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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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Voor mijn ouders en Wia

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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 phosphorylated 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;

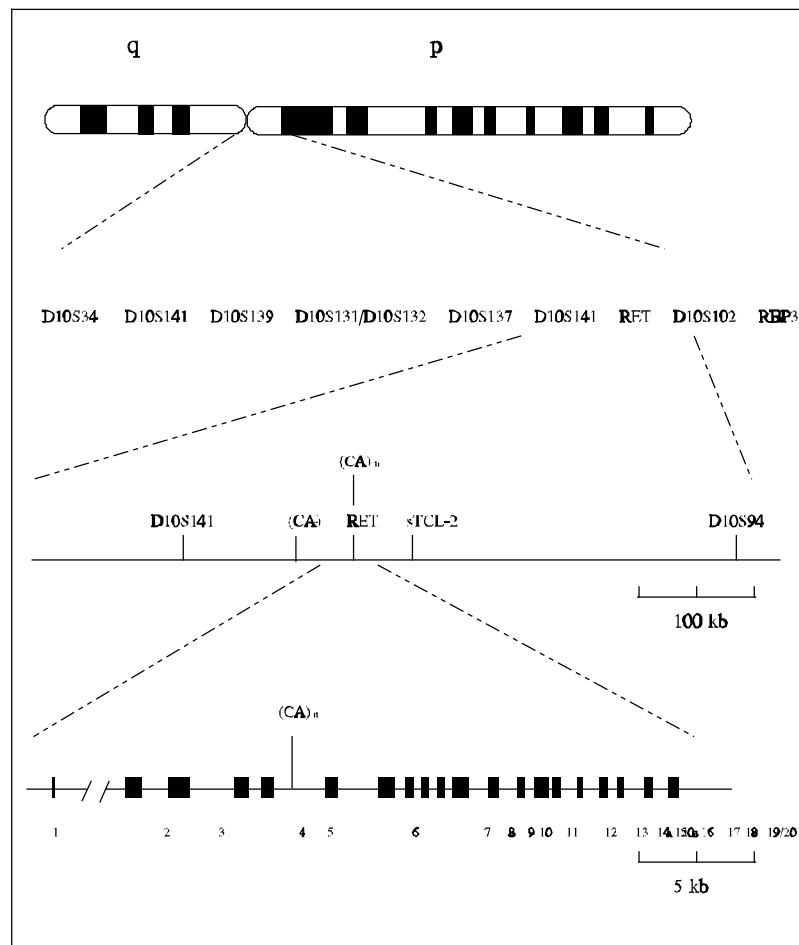


Figure 1. Map of the centromeric region of chromosome 10 giving the order of a number of loci including *RET* (Hofstra et al., in press), the subregion where the *RET* gene is located and a diagram of the intron-exon structure of *RET* (Ceccherini et al., 1993, Pasini et al., submitted).

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 *EcoRI* 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).

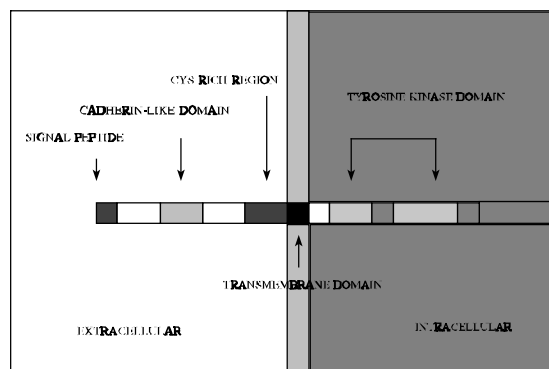


Figure 2. Schematic representation of the RET protein.

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-PTC1* (Fusco *et al.*, 1987), *RET-PTC2* (Bongarzone *et al.*, 1993), and *RET-PTC3* (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-PTC1* is composed of *H4* (D10S170) and *RET*, *RET-PTC2* of *RI α* and *RET* and *RET-PTC3* 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

