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Somatic Mutations in *RET* Exons 12 and 15 in Sporadic Medullary Thyroid Carcinomas: Different Spectrum of Mutations in Sporadic Type from Hereditary Type

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Germline mutations in the *RET* proto-oncogene are responsible for multiple endocrine neoplasia type 2 (MEN 2A and 2B) and familial medullary thyroid carcinoma (FMTC). Point mutations or in-frame deletions of exons 10, 11, 13, 14 and 16 are associated with sporadic medullary thyroid carcinoma (MTC). To understand further the role of the *RET* gene in sporadic MTC, we examined mutations in exons 12 and 15 of *RET* in patients with sporadic MTC. DNAs were extracted from 39 formalin-fixed tumor tissues and corresponding normal thyroid tissues or peripheral blood leukocytes. DNA sequencing was used to identify mutations in exons 12 and 15 of *RET*. In this study, one novel somatic mutation was found in exon 12 and five novel mutations or deletions were found in exon 15. Of the patients with mutations, one had an in-frame 12-bp deletion (nt. 2625–2636), one had point mutations in both codons 884 and 908, and the remaining three had point mutations in codons 748, 876 and 901, respectively. Together with our previous identification of somatic mutations in exons 10, 11, 13, 14 and 16, somatic alterations were found in 10 out of 39 (25.6%) sporadic MTCs. There was no association of *RET* gene mutations with tumor recurrence or prognosis. These results suggest that mutations occur frequently in the *RET* coding region in addition to the previously reported mutation hot spots, and there is a different spectrum of mutations between sporadic and hereditary MTC.

Key words: Sporadic medullary thyroid carcinoma — *RET* gene — Point mutation — Multiple endocrine neoplasia — Tyrosine kinase domain

The *RET* proto-oncogene on human chromosome 10q11.2 encodes a protein receptor tyrosine kinase that includes an extracellular region with a cysteine-rich domain, a transmembrane region and an intracellular region with a tyrosine kinase domain. Germline point mutations in *RET* have been found to cause MEN 2A, 2B and FMTC.[1,2] *RET* gene was first isolated by transfection of NIH 3T3 cells with a human T-cell lymphoma DNA, and was activated by fusion of the tyrosine kinase domain of this gene to the 5′-terminal portion of another gene,[3] i.e., H4 gene (*RET/PTC1*)[4] the regulatory subunit RIα of cAMP-dependent protein kinase A (*RET/PTC2*),[5] *ELE1* gene (*RET*/*PTC3* and *RET*/*PTC4*)[6–8] and *RFG5* gene (*RET*/*PTC5*).[9] In MEN 2A families, germline mutations are concentrated in five cysteine codons: codons 609, 611, 618 and 620 in exon 10, and codon 634 in exon 11.[10] A small number of MEN2A families has germline mutations at codons 790.[11] In MEN 2B, approximately 95% of families have an M918T germline mutation in exon 16,[12–14] and recent research revealed A883F and V804M with Y806C germline mutation as another cause of MEN 2B.[15–17] Germline mutations in *RET* have been found in 88% of FMTC families, and the majority of mutations in FMTC occur in the same five cysteine codons altered in MEN 2A.[10] C630F, C630Y, E768D, L790F, Y791F, V804L, V804M and S891A germline mutations have been found in some FMTC families.[11, 18–23]

Various somatic mutations in the cysteine-rich domain and tyrosine kinase domain of *RET* have been identified in sporadic MTCs. In the cysteine-rich domain, mutations in codons 630 and 634, and deletion of nucleotides have been reported.[24–29] In the intracellular tyrosine kinase domain, somatic mutations in codons 768 and 883 have been found in sporadic MTC, and are also found in FMTC and MEN 2B, respectively.[20, 30] In approximately one-third of sporadic MTCs, a somatic M918T mutation, which is also found in most MEN 2B families, has been reported,[12–14, 18, 22, 31, 32] and this mutation has been corre-

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Abbreviations used are: MEN, multiple endocrine neoplasia; FMTC, familial medullary thyroid carcinoma; MTC, medullary thyroid carcinoma; PCR, polymerase chain reaction.

lated with tumor recurrence or poor prognosis.[25, 33, 34] In contrast, we detected the somatic M918T mutation in only 6% (2/34) of sporadic MTCs, and correlation of this mutation with poor prognosis could not be confirmed.[35, 36] This low incidence of somatic mutations in codon 918 led us to examine *RET* mutations in exons 12 and 15. Here we report novel somatic point mutations in *RET* exons 12 and 15 in sporadic MTCs, and a different spectrum of mutations in sporadic MTCs compared with hereditary MTCs.

MATERIALS AND METHODS

Patients Thirty-nine cases of sporadic MTC were analyzed for *RET* gene mutations. Thirty-four patients had undergone surgery between 1965 and 1996 at Noguchi Thyroid Clinic and Hospital Foundation, and five at the Department of Surgery, Kitakyushu Municipal Medical Center. There were no apparent family histories of MTC, pheochromocytoma or parathyroid disease when patients were evaluated initially. No other diseases including pheochromocytoma, hyperparathyroidism, skeletal abnormalities, mucosal neuromas or Hirschsprung's disease coupled with MTC were detected in these cases, and no germline *RET* mutations were found.

