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ORIGINAL ARTICLE

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Ret/PTC3 is the most frequent form of gene rearrangement in papillary thyroid carcinomas in Japan

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Abstract Rearrangements of the *RET* and *TRK* proto-oncogenes, which generate fusion oncogenes, are frequent in papillary thyroid carcinomas in Caucasian populations. To determine the spectrum of gene rearrangements in Japanese patients, we systematically examined 40 papillary thyroid carcinomas for all possible types of gene fusion events involving *RET* or *TRK* genes. *RET* rearrangements were found in ten tumors (25%): ret/PTC1 had occurred in two tumors, ret/PTC2 in one, ret/PTC3 in six, and a novel *RET* rearrangement in the remaining patient. In this last patient, the 5′ novel sequence was fused in-frame to the *RET* amino acid sequence; thus, the fusion gene may encode a protein with a *RET* kinase domain at the carboxy terminus. The *RET* gene was fused to 5′ donor sequences at the beginning of exon 12 in all ten tumors. No rearrangements involving the *TRK* gene were found in this panel of carcinomas. Our results indicated that constitutive activation of the *RET* by gene rearrangement is a frequent mechanism of papillary thyroid carcinogenesis in Japanese adults.

Key words *RET* proto-oncogene · *TRK* proto-oncogene · Papillary thyroid carcinoma · Rearrangement

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Introduction

The inactivation of tumor suppressor genes and the activation of oncogenes play important roles in the carcinogenesis of a variety of solid tumors in humans (Nakamura 1993; Nakamura 1996), while chromosomal rearrangements that produce chimeric oncogenes are occasionally associated with hematologic malignancy and some sarcomas (Rabbits 1994). An exception to this general pattern is seen in one type of carcinoma, papillary thyroid carcinoma, in which rearrangements of tyrosine kinase domains in the *TRK* and *RET* proto-oncogenes occur with more than random frequency (Bongarzone et al. 1989). The *TRK* proto-oncogene is a receptor for nerve growth factor (Klein et al. 1991); the *RET* proto-oncogene product forms a receptor-complex for glial cell line-derived neurotrophic factor (GDNF) (Jing et al. 1996) and neurturin (NTN) (Klein et al. 1997). *TRK* chimeric oncogenes are generated by juxtaposition of the tyrosine kinase domain of the *TRK* proto-oncogene on chromosome 1q to 5′ end sequences of different donor genes. The three forms of *TRK* oncogenes found to date are TRK, TRK-T1, and TRK-T3, which, respectively, involve fusion to the tropomyosin gene on 1q (Martin-Zanca et al. 1986), to the translocated promoter region (TPR) on 1q (Greco et al. 1992), and to the *TRK*-fused gene (TFG) on chromosome 3 (Greco et al. 1995). *RET* chimeric oncogenes (ret/PTC oncogenes) encode fusion proteins in which *RET* tyrosine kinase domains are fused to 5′ end sequences of different donor genes. Three forms of the ret/PTC oncogene have been identified, ret/PTC1, ret/PTC2, and ret/PTC3; in these forms the *RET* proto-oncogene is fused, respectively, to the *H4* gene on chromosome 10q21 (Grieco et al. 1990), to the regulatory subunit RIα of the cyclic adenosine monophosphate (cAMP) dependent protein kinase A gene on 17q23 (Bongarzone et al. 1993), and to the *Ele 1* gene on 10q11.2 (Bongarzone et al. 1994; Jhiang et al. 1994; Santoro et al. 1994).

Ret/PTC oncogenes have been reported in more than 34% of papillary thyroid carcinomas examined in European populations (Bongarzone et al. 1989; Viglietto et al. 1995;

Williams et al. 1996). These phenomena have seldom been detected in papillary thyroid carcinomas (less than 3%) in Japan, although until now Japanese investigators have examined this type of carcinoma only for the ret/PTC1 chimeric gene (Namba et al. 1991; Wajjwalku et al. 1992). The discrepancy in the reported frequency of ret/PTC oncogenes could be due to environmental, racial, or methodological factors. To establish the frequency of *RET* and *TRK* activation in papillary thyroid carcinomas in Japan and to understand the molecular basis for the apparent differences among populations, we screened 40 papillary thyroid carcinomas from Japanese patients and characterized all possible types of rearrangements involving *RET* and *TRK* genes, using advanced techniques of 5′-rapid amplification of cDNA ends (RACE) and the reverse transcriptase-polymerase chain reaction (RT-PCR).

