 3 together with the expected sizes of the restricted fragments (constant bands are not shown). Such a restriction analysis allowed us to assess the polymorphic nature of 6 out of 9 SSCP variants and to estimate the frequency of the corresponding alleles (Table 3). No genotype frequency for each of the 6 polymorphic loci was found to deviate from the expected value calculated according to Hardy Weinberg equilibrium. PIC values have also been calculated according to Hearne *et al*. (1992) and are shown in the same Table. Despite their low information content these polymorphisms may be useful for population genetic studies. The study of a possible linkage

Table 1. Sequences flanking the 20 exons of the RET proto-oncogene and couples of primers designed for their amplification (indicated as underlined or in capital letters)

gctgagtgccccggaacgtgcgtcgcgcccccagtgtccgtcgcgtccgccgcgccccgggcggggatggggcggccagactgagcgccgcaccc-
gccatccagacccgccggccctagccgcagtccc tccagccgtggccccagcgcgcacgggcg(1)
EXON1gtgagttctgccggccgccggctcccgcagggccagggcgaagttggcgccgagcagcggacggcgcgttcagaagcgcctttctgtttgcc
gtgggcacgcgggctgtgcggtccccggccggccacccggcctcggctgggagcgcagtggctgcggcgggc-
gggggcggcgggcaggacgcctcgggccgggcgcctcgggccggggctgggcgggtctcgggcgggagcgga

tccntgaagaagccatattctcaccatccctcactcacatccctacttcccacag**EXON2**gtaagggagccgccccaacacccaccccgtgcccc-
accccacccccttcctcaagccgcccttatcacagccgctgacactgaagcttggcatggcttcccccccaccgctggtgtggaaggcgtcnaggg-
gttaagtgaggctggcctgcctctgtgtccmgccyggagagaagccaggacaagcttcagggctgcgtgaggctgtaggagtttggagacacacg-
ggantcatccgggtacactcttcctgccagancccgaatccctcttccag

ttgtggaccttggtggggaccagggtttacaccagccctggagctcctgcctcnnccccattcccgactgcctggcagatgtggccgatgcccc-
acagacctgacttctctctgcag**EXON3**gtgagtgccgaccttgtggggccgccccacagtgcctgctactgctggtcttgctctgcgagccctt-
gacacaagccatctggtttattcttcaccttcatgccatcagttcattcaatattccagagtacctctgacatactgcagg

tctgaccgcagagccccccttcccgaggaaagcggctggcccggtcccggctggtgatcacgcggggcccctgtctgcttggtgcgcag**EXON4**gt-
gcttgtccgcgcgtgctgtggtctacccagtgtctgtctccggccacagttcgtttctcggtcggtttagtgtccgtgtagccacccaacccg

tggctctacaacacacatctggtccacctatgggctgtgtggacgtgcagcatCCTAAGGTCTCTGGTTTTGGggggtctgaggggnc-
catctcgcctgcactgaccaacgccctctgcatcctgcag**EXON5**gtaagaggggctggtggcacggcctggctaggcccccca-
gGAAATGAGGTGCTCGCTCTTcatgggcaagcagcaccctacacacatgcacacctggcatggccctctgt

tgcgtacacatgcacacacacaggctgcctcaaattgagaagggttccttggactttcagttcagtaaatcccaacgtttgaacattggtgctaa-
cttaggaccagccccaggcctgttgcatggcactgtatgtgtgaaagtgcgtgtttgcaccagtgtgagtgcggggctgtgtct-
gggaagaggtgtgctacaCATGAGGAAGCAGCCAGAGCagcttggtggtcattgttgtgcccctacctgcag
EXON6gtgagcccatacctattgcctgtctggggaagattgaaaggccaagggacatgggggcACAGGGAGGCAGGTGACACTgc
ctcttggcccaaccagcacagagtagactg

tctaccctcaggccattacaggccggtccagctgcctggctaaggtgttcccctgtgcccccctag**EXON7**gtgagtgcctgctccagggaggga-
gggtcggggtctgggagcttctggagcctgggcct

atccgtgggcagntcagctggtgctgttccctgtccttgggcactagctggacgctgggcccaggccagccccctgtgaccctgcttgtctgcca-
cctgcag**EXON8**gtaagccctggaaacgcccaagggaggcctgcaggggcgatggcaccggtggaaacngggtcct

tctgcctagagtgtggggtgtggcggggctcccacatgggtgacagtctgctgtgtgtcctgtgcag**EXON9**gtaagcagggtttaatcagggca-
tgggaacaggtaggagatagtaggggaaacctggatcccacaggcacttcagccagaggttgccagggctgtcagttctatgcatcangctgagc-
tctgtgcattgcatcctgcat

gcgccccaggaggctgagtgggctacgtctgcctcag**EXON10**gtgagtgggtggcggccgggaccaccaccacctcccag-
ccccacagaggtctcaacagcacatctgaggt

cctctgcggtgccaagcctcacaccacccccacccacag**EXON11**gtgagggtccctgcgggcagggaagatcccctgccct-
ccccagctgccttccagggagggaggccagctggggagacagaggccatcctgtgaggggctgccaacgctgggcagacgagg-
cctgtgttctgcccccatttccatagggcgctgtgtggggacagtctgtggggtgggactatgatgaggtgccgttcccatctaggtgagaggcagtg-
gtcagggtcacagcatcgggcnggggagcagcagtgtggatngaggggcaccgaagtc

tcagatgacagccggttctctgcacattggaacttgtccatggggcctcctttaagggtcttGCCTTCTTCCTCCCCTGTCATcctcacactt-
ttcccccctcttctccccttccctcatttccaacatag**EXON12**gtacctgccaggcacaggcACAGTGCCCCTGGGGGAGTCtc-
cggggcggggggcgggtgaggcccctcctgcccagcatgggaccctgaaga

tgatgagcccctgtccactgatcccaaaggctgggagaagcctcaagcagcatcgtctttgcaggcctctctgtctgAACTTGGGCAA-
GGCGATGCAggtccatcctcacctggtatggtcatggaaggggcttccaggagcgatcgtttgcaacctgctctgtgctgcatttcag-
EXON13gtaaggccagctgcagggtgaggtgggcagccactgcacccaggctggggGCTCCATACAGCCCTGTTCTccctcttt-
ctccct

cctggctcctggaagacccaagctgcctgacccgcacgcccagggcccctctctccgcccccag**EXON14**gtgcgtgcatat-
ggctctgcacccagccagccccggccaggccacaccctgacccaccacg

catgtcacaccctgactccaccacgcccctgccatgccacacccccggcccaggtctcaccaggccgctacccgggccacacacc-
acccctctgctggtcacaccaggctgagccagTGACCGCTGCTGCCTGGCCATggcctgacgactcgtgctatttttcctcacag**EX-
ON15**gtgcccagtcccggggatgaggcggggctcccagggatcccaggtgcaccatgggGCAGGCAGTCCTTGGGAAGC-
ctaggaaagataccgaagattagtggagctctaagc

AGGGATAGGGCCTGGGCTTCtccnttacccctccttcctagagagttagagtaacttcaatgtctttattccatcttctc-
tttag**EXON16**gtaagtgtgggtgttgCTCTCTTGGGGTGGAGGTTAcagaaacacccttatacatgtagtggggccacgacncc-
cgtctgtgcagcttggccagggaattgcactgg

tctgtgagggccaggtggagccactcactggtccttcactctctgcag**EXON17**gtgagcggggactggctttggcccagcctc-
acttgggaaggaaggggacatctgtgtgcattccctccccatccagagcagccccaggagaaaccaggagaagtgggggtggg-
gagtgggcagggcagaggttagagag

ccccagtcccacgaggctcagagatgtcagcgatgcagaaatagctttggagttggagacagagcacactgggcccagggtacag-
ggcagggtgcgatggctgtggtgggctgtccttctgagacctggccctgcttggatcatattggcctgtctgctcttcccaccag
EXON18gtgagtgcctgggtccaattcccacaagctgaaagtggcttggggagactccagcctcaccccagggcagtagt-
tttagccctcagagttcccagtgtggggccacagtgggattgtgcagagagagagagtcatgctctcccctgcatgcatacagcagatt

ctgagttgtatcTAGTTGTGGCACATGGCTTGgagtgaccggccatctctgtcttccag**EXON19GTA-
GAATTTCCCATGCATTTACTAGATTC**[2]tagcaccgctgtccccTCTGCACTATCCTTCCTCtctgtgatgcttttt-
aaaaatgtttctggtctgaacaaaaccaaagtctgtgctctgaaccttttatttgtaaatgtctgacttttgcatccagtttacatttaggcattattgcaactagtt-
ttctaaaaggt

tgccgaccagtggtttgaacatcaaagggagttttgccaaggccttactgtctgcacttgaagttttggttcttcagtgcagaacaaatga-
tctgttttcatttttag**EXON20**taacatttctttgtgaaaggtaatggactcacaaggggaagaaacatgctgagaatggaaag-
tc[1]taccggccctttctttgt

(1)The 5’ and 3’ transcribed untranslated sequences had been already reported together with those corresponding to each exon (not shown) (see Takahashi et al., 1988, 1989 & Ceccherini et al., 1993). (2) The bold sequence downstream exon 19 encodes for the last nine amino acids of the short form of the RET protein. Alternatively 51 amino acids encoded by exon 20 are incorporated in the long form of the RET protein.

Table 2 PCR conditions for the amplification of each of the 20 exons of the RET proto-oncogene

	PCR conditions[a]				
Exon	Annealing temp.(oC)	Mg conc. (mM)	Product size (bp)	Restriction cleavage	Size of the fragments
1[b]	68	1	166		
2	57	1.5	387	Sau3AI	163+224
3	65	1	375	SacI	182+192
4	61	1	342	HinfI	145+197
5	60	1	275		
5[c]	50	1.5	329	AvaI	151+178
6	60	1.2	251		
6[c]	56	1.5	333	HaeIII	139+194
7	62	1.2	367	BamHI	176+191
8	63	1.2	262		
9	60	1.2	160		
10	68	1.5	187		
11	65	1.5	416	StuI	204+212
12	61	1.5	225		
12[c]	58	1.5	267		
13	60	1.2	239		
13[c]	57	1.5	277		
14	65	1.5	328	StyI	127+201
15	60	1.2	234		
15[c]	60	1.5	251		
16	53	1.2	135		
16[c]	58	1.5	192		
17	60	1.2	231		
18	60	1.2	234		
19	59	1.2	229		
19[c]	55	1.5	260		
20	55	1	266		

[a]All reactions were set up at 30 cycles following previously described general conditions (Romeo et al., 1994). [b]10% DMSO and a 1:100 ratio between forward and reverse primer were also neccessary. [c]PCR condition to be applied when the corresponding primers printed in capitals in Table 1 are used.

disequilibrium between these polymorphic alleles and the most common causative mutations of the *RET* gene observed in some neurocristopathies might be of interest.