DNA extraction Genomic DNA was isolated from 39 formalin-fixed tumor samples. For controls, formalin-fixed non-neoplastic thyroid tissues from 39 cases were selected and in addition, peripheral blood samples from 17 patients were obtained. Two 50-μm sections were sliced from each block of formalin-fixed tissue, and in cases of tumor tissue, a portion containing only the stroma of cancer cells as determined by hematoxylin and eosin staining was collected. DNA was extracted as described previously.[35]

Primer sequences and PCR conditions Oligonucleotide primers for PCR amplification of exons 12 and 15 of the *RET* gene were synthesized.[37] These primers were: 5′-GCCTTCTTCCTCCCCTGTCAT-3′ and 5′-GAGACTC-CCCCAGGGGCACTGT-3′ for exon 12, and 5′-TGAC-CGCTGCTGCCTGGCCAT-3′ and 5′-GCTTCCCAA-GGACTGCCTGC-3′ for exon 15. PCR amplification was carried out in a 50 μl reaction volume containing 100 ng of template DNA, 1.0 or 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 5 pmol of each sense and antisense primer, and 1 unit of AmpliTaq Gold (Perkin Elmer, Foster City, CA) with a PC-700 programmed thermal cycler (Astec, Tokyo). After initial denaturation at 94°C for 12 min, 35 cycles consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55 to 67°C and extension for 1 min at 72°C were done followed by a final extension of 7 min at 72°C. All PCR reactions were performed repeatedly and we confirmed the presence or absence of *RET* mutation.

Sequencing For nonisotopic sequencing, DNA products were purified with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany). PCR products were then subjected to 25 cycles with sense or antisense primer using fluorescence-based dideoxy terminator cycle sequencing (Perkin Elmer). The products were eluted through a Centri-sep spin-column (Perkin Elmer) and subjected to capillary gel electrophoresis. Data collection, and analysis were performed on an automated DNA sequencer (ABI PRISM310, Perkin Elmer).

RESULTS

Somatic point mutations or deletions in exons 12 and 15 were found in 5 out of 39 cases (12.8%). One patient had a G748C mutation in exon 12 (Fig. 1A). In exon 15, four somatic mutations were found: A876V, E884K, E901K and R908K (Fig. 1, B and C). One patient had both the E884K and R908K mutations. In addition to these point mutations, one patient had a 12-bp deletion of nucleotides 2625 to 2636, which included codons 898 to 902. This deletion resulted in an in-frame deletion of four amino acids (Fig. 1D). Among non-neoplastic thyroid tissues from 39 cases, no specific mutations or PCR errors was found. Furthermore, we confirmed the results of sequencing in every case by independent PCR analysis.

Together with the results from 34 previous cases and 5 additional patients,[35] somatic alterations in exons 10–16 were found in 10 out of 39 (25.6%) sporadic MTCs. Somatic mutations were distributed throughout exons 11 to 16, and were found in both the cysteine-rich and tyrosine kinase domains (Table I). The M918T mutation was found in only two patients (2/39, 5.1%). One patient had three point mutations (codons 766, 884 and 908), and one had mutations in both codons 918 and 919.

There was no association of somatic *RET* mutations with tumor recurrence or prognoses of patients (Table II). Median follow-up period was 12 years, and tumor recurrence was found in four patients, one of whom died of the disease. Three patients died of other causes, and the remaining 35 are alive and well.

DISCUSSION

In this study, we found somatic mutations of *RET* exons 12 and 15 in 5 out of 39 patients (12.8%) with sporadic MTC. Most other reports on sporadic MTC examined exons 10, 11, 13, 14 and 16 of *RET*, and codon 918 of exon 16 is a well-known mutation hot spot.[12, 18, 22, 31] In contrast, in our series only 5.1% of sporadic MTCs showed somatic mutations in codon 918. Somatic mutations in exons 12 and 15 are believed to be rare, and there have been few studies that examined exon 12 or 15 in sporadic MTC.[24, 27, 30, 38]

Fig. 2 illustrates the spectrum of *RET* mutations in hereditary and sporadic MTCs. Somatic mutations in cysteine codons 630 and 634, which are also detected in