Patients and methods

Tumor samples and RNA extraction

Papillary thyroid carcinomas were obtained from 40 adult patients during surgery. The specimens were frozen immediately and stored at −70°C until analysis. Total RNA was extracted by the guanidine thiocyanate method (Chomczynski and Sacchi 1987), using an Isogen RNA extraction kit (Nippon Gene, Tokyo, Japan).

PCR primers and hybridization probes

The sequences of the oligonucleotide primers (T1-T21) and the hybridization probes (A-H) used in the following experiments are listed in Table 1. The positions of these sequences on ret/PTC and TRK oncogenes are shown in Fig. 1.

Table 1 Oligonucleotide primers and probes used in this study

Primers	
T1	5′ CTTTCAGCATCTTCACGG 3′
T2	5′ GTAGGAATTCGGGTTGTAGGGAGGTCGACATGCC 3′
T3	5′ GGCAATGTCGACCTCCCTACAAC 3′
T4	5′ CTCCCTAGAACCCGAATTTCCTAC 3′
T5	5′ CGTTGCCTTGACCACTTTTC 3′
T6	5′ CGGTAATAGTCGGTGCTGTA 3′
T7	5′ CCTTGACAGCCACCAGCATC 3′
T8	5′ GTCGGGGGGCATTGTCAT 3′
T9	5′ GTTTTCGGTCTCCTTTATCG 3′
T10	5′ TGGAGAAGAGTGGCTGTATC 3′
T11	5′ CTTTCAGCATCTTCACGG 3′
T12	5′ GCCTTCTCCTAGAGTTTTTC 3′
T13	5′ GAGAACAAGGTGCTGAAGAT 3′
T14	5′ AACCAGTGTTGGGGAAGGAG 3′
T15	5′ TTGAGCGAATGGCTCCTTG 3′
T16	5′ GTGTCTGAGTGCTGCCGAAGC 3′
T17	5′ AGAAAGCAATACAACAAAGG 3′
T18	5′ GTTATGGCAGCAAGTATGTC 3′
T19	5′ CAAACTTGTTTCTCCGTCCAC 3′
T20	5′ GGCACTCAGCAAGGAAGACC 3′
T21	5′ AAGGAAGAGGCAGGCAAAGA 3′
Probes	
Probe A	5′ GAGGATCCAAAGTGGGAATT 3′
Probe B	5′ TGGAGACCTACAAACTGAAGTGCAAGGCACT-GC 3′
Probe C	5′ TGTGGGGCATCGACCGAGACAGCTATAGAAG-AA 3′
Probe D	5′ AGCACCGACCCCCAGGACTGGCTTACCCAAA-AG 3′
Probe E	5′ TCAAGTGGGAGCTGGGGGAGGGCGCCTTTG 3′
Probe F	5′ GCTGAGTTTGCTGAGAGATCGGTACCAAGC 3′
Probe G	5′ AGCTTCTGATGTTTCTGTTAAGTATCGAGA 3′
Probe H	5′ AATGTTATGTCAGCGTTTGGCTTAACAGAT 3′

