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Tumor-Specific Mutations in the Tyrosine Kinase Domain of the *RET* Proto-Oncogene in Pheochromocytomas of Sporadic Type

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Abstract. Sporadic pheochromocytomas, sporadic medullary thyroid carcinomas (MTCs), pheochromocytomas and/or MTCs in multiple endocrine neoplasia (MEN) 2A or 2B were screened for mutations in the tyrosine kinase domain of the *RET* proto-oncogene by direct sequencing of PCR-amplified products or sequencing subcloned DNAs from PCR-products. All tumors of 4 MEN 2B patients were confirmed to contain a heterozygous missense mutation at codon 918 (ATG→ACG; Met→Thr) of the *RET* proto-oncogene as well as their leukocytes. The same tumor-specific mutations at codon 918 were also found in 5/16 (31%) sporadic pheochromocytomas. These results suggest that mutations of the *RET* proto-oncogene in its tyrosine kinase domain play a role not only as the predisposing gene for MEN 2B, but also as a tumorigenic factor for pheochromocytomas of sporadic type.

Key words: *RET* proto-oncogene, Mutation, Pheochromocytoma, MEN 2A, MEN 2B

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THE *RET* proto-oncogene encodes a protein structurally related to transmembrane receptors with cytoplasmic tyrosine-kinase domains of which the putative ligand has not yet been identified [1, 2]. Several lines of evidence have implicated the *RET* gene as a dominant transforming gene in human malignancies. The *RET* oncogene was originally isolated during the transfection of NIH/3T3 cells with a human T-cell lymphoma DNA [3]. The discovery of the *RET* oncogene originated from recombination events of the *RET* proto-oncogene with 5′-terminal sequences of different genes. These rearrangements, however, were shown to have occurred *in vitro* during the transfection assay. Direct evidence for the involvement of the *RET* proto-oncogene in human tumors was obtained when it was found that the *RET* proto-oncogene was activated *in vivo* as the oncogene in human thyroid papillary carcinomas [4].

The loci of three syndromes, including MEN 2A, familial thyroid medullary carcinoma (FMTC), and MEN 2B, have been assigned by linkage to the chromosome 10q11.2 region, where the *RET* proto-oncogene had also been mapped [5, 6]. Recently, germline mutations of the *RET* proto-oncogene have been described in patients affected with MEN 2A, FMTC, and MEN 2B [7–13]. Nonconservative substitution of the 6 cysteine

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residues located in the extracellular domain adjacent to the transmembrane segment of the *RET* protein was detected in MEN 2A patients with MTCs, pheochromocytomas, and parathyroid tumors, and FMTC patients with MTC as a sole clinical manifestation [7–9]. In MEN 2B characterized by the combined occurrence of MTC, pheochromocytomas, mucosal neuromas, and skeletal abnormalities, a mutation at codon 918 causing the substitution of a threonine for a methionine within exon 16 of the tyrosine kinase domain of the *RET* protein was detected at the germline level [11–13]. In addition, the same mutations at codon 918 were found in 33% of sporadic MTCs [11]. Because high-level *RET* mRNA was found in pheochromocytomas as in MTC [14], we analyzed mutations of codon 918 of the *RET* proto-oncogene in pheochromocytomas of sporadic type and of MEN 2A. The analysis presented here supports a model of dominant oncogenic activity for the *RET* protein even in sporadic pheochromocytomas.

Materials and Methods

Tissue samples

Tissue samples were obtained at surgical operation or from paraffin-embedded sections. The diagnosis of pheochromocytoma or MTC was confirmed by histological examinations of the tumors. Peripheral blood samples were collected at surgical operation or retrospectively. These included 5 MEN 2A patients, 1 FMTC patient, 4 MEN 2B patients (case numbers 3, 19, 20, and 21 in [15]), 3 neurofibromatosis type 1 (NF1) patients with pheochromocytomas, 16 patients with sporadic pheochromocytomas, and 2 patients with sporadic MTCs. The sporadic tumors were considered sporadic because patients did not have a family history suggestive of MEN 2A, FMTC, MEN 2B, NF1, or von Hippel-Lindau syndrome, and because they did not have both pheochromocytomas and MTCs. The clinical data are listed in Table 1.

DNA preparation

DNA was isolated from frozen tumor sections obtained at surgical operation, leukocytes, and paraffin-embedded specimen, as previously described [16, 17]. For paraffin-embedded specimens, 10-μm sections were carefully prepared and placed in sterile Eppendorf tubes. New gloves, sterile forceps, and a microtome blade cleaned with xylene between sample preparations were used for every tumor block to avoid cross contamination and to minimize preparation artifacts.

