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NOTE

Absence of Mutations at Codon 768 of the *RET* Proto-Oncogene in Sporadic and Hereditary Pheochromocytomas

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Abstract. Sixteen sporadic pheochromocytomas, 3 pheochromocytomas in neurofibromatosis 1, and 4 pheochromocytomas in multiple endocrine neoplasia (MEN) 2A or 2B were screened for mutations at codon 768 of the *RET* proto-oncogene by *Alu*I digestion of polymerase chain reaction (PCR) products and mutations in exon 13 by PCR-single strand conformation polymorphism (SSCP) analysis. Although mutations at codon 768 (GAG→GAC; Glu→Asp) of the *RET* proto-oncogene were recently reported to be found in 40% of sporadic medullary thyroid carcinomas (MTCs), the absence of missense mutations at codon 768 was confirmed both with PCR-restriction fragment length polymorphism (RFLP) and PCR-SSCP analysis in all examined cases of pheochromocytomas. These results suggest that mutations at codon 768 of the *RET* proto-oncogene do not represent a frequent mechanism of tumorigenesis for both sporadic and hereditary pheochromocytomas.

Key words: *RET* proto-oncogene, Mutation, Codon 768, Exon 13, Pheochromocytoma

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THE *RET* proto-oncogene encodes a protein structurally related to transmembrane receptors with cytoplasmic tyrosine-kinase domains [1]. The *RET* protein spans the cell membrane, and is involved in the inward transduction of transmembrane signals. The protein has three regions: an extracellular portion which interacts with ligands; a transmembrane region; and a cytoplasmic tyrosine kinase region.

The predisposing genes for MEN 2A, familial thyroid medullary carcinoma (FMTC) and MEN 2B have been shown to be assigned to the *RET* proto-oncogene [2, 3]. Recently, germline mutations of the *RET* proto-oncogene have been described in patients affected with MEN 2A, FMTC or MEN 2B [2–7]. In families with MEN 2A or FMTC, detected mutations affect one of five cysteine residues in the extracellular region adjacent to the transmembrane segment of the *RET* protein [2, 4, 5]. In MEN 2B, a mutation at codon 918 causing the substitution of threonine for methionine of the tyrosine kinase domain of the *RET* protein was detected in almost every case [3, 6, 7].

A missense mutation at codon 768 in the tyrosine kinase domain of the *RET* proto-oncogene was recently described in a family with FMTC, which does not have a cysteine codon mutation [8]. The same mutation was also detected in 40% of sporadic MTCs which do not have codon 918 mutations. With respect to sporadic pheochromocytomas, we detected codon 918 mutations in 31% of sporadic pheochromocytomas [9]. In the present study, we analyzed sporadic pheochromocytomas and pheochromocytomas with neurofibromatosis 1 (NF1), MEN 2A, or MEN 2B for mutations of codon 768 of the *RET* proto-oncogene.

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Table 1. A list of analyzed cases