5′ RACE procedures

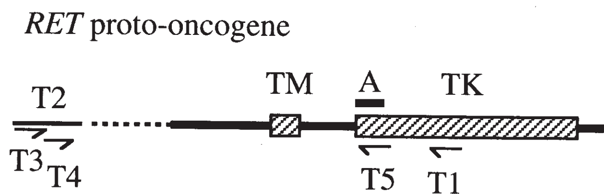
To screen for *RET* fusions, we used 5′ RACE according to the improved method of Chen (1996). Briefly, 4 μg of total RNA was reverse-transcribed with Superscript II (Life Technology, Rockbille, MD, USA) for 50 min at 42°C in a 40-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM dNTP, and 5 pmol of *RET* specific primer T1. The RNA was then digested with 4 U of RNaseH (Takara, Tokyo, Japan) at 37°C for 30 min. A single-stranded oligonucleotide adapter, T2, was ligated to the 3′ end of the first cDNA, using T4 RNA ligase (NEB, Beverly, MA, USA). The ligated cDNA was used as a template for the first PCR in a total volume of 10 μl containing 30 mM Tris-HCl (pH 8.8), 50 mM of KCl, 2 mM of $MgCl_2$, 5 mM of 2-mercaptoethanol, 100 μM of each dNTP, 1.6 pmol each of primers T1 and T3, and 0.25 units of Taq polymerase. Cycle conditions were 94°C for 2 min, then 14 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 120 s, with a final extension step 5 min at 72°C, in a Gene Amp PCR 9600 System (Perkin Elmer Cetus, Foster City, CA, USA). Nested PCR was carried out with an annealing temperature of 55°C in 40 cycles, with primers T4 and *RET*-specific primer T5. To screen *TRK* fusions, 5′ RACE was carried out using *TRK*-specific primer T6 and an inner *TRK* primer (T7), following the procedures described above.

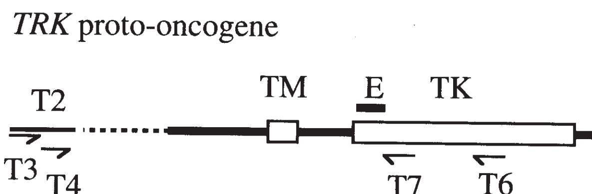
RT-PCR procedures

RET gene. RT-PCR was carried out to detect the expression of chimeric ret/PTC1, ret/PTC2, and ret/PTC3 mRNAs. Five microliters of total RNA was reverse-transcribed using *RET*-specific primer T1, followed by digestion with RNaseH. The first PCR was done with sense primers (T8, T9, and T10) specific for the H4, RIα, and Ele1 sequence, respectively, and the *RET* anti-sense primer T11, chosen to flank the breakpoint. Nested PCR was performed with inner primers of both *RET* (T12) and the respective fusion-gene sequences (T13, T14, and T15, corresponding to the H4, RIα, and Ele1 sequences, respectively). The PCR conditions were as described above.

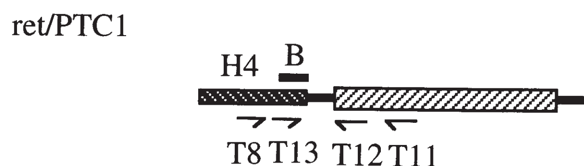
TRK gene. To detect the expression of chimeric TRK, TRK-T1, and TRK-T3 mRNAs, RT-PCR was performed following the procedures described above. Total RNA was reverse-transcribed using a *TRK*-specific primer (T6). The

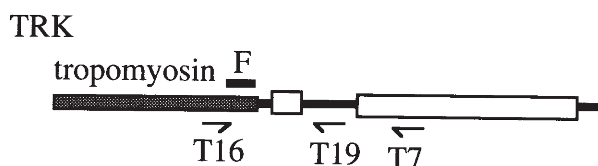
A. (5' RACE)

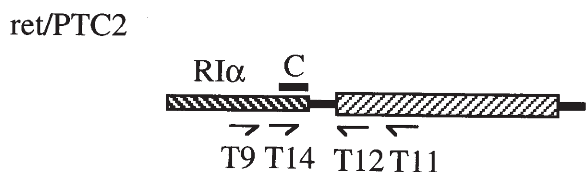


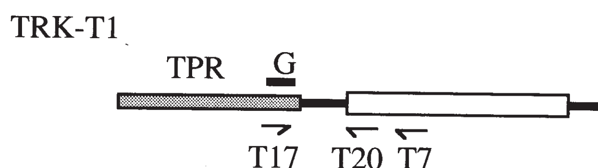


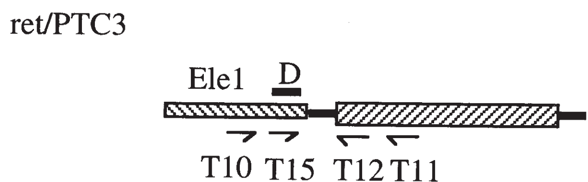
B. (RT-PCR)











