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# Somatic Mutations in the *RET* Protooncogene in Japanese and Chinese Sporadic Medullary Thyroid Carcinomas

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**Despite advances in the understanding of the genotype-phenotype correlation in multiple endocrine neoplasia type 2A and 2B (multiple endocrine neoplasia (MEN) 2A, MEN 2B), and familial medullary thyroid carcinoma (FMTC), the frequency and prognostic relevance of *RET* protooncogene mutations in sporadic medullary thyroid carcinomas (MTCs) remain controversial. To study somatic mutations in the *RET* protooncogene in Japanese and Chinese sporadic MTCs and to analyze comparatively the correlation between *RET* mutation and tumor differentiation, we investigated somatic mutations in the *RET* protooncogene in 20 Japanese and 20 Chinese sporadic MTCs by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Of the 40 sporadic MTCs, 13 had a point mutation in codon 918 of exon 16, a frequency of 32.5%. There was no significant difference in the frequency between Japanese and Chinese sporadic MTCs, as 30% of the Japanese and 35% of the Chinese sporadic MTCs contained this mutation. We did not observe any correlation between the presence or absence of codon 918 mutation and tumor differentiation in either Japanese or Chinese sporadic MTCs. Our findings indicate that the frequency of *RET* somatic mutations is similar in Japanese and Chinese sporadic MTCs, and the presence or absence of *RET* mutation does not correlate with the differentiation of sporadic MTCs.**

Key words: *RET* protooncogene — Mutation — Sporadic medullary thyroid carcinoma — Japanese — Chinese

Mutation of the *RET* protooncogene is involved in the pathogenesis not only of hereditary medullary thyroid carcinomas (MTCs), such as multiple endocrine neoplasia type 2 (multiple endocrine neoplasia (MEN) 2) and familial medullary thyroid carcinoma (FMTC), but also of sporadic MTC.[1–3] Recent studies have revealed that *RET* mutation genotypes are correlated with the phenotypes of MTC. 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.[4,5] Like MEN 2B, sporadic MTCs also mainly harbor somatic mutations in codon 918.[6,7] Despite recent advances in the understanding of the genotype-phenotype correlation, the frequency and prognostic relevance of the somatic mutations in sporadic MTCs remain controversial. First, the less favorable clinical outcome of the MEN 2B phenotype 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. Some studies have shown a significant difference in the clinical outcome of sporadic MTC with or without a codon 918 mutation.[8] However, in other studies, no significant difference has been found.[9] Second, the frequency of codon 918 somatic mutation in sporadic MTC has varied greatly in the literature from 23% to 85%,[10,11] for reasons which are still not clear. Some authors have suggested that the difference in frequency is due to ethnic or environmental factors or simply due to differences in detection methods or techniques.[12] In our previous studies,[13–15] we have classified sporadic MTCs into well and poorly differentiated types by investigating the relationship between the clinical outcome of sporadic MTC and the pathological features. In this report, we present our studies on codon 918 somatic mutation in Japanese and Chinese sporadic MTCs and comparatively analyze the correlation between mutation in codon 918 and differentiation of sporadic MTCs.

## MATERIALS AND METHODS

**Tumor specimens** We analyzed 40 sporadic MTCs in this study, comprising 20 Japanese and 20 Chinese sporadic MTCs. In addition, 2 MTCs associated with MEN 2A and two associated with FMTC were also analyzed as

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negative controls of exon 16 mutation. All were formalin-fixed and paraffin-embedded. These specimens were obtained from the archival files of the Pathology Department of Wakayama Medical College, Japan, and Shandong Medical University, China. The 40 MTCs were considered to be sporadic because of the absence of any evidence of association with hereditary MTC, either clinically or pathologically. All tumors were divided into two groups, well and poorly differentiated MTCs (Table I), according to the criteria of our classification. The criteria are briefly summarized in Table II. A poorly differentiated MTC would be classified as such if it possessed the following features: small cell variant with increased nucleocytoplasmic (N/C) ratio, increased mitotic activity (more than 0.5 per ×400 field), evident tumor necrosis, lymphatic and vascular invasion, less amyloid deposits and stroma, and weak or focal calcitonin (CT) immunoreactivity or showing adrenocorticotrophic hormone (ACTH) reaction by immunohistochemistry.

