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Novel Point Mutations and Allele Loss at the *RET* Locus in Sporadic Medullary Thyroid Carcinomas

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Germline mutations in the *RET* proto-oncogene have been shown to be the underlying cause of multiple endocrine neoplasia type 2 (MEN 2A and 2B) and familial medullary thyroid carcinoma (FMTC). Some cases of sporadic medullary thyroid carcinoma (sporadic MTC) are reported to have specific codon 918, 883 and 768 mutations of the *RET* gene in tumor tissues. We examined *RET* gene mutations in 40 Japanese cases who had previously undergone surgery for sporadic MTC. DNA extracted from formalin-fixed tumor tissues and corresponding normal thyroid tissues or peripheral blood leukocytes was analyzed for mutations of exon 10, 11, 13, 14 and 16 of the *RET* gene by DNA sequencing and by mutation-specific restriction enzyme analysis. Germline *RET* point mutations were found in six of 40 cases (15%), cysteine residues at codon 618 in two, codon 634 in three and valine residue at codon 804 in one, and were newly identified as heritable MTC. Of the remaining 34 sporadic MTC cases, four (12%) had tumor-specific *RET* point mutations. Two were found in exon 16; one case showed an ATG to ACG (Met to Thr) mutation at codon 918, and the other showed two point mutations, ATG to ACG (Met to Thr) at codon 918 and GCA to GTA (Ala to Val) at codon 919 with loss of the wild-type allele, suggesting that both alleles at the *RET* locus were altered. The other two were found in exon 13; one case showed a CCG to TCG (Pro to Ser) mutation at codon 766 and the other showed a silent mutation, GTC to GTT (Val) at codon 778 with loss of the wild-type allele. There was no association of sporadic mutations with recurrence or prognosis in patients with sporadic MTCs. The low rate of somatic *RET* mutation at codon 918 in our sporadic MTC suggests that as yet unknown factors may be involved. Genetic alterations in both alleles may have an important role in a small fraction of sporadic MTCs.

Key words: Sporadic medullary thyroid carcinoma — *RET* gene — Point mutation — Allele loss — Multiple endocrine neoplasia

Specific germline mutations in the *RET* proto-oncogene on chromosome 10q11.2 have been shown to be the underlying cause of multiple endocrine neoplasia (MEN) 2A, 2B and familial medullary thyroid carcinoma (FMTC).[1,2] Mutations in MEN 2A and FMTC occur in a cysteine-rich extracellular region and are concentrated on cysteine residues at codons 609, 611, 618, 620 or 634 of exon 10 or 11.[3,4] In MEN 2B, mutations are found in the intracellular tyrosine kinase domain at codon 918 of exon 16.[5–7] In addition, mutations at codon 768 of exon 13 and codon 804 of exon 14 were reported in some FMTC families.[8,9]

More than half of all MTC cases are sporadic MTC. Clinically, sporadic MTC is characterized by negative family history in patients who are usually in their fifties or sixties when MTC is diagnosed. Occasionally, patients who undergo thyroidectomy for benign thyroid disease are diagnosed as MTC postoperatively. Before the susceptibility gene for MEN 2 was discovered, patients without a family history of MTC or pheochromocytoma were likely to be regarded as having the sporadic type. Recently, examination of *RET* germline mutations from peripheral blood leukocytes has enabled discrimination between hereditary and sporadic MTC.[10] Tumor-specific ATG to ACG mutations at codon 918 are found in about one-third of European and American sporadic MTCs [5,6,10–14] and this mutation has been reported to correlate with tumor recurrence or poor prognosis.[15–17] Furthermore, a GAG to GAC mutation at codon 768 and a GCT to TTT mutation at codon 883 were reported in some sporadic MTCs.[5,8] Interestingly, deletion of 3–48 nucleotide sequences in the cysteine-rich domain is also found in some sporadic MTCs.[4,18–20]

Here we report *RET* gene mutations in cases surgically treated as sporadic MTC and in addition, novel somatic point mutations and the loss of the wild-type allele at the *RET* gene locus in sporadic MTCs.