Since each of the remaining 3 SSCP variants was observed in only one chromosome out of the 246 alleles screened, the corresponding nucleotides changes have been considered as private variants (Table 3).

The presence of silent mutations found in exons 13 and 15 of the *RET* proto-oncogene was already reported (Mulligan *et al*., 1993), although the frequency of the corresponding polymorphisms had not been investigated.

The PCR products used in this study for the SSCP analysis of the *RET* gene may be also used efficiently with either modified SSCP protocols, like RNA-SSCP (Danenberg *et al.*, 1992), dideoxy fingerprinting (Sarkar *et al*., 1992), amplification refractory mutation system (ARMS)-SSCP (Lo *et al*., 1992), or other procedures, suitable for the screening of point mutations, like denaturing gradient gel electrophoresis (DDGE) (Myers *et al*., 1985a), mismatch cleavage by RNAse or chemical agents (Myers *et al*., 1985b; Cotton *et al*., 1988), electrophoresis of heteroduplex (White *et al*., 1992).

The intronic sequences and the 27 sets of primers reported here together with the PCR conditions suitable to amplify the corresponding DNA fragments, represent therefore a valuable tool for the overall screening of the coding sequence of the *RET* proto-oncogene. Moreover mutations affecting both the consensus sequences for the RNA splicing and the sequences coding the last 9 amino acids of the short form of the RET protein can also be detected.

Table 3 Nucleotide changes (polymorphisms and private variants) detected in the coding sequence of RET

EXON	Nucleotide change	Amino acid substitution	Restriction sitechanged	Size of the alleles (1)[a]	(2)[a]	controls tested (N)	Allele 1 frequency	PIC[b]
polymorphic nucleotide changes								
2	GCG GCA	Ala 45	EagI	104+283	387	52	0,71	0.41
3	GTC GTA	Val 125	MboII	64+72	136	49	0.98	0.04
7	GCG GCA	Ala 432	BsmI	32+97	129	45	0.29	0.41
11	GGT AGT	Gly 691 Ser	BanI	185+223	408	53	0.79	0.33
13	CTT CTG	Leu 769	TaqI	106+133	239	46	0.74	0.38
15	TCC TCG	Ser 904	RsaI	81+130	211	48	0.21	0.33
Private nucleotide changes								
6	TCG TCA	Ser 396				123		
7	TCG TCA	Ser 462				123		
11	ATC ATT	Ile 647				123		

[a]Allele 1 is always defined as the one with the restriction site present, allele 2 with the restriction site absent. [b]PIC: polymorphism information content calculated as described by Hearne et al.(1992)

The point mutation analysis of the *RET* gene, now feasible for its whole coding sequence and exon-intron junctions, will contribute to the identification of genotype-phenotype correlations in MEN 2A, MEN 2B, MTC and HSCR patients, thus improving the comprehension of the biological role of *RET* in differentiation and the maintenance of the differentiated state of cells of neural crest origin. To the same end patients with other neurocristopathies and families

showing recurrence of HSCR and MEN 2 cosegregating in the same members (Verdy *et al.*, 1982; Mahaffey *et al.*, 1990) should also be considered for mutation screening of the *RET* gene.

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Oncogene (1995) **10**, 1257

Erratum PIC values reported in Table 3 should be read as heterozygosity values.

Appendix 4

A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma

Hofstra, R.M.W.[1], Landsvater, R.M.[2], Ceccherini, I.[3], Stulp, R.P.[1], Stelwagen, T.[1], Yin Luo[3], Pasini, B.[3], Höppener, J.W.M.[2], Ploos van Amstel, H.K.[2], Romeo, G.[3], Lips C.J.M.[2] & Buys C.H.C.M.[1]

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Nature, 367, 375-376, 1994.

Abstract

Multiple endocrine neoplasia type 2 (MEN 2) comprises three clinically distinct dominantly inherited cancer syndromes. MEN 2A patients develop medullary thyroid carcinoma (MTC) and phaeochromocytoma. MEN 2B patients show in addition ganglioneuromas of the gastro-intestinal tract and skeletal abnormalities. In familial MTC, only the thyroid is affected. Germ-line mutations of the RET proto-oncogene have recently been reported in association with MEN 2A and familial MTC[1,2]. All mutations occurred within codons specifying cysteine residues in the transition point between the RET protein extracellular and transmembrane domains. We now show that MEN 2B is also associated with mutation of the RET proto-oncogene. A mutation in codon 918, causing the substitution of a threonine for a methionine in the tyrosine kinase domain of the protein, was found in all nine unrelated MEN 2B patients studied. The same mutation was found in six out of 18 sporadic tumours.

As the MEN 2 syndromes resemble several other hereditary cancers in occurring both in a familial form, characterised by a dominant pattern of inheritance, and in a sporadic form, involvement of tumour-suppressor genes seemed likely[3]. The disease locus for both MEN 2A and MEN 2B has been assigned to chromosome 10 (refs 4-6). Still, MTCs and phaeochromocyt-omas seldom show allelic losses for this chromosome, although these would be expected in the case of a tumour-suppressor mechanism[7,8]. Recently, mutations in the *RET* proto-oncogene were described in association with MEN 2A[1,2]. If these mutations underlie the MEN 2A phenotype, a dominant or dominant-negative mechanism is a more probable explanation for this syndrome. Thusfar, no mutations have been reported to be associated with MEN 2B.

Determination of the gene structure of *RET*[9] allowed us to design specific intronic primer pairs for almost all exons. When these were used in a single-strand conformational polymorphism (SSCP) analysis on constitutive DNA from MEN 2B patients, a variant pattern for exon 16 was found in the DNA from all 9 MEN 2B patients, but not in DNA from 70 indepen-dent persons, nor in DNA available from the parents of three of the MEN 2B patients (Fig. 1). Sequence analysis of the polymerase chain reaction (PCR) products of exon 16 revealed a T→C transition at position 2753 (appendix 1) in one of the alleles of all the MEN 2B patients. A threonine (ACG) is thereby substituted for a methionine (ATG) at codon 918. As the mutation eliminates a *Fok*I restriction site (GGATG(N)9/13→GGACG(N)9/13), digestion of the PCR products of exon 16 by this restriction enzyme was used to confirm the presence of the mutation in all MEN 2B patients (Fig. 2). As the MEN 2B patients analysed originated from The Netherlands, Italy and North America, our *1 2 3 4 5 6 7 8 9*

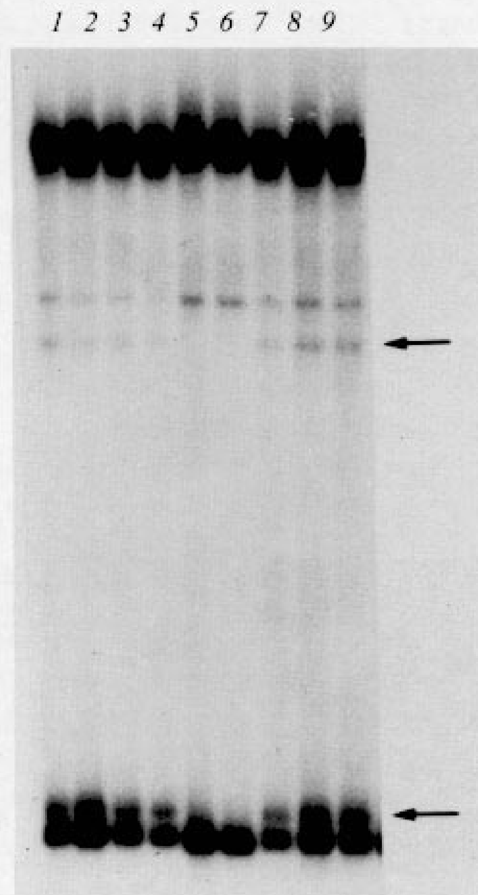

FIG. 1 Single-strand conformational polymorphism (SSCP) analysis[14] of RET. The autoradiogram shows SSCP patterns of PCR-amplified products of exon 16 of the RET gene. MEN 2B patients are indicated as 1-4 and 7-9, the parents of 4 are indicated as 5 and 6. Arrows indicate the positions of the SSCP variant bands.
METHODS.
The PCR primers designed were fRET 16 (5'-AGGGATAGGGCCTGGGCTTC-3') and rRET 16 (5'-TAACCTCCACCCCAAGAGAG-3'). They give a PCR product of 192 base pairs (bp) containing the entire exon 16 and part of the flanking intronic sequences. Radioactive PCR amplification was carried out on 150 ng of DNA in a total volume of 30 μl for 25 cycles at 92°C for 35 s, 58°C for 35 s and 72°C for 60 s using (α-^{32}P)dCTP. SSCP analysis was carried out under three different sets of conditions. The variant pattern could be detected in all. The figure shows an autoradiogram of a 6% acrylamide gel containing 10 % glycerol. DNA was electrophoresed in 45 mM Tris-Borate, 45 mM Boric acid, 1 mM EDTA, pH 8.0, at 20°C.

results suggest that a single *RET* mutation may underlie most, if not all, of the MEN 2B cases.

When analysing tumour DNA from 18 sporadic MTC patients for this mutation, we detected in six cases the same SSCP variant, *Fok*I restriction pattern and sequence as found in the MEN 2B patients. No indication for an exon 16 mutation was obtained for five sporadic phaeochromocytomas. The same held true for 15 independent MEN 2A patients.