No.	Age	sex	Type	Tumors analyzed	Location, size and weight
1.	50	M	sporadic	pheochromocytoma	right (4.3 × 5.4 cm)
2.	47	F	sporadic	pheochromocytoma	left (7 × 5 × 3.5 cm, 50 g)
3.	51	F	sporadic	pheochromocytoma	right (4.3 × 5.4 cm)
4.	53	F	sporadic	pheochromocytoma	left (5 × 5 cm)
5.	48	M	sporadic	pheochromocytoma	left (3 × 3 cm)
6.	69	F	sporadic	pheochromocytoma	left (32.2 g)
7.	70	F	sporadic	pheochromocytoma	right (3 × 3 cm)
8.	42	F	sporadic	pheochromocytoma	right (?)
9.	19	M	sporadic	pheochromocytoma	right (?)
10.	23	F	sporadic	pheochromocytoma	?
11.	64	F	sporadic	pheochromocytoma	right (68 g)
12.	?	F	sporadic	pheochromocytoma	?
13.	41	F	sporadic	pheochromocytoma	right (3 × 4 cm)
14.	60	M	sporadic	pheochromocytoma	right (6.5 × 5.3 × 3.7 cm)
15.	45	F	sporadic	pheochromocytoma	right (5 × 6 cm)
16.	45	F	sporadic	pheochromocytoma (malignant)	left (5.3 × 5.0 × 4.7cm)
17.	44	F	NF1	pheochromocytoma	left (7 × 6 × 5cm)
18.	19	F	NF1	pheochromocytoma	left (5 × 4 × 3cm)
19.	34	M	NF1	pheochromocytoma	left (9 × 7.2 × 6.5cm)
20.	32	M	MEN 2A	pheochromocytoma	bilateral right (1.8 × 1.6 × 0.8 cm) left (2.9 × 2.6 × 1.7 cm)
21.	25	F	MEN 2A	pheochromocytoma	bilateral right (8.2 × 7.1 × 5.8 cm) left (0.5 × 0.4 cm)
22.	46	F	NEM 2A	pheochromocytoma	bilateral right (460 g) left (20 g)
23.	18	F	MEN 2B	pheochromocytoma	bilateral right (26.5 g) left (12.5 g)

?: information unavailable.

Materials and Methods

Tissue samples

Tissue samples were obtained at surgical operations or from paraffin-embedded sections. The diagnosis of pheochromocytoma was confirmed by histological examinations of the tumors. Peripheral blood samples were collected at surgical operations or retrospectively. These included 16 patients with sporadic pheochromocytomas, 3 NF1 patients with pheochromocytomas, 3 MEN 2A patients, and 1 MEN 2B patient. The sporadic tumors were considered sporadic because patients did not have a family history suggestive of MEN 2A, MEN 2B, NF1 or von Hippel-Lindau syndrome. The clinical data are listed in Table 1.

Extraction of DNA from tissues

DNA was isolated from frozen tumor sections obtained at surgical operations, leukocytes and paraffin-embedded specimen, as previously described [10].

PCR amplification and mutation analysis

For PCR-RFLP of codon 768, genomic DNA was amplified with primers oRB1395 (5'-TCCAGGAGCGATCGTTTGCA-3') and oRB1396 (5'-GACATGTGGGTGGTTGACCT-3'). The PCR amplified a 121 bp fragment containing a part of exon 13 of the *RET* proto-oncogene. 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. The products were digested with *Alu*I 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.

For PCR-SSCP analysis of exon 13, a pair of intron-based primers, [oRB1395 (5'-TTCCAGGAGCGATCGTTTGCA-3') and oRB1508 (5'-GGAGAACAGGGCTGTATGGA-3')] were used to generate a 223 bp PCR product including exon 13. PCR was performed with 50 ng of genomic DNA in a total volume of 5 μl containing 0.5 μl of [α-^{32}P]dCTP (3000 Ci/mmol; 10 mCi/ml). Thirty cycles consisting of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C were performed. The electrophoresis and exposure to X-ray films were performed as described previously [10].

Results

Genomic DNAs obtained from pheochromocytomas were tested for codon 768 mutations of the *RET* proto-oncogene by *Alu*I digestion of PCR products. Because the mutation of codon 768 (GAG →GAC; Glu→Asp) causes loss of an *Alu*I restriction site, *Alu*I restriction digestion was performed for exon 13 amplicons. No mutations causing loss of *Alu*I restriction site at codon 768 were detected in 16 sporadic pheochromocytomas, 3 pheochromocytomas with NF1, 3 pheochromocytomas with MEN 2A or 1 pheochromocytoma with MEN 2B (Fig. 1).

PCR-SSCP analysis of exon 13 from genomic DNA of pheochromocytomas showed 3 different patterns (Fig. 2). The difference of mobility was found to be caused by polymorphic nucleotide changes at codon 769 (data not shown) [11]. No extra bands except those caused by polymorphism were detected in all examined tumors.

