Obvious mRNA and protein expression but absence of mutations of the RET proto-oncogene in parathyroid tumors

Takehiko Kimura¹, Katsuhiro Yoshimoto², Chisato Tanaka², Toshihiro Ohkura¹, Hiroyuki Iwahana², Akira Miyauchi³, Toshiaki Sano¹ and Mitsuo Itakura¹

First Department of Internal Medicine¹, Otsuka Department of Clinical and Molecular Nutrition² and Department of Pathology¹, School of Medicine, The University of Tokushima, Tokushima, Japan; Second Department of Surgery³, Kagawa Medical School, Kagawa, Japan


The study on the expression of the RET proto-oncogene in parathyroid tumors disclosed obvious mRNA expression by the reverse transcription (RT)-polymerase chain reaction (PCR) method and protein expression by Western blotting. To find out whether mutations in the cysteine-rich regions or tyrosine kinase domain of the RET proto-oncogene are etiological for parathyroid tumorigenesis, sporadic parathyroid adenomas and carcinomas, parathyroid tumors from multiple endocrine neoplasia 1, familial isolated hyperparathyroidism or hereditary hyperparathyroidism-jaw tumor syndrome were screened by PCR-single strand conformation polymorphism and PCR restriction fragment length polymorphism. Missense mutations in the cysteine-rich region, or codons 768 or 918 in the tyrosine kinase domain of the RET proto-oncogene, were not detected in any of the examined cases of parathyroid tumors. These results suggest that mutations of the RET proto-oncogene do not represent a frequent mechanism of tumorigenesis for parathyroid tumors.

Mitsuo Itakura. Otsuka Department of Clinical and Molecular Nutrition, School of Medicine, The University of Tokushima, 3-18-15, Karamoto-cho, Tokushima-city, 770 Japan

Parathyroid tumors can occur either sporadically or as a part of inherited cancer syndromes such as multiple endocrine neoplasia (MEN) 1, MEN 2A, familial isolated hyperparathyroidism (FIHP) and hyperparathyroidism-jaw tumor syndrome (HPT-JT). Primary hyperparathyroidism is an important feature of MEN 1 and occurs in more than 95% of patients with MEN 1. In MEN 2A, primary hyperparathyroidism is observed in 10–25% of patients, but hyperparathyroidism is not associated with MEN 2B (1).

Recently, development of MEN 2A and 2B was shown to be related to specific mutations in the RET proto-oncogene. Non-conservative substitution of the five cysteine residues located in the extracellular domain adjacent to the transmembrane segment of the RET protein was detected in patients with MEN 2A and patients with familial medullary thyroid carcinoma (FMTC) (2). In patients with MEN 2B, a mutation at codon 918 causing the substitution of threonine for methionine within exon 16 of the tyrosine kinase domain of the RET protein was detected at the germline level (3). A missense mutation at codon 768 in the tyrosine kinase domain of the RET proto-oncogene was described recently in a family with FMTC that does not have a cysteine codon mutation (4).

Medullary thyroid carcinomas (MTCs), pheochromocytomas and parathyroid tumors are component tumors of MEN 2A and MTCs and pheochromocytomas are those of MEN 2B, but they also occur as a sporadic type. In sporadic MTCs and pheochromocytomas, codon 918 mutations were detected frequently (3, 5, 6), but mutations in the cysteine-rich region of the RET proto-oncogene were found to be rare (7, 8). In addition, the codon 768 mutation was detected in 40% of sporadic MTCs that did not have codon 918 mutations (4).

High-level RET mRNA was found in pheochromocytomas and MTCs (9). With respect to parathyroid glands, RET expression by an in situ hybridization method was reported in the endoderm of the posterior branchial arches from which parathyroid progenitor cells are derived (10). Because there are no reports on the expression of the RET proto-oncogene in parathyroid tumors, we have investigated its expression by the reverse transcription-polymerase chain reaction (RT-PCR) method and Western blotting in this study. By analogy to MTCs and pheochromocytomas, somatic mutations corresponding to germline mutations may make RET a candidate oncogene for sporadic parathyroid tumors. In this study, we first studied the expression of the RET proto-oncogene in parathyroid tumors and next screened for mutations in the cysteine-rich regions and tyrosine kinase domain of the RET proto-oncogene in parathyroid tumors.
Materials and methods

Tissue samples

Tissue samples were obtained at surgical operations or from paraffin-embedded sections. Peripheral blood samples were collected at surgical operations or retrospectively. These included 14 patients with sporadic primary hyperparathyroidism (ten adenomas and four carcinomas), six patients with MEN 1 (eight tumors), three patients with FIHP (three adenomas and one carcinoma) and one patient with HPT-JT (one tumor) (Table 1).

