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Akihiro Miya

Masayuki Yamamoto

Hideki Morimoto

Norifumi Tanaka

Esei Shin

See next page for additional authors

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Expression of the *ret* Proto-oncogene in Human Medullary Thyroid Carcinomas and Pheochromocytomas of MEN 2A

Akihiro Miya,[*] Masayuki Yamamoto,[*] Hideki Morimoto,[*] Norifumi Tanaka,[*] Eisei Shin,[*] Katsu Karakawa,[*] Kumao Toyoshima,[†] Yukihito Ishizaka,[‡] Takesada Mori,[*] and Shin-Ichiro Takai[§]

We studied the expression of the ret *proto-oncogene (proto-*ret*) in human medullary thyroid carcinomas (MTCs) and pheochromocytomas of multiple endocrine neoplasia type 2A (MEN 2A) by Northern blot analysis. Expression of the normal-sized transcripts was detected in all 12 MTCs and in 6 of 8 pheochromocytomas. In situ localization of proto-*ret *mRNA revealed that the signal was confined to the cytoplasm of MTC cells. By Southern blot analysis neither amplification nor gross genetic changes of proto-*ret *were found in the tumors. Although no transcripts were detected in the normal portion of the thyroid from one MEN 2A patient, faint signals were detected in normal adrenal glands by Northern blot analysis, probably due to minor populations of C-cells and chromaffin cells in specimens from which MTC and pheochromocytoma might later develop. Proto-*ret *may play an important role in differentiation of a specific cell lineage from neuroectoderm, and it may be involved in development of MEN 2A tumors. (Henry Ford Hosp Med J 1992;40:215-9)*

The gene responsible for multiple endocrine neoplasia type 2A (MEN 2A) has been mapped to the pericentromeric region of chromosome 10 by linkage analysis using polymorphic DNA markers (1-3). The activated *ret* oncogene was first isolated by DNA transfection of a human T-cell lymphoma DNA into NIH3T3 cells (4). Two different activated forms of the *ret* proto-oncogene, *ret* (4) and *ret*-II (5), have been reported. The activation in these cases occurred by the rearrangement of the 5′-half of proto-*ret* during the transfection assay. Proto-*ret* was mapped to chromosome 10q11.2 near the probable MEN2A locus by in situ hybridization (6). We have confirmed by linkage analysis that proto-*ret* is located near the MEN2A locus (7). Expression of proto-*ret* has been examined in human tumor cell lines and its overexpression was detected in a neuroblastoma cell line (SK-N-SH), a promyelocytic leukemia cell line (HL-60), and a monocytic leukemia cell line (THP-1) (8). Ikeda et al (9) found that expression of proto-*ret* was detected in all 11 neuroblastoma cell lines examined. Nagao et al (10) found specific expression of proto-*ret* mRNA in all 29 neuroblastomas studied. Moreover, Santoro et al (11) reported that among several normal and tumorous human tissues, proto-*ret* was consistently expressed in pheochromocytomas and medullary thyroid carcinomas (MTCs). MTC, pheochromocytoma, and neuroblastoma are tumors derived from neural crest. These findings suggest a possible relationship between activation or overexpression of proto-*ret* and the oncogenic mechanism in MEN 2.

One of the seven MTCs examined by Santoro et al (11) was from a patient with the MEN 2B syndrome, while the other six were of the nonhereditary type. Their report prompted us to study expression of proto-*ret* in MTCs and pheochromocytomas, especially from patients with MEN 2A. Murotani et al (12) reported that microwave (MW)-fixed tissue samples were applicable to in situ hybridization as well as to the immunohistochemical analysis of the expression of various oncogenes.

In the present study, we report expression of the proto-*ret* in the tumors of the MEN 2A syndrome. Moreover, we show the localization of proto-*ret* mRNA in MTC cells by in situ hybridization in MW-fixed thyroid tissues using a digoxigenin-labeled cDNA probe.

Methods

Tumor samples and normal tissues

Surgical specimens of 12 MTCs (8 from MEN 2A patients and 4 from nonhereditary patients) and 8 pheochromocytomas (6 from MEN 2A patients and 2 from nonhereditary patients) were used. Normal thyroid tissues were nontumorous parts of thyroid glands resected from MEN 2A patients. Normal adrenal tissues were obtained from widely resected specimens in the radical operation for renal cell carcinoma. All samples were frozen immediately and kept at −80 °C until use.