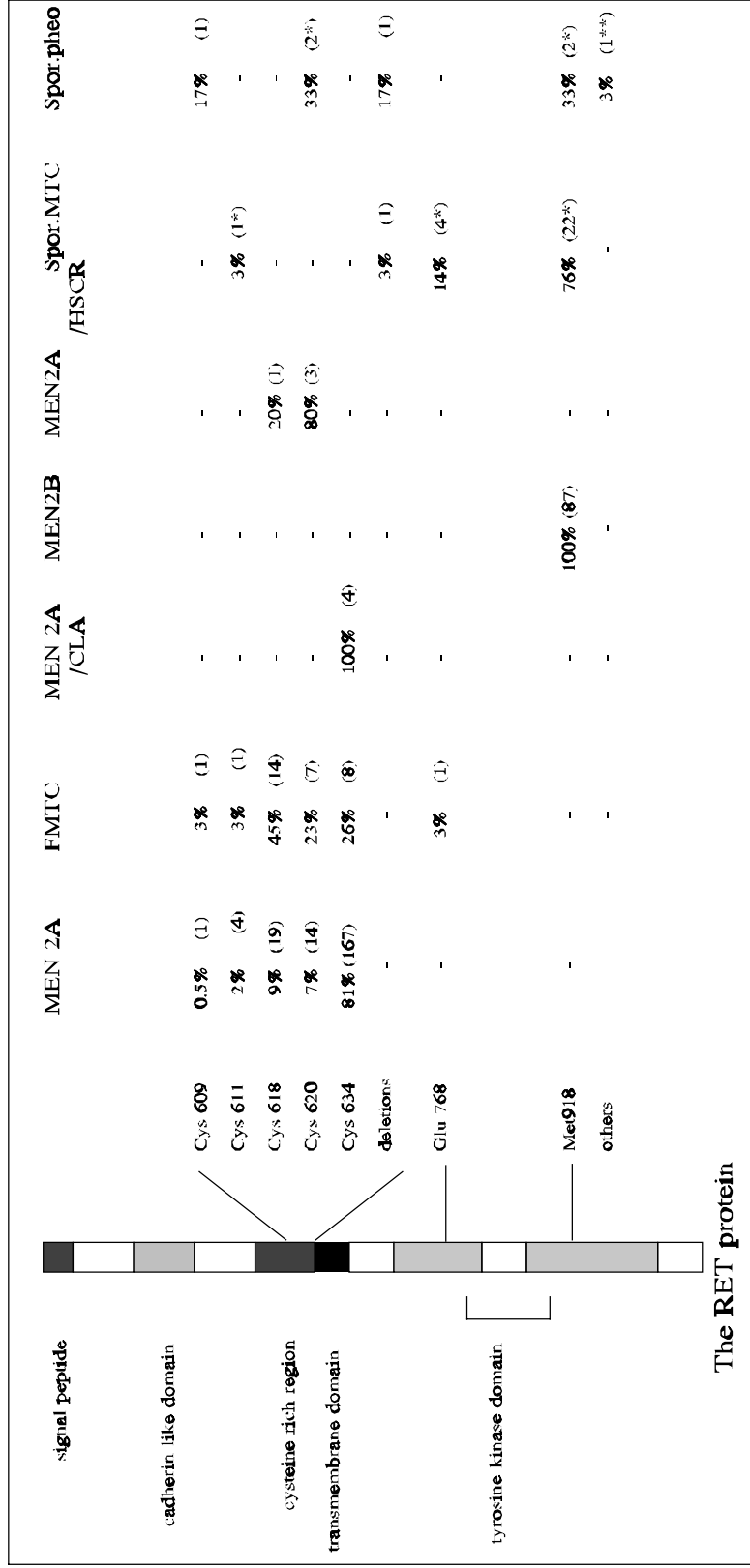
Multiple endocrine neoplasia type 2 (MEN 2) comprises at least two clinically distinct dominantly inherited cancer syndromes. MEN 2A patients develop medullary thyroid carcinoma (MTC), pheochromocytoma and parathyroid hyperplasia. MEN 2B patients do not have hyperplasia of the parathyroid, but in addition 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, so far, 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).*



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.

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 an 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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A*ppendix 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.