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PATIENTS AND METHODS

Patients Forty cases surgically treated as sporadic MTC were analyzed for *RET* gene mutations. These cases had undergone surgery between 1965 and 1996 at Noguchi Thyroid Clinic and Hospital Foundation. There was no apparent family history of hereditary MTC, pheochromocytoma or parathyroid disease at the time of initial evaluation. No other diseases, including pheochromocytoma, hyperparathyroidism, skeletal abnormalities, mucosal neuromas or Hirschsprung's disease, coupled with MTC had been detected in these cases.

DNA extraction and polymerase chain reaction (PCR) conditions DNA was extracted from formalin-fixed tumor tissues and corresponding nonneoplastic thyroid tissues or peripheral blood leukocytes. Formalin-fixed tumor tissues from 40 cases were used in this study. As nonneoplastic samples, formalin-fixed nonneoplastic thyroid tissues from 17 cases and peripheral blood samples from 23 cases were obtained. These patients gave written informed consent for the *RET* gene study. For formalin-fixed tissue samples, two 50-μm sections were sliced from each block, and in the case of tumor tissue, a portion containing only the stroma of cancer cells as determined by hematoxylin and eosin staining was collected. The paraffin was removed by placing the specimen in xylene for 30 min followed by dehydration with 100% ethanol and drying. The preparations were then incubated with 675 μl of Tris-disodium ethylenediaminetetraacetate (Tris-EDTA, pH 9.0), 0.95% sodium dodecyl sulfate, and 500 μg of proteinase K at 48°C with shaking for three days. DNA was extracted by the phenol/chloroform method as described previously.[21] High-molecular-weight DNA from peripheral blood leukocytes was extracted by use of a DNA extractor WB kit (Wako Chemicals, Osaka).

Oligonucleotide primers for exons 10, 11, 13, 14 and 16 of the *RET* gene for PCR amplification were synthesized as described by Ceccherini *et al.*[22] [5′-CTC AGG GGG CAG CAT TGT T-3′ and 5′-CAC TCA CCC TGG ATG TCT T-3′ for exon 10, 5′-CCT CTG CGG TGC CAA GCC TC-3′ and 5′-TGT GGG CAA ACT TGT GGT AGC A-3′ for exon 11, 5′-GCA GGC CTC TCT GTC TGA ACT T-3′ and 5′-GGA GAA CAG GGC TGT ATG GA-3′ for exon 13, 5′-CCT GGC TCC TGG AAG ACC CA-3′ and 5′-AGT GGT GGG TCA GGG TGT GG-3′ for exon 14, and 5′-AGG GAT AGG GCC TGG GCT T-3′ and 5′-TAA CCT CCA CCC CAA GAG-3′ for exon 16] and purified on an oligonucleotide purification column. The PCR amplification reaction was carried out in a 50 μl mixture containing 100 ng of template DNA, 1.0 or 1.5 mM $MgCl_2$, 0.2 mM dNTP, 5 pmol of each sense and antisense primer, and 1 unit of Ampli Taq Gold (Applied Biosystems, CA) with a PC-700 programmed thermal cycler (Astec, Tokyo). After initial denaturation at 94°C for 12 min, PCR was carried out as follows: 35 cycles each consisting of denaturation for 1 min at 94°C, annealing reaction for 1 min at 55 to 67°C and polymerase reaction for 1 min at 72°C, followed by 7 min of final extension at 72°C.

Sequencing and mutation-specific restriction enzyme analysis For nonisotopic sequencing, DNA products were purified with a QIA quick PCR purification kit (Qiagen, Germany). These purified products were subjected to a further 25 PCR cycles with sense or antisense primer by fluorescence-based dideoxy terminator cycle sequencing (Applied Biosystems). The products were then eluted through a Centri-sep spin-column (Applied Biosystems) and subjected to capillary gel electrophoresis. Data collection and analysis were performed on an automated DNA sequencer (Model 310, Applied Biosystems).

