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Alterations of *RET* Oncogene in Human Adrenal Tumors

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Previous studies have revealed specific activations of the *RET* oncogene in multiple endocrine neoplasia type 2 (MEN 2) and thyroid tumors. To understand the role of the *RET* proto-oncogene activation in sporadic adrenal tumors, we analyzed the alterations of the *RET* proto-oncogene in the cysteine-rich extracellular domain (exons 6 and 10), the terminal region of the extracellular domain and transmembrane domain (exon 11) and the tyrosine kinase domain (exons 12–17) in 35 cases of adrenal tumors (including 18 Conn's syndrome, 3 Cushing's syndrome, 2 non-functional adrenocortical tumor and 12 pheochromocytomas by polymerase chain reaction-single strand conformational polymorphism and sequencing methods. One case with pheochromocytoma and one with Conn's syndrome had point mutation. We also detected the rearrangement of the *RET* gene by reverse transcription-polymerase chain reaction and Southern hybridization. One case with Conn's syndrome and one with Cushing's syndrome were found to harbor RET/PTC1 (*RET* tyrosine kinase domain rearranged with *H4* gene). The above results indicate that *RET* proto-oncogene mutations and RET/PTC1 are involved in the pathogenesis of sporadic adrenal tumors. Mutations at codon 634 of the *RET* gene were also found in adrenal tumors. This suggests that the *RET* oncogene may also play a role in the tumorigenesis of adrenal tumors, and this possibility requires further investigation.

Key words: *RET* oncogene — Adrenal tumors

The *RET* gene was first identified as a proto-oncogene a decade ago on the basis of its ability to transform NIH 3T3 mouse cells in culture.[1)] The transforming sequences that were initially recovered actually represented the product of a rearrangement between *RET* and another gene that had occurred during the transfection assay. Sequence analysis of the *RET* proto-oncogene proved that it is a member of the tyrosine kinase receptor gene family.[2, 3)] The *RET* proto-oncogene has a calcium-binding cysteine-rich extracellular domain along with a cadherin-like ligand binding site, a tyrosine kinase-containing intracellular domain, and a short transmembrane domain.[4, 5)] Previous studies have shown that rearrangement of the *RET* gene in papillary thyroid carcinomas results in a protein with altered or novel tyrosine kinase function.[6, 7)] The portion of the *RET* gene encoding the tyrosine kinase domain is juxtaposed to sequences from one of three other genes through chromosomal rearrangements.[8, 9)] Two of the gene fusions result from rearrangement involving sequences on the same chromosome as *RET* (the PTC1 and PTC3 chimeras), and the third (PTC2) results from an interchromosomal rearrangement.[10–12)] The frequency of *RET* rearrangements differs in different geographical areas,[13–18)] but whether the differences can be ascribed to environmental factors, or merely to sampling errors or differences in the techniques used, remains to be elucidated. In addition, point mutation of the *RET* proto-oncogene has so far only been found in multiple endocrine neoplasia type 2A (MEN 2A), MEN 2B, familial medullary thyroid carcinoma (FMTC), sporadic MTC, sporadic pheochromocytomas and Hirschsprung's disease. The point mutation sites are located in codons specifying cysteine residues, tyrosine kinase domains and intracellular domains.[19)] According to the above studies, the *RET* proto-oncogene is specifically activated in endocrine tumors. Adrenal tumors are also a kind of endocrine tumor, but no study has yet indicated that *RET* oncogene activation occurs in adrenal tumors, except for pheochromocytoma. In general, functional adrenal tumors are small and characterized as benign, as well as being active hormone-producing tumors. Malignant functional tumors are relatively rare.[20, 21)] Presentation of typical feature of uncontrollable overproduction of hormones makes for easy clinical diagnosis of functional adrenal tumor with the use of modern biochemical analysis or imaging technology. Because functional tumors can be diagnosed very easily and resected when the tumor is small in size, they can be used for studies. In contrast, non-functional adrenal tumors are hard to obtain. We were able to collect 2 cases of non-functional adrenal tumor as controls for our study. In order to clarify the role of the *RET* gene in the tumorigenesis of human adrenal tumors, we performed molecular studies in 35 adrenal tumor tissues. Our results suggest that the RET gene may be related to the development of these tumors.

