[CANCER RESEARCH 56, 4853–4855, November 1, 1996]

Advances in Brief

Somatic Mutations of the *RET* Proto-oncogene Are Not Required for Tumor Development in Multiple Endocrine Neoplasia Type 2 (*MEN 2*) Gene Carriers[1]

Rudy M. Landsvater,[2] Mireille J. de Wit, Richard A. Zewald, Robert M. W. Hofstra, Charles H. C. M. Buys, Hans-Kristian Ploos van Amstel, Jo W. M. Höppener, and Cornelis J. M. Lips

Departments of Internal Medicine [R. M. L., M. J. d. W., R. A. Z., J. W. M. H., C. J. M. L.] and Pathology [R. M. L., M. J. d. W., R. A. Z., J. W. M. H.], University Hospital Utrecht, P.O. Box 85500, 3508 GA Utrecht, the Netherlands; Department of Medical Genetics, University of Groningen, Antonius Deusinglaan 4, 9713 AW Groningen, the Netherlands [R. M. W. H., C. H. C. M. B.]; and DNA Laboratory, Clinical Genetics Center Utrecht, P. O. Box 85500, 3508 GA Utrecht, the Netherlands [H-K. P. v. A.]

Abstract

Germ line mutations in one allele of the *RET* proto-oncogene predispose to the multiple endocrine neoplasia type 2 (MEN 2) syndromes. To investigate whether these inherited mutations alone can cause the development of tumors *in vivo* (oncogene model) or whether somatic mutations in the homologous *RET* allele are required for tumorigenesis (tumor suppressor gene model), we analyzed the entire coding region of both alleles of the *RET* gene in two MEN 2A and two MEN 2B tumors by reverse transcription-PCR and direct sequencing. No tumor-specific mutations could be detected in either allele of the *RET* gene in these tumors. Unlike the molecular mechanism in other hereditary tumor syndromes, somatic mutations in the homologous allele are apparently not required in MEN 2 tumorigenesis. Thus, *RET* genes with MEN 2-specific germ line mutations act as dominantly transforming oncogenes *in vivo*.

Introduction

The dominantly inherited cancer syndromes MEN[3] 2A and MEN 2B were found to be associated with specific germ line mutations in the *RET* gene (1–5). MEN 2A is characterized by MTC, pheochromocytoma (in about 50% of gene carriers), and sometimes parathyroid adenoma (6). MEN 2B is also characterized by MTC and pheochromocytoma, but with an earlier age of onset, and also by mucosal ganglioneuromas and a marfanoid *habitus* (7). MEN 2 resembles other hereditary tumor syndromes in the dominant pattern of inheritance. A germ line mutation of one allele of a tumor suppressor gene leads to a cancer predisposition in these other diseases. However, tumors will only develop after functional loss of the homologous allele (8). It was conceivable that the *MEN2* gene would also act as such a tumor suppressor gene. However, loss of heterozygosity for markers near the disease gene locus, characteristic of tumor suppressor genes, was found in only a small percentage of MEN 2-related tumors (9, 10). The *RET* locus itself was also investigated for loss of constitutional heterozygosity in MEN 2 tumor DNA, but again no gross abnormalities could be detected (11). Apparently, the etiology of MEN 2 at a chromosomal level was different from what had been found in other hereditary tumor syndromes.

The *RET* gene encodes a transmembrane receptor tyrosine kinase and had been identified as a proto-oncogene (12). Thus, the finding of congenital mutations in the *RET* gene in families with MEN 2A and MEN 2B suggested that the classical tumor suppressor gene model might not apply for these traits (1, 13). In almost all MEN 2A families investigated, a missense mutation in either exon 10 or exon 11 of the *RET* gene has been detected, invariably affecting one of five cysteine residues in the extracellular region of the Ret protein (1, 2, 14). In almost all MEN 2B-families, as well as in *de novo* MEN 2B patients, a specific missense mutation in exon 16 affecting the tyrosine kinase domain 2 of the Ret protein has been described (3–5). A schematic representation of the *RET* gene with the positions of the MEN 2-specific germ line mutations is given in Fig. 1. *In vitro* studies by Santoro *et al.* (15) and Asai *et al.* (16) have shown that in NIH 3T3 cells transfected with expression vectors harboring RET cDNA with MEN 2 mutations, Ret kinase activity is constitutively stimulated and associated with cellular transformation. In these transfected cell lines, the mutant *RET* genes thus act as dominantly transforming oncogenes.