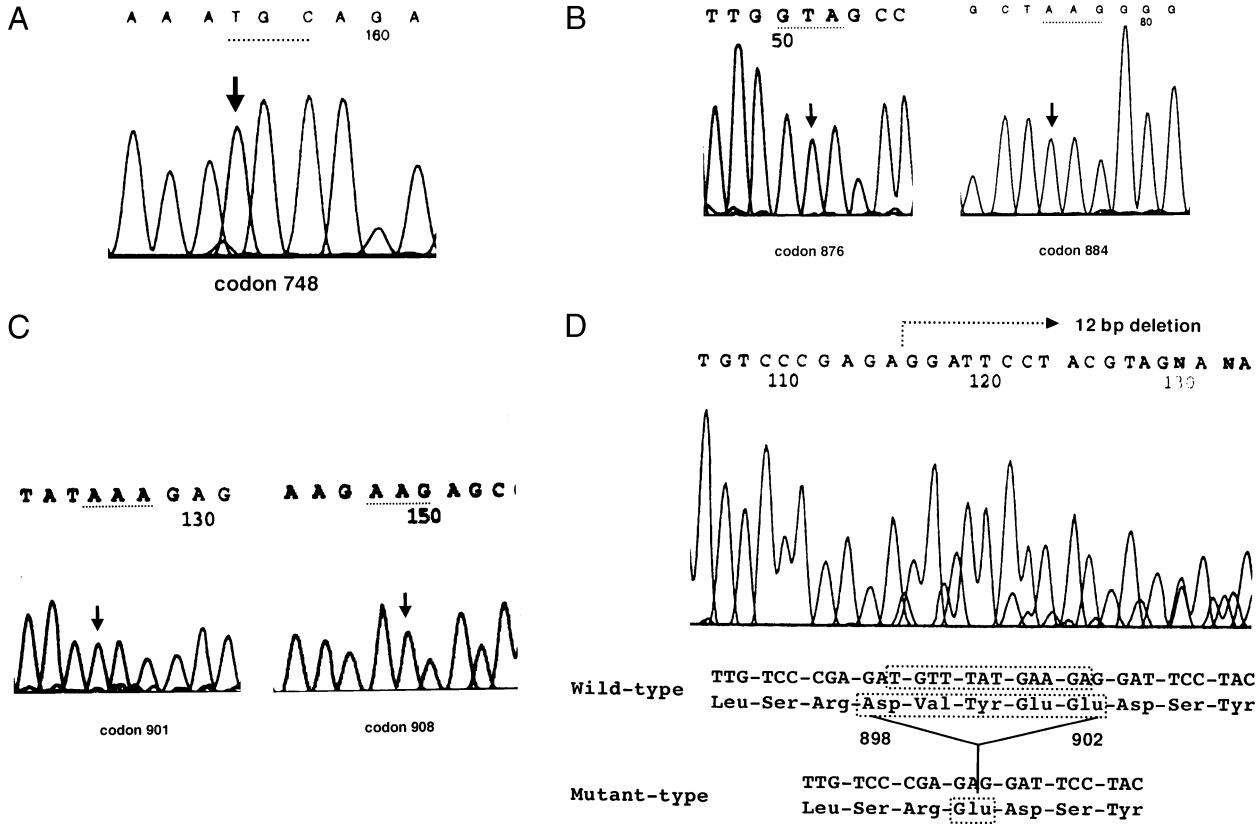

Fig. 1. *RET* DNA sequences from sporadic MTC. A, Tumor tissue of sporadic MTC from a 33-year-old female had a GGC-to-TGC (Gly to Cys) mutation at codon 748. B, The left side shows a GCA-to-GTA (Ala to Val) mutation at codon 876 in tumor tissue of sporadic MTC from a 61-year-old female and the right side shows a GAG-to-AAG (Glu to Lys) mutation at codon 884 in tumor tissue of sporadic MTC from a 49-year-old female. C, The left side shows a GAA-to-AAA (Glu to Lys) mutation at codon 901 in tumor tissue of sporadic MTC from a 62-year-old female and the right side shows a AGG-to-AAG (Arg to Lys) mutation at codon 908 in tumor tissue of sporadic MTC from a 49-year-old female. Arrowheads in A, B and C indicate the nucleotide substitutions. Both the E884K and R908K mutations were found in the same case. D, Tumor tissue of sporadic MTC from a 56-year-old female had a 12-bp deletion of nt. 2625–2636 that removed four amino acid residues (Asp-Val-Tyr-Glu).

Table I. Summary of Somatic *RET* Mutations in Sporadic MTCs

Exon	11	11	12	13	15	15	15	15	15	16	16
Codons	634	634	748	766	876	884	898–902 (nt. 2625–2635)	901	908	918	919
Wild-type	TGC	TGC	GGC	CCG	GCA	GAG	GATGTTTATGAAGAG	GAA	AGG	ATG	GCA
Amino acid	Cys	Cys	Gly	Pro	Ala	Glu	Asp-Val-Tyr-Glu-Glu	Glu	Arg	Met	Ala
Mutant-type	TGG	TAC	TGC	TCG	GTA	AAG	GAG (12 bp deletion)	AAA	AAG	ACG	GTA
Amino acid	Trp	Tyr	Cys	Ser	Val	Lys	Glu	Lys	Lys	Thr	Val
Number of cases	1	2	1	1[a)]	1	1[a)]	1	1	1[a)]	2[b)]	1[b)]

a) One case showed three point mutations (codons 766, 884 and 908).
b) One case showed mutations in codons 918 and 919.