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MATERIALS AND METHODS

Patients and tissues Thirty-five adrenal tumors and their paired remnant normal adrenal tissues were obtained from patients who underwent surgery for adrenal lesions (Table I). These included 18 patients with primary aldosteronism, 3 patients with adrenal Cushing's syndrome, 12 patients with pheochromocytoma, and 2 patients with nonfunctional adrenal tumor. The diagnosis of primary aldosteronism was made on the basis of the clinical features, including hypertension, hypokalemia, suppression of the serum renin and the biochemical examination of increased aldosterone in blood. Further analysis by computer tomography proved the existence of unilateral adenoma. With regard to Cushing's syndrome, the diagnosis was made on the basis of the clinical features including obesity, hypertension, hirsutism, and facial plathora. Biochemical examination showed increased cortical secretion in blood. Computer tomographic scanning and pathologic findings confirmed the existence of adrenocortical tumor. The diagnosis of pheochromocytoma depended on the analysis of computer tomographic scans, pathological findings, and vanillylmandelic acid. All tissue samples were frozen at −80°C until analyzed.

DNA extraction Genomic DNA was extracted from tissues by proteinase-K digestion and phenol-chloroform extraction according to Bline and Stanffard.[22)]

Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis To search for mutations of the *RET* gene using PCR-SSCP analysis, 9 different sets of primers were prepared to amplify regions including all of the coding exons (exons 6, 10–17), as summarized in Table II. Oligonucleotides used as primers for PCR were synthesized based on the published *RET* gene sequence.[3)] The reaction mixture contained 50 pmol of each primer, 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany), 100 m*M* of each deoxy-nucleotide 5′-triphosphate (NTP), [α-^{32}P]deoxy-cytidine 5′-triphosphate (3000 Ci/mmol, 10 mCi/ml, New England Nuclear Research Products, Boston, MA), 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.3), and gelatin at 10 μg/ml. A programmable thermal cycler (PTC-100, MJ Research, Watertown, MA) was used to perform 35 cycles of denaturation for 15 s each at 94°C and annealing for 15 s at 55°C for exons 6, 10, 12, 14–17, or at 60°C for exons 11 and 13, with an extension for an additional 30 s at 72°C. The total final extension time was 5 min at 72°C. The PCR-SSCP reactions were electrophoresed on 6% neutral polyacrylamide gels.

Cloning and sequencing Sequence analysis of PCR-amplified *RET* exons 6 and 11 was performed after subcloning the amplified fragment in pCR-Script cloning vector (Stratagene, La Jolla, CA). Dideoxynucleotide sequencing was done with a Sequenase kit (U.S. Biochemical, Cleveland, OH), using [α-^{35}S]dATP (10 μCi/μl, New England Nuclear Research Products). Aliquots of the sequencing reaction mixtures were electrophoresed on 8% denaturing polyacrylamide gels. For accuracy, we collected 10 independent clones and performed bidirectional sequencing with the T3 and T7 sequencing primers.

RT (reverse transcription)-PCR The total RNA was isolated using the acid-guanidine isothiocyanate-phenol-chloroform method[23)] and used as a template for cDNA

Table I. Alterations of the *RET* Oncogene in Human Adrenal Tumors

P't	Sex	Disease	*RET*		Rearranged form
			Codon/Base	Amino acid	
1	F	Conn	N	—	N
2	M	Conn	N	—	ND
3	M	Conn	N	—	ND
4	M	Conn	N	—	N
5	F	Conn	N	—	ND
6	M	Conn	634/TGC→TGG	Cys→Trp	N
7	F	Conn	N	—	N
8	F	Conn	N	—	N
9	M	Conn	N	—	ND
10	F	Conn	N	—	N
11	F	Conn	N	—	N
12	F	Conn	N	—	N
13	F	Conn	N	—	N
14	M	Conn	N	—	N
15	M	Conn	N	—	N
16	F	Conn	N	—	RET/PTC1
17	M	Conn	N	—	ND
18	F	Conn	N	—	ND
19	F	Cushing	N	—	RET/PTC1
20	F	Cushing	N	—	N
21	F	Cushing	N	—	ND
22	M	NFA	N	—	ND
23	F	NFA	N	—	N
24	F	pheo	N	—	ND
25	F	pheo	N	—	ND
26	M	pheo	N	—	ND
27	F	pheo	N	—	ND
28	F	pheo	N	—	N
29	F	pheo	393/TTC→TCC 634/TGC→TGG	Phe→Ser Cys→Trp	N
30	F	pheo	N	—	ND
31	F	pheo	N	—	N
32	M	pheo	N	—	N
33	M	pheo	N	—	ND
34	M	pheo	N	—	ND
35	M	pheo	N	—	N

N, normal; ND, not detected; Conn, Conn's syndrome; Cushing, Cushing's syndrome; Pheo, pheochromocytoma; NFA, non-functional adrenal tumor.

