Jpn. J. Cancer Res. **92**, 645–648, June 2001

Large-scale Analysis of Mutations in *RET* Exon 16 in Sporadic Medullary Thyroid Carcinomas in Japan

Toru Takano,[1,3] Akira Miyauchi,[2] Hiroshi Yoshida,[2] Yukiko Hasegawa,[1] Kanji Kuma[2] and Nobuyuki Amino[1]

[1]Department of Laboratory Medicine, Osaka University Medical School, D2 2-2 Yamadaoka, Suita, Osaka 565-0871 and [2]Kuma Hospital, 8-2-35 Simoyamate-dori, Chuo-ku, Kobe, Hyogo 650-0011

Germline mutations in the *RET* proto-oncogene are the cause of multiple endocrine neoplasia type 2 (MEN 2A and 2B) and familial medullary thyroid carcinoma (FMTC). Some cases of sporadic medullary thyroid carcinoma (MTC) have also been reported to have mutations in the *RET* gene. However, two previous reports have given discrepant results on the frequency of the mutations in *RET* in sporadic MTCs in Japan. To clarify this problem, we analyzed mutations in *RET* exon 16 in 72 sporadic MTCs by means of the two methods used in the previous studies, direct sequencing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Mutations in exon 16 were detected in only 2 of 72 cases of sporadic MTC. These results suggest that when a MTC has a mutation in *RET* exon 16, it is more likely to be a hereditary MTC than a sporadic one in Japan.

Key words: Somatic mutation — Thyroid medullary carcinoma — *RET* — Japanese — PCR-RFLP

Sporadic medullary thyroid carcinoma (MTC) is characterized by a negative family history in patients who are usually in their fifties or sixties when MTC is diagnosed.[1)] Mutation of the *RET* proto-oncogene is involved in the pathogenesis not only of hereditary MTCs, such as multiple endocrine neoplasia (MEN) type 2 and familial medullary thyroid carcinoma (FMTC), but also of sporadic MTC.[2, 3)] Recent studies have revealed that germline mutations in codons 609, 611, 618, 620, 634, and 768 have been discovered predominantly in MEN 2A and FMTC, whereas germline mutations in codon 918 are common in MEN 2B.[4–6)] Sporadic MTCs have also been reported to have somatic mutations in codon 918.[7)] For example, tumor-specific ATG to ACG mutations at codon 918 are found in about one-third of European and American sporadic MTCs.[8–12)] The fact that the MEN 2B phenotype has a less favorable clinical outcome than the MEN 2A and FMTC phenotypes has raised the possibility that a codon 918 mutation may be related to much more severe phenotypes of sporadic MTC, such as those with early onset, rapid progress or poor prognosis.[13, 14)] However, whether or not there is a difference in the clinical outcome of sporadic MTC with or without a codon 918 mutation is still controversial. In addition, the frequency of codon 918 somatic mutation in sporadic MTC has varied greatly in the literature for reasons, which are still not clear, although some authors have speculated that this discrepancy is due to ethnic or environmental factors or simply due to differences in detection methods or techniques. In the Japanese population, there have been two studies that have reported discrepant results concerning the frequency of codon 918 mutation in the *RET* gene.[15, 16)] One reported a frequency of 5.9% and the other that of 30%. Further, these two studies used different methods; one used direct sequencing and the other used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). In this study, in order to analyze the frequency of mutations in exon 16 in the *RET* gene in 72 sporadic medullary carcinomas, we performed both direct sequencing and PCR-RFLP, and the reason for the discrepancy in the previous reports is discussed.

[3] To whom correspondence should be addressed.
E-mail: ttakano@labo.med.osaka-u.ac.jp

PATIENTS AND METHODS

Patients Seventy-two cases (between 20 and 76 years of age, 11 males and 61 females) surgically treated as sporadic MTC were analyzed for *RET* gene mutations. These cases had undergone surgery between 1967 and 1999 at Kuma Hospital. 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 Genomic DNA was extracted from paraffin blocks using a TaKaRa DEXPAT kit (TaKaRa, Otsu) according to the manufacturer's protocols. Corresponding nonneoplastic thyroid tissues were used as controls to determine the presence or absence of germline mutations.