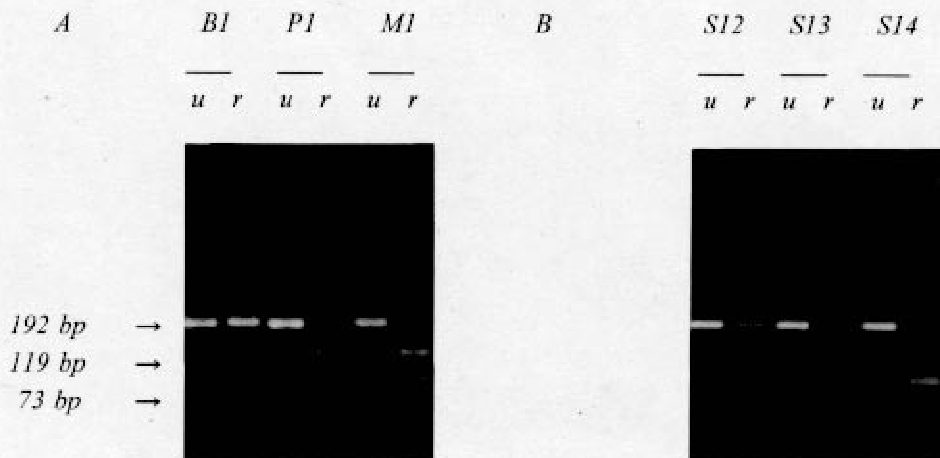

FIG. 2 Restriction patterns of PCR products of exon 16 with FokI. The described mutation eliminates a FokI restriction site. Whereas normally both alleles are restricted, in the presence of the mutation the mutated allele will not be restricted. A, B, The undigested (u) and the restricted (r) PCR products of constitutive DNA from a MEN 2B patient, designated B1, and from his parents designated P1 and M1 (A), and tumour DNA from 3 sporadic MTC patients, S12-S14. As can be seen, S12 has the mutation whereas S13 and S14 do not (B).

METHODS. A non radioactive PCR was carried out described in Fig. 1 legend. The PCR products were purified in low-melting-point agarose, isolated (Sephaglas BandPrep kit; Pharmacia) and digested for 1 h using 2 U FokI (Boehringer) in the restriction buffer recommended by the manufacturer. The samples were run in a 1 % normal agarose/2 % low-melting-point agarose gel.

The codon 918 mutation affects the intracellular tyrosine kinase domain of RET[10], whereas the mutations associated with MEN 2A are all in the extracellular region, close to the transmembrane domain. This may account for the different phenotypes of MEN 2A and MEN 2B. Occurrence of a somatic mutation of the *RET* proto-oncogene may lead to neoplasia of the tissue involved. This explains the finding in sporadic MTC of mutations affecting the same codons as in MEN 2A[2] and in MEN 2B (the present study). No constitutive DNA was available for our patients with sporadic MTC. However, the absence of additional MEN 2B symptoms makes a germ-line mutation unlikely.

The T→C transition in codon 918 of the *RET* gene affects the protein kinase domain of the gene product in subdomain VIII, one of the major conserved subdomains of the protein kinases. In some of the subfamilies of protein tyrosine kinases, including the platelet-derived growth-factor receptor subfamily to which the RET protein belongs, there is a methionine at the relevant peptide position in the kinase domain[11]. In the remaining subfamilies, this position is occupied by a threonine in the large majority of cases. The protein serine/threonine kinase class shows a substantial diversity at this position, but no occurrence of threonine[11]. Remarkably, the *RET* mutation in MEN 2B leads to the substitution of a threonine, mainly found in the other set of protein tyrosine kinases, for a methionine, normally found in the set to which RET belongs. We therefore, expect that the mutation causes some change in substrate specificity or perhaps in mode of regulation rather than in catalytic function.

As protein tyrosine kinases do not occur in yeast and as a protein tyrosine kinase catalytic domain is part of many growth factor receptors, tyrosine specificity may have evolved in multicellular eukaryotes to play a role in cell-to-cell communication[11]. For RET, this suggestion is corroborated by similarities between its extracellular receptor domain and cadherins, transmembrane proteins that mediate cell-cell adhesion[12]. A critical role in mammalian embryogenesis, notably in migrating neural crest cells of the developing peripheral nervous and excretory system, is suggested by the results of *RET* expression studies and gene targeting experiments in mice [13]. From these observations we are now beginning to understand the various clinical symptoms of the syndromes in which RET plays a role.

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The base pair and amino acid numbering in this Appendix is according to Appendix 1

Appendix 5

Mutation scanning of *RET* in sporadic Medullary Thyroid Carcinoma and of *RET* and *VHL* in sporadic Pheochromocytoma

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Abstract

Sporadic medullary thyroid carcinoma (MTC) and pheochromocytoma (PC) have been reported to be associated with some specific RET gene mutations. A complete mutation analysis of the whole coding sequence, including the intron-exon junctions, has only recently become possible. In order to assess the role of RET in the development of MTC and PC we have screened 15 sporadic MTC and 5 sporadic PC cases for RET mutations by a systematic analysis of all exons. Apart from the Met918→Thr mutation which we had detected earlier in 6 of the MTC cases, we found a Cys634→Trp mutation in only one additional MTC, the claimed sporadic nature of which could not be confirmed. We conclude that; (i) a somatic Met918→Thr mutation of Ret is sufficient for MTC development; (ii) the majority of sporadic MTC is likely due to mutations in (an) unidentified gene(s) other than RET. Since PC is a frequent complication in families suffering from von Hippel Lindau disease, for which mutations of the VHL gene are responsible, we screened the 5 sporadic PC cases also for VHL mutations. This revealed a Gly164→Ser mutation in one specimen. Thus in PC, most tumors are presumably due to mutations in (an) unidentified gene(s) other than RET and VHL.

Introduction

An estimated 20-25% of medullary thyroid carcinomas (MTC) appear in the context of inherited disease, i.e. in the multiple endocrine neoplasia type 2 (MEN 2) syndromes and the familial form of medullary thyroid carcinoma. (Saad *et al*., 1984; Raue *et al*., 1993). For pheochromocytomas (PC), the percentage of cases being part of an inherited neoplastic syndrome is similar as for MTC (Neumann *et al*., 1993). They occur as the only type of tumor in familial PC (Calkins & Howard, 1947; Kaufman & Franklin, 1979), in addition to MTC in the MEN 2 syndromes, or as one of several types of tumors in Von Hippel Lindau disease. Their occurrence, albeit at a very low frequency, is also known in neurofibromatosis type I. The large majority of PC, however, consists of sporadic cases.

MEN 2A, familial MTC (Mulligan *et al*., 1993a; Donis-Keller *et al*., 1993; Mulligan *et al*, 1994; Eng *et al*., 1995a), and MEN 2B (Hofstra *et al*., 1994; Carlson *et al*., 1994; Eng *et al*., 1994) are associated with specific constitutive *RET* mutations in exons 10, 11, 13 and 16, respectively. By examining only exon 16 for mutations we detected the mutation which constitutively occurs in MEN 2B (Met918→Thr) somatically in one third of sporadic MTC (Hofstra *et al*., 1994). Comparable results have been reported by others (Eng *et al*.,1994; Eng *et al*., 1995b; Zedenius *et al*., 1994; Blaugrund *et al*., 1994). Sofar, three other *RET* mutations in sporadic MTC have been described, namely in one case a 6 base pair deletion in exon 11 encompassing codon 630 (Donis-Keller *et al*., 1993), a mutation affecting codon 768 (Glu768→Asp) of exon 13, found in several sporadic MTC (Eng *et al*., 1995a), and according to preliminary data of Eng *et al*. (1995b) a somatic mutation in exon 15 also in several cases. Constitutive mutations of codon 630 in exon 11 or of exon 15 have never been reported to occur in MEN 2 or familial MTC patients, whereas the exon 13 mutation has also been found in one family with familial MTC (Eng *et al*., 1995a). Several studies describe an analysis of sporadic

MTC cases (Mulligan *et al*.,1993a; Donis-Keller *et al*., 1993; Zedenius *et al*., 1994; Blaugrund *et al*., 1994; Eng *et al*., 1995a). No somatic *RET* mutations like those found in MEN 2A patients are presented in these reports, but results from a recent study (Eng *et al*., 1995b) include two sporadic MTC with exon 10 mutations affecting codons 611 and 620, respectively, known to occur in MEN 2A families. However, in the first case constitutive DNA was not available, whereas in the second it was, but turned out to also contain the mutation. Therefore, it is still doubtful whether there do exist sporadic MTC with somatic *RET* mutations like those in MEN 2A. It may be noted that in several of these reports mutation analysis had been restricted to *RET* exons 10, 11, 13 and 16.

Reports on mutation analysis of *RET* in sporadic PC revealed mutations in three exons. In exon 16 a mutation like the one found in MEN 2B, was detected in two PC (Eng *et al*., 1994; Lindor *et al*., 1995). One of these was accompanied by a second mutation in exon 16 a G→C transversion affecting codon 925 (Lindor *et al*., 1995). In exon 11 a 6 base pair deletion was detected, in the tumor only. The deletion encompassed codons 632 and 633 (Lindor *et al*., 1995). In exon 10 a mutation affecting codon 609 was found somatically in one PC (Lindor *et al*., 1995). The codon has previously been reported to be mutated in MEN 2A. For the two sporadic PC that Eng *et al*.,(1994) reported to have mutations in codon 620 of exon 10, a codon also known to be mutated in MEN 2A as mentioned before, a somatic nature could not be proven, as constitutive DNA was not available.

Pheochromocytoma (PC) is a frequent complication in families suffering from von Hippel Lindau disease. Therefore, in sporadic PC, somatic mutations of VHL, the gene responsible for this hereditary disease (Latif *et al*., 1993), should not be excluded .

In order to assess the role of *RET* in the development of sporadic MTC and PC we have carried out a systematic scanning for mutations of all exons of *RET* in 15 sporadic cases of MTC, i.e. 13 primary tumors and two cell lines, and in 5 sporadic cases of PC. In the latter all VHL exons have also been scanned for mutations. Our results are reported here.

Materials and Methods

Patients

High molecular weight DNA was prepared according to standard methods from tumor tissue and, when available matched blood samples from 13 patients with MTC and from 5 patients with PC only. In none of these cases there was a family history of familial MTC, MEN 2A, MEN 2B, Von Hippel Lindau disease or neurofibromatosis type I. One of the patients with MTC had a diffuse C cell hyperplasia and another patient had bilateral MTC. Furthermore two cell lines were investigated: TT, a cell line reported to derive from a sporadic MTC (Leong *et al*., 1981) and MZ-CRC-1, a cell line derived from a malignant pleural effusion from a patient with metastatic sporadic MTC (Taylor *et al*., 1989).