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*Department of Surgery II, Osaka University Medical School, Osaka, Japan.
†Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.
‡Carcinogenesis Division, National Cancer Center Research Institute, Tokyo, Japan.
§Department of Medical Genetics, Biomedical Research Center, Osaka University Medical School, Osaka, Japan.
Address correspondence to Dr. Takai, Department of Medical Genetics, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita-city, Osaka 565, Japan.

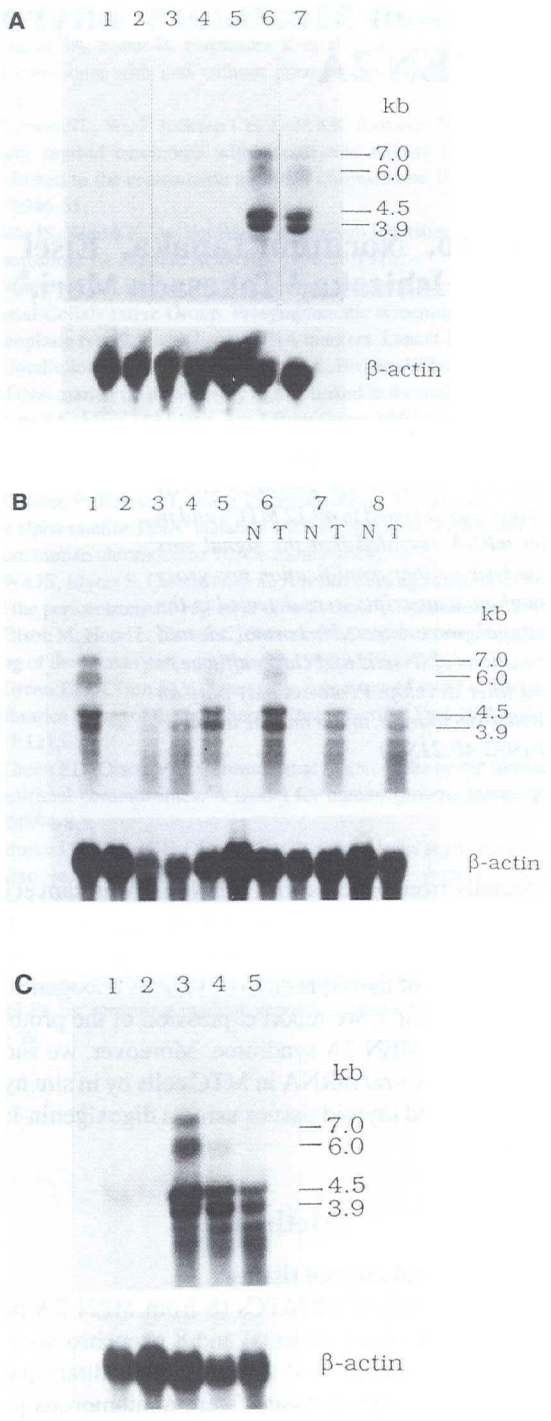

*Fig 1—Northern blot analysis of proto-*ret *mRNA in human MTCs and pheochromocytomas. A) Expression in human thyroid tumors. The samples were as follows: follicular adenoma (lanes 1, 2); papillary carcinoma (lanes 3, 4); anaplastic carcinoma (lane 5); nonhereditary MTC (lane 6); and MTC from MEN 2A patient (lane 7). B) The samples were as follows: pheochromocytoma from MEN 2A patient (lane 1); nonhereditary pheochromocytoma (paraganglioma) (lane 2); MTCs from MEN 2A patients (lanes 3, 4, 5, 6T, 7T, 8T); and nontumorous thyroid glands (lanes 6N, 7N, 8N). C) The samples were as follows: normal adrenal gland (lanes 1, 2) and pheochromocytoma from MEN 2A patients (lanes 3, 4, 5).*

Northern blot analysis

Total cellular RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method (13). Total RNA, 20 μg, was electrophoresed in 1% formaldehyde-agarose denaturing gel and blotted onto nylon membrane (Hybond-N, Amersham). Prehybridization was performed at 42 °C for 4 hours under stringent conditions (5 × SSPE, 50% formamide), and the membrane hybridized with the radiolabeled cDNA for 16 hours.