Affected MEN 2 family members are heterozygous for the family-specific *RET* mutation also in their tumor DNA. However, it was conceivable that other mutations, perhaps in the initially unaffected (homologous) *RET* allele, are a prerequisite for tumors to develop, in which case the "two-hit" model for tumor suppressor genes would still apply. Therefore, we studied expression of the *RET* gene in tumors from MEN 2 patients by reverse transcription-PCR and direct sequencing to investigate whether the MEN 2-specific germ line mutations are the only *RET* gene mutations responsible for tumor development in *MEN 2* gene carriers *in vivo*. We have analyzed the entire coding region of both *RET* gene alleles in four tumor specimens derived from MTC and pheochromocytoma from two different MEN 2A patients and from one MEN 2B patient, as well as in a normal thyroid gland.

PATIENTS AND METHODS

Patients. The four investigated tumors were specimens from MEN 2A and MEN 2B patients. The MEN 2A MTC was from a patient belonging to a family with a germ line mutation of codons 634 and 635 (TGC CGC to TGG GGC) of the *RET* proto-oncogene. The MEN 2A pheochromocytoma came from a patient with a germ line mutation of codon 634 (TGC to CGC). The other two tumors were an MTC and a pheochromocytoma from one MEN 2B patient. This patient had a *de novo* germ line mutation of RET codon 918 (ATG to ACG). The normal thyroid gland, investigated as a control for analytical procedures and providing a reference RET cDNA sequence, was an autopsy specimen from a non-MEN 2 subject.

RNA Isolation. MTC, pheochromocytoma, and normal thyroid gland tissue were snap-frozen in liquid nitrogen after surgery or autopsy. Tissue specimens (300 mg) were homogenized in 3 ml of RNA-SR (Biogenesis, Bournemouth, United Kingdom) using an Ultra-Turrax (IKA Labortechnik, Staufen, Germany). Chloroform (0.1 volume) was added and mixed, and the

Received 7/30/96; accepted 9/19/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Grant 28–1367-2 from the Prevention Fund, the Netherlands (to R. M. L., M. J. d. W., R. A. Z.) and Grant 91–14 from the Dutch Cancer Society (to R. M. W. H.).

[2] To whom requests for reprints should be addressed, at Department of Pathology H04.313, University Hospital Utrecht, P.O. Box 85500, 3508 GA Utrecht, the Netherlands. Phone: 31-302506557; Fax: 31-302544990; E-mail: R.M.Landsvater@lab.azu.nl.

[3] The abbreviations used are: MEN, multiple endocrine neoplasia; MTC, medullary thyroid carcinoma.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/21/4853/2462126/cr0560214853.pdf by guest on 29 May 2022