-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																					
-48	CGG	CCC	TAG	CCG	CAG	TCC	CTC	CAG	CCG	TGG	CCC	CAG	CGC	GCA	CGG	GCG	ATG	GCG	AAG	GCG	4
																					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	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

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

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

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

A*ppendix 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
gcacggg	cgATGGCGAAGG (1)	exon 1 73 bp	CTAGGCAAAGgtgagttctg		24000 bp
cttccca	cagTGGCATTGGG (74)	exon 2 264 bp	AGTGTCCGCAgtaaggggagc		1650 bp
ctctctg	cagACCGCGGCTT (338)	exon 3 288 bp	CTCCTGGAGGgtgagtgccg		2350 bp
tggtg	cagGTGAGGGTCT (626)	exon 4 242 bp	AGCGGAAGGAgtgcttgtcc		800 bp
catcctg	cagGACACCGTGG (868)	exon 5 196 bp	CATGACTATAgtaagagggg		2400 bp
ctacctg	cagGGCTGGTTCT (1064)	exon 6 200 bp	ATTTGCCAGgtgagcccat		2000 bp
gccccct	tagATCGGAAAG (1264)	exon 7 259 bp	GAGGGTCATgtgagtgcc		680 bp
ccacctg	cagATGTGGCCGA (1523)	exon 8 126 bp	GATGGCAAAGgtaagccctg		620 bp
tcctgtg	cagGGATCACCAG (1649)	exon 9 111 bp	GACTGCCTCCgtaagcaggg		610 bp
tctgcct	cagGGGGCAGCAT (1760)	exon 10 120 bp	GACATCCAGGgtgagtggg		850 bp
ccaccac	cagATCCACTGTG (1880)	exon 11 257 bp	CAAGATCCTGgtgaggggtcc		1650 bp
tccaaca	tagGAGGATCCAA (2137)	exon 12 148 bp	GATGCTGAAAgtaacctgcca		1600 bp
tgcat	ttcagGAGAACGCCT (2285)	exon 13 108 bp	AGCCAGGATGgtaaggccag		1100 bp
ccgcccc	cagGCCCGCTCCT (2393)	exon 14 215 bp	CGAGATGAAGgtgctgcat		350 bp
ttcctca	cagCTCGTTCATC (2608)	exon 15 123 bp	GAGGAGCCAGgtgccagtc		1700 bp
ttctctt	tagGGTCCGATTC (2731)	exon 16 71 bp	AAAGTGATGTgtaagtgtgg		1650 bp
ctctctg	cagATGGTCTTTT (2802)	exon 17 138 bp	GCGAGGAGATgtgagcgggg		1100 bp
ttcccac	cagGTACCGCCTG (2940)	exon 18 100 bp	TAAGAGGAGAgtgagtgcc		1600 bp
tgtcttc	cagGACTACTTGG (3040)	exon 19 148 bp	AAACTCTATATG		0 bp
	GTAGAATTTCCCATGCATTTACTAGATTctagcaccgct (3188)	29 bp			1350 bp
tcatttt	tagGCATGTCAGA (3188)	exon 20b 154 bp	CTTTGATAGTtaacatttct		

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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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 nondenaturing 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)

gctgagtgccccggaacgtgcgtcgcgccccagtgctcgcgtccgccgcgccccggcggggatggggcgccagactgagcgccgcccc-
gccatccagaccgcccggccctagccgcagtccc tccagccgtggccccagcgcgcacgggcg(1)

EXON1gtgagttctgccggccggtctccgcagggccagggcgaagtggcgccgagcagcggacggcgcttcagaagcgcctttctgtttgcc
gtgggcaacgcggtgtgtgctgccccggccgcccaccgcccctcggctgggagcgcagtgctgcggcgggc-
ggggcgggggcaggacgctcggccgggcccctcggccggggctgggcccgggtctcgggcccgggagcgga

tcctgaagaagccatattctaccatcctcactcacatccctacttcccacag**EXON2**gtaggagcggccccaacaccaccctgcccc-
accacccttcctcaagccgcccattacacagccgctgacactgaagcttggcatggctccccccaccgctggtgtggaaggcgtcnaggg-
gtaagtgaggtggcctgcctctgtgtccmccyggagagaagccagacaagcttcaggctgcgtgaggtgtaggagttggagacacag-
ggantcatccgggtacacttctctccaganccgaatccctctccag

ttgtggaccttggtggggaccagggtttacaccagccctggagctcctgctcnnccccattcccagctcctggcagatgtgcccgatcccc-
acagacctgacttctctctgcag**EXON3**gtgagtgccgacctgtggggcccacagtcctgctactgctggtcttctctgcgagccctt-
gacacaagccatctggtttattctacctcatgcccagctcattcaatattccagagtacctctgacatactgcagg