The probe used for detecting proto-*ret* mRNA was a 0.4 kb EcoRI-NcoI fragment of *ret*-II cDNA, which contained a portion of the kinase domain (10). As a control, a complete 2 kb human β-actin cDNA (Clontech Laboratories, Inc., Palo Alto, CA) was used. Each probe was labeled with [α^{32}P]dCTP (Amersham, 3,000 Ci/mmoL) to a specific activity of 1–3 × 10^8 cpm/μg DNA, using a Multiprime Labeling Kit (Amersham). The membrane was washed in 2 × SSPE, 0.1 × SDS at 42 °C for 15 minutes twice, in 1 × SSPE, 0.1 × SDS at 65 °C for 30 minutes, and finally in 0.1 × SSPE, 0.1 × SDS at 65 °C for 15 minutes twice. Blots were exposed to x-ray film at –80 °C with an intensifying screen for 24 to 120 hours.

Southern blot analysis

High molecular weight DNA was extracted as described previously (14). Five μg DNA of each sample was digested with BamHI, electrophoresed in 0.8% agarose gel, and blotted onto nylon membrane. Hybridization was performed with a 0.6 kb SalI-BamHI fragment of proto-*ret* cDNA (pN6) as described previously (15).

In situ hybridization on MW-fixed specimen

Fixation of tissues and preparation of sections—Specimens obtained at surgery were immediately cut into pieces of about 5 cubic mm. They were fixed by MW irradiation, embedded in paraffin, and sliced into 4 μm sections as described by Murotani et al (12).

Immunohistochemical analysis—One of the serial sections was stained by the avidin-biotin peroxidase complex (ABC) method with the murine anti-CEA monoclonal antibody (CM-010, Mochida Pharmaceutical Co., Tokyo) (16) to identify foci of MTC.

*Localization of proto-*ret *mRNA by in situ hybridization*—Sections adjacent to the one for CEA staining were used for in situ hybridization. A 0.2 kb BamHI fragment of proto-*ret* cDNA (pN6) which contained a portion of the transmembrane domain (9) was labeled with digoxigenin (17) by Genius™ Nonradioactive DNA Labeling and Detection Kit (Boehriger Mannheim Biochemicals) and used as a probe. KS vector (Stratagene, CA), which was used to subclone proto-*ret* cDNA, was labeled and used as a negative control.

Results

Expression of the proto-*ret* in human MTCs and pheochromocytomas

We examined expression of proto-*ret* in various human thyroid tumors, including two follicular adenomas, two papillary carcinomas, four anaplastic carcinomas, and 12 MTCs. Among

these tumors, all 12 MTCs expressed the 7.0, 6.0, 4.5, and 3.9 kb mRNA as shown in Fig 1, regardless of MEN 2A (Fig 1A, lane 7; Fig 1B, lanes 3, 4, 5, 6T, 7T, 8T) or nonhereditary type (Fig 1A, lane 6). Other types of thyroid tumors (Fig 1A, lanes 1-5) showed no detectable signals of proto-*ret* transcripts in autoradiogram even after 5-day exposure of the film (data not shown).

We examined expression of proto-*ret* in pheochromocytomas of MEN 2A. Proto-*ret* mRNA was expressed in 6 of 8 pheochromocytomas. Representative results are shown in Fig 1B (lane 1) and Fig 1C (lanes 3-5). The two negative cases were MEN 2A tumors which were the oldest specimens kept for more than five years. The negative result was probably related to RNA degradation because mRNA of β-actin was degraded in these cases. In an ectopic pheochromocytoma (paraganglioma) we detected low expression (Fig 1B, lane 2). In three cases of MEN 2A we could compare the expression of the proto-*ret* in MTC and in the normal portion of the thyroid gland, and no expression was detected in the latter (Fig 1B, lanes 6N, 7N, 8N). We found only faint signals of mRNA in normal adrenal glands (Fig 1C, lanes 1, 2).