Reverse transcription-PCR

For RNA study, a parathyroid adenoma and a parathyroid carcinoma from FIHP were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated by the guanidium isothiocyanate followed by phenol–chloroform method (11). Complementary DNA was made from 2 μg of total RNA using MMLV reverse transcriptase (Promega, Madison, WI) and random hexamers. The cDNAs were then amplified by PCR in 30 cycles using two primers: 5'-GGGGGATTAAGCTGGCATA-3' (exon 10) and 5'-TGGCTTGTGGGCAACTTGT-3' (exon 11). The primer pair was chosen to span intron 10. The PCR condition was as follows: 1 min of denaturation at 94°C, 1 min of annealing at 60°C and 1 min of extension at 72°C. The PCR products were electrophoresed on a 10% polyacrylamide gel, followed by ethidium bromide staining. The gels were photographed using an ultraviolet transilluminator. The specificity of RET amplification was determined by sequencing a band (203 bp) extracted from a polyacrylamide gel.

Western blotting

Plasma membrane samples (25 or 100 μg per lane) from the parathyroid adenoma and carcinoma, NIH3T3 cells and human MTC cell line of TT cells were applied to 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes (BIO-RAD) and immunoblotted with affinity-purified

Table 1. List of cases analyzed.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Location (size)</th>
<th>Serum Ca (mg/dl)</th>
<th>Complication, etc.</th>
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<tbody>
<tr>
<td>Sporadic adenoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>F</td>
<td>LL (19 x 13 x 10)</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>73</td>
<td>F</td>
<td>RL (42 x 40 x 25)</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>53</td>
<td>M</td>
<td>RL (20 x 12 x 17)</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>71</td>
<td>F</td>
<td>RL (12 x 8 x 4)</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>M</td>
<td>LL (420 mg)</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>M</td>
<td>LU (13 x 12 x 9)</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>F</td>
<td>RU (33 x 18 x 9)</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>59</td>
<td>F</td>
<td>LL (16 x 13 x 8)</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>47</td>
<td>F</td>
<td>R (30 x 20)</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td>F</td>
<td>LU (15 x 9 x 6)</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td>Sporadic carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>61</td>
<td>F</td>
<td>RU (34 x 26 x 20)</td>
<td>11.9</td>
<td>Extracapsular invasion</td>
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<tr>
<td>12</td>
<td>84</td>
<td>F</td>
<td>RU (25 x 19 x 18)</td>
<td>10.8</td>
<td>Recurrent laryngeal nerve invasion</td>
</tr>
<tr>
<td>13</td>
<td>72</td>
<td>M</td>
<td>?</td>
<td>?</td>
<td>Cervical lymph node metastasis</td>
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<tr>
<td>14</td>
<td>43</td>
<td>F</td>
<td>RU (22 x 17 x 12)</td>
<td>11.4</td>
<td>Multiple lung metastases</td>
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<td>MEN 1</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td>59</td>
<td>F</td>
<td>RU (30 x 10 x 19)</td>
<td>11.1</td>
<td>Pituitary adenoma, follicular thyroid adenoma</td>
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<tr>
<td>16</td>
<td>35</td>
<td>F</td>
<td>LU (30 x 15 x 10)</td>
<td>11.1</td>
<td>Pituitary adenoma, insulinaoma</td>
</tr>
<tr>
<td>17</td>
<td>67</td>
<td>F</td>
<td>LU (32 x 12 x 12)</td>
<td>11.4</td>
<td>Gastrinoma</td>
</tr>
<tr>
<td>18</td>
<td>30</td>
<td>F</td>
<td>R (32 x 10)</td>
<td>11.0</td>
<td>Pituitary adenoma</td>
</tr>
<tr>
<td>19</td>
<td>32</td>
<td>F</td>
<td>LL (18 x 35 x 9)</td>
<td>12.8</td>
<td>Pituitary adenoma</td>
</tr>
<tr>
<td>20</td>
<td>59</td>
<td>M</td>
<td>RL (13 x 11)</td>
<td>10.9</td>
<td>Gastrinoma</td>
</tr>
<tr>
<td>FIHP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>34</td>
<td>F</td>
<td>RU (30 x 20)b</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>36</td>
<td>F</td>
<td>RL (8 x 5 x 13)</td>
<td>13.1</td>
<td>Elder sister of case 21</td>
</tr>
<tr>
<td>23</td>
<td>29</td>
<td>F</td>
<td>RU (20 x 12 x 9)</td>
<td>12.3</td>
<td>Cousin of case 21</td>
</tr>
<tr>
<td>HPT-JT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>53</td>
<td>F</td>
<td>RU (9 x 7 x 4)</td>
<td>12.8</td>
<td>Papillary thyroid carcinoma</td>
</tr>
</tbody>
</table>