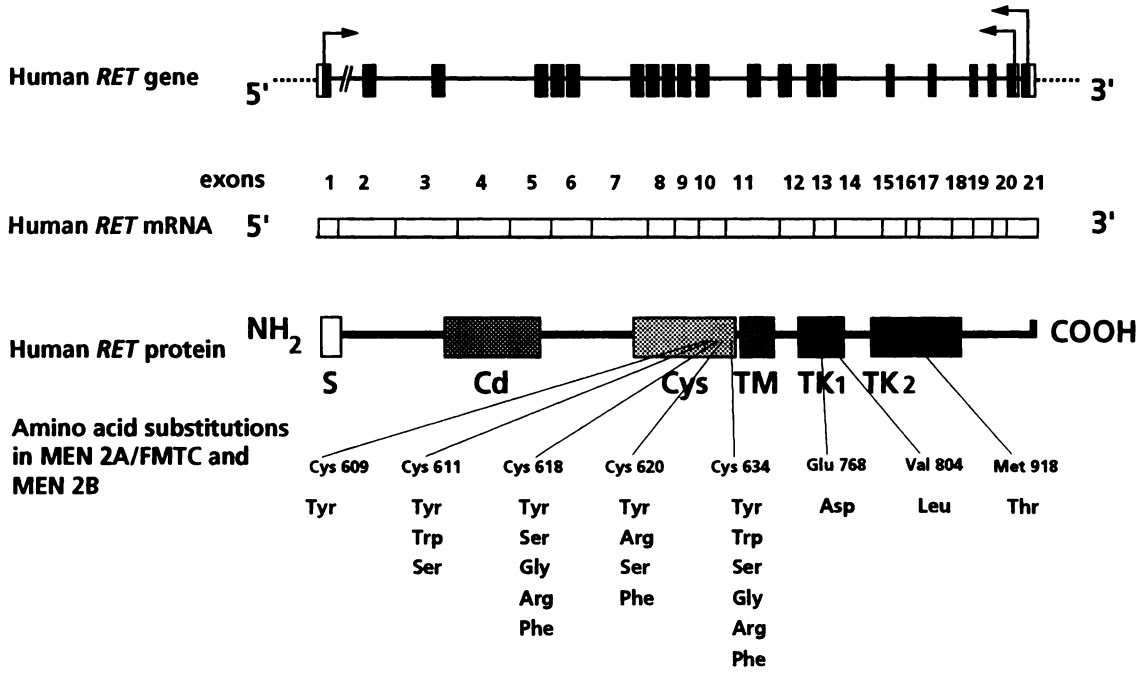

Fig. 1. Schematic representation of the *RET* proto-oncogene, mRNA, and protein. The intron-exon structure of the *RET* gene is shown in the *upper panel*. Exons, ■. Noncoding regions within exons are *white*. The Ret protein structure is shown in the *lower panel*, where the positions of the amino acids mutated in MEN 2A and MEN 2B are indicated. *S*, signal peptide; *Cd*, cadherin-like domain; *Cys*, cysteine-rich domain; *TM*, transmembrane domain; *TK1* and *TK2*, tyrosine kinase subdomains 1 and 2, respectively.

mixture was left on ice for 15 min. The homogenates were centrifuged at 12,000 × *g* for 15 min. The aqueous phase was transferred to a clean tube, 1 volume of 2-propanol was added, and the RNA was pelleted by centrifugation and washed with 70% ethanol. Total cellular RNA was dissolved in 10 mM Tris-HCl and 1 mM EDTA (pH 8).

Reverse Transcription-PCR and Nucleotide Sequence Analysis. cDNA was synthesized on 3 μg of total cellular RNA with SuperScript RNaseH$^-$ reverse transcriptase (Life Technologies, Inc., Bethesda, MD) at 45°C, using a mixture of oligo $d(T)_{12-18}$ and random hexanucleotides or RET-specific oligonucleotides as primers. A 2-μl aliquot of the cDNA reactions was used for 33 cycles (1 min at 94°C, 1 min at 55°C, and 3 min at 72°C) of PCR with AmpliTaq polymerase (Perkin Elmer Cetus). PCR products were visualized on ethidium bromide-stained 3% agarose gels. If PCR reactions did not result in sufficient product for sequencing reactions, another PCR with nested primers was performed for 10–30 cycles. PCR fragments were directly sequenced after excision from 1% ultra low-melting-point agarose gels (Sigma Chemical Co., St. Louis, MO), using a pUC sequencing kit (Boehringer Mannheim) with dideoxy chain termination and ^{35}S-labeled dATP (specific activity = 600 Ci/mmol) as label. Sequence reaction products of the four tumors and the control tissue (normal thyroid gland) were run on 8% polyacrylamide gels. The 32 primers used for cDNA synthesis, PCR, and sequencing reactions were evenly spread over the coding region of the *RET* gene and were designed to give overlapping PCR products and nucleotide sequences. Detailed information on primers is available on request.