tctgaccgcagagccccctcccaggaagcggctggccccgtcccggctggtgatcacgcggggcccctgtctgcttggtgcgcag**EXON4**gt-
cctgtcccgcgctgctgtgtctaccagtgctgtctccggccacagttcgtttctcgggttagtgctgtagccaccaaccg

tggtctacaacacacatctggtccacctatgggctgtgtggacgtgcagcatCCTAAGGTCTCTGGTTTTGGggggtctgaggggnc-
catctcgcctgactgaccaacgcccctctgcacctctgcag**EXON5**gtaaagggggctggtggcacggcctggctagggcccca-
gGAAATGAGGTGCTCGCTCTTcatgggaagcagcaccctacacacatgcacacctggcatgcccctctg

tgcgtacacatgcacacacacaggctgcctcaattgagaagggctccttgacttccagttcagtaaatcccaacgttgaacattggtgctaa-
cttaggaccagccccagcctggttgcactgtatgtgtgaaagtgcgtgtttgaccagtgtgagtgccgggctgtgtct-
gggaagaggtgtgctacaCATGAGGAAGCAGCCAGAGCagcttgggtgctattgttgccccctacctgcag
EXON6gtgagccatactattgctgctgggaagattgaaaggccaaggacatgggggcACAGGGAGGCAGGTGACTgc
ctctggcccaaccagcacagtagactg

tctaccctcagccattacagccggtccagctgcctggctaaggtgttcccctgtgccccctag**EXON7**gtgagtgctcctcaggaggga-
gggctggggtctgggagcttctggagcctggcct

atccgtgggcagntcagctggtgctgttccctgtccttgggcaactagctggacgctggcccaggccagccccctgtaccctgctgtctgcca-
cctgcag**EXON8**gtaaagccctggaacgcccaaggagggcctgcagggcgatggcaccggtggaaacngggtcct

tctgcttagagtgtggggtgtggcggggctcccacatgggtgacagtctgctgtgtcctgtgcag**EXON9**gtaaagcagggttaacaggggca-
tgggaacaggtaggagatagtaggggaaacctggatcccacaggcacttcagccagaggttccagggctgtcagttctatgcatcangctgagc-
tctgtgcattgcatcctgcat

gccccagggaggtgagtgggctacgtctgcctcag**EXON10**gtgagtggtggcgccggggaccaccaccctcccag-
ccccacagaggtctcaacagcactctgaggt

cctctgcggtgccaagcctcacaccaccaccaccacag**EXON11**gtgagggtcctgcccggcagggaaagatcccctgcct-
ccccagctgccttcagggagggagggccagctggggagacagaggccatctgtgagggctgccaacgctgggcagacgagg-
cctgttctgccccatttccataggcgctgtgtggggacagctgtgggggtgggactatgataggtgccgttccatctaggtgagaggcagtg-
gtcagggtcacagatcgggcngggagcagcaggtgtggatngaggggcaccgaagtc

tcagatgacagcgggttctctgcacattggaactgtccatggggcctctttaagggtcttGCCTTCTTCCTCCCCTGTCACTcctcacatt-
tccccctcttctccccttcccctatttccaacatag**EXON12**gtacctgccaggcacagcACAGTGCCCCCTGGGGGAGTCTc-
cggggcgggggcggtgaggcccctcctgccagcatgggacctgaaga

tgatgagcccctgtccactgatcccaaaggctgggagaagcctcaagcagcatcgtctttgagggcctctctgtctgAACTTGGGCAA-
GGCGATGCAGgtccatcctcacctggatggtcatggaaggggctccaggagcgcggttgcaacctgctctgtgctgatttcag-
EXON13gtaaggccagctgcagggtgaggtgggcagccactgcaccaggctggggGCTCCATACAGCCCTGTTCTcctcttt-
ctcct

cctggctcctggaagacccaagctgcctgaccgcacgcccagggccctctctccgccccag**EXON14**gtgctgcatat-
gctctgcaccagccagccccggccaggccacacctgaccaccag

catgtcacaccctgactccaccagcccctgceatgceacacccccggcccagggtctcaccaggccgctaccggggccacacacc-
accctctgctggtcacaccaggctgagccagTGACCGCTGCTGCCTGGCCATggcctgacgactcgtctatcttctcag**EX-**
ON15gtgccagctccgggggatgaggggggctcccagggatcccagggtgcacatgggGCAGGCAGTCCTTGGGAAGC-
ctaggaagataccgaagattagtgagcttaagc