**Detection of codon 918 point mutation in *RET* exon 16** Somatic mutations in codon 918 were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Genomic DNA was extracted from paraffin blocks using a QIAamp tissue kit (QIAGEN GmbH, Hilden, Germany) or a Takara DEXPAT kit (Takara, Otsu) according to the manufacturers' protocols. Lymph nodes or thyroid tissues from the same patients were used as controls to determine the presence or absence of germline mutations. PCR amplifications were carried out in a final volume of 50 $\mu$l using the primers 5′-AGGGATAGGGCCTGGGCTT-3′ and 5′-TAACCTCCACCCCAAGAG-3′ for the first 40 cycles. For the second 40 cycles, 2 $\mu$l of the PCR product was used as a template with the nested primers 5′-AGAGTTAGAGTAACTTCAATGTC-3′ and 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. After the PCR products were assessed by agarose gel electrophoresis (2% Nusieve GTG and 1% Seakem, FMC, Rockland, ME), 6 $\mu$l of the PCR product was digested for 4 h with 20 units of the restriction 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.

**Statistical analysis** The statistical significance of the difference between any two groups was determined by using Fisher's exact test.

Table I. RET Codon 918 Mutation in Sporadic MTC

| Samples | Cases | Cases with mutation | % | |
|---|---|---|---|---|
| Sporadic MTC | | | | |
| Japanese | 20 | 6 | 30 | * |
| Chinese | 20 | 7 | 35 | * |
| Total | 40 | 13 | 32.5 | |
| MEN 2A | 2 | 0 | 0 | |
| FMTC | 2 | 0 | 0 | |

* $P$>0.05.

Table II. Pathological and Immunoreactive Features of Poorly Differentiated MTCs

Pathological
1. Nuclear pleomorphism, increased N/C ratio and small cell variant
2. Increased mitotic figures (more than 0.5 per ×400 field)
3. Presence of tumor necrosis
4. Lymphatic and vascular invasion
5. Medullary growth with little or no amyloid stroma
6. Loss of cellular differentiation
   a. few secretory granules
   b. increased free-ribosomes and polyribosomes
7. Dedifferentiation
   ectopic hormone production, ectopic ACTH syndrome

Immunoreactivity
1. Weak or focal CT reaction
2. ACTH positive reaction

## RESULTS

**Frequency of somatic mutation in Japanese and Chinese sporadic MTCs** The results of exon 16 mutations are summarized in Table I (Fig. 1). Of the 20 Japanese sporadic MTCs, 6 harbored point mutations in exon 16, a frequency of 30%. Similarly, of the 20 Chinese sporadic MTCs, 7 had point mutations, a frequency of 35%. Germline mutation was absent in the normal control tissue of

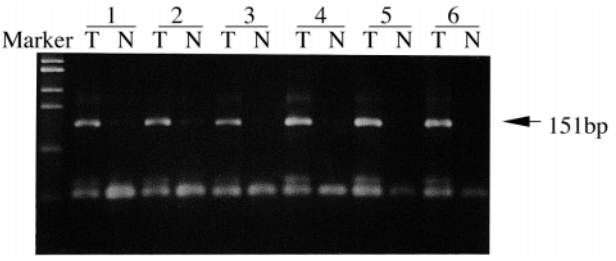


Fig. 1. Representative analysis of codon 918 mutation in 6 sporadic MTCs, showing an uncleaved band of 151 bp in the tumor tissues, but not in the corresponding normal tissues. Tumors No. 1 to 4 were obtained from Japanese patients and tumors No. 5 and 6 were from Chinese patients. T, tumor; N, normal tissue; Marker, PhiX174 RF DNA *Hae*III digest.