Results and Discussion

Five DNA sequence polymorphisms in the coding region of the *RET* gene were revealed by sequencing this small panel of five tissues from four individuals (Table 1). One of these polymorphisms involves an amino acid substitution at codon 691 but is most probably not related to tumor development because it was also demonstrated in DNA from control subjects. However, because it is not known which amino acid residues are essential for proper functioning of the Ret protein, it is conceivable that one or more of such amino acid substitutions can modulate disease phenotype in *MEN 2* gene carriers. Apart from the five polymorphisms revealed in this study, several more have been reported (1, 17).

Heterozygosity for DNA sequence polymorphisms in the cDNA amplicons demonstrated that there was no loss of heterozygosity for the *RET* locus in these tumors and that both alleles of the *RET* gene were transcribed with similar efficiency in all tumors investigated, as well as in the normal thyroid gland. In earlier family studies (3, 14, 18), we had identified the germ line *RET* gene mutations in peripheral blood DNA of the patients investigated in the present study. These germ line mutations were also observed in the cDNA of the respective tumors. However, no tumor-specific mutations were found in the

Table 1 *DNA sequence polymorphisms revealed by sequencing the coding region of RET cDNA from four MEN 2 tumors and a normal thyroid gland*

Codon[a]	Nucleotide change	Restriction site[b]	Amino acid change	Allele frequencies[c]
45	GCG → GCA	− *Hae*III	None	ND
432	GCG → GCA	+ *Bsm*I	None	0.72/0.28
691	GGT → AGT	+ *Bsr*I	Gly → Ser	0.78/0.22
769	CTT → CTG	− *Taq*I	None	ND
904	TCC → TCG	+ *Rsa*I	None	ND

[a] Codon number 1 is the Met translational start codon at positions 196–198 of the RET cDNA, according to Ref. 12.
[b] +, the generation of a new cleavage site for the restriction endonuclease; −, the disappearance of an existing cleavage site.
[c] The first number is the frequency of the allele without the respective restriction site; the second number is the frequency of the allele with the respective restriction site. Allele frequencies were determined by restriction enzyme digestion of PCR products generated on DNA from 35 (for codon 432) and 75 (for codon 691) random subjects, respectively. Mendelian inheritance of the polymorphisms was confirmed in three families. ND, not determined.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/21/4853/2462126/cr0560214853.pdf by guest on 29 May 2022

coding region of the RET cDNA sequence in the four tumors investigated. The *RET* gene promoter and intronic sequences were not screened for mutations. However, it is unlikely that such mutations were present because both alleles of the *RET* gene were expressed at similar levels, and no indications for tumor-specific RNA splice-variants were obtained. *In vitro* studies by Santoro *et al.* (15) and Asai *et al.* (16) have shown that Ret proteins harboring MEN 2A- or MEN 2B-specific missense mutations are constitutively activated in NIH 3T3 cells transfected with the respective mutant RET cDNAs. In addition, the mutant cDNAs in these cell lines have transforming activity. Our present results also demonstrate that *in vivo*, in MTC and pheochromocytoma from both MEN 2A and MEN 2B patients, the mutated *RET* genes act as dominantly transforming oncogenes.