a: ? = information unavailable.

b: Parathyroid carcinoma.
anti-RET polyclonal antibody (IBL, Fujioka, Japan) (12). The TT cells were reported to express both the wild-type and the mutant RET allele (13). Immunoreactive bands were visualized using a horseradish peroxidase conjugated anti-rabbit antiserum and ECL detection reagents (Amersham, Buckinghamshire, UK).

**Deoxyribonucleic acid preparation**

Deoxyribonucleic acid was isolated from frozen tumor sections obtained at surgical operations, leukocytes and paraffin-embedded specimens, as described previously (14).

**Polymerase chain reaction amplification and mutation analysis**

**Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP).** Polymerase chain reaction amplification was performed using the oligonucleotide primers 5'-GGTGCCAAGCCTC-3' and 5'-GACATGTGGGTGGTTGACCT-3' for exon 10 and 5'-CGT-GGTGGTCCCGGCCGCC-3' for exon 11. Amplified DNA fragments showed expected sizes of 187 and 234 bp for exons 10 and 11, respectively. The PCR proceeded in Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan) with 50 ng of genomic DNA in a total volume of 5 µl containing 1.5 µCi of [α-32P]dCTP (3000 Ci/mmol; 10 mCi/ml). Thirty-five cycles consisted of 30 s at 94°C for denaturation, 30 s at 68°C for exon 10 or 65°C for exon 11 for annealing, and 1 min at 72°C for extension. The reaction mixture in 5 µl was heated with 95 µl of dye (95% formamide/20 mmol/l EDTA/0.05% bromophenol blue/0.05% xylene cyanol), then 1 µl of the mixture was applied to a 5% polyacrylamide gel containing 45 mmol/l TRIS-borate (pH 8.3) and 4 mmol/l EDTA. Glycerol (5 or 10%) was added when specified, as described previously (14). Electrophoresis proceeded at 30 W for 4–6 h at room temperature. The gel was dried and exposed to X-ray films with intensifying screens at −70°C for 12–24 h.

**Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP).** For PCR-RFLP of exon 768, genomic DNA was amplified using primers oRB1395 (5'-GCCAGGAGCATGTGTTGCA-3') and oRB1396 (5'-GACATGTGTTGTTGACCT-3'). For PCR-RFLP of exon 918, genomic DNA was amplified using primers oRB912 (5'-AGGGATAGGGCCTGGGCTTC-3') and oRB913 (5'-TACCTGCCACCCAAAGAGG-3'). The PCR amplified a fragment of 121 bp for exon 13 and a fragment of 192 bp for exon 16 of the RET proto-oncogene. The reaction was carried out for 30 cycles at 94°C for 1 min, 60°C for both exons 13 and 16 for 1 min and 72°C for 2 min. The products were digested with