SSCP analysis
DNA amplification was carried out on 150 ng of DNA in a total volume of 30 µl 1x Super Taq reaction buffer containing 0.125 unit Super Taq (HT Biotechnology LTD, Cambridge, UK), 20µM dCTP, 200µM dATP/TTP/GTP and 1µCi [α-^{32}P]dCTP. The PCR consisted of 30 cycles of 92ºC for 40 s, 60 s, at the appropriate annealing temperature and another 60 s, at 72ºC. The primers (100ng of each primer) used for each exon of the *RET* gene, the annealing temperature, and the specific conditions were as previously described (Ceccherini *et al*., 1994). Electrophoresis was carried out in a 6% PAA gel using at least two different conditions. Glycerol concentrations used were 0%, 5%, or 10%, at 4ºC, 20ºC, or 30ºC, respectively. We also used MDE gel solution (AT Biochem, Malvern, USA) as a replacement for acrylamide and glycerol, running the gels at 30ºC. All gels were run in 0.5xTBE buffer at max. 1750 volts and max. 60 Watts, in a temperature- regulating LKB 2010 Macrophore electrophoresis unit.
Sequence analysis of RET
Sequence analysis was carried out on exons 10, 11, 15, and 16 for all MTC and pheochromocytoma cases. Furthermore, all SSCP variants observed have been sequenced. For SSCP and sequence analysis the same primer pairs were used, but for sequence analysis one of the primers from each primer pair was biotinylated. DNA amplification was carried out as described above. PCR products were separated in a 2% low melting point agarose gel. After ethidium bromide staining, bands were cut out and isolated using the Sephaglas™ BandPrep kit (Pharmacia, Biotech). With Dynal beads (DYNAL AS, Oslo, Norway) the two single strands could be separated. They were sequenced with the T7 sequencing kit (Pharmacia, Biotech) and [α-^{32}P]dCTP. For electrophoresis, a 6% sequencing gel was used.
Restriction analysis of the Gly768→Asp mutation
As this exon 13 mutation eliminates an AluI site, restriction analysis of the PCR product with this enzyme was used to detect possible mutations.
SSCP and sequence analysis of VHL
SSCP and sequence analyses were carried out as described above using earlier reported *VHL* primers and conditions (Crossey *et al*., 1994).
RNA isolation and cDNA synthesis of the TT cell line
RNAzol™B (Cinna/Biotecx laboratories, Houston, USA) was used to isolate RNA from the cell line TT. cDNA synthesis was performed using the Ready to go, T-primed first strand kit (Pharmacia, Biotech).
Cloning of PCR products
PCR products were cloned using the TA cloning kit (InVitrogen, San Diego, USA).

Results

Mutation analysis of the RET gene

Single strand conformation polymorphism (SSCP) analysis of the *RET* gene performed on all sporadic MTC and PC revealed 6 identical SSCP variants in exon 16, one in exon 11, and one in exon 18, besides already known polymorphisms (Ceccherini *et al*., 1994). Sequence analysis

of the variants showed that all cases with an exon 16 variant had the same mutation, a T2753→C transition substituting a threonine for a methionine (Met918→Thr). The exon 11 variant found in the cell line TT was a C1903→G transversion causing Cys634→Trp, while the exon 18 variant present in the same cell line was a C2944→T transition resulting in Arg982→Cys (Table 1). Exon 10 and exon 19 primers were used in a PCR reaction on first strand cDNA of the TT cell line to make a PCR product that spanned both mutations in this cell line. Sequence analysis of these cloned PCR products showed that both mutations are present on the same allele. In ninety control DNA samples from the Centre d'Etude du Polymorphisme Humain (CEPH) screened for the Arg918→Cys mutation two persons proved to be heterozygous for this mutation.

Exons 10, 11, and 16, known to harbour the MEN 2A and MEN 2B mutations, and exon 15 preliminary suggested to contain somatic mutations in sporadic MTC, have been sequenced in all samples. No mutations were detected other than those already found by SSCP analysis.

Screening for the exon 13 mutation found in codon 768 (Gu768→Asp) by an AluI restriction analysis did not reveal the presence of this mutation in any of the cases.

Mutation analysis of the VHL gene

SSCP analysis of the VHL gene for the PC cases revealed in one case a variant in exon 1 which was caused by a transition G490→A in codon 164 substituting a serine for a glycine (Table 1). The mutation was not present in constitutive DNA from the patient nor in 30 control DNA samples (CEPH).

To look for a possible loss of the other allele in the tumor we used microsatellites markers in a loss of heterozygosity analysis of the tumor and matched normal tissue. Allelic losses were found in the VHL region (D3S1317) and in 3p21 (D3S1029 and D3S1235).

tumor	"MEN 2A" mutation	"MEN 2B" mutation	any other RET mutation	VHL mutation
sporadic MTC	0/13	5/13	0/13	nd
cell line TT	C634W	-	R982C	nd
cell line MZ-CRC-1	-	M918T	-	nd
sporadic pheochromocytoma	0/5	0/5	0/5	1/5 (G164S)

Mutations found using SSCP and sequence analysis of VHL and/or RET. Occurrence of mutations found to date in MEN 2A and MEN 2B is indicated, as are other possibly causative RET and VHL mutations. (C=cysteine, W=tryptophan, R= arginine, M= methionine, T= threonine, G= glycine, S= serine, nd=not determined).

Discussion

We have investigated sporadic MTC and PC for the presence of causative mutations of the *RET* gene by three approaches: (1) sequencing those exons that have been reported to contain mutations in MEN 2A (exons 10 and 11), MEN 2B (exon 16) and sporadic MTC (exons 11, 15 and 16); (2) AluI restriction analysis for the exon 13 (Gly768→Asp) mutation; (3) SSCP analysis of all exons for all samples using at least two different conditions.

Our screening of 13 sporadic primary MTC and 5 sporadic PC cases revealed in 5 MTC tumors the heterozygous mutation reported to be associated with MEN 2B, Met918→Thr. In three cases we could confirm the somatic nature of the mutation, in the remaining two cases constitutive DNA was not available. In these 5 cases no other *RET* mutation could be found by DNA sequencing or SSCP analysis. In the remaining 8 MTC and 5 PC cases, no causative *RET* mutations could be detected apart from several already known polymorphisms (Ceccherini *et al*., 1994). All mutations found by sequence analysis were also found by SSCP analysis.

Among the 13 sporadic MTC patients, two were considered to be at risk for MEN 2, as they had bilateral MTC and a diffuse C cell hyperplasia, respectively. The patient with bilateral MTC showed a Met918→Thr mutation in the tumor. This mutation did not occur constitutively. We therefore concluded that this patient had a sporadic MTC. Bilateral occurrence of the tumor could be due to metastases. The patient with diffuse C cell hyperplasia together with MTC did not show any *RET* mutation and is therefore also not considered to be a MEN 2 patient. This illustrates, as we have shown previously (Landsvater *et al*., 1993; Lips *et al*., 1994), that C cell hyperplasia cannot always be considered as an indication of MEN 2.

We also screened two reportedly sporadic MTC cell lines, named MZ-CRC-1 and TT, for *RET* mutations. MZ-CRC-1 contained the Met918→Thr mutation. TT appeared to contain two mutations, a Cys634→Trp mutation, previously also found in several MEN 2A patients, and an Arg982→Cys. Constitutive DNA of the patients from whom the cell lines were derived was not available. Therefore, the sporadic nature of these cases cannot be confirmed. We investigated whether both mutations were on the same allele and found that to be the case. When we checked whether the amino acid substitution at codon 982 also occurs in the normal population, two out of ninety normal individuals (CEPH) showed the same heterozygous pattern as present in the TT cell line. This and our finding that both mutations are on the same allele, point to the noncausative polymorphic nature of the Arg982→Cys mutation.

MEN 2B is the more aggressive of the two hereditary neoplastic syndromes MEN 2A and MEN 2B. The Met918→Thr mutation, found constitutively in almost all MEN 2B patients (Hofstra *et al*., 1994; Carlson *et al*., 1994; Eng *et al*., 1994), is present in one third of the sporadic MTC's. Its occurrence in a single cell in the thyroid gland may be sufficient to cause development of an MTC. The likely absence of somatic mutations identical to constitutive mutations of MEN 2A patients, suggests that the MEN 2A mutations cannot directly cause MTC development. In MEN 2A patients a constitutive *RET* mutation may be a necessary but not sufficient condition for the development of a malignant tumor. An additional mutation at a second locus may be necessary. This idea is supported by loss of heterozygosity (LOH) studies.

Analysis of MTCs from MEN 2 families, showed losses of the short arm of chromosome 1 in 6/18 cases (Mulligan *et al*., 1993b). In PC the situation seems to be somewhat different. A codon 609 mutation found by Lindor *et al*.(1995) as a somatic event, may also rarely occur in MEN 2A (Mulligan *et al*., 1994). There is, however, a notable difference in malignancy between PC and MTC. Whereas the latter may metastasize to the lungs, to the liver and to bones, PC may easily remain unnoticed since many PC patients are asymptomatic. LOH analysis of PC suggests that also in this tumor additional mutations occur, as PC from MEN 2A (14 cases) and MEN 2B patients (5 cases) showed allelic losses at 1p and at 3q in all cases (Moley *et al*., 1994; Dou *et al*., 1994).

Scanning all VHL exons by SSCP analysis revealed in one of five PC a single missense mutation in codon 164 substituting a serine for a glycine (GGC→AGC). This mutation was not present in constitutive DNA of this patient nor in 30 control samples (CEPH). As the VHL gene is a tumor suppressor gene, a mutation of the other allele should also have occurred. Our finding of LOH of the VHL region therefore supports the idea that the observed VHL missense mutation may be a tumorigenic one. It has already been reported that notably missense mutations of VHL seem to be associated with the occurrence of pheochromocytoma in von Hippel Lindau families (Crossey *et al*., 1994; Chen *et al*., 1995).

In summary, *RET* seems to be involved only in a minority of sporadic MTC and sporadic PC. The same holds true for the involvement of the VHL gene in sporadic PC. Other genes, as yet unidentified, must be responsible for development of the large majority of these tumors.

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Appendix 6

RET mutation scanning in sporadic and hereditary neuroblastoma.

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Abstract

Neuroblastoma occasionally occurs in diseases associated with abnormal neurocrest differentiation, e.g. Hirschsprung disease. According to expression studies in developing mice the proto-oncogene RET may play a role in neurocrest differentiation. In humans RET expression is limited to some tumor types, including neuroblastoma, that derive from migrating neural crest cells. Mutations of RET are found associated with Hirschsprung disease. This data prompted us to an investigation of expression of RET and a search for gene mutations in neuroblastoma. Out of 16 neuroblastoma cell lines analyzed, 9 show a clear expression of RET in a Northern blot analysis. In an SSCP analysis of all exons, no mutations were detected other than neutral polymorphisms, including a new one, Arg982→Cys. In a patient with neuroblastoma from a family in which different neurocristopathies occurred, including neuroblastoma and Hirschsprng disease, we also failed to detect RET mutations. Possibly, expression of RET in neuroblastoma just reflects the differentiation status of the tumor cells. The absence of mutations suggests that RET does not play a crucial role in the tumorigenesis of neuroblastoma.