MEN 2A, were found in a subset of sporadic MTCs.[24, 25)] In our study, C634W and C634Y mutations were found in one and two cases, respectively.[36)] In these cases, the same mechanism might be operative as for MEN 2A mutation; wild-type RET exists normally as an undimerized form and mutant RET protein with these cysteine residues

Table II. Tumor Recurrence and Prognoses of Patients with Sporadic MTC with or without Somatic *RET* Mutations

RET mutation	Number of patients	Alive (%)	Tumor recurred (%)	Died of MTC (%)	Died of other disease (%)
Present	10	9 (90.0)	1 (10.0)	0 (0)	1 (10.0)
Absent	28	25 (89.3)	3 (11.7)	1 (3.6)	2 (7.1)

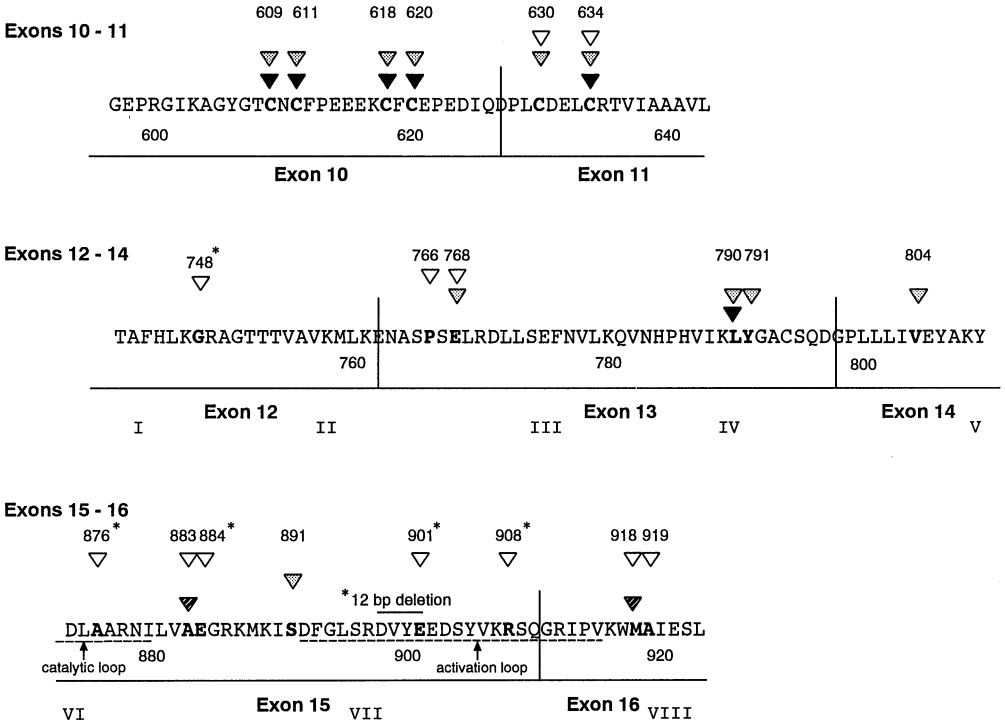

Fig. 2. Spectrum of mutations in the *RET* gene in hereditary and sporadic MTCs. ▼, ▽ and ▽ indicate the locations of germline *RET* mutations in MEN 2A, 2B and FMTC, respectively. ▽ shows the location of somatic *RET* mutations in sporadic MTCs. Mutations marked with * are described in this paper. Roman numerals indicate the positions of highly conserved subdomains of the tyrosine kinase family.

forms aberrant disulfide bonds with other mutant RET molecules, and thus creates a RET homodimer with constitutive activation of its kinase and transforming activities.[39, 40]

Deletion of nucleotides in the cysteine-rich domain is also found in certain sporadic MTCs; a 6-bp deletion that includes codon 630 and a 3-bp deletion that spans codons 632 and 633 have been reported.[26, 27] Ceccherini *et al*.[28] found one case of sporadic MTC with a 6-bp deletion that removed codons 632 and 633 and one case with a 48-bp deletion that removed codons 592 to 607. They also showed that expression of *RET* carrying a deletion resulted in strong transforming activity in NIH 3T3 cells. Alemi *et al*.[29] reported that 14 of 15 sporadic MTCs had positions 1825 and 1830 to 1837 within the cysteine-rich domain deleted, resulting in the loss of codons 633 to 635 (Leu-Cys-Arg) and amino acid substitutions in codons 632 (Glu to Ser) and 636 (Thr to Ser). These are all in-frame mutations, and it is hypothesized that deletions in the cysteine-rich region cause constitutive activation of the tyrosine kinase activity of *RET*.