In Figs. 1 and 2, representative results are shown for sporadic pheochromocytomas (case 1 to 9 in Table 1) and pheochromocytomas with NF1 (case 17 in Table 1) or MEN 2A (case 20 in Table 1). Results for other cases are omitted because the results are the same as those in Figs. 1 and 2.

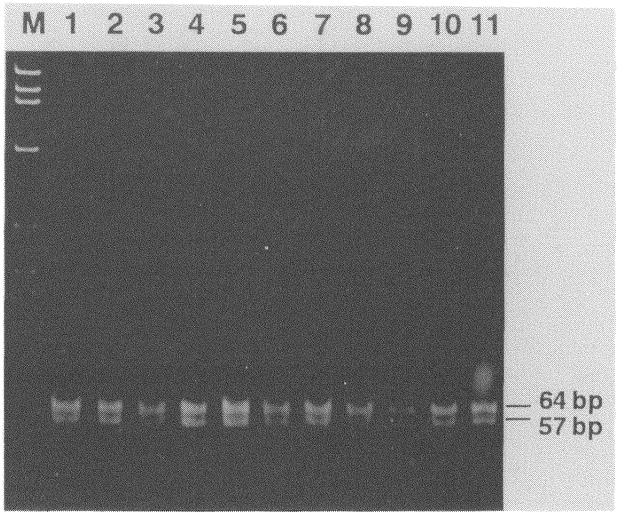

Fig. 1. *Alu*I restriction digestion of exon 13 of the *RET* proto-oncogene for pheochromocytomas. Genomic DNAs from sporadic pheochromocytomas and pheochromocytomas with NF1 or MEN 2A were amplified by PCR with primers for exon 13 of the *RET* proto-oncogene. Products 121 bp in size were cleaved with *Alu*I, size-fractionated by electrophoresis through a 10% polyacrylamide gel and stained with ethidium bromide. M, øX174 *Hae*III-digested DNA fragments used as molecular markers; lanes 1–9, sporadic pheochromocytomas (cases 1 to 9 in Table 1); lane 10, pheochromocytoma with NF1 (case 17 in Table 1); lane 11, pheochromocytoma with MEN 2A (case 20 in Table 1). Lanes 1-11 revealed no point mutations causing loss of *Alu*I restriction site at codon 768.

To avoid possible false-negative results from PCR-RFLP and PCR-SSCP analysis, DNA from several tumors was subcloned into plasmid vectors and sequenced. DNA sequencing showed no mutations in exon 13 of the *RET* proto-oncogene in these tumors (data not shown).

Discussion

Missense mutations in the *RET* proto-oncogene have been found in the constitutional DNA of the majority of patients with MEN 2A, FMTC, or MEN 2B [2–7]. In MEN 2A and FMTC, the mutations alter 1 of 5 cysteine residues in exon 10 and 11 within the cysteine-rich domain of the *RET* proto-oncogene. These mutations induced ligand-independent dimerization of the *RET* protein, leading to activation of tyrosine kinase [12, 13]. In