Neuroblastoma, a tumor from the sympathetic nervous system, is the most common extracranial solid tumor in children. Multiple genetic events seem to be involved in the development and progression of neuroblastomas. Deletions of 1p with a smallest region of overlap at 1p36 (Fong *et al*., 1989; Weith *et al*., 1989; Caron *et al*., 1993) and N-myc amplification (Seeger *et al*., 1985) are found in particular in advanced disease. Additional 17q material is also frequently present (Caron *et al*., 1994; Savelyeva *et al*., 1994). Most cases are sporadic, but rare familial cases do occur. Occasionally neuroblastoma occurs in diseases associated with abnormal neurocrest differentiation such as neurofibromatosis type I and Hirschsprung disease (Clausen *et al*., 1992; Verloes *et al*., 1993; van Dommelen *et al* 1994). Genetic alterations underlying the abnormal neural crest differentiation may therefore also be involved in neuroblastoma development. *RET*, a gene coding for a tyrosine kinase receptor, may according to expression studies in mice (Pachnis *et al*., 1993), play a role in neuronal cell differentiation. Studies of human neoplasia show that *RET* expression is limited to some tumor types that also derive from migrating cells of the neural crest such as neuroblastomas (Ikeda *et al*., 1990; Nagao *et al*., 1990; Tahira *et al*., 1991; Takahashi *et al*., 1991), medullary thyroid carcinoma and pheochromocytoma (Santoro *et al*., 1990; Itoh *et al*., 1992; Miya *et al*., 1992). These latter tumor types are known to occur in the hereditary cancer syndromes multiple endocrine neoplasia (MEN) types 2A and 2B that are associated with *RET* mutations (Donis-Keller *et al*., 1993; Mulligan *et al*., 1993; Hofstra *et al*., 1994; Carlson *et al*., 1994; Eng *et al*., 1994).

Mutations of *RET* are also found associated with Hirschsprung disease (Edery *et al.*, 1994, Romeo *et al.*, 1994) which is a congenital disorder characterized by the absence of intramural ganglion cells along the hindgut. This is most likely a consequence of a disturbance of the migration of hindbrain neural crest cells during early embryonic life. Furthermore, mice that carried a metallothionein/*RET* fusion gene were found to develop neuroblastoma (Iwamoto *et al.*, 1993). Therefore, we have carried out expression studies and mutation analysis of *RET* in a number of neuroblastoma cell lines to investigate a possible role of *RET* in the development of neuroblastoma.

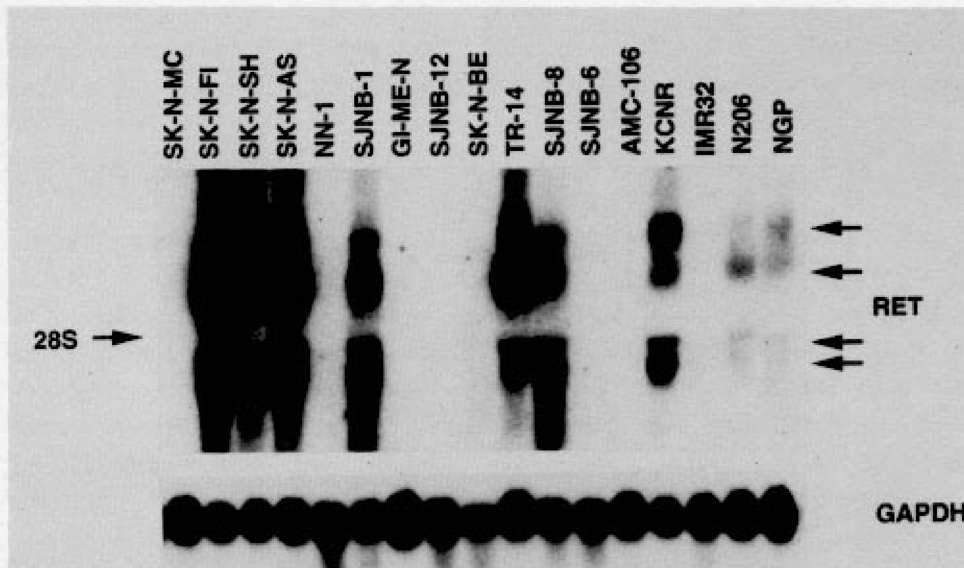

Figure 1. Northern blot analysis of RET expression in human neuroblastoma cell lines with RET (cell lines have been described in Biedler et al., 1973, Brodeur et al., 1977, Cheng et al., 1995). RNA was extracted as described previously (Auffray & Rougeon, 1980). Total RNA (15 μg) was size-fractioned in a denaturating formaldehyde- agarose gel, blotted onto Hybond-N (Amersham) and hybridized with a 504 bp PCR product containing exons 11-14 of RET. A human GAPDH probe was used as a control for RNA quantification. Hybridization was carried out as described before (Cheng et al., 1995).

Detection of human RET transcripts in neuroblastoma cell lines.

Total RNA was extracted from 16 neuroblastoma cell lines and analyzed by Northern blot analysis using as a probe a 504 bp long PCR product containing *RET* exons 11, 12, 13 and 14. This product was obtained by amplifying by PCR reverse-transcribed RNA from TT, an MTC-derived cell line, which expresses *RET*. Due to the alternative splicing of *RET* the Northern blot showed four different bands (Fig. 1). The sizes of the transcripts (7.0, 6.0, 4.5 and 3,9 kb) were consistent with those reported previously (Takahashi *et al*., 1987). As shown in figure 1, nine out of sixteen cell lines have the *RET* gene expressed. This finding differs from those published earlier, where expression was reported to occur in all cell lines and tumors examined (Ikeda *et al*., 1990; Nagao *et al*., 1990; Tahira *et al*., 1991). *RET* expression as assessed by Northern blot analysis is apparently not a consistent feature of neuroblastoma. As *RET* is expressed in normal differentiating neuroblasts, the variable expression in our neuroblastoma cell lines could also reflect different stages of neuroblast differentiation.

Systematic Single Strand Conformation Polymorphism (SSCP) analysis of the whole RET gene in neuroblastomas cell lines.

High molecular weight DNA from 16 neuroblastoma cell lines was used for a radioactive amplification of all exons of the *RET* gene. PCR products were electrophoresed in a 6% PAA gel under at least two different conditions, i.e. with glycerol in concentrations of 5% or 10 % and in an MDE gel (AT Biochem, Malvern, USA) replacing acrylamide and glycerol. All gels were run in 0.5xTBE at 30ºC using maximally 1750 volts and 60 Watts in a temperature-regulating LKB 2010 Macrophore electrophoresis unit. Primers for each *RET* exon, annealing temperatures and specific conditions were as previously described (Ceccherini *et al*., 1994). Several already known polymorphisms (Ceccherini *et al*., 1994) were found, but also one new variant, leading to the substitution of a cysteine for a arginine in codon 982 (exon 18). This variant occurred in two cell lines (SK-N-BE and Gi-MEN) that did not express *RET*. Analysis of 120 primary neuroblastoma tumors for the presence of this variant revealed two additional heterozygous cases. Both tumor and constitutive DNA of these patients, as well as DNA from both parents of one patient showed this variant. Therefore, this polymorphism can not be considered as a causative one.

It might be that the neuroblastoma situation is comparable with that of medullary thyroid carcinoma (MTC), where the large majority of sporadic cases do not have *RET* mutations (Hofstra *et al*., submitted), but inherited MTC have (Mulligan *et al*., 1993; Donis-Keller *et al*., 1993). We had available DNA from a patient with neuroblastoma, whose sister also had neuroblastoma, while her brother had neurofibromatosis and their mother ganglioneuroma (Clausen *et al*., 1992). In other branches of this kindred Hirschsprung disease occurred. DNA from our neuroblastoma patient was also subjected to the same systematic SSCP analysis as the 16 cell lines of sporadic cases.

We did not detect any *RET* mutations responsible for the development of the hereditary neuroblastoma. The same result was obtained for the sporadic cases. As we were unable to detect any mutation it could be argued that mutations are missed, as SSCP does not detect all mutations. The maximal fraction of mutations detectable by this technique was estimated to be 80% (Sarkar *et al*., 1992). However, in an analysis of *RET* mutations in sporadic MTC none of

the mutations detected by sequence analysis was missed by SSCP analysis (Hofstra *et al.*, submitted).

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Appendix 7

RET mutation screening in familial cutaneous lichen amyloidosis and in skin amyloidosis associated with multiple endocrine neoplasia

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Abstract

In several families multiple endocrine neoplasia type 2A (MEN 2A) has been found in association with cutaneous lichen amyloidosis (CLA). It has, however, been debated whether the skin amyloidosis found in MEN 2A families, localised exclusively in the interscapular area, represents the same anomaly as that found in autosomal dominant familial CLA (FCLA), which is more generalized. We screened 2 MEN 2A families with associated skin amyloidosis for germline RET mutations and found only a mutation characteristic for MEN 2A. We also screened probands from three pedigrees with FCLA for RET mutations. In none of the RET coding and flanking intronic sequences a mutation was detected. This most likely indicates that skin amyloidosis found in some MEN 2A families and FCLA are different conditions. Consequently, apparent FCLA patients do not appear to be at risk for MEN 2A.

Introduction

Cutaneous lichen amyloidosis (CLA) is a rare disorder, characterised by deposit of amyloid in the papillary dermis. Sporadic as well as autosomal dominant hereditary forms have been documented. Gagel et al. (1) reviewed 63 of these hereditary cases. Here we refer to the hereditary form as familial cutaneous lichen amyloidosis (FCLA).

CLA-like skin lesions have also been found in patients with multiple endocrine neoplasia, type 2A (MEN 2A) (1-7). MEN 2A is a neoplastic syndrome characterized by C-cell hyperplasia, medullary thyroid carcinoma, pheochromocytoma and parathyroid hyperplasia. The disorder is caused by specific germline mutations in the *RET* proto-oncogene (8,9). This gene encodes a transmembrane tyrosine kinase receptor which is involved in the differentiation of neural crest cell-derived tissues, including parts of the nervous system.