Table II. PCR-SSCP Primers for *RET* Gene Analysis

	Forward primer	Reverse primer
Exon 6	5′-TGG TCA ATG ACT CAG ACT TC-3′	5′-ATC TGG GCA AAT CGG CGA GC-3′
Exon 10	5′-ATT AAA GCT GGC TAT GGC AC-3′	5′-CAC TCA CCC TGG ATG TCT TC-3′
Exon 11	5′-ATC CAC TGT GCG ACG AGC TG-3′	5′-GAA GGT CAT CTC AGC TGA GG-3′
Exon 12	5′-TTT CCA ACA TAG GAG GAT CC-3′	5′-CCT GGC AGG TAC CTT TCA GC-3′
Exon 13	5′-TGT GCT GCA TTT CAG AGA AC-3′	5′-TGG CCT TAC CAT CCT GGC-3′
Exon 14	5′-TCC TCC TCA TCG TGG AGT AC-3′	5′-TAT GCA CGC ACC TTC ATC TC-3′
Exon 15	5′-TCC TCA CAG CTC GTT CAT CG-3′	5′-CTC CTC TTC ACG TAG GAA TC-3′
Exon 16	5′-TCT CTT TAG GGA CGG ATT CC-3′	5′-CAC ACT TAC ACA TCA CTT TG-3′
Exon 17	5′-TTC ACT CTC TGC AGA TGG TC-3′	5′-CTC GCT GCA GTT GTC TGG CC-3′

Table III. Primers for Analysis of *RET* Gene Rearrangement

	Forward primer	Reverse primer	Size
RET/PTC1	5′-ACT GAA GTG CAA GGC ACT-3′	5′-AAG TTC TTC CGA GGG AAT TC-3′	96 bp
RET/PTC2	5′-AAG CAA ACC TGC CAG TGG-3′	5′-CTT TCA GCA TCT TCA CGG-3′	363 bp
RET/PTC3	5′-CAT GCC AGA GCA GAA GTC A-3′	5′-CTG CTT CAG GAC GTT GAA-3′	241 bp
c-*raf*-1	5′-GAT TTC CTG GAT CAT GTT-3′	5′-GCT GGC ACG GGG GTT TTC-3′	536 bp

synthesisusing 2 μg of total RNA in a 10 μl reaction mixture containing 10 pmol Oligo (dT)-15mer primer (Boehringer Mannheim GmbH), 10 m*M* dithiothreitol, 0.5 m*M* deoxy-NTP mix, acetylated bovine serum albumin (0.1 mg/ml), and reverse transcriptase from Moloney murine leukemia virus. Rearranged forms of the *RET* gene were amplified by using PCR with specific primers for RET/PTC1, RET/PTC2 and RET/PTC3 (Table III) and *Taq* DNA polymerase (Boehringer Mannheim GmbH).

Southern analysis Purified genomic DNA was digested with the restriction enzyme *Hin*dIII, *Eco*RI, or *Bam*HI for 6 h at 37°C. The digested DNA was size-fractionated on 1% agarose gel and transferred to a transfer membrane (Schleicher and Schuell, Dassel, Germany). The membrane was hybridized with a fluorescein-labeled 1 kb *Bam*HI-*Bgl*II DNA fragment of *RET* gene as a probe in Express Hyb solution (Clontech Laboratories Inc., CA) for 1 h at 60°C. This probe, located between the transmembrane and tyrosine kinase domains of the *RET* gene, is able to detect the region within the *RET* gene where the rearrangement can occur.[24] After high stringency washing, the membrane was exposed to Kodak XAR autoradiographic film (Eastman Kodak, Rochester, NY) according to the standard method.[25]

RESULTS

PCR-SSCP One of 12 (8.3%) pheochromocytomas and 1 of 18 (5.6%) patients with primary aldosteronism showed an apparent electrophoretic mobility shift between the tumor and its paired adjacent normal tissue (Fig. 1), implying the existence of a mutation. Such differences were detected in exon 6 (1 case) and exon 11 (2 cases). A mobility shift of the amplified exon 11 could be clearly detected in cases 6 and 29 (Fig. 1, B and C). In addition, a mobility shift of the amplified exon 6 was detected in case 29 (Fig. 1A). All cases with mutation still showed a normal allelic band. In total, 2 of 35 (5.7%) cases of adrenal tumors had conformational alteration.

