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NOTE

Infrequent Detectable Somatic Mutations of the RET and Glial Cell Line-derived Neurotrophic Factor (GDNF) Genes in Human Pituitary Adenomas

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Abstract. RET is a receptor tyrosine kinase expressed in neuroendocrine cells and tumors. RET is activated by a ligand complex comprising glial cell line-derived neurotrophic factor (GDNF) and GDNF receptor-α (GDNFR-α). Activating mutations of the RET proto-oncogene were found in multiple endocrine neoplasia (MEN) 2 and in sporadic medullary thyroid carcinoma and pheochromocytoma of neuroendocrine origin. Mutations of the RET proto-oncogene and the glial cell line-derived neurotrophic factor (GDNF) gene were examined in human pituitary tumors. No mutations of the RET proto-oncogene including the cysteine-rich region or codon 768 and 918 in the tyrosine kinase domain were detected in 172 human pituitary adenomas either by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) or by PCR-restriction fragment length polymorphism (RFLP). Further, somatic mutations of the GDNF gene in 33 human pituitary adenomas were not detected by PCR-SSCP. One polymorphism of the GDNF gene at codon 145 of TGC or TGT was observed in a prolactinoma. The RET proto-oncogene message was detected in a normal human pituitary gland or 4 of 4 human pituitary adenomas with reverse transcription (RT)-PCR, and in rodent pituitary tumor cell lines with Western blotting. The expression of GDNF gene was detected in 1 of 4 human somatotroph adenomas, 1 of 2 corticotroph adenomas, and 2 of 6 rodent pituitary tumor cell lines with RT-PCR. Based on these, it is concluded that somatic mutations of the RET proto-oncogene or the GDNF gene do not appear to play a major role in the pituitary tumorigenesis in examined tumors.

Key words: RET proto-oncogene, Glial cell line-derived neurotrophic factor (GDNF) gene, Mutations, Expression, Pituitary adenomas

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MEN 2A and 2B has been shown to be caused by specific mutations of the RET proto-oncogene [1]. Nonconservative substitution of the cysteine residues located in the extracellular domain adjacent to the transmembrane segment of the RET protein is responsible for MEN 2A and familial medullary thyroid carcinoma (FMTC) [1]. MEN 2B is caused by a mutation causing the substitution at codon 918 (methionine to threonine) within exon 16 of the tyrosine kinase domain of the RET protein at the germline level [1]. A missense mutation at codon 768 or 804 in the tyrosine kinase domain of the RET proto-oncogene was recently described in families with FMTC [1].

Glial cell line-derived neurotrophic factor (GDNF) is one of natural ligands of the RET and acts via a multimeric receptor complex, which includes a GDNFR-α [2]. It has been shown that

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GDNF is the ligand for a heterotetrameric complex of RET and GDNFR-α. GDNF knockout mice exhibit a similar phenotype to that of RET knockout mice such as renal agenesis and absence of enteric neurons [3]. In some cases of Hirschsprung disease, germline GDNF mutations with loss of function were reported [1, 4]. Because both GDNF and GDNFR-α are involved in the RET signaling pathway in addition to neurturin (NTN) and NTN receptor-α [5], their gain of function mutations represent candidates for the pathogenesis of sporadic neuroendocrine tumors.

The gene expression of GDNF and GDNFR-α was detected in epithelial cells of Rathke's pouch [6] and the embryonic rat pituitary gland [3], respectively. RET mRNA was found at a high level in pheochromocytomas, medullary thyroid carcinomas (MTCs) and neuroblastomas [7, 8]. Expression of the RET proto-oncogene was detected in the normal tissue derived from the neural crest [9]. Pituitary tumors are typical neuroendocrine tumors as pheochromocytomas, MTCs, neuroblastomas, paragangliomas, small cell lung carcinomas, gastrointestinal neuroendocrine tumors and pancreatic neuroendocrine tumors. Although somatic mutations of the RET proto-oncogene were detected in a subset of sporadic pheochromocytomas, MTCs, small cell lung carcinomas and neuroblastomas which express the RET proto-oncogene at a high level do not have mutations [1, 10–12].