When a mutation was present, samples were subjected to mutation-specific restriction enzyme analysis. In addition, we performed *Fok*I digestion in the PCR products of exon 16 of all cases examined in this study to confirm the presence of codon 918 mutation. PCR products were restricted in a volume of 50 μl of low-salt buffer with the restriction enzyme *Fok*I, *Mun*I (Takara Shuzo, Tokyo) *Xho*I or *Eco*NI (Daiichi Pure Chemicals, Tokyo) for 2 h at 37°C. Restriction fragments were analyzed by 4% agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Germline and somatic mutations of the *RET* gene in this study are summarized in Table I. We identified germline *RET* point mutations in six of 40 cases (15%). Mutations at codon 618 were found in two; TGC to GGC (Cys to Gly) and AGC (Cys to Ser). Three mutations were found at codon 634; TGC to AGC, TAC, and TCC (Cys to Ser, Tyr and Ser, respectively). One case had a GTG to ATG (Val to Met) mutation at codon 804. All 6 cases were identified as heritable MTC. It is possible that these germline mutations are de novo. Four of the 6 mutations are also present in first-degree relatives and we have not yet examined family members of the remaining 2 families. None of the 6 cases had received total thyroidectomy. In two of these subjects, recurrent MTC was demonstrated. One case, a 74-year-old woman with a TGC to AGC mutation at codon 634, had a hemithyroidectomy at the age of 55, recurrence in the residual thyroid six months later and subsequently, a total thyroidectomy. After genetic diagnosis, adrenal examination revealed pheochromocytoma of the left adrenal gland and left adrenalectomy was performed. The other, a 28-year-old woman with a TGC to TCC mutation at codon 634, had a subtotal thyroidectomy at the age of 16. High levels of serum calcitonin were found after genetic diagnosis and she is scheduled to undergo total thyroidectomy. No pheo-

Table I. Summary of *RET* Gene Mutations in Patients Treated as Sporadic MTC

Codons Wild-type	618 TGC	634 TGC	804 GTG	766 CCG	778 GTC	918 ATG	919 GCA
Base changes	GGC (1)[a)] AGC (1)	AGC (1) TAC (1) TCC (1)	ATG (1)	TCG (1)	GTT (1)	ACG (2)	GTA (1)
Total	2	3	1	1	1	2[b)]	1[b)]
Type of mutation	G[c)]	G	G	S[d)]	S	S	S

a) Numbers in parenthesis are numbers of cases.
b) One case, case B, had both 918 and 919 mutations.
c) Germline mutation.
d) Somatic mutation.

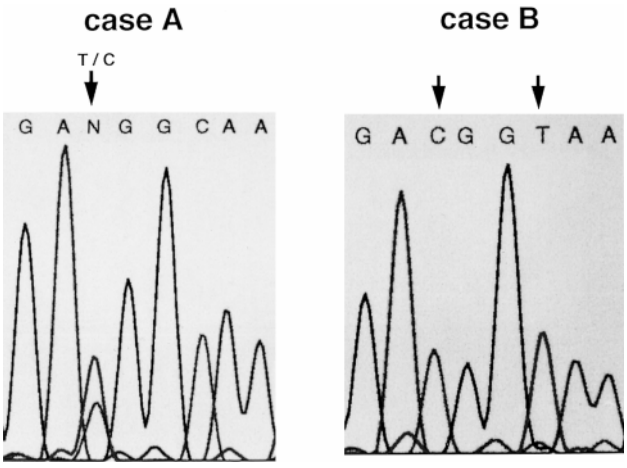

Fig. 1. Sequencing results of DNA from sporadic MTC tissue of cases A and B at exon 16 in the *RET* gene. Sequencing of case A showed ATG to ACG (Met to Thr) mutation at codon 918. In case B, two point mutations, ATG to ACG (Met to Thr) at codon 918 and GCA to GTA (Ala to Val) at codon 919, with loss of the wild-type allele, were detected.