The differences in the clinical manifestation of disease among MEN 2A patients, however, are not readily explained by a single *RET* mutation. Different phenotypes of MEN 2A in different families, and even within one family, can be observed for one specific germ line *RET* mutation. Variability is observed with respect to the aggressiveness of tumor growth and the prevalence of pheochromocytoma and hyperparathyroidism, but also to concurrent manifestation of cutaneous lichen amyloidosis and Hirschsprung's disease (19). Although the inherited mutations may also result in constitutively activated Ret kinase activity *in vivo* and thus predispose to tumor development, these inherited mutations may not be sufficient for aggressive tumor growth. Additional mutations of the *RET* proto-oncogene, *in casu* somatic mutations of Met codon 918, were recently described for 2 of 14 investigated MEN 2-related MTCs as well as 1 case of MEN 2-related C-cell hyperplasia (20). It was not clear whether the allele harboring the germ line *RET* mutation or the homologous allele acquired this additional mutation. Although somatic *RET* gene mutations may contribute to MEN 2 tumor progression by further impeding normal Ret function, our data show that such additional mutations of the *RET* gene are not required for tumorigenesis. Phenotypic variability observed both between and within MEN 2A families may be due to inherited conditions other than a mutated *RET* gene, somatic mutations at other loci in the genome, or nurture. For example, in MTC and pheochromocytoma, frequent loss of heterozygosity has been observed for loci on chromosomes 1p and 22 (21). The finding that the inherited mutations are the only *RET* gene mutations required for tumor development in MEN 2 has clinical consequences. All the MEN 2 target cells in which the constitutively activated Ret kinase is already expressed during prenatal development (22) are prone to neoplastic changes. Therefore, presymptomatic removal of the thyroid gland at a young age is indicated in all *MEN 2* gene carriers.