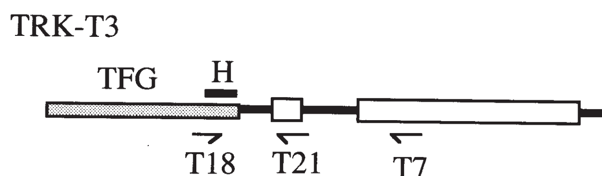


Fig. 1A,B Positions of oligonucleotide primers and hybridization probes on ret/PTC oncogenes and TRK oncogenes for **A** 5′-rapid amplification of cDNA ends (RACE) and **B** reverse transcriptase-polymerase chain reaction (RT-PCR) procedures. *Arrows* indicate orientation of oligonucleotide primers (T1, T3–T21) and an adapter (T2). *Short horizontal bars* represent hybridization probes (probes A–H). *TM box*, Transmembrane domain; *TK box*, tyrosine kinase domain, 5′ end boxes, 5′ end sequences of donor genes for ret/PTC and TRK fusion genes

first PCR employed anti-sense *TRK* primer T7 and the sense primers (T16, T17, and T18) specific for tropomyosin, TPR, and TFG sequence, respectively. Internal *TRK* primers T19, T20, and T21, together with donor-gene primers T16, T17, and T18, respectively, were used for nested PCR.

Oligonucleotide hybridization

The nested PCR products from the 5′ RACE and RT-PCR experiments were electrophoresed on 3% NuSieve agarose gels (FMC, Rockland, ME, USA) and transferred to nylon membranes. A *RET* gene-specific probe (A), H4 gene-specific probe (B), RIα gene-specific probe (C), Ele1 gene-specific probe (D), *TRK* gene-specific probe (E), tropomyosin gene-specific probe (F), TPR gene-specific probe (G), and TFG gene-specific probe (H) were each end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase. Hybridization of blotted membranes to oligonucleotide probes was performed according to the procedures described previously (Tsukamoto et al. 1998).

Sequence analysis

To characterize the structure of *RET* fusion transcripts, the 5′ RACE and RT-PCR products positive for hybridization were cloned into plasmid vector pBluescript II and sequenced with a Thermo-sequenase cycle sequencing kit (Amersham International Life Science, Cleveland, OH, USA).

Results

Total RNAs from 40 papillary thyroid carcinomas were first screened for the presence of ret/PTC oncogenes by 5′ RACE, according to the experimental strategies outlined in Fig. 1. In ten of the tumors, the 5′ RACE products hybridized to a *RET*-specific oligonucleotide probe (A) (cases 26, 28, 56, 60, 70, 76, 80, 86, 88, and 96). The same panel of tumors was then submitted to separate RT-PCR experiments, using the *RET*-specific primer together with primers