In the intracellular tyrosine kinase domain, somatic E768D mutation, which is also found in some FMTCs, has been found.[20] In exon 12, we found a G748C mutation in a case of sporadic MTC, and we previously reported a P766S mutation of exon 13 in one case.[35] These mutations are within subdomains I and II of the highly conserved region, respectively.[41] Although the effects of these mutations have not been thoroughly investigated, it is thought that these mutations modify tyrosine kinase activity by changing the substrate specificity or the ATP-binding capacity of the receptor.[42]

In the tyrosine kinase domain, codon 918 is frequently mutated in sporadic MTCs. The methionine at codon 918 is highly conserved and lies within the substrate binding pocket of the central catalytic core of the tyrosine kinase domain. It is thought that the M918T mutation causes constitutive activation of tyrosine kinase activity because the affinity of the substrate binding pocket is altered and phosphorylation is increased.[39, 43] One sporadic MTC case in our series showed point mutations in both codons 918 and 919.[35]

The M918T mutation has been reported to correlate with tumor recurrence and poor prognosis.[33, 34] In contrast, there are reports of no correlation between the M918T mutation and clinicopathological characteristics or patients' prognoses.[30, 44] In this study, only four patients had recurrence of MTC, and none of them had mutation at codon 918. Two patients with somatic M918T mutations were free of disease for 15 years and 25 years after thyroidectomy, respectively. Our findings suggest no association of the M918T mutation with tumor recurrence or prognosis. Other reports examining sporadic MTC in Japanese patients also reported no correlation of the M918T mutation with tumor differentiation or clinical features.[45, 46] Factors other than somatic *RET* mutations might play an important role in sporadic MTC progression in Japanese patients. Because of the small number of cases in this study, the frequency of 918 mutation and the association of somatic 918 mutation with tumor recurrence or prognosis must be confirmed in a larger series of Japanese MTC cases.

The M918T, A919V and A883F mutations of the *RET* gene lie within highly conserved domains VI to VIII.[41] The A883F mutation lies within a region involved in substrate recognition. The A876V, E884K, E901K and R908K point mutations and the 12-bp deletion (nucleotides 2625 to 2636) found in this study also lie within the highly conserved VI to VIII region. Mutations in the activation loop are expected to significantly change tyrosine kinase activation.[16] The E901K and R908K mutations and 12-bp deletion occurred within this activation loop. Mutations in the activation loop of other tyrosine kinases have been reported to cause ligand-independent activation.[47, 48] The A876V mutation lies within the catalytic loop of the tyrosine kinase, and the E884K mutation lies within the substrate recognition site. Mutations in these areas might activate the tyrosine kinase and change the substrate specificity.

To establish whether the observed mutations in this study truly represent oncogenic alterations of the *RET* gene, we are now examining the significance of these new mutations by further verification of their oncogenicity in NIH3T3 transformation assays or by examining possible changes in biochemical properties such as dimerization of mutant RET proteins or tyrosine kinase activation.[49] In conclusion, *RET* gene mutations are found frequently in the cysteine-rich and tyrosine kinase domains in addition to the previously identified mutation hot spots. The difference in the spectrum of mutations between sporadic and hereditary MTC shows that tumor development or progression may be different between these two forms of MTC.

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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. Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. *Nature*, **363**, 458–460 (1993).
2) Goodfellow, P. J. and Wells, S. A., Jr. *RET* gene and its implications for cancer. *J. Natl. Cancer Inst.*, **87**, 1515–1523 (1995).
3) Takahashi, M., Ritz, J. and Cooper, G. M. Activation of a novel human transforming gene, *RET*, by DNA rearrangement. *Cell*, **42**, 581–588 (1985).
4) Pierotti, M. A., Santoro, M., Jenkins, R. B., Sozzi, G., Bongarzone, I., Grieco, M., Monzini, N., Miozzo, M., Herrmann, M. A., Fusco, A., Hay, I. D., Della Porta, G. and Vecchio, G. Characterization of an inversion on the long arm of chromosome 10 juxtaposing D10S170 and *RET* and creating the oncogenic sequence *RET*/*PTC*. *Proc. Natl. Acad. Sci. USA*, **89**, 1616–1620 (1992).
5) Bongarzone, I., Monzini, N., Borrello, M. G., Carcano, C., Ferraresi, G., Arighi, E., Mondellini, P., Della Porta, G. and Pierotti, M. A. Molecular characterization of a thyroid tumor-specific transforming sequence formed by the fusion of *RET* tyrosine kinase and the regulatory subunit RIα of cyclic AMP-dependent protein kinase A. *Mol. Cell. Biol.*, **13**, 358–366 (1993).
6) Bongarzone, I., Butti, M. G., Coronelli, S., Borrello, M. G., Santoro, M., Mondellini, P., Pilotti, S., Fusco, A., Della Porta, G. and Pierotti, M. A. Frequent activation of *RET* protooncogene by fusion with a new activating gene in papillary thyroid carcinomas. *Cancer Res.*, **54**, 2979–2985 (1994).
7) Jhiang, S. M., Smanik, P. A. and Mazzaferri, E. L. Development of a single-step duplex RT-PCR detecting different forms of *RET* activation, and identification of the third form of *in vivo RET* activation in human papillary thyroid carcinoma. *Cancer Lett.*, **78**, 69–76 (1994).
8) Fugazzola, L., Pierotti, M. A., Vigano, E., Pacini, F.,

Vorontsova, T. V. and Bongarzone, I. Molecular and biochemical analysis of *RET*/*PTC4*, a novel oncogenic rearrangement between *RET* and *ELE1* genes, in a post-Chernobyl papillary thyroid cancer. *Oncogene*, **13**, 1093–1097 (1996).