AGGGATAGGGCCTGGGCTTctcenttaccctccttctagagagtttagagtaactcaatgtctttattccatcttctc-
tttag**EXON16**gtaagtgtgggtgtgCTCTCTTGGGGTGGAGGTTAcagaaacaccttatacatgtagtgggccacgacncc-
cgtctgtcagctggccagggaattgactgg

tctgtgagggccaggtggagccactcactggtccttactctctcag**EXON17**gtgagcggggactggctttggcccagcctc-
acttgggaaggaaggggacatctgtgcttccctcccacccagagcagccccaggagaaaccaggagaagtggggggtggg-
gagtggcaggggcagaggttagagag

ccccagtcccacgaggctcagagatgtcagcgatgcagaaatagctttggagttggagacagagcacactgggcccagggtacag-
ggcagggtgcatggctgtggtgggctgccttctgagacctggccctgcttgatcatattggcctgctgctcttcccaccag
EXON18gtgagtgcctgggtccaattcccacaagctgaaagtggcttggggagactccagcctcaccaccagggcagtagt-
tttagccctcagagttcccagtggtggccacagtgggattgtcagagagagagatcatgctctcccctgcatgcatacagcagatt

ctgagttgtatcTAGTTGTGGCACATGGCTTGgagtgaccggccatctctgtcttccag**EXON19**GTA-
GAATTTCCCATGCATTTACTAGATTC⁽²⁾tagcaccgctgtccccTCTGCACTATCCTTCCTCctgtgatgctttt-
aaaaatgtttctgctgaacaaaacaaagtctgtgctctgaaccttttatttgtaaagtctgacttttcatccagtttacatttaggcattattgcaactagtt-
ttctaaaaggt

tgccgaccagtggttgaaatcaaaaggagttttgccaaggccttactgtctgcacttgaagttttggttcttcagtgagacaacaaatga-
tctgttttcatttttag**EXON20**taacatttctttgtgaaaggtaatggactcacaaggggaagaacatgctgagaatggaag-
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. (°C)	Mg conc. (mM)	Product size (bp)	Restriction cleavage	Size of the fragments
1 _b	68	1	166		
2	57	1.5	387	Sau3A I	163+224
3	65	1	375	Sac I	182+192
4	61	1	342	Hinf I	145+197
5	60	1	275		
5 _c	50	1.5	329	Ava I	151+178
6	60	1.2	251		
6 _c	56	1.5	333	Hae III	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	Stu I	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	Sty I	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		

^aAll reactions were set up at 30 cycles following previously described general conditions (Romeo *et al.*, 1994). ^b10% DMSO and a 1:100 ratio between forward and reverse primer were also necessary. ^cPCR 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 site	Size of the alleles (1)	(2)	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		

^aAllele 1 is always defined as the one with the restriction site present, allele 2 with the restriction site absent. ^bPIC: 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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Erratum PIC values reported in Table 3 should be read as heterozygosity values.

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

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Multiple endocrine neoplasia type 2 (MEN 2) comprises three clinically distinct dominantly inherited cancer syndromes. MEN 2A patients develop medullary thyroid carcinoma (MTC) and pheochromocytoma. MEN 2B patients show in addition ganglioneuromas of the gastrointestinal 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 likely³. The disease locus for both MEN 2A and MEN 2B has been assigned to chromosome 10 (refs 4-6). Still, MTCs and pheochromocytomas 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 independent 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 *FokI* 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