Sequence analysis To examine the type of mutation, the exon 6 and 11 region was cloned from tumor specimens of case 6 and case 29. The sequencing data revealed a substitution from phenylalanine (TTC) to serine (TCC) at codon 393 (exon 6) in case 29 and a substitution from cysteine (TGC) to tryptophan (TGG) at codon 634 (exon 11) in cases 6 and 29 (Fig. 2). The results of bidirectional sequencing of 10 independent clones confirmed that these sites were mutated in the adrenal tumor specimens we collected.

RT-PCR To investigate whether *RET* gene rearrangement forms, including RET/PTC1, RET/PTC2 and RET/PTC3, were also present in adrenal tumors, we performed RT-PCR on 20 adrenal tumor samples. These samples were collected from 12 patients with Conn's syndrome, 2 patients with Cushing's syndrome, 5 patients with pheochromocytoma and 1 patient with non-functional adrenal tumor. In all the cases studied before with *RET* gene rearrangement, the breakpoint of the *RET* gene occurred in an intronic sequence between the tyrosine-kinase and transmembrane encoding domains. This rearrangement was

screened by amplification of a cDNA fragment including the chimeric point. A 96 bp fragment of RET/PTC1, a 363 bp fragment of RET/PTC2 and a 241 bp fragment of RET/PTC3 were amplified. Among 20 cases, we found RET/PTC1 present in cases 16 and 19 (1 Conn's syndrome and 1 Cushing syndrome). The cDNA of c-*raf*-1 was also amplified to evaluate the quality of each RNA sample (Fig. 3). RET/PTC2 and RET/PTC3 were not found in any of the tested samples.

Southern blot analysis To confirm the presence of RET/PTC1 in cases 16 and 19 and to rule out the possibility of errors in PCR, we performed Southern blot hybridization using a 1 kb *Bgl*II-*Bam*HI *RET* specific DNA fragment as the probe. This fragment was able to detect the region within the *RET* gene where the rearrangement had occurred. This probe detected a 6.3 kb fragment after digestion with *Eco*RI, a 3.7 kb fragment after digestion with *Bam*HI, and a 9.3 kb fragment after digestion with

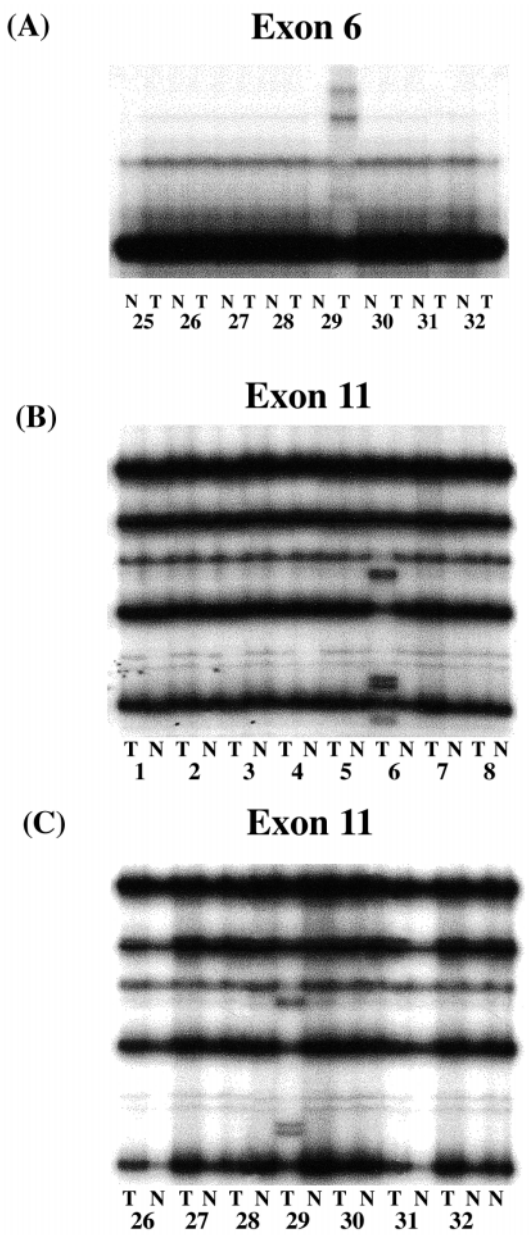

Fig. 1. PCR-SSCP analysis of *RET* oncogene mutations in human adrenal tumors. Representative samples are shown for exon 6 (A) and exon 11 (B and C). An electrophoretic mobility shift of the bands between the tumor (T) and its paired normal tissue (N) implies a different conformation of the fragment, suggesting the presence of mutation in these exons. The PCR-SSCP analysis of exons 11 and 6 showed an apparent mobility shift in cases 6 and 29.