Since in several MEN 2A families patients have been found with CLA lesions, it has been suggested that patients having sporadic or familial CLA should be considered at risk for the MEN 2A syndrome, and therefore be tested for MEN 2A mutations. Based upon the association of both these conditions *RET* gene mutations have been thought responsible for the skin amyloidosis found in MEN 2A patients. A limited *RET* mutation screening has been reported in a single MEN 2A family with CLA-like lesions (10). A Cys634→Tyr mutation, as found in several MEN 2A families, is co-segregating with both the MEN 2A and the MEN 2A/CLA phenotype in that family.

Since *RET* mutations have been reported to cause a variety of phenotypes, namely MEN 2A and FMTC, (9-11), MEN 2B (12-14), MEN 2A/Hirschsprung disease (15,16) and Hirschsprung disease (17,18), it is possible that specific *RET* mutations cause MEN 2A/CLA and/or FCLA. We therefore screened two MEN 2A/CLA families and three FCLA families for *RET* germline mutations.

Materials and Methods

FCLA families

The three families participating in this study featured CLA in at least 2 generations (Fig. 1). All affected family members were examined by a dermatologist. Light microscopic and

electron microscopic evidence for amyloid was found in skin biopsies from at least two individuals in each of the families CLA2 and CLA3. Although no EM analysis was performed on patient material from family CLA1, the diagnosis in this family was based on a characteristic clinical picture and on histopathologic and immunofluorescence examination of skin biopsies. In all three families the CLA lesions were found mainly on arms and legs.

Figure 1. Pedigrees of families featuring CLA and participating in this study. Symbols are squares for males, circles for females, solid symbols for individuals affected with CLA and open symbols for unaffected individuals.

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MEN 2A/CLA families

Families MEN2A/CLA1 and 2 have been described before. Fig. 2 shows the relevant parts of the pedigrees. Family MEN2A/CLA1 has been reported by Kousseff et al. (4,19), who gave a detailed description of the CLA lesions. Family MEN2A/CLA2 has been described as family B by Lips et al. (20). Some of the patients in this family appeared with lesions in the interscapular region only and were clinically diagnosed as CLA patients upon examination by a dermatologist. Light microscopic evaluation of biopsies of the lesions failed, however, to detect amyloid.

Fig. 1

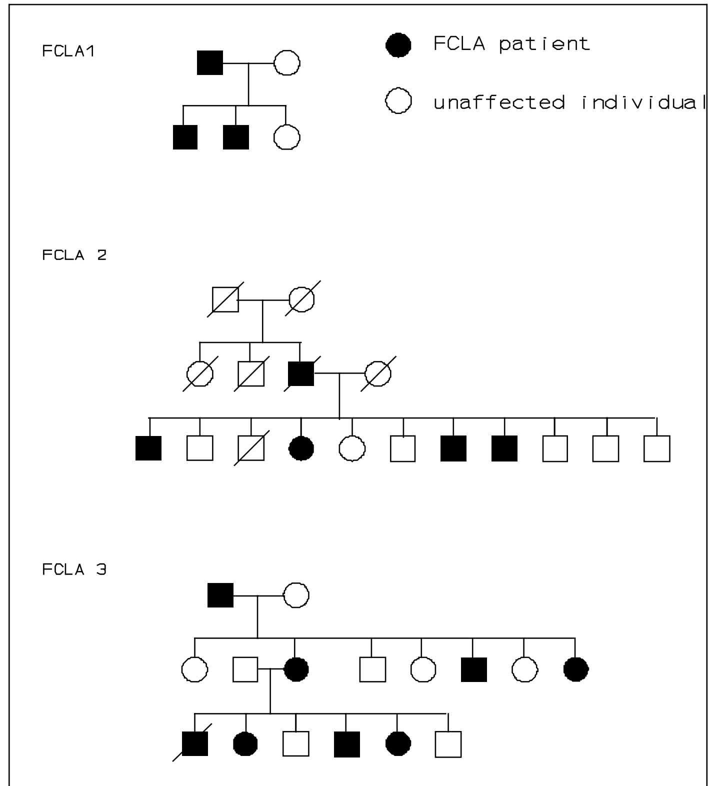

Fig. 2

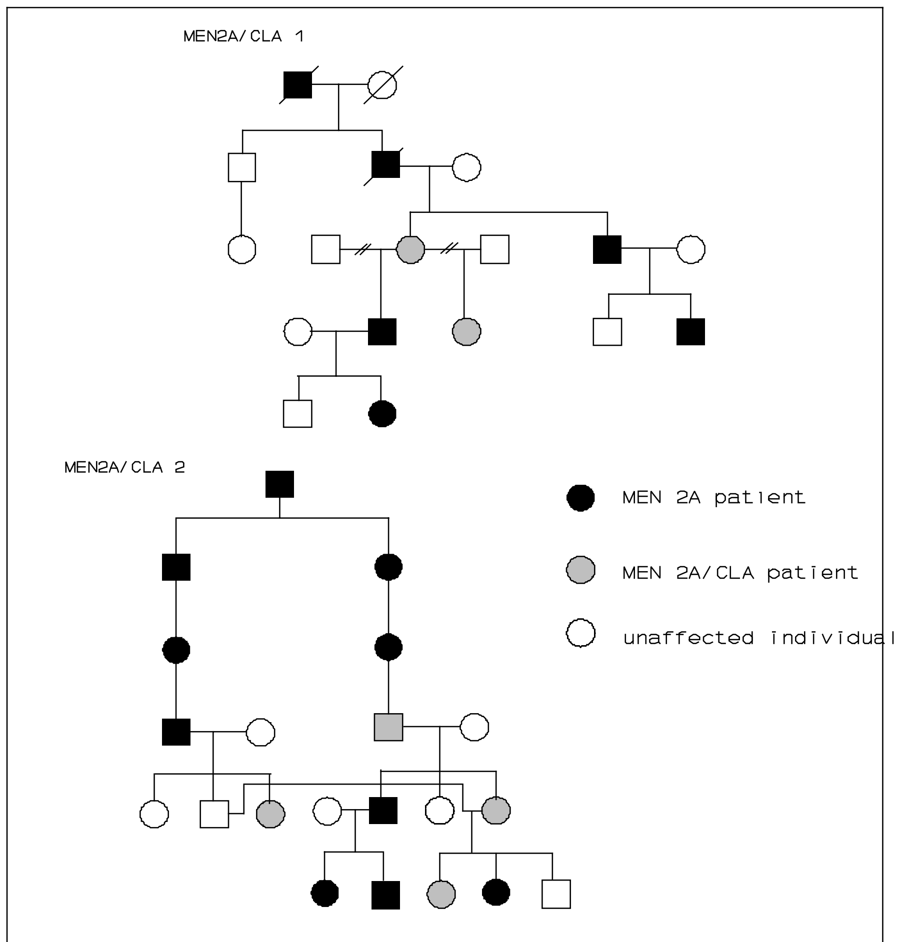

Figure 2. Pedigrees of families featuring MEN 2A and CLA and participating in this study. Symbols as in Fig. 1 with the exception of solid symbols representing individuals affected with MEN 2A and hatched symbols for individuals affected with both MEN 2A and CLA.

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Single strand conformation polymorphism (SSCP) analysis

High molecular weight DNA from patients from families MEN2A/CLA1, CLA1, CLA2 and CLA3 was used for an SSCP analysis of all 20 *RET* exons. DNA amplification was carried out on 150 ng of DNA in 1x Super Taq reaction buffer using 1/8 unit Super Taq (HT Biotechnology LTD, Cambridge, UK) in a total volume of 30μl containing 20μM dCTP, 200μM dATP/TTP/GTP and 1μCi (α-^{32}P)dCTP. The PCR consisted of 30 cycles of 92ºC for 40 s, 72ºC for 60 s and another and 60 s for annealing at the appropriate temperature. The primers used (100ng of each primer) for each exon of the *RET* gene, the annealing temperature and the specific conditions were as previously described (21). Electrophoresis was carried out in a 6% PAA gel using at least two different conditions. Glycerol concentrations used were 0%, 5%, or 10%, at 4ºC, 20ºC or 30ºC, respectively. We also used MDE gel solution (At Biochem, Malvern, USA) as a replacement for acryl-amide and glycerol, running the gels at 30ºC. All gels were run in 0.5xTBE buffer at max. 1750 volts and max. 60 Watts. in a temperature regulating LKB 2010 Macrophore electrophoresis unit.

Sequence analysis

For all families sequence analysis was carried out on exons 10 and 11 that are known to contain all mutations found sofar for MEN 2A. In addition, all SSCP variants observed have been sequenced. For SSCP and sequence analysis the same primer sets were used. For sequence analysis, however, one of the primers from each set was biotinylated. DNA amplification was carried out as described above. PCR products were separated in a 2% low melting point agarose gel. After ethidium bromide staining, bands were cut out and isolated using the Sephaglas™ BandPrep kit (Pharmacia, Biotech). The two single strands were separated with the use of Dynal beads (DYNAL AS, Oslo, Norway) . They were sequenced using the T7 sequencing kit (Pharmacia, Biotech) and (α-^{32}P)dCTP. For electrophoresis a 6% sequencing gel was used.

Results

RET mutation screening in MEN2A/CLA families

A search for mutations throughout the entire *RET* gene by means of SSCP revealed in one family (MEN2A/CLA1) a conformation variant in exon 11 in all affected family members (MEN 2A and MEN 2A/CLA patients). Upon sequence analysis this appeared to be caused by a transition T1900→C, resulting in the substitution of a arginine for a cysteine at codon 634 (Cys634→Arg).

Sequence analysis of exon 10 and 11 of the *RET* gene showed the same mutation, a T1900→C, in family MEN2A/CLA2 in all affected family members (MEN 2A and MEN 2A/CLA patients).

RET mutation screening in CLA families

SSCP analysis of all exons of the *RET* gene did not show any causative *RET* mutation nor did sequence analysis of exons 10 and 11.