PCR PCR amplifications were carried out in a final vol-

ume of 50 μl using the primers 16F: 5′-AGGGATAGGGCCTGGGCTT-3′ and 16R: 5′-TAACCTCCACCCCAAGAG-3′ for the first 40 cycles. For the second 40 cycles, 2 μl of the PCR product was used as a template with the nested primers 16NF: 5′-AGAGTTAGAGTAACTTCAATGTC-3′ and 16NR: 5′-TAACCTCCACCCCAAGAGA-3′. The fragment sizes of the PCR products were 192 bp and 151 bp, and the annealing temperatures were 58°C and 55°C, respectively.

Direct sequencing After 2% agarose gel electrophoresis and ethidium bromide staining, the PCR products were cut out from the gel and purified with SUPREC-01 (TaKaRa). For nonisotopic sequencing, PCR products were subjected to 25 PCR cycles with either primer 16NF or 16NR by fluorescence-based dideoxy terminator cycle sequencing (Applied Biosystems, Foster City, CA). Data collection and analysis were performed on an automated DNA sequencer (Model 373S, Applied Biosystems). When a mutation was present, DNA from corresponding nonneoplastic thyroid tissues were similarly examined.

RFLP The PCR products (6 μl) were digested for 4 h with 20 U of the restricted enzyme *Fok*I (TaKaRa). Restriction fragments were analyzed by 8% polyacrylamide gel electrophoresis and ethidium bromide staining. If the 151 bp fragment was determined to be not cut, it was considered mutant.

RFLP after gel-purification The PCR products were run on 2% agarose, and then they were cut out from the gel and purified with SUPREC-01. The purified PCR products were digested for 4 h with 20 U of *Fok*I then analyzed by 8% polyacrylamide gel electrophoresis (PAGE).

RESULTS

A somatic mutation in *RET* exon 16 was found in two cases by direct sequencing (Fig. 1). Both were an ATG to ACG (Met to Thr) mutation at codon 918. This mutation was not found in the corresponding normal thyroid tissue. By means of RFLP, 26 of 72 samples were found to have

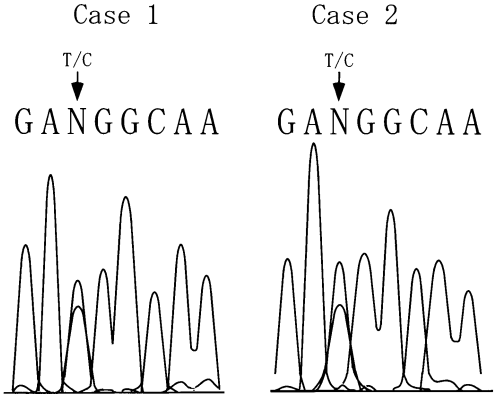

Fig. 1. Sequencing results of DNA from sporadic MTC tissues with a mutation in exon 16 in the *RET* gene. Case 1 is a 62-year-old man and case 2 is a 42-year-old woman. Sequencing of both cases showed an ATG to ACG (Met to Thr) mutation at codon 918.

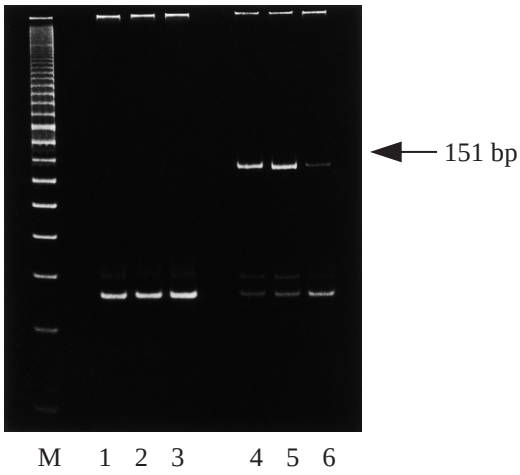

Fig. 2. Representative data of PCR-RFLP analysis. DNAs extracted from a normal thyroid (lanes 1, 4) and two sporadic MTCs (lanes 2, 3, 5, 6) were amplified by PCR, then before (4, 5, 6) and after (1, 2, 3) gel purification, PCR products were digested with *Fok*I and analyzed by 8% PAGE. M: 20 bp ladder marker (TaKaRa).