In situ localization of the proto-*ret* mRNA

Localization of proto-*ret* mRNA in the thyroid gland of one MEN 2A patient was clearly confined to the cytoplasm of MTC cells identified immunohistochemically on the adjacent section using anti-CEA monoclonal antibody (Fig 2, left half). Treatment of the sections with RNase greatly decreased or abolished these cytoplasmic signals, and no signals were detected by in situ hybridization using a nonspecific vector probe instead of proto-*ret* cDNA (data not shown).

Southern blot analysis of the structure of proto-*ret*

To examine amplification or gross structural changes in proto-*ret*, Southern blot analysis was performed. A single band of 7.8 kb was detected by BamHI in all MTCs and pheochromocytomas from the MEN 2A patients examined. Neither amplification nor rearrangements were detected in any case (Fig 3).

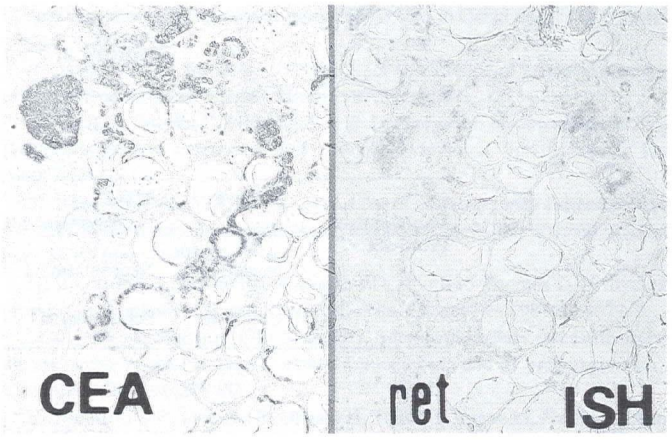

*Fig 2—Left half: Immunohistochemical detection of CEA in MTC. A small focus of MTC was clearly demonstrated by CEA staining. Right half: Expression of proto-*ret *in MTC. Proto-*ret *expression in MTC cells was demonstrated by in situ hybridization (ISH). In the normal follicular epithelial cells, no evidence of hybridization was detectable.*

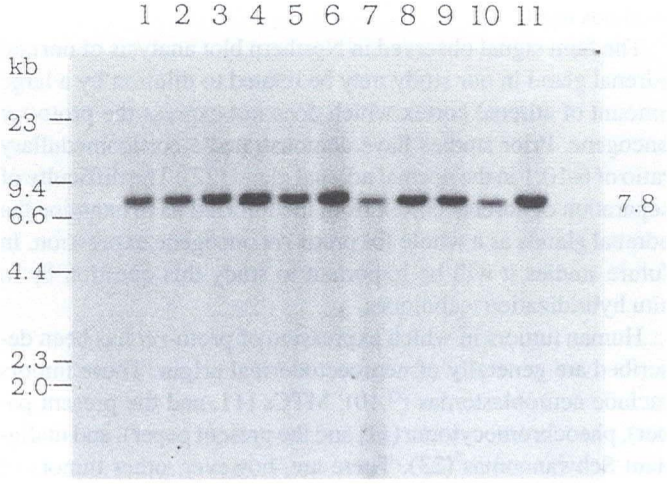

*Fig 3—Southern blot analysis of proto-*ret *in MTCs and pheochromocytomas from MEN 2A patients. DNA was digested with BamHI. A 0.6 kb SalI-BamHI fragment of proto-*ret *cDNA (pN6) was used as a probe. The samples were as follows: MTCs (lanes 1-5); pheochromocytomas (lanes 6-10); and placenta DNA, as a control (lane 11). A single 7.8 kb band was detected from the MEN 2A tumors.*

Discussion

It is generally accepted that growth of a neoplasm results from the accumulation of several genetic changes in a particular cell type. These changes could include activation or overexpression of oncogenes or inactivation of one or more tumor-suppressor genes. The predisposing gene for MEN 2A has been mapped to the pericentromeric region of chromosome 10; however, the nature of the genetic defect has not yet been defined. It is likely, however, that other genetic changes besides the MEN 2 locus will be involved in the oncogenesis of MTC and pheochromocytoma in this disorder. Studies on the loss of heterozygosity in these two tumors suggest the involvement of putative tumor-suppressor genes on chromosome 1, 3, 17, and 22 (18-20). No specific oncogene has yet been implicated in either MTC or pheochromocytoma, although there have been reports of *myc* expression (21) or *ras* activation (14) in a few cases.