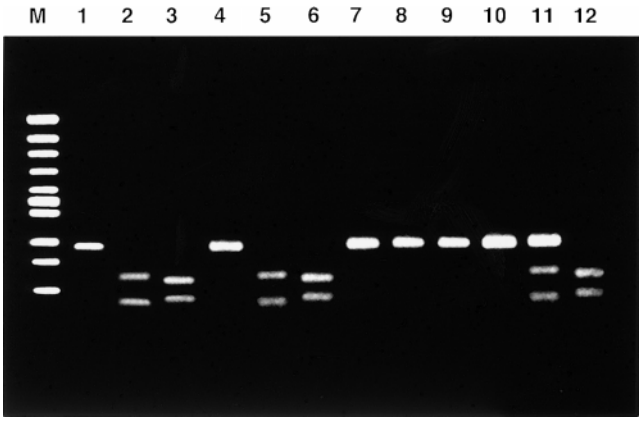

Fig. 2. Results of agarose gel electrophoresis for mutation specific-restriction enzyme analysis of exon 16 in the *RET* gene. PCR products of exon 16 (193 bp) were cut with *Fok*I or *Mun*I and then electrophoresed in 4% agarose and stained with ethidium bromide. The wild-type *RET* gene was cut into 118 and 75 bp fragments with *Fok*I and 111 and 82 bp fragments with *Mun*I, respectively, but specific mutations at codons 918 and 919 eliminate these restriction enzyme sites. Lanes 1–3, peripheral blood from healthy human; lanes 4–6, peripheral blood from case B; lanes 7–9, tissues from MTC of case B; lanes 10–12, tissues from MTC of case A; lanes 1, 4, 7 and 10, undigested PCR products; lanes 2, 5, 8 and 11, products digested with *Fok*I; lanes 3, 6, 9 and 12, products digested with *Mun*I, lane M, molecular size markers. In lanes 8 and 9, codon 918 and 919 mutations eliminate the *Fok*I and *Mun*I restriction sites with loss of the wild-type allele, respectively. In lane 11, codon 918 mutation eliminates the *Fok*I site without loss of heterozygosity.

chromocytoma, parathyroid disease or recurrent MTC was apparent in the other four subjects, one of whom died in a traffic accident without recurrent MTC five years after surgery. The patient with germline mutation at codon 804, whose preoperative clinical diagnosis was Graves' disease, underwent subtotal thyroidectomy, and a microscopic MTC was found after postoperative histological examination. No simultaneous somatic mutation besides germline mutation was found in six cases newly identified as heritable MTC.

Of the remaining 34 sporadic MTC cases, four had somatic (tumor-specific) *RET* mutations (12%). One case, case A, showed ATG to ACG (Met to Thr) mutation at codon 918 (Fig. 1, case A). The PCR product of exon 16 in this case was subjected to restriction analysis and the *Fok*I restriction site was found to have been eliminated by the codon 918 mutation (Fig. 2, lanes 10–12). Constitutional DNA (nonneoplastic thyroid tissue) did not have this mutation and the wild-type allele of the *RET* gene in the tumor DNA was retained, based on the results of

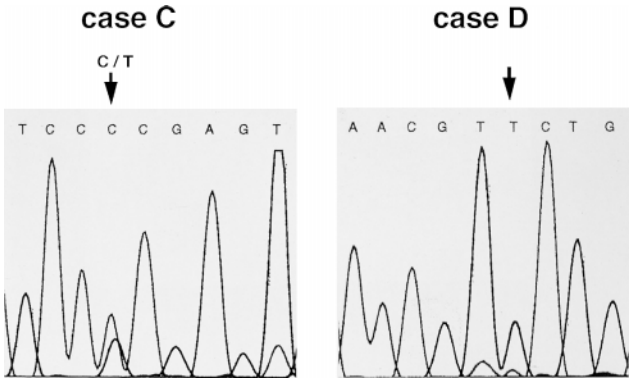

Fig. 3. Sequencing results of DNA from sporadic MTC tissue of cases C and D at exon 13 in the *RET* gene. Sequencing of case C showed CCG to TCG (Pro to Ser) mutation at codon 766. In case D, a silent mutation, GTC to GTT (Val) at codon 778, and loss of the wild-type allele were detected.