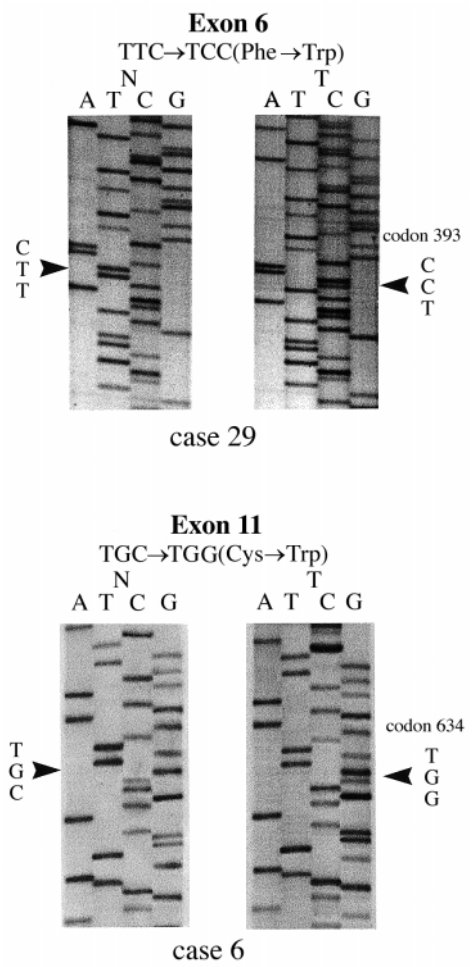

Fig. 2. Sequencing analysis of the *RET* oncogene (exons 11 and 6) in human adrenal tumor. TGC→TGG (Cys→Trp) and TTC→TCC (Phe→Ser) changes were found at codon 634 and codon 393.

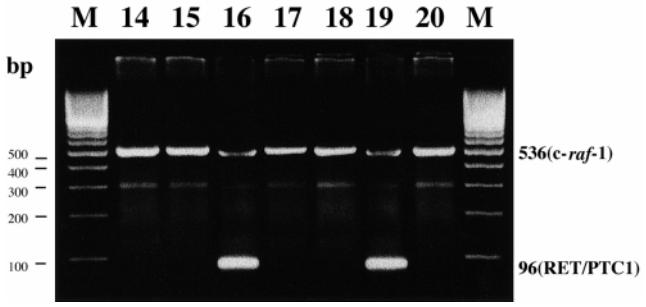

Fig. 3. Representative results of RT-PCR. For evaluating the quality of each RNA, a part of the c-*raf*-1 gene was amplified. RET/PTC1 was detected in tumor tissue of cases 16 and 19.

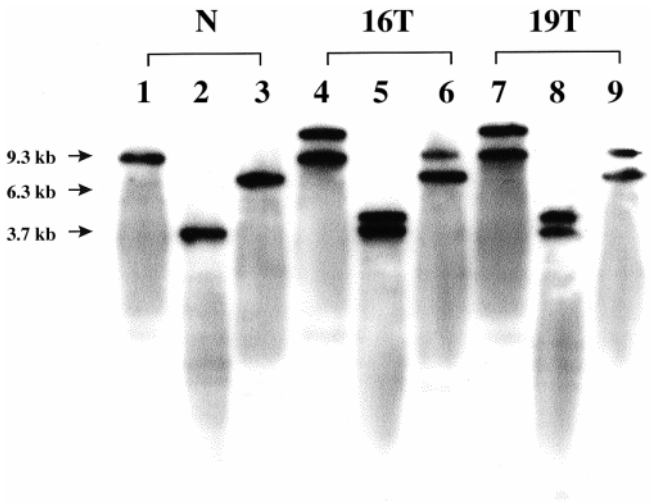

Fig. 4. Activation of the *RET* oncogene (RET/PTC1) in adrenal tumors. Genomic DNA extract from normal adrenal gland of case 1 and tumor tissue of cases 16 and 19 were digested with *Eco*RI (lanes 3, 6, 9), *Bam*HI (lanes 2, 4, 6), or *Hin*dIII (lanes 1, 4, 7) and hybridized to a 1.0 kb *Bam*HI-*Bgl*II *RET*-specific DNA probe. Both *RET* proto-oncogene and the RET/PTC rearrangement band were seen in tumor tissue of cases 16 and 19, indicating that the tumors are both heterozygotic for the rearrangement.