9) Klugbauer, S., Demidchik, E. P., Lengfelder, E. and Rabes, H. M. Detection of a novel type of *RET* rearrangement (*PTC5*) in thyroid carcinomas after Chernobyl and analysis of the involved *RET*-fused gene *RFG5*. *Cancer Res.*, **58**, 198–203 (1998).
10) Eng, C., Clayton, D., Schuffenecker, I., Lenoir, G., Cote, G., Gagel, R. F., van Amstel, H. K. P., Lips, C. J. M., Nishisho, I., Takai, S., Marsh, D. J., Robinson, B. G., Frank-Raue, K., Raue, F., Xue, F., Noll, W. W., Romei, C., Pacini, F., Fink, M., Niederle, B., Zedenius, J., Nordenskjöld, M., Komminoth, P., Hendy, G. N., Gharib, H., Thibodeau, S. N., Lacroix, A., Frilling, A., Ponder, B. A. J. and Mulligan, L. M. The relationship between specific *RET* proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International *RET* mutation consortium analysis. *JAMA*, **276**, 1575–1579 (1996).
11) Berndt, I., Reuter, M., Saller, B., Frank-Raue, K., Groth, P., Grubendorf, M., Raue, F., Ritter, M. M. and Höppner, W. A new hot spot for mutations in the *ret* protooncogene causing familial medullary thyroid carcinoma and multiple endocrine neoplasia type 2A. *J. Clin. Endocrinol. Metab.*, **83**, 770–774 (1998).
12) Hofstra, R. M. W., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Höppener, J. W. M., van Amstel, H. K. P., 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*, **367**, 375–376 (1994).
13) 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* protooncogene is associated with multiple endocrine neoplasia type 2B. *Proc. Natl. Acad. Sci. USA*, **91**, 1579–1583 (1994).
14) Blaugrund, J. E., Johns, M. J., Eby, Y. J., Ball, D. W., Baylin, S. B., Hruban, R. H. and Sidransky, D. *RET* proto-oncogene mutations in inherited and sporadic medullary thyroid cancer. *Hum. Mol. Genet.*, **3**, 1895–1897 (1994).
15) Gimm, O., Marsh, D. J., Andrew, S. D., Frilling, A., Dahia, P. L., Mulligan, L. M., Zajac, J. D., Robinson, B. G. and Eng, C. Germline dinucleotide mutation in codon 883 of the *RET* proto-oncogene in multiple endocrine neoplasia type 2B without codon 918 mutation. *J. Clin. Endocrinol. Metab.*, **82**, 3902–3904 (1997).
16) Smith, D. P., Houghton, C. and Ponder, B. A. J. Germline mutation of *RET* codon 883 in two cases of de novo MEN 2B. *Oncogene*, **15**, 1213–1217 (1997).
17) Miyauchi, A., Futami, H., Hai, N., Yokozawa, T., Kuma, K., Aoki, N., Kosugi, S., Sugano, K. and Yamaguchi, K. Two germline missense mutations at codons 804 and 806 of the *RET* proto-oncogene in the same allele in a patient with multiple endocrine neoplasia type 2B without codon 918 mutation. *Jpn. J. Cancer Res.*, **90**, 1–5 (1999).
18) Komminoth, P., Kunz, E. K., Matias-Guiu, X., Hiort, O., Christiansen, G., Colomer, A., Roth, J. and Heitz, P. U. Analysis of *RET* protooncogene point mutations distinguishes heritable from nonheritable medullary thyroid carcinomas. *Cancer*, **76**, 479–489 (1995).
19) Kitamura, Y., Goodfellow, P. J., Shimizu, K., Nagahama, M., Ito, K., Kitagawa, W., Akasu, H., Takami, H., Tanaka, S. and Wells, S. A., Jr. Novel germline *RET* proto-oncogene mutations associated with medullary thyroid carcinoma (MTC): mutation analysis in Japanese patients with MTC. *Oncogene*, **14**, 3103–3106 (1997).
20) Eng, C., Smith, D. P., Mulligan, L. M., Healey, C. S., Zvelebil, M. J., Stonehouse, T. J., Ponder, M. A., Jackson, C. E., Waterfield, M. D. and Ponder, B. A. J. A novel point mutation in the tyrosine kinase domain of the *RET* proto-oncogene in sporadic medullary thyroid carcinoma and in a family with FMTC. *Oncogene*, **10**, 509–513 (1995).
21) Bolino, A., Schuffenecker, I., Luo, Y., Seri, M., Silengo, M., Tocco, T., Chabrier, G., Houdent, C., Murat, A., Schlumberger, M., Tourniaire, J., Lenoir, G. M. and Romeo, G. *RET* mutations in exons 13 and 14 of FMTC patients. *Oncogene*, **10**, 2415–2419 (1995).
22) Fink, M., Weinhäusel, A., Niederle, B. and Haas, O. A. Distinction between sporadic and hereditary medullary thyroid carcinoma (MTC) by mutation analysis of the *RET* proto-oncogene. *Int. J. Cancer*, **69**, 312–316 (1996).
23) Hofstra, B. M. W., Fattoruso, O., Quadro, L., Wu, Y., Libroia, A., Verga, U., Colantuoni, V. and Buys, C. H. C. M. A novel point mutation in the intracellular domain of the *ret* protooncogene in a family with medullary thyroid carcinoma. *J. Clin. Endocrinol. Metab.*, **82**, 4176–4178 (1997).
24) Bugalho, M. J., Frade, J. P., Santos, J. R., Limbert, E. and Sobrinho, L. Molecular analysis of the *RET* proto-oncogene in patients with sporadic medullary thyroid carcinoma: a novel point mutation in the extracellular cysteine-rich domain. *Eur. J. Endocrinol.*, **136**, 423–426 (1997).
25) Romei, C., Elisei, R., Pinchera, A., Ceccherini, I., Molinaro, E., Mancusi, F., Martino, E., Romeo, G. and Pacini, F. Somatic mutations of the *RET* protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. *J. Clin. Endocrinol. Metab.*, **81**, 1619–1622 (1996).
26) Donis-Keller, H., Dou, S., Chi, D., Carlson, K. M., Toshima, K., Lairmore, T. C., Howe, J. R., Moley, J. F., Goodfellow, P. and Wells, S. A., Jr. Mutations in the *RET* proto-oncogene are associated with MEN 2A and FMTC. *Hum. Mol. Genet.*, **2**, 851–856 (1993).
27) Hofstra, R. M. W., Stelwagen, T., Stulp, R. P., de Jong, D., Hulsbeek, M., Kamsteeg, E. J., van den Berg, A., Landsvater, R. M., Vermey, A., Molenaar, W. M., Lips, C. J. M. and Buys, C. H. C. M. Extensive mutation scanning of *RET* in sporadic medullary thyroid carcinoma and of *RET* and *VHL* in sporadic pheochromocytoma reveals