To investigate the role of the abnormal RET signaling pathway including GDNF for the pathogenesis of pituitary tumors, we screened for mutations in the cysteine-rich regions and tyrosine kinase domain of the RET proto-oncogene and the GDNF gene in 172 and 33 human pituitary adenomas, respectively. We further examined the expression of the RET proto-oncogene and GDNF gene in the human pituitary gland, human pituitary adenomas, and rodent pituitary tumor cell lines.

Materials and Methods

Tissue samples

One hundred seventy-two pituitary adenoma tissues were obtained at the time of transsphenoidal surgery or paraffin-embedded sections. All tissues were fixed in formalin and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin for histological evaluation and analyzed for immunoperoxidase staining with antibodies to human GH, PRL, ACTH, βTSH, βFSH, βLH and α-subunit of glycoprotein hormones, as previously described [13]. The types of 172 human pituitary adenomas examined in this study are listed in Table 1. Peripheral blood samples were collected at or after surgery.

Cell lines

TT (human MTC), neuro2a (mouse neuroblastoma), NIH/3T3 (mouse fibroblast), AtT20 (mouse ACTH-secreting pituitary tumor), GH1 (rat GH-secreting pituitary tumor), GH3 (rat GH/PRL-secreting pituitary tumor), MtT/S (rat GH-secreting pituitary tumor), MtT/SM (rat GH/PRL-secreting pituitary tumor), αTSH (mouse thyrotroph tumor) and αT3-1 (mouse gonadotroph tumor) cell lines were cultured in DMEM medium supplemented with 10% fetal calf serum. Cell lines of AtT20, GH1 and GH3 were supplied by the Japanese Cancer Research Resources Bank. Cell lines of MtT/S and MtT/SM were obtained from RIKEN Cell Bank. Cell lines of αTSH and αT3-1 were provided by Dr. Mellon of the University of California, San Diego. A cell line of neuro2a was provided by Dr. Takahashi of the University of Nagoya, Aichi, Japan.

DNA preparation and mutation analysis

DNA was isolated from frozen tumor sections obtained at surgical operation, leukocytes and paraffin-embedded specimens, as previously described [14]. Mutation analyses of the RET proto-oncogene and GDNF gene were performed on 172 and 33 human pituitary adenomas, respectively. PCR amplification was performed with the oligonucleotide primers shown in Table 2. Amplified DNA fragments were all of expected sizes. PCR proceeded in a 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-^{32}P]dCTP (3,000 Ci/mmol; 10 mCi/ml). The PCR products in 5 μl were heated with 3 μl of dye solution (66% formamide/167 mM sodium hydroxide/17 mM EDTA/0.03%

Table 1. Summary of the mutations and expression of the RET proto-oncogene and GDNF gene in human pituitary adenomas

Types of pituitary adenomas	Positive numbers/Numbers analyzed
RET mutations	
GH producing adenomas	0/93
mixed GH/PRL producing adenomas	0/11
PRL producing adenomas	0/16
TSH producing adenomas	0/16
ACTH producing adenomas	0/10
non-functioning adenomas	0/26
RET expression by RT-PCR	
normal pituitary gland	1/1
GH producing adenomas	1/1
PRL producing adenomas	1/1
TSH producing adenomas	1/1
non-functioning adenomas	1/1
GDNF mutations	
GH producing adenomas	0/12
mixed GH/PRL producing adenomas	0/2
PRL producing adenomas	1*/6
TSH producing adenomas	0/2
ACTH producing adenomas	0/1
non-functioning adenomas	0/10
GDNF expression by RT-PCR	
GH producing adenomas	1/4
ACTH producing adenomas	1/2
non-functioning adenomas	0/2

* The silent mutation (TGC to TGT, cysteine to cysteine) was also found in her leukocytes.

bromophenol blue/0.03% xylene cyanol), and then 1 μl of the mixture was applied to two 5% polyacrylamide gels containing 0 or 5% glycerol. 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 to 24 h. The method of DNA sequencing showing aberrantly shifted bands in PCR-SSCP was described previously [15]. The PCR products were digested with *Alu*I for codon 768 or *Fok*I 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.