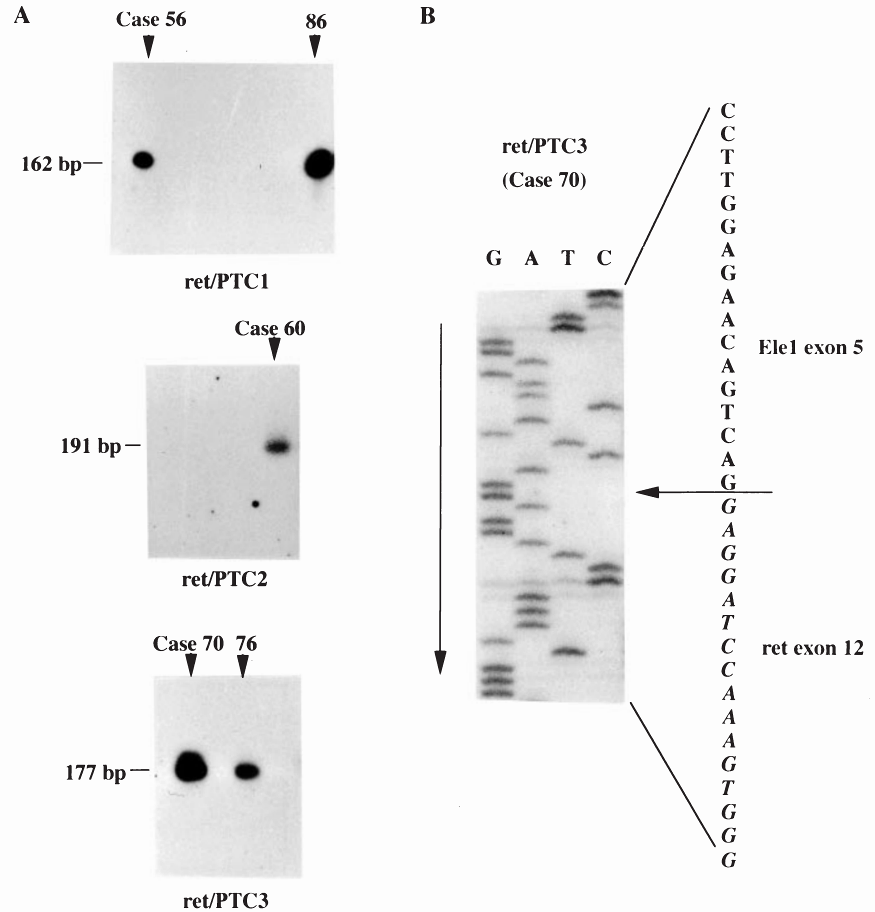


Fig. 2A Representative autoradiograms from RT-PCR, examined in Japanese papillary thyroid carcinomas. RT-nested PCR products of ret/PTC1, ret/PTC2, and ret/PTC3 were shown to hybridize to H4, RIα, and Ele1-specific probes, respectively. *Arrowheads* indicate cases positive for hybridization. **B** Sequence analysis of the *ret/*PTC oncogene. A representative sequence of the ret/PTC3 transcript is shown. The fusion point is indicated by a *horizontal arrow*, where the 5′ end of *RET* exon 12 is fused to the 3′ end sequence of Ele1 exon 5. *Vertical arrow* indicates the direction of transcription

Fig. 3 Nucleotide and predicted amino acid sequence of novel fusion cDNA involving the *RET* gene detected in tumor 28. The novel sequence in the 5′ portion is *underlined*. The 3′ portion represents the *RET* sequence starting from exon 12, *Arrowheads* indicates the fusion point

specific for H4, RIα, or Ele1 gene sequences. Figure 2A shows representative autoradiograms from these experiments; RT-PCR products hybridized to the H4 probe in two cases (56 and 86), to the RIα probe in one case (60), and to the Ele1 probe in six cases (26, 70, 76, 80, 88, and 96). On the basis of these results, we assumed that nine of the ten fusion transcripts resulted from rearrangements of the *RET* proto-oncogene, namely, ret/PTC1, ret/PTC2, and ret/PTC3, respectively. Nucleotide sequencing of the cloned PCR products from the nine cases confirmed that the 5′ donor sequences were from the *H4*, *RIα*, or *Ele1* genes (reprensentative autoradiogram shown in Fig. 2B). In the remaining tumor (No. 28), a novel type of *RET* rearrangement was identified in the 5′ RACE product. The nucleotide and amino acid sequence of this novel fusion 5′ RACE product, 180 bp in size, is shown in Fig. 3. In this tumor, the 5′ novel sequence was fused in-frame to the *RET* amino acid sequence starting from exon 12 of the *RET* gene. Thus, the fusion gene may encode a protein with a *RET* kinase domain at the carboxy terminus. The 5′ portion of the fusion product had hydrophobic amino acid residues periodically every seven residues, suggesting that the product may form heptad repeats in the helical-domain structure. In our 40 papillary thyroid carcinomas from Japanese patients, therefore, ret/PTC1 was identified in 2, ret/PTC2 in 1, ret/PTC3 in 6, and a novel *RET* rearrangement in 1. Table 2 summarizes the genetic abnormalities identified in our panel of tumors. No products from the 5′ RACE and RT-PCR experiments hybridized to a *TRK*-specific probe or to tropomyosin, TPR, or TFG-specific probes.