Table I. Summary of the PCR-RFLP of Codon 918

	Gel purification	
	+	–
Cleaved	70 (97.2%)	46 (63.9%)
Uncleaved	2 (2.8%)	26 (36.1%)

Table II. Summary of the Reports on Mutations in Codon 918

	Case	Method	Mutation in codon 918 (%)
Uchino *et al.* (1998)	34	Direct sequencing and PCR-RFLP	5.9
Shan *et al*. (1998)	20	PCR-RFLP	30
Takano *et al.* (this work)	72	Direct sequencing	2.8
		PCR-RFLP[a)]	36.8

a) The result of PCR-RFLP without gel purification.

an uncleaved band. However, after gel purification, these bands disappeared except in two cases (Table I, Fig. 2). These cases were identical with those in which mutations were detected by direct sequencing.

DISCUSSION

In this report, we analyzed the somatic mutation in *RET* exon 16 in 72 sporadic MTCs. We detected mutation in only two of 72 cases. The comparison of the present results with those of the previous studies is summarized in Table II. Interestingly, our study using direct sequencing and PCR-RFLP gave similar results to those of Uchino *et al.*[16] and Shan *et al.*,[15] respectively. Further, after gel purification, most of the uncleaved band was cleaved by *Fok*I, indicating that the enzymatic activity of *Fok*I was inhibited by something in the PCR reaction mixture. After gel purification, both methods, direct sequencing and PCR-RFLP, gave identical results. Considering the fact that Shan *et al.* used clinical materials obtained in a region similar to ours in Japan (Kansai area), it is possible that the PCR-RFLP data obtained by Shan *et al.* may have been modified by the inhibition of the enzymatic activity of *Fok*I, at least to some extent.

The results of the present study indicate that mutation in *RET* exon 16, which is frequently observed in hereditary MTCs, is rare in sporadic MTCs, and this suggests that when we find a *RET* exon 16 mutation in an MTC, the MTC is more likely to be hereditary than sporadic. In other words, when a *RET* exon 16 mutation is found in an MTC, we should carefully check the family history and check for other diseases such as pheochromocytoma and hyperparathyroidism to exclude the possibility of hereditary MTC. In addition, it is recommended that a sequence analysis of the *RET* gene using DNA from the peripheral blood or the corresponding normal thyroid tissues be performed in order to confirm the germline mutation.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (to T. T.; No.12771474) from the Ministry of Education, Science, Sports and Culture of Japan.

(Received February 19, 2001/Accepted March 26, 2001)

REFERENCES

1) Marsh, D. J., Learoyd, D. L. and Robinson, B. G. Medullary thyroid carcinoma: recent advances and management update. *Thyroid*, **5**, 407–424 (1995).
2) 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).
3) Goodfellow, P. J. and Wells, S. A., Jr. RET gene and its implications for cancer. *J. Natl. Cancer Inst.*, **87**, 1515–1523 (1995).
4) Mulligan, L. M., Kwok, J. B., 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).
5) Mulligan, L. M., Eng, C., Healey, C. S., Clayton, D., Kwok, J. B., Gardner, E., Ponder, M. A., Frilling, A., Jackson, C. E., Lehnert, H., Neumann, H. P. H., Thibodeau, S. N. and Ponder, B. A. J. Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. *Nat. Genet.*, **6**, 70–74 (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., 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) Hofstra, R. M., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W., 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*, **367**, 375–376 (1994).
9) 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).
10) Fink, M., Weinhusel, 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).
11) Blaugrund, J. E., Johns, M. M., Jr., 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).
12) 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. and Robinson, B. G. Germline and somatic mutations in an oncogene: RET mutations in inherited medullary thyroid carcinoma. *Cancer Res.*, **56**, 1241–1243 (1996).

13) 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).
14) 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).
15) 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).
16) 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).