Direct sequencing of the PCR products, or sequencing after subcloning of PCR products into a plasmid vector

The 5′ primer used for PCR was oRB905 (5′-TGTAAAACGACGGCCAGTAGGGATAGGGC-CTGGGCTTC-3′) that contained an M13 tail of 18 bp as underlined. The 3′ primer was oRB914 (5′-biotin-TAACCTCCACCCCAAGAGAG-3′). The polymerase chain reaction (PCR) amplified a 210 bp fragment containing a 192 bp sequence of the *RET* proto-oncogene, in addition to the 18 bp M13 sequence. PCR was performed in a 10 μl reaction mixture containing 0.1 μg of genomic DNA, 200 μM each of deoxynucleotide triphosphate, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 10 pmol of PCR primers, and 0.25 unit of Taq DNA polymerase. The reaction was carried out in a Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan) for 30 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. Dynabeads M-280 magnetic beads (Dynal AS, Oslo, Norway) were used to prepare single-stranded, immobilized templates following the method recommended by the manufacturer.

In some samples, PCR fragments were cloned into the pCR™II vector with a TA Cloning kit (Invitrogen Co., San Diego, CA). The accuracy of our sequencing data in cloned DNAs was confirmed by analyzing at least 10 independent clones.

DNA sequences of the immobilized PCR products and/or the cloned PCR products were determined by fluorescence-based dideoxy sequencing with Taq DNA polymerase in a thermal cycler, and fluorescently-labeled M13 universal sequencing primer, followed by gel electrophoresis, data collection and analysis on an Applied Biosystems model 373A automated sequencer (Perkin Elmer, division of Applied Biosystems, Foster City, CA).

Results

Genomic DNAs obtained from MTCs and pheochromocytomas were tested for sequence variation within the exon 16 of the *RET* proto-oncogene by direct sequencing or sequencing subcloned PCR products. The results are summarized in Table 1.

Mutations of codon 918 of the *RET* proto-oncogene were confirmed in MTCs and/or a pheochromocytoma of MEN 2B. Codon 918 of ATG for methionine was mutated to ACG for threonine by a T to C transition of the second letter in one allele, and normal *RET* allele was also found in MTCs in all 4 cases. We also confirmed the same mutations in their leukocyte DNAs in 4 cases with MEN 2B.

When tumor DNAs of 3 pheochromocytomas of NF1, 16 sporadic pheochromocytomas, and 2 sporadic MTCs were analyzed for this mutation, we

Table 1. List of cases analyzed

No.	Age	Sex	Type	Tumors analyzed	Location, size and weight	RET mutation at codon 918 *in tumors*	*in WBC*
1.	32	M	MEN 2A	pheochromocytoma	bilateral right (1.8 × 1.6 × 0.8 cm) left (2.9 × 2.6 × 1.7 cm)	−2)	−1)
2.	25	F	MEN 2A	pheochromocytoma	bilateral right (8.2 × 7.1 × 5.8 cm) left (0.5 × 0.4 cm)	−2)	−1)
3.	33	F	MEN 2A	pheochromocytoma	bilateral right (5.1 × 4.4 × 3.4 cm) left (0.6 × 0.4 cm)	−2)	−1)
4.	46	F	MEN 2A	MTC	bilateral	−1)	nd
				pheochromocytoma	bilateral right (460 g) left (20 g)	−1)	
5.	42	F	MEN 2A	MTC	bilateral	−1)	nd
6.	60	M	FMTC	MTC	bilateral	−1)	nd
7.	18	F	MEN 2B	MTC	bilateral	+1, 2)	+1)
				pheochromocytoma	bilateral right (26.5 g) left (12.5 g)	+1, 2)	
8.	10	F	MEN 2B	MTC	unilateral	+1, 2)	+1
9.	8	F	MEN 2B	MTC	unilateral	+1, 2)	+1)
10.	18	M	MEN 2B	MTC	bilateral	+1, 2)	+1)
11.	44	F	NF 1	pheochromocytoma	left (7 × 6 × 5 cm)	−1)	nd
12.	19	F	NF 1	pheochromocytoma	left (5 × 4 × 3 cm)	−1)	nd
13.	34	M	NF 1	pheochromocytoma	left (9 × 7.2 × 6.5 cm)	−1)	nd
14.	41	F	sporadic	pheochromocytoma	right (3 × 4 cm)	+1, 2)	−1, 2)
15.	60	M	sporadic	pheochromocytoma	right (6.5 × 5.3 × 3.7 cm)	+1, 2)	−1, 2)
16.	45	F	sporadic	pheochromocytoma	right (5 × 6 cm)	+1,2)	−1, 2)
17.	45	F	sporadic	pheochromocytoma (malignant)	left (5.3 × 5.0 × 4.7 cm)	+2)	−1, 2)
18.	67	F	sporadic	pheochromocytoma (malignant)	left (3.2 × 2.8 × 1.8 cm) (extraadrenal)	+2)	−1, 2)
19.	64	F	sporadic	pheochromocytoma	right (68 g)	−1)	−1)
20.	?	F	sporadic	pheochromocytoma	?	−1)	−1)
21.	50	M	sporadic	pheochromocytoma	right (4.3 × 5.4 cm)	−1)	nd
22.	47	F	sporadic	pheochromocytoma	left (7 × 5 × 3.5 cm, 50 g)	−1)	nd
23.	51	F	sporadic	pheochromocytoma	right (4.3 × 5.4 cm)	−1)	nd
24.	53	F	sporadic	pheochromocytoma	left (5 × 5 cm)	−1)	nd
25.	48	M	sporadic	pheochromocytoma	left (3 × 3 cm)	−1)	nd
26.	69	F	sporadic	pheochromocytoma	left (32.2 g)	−1)	nd
27.	70	F	sporadic	pheochromocytoma	right (3 × 3 cm)	−1)	nd
28.	42	F	sporadic	pheochromocytoma	right (?)	−1)	nd
29.	19	M	sporadic	pheochromocytoma	right (?)	−1)	nd
30.	94	M	sporadic	MTC	right	−1)	nd
31.	36	F	sporadic	MTC	left	−1)	nd