Discussion

Phenotypic diversity due to mutations affecting different domains of a gene product is a frequent phenomenon, genetically known as allelic heterogeneity. The *RET* gene is a well known example. Base pair substitutions affecting one of five highly conserved cysteine residues in the extracellular part of the protein are associated with MEN 2A and familial medullary thyroid carcinoma (9-11). Substitution of one of these five codons, codon 634, strongly correlates with parathyroid hyperplasia and occurrence of pheochromocytoma in MEN 2A families (22,23). Furthermore, a missense mutation substituting threonine for methionine at codon 918 in the tyrosine kinase domain of the protein has been found uniquely associated with MEN 2B (12-14). Mutations of one of the two alleles presumably leading to inactivation of the protein have been found responsible for a proportion of patients suffering from Hirschsprung disease (17,18). These mutations have been found all over the gene (17,18,24,25). Patients suffering from both MEN 2A and Hirschsprung disease have *RET* mutations in the exon 10 codons 618 and 620 (15,16). The combined occurrence of both MEN 2A and CLA in some families and patients might also be associated with specific *RET* mutations. We therefore analyzed two families. In patients of one of these, MEN2A/CLA1, presence of amyloid could be clearly demonstrated. The MEN2A/CLA patients in the other family showed the same clinical symptoms, comparable to those described for a number of such families that have fully been published earlier (1-7). Presence of amyloid could not be demonstrated in biopsies from the lesions, but is also not a consistent feature of presumed CLA patients in the previously reported MEN2A/CLA families (1-7). Because all lesions were limited to the interscapular region, which is generally considered characteristic for the association of MEN 2A and CLA, family MEN2A/CLA2 was included in this study. In the two families we found the same *RET* mutation in codon 634 (Cys634→Arg). The mutation was present in all MEN 2A patients some of which also had CLA. A Cys634→Tyr mutation (G1901→A) has been reported previously in another family with MEN 2A and CLA (10). Although all the mutations affect codon 634, different amino acid substitutions result. The mutations found also occur in MEN 2A families without CLA lesion. In fact, mutations of codon 634 and the amino acid substitution arginine for cysteine, which is found in both families, are the most frequent changes occurring in MEN 2A patients (22,23). Although an association between MEN 2A/CLA and mutations in codon 634 may be postulated, the above-mentioned arguments make it hard to suggest a correlation between a specific *RET* mutation and the MEN 2A/CLA phenotype.

It might be suggested that the joint occurrence of both MEN 2A and CLA would be due to the interaction of an apparently non-causative polymorphism and a disease- causing mutation,

as has been described for the prion gene (26). In the *RET* gene several non-causative polymorphisms have been found (21, Hofstra et al., submitted), two of these, in exons 11 and 18, leading to amino acid substitutions. A haplotype analysis of all intragenic polymorphisms was made. None of the polymorphisms, however, seemed to co-segregate with the MEN 2A/CLA phenotype.

Thus, for the intrafamilair phenotypic variability there might be a need to look beyond the mutational-polymorphic genotype. A differential handling of the gene product by the paracrine growth mechanism of a particular individual may alter the pathogenesis of the condition and cause the pleiotropy of the phenotype (4,19,27)

A search for *RET* mutations in patients from three "CLA only" families did not reveal any mutation other than already known non-causative polymorphisms. Also in these families we looked for possible co-segregation of these intragenic polymorphisms with the cutaneous phenotype, but did not find that. We, therefore, conclude that *RET* is not involved in these cases of FCLA.

Our findings raise the question whether or not CLA found in MEN 2A and FCLA are similar conditions from an etiological point of view. Clinically, there is a distinction in the affected sites. In MEN 2A patients skin lesions are always found in the interscapular region, whereas in FCLA patients skin lesions are more generalized (6). Dysfunction of the RET gene, which in developing mice is expressed in the peripheral nervous system (28) might lead to pruritus, and subsequently to scratching and degeneration of keratinocytes. It has been suggested that prolonged mechanical friction may produce a macular amyloidosis, "friction amyloidosis" (6,29). However, since many chronic pruritic skin conditions do not feature skin amyloidosis, this etiological model might be an oversimplification.

The present results lend support to the idea that skin lesions in FCLA and MEN 2A/CLA patients, respectively, are different from a genetic, a clinical, and an etiological point of view. Consequently, FCLA patients do not appear to be at risk for MEN 2A. In order to settle this issue definitely, however, more data are welcome. Mainly for this reason, physicians of (apparent) FCLA patients may still consider to have their patients screened for *RET* mutations.

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Summary / Samenvatting

Summary

This thesis starts with a brief description of protein kinases, a large family of proteins involved in cell proliferation and differentiation and also in a number of cancer types and hereditary diseases (chapter 1), and subsequently discusses in greater detail the receptor protein kinase RET (chapter 2) and its involvement in several diseases (chapter 3). Furthermore, our own data on the *RET* gene and its role in diseases as well as results obtained in collaborative efforts with other groups are presented in the appendices 2-7.

The RET protein is involved in the normal development and the neoplastic growth of neural crest lineages. The ligand of the receptor is as yet unidentified. During embryogenesis, *RET* expression is high in neuroectodermal tissues, suggesting a function of RET in the proliferation, the migration and the differentiation of these cell types. In adult tissues the gene is hardly expressed. Expression is high in several tumor types derived from neural crest cells (chapter 2).

Transfection studies with DNA from different tumors revealed focal proliferation due to the presence of different DNA sequences that, however, shared a common part called *RET*. The original *RET* gene turned out to be rearranged in such a way that the sequences coding for the extracellular part of its protein product were replaced by sequences from elsewhere, resulting in a rearranged protein with a steady tyrosine kinase activity. The same rearrangement occurs in papillary thyroid carcinoma (PTC) (chapter 3).

After the genes involved in multiple endocrine neoplasia types 2A (MEN 2A) and 2B (MEN 2B) had been mapped to the centromeric region of chromosome 10 by linkage analysis, mutations of *RET*, a gene present in this very region, appeared responsible for the development of MEN 2A. The establishment of the intron-exon junctions of the *RET* gene and the determination of the flanking intronic sequences in a collaborative effort with the group of professor Romeo (Genua, Italy), made it possible to design primers and to develop PCR conditions for SSCP analysis (Appendices 2 and 3). Using this mutation detection system (Appendix 3) we found that a single *RET* mutation is uniquely associated with MEN 2B (Chapter 3 and Appendix 5).

In some families, MEN 2A is also found associated with cutaneous lichen amyloidosis (CLA), a rare skin disorder. We screened two of these families for *RET* mutations to determine whether specific mutations are involved in these families. A Cys634→Arg mutation was found. Though the same codon was affected in an earlier described family, the mutation in that family was different. This makes it hard to suggest a correlation between MEN 2A associated with CLA and a specific *RET* mutation. Because of the association of CLA with MEN 2A, *RET* might also be involved in hereditary "CLA only". We, therefore, screened *RET* in three families with hereditary CLA, but did not find any mutation. We concluded that the CLA lesions found in MEN 2A patients and those found in inherited CLA without MEN 2A must be caused by different genes. (chapter 3 and appendix 7).

An estimated 25% of medullary thyroid carcinomas (MTC) appear in the context of inherited disease (MEN 2 syndromes and familial MTC). For pheochromocytoma, the percentage of cases being part of an inherited neoplastic syndrome (MEN 2A, von Hippel Lindau,

neurofibromatosis I) is similar to that in MTC. The large majority of MTC and pheochromocytoma, however, consists of sporadic cases. We analyzed *RET* in sporadic MTC and *RET* and the von Hippel Lindau gene (*VHL*) in pheochromocytoma for the presence of mutations. In sporadic MTC and in sporadic pheochromocytoma *RET* mutations appeared to account for only a proportion of cases. The same could be concluded for the *VHL* gene in pheochromocytoma (chapter 3 and appendix 5).

Besides the gene for the neoplastic syndromes MEN 2A and MEN 2B, the gene for Hirschsprung disease could also be mapped by linkage analysis to the same small region of chromosome 10. Using the *RET* mutation detection system described in appendix 3, the Romeo group was one of two research groups who demonstrated that HSCR was also associated with *RET* mutations. The mutations appeared to be scattered all over the gene (chapter 3).

Publications in the recent literature on *RET* explain how these different diseases can be caused by one single gene. These are discussed in chapter 3. Mutations causing MEN 2A and MEN 2B activate the protein product, whereas mutations for HSCR result in a loss of function of the translated protein (chapter 3).

Based on the involvement of *RET* in the development of neural crest-derived tissues and on the association of *RET* mutations with neurocristopathies such as the MEN 2 syndromes and HSCR, a search for *RET* mutations in other neurocristopathies seems justified. Neuroblastoma occasionally occurs in diseases associated with abnormal neurocrest differentiation, e.g. Hirschsprung disease. Furthermore, neuroblastomas express *RET*. We therefore scanned the entire *RET* gene in 16 neuroblastoma cell lines and in a neuroblastoma patient belonging to a family in which different neurocristopathies occurred, including Hirschsprung disease and ganglioneuroma. We did not find any *RET* mutation. Therefore, expression of *RET* in neuroblastoma might just reflect the differentiation status of the tumor cells, rather than indicating an involvement in the tumorigenesis of neuroblastoma (chapter 3 and appendix 7).

We may conclude that *RET*, as a gene in which different mutations lead to different diseases, is a good example of allelic heterogeneity. On the other hand, in some diseases *RET* plays a role in only a proportion of the cases and other, yet unidentified, genes account for the remaining cases. Therefore, *RET* is also a good example of non-allelic heterogeneity.

Samenvatting

De erfelijke eigenschappen van de mens worden bepaald door genen. Genen bestaan uit DNA. DNA is een lange aaneenschakeling van bepaalde bouwstenen, basenparen genoemd, waarvan 4 verschillende typen bestaan. In totaal bevat elke cel van de mens twee sets van 3 miljard basenparen. De genen, waarvan wij er naar schatting 60.000-70.000 hebben vormen maar een paar procent van ons DNA. Dat DNA is opgedeeld in verschillende stukken, de chromosomen. In elke menselijke cel bevinden zich 22 chromosomen (autosomen) in tweevoud, plus 2 geslachtschromosomen. Deze laatste zijn verschillend bij mannen en bij vrouwen. Mannen hebben één X chromosoom en één Y chromosoom, terwijl vrouwen twee X chromosomen hebben. In totaal heeft de mens dus 46 chromosomen in iedere cel. De helft daarvan, nl. één van elk van de autosomen plus één X is van moederlijke oorsprong, de andere helft, eveneens 22 autosomen plus één X of één Y chromosoom is van vaderlijke oorsprong.