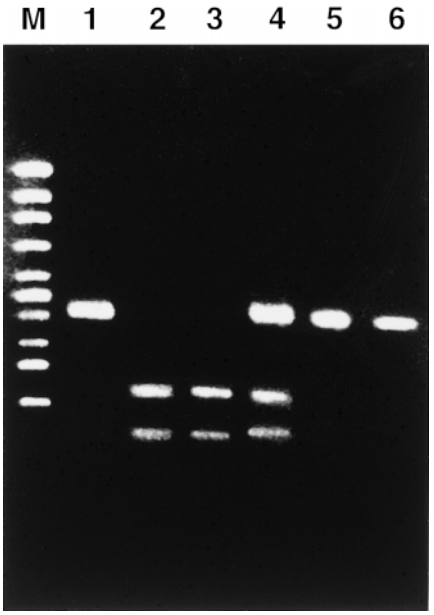

Fig. 4. Results of agarose gel electrophoresis for mutation specific-restriction enzyme analysis of exon 13 in the *RET* gene. PCR products of exon 13 (297 bp) were cut with *Eco*NI or *Xho*I and then electrophoresed in 4% agarose and stained with ethidium bromide. Wild-type *RET* gene was cut into 170 and 127 bp fragments with *Eco*NI and has no restriction site for *Xho*I. Specific GTC to GTT mutation at codon 778 eliminated the *Eco*NI site. A CCG to TCG mutation at codon 766 made a new restriction site for *Xho*I giving fragments of 169 and 128 bp. Lane 1, tissue from MTC of case D; lane 2, peripheral blood from case D; lane 4, tissue from MTC of case C; lane 5, peripheral blood from case C; lanes 3 and 6, peripheral blood from healthy human; lanes 1–3, products digested with *Eco*NI; lanes 4–6, products digested with *Xho*I; lane M, molecular size markers.

sequencing and restriction enzyme analysis. The maximum diameter of this tumor was 8.0 cm and subtotal hemithyroidectomy without lymph node dissection was performed. This patient, who had been disease-free, died of cerebral infarction 14 years after thyroidectomy.

The tumor from case B showed two point mutations, ATG to ACG (Met to Thr) at codon 918 and GCA to GTA (Ala to Val) at codon 919 (Fig. 1, case B). Restriction enzyme analysis revealed that the codon 918 and 919 mutations had eliminated the *Fok*I and *Mun*I restriction sites, respectively (Fig. 2, lanes 7–9), and constitutional DNA (peripheral blood leukocyte and nonneoplastic thyroid tissue) had neither mutation (Fig. 2, lanes 4–6). As shown in Figs. 1 and 2, the wild-type band was lost in this tumor. Furthermore, sequencing of exon 13 in peripheral blood leukocytes and nonneoplastic thyroid tissue from case B showed CTT/CTG polymorphism at codon 769, but only the CTT sequence was found in tumor tissue (data not shown). These results imply that this tumor has two point mutations in one allele at the *RET* gene locus and the opposite wild-type allele was lost. The maximum diameter of this tumor was 3.0 cm and partial thyroidectomy without lymph node dissection was performed. This patient remains disease-free 24 years after thyroidectomy.

Case C showed a CCG to TCG (Pro to Ser) mutation at codon 766 (Fig. 3, case C). Restriction enzyme *Xho*I analysis revealed a new restriction site generated by the codon 766 mutation and the wild-type allele of the *RET* gene in the tumor DNA was retained (Fig. 4, lane 4). The maximum diameter of this tumor was 1.7 cm and partial thyroidectomy with modified neck dissection was performed. This patient remains disease-free 14 years after thyroidectomy.

Case D showed a silent mutation, GTC to GTT (Val) at codon 778 (Fig. 3, case D) and the *Eco*NI restriction site was found to have been eliminated (Fig. 4, lane 1). The wild-type allele of the *RET* gene in this tumor DNA was lost, based on the results of sequencing of codon 778 and codon 769 polymorphic sites and restriction enzyme analysis. The maximum diameter of this tumor was 2.9 cm and subtotal thyroidectomy with central lymph node dissection was performed. This patient remains disease-free 3 years after thyroidectomy.

Among 30 cases without somatic *RET* mutation, median follow-up time was 11 years and tumor recurrence was found in four, one of whom died of the disease. Three patients died of other diseases while the other 26 are alive and well.