involvement of these genes in only a minority of cases. *J. Clin. Endocrinol. Metab.*, **81**, 2881–2884 (1996).
28) Ceccherini, I., Pacini, B., Pacini, F., Gullo, M., Bongarzone, I., Romei, C., Santamaria, G., Matera, I., Mondellini, P., Scopsi, L., Pinchera, A., Pierotti, M. A. and Romeo, G. Somatic in-frame deletions not involving juxtamembranous cysteine residues strongly activate the *RET* proto-oncogene. *Oncogene*, **14**, 2609–2612 (1997).
29) Alemi, M., Lucas, S. D., Sällström, J. F., Bergholm, U., Åkerström, G. and Wilander, E. A complex nine base pair deletion in *RET* exon 11 common in sporadic medullary thyroid carcinoma. *Oncogene*, **14**, 2041–2045 (1997).
30) Marsh, D. J., Learoyd, D. L., Andrew, S. D., Krishnan, L., Pojer, R., Richardson, A. L., Delbridge, L., Eng, C. and Robinson, B. G. Somatic mutations in the *RET* proto-oncogene in sporadic medullary thyroid carcinoma. *Clin. Endocrinol. (Oxf)*, **44**, 249–257 (1996).
31) Eng, C., Mulligan, L. M., Smith, D. P., Healey, C. S., Frilling, A., Raue, F., Neumann, H. P. H., Pfragner, R., Behmel, A., Lorenzo, M. J., Stonehouse, T. J., Ponder, M. A. and Ponder, B. A. J. Mutation of the *RET* protooncogene in sporadic medullary thyroid carcinoma. *Genes Chromosom. Cancer*, **12**, 209–212 (1995).
32) 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).
33) Zedenius, J., Larsson, C., Bergholm, U., Bovee, J., Svensson, A., Hallengren, B., Grimelius, L., Backdahl, M., Weber, G. and Wallin, G. Mutations of codon 918 in the *RET* proto-oncogene correlate to poor prognosis in sporadic medullary thyroid carcinomas. *J. Clin. Endocrinol. Metab.*, **80**, 3088–3090 (1995).
34) Jhiang, S. M., Fithian, L., Weghorst, C. M., Clark, O. H., Falko, J. M., O'Dorisio, T. M. and Mazzaferri, E. L. *RET* mutation screening in MEN2 patients and discovery of a novel mutation in a sporadic medullary thyroid carcinoma. *Thyroid*, **6**, 115–121 (1996).
35) Uchino, S., Noguchi, S., Adachi, M., Sato, M., Yamashita, H., Watanabe, S., Murakami, T., Toda, M., Murakami, N. and Yamashita, H. Novel point mutations and allele loss at the *RET* locus in sporadic medullary thyroid carcinomas. *Jpn. J. Cancer Res.*, **89**, 411–418 (1998).
36) Uchino, S., Noguchi, S., Sato, M., Adachi, M., Yamashita, H., Watanabe, S., Murakami, T., Toda, M., Murakami, N. and Yamashita, H. *RET* gene analysis of latent small medullary thyroid carcinoma associated with Graves' disease. *Thyroidol. Clin. Exp.*, **10**, 279–281 (1998).
37) Ceccherini, I., Hofstra, R. M. W., Luo, Y., Stulp, R. P., Barone, V., Stelwagen, T., Bocciardi, R., Nijveen, H., Bolino, A., Seri, M., Ronchetto, P., Pasini, B., Bozzano, M., Buys, C. H. C. M. and Romeo, G. DNA polymorphisms and conditions for SSCP analysis of the 20 exons of the *RET* proto-oncogene. *Oncogene*, **9**, 3025–3029 (1994).