Table 2 Molecular structure of the *RET* rearrangements identified in this study

RET fusion gene	Frequency	Case no.	Characterization of the fusion point: 5′ Terminal sequence	3′ Terminal sequence
ret/PTC1	2/40	56, 86	3′ End of exon 1 of H4	5′ End of exon 12 of *RET*
ret/PTC2	1/40	60	Codon 1-236 of RIα	5′ End of exon 12 of *RET*
ret/PTC3	6/40	26, 70, 76, 80, 88, 96	3′ End of exon 5 of Ele1	5′ End of exon 12 of *RET*
Unknown	1/40	28	Undescribed sequence	5′ End of exon 12 of *RET*
Total	10/40 (25%)			

Discussion

Thyroid cancers are classified as either medullary, papillary, follicular, or anaplastic carcinomas. Medullary carcinoma is derived from parafollicular C cells; the other types originate from follicular cells of the thyroid gland. Germline missense mutations that activate the *RET* proto-oncogene initiate familial medullary thyroid carcinomas, including those found in multiple endocrine neoplasia type 2 (Donis-Keller et al. 1993; Mulligan et al. 1993; Kitamura et al. 1995; Kitamura et al. 1997). Somatic missense mutations of the *RET* gene are found in sporadic medullary thyroid carcinomas as well (Hofstra et al. 1994; Kitamura et al. 1997). The loss of heterozygosity (LOH) in specific chromosomal regions that is frequently detected in follicular (Tung et al. 1997) and anaplastic thyroid carcinomas, but not in medullary or papillary thyroid carcinomas, implicates a number of different tumor suppressor genes in those tumors. However, there is no evidence to support the idea that inactivation of these tumor suppressors plays a role in the development of papillary thyroid carcinomas, which comprise the majority of thyroid cancers (Ward et al. 1998). This remarkable contrast in LOH frequencies suggests a fundamental difference in the genetic basis of tumorigenesis in papillary thyroid carcinomas and other types of thyroid cancer.

Three forms of *TRK* rearrangement have been reported in papillary thyroid carcinomas (Bongarzone et al. 1989), but in our panel of tumors we detected no rearrangements involving the *TRK* gene. Three forms of somatic rearrangement previously reported in the *RET* gene (ret/PTC1, ret/PTC2, and ret/PTC3), however, did appear in our papillary thyroid carcinomas, in which the tyrosine kinase domain of *RET* was fused to the 5′ terminal sequences of genes encoding H4, regulatory subunit RIα of protein kinase A, and Ele1, respectively. The ret/PTC1 and ret/PTC3 oncogenes result from a paracentric inversion of the long arm of chromosome 10 (Pierotti et al. 1992; Minoletti et al. 1994). The genomic breakpoints of the *RET* proto-oncogene in all three forms of *RET* rearrangement occur within intron 11; the genomic breakpoints of ret/PTC1 and ret/PTC3 are known to occur within intron 1 of the *H4* gene and intron 5 of the *Ele1* gene, respectively (Smanik et al. 1995). At the cDNA level, therefore, exon 12 of RET (the first of the exons encoding the *RET* tyrosine kinase domain) is fused to 5′ coding sequences of the donor genes (exon 1 of H4, methionine at position 236 of RIα, and exon 5 of Ele1). Recently, an exceptional case, ret/PTC4 in which exon 11 of *RET*, instead of exon 12, rearranged with exon 5 of Ele1, was observed in papillary thyroid carcinomas from children in Belarus after the Chernobyl reactor accident (Fugazzola et al. 1996; Klugbauer et al. 1996). In our series, the cDNAs of all ret/PTC1, ret/PTC2, and ret/PTC3 oncogenes identified had identical *RET* fusion points at the start of exon 12.