DISCUSSION

Among 40 cases surgically treated as sporadic MTC with no family history of MEN 2A, 2B or FMTC, germline *RET* mutations at codons 618, 634 and 804 were found in 6 and newly identified as heritable MTC. Though reoperative total thyroidectomy may be necessary for these cases, 4 of 6 have not recurred since initial operation. The incidence of *RET* germline mutations in apparent sporadic MTC ranges from 1.5 to 24.0%.[10, 11, 15, 23–25] Cases of hereditary MTC may be treated as sporadic MTC, and therefore genetic testing for *RET* mutations in peripheral blood leukocytes and tumor DNA is indispensable preoperatively or postoperatively. We should not operate on MTC until we have a genetic diagnosis. In our institute, we now employ a *RET* gene screening system for preoperative patients with MTC using peripheral blood leukocytes or aspiration cytology samples.

Several European and American studies report a high incidence of somatic methionine to threonine *RET* gene mutation at codon 918 in sporadic MTC.[5, 10–12] Somatic mutations at codon 918 was found in 6 of 18 (33%) sporadic MTCs from Holland,[5] 5 of 12 (38%) sporadic MTCs from Switzerland and Spain,[10] and 15 of 65 (23%) sporadic MTCs from the Human Cancer Genetics Research Group.[11] In contrast, in the present study, only 2 of 34 (6%) sporadic MTCs had somatic mutations at codon 918. The incidence of *RET* somatic mutations at codon 918 in our study was very low and this result suggests that different, as yet unknown, factors may be associated with sporadic MTCs.

Mutation at codon 918 has also been reported to correlate with tumor recurrence and poor prognosis. Zedenius *et al*.[15] reported somatic mutations at codon 918 in 29 of 46 (63%) sporadic MTCs and the presence of the somatic 918 mutation in a tumor was significantly correlated with a poor outcome. Jhiang *et al*.[16] also reported somatic 918 mutations in 6 of 6 apparent sporadic MTC tumors and almost all of the cases with somatic 918 mutation had shown recurrent disease. In this study, only 4 cases showed recurrence of MTC, of which none had mutations at codon 918, and 2 cases with somatic 918 mutation were free of disease for 14 years and 24 years after thyroidectomy, respectively. Our findings contradict the reported association of somatic codon 918 mutation with tumor recurrence or prognosis. Because of the small number of cases, association of *RET* mutation with somatic codon 918 mutation needs to be confirmed in a larger series of Japanese cases. Some factor(s) other than somatic codon 918 mutations probably plays an important role in Japanese sporadic MTC progression.

Although single germline or somatic *RET* gene mutation apparently contributes to C-cell hyperplasia and MTC development, it is not clear whether the allele with the *RET* mutation or the wild-type allele requires additional *RET* alteration. In the study of Landsvater *et al.*,[26] no somatic mutations were found on the wild-type allele by examining the entire coding region of the *RET* gene in some MEN 2A and 2B tumors. However, Marsh *et al*.[14] reported both simultaneous germline mutations in exon 10 or 11 and somatic mutations at codon 918 of *RET* in 3 of 15 MTCs and in a sample with C-cell hyperplasia. Miyauchi *et al*.[27] also found both simultaneous germline codon 768 mutation and somatic mutation at codon 919 in an FMTC. However, they did not clarify on which allele the additional somatic mutation occurred. In the present study, a sporadic MTC showed two mutations at codons 918 and 919 with loss of the wild-type *RET* allele, which seems consistent with loss of function as proposed by Knudson's two-hits theory for tumor suppressor genes.[28] This is the first report of both somatic mutations and loss of the wild-type allele of the *RET* gene in sporadic MTCs. *RET* gene encodes a receptor tyrosine kinase and binding of this receptor with a ligand causes signal transduction for cell growth and differentiation. We have not yet clarified the biological significance of allelic deletion of the *RET* gene (e.g., change of phosphorylation level of the RET protein or tyrosine kinase activation). Wild-type and mutant RET transcripts are supposed to be expressed at similar levels in a single tumor.[30] The codon 918 mutation which changes methionine to threonine would result in RET autophosphorylation even if an additional codon 919 mutation is present.[29] In our case with two somatic 918 and 919 mutations and loss of heterozygosity (LOH), all RET molecules would be mutants and the phosphorylation level in this tumor may be very high compared to that in tumors with codon 918 mutation without LOH. Furthermore, we found a case with a silent mutation at codon 778 with loss of the wild-type RET allele. This tumor may have another mutation with amino acid change or frame-shift change in another exon besides those we examined in this study. Somatic or germline mutation of the *RET* gene causes continuous inappropriate activation of RET protein and the subsequent loss of the *RET* locus itself may lead to MTC development. Thus, a second *RET* genetic alteration may exist in MTC.