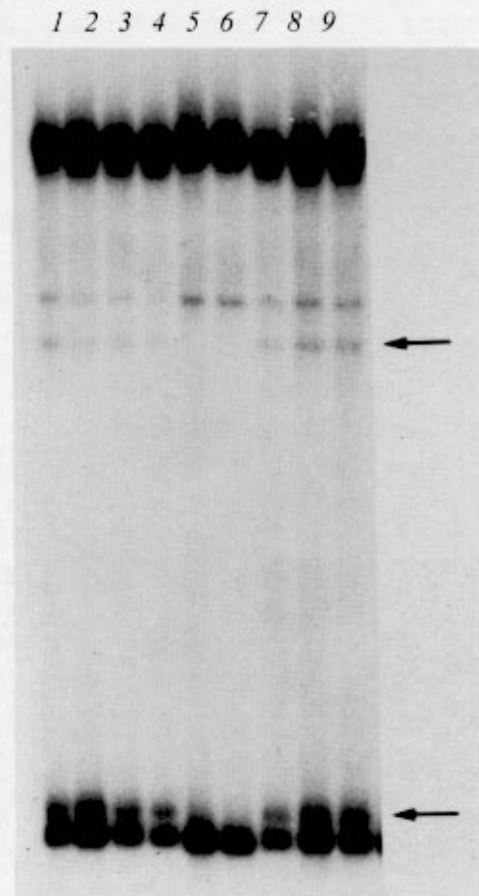


FIG. 1 Single-strand conformational polymorphism (SSCP) analysis¹⁴ of RET. The autoradiogram shows SSCP patterns of PCR-amplified products of exon 16 of the RET gene. MEN 2B patients are indicated as 1-4 and 7-9, the parents of 4 are indicated as 5 and 6. Arrows indicate the positions of the SSCP variant 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 μ l for 25 cycles at 92°C for 35 s, 58°C for 35 s and 72°C for 60 s using (α -³²P)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.

Appendix 4

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, *FokI* restriction pattern and sequence as found in the MEN 2B patients. No indication for an exon 16 mutation was obtained for five sporadic pheochromocytomas. The same held true for 15 independent MEN 2A patients.

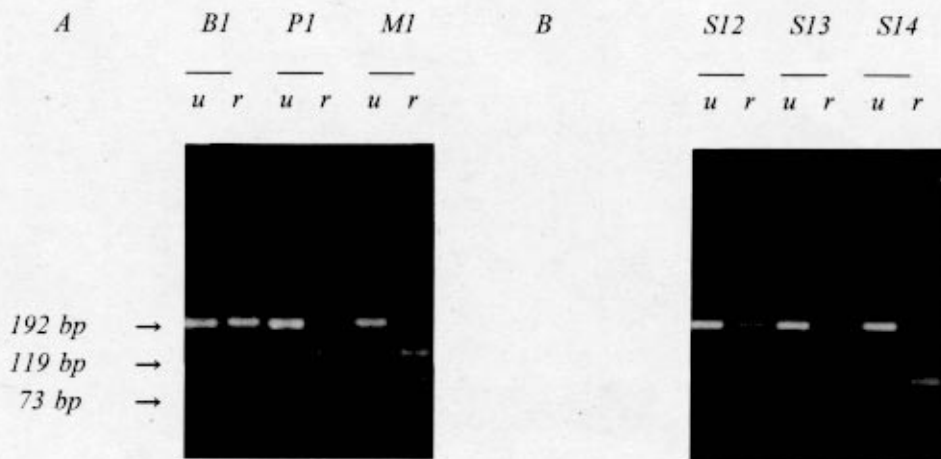


FIG. 2 Restriction patterns of PCR products of exon 16 with *FokI*. The described mutation eliminates a *FokI* restriction site. Whereas normally both alleles are restricted, in the presence of the mutation the mutated allele will not be restricted. A, B, The undigested (u) and the restricted (r) PCR 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 RET¹⁰, 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 2A² 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 domain¹¹. 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 threonine¹¹. 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 communication¹¹. For RET, this suggestion is corroborated by similarities between its extracellular receptor domain and cadherins, transmembrane proteins that mediate cell-cell adhesion¹². 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 mice¹³. 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

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

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(Submitted)

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). So far, 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 μ l 1x Super Taq reaction buffer containing 0.125 unit Super Taq (HT Biotechnology LTD, Cambridge, UK), 20 μ M dCTP, 200 μ M dATP/TTP/GTP and 1 μ Ci [α -³²P]dCTP. The PCR consisted of 30 cycles of 92°C for 40 s, 60 s, at the appropriate annealing temperature and another 60 s, at 72°C. 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 4°C, 20°C, or 30°C, respectively. We also used MDE gel solution (AT Biochem, Malvern, USA) as a replacement for acrylamide and glycerol, running the gels at 30°C. All gels were run in 0.5xTBE buffer at max. 1750 volts and max. 60 Watts, in a temperature-regulating LKB 2010 Macrochore 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 SephaglasTM 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 -³²P]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

RNAzolTMB (Cinna/Biotech 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 Z-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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***RET* mutation scanning in sporadic and hereditary neuroblastoma.**

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(Submitted)

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 Hirschsprung 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.