RT-PCR

For RNA study, mouse pituitary glands, a human pituitary gland obtained at autopsy, human pituitary adenomas and rodent pituitary tumor cell lines of AtT20, GH1, MtT/S, GH3, αTSH and αT3-1 were snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated with guanidium isothiocyanate followed by the phenol-chloroform method [16]. cDNA was produced from 2 μg of total RNA with MMLV reverse transcriptase (Promega, Madison, WI) and random hexamers. The cDNAs were then amplified with PCR in 30 cycles. The primer pairs which flank at least one intron were designed to avoid the amplification from the contaminated DNA (Table 2). The PCR products were electrophoresed on a 10% polyacrylamide gel, followed by ethidium bromide

Table 2. PCR primers used in this study

Primer sequence	PCR product (bp)	Annealing temp
PCR-SSCP		
The RET proto-oncogene		
exon 10		
5′-GCGCCCCAGGAGGCTGAGTG-3′	187 bp	68 °C
5′-CGTGGTGGTCCCGGCCGCC-3′		
exon 11		
5′-CCTCTGCGGTGCCAAGCCTC-3′	234 bp	65 °C
5′-CACCGGAAGAGGAGTAGCTG-3′		
The GDNF gene*		
exon 1		
5′-TTCTCTCCCCCACCTCCCGCC-3′	223 bp	58 °C
5′-GGAACGGTTCTTACAGTCACT-3′		
exon 2a		
5′-CAAATATGCCAGAGGATTATC-3′	270 bp	58 °C
5′-TCAGTTCCTCCTTGGTTTCAT-3′		
exon 2b		
5′-CATTTAAATGTCACTGACTTG-3′	267 bp	58 °C
5′-TCAGATACATCCACACCTTTT-3′		
PCR-RFLP		
codon 768 of the RET proto-oncogene		
5′-TCCAGGAGCGATCGTTTGCA-3′	121 bp	60°C
5′-GACATGTGGGTGGTTGACCT-3′		
codon 918 of the RET proto-oncogene		
5′-AGGGATAGGGCCTGGGCTTC-3′	192 bp	60 °C
5′-TAACCTCCACCCCAAGAGAG-3′		
RT-PCR		
The RET proto-oncogene		
human		
5′-GGGGGATTAAAGCTGGCATA-3′(exon 10)	203 bp	60 °C
5′-TGGCTTGTGGGCAAACTTGT-3′ (exon 11)		
mouse		
5′-TTCGGGCAACCATGCACAAT-3′ (exon 5)	322 bp	60 °C
5′-CCTAGGGCAGTGCAGTTGAT-3′ (exon 7)		
The GDNF gene		
human, mouse, rat		
5′-AAGTTATGGGATGTCGTGGC-3′ (exon 1)	237 bp	60 °C
5′-ATCTGGTGACCTTTTCAGTC-3′ (exon 2)	(157 bp)	

*DNA sequences were derived from GenBank accession numbers L19062 and L19063, except exon 1 the sequence of which was derived from Angrist *et al*, 1996, reference 4.

staining. The gels were photographed with an ultraviolet transilluminator. Negative controls included PCR with samples without RT or a water control instead of cDNA as templates in PCR.

Western blotting

Expression of RET protein in mouse, rat and human pituitary glands, and rodent pituitary tumor cell lines of AtT20, GH1, MtT/S, GH3, MtT/SM, αTSH and αT3–1 was analyzed with neuro2a and TT as positive controls. Cells were solubilized on ice in lysis buffer containing phosphate-buffered saline, 1% Triton X-100, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 50 mg/L leupeptin and 50 mg/L aprotinin. The lysates were

separated by 7–15% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and immunoblotted with affinity-purified anti-RET polyclonal antibody (IBL, Fujioka, Gunma, Japan) [17]. Immunoreactive bands were visualized with a horseradish peroxidase conjugate anti-rabbit antiserum and ECL™ detection reagents (Amersham, Bucks, UK).