*Hin*dIII in normal human DNA. Rearranged bands were found in cases 16 and 19 (Fig. 4).

DISCUSSION

A number of studies have been done on the alterations of the *RET* gene in MEN2A and MEN2B. They have shown that 95% of the *RET* mutations occur in exons 10 and 11 in MEN2A, whereas more than 90% of them occur in exon 16 (codon 918) in MEN2B.[26] Recent studies have revealed that the frequency of *RET* gene mutation was 97% and the mutation hot spot was at codon 634 (86%) in MEN2A.[27, 28] This suggests that mutation at codon 634 is associated with the transforming ability of the *RET* gene. Furthermore, Santoro *et al.*[29] have indicated that the dimerization of the *RET* oncoprotein caused the activation of the *RET* gene in MEN2A. For the above reasons, the *RET* gene plays an important role in MEN2A. To understand the role of the *RET* gene in adrenal tumors, we examined 18 cases with Conn's syndrome, 3 cases with Cushing's syndrome, and 12 cases with pheochromocytoma. The results showed that 2 patients, one with Conn's syndrome and the other with pheochromocytoma, had *RET* gene mutations at codon 634, resulting in a cysteine-to-tryptophan substitution. Codon 634 is located in the extracellular domain close to the transmembrane domain. Mutations of codon 634 may influence the structure of the *RET* protein receptor,[29] leading to loss of specificity of ligand binding or activation of the *RET* protein in the absence of ligand binding. Uncontrolled *RET* protein activation would contribute to cell malignancy. This suggests that the mutations at codon 634 of the *RET* gene may be involved in the development of adrenal tumors. On the other hand, one of the 2 cases showed a phenylalanine-to-serine substitution at codon 393, which was also found in Hirschsprung's disease. In addition, a loss-of-function effect of missense mutation at codon 393 of the *RET* protein has been demonstrated.[30, 31] With regard to rearrangement of the *RET* oncogene, this was found in thyroid carcinomas, with most of them being RET/PTC1. RET/PTC1 is the rearrangement between the *RET* gene and H4 (D10S170) gene. The chimera point is located at 1843 bp of the *RET* gene, flanking intron 11, which is upstream of the tyrosine kinase domain. RET/PTC1 was found in most thyroid tumors. The prevalence of RET/PTC1 varies in different geographical areas and in different ethnic populations.[6–18] Previous studies have suggested that RET/PTC1 rearrangement may induce activation of the *RET* oncogene in three situations.[9] 1. When the rearrangement occurs at the N-terminus, the ligand binding sites change, so the foreign gene insertion might activate *RET* tyrosine kinase and cause the *RET* oncoprotein activation. 2. When the replaced region of the *RET* gene contains a transmembrane domain, the rearrangement will change the domain to a free protein in the cytoplasm, so the protein can easily be activated by other proteins. 3. When deletion of the *RET* protein receptor occurs, RET/PTC1 might be autophosphorylated without any ligand stimulation. These hypotheses need to be studied further to determine which is the real mechanism of *RET* oncogene activation. In this study, we found that RET/PTC1 was also present in adrenal tumors, indicating that rearrangement of the *RET* oncogene also plays a role in tumorigenesis in adrenal tumors. *RET* gene mutation and rearrangement were not

found in many of the cases we analyzed; however, it is known that the mutation site (codon 634) and rearrangement form (RET/PTC1) are associated with the transforming activity of the *RET* gene. This indicates that alterations of the *RET* gene may contribute to adrenal tumor development. Additionally, our results show that the occurrence of the *RET* gene mutation and rearrangement are not associated with the tumor size, indicating that they may be initial events in adrenal tumor development. Furthermore, numerous studies have shown that the *RET* proto-oncogene is involved in neurodifferentiation and the nervous system is associated with the secretion of adrenocortical cells. Whether the *RET* gene also plays a crucial role in hormone overexpression is something we are still investigating.

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