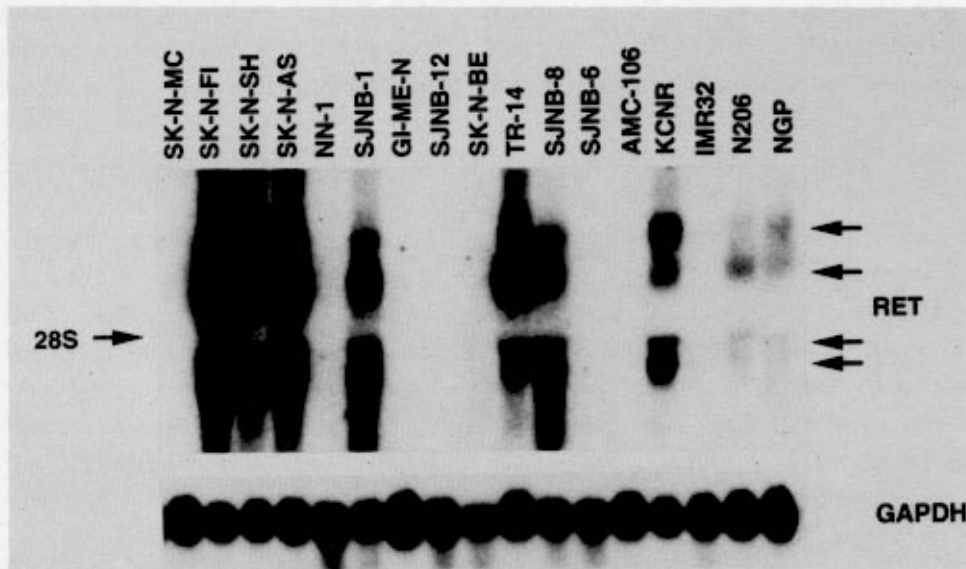


Figure 1. Northern blot analysis of *RET* expression in human neuroblastoma cell lines with *RET* (cell lines have been described in Biedler *et al.*, 1973, Brodeur *et al.*, 1977, Cheng *et al.*, 1995). RNA was extracted as described previously (Auffray & Rougeon, 1980). Total RNA (15 μ g) was size-fractionated in a denaturing 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 30°C using maximally 1750 volts and 60 Watts in a temperature-regulating LKB 2010 Macrochore 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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***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