Results

Mutations of the RET proto-oncogene in human pituitary adenomas

Genomic DNAs obtained from human pituitary adenomas were tested for mutations within exons 10 and 11 including the cysteine-rich region 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. No extra bands were detected by PCR-SSCP of exons 10 and 11 in 2 different electrophoresis conditions in any tumor examined. The mutations of codon 768 (GAG to GAC) and codon 918 (ATG to ACG) cause the loss of an *Alu*I and a *Fok*I restriction site, respectively. No mutation causing the loss of an *Alu*I restriction site at codon 768 or of a *Fok*I restriction site at codon 918 was detected.

Mutations of the GDNF gene in human pituitary adenomas

PCR-SSCP analysis of exon 2b of the GDNF gene showed an extra band relative to those amplified from leukocytes of healthy subjects in one prolactinoma (Fig. 1A). A silent mutation of TGC or TGT coding for cysteine at codon 145 was observed in DNAs from the prolactinoma (Fig. 1B) and the patient's leukocytes (data not shown). No

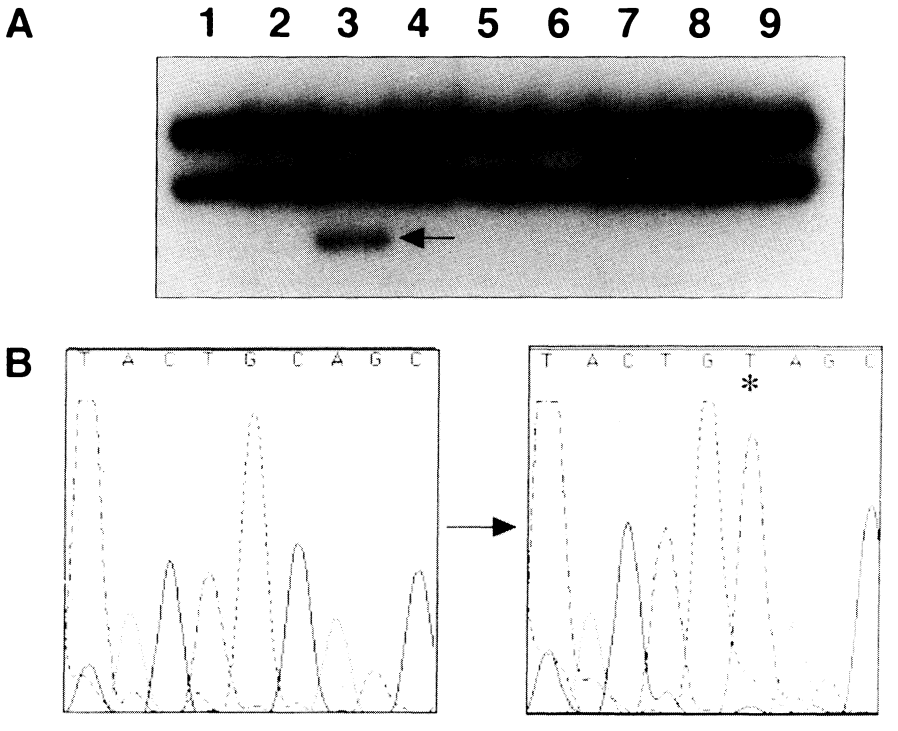

Fig. 1. PCR-SSCP analysis and nucleotide sequence analysis of exon 2b of the GDNF gene in human pituitary adenomas. A. Electrophoresis was performed in an 8% polyacrylamide gel with 5% glycerol at room temperature. Lane 1, leukocytes from normal subjects; lanes 2–9, pituitary adenomas. An extra band in lane 3 is indicated with an arrow. B. The left panel shows the normal sequence of codons 144–146 of the human GDNF gene. The right panel shows the sequence of the variant SSCP allele in a prolactinoma with C to T transition at codon 145. A mutated base is indicated by an asterisk.