Table III. Correlation of Mutation and Tumor Differentiation

| Samples | Differentiation | Cases | Cases with mutation | % |
|---|---|---|---|---|
| Japanese | well | 15 | 4 | 26.7 |
| | poorly | 5 | 2 | 40.0 |
| Chinese | well | 11 | 4 | 36.4 |
| | poorly | 9 | 3 | 33.3 |
| Total | well | 26 | 8 | 30.8 |
| | poorly | 14 | 5 | 35.7 |

$P>0.05$ between any two groups.

each case. There was no statistically significant difference for the frequency of mutation between Japanese and Chinese sporadic MTCs ($P>0.05$). Of the 4 cases associated with MEN 2A and FMTC, none showed mutations in exon 16. The 4 cases showed germline mutations in exons 10 and 11 by nonradioactive PCR-single strand conformation polymorphism (SSCP) analysis (data not shown).

**Correlative analysis between mutation and tumor differentiation** By analyzing the relationship between clinical information and the morphological features, CT immunoreactivity and ultrastructural characteristics of sporadic MTC, we divided sporadic MTCs into well and poorly differentiated types as mentioned above. This classification correlated relatively well with the clinical progress and prognosis of the sporadic MTCs. In the present study, the 40 tumors were divided into 26 well and 14 poorly differentiated types, and we did not observe a correlation between mutation and tumor differentiation (Table III). Eight of the 26 well differentiated MTCs had somatic mutation (30.8%) and 5 of the 14 poorly differentiated MTCs had somatic mutation (35.7%). Similarly, no correlation was observed in either the Japanese MTC or the Chinese MTC.

## DISCUSSION

Germline mutations in exons 10, 11 and 16 of the *RET* protooncogene have been identified as the causative genetic alterations of MEN 2 and FMTC. Somatic mutations of the same gene, exclusively associated with codon 918, have also been characterized in sporadic MTC, but with large variations in frequency. Whether the differences are due to ethnic or environmental factors or simply due to different experimental methods remains unknown.[10, 11] In the present study, we have analyzed only codon 918 mutation, because other codon mutations are extremely rare. There was no significant difference in the mutation frequency between Japanese and Chinese sporadic MTCs, suggesting that the variations in frequency reported in the literature are most likely unrelated to ethnic or environmental factors. Recently, Eng *et al.* have found that somatic mutations in the *RET* protooncogene may be present in subpopulations of MTC or in metastatic lesions, but not in primary MTC.[16] These findings suggest that the distribution of *RET* mutations is heterogeneous, rather than homogeneous. Therefore, sampling method may also be a factor in the variations in frequency found in the literature.

Recent evidence has shown that MEN 2B mutation *in vitro* can result in a shift in peptide substrate specificity, resulting in downstream signaling to proteins not normally activated by *RET*.[17, 18] This may be the reason why MEN 2B tumors are more aggressive than MEN 2A and FMTC tumors. However, the relevance of this in sporadic MTC is still controversial. Zedenius *et al.* have reported that mutation in codon 918 in sporadic MTC is significantly correlated with a poor outcome, with regard to distant metastasis or tumor recurrence.[19] A similar association between poor prognosis and the presence of somatic mutation in sporadic MTC has been reported by Romei *et al.*[8] In contrast, Marsh *et al.* found no correlation between the presence or absence of codon 918 mutation and age at diagnosis, tumor size, presence or absence of metastasis, MTC-related morbidity, and base-line CT levels at diagnosis or most recent follow-up.[9] Similarly, no correlation was found by Komminoth *et al.* in the clinical course or survival of sporadic MTC patients with or without *RET* mutations.[20] In our study, we found no correlation of tumor differentiation with the somatic mutation either in Japanese or in Chinese sporadic MTCs. The reasons for these discrepancies are not clear. Our results indicate that somatic mutation is responsible for tumorigenesis in a subset of sporadic MTC, but not for tumor differentiation. Somatic and germline mutations may lead to tumor development via different mechanisms, even though they show a similar nucleotide change in codon 918.

In summary, we have analyzed the somatic mutation in codon 918 in Japanese and Chinese sporadic MTCs and examined the relation between the presence of the mutation and tumor differentiation. A similar mutation frequency was observed in Japanese and Chinese sporadic MTCs. There was no correlation between codon 918 mutation and tumor differentiation in sporadic MTC.

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