Tumor suppressor genes are well-known to be involved in inherited cancers, including Li-Fraumeni syndrome, retinoblastoma, and adenomatous polyposis coli (*p53*, *Rb* and *APC* genes are responsible, respectively). Tumor suppressor genes are inactivated by the loss of one allele and the mutation of the remaining allele.[31–34] On the other hand, specific germline or somatic mutations have been found in only one allele at the *RET* locus, and so this gene is considered to be activated in a dominant manner. No genetic change(s) of the *RET* gene besides single mutation at previously well-known hot spots has been found in sporadic MTC. In MTC as in other tumors, accu-

mulation of genetic alterations, functional loss of tumor suppressor genes, and activation of oncogenes contribute to tumor development. Mulligan *et al*.[35] found that frequent LOH on chromosomes 1p, 3p, 3q and 22q occurred somatically in MTCs and pheochromocytomas of MEN type 2 cases. LOH on chromosomes 1, 3, 11, 17 and 22 in MTCs or pheochromocytomas has also been reported.[36–41] Alterations in other gene(s) may also contribute to the progression of MTC and phenotypic variation among families with MEN 2A with the same *RET* germline mutation. It is necessary to investigate alterations in other coding and intronic regions throughout the entire *RET* gene, LOH at the site of candidate tumor suppressor genes and activation of other oncogenes in sporadic MTCs.