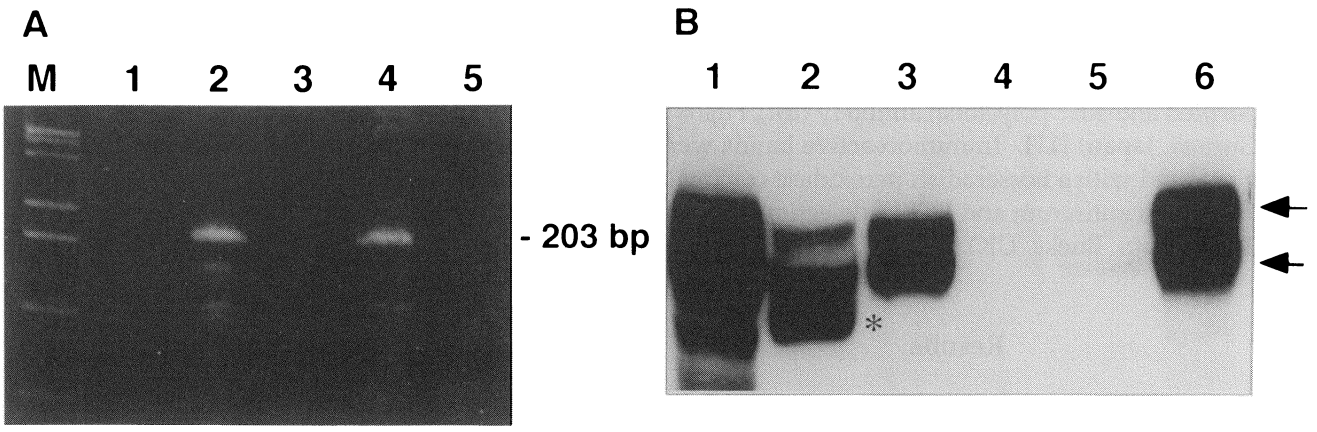

Fig. 2. Expression of the RET proto-oncogene in the pituitary gland, pituitary adenomas and rodent pituitary tumor cell lines. A: RT-PCR detection of transcripts of the RET proto-oncogene. Total RNA extracted from the human pituitary gland and a human somatotroph adenoma was reverse-transcribed, and 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, ØX174 *Hae*III-digested DNA fragments used as molecular markers; lane 1, template free; lane 2, RT treatment of the human pituitary gland; lane 3, no RT treatment of the human pituitary gland; lane 4, RT treatment of a human somatotroph adenoma; lane 5, no RT treatment of a human somatotroph adenoma. B: Detection of RET protein by western blotting. Cell lysates from cell lines from pituitary tumors were incubated with the anti-RET and a horseradish peroxidase conjugate anti-rabbit antiserum. Lane 1, a human MTC cell line, TT cell; lane 2, a mouse ACTH-secreting cell line, AtT20 cell; line 3, a rat GH-secreting cell line, MtT/S; lane 4, mouse αTSH of a thyrotroph cell line; lane 5, mouse αT3–1 of a gonadotroph cell line; lane 6, a mouse neuroblastoma cell line, neuro2α. The 170 and 150 kDa RET proteins are indicated with arrows. A 130 kDa protein indicated with an asterisk in AtT20 cells is supposed to be a degradated product of RET proteins.

abnormal band shift in exon 1 or 2a of the GDNF gene was observed in any sample. Two samples were sequenced for each exon, and no mutations was found.

Expression of the RET proto-oncogene in pituitary tumors

To examine the expression of the RET proto-oncogene in the pituitary gland and pituitary tumors, we extracted RNA from the mouse pituitary glands, the human pituitary gland, human pituitary adenomas including somatotroph adenoma, lactotroph adenoma, thyrotroph adenoma, corticotroph adenoma, non-functioning adenoma, and an AtT20 cell line (Table 1). RT-PCR of RNA derived from all of these tissues and the cell line revealed transcript signals of the predicted size of 203 bp for human and 322 bp for mouse of which the sequences were identical to the published sequences of the RET proto-oncogene (GenBank Accession Numbers, X12949, M57464 and X67812). Representative results in a normal human pituitary gland and a human somatotroph adenoma are shown in Fig. 2A. RET proto-oncogene transcripts were not detected in NIH/3T3 cells even by the RT-PCR method.