**Fig. 1. Expression of the RET proto-oncogene in human parathyroid tumors.** (A) Reverse transcriptase polymerase chain reaction (RT-PCR) detection of transcripts of the RET proto-oncogene. Total RNA extracted from a parathyroid adenoma (a) and carcinoma (b) were reverse transcribed, and the resulting products were amplified by PCR with primers located in exons 10 and 11. The PCR products were electrophoresed on a polyacrylamide gel and stained with ethidium bromide: M = φX174 HaeIII-digested DNA fragments used as molecular markers; lane 1 = template free; lane 2 = cDNA as a template. (B) Detection of RET protein by Western blotting. Plasma membrane samples from parathyroid tumors (each 100 µg), NIH 3T3 cells (100 µg) and TT cells (25 µg) were reacted with the anti-RET and a horseradish peroxidase conjugated anti-rabbit antiserum. Lane 1 = a parathyroid carcinoma; lane 2 = a parathyroid adenoma; lane 3 = NIH 3T3 cells as a negative control; lane 4 = TT cells as a positive control.
AhI for codon 768 or FokI for codon 918 according to the manufacturer's recommendations (Takara Shuzo, Kyoto, Japan) and electrophoresed on a 10% polyacrylamide gel, followed by ethidium bromide staining. The gels were photographed with an ultraviolet transilluminator.

Results

Identification of expression of the RET proto-oncogene in parathyroid tumors by RT-PCR and Western blotting

To examine the expression of the RET proto-oncogene in parathyroid tumors, we extracted RNA from a parathyroid adenoma and a parathyroid carcinoma. Reverse transcription PCR of RNA derived from parathyroid tumors revealed transcript signals of the predicted size of 203 bp (Fig. 1A). Sequencing of the RT-PCR products confirmed that they had the published sequence.

The antibody detected 170-kD RET protein in plasma membrane samples of the parathyroid adenoma, parathyroid carcinoma and TT cells (Fig. 1B). The 150-kD protein in TT cells was derived from a non-glycosylated form of RET protein in cytosol (12).

Screening for mutations of the RET proto-oncogene in parathyroid tumors

Genomic DNAs obtained from parathyroid tumors were tested for mutations within exons 10 and 11 and those of codon 768 in exon 13 and codon 918 in exon 16 of the RET proto-oncogene by PCR-SSCP analysis or PCR-RFLP. The PCR-SSCP analysis of exons 10 and 11 in a 5% polyacrylamide gel containing 5% glycerol showed no extra bands relative to those amplified from leukocytes from a healthy subject (Fig. 2A). In addition, the SSCP with two other gel conditions of 5% polyacrylamide gel containing 0 or 10% glycerol also did not reveal any extra bands.

Fig. 2. Screening for mutations of the RET proto-oncogene. (A) Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of exon 11 of the RET proto-oncogene. Electrophoresis was performed in a 5% polyacrylamide gel with 5% glycerol at room temperature. Lanes 1–10 = sporadic parathyroid adenomas (cases 1–10 in Table 1); lane 11 = sporadic parathyroid carcinoma (case 11 in Table 1). Lanes 1–11 revealed no extra bands. (B) The FokI restriction digestion of exon 16 of the RET proto-oncogene. Genomic DNA from sporadic parathyroid tumors was amplified by PCR using primers for exon 16 of the RET proto-oncogene. The products of 192 bp were cleaved with FokI, size-fractionated by electrophoresis through a 10% polyacrylamide gel and stained with ethidium bromide. M = φX174 HaeIII-digested DNA fragments used as molecular markers; lanes 1–8 = sporadic parathyroid adenomas (cases 1–8 in Table 1); lane 9 = leukocytes from a patient with MEN 2B. Lanes 1–8 revealed no loss of FokI restriction site at codon 918.
Genomic DNAs obtained from parathyroid tumors were tested for codon 768 and 918 mutations of the RET proto-oncogene by AluI and FokiI digestion of PCR products, respectively, because the mutations of codon 768 (GAG → GAC) and codon 918 (ATG → ACG) cause loss of an AluI and a FokiI restriction site, respectively (3, 4). No mutations causing loss of AluI restriction site at codon 768 and loss of FokiI restriction site at codon 918 were detected in any of the tumors examined (Fig. 2B).