References

1. Mulligan, L. M., Kwok, J. B. J., Healey, C. S., Elsdon, M. J., Eng, C., Gardner, E., Love, D. R., Mole, S. E., Moore, J. K., Papi, L., Ponder, M. A., Telenius, H., Tunnacliffe, A., and Ponder, B. A. J. Germline mutations of the *RET* proto-oncogene in multiple endocrine neoplasia syndrome type 2A. Nature (Lond.), *363:* 458–460, 1993.
2. Donis-Keller, H., Dou, S., Chi, D., Carlson, K. M., Toshima, K., Lairmore, T. C., Howe, J. R., Moley, J. F., Goodfellow, P. J., and Wells, S. A. Mutations in the *RET* proto-oncogene are associated with MEN 2A and FMTC. Hum. Mol. Genet., *2:* 851–856, 1993.
3. Hofstra, R. M. W., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Höppener, J. W. M., Ploos van Amstel, H-K., Romeo, G., Lips, C. J. M., and Buys, C. H. C. M. A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature (Lond.), *367:* 375–376, 1994.
4. Carlson, K. M., Dou, S., Chi, D., Scavarda, N., Toshima, K., Jackson, C. E., Wells, S. A., Jr., Goodfellow, P. J., and Donis-Keller, H. Single missense mutation in the tyrosine kinase catalytic domain of the *RET* proto-oncogene is associated with multiple endocrine neoplasia type 2B. Proc. Natl. Acad. Sci. USA, *91:* 1579–1583, 1994.
5. Eng, C., Smith, D. P., Mulligan, L. M., Nagai, M. A., Healey, C. S., Ponder, M. A., Gardner, E., Scheumann, G. F. W., Jackson, C. E., Tunnacliffe, A., and Ponder, B. A. J. Point mutation within the tyrosine kinase domain of the *RET* proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. Hum. Mol. Genet., *3:* 237–241, 1994.
6. Sipple, J. H. The association of pheochromocytoma with carcinoma of the thyroid gland. Am. J. Med., *31:* 163–166, 1961.
7. Schimke, R. N., Hartmann, W. H., Prout, T. E., and Rimoin, D. L. Syndrome of bilateral pheochromocytomas, medullary thyroid carcinoma, and multiple neuromas. A possible regulatory defect in the differentiation of chromaffin tissue. N. Engl. J. Med., *279:* 1–7, 1968.
8. Knudson, A. G., Jr. Hereditary cancer, oncogenes, and anti-oncogenes. Cancer Res., *45:* 1437–1443, 1985.
9. Landsvater, R. M., Mathew, C. G. P., Smith, B. A., Marcus, E. M., Te Meerman, G. J., Lips, C. J. M., Geerdink, R. A., Nakamura, Y., Ponder, B. A. J., and Buys, C. H. C. M. Development of multiple endocrine neoplasia type 2A does not involve substantial deletions of chromosome 10. Genomics, *4:* 246–250, 1989.
10. Nelkin, B. D., Nakamura, Y., White, R. W., de Bustros, A. C., Herman, J., Wells, S. A., and Baylin, S. B. Low incidence of loss of chromosome 10 in sporadic and hereditary human medullary thyroid carcinoma. Cancer Res., *49:* 4114–4119, 1989.
11. Mulligan, L. M., Gardner, E., Smith, B. A., Mathew, C. G. P., and Ponder, B. A. J. Genetic events in tumour initiation and progression in multiple endocrine neoplasia type 2. Genes Chromosomes & Cancer, *6:* 166–177, 1992.
12. Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H., and Hiai, H. Cloning and expression of the *RET* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. Oncogene, *3:* 571–578, 1988.
13. Nelkin, B. D., and Baylin, S. B. *RET* oncogene responsible for MEN 2A. Curr. Biol., *3:* 477–480, 1993.
14. Landsvater, R. M., Jansen, R. P. M., Hofstra, R. M. W., Buys, C. H. C. M., Lips, C. J. M., and Ploos van Amstel, J. K. Mutation analysis of the *RET* proto-oncogene in Dutch families with MEN 2A, MEN 2B, and FMTC: two novel mutations and one *de novo* mutation for MEN 2A. Hum. Genet., *97:* 11–14, 1995.
15. Santoro, M., Carlomagno, F., Romano, A., Bottaro, D. P., Dathan, N. A., Grieco, M., Fusco, A., Vecchio, G., Matoskova, B., Kraus, M. H., and Di Fiore, P. P. Activation of *RET* as a dominant transforming gene by germline mutations of MEN 2A and MEN 2B. Science (Washington DC), *267:* 381–383, 1994.
16. Asai, N., Iwashita, T., Matsuyama, M., and Takahashi, M. Mechanism of activation of the *RET* proto-oncogene by multiple endocrine neoplasia 2A mutations. Mol. Cell. Biol., *15:* 1613–1619, 1995.
17. Ceccherini, I., Hofstra, R. M. W., Luo, Y., Stulp, R. P., Barone, V., Stelwagen, T., Bocciardi, R., Nijveen, H., Bolino, A., and Seri, M. DNA polymorphisms and conditions for SSCP analysis of the 20 exons of the *RET* proto-oncogene. Oncogene, *9:* 3025–3029, 1994.
18. Lips, C. J. M., Landsvater, R. M., Höppener, J. W. M., Geerdink, R. A., Blijham, G. H., Jansen-Schilhorn van Veen, J. M., Van Gils, A. P. G., De Wit, M. J., Zewald, R. A., Berends, M. J. H., Beemer, F. A., Brouwers-Smalbraak, J., Jansen, R. P. M., Ploos van Amstel, J. K., Van Vroonhoven, T. J. M. V., and Vroom, T. M. Clinical screening as compared with DNA analysis in families with multiple endocrine neoplasia type 2A. N. Engl. J. Med., *331:* 828–835, 1994.
19. Höppener, J. W. M., and Lips, C. J. M. Ret receptor tyrosine kinase gene mutations: molecular biological, physiological, and clinical aspects. Eur. J. Clin. Invest., *26:* 613–624, 1996.
20. Marsh, D. J., Andrew, S. D., Eng, C., Learoyd, D. L., Capes, A. G., Pojer, R., Richardson, A-L., Houghton, C., Mulligan, L. M., Ponder, B. A. J., and Robinson, B. G. Germline and somatic mutations in an oncogene: *RET* mutations in inherited medullary thyroid carcinoma. Cancer Res., *56:* 1241–1243, 1996.
21. Ponder, B. A. J., Smith, B. A., Marcus, E. M., Nakamura, Y., Landsvater, R. M., Buys, C. H. C. M., and Mathew, C. G. P. Genetic events in tumorigenesis in multiple endocrine neoplasia type 2. Cancer Cells (Cold Spring Harbor), *7:* 219–221, 1989.
22. Pachnis, V., Mankoo, B., and Constantini, F. Expression of the c-*ret* proto-oncogene during mouse embryogenesis. Development (Camb.), *119:* 1005–1017, 1993.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/56/21/4853/2462126/cr0560214853.pdf by guest on 29 May 2022