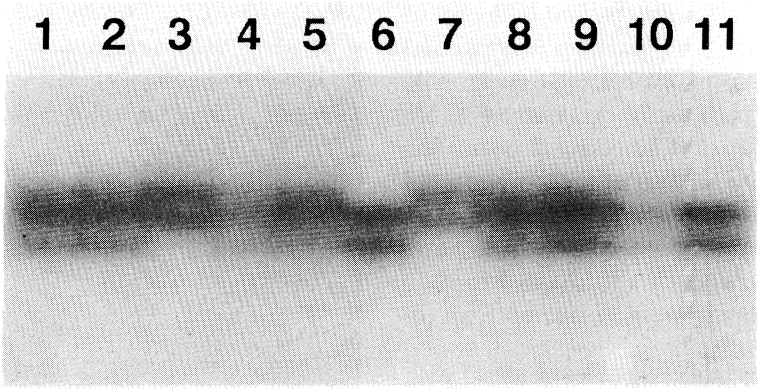

Fig. 2. PCR-SSCP analysis of exon 13 of the *RET* proto-oncogene. Electrophoresis was performed in a 5% polyacrylamide gel with 5% glycerol at room temperature. Lanes 1–9, sporadic pheochromocytomas (cases 1 to 9 in Table 1); lane 10, pheochromocytoma with NF1 (case 17 in Table 1); lane 11, pheochromocytoma with MEN 2A (case 20 in Table 1). Three different polymorphic patterns are shown: lanes 3 and 7, CTT at codon 769; lanes 6, 10, and 11, CTG at codon 769; lanes 1, 2, 4, 5, 8, and 9, heterozygous for both alleles. Lanes 1–11 revealed no extra bands except those caused by polymorphism in exon 13.

patients with MEN 2B, a single missense mutation affecting a methionine (ATG) to threonine (ACG) change at codon 918 has been found. The codon 918 mutation altered *RET* catalytic properties both quantitatively and qualitatively, and resulted in constitutive activation of tyrosine kinase [12].

Although MTC and pheochromocytomas are component tumors of the MEN 2 syndromes, they also occur as sporadic types. By analogy applied to other inherited cancer syndromes, somatic mutations corresponding to germline mutations were observed in MEN 2A, FMTC and MEN 2B. In sporadic MTC, 23–40% had codon 918 mutations [3, 14, 15], but mutations in the cysteine-rich region were rare [4, 16]. Recently, a missense mutation at codon 768 in the tyrosine kinase domain of the *RET* proto-oncogene was also detected in 40% of sporadic MTCs which do not have codon 918 mutations [8]. In sporadic pheochromocytomas, no mutations except 1 mutation at codon 609 and 2 mutations at codon 620 in the cysteine-rich region have been reported [4, 7, 17]. Although Lindor *et al.* [17] reported a 6bp-deletion at codons 632 and 633 and a mutation at codon 925 in sporadic pheochromocytomas, the significance of these mutations in tumorigenesis of pheochromocytomas is unclear. We also found no mutations in the cysteine-rich region in 12 sporadic pheochromocytomas and 3 pheochromocytomas with NF1 [16]. Recently, we found codon 918 mutations in 31% of sporadic pheochromocytomas (cases 13–16 in Table 1 in this study), for which mutations were heterozygous and tumor-specific [9]. In this study, codon 768 mutations were shown to be rare in sporadic pheochromocytomas in contrast to sporadic MTCs. In addition, pheochromocytomas with NF1 and MEN 2A/2B were shown to have no mutations at codon 768.

Codon 768 was located in the subdomain III implicated in ATP binding within the tyrosine kinase domain of *RET* protein. The mutation of codon 768 from glutamine to aspartic acid would affect both substrate interactions and ATP-receptor binding [8]. Results obtained by Eng *et al.* [8] showed that the somatic mutation at codon 768 contributed to tumorigenesis of thyroid C-cells. The reason for the difference between the frequency of mutations at codon 768 in sporadic MTCs and pheochromocytomas was unknown. But all patients with MEN 2A and 2B develop MTCs, and approximately 50% of patients develop pheochromocytomas [18]. These results suggest that mutations of the *RET* proto-oncogene are not sufficient, and other genetic changes are required in the tumorigenesis of pheochromocytomas. This situation may be applicable to sporadic MTCs and

pheochromocytomas. In addition, it is possible that the frequency of MTCs and pheochromocytomas may also depend on the number of *RET*-positive cells and expression levels of *RET* protein in thyroid C-cells and adrenal chromaffin cells [19]. In future, the study on transgenic mice carrying the mutated *RET* proto-oncogene at codon 768 with tissue-specific promoters should provide further insight into the role of the *RET* proto-oncogene in the development of sporadic MTCs and pheochromocytomas.

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