Our studies have demonstrated expression of the proto-*ret* oncogene in 12 MTCs and 6 of 8 pheochromocytomas when analyzed by Northern analysis, results in accordance with the data of Santoro et al (11). These results identify the proto-*ret* oncogene as a possible candidate oncogene in MTC or pheochromocytoma.

To understand the role of proto-*ret* in oncogenesis, the expression of proto-*ret* in normal tissues must be studied. Santoro et al (11) did not find proto-*ret* expressed in normal human meninges, testis, ovary, adult brain, fetal brain, pancreas, liver, or placenta. However, they found low levels of expression in normal thyroid using poly(A)$^+$mRNA. We studied nontumorous portions of the thyroid gland from MEN 2A patients and found

Table
Tumors of Neuroectodermal Origin

		Expression of Proto-ret		
		Positive	Negative	Unknown
Expression of Proto-dbl	Positive	MTC Pheochromocytoma	Meningioma Ewing's Sarcoma	
	Negative	Neuroblastoma	Glioma	Ganglioneuroma Neuroepithelioma
	Unknown	Schwannoma	Melanoma	

that RNA transcripts of proto-*ret* were not detectable by Northern blot analysis using total RNA. In our study, normal thyroid follicular cells were not stained by in situ hybridization (perhaps low levels are not stained by in situ hybridization). It is possible that low levels of proto-*ret* expression in the normal thyroid detected by Santoro et al (11) are due to the expression in C-cells in the specimen. The discrepancy between our results and those of Santoro et al (11) may have resulted from the different detection methods used.

The faint signal observed in Northern blot analysis of normal adrenal gland in our study may be related to dilution by a large amount of adrenal cortex which does not express the proto-*ret* oncogene. Prior studies have demonstrated a corticomedullary ratio of 6-10:1 in the normal adrenal gland (22). The difficulty of separation of adrenal cortex from medulla led us to examine the adrenal glands as a whole for proto-*ret* oncogene expression. In future studies it will be important to study this question by in situ hybridization techniques.

Human tumors in which expression of proto-*ret* has been described are generally of neuroectodermal origin. These tumors include neuroblastomas (9,10), MTCs (11, and the present paper), pheochromocytoma (11, and the present paper), and malignant Schwannomas (23). There are, however, other tumors of neuroectodermal origin such as melanomas, gliomas, and Ewing's sarcoma which do not express the proto-*ret* oncogene (11,24).

Recently, de Franciscis et al (25) reported expression of the *dbl* proto-oncogene (proto-*dbl*) in some tumors of neuroectodermal origin. These tumors include meningiomas, Ewing's sarcomas, MTCs, and pheochromocytomas. Neuroblastomas and gliomas do not express *dbl* (25). One could conclude from these studies that expression of *dbl* is important in differentiation of certain cell types from neuroectoderm; however, it cannot be the MEN 2 predisposition gene because of the *dbl* location on the X chromosome (26).

We would like to propose a classification of tumors of neuroectodermal origin into four classes as outlined in the Table: group 1 = cells requiring expression of proto-*ret* for differentiation, group 2 = cells requiring expression of proto-*dbl* for differentiation, group 3 = cells requiring expression of both proto-*ret* and proto-*dbl* for differentiation, and group 4 = cells requiring neither for differentiation.

MTC and pheochromocytoma, the two main component tumors of the MEN 2 syndrome, appear to belong to the class of neuroectodermal cells which require expression of proto-*ret* and proto-*dbl* for differentiation. We hypothesize that mutation of the MEN 2 gene might initiate transformation only in those cells in which both proto-*ret* and proto-*dbl* are expressed. This hypothesis would provide an explanation for organ specificity of tumors in MEN 2A.

It will be important and interesting to study the structural alteration of the proto-*ret* oncogene in MTC to more fully define the role of this gene in the development of tumors associated with MEN 2.

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