The antibody detected RET protein of 170 kDa (a glycosylated form) and 150 kDa (a non-glycosylated form) in an MtT/S cell line and an AtT20 cell line (Fig. 2B). The quantitation analysis of the 170 kDa protein in MtT/S and AtT20 cell lines showed 3 and 17% of the 170 kDa protein in TT cells, respectively. The lower levels of RET protein expression compared to MtT/S and AtT20 cell lines were detected in an αTSH cell line and an αT3-1 cell line. Western blotting with crude plasma membrane showed positive signals in αTSH cells and αT3-1 cells (data not shown). RET protein was not detected in those of the mouse pituitary glands, the rat pituitary gland, the human pituitary gland or cell lines of GH1, GH3 or MtT/SM (data not shown).

Identification of expression of the GDNF gene in pituitary tumors by RT-PCR

RT-PCR of human pituitary adenomas and

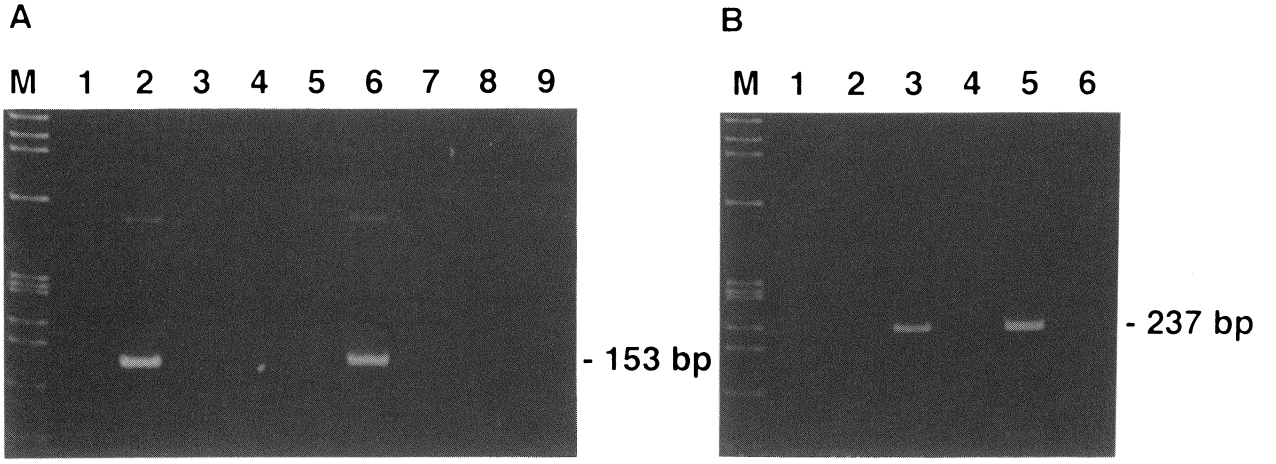

Fig. 3. Gene expression of the GDNF gene in human pituitary adenomas and rodent pituitary tumor cell lines. RT-PCR detection of transcripts of the human and rodent GDNF gene. Total RNA extracted from human pituitary adenomas and rodent pituitary tumor cell lines was reverse-transcribed, and the resulting products were amplified by PCR with the primers shown in Table 1. The PCR products were electrophoresed on a polyacrylamide gel and stained with ethidium bromide. A. RT-PCR detection of transcripts of the human GDNF gene. M, ØX174 *Hae*III-digested DNA fragments used as molecular markers; lane 1, template free; lane 2, RT treatment of a human somatotroph adenoma; lane 3, no RT treatment of a human somatotroph adenoma; lane 4, RT treatment of another human somatotroph adenoma; lane 5, no RT treatment of another human somatotroph adenoma; lane 6, RT treatment of a human corticotroph adenoma; lane 7, no RT treatment of a human corticotroph adenoma; lane 8, RT treatment of another human corticotroph adenoma; lane 5, no RT treatment of another human corticotroph adenoma. B. RT-PCR detection of transcripts of the rodent GDNF gene. M, ØX174 *Hae*III-digested DNA fragments used as molecular markers; lane 1, RT treatment of GH1 cells; lane 2, no RT treatment of GH1 cells; lane 3, RT treatment of AtT20 cells; lane 4, no RT treatment of AtT20 cells; lane 5, RT treatment of αT3-1 cells; lane 6, no RT treatment of αT3–1 cells.

rodent pituitary tumor cell lines revealed a transcript signal of a 237 bp GDNF or a 153 bp splicing variant GDNF in 1 of 4 human somatotroph adenomas and 1 of 2 corticotroph adenomas, AtT20 cells and αT3-1 cells (Fig. 2 and Table 2), the sequences of which were identical to the published sequences of the GDNF genes [18].

Discussion