38) Komminoth, P., Roth, J., Muletta-Feurer, S., Saremaslani, P., Seelentag, W. K. F. and Heitz, P. U. *RET* proto-oncogene point mutations in sporadic neuroendocrine tumors. *J. Clin. Endocrinol. Metab.*, **81**, 2041–2046 (1996).
39) 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 MEN2A and MEN2B. *Science*, **267**, 381–383 (1995).
40) Xing, S., Smanik, P. A., Oglesbee, M. J., Trosko, J. E., Mazzaferri, E. L. and Jhiang, S. M. Characterization of RET oncogenic activation in MEN2 inherited cancer syndromes. *Endocrinology*, **137**, 1512–1519 (1996).
41) Hanks, S. K., Quinn, A. M. and Hunter, T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52 (1988).
42) Eng, C. The *RET* proto-oncogene in multiple endocrine neoplasia type 2 and Hirschsprung's disease. *N. Engl. J. Med.*, **335**, 943–951 (1996).
43) Borrello, M. G., Smith, D. P., Pasini, B., Bongarzone, I., Greco, A., Lorenzo, M. J., Arighi, E., Miranda, C., Eng, C., Alberti, L., Bocciardi, R., Mondellini, P., Scopsi, L., Romeo, G., Ponder, B. A. J. and Pierotti, M. A. RET activation by germline MEN2A and MEN2B mutations. *Oncogene*, **11**, 2419–2427 (1995).
44) Komminoth, P., Muletta-Feurer, S., Saremaslani, P., Kunz, E. K., Matias-Guiu, X., Hiort, O., Schroder, S., Seelentag, W. K. F., Roth, J. and Heitz, P. U. Molecular diagnosis of multiple endocrine neoplasia (MEN) in paraffin-embedded specimens. *Endocr. Pathol.*, **6**, 267–278 (1995).
45) Shan, L., Nakamura, M., Nakamura, Y., Utsunomiya, H., Shou, N., Jiang, X., Jing, X., Yokoi, T. and Kakudo, K. Somatic mutations in the *RET* protooncogene in Japanese and Chinese sporadic medullary thyroid carcinomas. *Jpn. J. Cancer Res.*, **89**, 883–886 (1998).
46) Maeda, S., Namba, H., Takamura, N., Tanigawa, K., Takahashi, M., Noguchi, S., Nagataki, S., Kanematsu, T. and Yamashita, S. A single missense mutation in codon 918 of the *RET* proto-oncogene in sporadic medullary thyroid carcinomas. *Endocr. J.*, **42**, 245–250 (1995).
47) Piao, X., Paulson, R., van der Geer, P., Pawson, T. and Bernstein, A. Oncogenic mutation in the *Kit* receptor tyrosine kinase alters substrate specificity and induces degradation of the protein tyrosine phosphatase SHP-1. *Proc. Natl. Acad. Sci. USA*, **93**, 14665–14669 (1996).
48) Webster, M. K., d'Avis, P. Y., Robertson, S. C. and Donoghue, D. J. Profound ligand-independent kinase activation of fibroblast growth factor receptor 3 by the activation loop mutation responsible for a lethal skeletal dysplasia, thanatophoric dysplasia type II. *Mol. Cell. Biol.*, **16**, 4081–4087 (1996).
49) Iwashita, T., Kato, M., Murakami, H., Asai, N., Ishiguro, Y., Ito, S., Iwata, Y., Kawai, K., Asai, M., Kurokawa, K., Kajita, H. and Takahashi, M. Biological and biochemical properties of Ret with kinase domain mutations identified in multiple endocrine neoplasia type 2B and familial medullary thyroid carcinoma. *Oncogene*, **18**, 3919–3922 (1999).