In a study reported by Bongarzone et al. (1994), *RET* rearrangements were detected in 18 of 52 papillary thyroid carcinomas from Italian patients (35%): there were 10 cases of ret/PTC1, 2 of ret/PTC2, and 6 of ret/PTC3. The ret/PTC1 oncogene is generally the most frequent form of *RET* rearrangement found in Caucasian populations, although the precise frequency varies with different studies (Jhiang et al. 1992; Santoro et al. 1992; Williams et al. 1996; Bounacer et al. 1997). In Japan, Namba et al. (1991) examined ten papillary thyroid carcinomas only for ret/PTC1 rearrangements and found no rearrangement; Ishizaka et al. (1991) found one ret/PTC1 rearrangement among 11 carcinomas, and Wajjwalku et al. (1992) found only one such alteration among 38 carcinomas. On the basis of those observations, *RET* gene rearrangements in papillary thyroid carcinomas were thought to be rare in the Japanese population. Recently, Motomura et al. (1998) detected previously described *RET* rearrangements (which included five cases of ret/PTC1 and two cases of ret/PTC3) in 4 of 11 Japanese adult patients and 3 of 10 Japanese children with papillary thyroid carcinomas. In our panel of 40 adult patients, ret/PTC3 was the most frequent *RET* rearrangement. This may be due to differences in the etiology of carcinogenesis between childhood and adult cancers.

As not only ret/PTC1 but also other types of gene rearrangements involving *RET* or *TRK* have been identified recently in papillary thyroid carcinomas in Caucasians, we systematically screened a panel of Japanese papillary thyroid carcinomas for all possible types of rearrangement involving *RET* or *TRK* genes, using 5′ RACE in addition to RT-PCR. The results reported here show that *RET* rearrangements are indeed, common (25%) in Japanese patients, and that ret/PTC3 is the most frequent type of fusion gene in this study population. No *TRK* rearrangement was detected in this study, although we cannot rule out the remote possibility of experimental failure that may have led to the apparent lack of *TRK* fusion. We also identified a novel type of fusion gene involving *RET*. Since the 5′ portion of this fusion product had hydrophobic amino acid

residues periodically at every seven residues, it is possible that this domain may form heptad repeats in the helical-domain structure, often observed in the coiled-coil structure of proteins that are capable of forming dimers. The 3′ *RET* portion of the fusion protein could have a *RET* kinase domain at the carboxy terminus. Future characterization of the novel gene will eventually clarify the detailed structural arrangement of this fusion event and its consequences, which may explain the carcinogenetic mechanisms in this type of rearrangement.

The follicular cells from which papillary thyroid carcinomas arise do not normally express *RET* transcripts (Fabien et al. 1992). However, ubiquitously expressed promoters of the *H4*, *RI*α, or *Ele1* genes that have been rearranged would likely result in the ectopic expression of the *RET* tyrosine kinase (Bongarzone et al. 1994) in tumors derived from follicular cells. Receptor-type tyrosine kinases are activated by dimerization (Ullrich and Schlessinger 1990). In the normal state, *RET* reversibly dimerizes, activating its kinase, only when its ligands (GDNF and NTN) stimulate their receptors that have complexed with *RET*. Constitutive dimer formation, mediated by the dimerization domain of the 5′ portion of each rearranged ret/PTC oncogene, would lead to the activation of *RET* tyrosine kinase at the 3′ end of the fused gene (Bongarzone et al. 1993). Therefore, the respective ret/PTC chimeric proteins would bring about constitutive activation of tyrosine kinase; subsequent autophosphorylation of key tyrosine residues (Bongarzone et al. 1993; Bongarzone et al. 1994) would ultimately lead to cellular transformation. Others have shown that thyroid tumors resembling human papillary thyroid carcinomas will arise when transgenic mice are engineered to overexpress the ret/PTC1 oncogene (Jhiang et al. 1996; Santoro et al. 1996). This functional evidence that the *RET* gene-fusion event participates in cellular transformation, together with the molecular characterization of rearrangements in a substantial proportion of primary papillary thyroid carcinomas in the present study and elsewhere, implicates the etiological significance of *RET* rearrangement in the development of papillary thyroid carcinoma, regardless of race or population.

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