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REFERENCES

1) Mulligan, L. M. and Ponder, B. A. J. Genetic basis of endocrine disease: multiple endocrine neoplasia type 2. *J. Clin. Endocrinol. Metab.*, **80**, 1989–1995 (1995).
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) 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).
4) 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).
5) 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).
6) 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).
7) 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).
8) 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).
9) 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).
10) 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).
11) 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 Chrom. Cancer*, **12**, 209–212 (1995).
12) 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).
13) Blaugrund, J. E., Johns, M. J., Eby, Y. J., Ball, D. W., Baylin, S. B., Hruban, R. H. and Sidransky, D. *RET* protooncogene mutations in inherited and sporadic medullary thyroid cancer. *Hum. Mol. Genet.*, **3**, 1895–1897 (1994).
14) 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).
15) 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).
16) 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).
17) 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).
18) 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).
19) 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).
20) 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).
21) Uchino, S., Tsuda, H., Noguchi, M., Yokota, J., Terada, M., Saito, T., Kobayashi, M., Sugimura, T. and Hirohashi, S. Frequent loss of heterozygosity at the *DCC* locus in gastric cancer. *Cancer Res.*, **52**, 3099–3102 (1992).
22) 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).
23) Decker, R. A., Peacock, M. L., Borst, M. J., Sweet, J. D. and Thompson, N. W. Progress in genetic screening of multiple endocrine neoplasia type 2A: is calcitonin testing obsolete? *Surgery*, **118**, 257–263 (1995).
24) Wohlik, N., Cote, G. J., Bugalho, M. M. J., Ordonez, N., Evans, D. B., Goepfert, H., Khorana, S., Schultz, P., Richards, C. S. and Gagel, R. F. Relevance of *RET* proto-oncogene mutations in sporadic medullary thyroid carcinoma. *J. Clin. Endocrinol. Metab.*, **81**, 3740–3745 (1996).
25) 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*, **26**, 3103–3106 (1997).
26) Landsvater, R. M., de Wit, M. J., Zewald, R. A., Hofstra, R. M. W., Buys, C. H. C. M., van Amstel, H. K. P., Höppener, J. W. M. and Lips, C. J. M. Somatic mutations of the *RET* proto-oncogene are not required for tumor development in multiple endocrine neoplasia type 2 (MEN 2) gene carriers. *Cancer Res.*, **56**, 4853–4855 (1996).
27) Miyauchi, A., Egawa, S., Futami, H., Kuma, K., Obara, T. and Yamaguchi, K. A novel somatic mutation in the *RET* proto-oncogene in familial medullary thyroid carcinoma with a germline codon 768 mutation. *Jpn. J. Cancer Res.*, **88**, 527–531 (1997).
28) Knudson, A. G. Antioncogenes and human cancer. *Proc. Natl. Acad. Sci. USA*, **90**, 10914–10921 (1993).
29) 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).
30) Quadro, L., Panariello, L., Salvatore, D., Carlomagno, F., Del Prete, M., Nunziata, V., Colantuoni, V., Di Giovanni, G., Brandi, M. L., Mannelli, M., Gheri, R., Verga, U., Libroia, A., Berger, N., Fusco, A., Grieco, M. and Santoro, M. Frequent *RET* protooncogene mutations in multiple endocrine neoplasia type 2A. *J. Clin. Endocrinol. Metab.*, **79**, 590–594 (1994).
31) Friend, S. H., Horowitz, J. M., Gerber, M. R., Wang, X. F., Bogenmann, E., Li, F. P. and Weinberg, R. A. Deletions of a DNA sequence in retinoblastomas and mesenchymal tumors: organization of the sequence and its encoded protein. *Proc. Natl. Acad. Sci. USA*, **84**, 9059–9063 (1987).
32) Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A. and Friend, S. H. Germ line *p53* mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*, **250**, 1233–1238 (1990).
33) Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., Paslier, D. L., Abderrahim, H., Cohen, D., Leppert, M. and White, R. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell*, **66**, 589–600 (1991).
34) Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I. and Nakamura, Y. Identification of *FAP* locus genes from chromosome 5q21. *Science*, **253**, 661–665 (1991).
35) Mulligan, L. M., Gardner, E., Smith, B. A., Mathew, C. G. P. and Ponder, A. J. P. Genetic events in tumor initiation and progression in multiple endocrine neoplasia type 2. *Genes Chrom. Cancer*, **6**, 166–177 (1993).
36) Mathew, C. G. P., Smith, B. A., Thorpe, K., Wong, Z., Royle, N. J., Jeffereys, A. J. and Ponder, B. A. J. Deletion of genes on chromosome 1 in endocrine neoplasia. *Nature*, **328**, 524–526 (1987).
37) Takai, S., Tateishi, H., Nishisho, I., Miki, T., Motomura, K., Miyauchi, A., Kato, M., Ikeuchi, T., Yamamoto, K., Okazaki, M., Yamamoto, M., Honjo, T., Kumahara, Y. and Mori, T. Loss of genes on chromosome 22 in medullary thyroid carcinoma and pheochromocytoma. *Jpn. J. Cancer Res.*, **78**, 894–898 (1987).
38) Yang, K., Nguyen, C. V., Castillo, S. G. and Samaan, N.

A. Deletion mapping on the distal third region of chromosome 1p in multiple endocrine neoplasia type 2A. *Anticancer Res.*, **10**, 527–533 (1990).
39) Yokogoshi, Y., Yoshimoto, K. and Saito, S. Loss of heterozygosity on chromosomes 1 and 11 in sporadic pheochromocytomas. *Jpn. J. Cancer Res.*, **81**, 632–638 (1990).
40) Khosla, S., Patel, V. M., Hay, I. D., Schaid, D. J., Grant, C. S., van Heerden, J. A. and Thibodeau, S. N. Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. *J. Clin. Invest.*, **87**, 1691–1699 (1991).
41) Moley, J. F., Brother, M. B., Fong, C.-T., White, P. S., Baylin, S. B., Nelkin, B., Wells, S. A., Jr. and Brodeur, G. M. Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. *Cancer Res.*, **52**, 770–774 (1992).