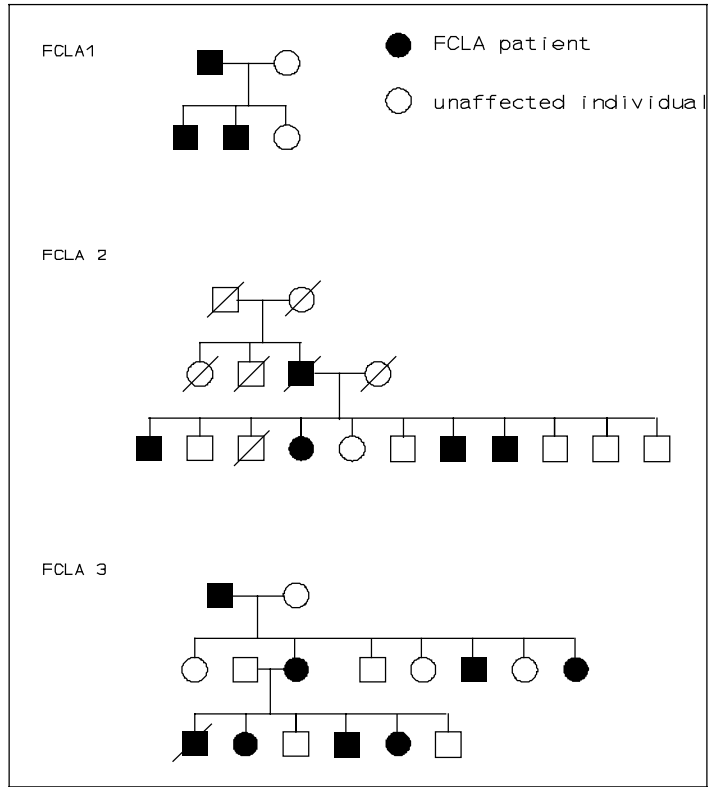


Fig. 2

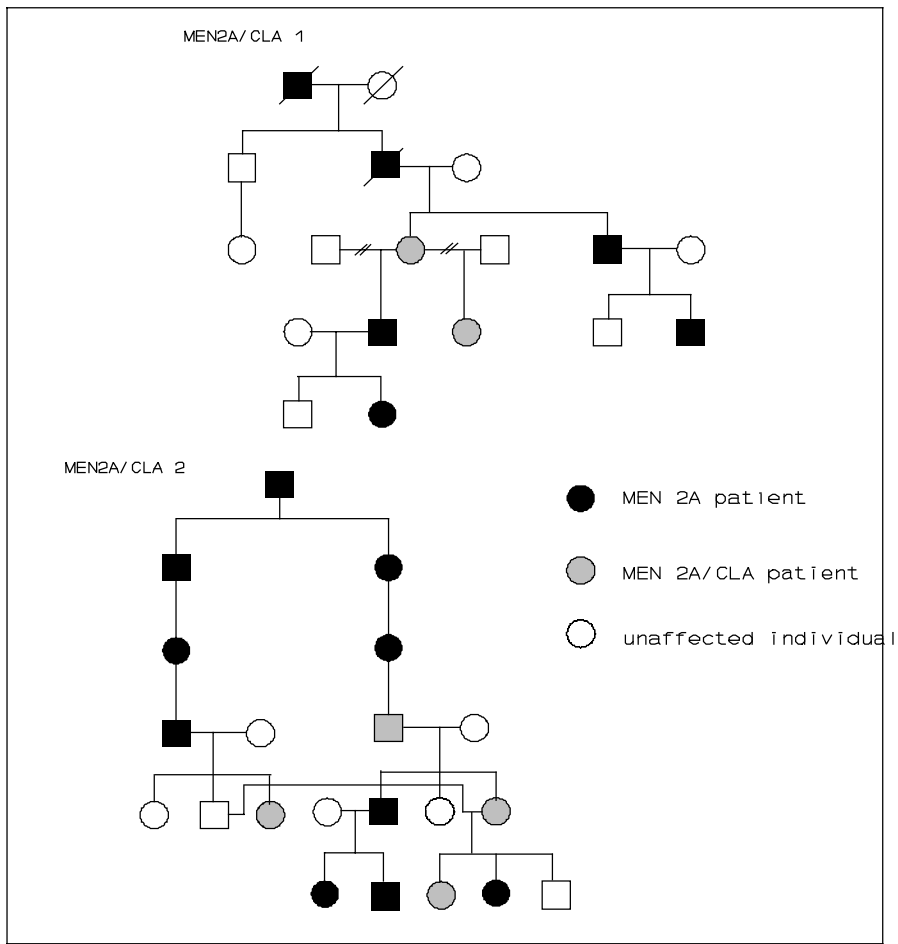


Figure 2. Pedigrees of families featuring MEN 2A and CLA and participating in this study. Symbols as in Fig. 1 with the exception of solid symbols representing individuals affected with MEN 2A and hatched symbols for individuals affected with both MEN 2A and CLA.

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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 -³²P)dCTP. The PCR consisted of 30 cycles of 92°C for 40 s, 72°C 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 4°C, 20°C or 30°C, respectively. We also used MDE gel solution (At Biochem, Malvern, USA) as a replacement for acryl-amide and glycerol, running the gels at 30°C. All gels were run in 0.5xTBE buffer at max. 1750 volts and max. 60 Watts. in a temperature regulating LKB 2010 Macrochore 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 so far 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 -³²P)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 intrafamilial 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.

Acknowledgements

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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 (Genoa, 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 lichaamcellen, 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 "proteïne 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 bijniere. 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 eiwitproduct 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 bijnier tumoren 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

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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 bijnier tumoren 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

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

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Research training:

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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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Neuteboom, B., Jekel, P., Hofstra, R.M.W., Sierdsema, S.J. & Beintema, J.J. Structure, function, and evolution of crustacean hemocyanins. (1990) In "*Invertebrate dioxygen carriers*", edited by G. Preaux and R Lontie. Leuven University Press. pp 85-88.

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