Bepaalde veranderingen in de base volgorde van de genen kunnen erfelijke ziekten of kanker tot gevolg hebben. Wanneer een dergelijke verandering optreedt in een voortplantingscel en wordt doorgegeven aan de volgende generatie, dan spreken we van een erfelijke ziekte. Treedt zo'n verandering op in een van onze gewone lichaamscellen, dan kan dat tot kanker leiden. Er bestaan ook erfelijke kankers. Erfelijke ziekten kunnen op verschillende manieren overerven. Bij de zogenoemde recessieve overerving ontstaat de ziekte, wanneer beide ouders een verandering in hetzelfde gen hebben en zij beiden de gen copie met de verandering aan een nakomeling doorgeven. De kans daarop is 1 op 4. Met één veranderde gen copie is men niet ziek, maar wordt men drager/draagster genoemd. Een dominante aandoening berust op één gen copie met een verandering. Het doorgeven daarvan heeft dus een kans van 1 op 2. In een familie waarin een erfelijke ziekte voorkomt, kan op grond van kennis van wie (een) veranderde gen copie(ën) draagt, voorspeld worden wie de ziekte wel en wie de ziekte niet gaat krijgen.

Dit proefschrift gaat over een gen dat *RET* genoemd wordt en dat betrokken is bij het ontstaan van bepaalde erfelijke ziekten en kankers. Het gen dient als blauwdruk voor een eiwit (RET), een zogenaamde "protein kinase", dat andere eiwitten kan activeren. Het RET eiwit steekt door de celwand heen zodat een deel zich in de cel bevindt (het intracellulaire gedeelte) en een deel door de celwand heen naar buiten steekt (het extracellulaire gedeelte). Op deze manier kan het eiwit signalen van buiten de cel opvangen en daar vervolgens op reageren. Dit reageren gebeurt in eerste instantie door een zogenoemde zelfactivatie. Het geactiveerde RET eiwit zorgt er vervolgens weer voor, dat in de cel allerlei processen in gang worden gezet. Men denkt, dat RET onder andere betrokken is bij de celdeling en de celdifferentiatie. Een en ander komt aan de orde in de eerste twee hoofdstukken van het proefschrift.

De betrokkenheid van *RET* in bepaalde erfelijke ziekten en kankers wordt behandeld in hoofdstuk drie en bijbehorende appendices. RET blijkt betrokken in de betrekkelijk zeldzame erfelijke kankersyndromen die worden samengevat als multiple endocriene neoplasie type 2 (MEN 2). Hierbij treden meerdere (multiple) gezwelachtige woekeringen (neoplasieën), goed- of kwaadaardig, op in endocriene klieren. Type 2 wordt onderverdeeld in twee subtypen, type 2A (MEN 2A) en type 2B (MEN 2B). Beide typen worden gekarakteriseerd door tumoren in de schildklier en bijnieren. MEN 2A kan als extra complicatie een bovenmatige groei van cellen

van de bijschildklier hebben, terwijl bij MEN 2B patiënten karakteristieke gezwellen optreden aan het uiteinde van de zenuwen op de tong en in en rond de mond. Deze erfelijke kankers hebben een dominante overerving. Onderzoek naar de lokalisatie van de genen voor zowel MEN 2A als MEN 2B gaf aan, dat deze in een bepaald stuk van chromosoom 10 zouden moeten liggen. In dat stuk ligt nu juist het *RET* gen. In een samenwerking met de groep van professor Romeo in Genua, hebben we het *RET* gen in kaart gebracht (Appendix 2) en hebben we een systeem opgezet om genetische veranderingen (mutaties) op te kunnen sporen in het gen. (Appendix 3). In 1993 toonde de groep van professor Ponder in Cambridge aan, dat specifieke mutaties in dit gen het MEN 2A kanker syndroom veroorzaken. De gevonden veranderingen bevinden zich allemaal in het extracellulaire gedeelte dat betrokken is bij het ontvangen van de signalen van buiten de cel. Wij zelf hebben kunnen aantonen dat ook MEN 2B wordt veroorzaakt door een verandering in het *RET* gen. Hierbij ging het echter om slechts *één* enkele specifieke mutatie. Deze zorgt voor een verandering van het intracellulaire gedeelte van het RET eiwit (Appendix 4).

Voor de MEN 2 families is het belang van deze bevindingen groot. Immers nu kan met zekerheid gezegd worden, wie de kanker zal ontwikkelen en wie niet. Op basis van de genetische analyse wordt nu presymptomatisch op jeugdige leeftijd de schildklier verwijderd, zodat volgroeide schildkliertumoren en uitzaaiingen van de tumor voorkomen worden. Wie geen mutaties heeft, hoeft nu ook niet meer het vroeger periodiek uitgevoerde belastende onderzoek te ondergaan, dat diende om tumoren in een vroeg stadium op te sporen.

Welke effecten deze mutaties hebben op het eiwitprodukt waar het gen voor codeert, is door andere onderzoeksgroepen aangetoond. Zij konden laten zien dat de veranderingen er bij zowel MEN 2A als MEN 2B voor zorgen, dat het RET eiwit zelfs zonder een stimulatie van buiten af al actief is, waardoor de normale regulatie ontbreekt en wildgroei kan optreden. De ontregeling van de functie van het RET eiwit verschilt bij MEN 2A en MEN 2B en leidt tot de verschillende ziektebeelden.

Schildklier- en bijniertumoren komen echter vaker op zich zelf voor, dan als onderdeel van de erfelijke MEN 2 syndromen. Het gaat dan om zogenoemde sporadische tumoren. Dat betekent echter niet, dat de cellen waaruit de tumoren ontstaan zijn, geen genetische veranderingen hebben ondergaan. Wij hebben gekeken of die sporadische schildklier- en bijschildkliertumoren ook veroorzaakt kunnen worden door mutaties in het *RET* gen. We vonden, dat alleen dezelfde verandering als gevonden bij MEN 2B patiënten, is terug te vinden in deze tumoren. Omdat dit echter slechts bij een minderheid van de sporadische tumoren het geval is, concluderen wij dat de meeste tumoren ontstaan door mutaties in een ander gen(en) (Appendix 5).

Verder hebben we gekeken naar MEN 2A patiënten die naast de bekende kenmerken (schildklier en bijnier tumoren) ook een huidaandoening hebben (cutaneous lichen amyloidosis). De vraag was of een specifieke verandering in het *RET* gen de oorzaak zou kunnen zijn van deze associatie. We vonden dat de onderzochte families een mutatie hadden in *één* bepaald "codon". Een codon is een drietal opeenvolgende basenparen dat codeert voor een bouwsteen van een eiwit, een aminozuur. Ook hebben we gekeken of *RET* mutaties misschien aanwezig

zijn in families met alleen de huidaandoening. Dit bleek niet het geval. Er zijn dus meerdere genen die kunnen leiden tot de cutaneous lichen amyloidosis (Appendix 7).

Behalve bij het ontstaan van kanker is *RET* ook betrokken bij een erfelijke aangeboren aandoening, de ziekte van Hirschsprung. De ziekte kenmerkt zich door het ontbreken van zenuwknopen over een variabele lengte in de darmwand. Daardoor kan de darm de normale peristaltische bewegingen niet maken en ontstaat ernstige obstipatie. Lokalisatie van het gen voor deze ziekte wees hetzelfde gebied aan als voor de MEN 2 syndromen. Screening van het *RET* gen leverde mutaties op die geassocieerd konden worden met de ziekte van Hirschsprung. Terwijl bij MEN 2 een activatie van het RET eiwit wordt gevonden, blijken bij de ziekte van Hirschsprung mutaties te worden gevonden die zorgen voor de aanmaak van een incompleet RET eiwit, wat dus eerder wijst op een inactivatie. Voor een aantal mutaties is aangetoond, dat ze de activiteit van het eiwit naar nul brengen.

Het RET eiwit is dus op verschillende manieren betrokken bij het ontstaan van een aantal erfelijke aandoeningen. Het effect van *RET* mutaties is daarbij specifiek zichtbaar in bepaalde weefsels. Al deze weefsels komen voort uit cellen die embryonaal afkomstig zijn van de neurale lijst. *RET* zou daarom ook betrokken kunnen zijn bij andere ziektebeelden waarbij weefsel afkomstig van de neurale lijst is aangedaan, zoals bijvoorbeeld bij neuroblastoom. Neuroblastomen zijn kwaadaardige woekeringen van cellen afkomstig van de neurale lijst en komen vooral voor op jeugdige leeftijd. *RET* mutaties komen voor dit type tumor verder in aanmerking om een aantal andere redenen. Eén daarvan is het aanwezig zijn van RET eiwitten in neuroblastoom cellen. In het algemeen geldt, dat lang niet alle genen altijd en overal tot expressie komen. *RET* komt maar in heel weinig weefsels en tumoren tot expressie. Met name betreft het de weefsels waaruit schildklier- en bijniertumoren ontstaan en de tumoren zelf. Een ander argument voor de mogelijke betrokkenheid van *RET* in neuroblastomen is het voorkomen van de ziekte van Hirschsprung in families waarin ook neuroblastomen voorkomen. Wij hebben daarom gekeken of mutaties in het *RET* gen een rol spelen in de ontwikkeling van deze neuroblastoom. We konden aantonen, dat er expressie van *RET* is, maar in de tumoren die we hebben onderzocht, bleken geen mutaties aanwezig. Het lijkt dus waarschijnlijk, dat RET niet direct betrokken is bij het ontwikkelen van deze tumoren van de kinderleeftijd (Appendix 6).

Samengevat kan gezegd worden, dat *RET* een mooi voorbeeld is van een gen waarbij verschillende mutaties tot heel verschillende ziekten kunnen leiden. Anderzijds blijken aan ziektebeelden waarbij *RET* een rol speelt, ook nog andere, nader te identificeren, genen ten grondslag te kunnen liggen.

Curriculum Vitae / List of publications

Curriculum Vitae

Name:	Robert Martinus Wouter Hofstra
Nationality:	Dutch
Date and place of birth:	March 14, 1962, Groningen, The Netherlands

Academic qualification:

1984	B.Sc., University of Groningen, The Netherlands
1988	M.Sc., University of Groningen, The Netherlands

Research training:

Jan. 1989-May 1989	Research position at BioIntermediair, a biotechnology company, specialized in the production of monoclonal antibodies and the construction of testkits based on the use of monoclonal antibodies
June 1989-Dec. 1990	Research position as conscientious objector on a project funded by the Dutch Cancer Society and supervised by Prof. Dr. C.H.C.M. Buys Identification and characterization of a gene responsible for the development of small cell lung carcinoma. Department of Medical Genetics, University of Groningen, The Netherlands
Jan. 1991-present	PhD student on a project funded by the Dutch Cancer Society and supervised by Prof. Dr. C.H.C.M. Buys Identification and characterization of the gene responsible for the development of the MEN 2A syndrome. Department of Medical Genetics, University of Groningen, The Netherlands. Max Gruber prize for the best 1994 Groningen paper in Biochemistry and Molecular Biology by a junior scientist.

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