Figure 2 shows representative results. Results of other cases were omitted because they were the same as those in Fig. 2.

Discussion

Few studies have assessed the expression of proto-oncogenes and tumor suppressor genes in parathyroid tumors. In most of the parathyroid tumors found in MEN 1 patients, allelic loss of chromosome 11q13 is observed (15). In sporadic parathyroid tumors, allelic loss of the MEN1 gene on chromosome 11 and those of chromosome 1 have been observed in 25% (15) and 40% (16), respectively. Activation of the PRAD1 gene by rearrangement has been reported in a small percentage of parathyroid tumors (17). Inactivation of the Rb gene has been reported to be a key factor in the pathogenesis of most parathyroid carcinomas (18). However, activation of the ras genes and inactivation of the p53 genes occur very rarely in parathyroid tumors (14, 19).

Numerous point mutations in the RET proto-oncogene have been identified recently in association with MEN 2A, FMTC and MEN 2B (2–4). These mutations in the cysteine-rich regions induced ligand-independent dimerization of the RET protein, leading to activation of tyrosine kinase (13, 20). The codon 918 mutation altered the RET catalytic properties both quantitatively and qualitatively, and resulted in constitutive activation of tyrosine kinase (13). These mutations were also detected in sporadic MTCs and pheochromocytomas (3–8).

Transcription of RET proto-oncogene was found to occur preferentially in neuroblastoma, pheochromocytoma and MTC, all of which originate from neural crest cells (9). Recently, Pachnis et al. (10) reported in situ hybridization data showing that RET proto-oncogene is expressed predominantly in the developing peripheral nervous systems during mouse embryogenesis. In addition, non-neural expression of the RET proto-oncogene was observed in developing kidneys, salivary glands, thymus, spleen and lymph nodes (10, 21). Expression of the RET proto-oncogene in the endoderm of posterior brachial arches, from which parathyroid progenitor cells are derived, suggests an important role of the RET proto-oncogene in the development of parathyroid glands (10). In this report, we demonstrated the expression of the RET proto-oncogene in parathyroid tumors by the RT-PCR method and Western blotting. Western blotting showed that the amount of the 170-kD RET protein in parathyroid tumors was 20–25% of that of the 170-kD RET protein in TT cells (Fig. 1B). Although the expression of RET proto-oncogene and somatic mutations in sporadic MTCs and pheochromocytomas as components of MEN 2A and 2B made RET a candidate oncogene for sporadic parathyroid tumors, mutations of the RET proto-oncogene were not detected in sporadic parathyroid tumors.

We did not find somatic mutations in exons 10 and 11 of the RET proto-oncogene by PCR-SSCP analysis. In general, the sensitivity of PCR-SSCP analysis is less than 100%. However, we detected mutations of the RET proto-oncogene in 3/3 patients with MEN 2A or FMTC, three sporadic MTCs and TT cells by the same conditions of PCR-SSCP analysis as in this study (22). Owing to the sensitivity of PCR-SSCP analysis and the limited number of sporadic parathyroid tumors examined in this study, we could not rule out the existence of mutations at a low frequency. In addition, the possibility of the presence of other types of mutations at codons 768 or 918 not detected by PCR-RFLP used in this study has not been ruled out.

With regard to hereditary parathyroid tumors, tumorigenesis in MEN 1 involves a constitutional inactivating mutation at the MEN1 locus. The finding that constitutional homozygosity for MEN1 does not result in a more severe hyperparathyroidism or endocrine pancreatic tumors, however, indicates that additional genetic events are involved in tumor development (23). With respect to the linkage study of FIHP, different results were reported: linkage to chromosome 11q13 in one family and exclusion of linkage to 11q13 in another family (24, 25). Szabó et al. (26) mapped HPT-JT gene to 1q21–q31. No mutations of the RET proto-oncogene were, however, detected in the examined hereditary parathyroid tumors in our study.

Our results suggest that mutations of the RET proto-oncogene do not have a primary role in the formation of sporadic parathyroid tumors or parathyroid tumors from MEN 1. FIHP and HPT-JT, in spite of obvious expression at mRNA and protein levels.

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References

et al. Germline mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. Nature 1993;363:458–60


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