?, information unavailable; nd, not determined. 1) direct sequencing of PCR products, 2) sequencing of subcloned DNA.

detected the same mutations at codon 918 in 5 sporadic pheochromocytomas as detected in tumors of MEN 2B. The mutations in 5 sporadic pheochromocytomas were confirmed in both sense and antisense strands of cloned PCR products. No mutations at codon 918 were detected in their leukocyte DNAs. No mutations at codon 918 were detected in 2 of 2 sporadic MTCs examined.

To reduce the possibility of cross-contamination, positive samples having *RET* mutations were re-examined with DNA templates extracted from re-cut paraffin-embedded specimens.

Discussion

Numerous point mutations in the *RET* proto-oncogene have recently been identified in association with MEN 2A and FMTC [7–10]. Mutations which result in the replacement of cysteines with other amino acids, could diminish or abolish the regulatory effect of ligand binding on the activity of the tyrosine kinase. In our studies, MEN 2A and FMTC patients were confirmed to have similar mutations at one of 6 cysteines in the extracellular domain, but no such mutations were detected in MTCs or pheochromocytomas of MEN 2B or of sporadic type by the method of PCR-single strand conformation polymorphism (Kimura T *et al.*, manuscript in preparation).

According to Hofstra *et al.* [11], Carlson *et al.* [12], and Eng *et al.* [13], sequencing of germline DNAs from MEN 2B patients revealed the existence of the point mutation at codon 918 in the *RET* proto-oncogene in all 9, all 34, and 26 of 28 MEN 2B patients, respectively. In this study, we confirmed the same germline mutations at codon 918 in all 4 Japanese MEN 2B patients. The codon 918 mutation affects the intracellular tyrosine kinase domain of *RET* protein, whereas the mutations associated with MEN 2A and FMTC are all in the extracellular region, close to the transmembrane domain.

MEN1 appears to be qualified as an antioncogene [18]. Such is not the case for *MEN2*, of which mutations predispose heterozygotes to two neural crest-derived tumors, MTC and pheochromocytoma [19]. A plausible scenario for the development of these tumors is that the *RET* mutations of either the extracellular cysteine-rich domain in MEN 2A or the tyrosine kinase domain in MEN 2B produce the preneoplastic hyperplasia of the adrenal medullary cells and thyroid C-cells, and increase the number of target cells available for transformation to neoplasia by mutation of other tumor suppresser genes probably located on chromosome 1p and/or 22q.

The occurrence of somatic mutations of the *RET* proto-oncogene may lead to sporadic tumors of the tissue involved. This explains the finding of mutations affecting the same codons in sporadic MTCs as in MEN 2A and MEN 2B. Donis-Keller *et al.* [8] and we (Kimura T *et al.*, manuscript in preparation) found 6 bp deletion in tumor DNA of sporadic MTC which removed the cysteine residue at codons 630 and 634, respectively. A recent report by Eng *et al.* [13] revealed 2 mutations at codon 620 among 13 sporadic MTCs and 12 pheochromocytomas. When analyzing tumor DNAs of 18 sporadic MTCs, Hofstra *et al.* [11] detected mutations of codon 918 in 6 cases, but no information about mutations in their constitutive DNAs was obtained. But they reported no mutations of codon 918 of the *RET* proto-oncogene in 5 sporadic pheochromocytomas. Recently, Eng *et al.* [13] reported that 5 of 13 sporadic MTCs and 1 of 12 sporadic pheochromocytomas had the same codon 918 mutation. In this study, we detected codon 918 mutations of the *RET* proto-oncogene in 5/16 (31%) sporadic pheochromocytomas, for which mutations were heterozygous and tumor-specific. Careful precautions and repeated examination excluded the possibility of contamination of the PCR products.

Neither the normal function nor the ligand of *RET* is yet known. In addition, the basis for the tissue specificity of these *RET* proto-oncogene mutations in sporadic tumors is unclear. Biological assays testing the mutant forms in cell culture and transgenic mice should provide further insight into the role of the *RET* proto-oncogene in the development of neoplasia.

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