Numerous point mutations of the RET proto-oncogene have recently been identified in association with MEN 2A, FMTC and MEN 2B [1]. These mutations in the cysteine-rich regions induce ligand-independent dimerization of the RET protein, leading to the activation of tyrosine kinase [19, 20]. The codon 918 mutation alters RET catalytic properties both quantitatively and qualitatively, and results in the constitutive activation of tyrosine kinase [19].

We looked for RET mutations in 172 human pituitary adenomas, but no mutation was found. Our results confirmed the report by Komminoth *et al.* [10] that RET mutations were not detected in 8 human pituitary adenomas. Because the sensitivity of SSCP analysis is less than 100% [21], we could not completely rule out the existence of mutations in exons 10 and 11 or in unexamined exons of the RET proto-oncogene.

Transcription of the RET proto-oncogene was found preferentially in neuroblastoma, pheochromocytoma and MTC, all of which originate in neural crest cells [7, 8]. Pachnis *et al.* [9] reported that RET proto-oncogene is expressed predominantly in the developing 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 [9, 22]. Recently we demonstrated the expression of the RET proto-oncogene in parathyroid tumors with RT-PCR and western blotting [23]. With regard to pituitary tumors as one type of typical neuroendocrine tumor, we detected the expression of the RET proto-oncogene in mouse pituitary gland, a human

pituitary gland and 4 of 4 human pituitary adenomas examined by RT-PCR. Levels of RET protein varied in pituitary tumor cell lines from rodents. Different levels of the expression of RET protein in pituitary cell lines from rodents may be related to secretory activity of hormones.

In addition a GDNFR-α, which forms a complex with RET protein for GDNF binding, was recently found to be expressed in an embryonic rat pituitary gland [2]. We detected expression of the GDNFR-α gene in rat pituitary tumor cell lines including GH1, GH3 and MtT/S by RT-PCR (unpublished results). The GDNF gene, the ligand of RET/GDNFR-α complex, was reported to be expressed in epithelial cells of Rathke's pouch [6]. We detected the gene expression of GDNF in AtT20 cells, αT3-1 cells, 1 of 4 human somatotroph adenomas and 1 of 2 corticotroph adenomas. Although GDNF was expressed in 1 of 4 human somatotroph adenomas and 1 of 2 human corticotroph adenomas, no mutations of the GDNF gene except one synonymous polymorphism were detected. This is consistent with the absence of mutations of the GDNF gene in sporadic pheochromocytomas, MTCs, parathyroid adenomas and small cell lung carcinomas (SCLC) in spite of its confirmed expression in 7 of 7 pheochromocytomas and 7 of 21 SCLC cell lines [24–26].

Our results suggest that the RET and GDNF genes do not play a major role in the formation of human pituitary adenomas, although the RET proto-oncogene is frequently expressed.

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