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Low Frequency of Rearrangements of the *ret* and *trk* Proto-oncogenes in Japanese Thyroid Papillary Carcinomas

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We investigated the frequency of rearrangements of the *ret* and *trk* proto-oncogenes in Japanese thyroid tumors. DNAs from 38 thyroid papillary carcinomas and 14 follicular adenomas were analyzed by Southern blotting. Rearrangements of the *ret* and *trk* proto-oncogenes were detected in one and two papillary carcinomas, respectively, but not in follicular adenomas. Analysis by a reverse transcriptase-polymerase chain reaction method showed that the *ret* rearrangement-positive tumor contained the PTC/*ret*^{TPC} chimeric transcript, which was reported to be found specifically in thyroid tumors and adenomatous goiter. We also found that rearranged mRNA of the *trk* proto-oncogene was expressed at high levels in one of two *trk* rearrangement-positive tumors. Our results indicated that the frequency of rearrangements of these proto-oncogenes in Japanese papillary carcinomas was much lower than that in Italian patients.

Key words: *ret* and *trk* proto-oncogenes — Rearrangement — Thyroid papillary carcinoma

DNA rearrangement is an important mechanism of oncogene activation. It is well known that rearrangements of the *myc*, *bcl*-2 and *abl* genes are caused by chromosome translocations in Burkitt lymphoma, follicular lymphoma and chronic myelogenous leukemia, respectively.[1–3] Rearrangement deregulates the expression of the *myc* and *bcl*-2 genes while rearrangement of the *abl* gene leads to synthesis of an altered gene product by fusion of the coding sequence of the *bcr* gene.

The *ret* and *trk* oncogenes, originally detected by transfection of NIH3T3 cells, were activated by DNA rearrangement of receptor-type tyrosine kinase genes with the *rfp* gene and the nonmuscle tropomyosin gene, respectively.[4–6] Recently, high frequency of activation of the *ret* and *trk* proto-oncogenes was found in human thyroid papillary carcinoma.[7,8] In this tumor, the kinase domain of the *ret* proto-oncogene was fused to the amino-terminal sequence of the H4 gene. This chimeric gene called the PTC/*ret*^{TPC} oncogene was also found in a papillary carcinoma cell line, TPC-1.[9] On the other hand, the involvement of the tropomyosin sequence was shown in three out of seven papillary thyroid carcinomas in which the *trk* rearrangement was detected.[10] These chimeric genes were generated by intrachromosomal rearrangements, because the *ret* proto-oncogene and the H4 gene were mapped to chromosome 10q,[11,12] and the *trk* proto-oncogene and the tropomyosin gene to chromosome 1q.[10,13] In the present study, we investigated the presence of rearrangements of the *ret* and *trk* proto-oncogenes in thyroid tumors from Japanese patients.

DNAs were extracted from 38 thyroid papillary carcinomas and 14 follicular adenomas. We also extracted DNA from a TPC-1 thyroid carcinoma cell line. DNAs were digested with *Eco*RI or *Bam*HI restriction endonuclease for Southern blotting. We used a 0.6 kb *Sal*I-*Eco*RI fragment as a probe (Fig. 1A), because the breakpoints of the *ret* proto-oncogene were reported to be present in a 6.3 kb *Eco*RI fragment in seven of nine rearrangement-positive papillary carcinomas from Italian patients[7,8] (the breakpoints in the other two cases have not been determined). As shown in Fig. 1B, this fragment detected 6.3 kb *Eco*RI and 3.6 kb *Bam*HI fragments of the *ret* proto-oncogene in DNAs of each of 38 papillary carcinomas and 14 follicular adenomas examined. In addition to these bands, 5.6 kb *Eco*RI and 8.0 kb *Bam*HI fragments and 1.8 kb *Eco*RI and 2.3 kb *Bam*HI fragments were found in DNAs of TPC-1 cells and of papillary carcinoma of one patient (patient 7), respectively. These additional fragments appear to represent rearranged fragments of the *ret* proto-oncogene but not polymorphism observed in the general population, because no such bands were found in tumor DNAs from 51 other patients.

To investigate the expression of the rearranged *ret* gene, we attempted to isolate poly(A)$^+$ RNAs from thyroid tumors, and obtained poly(A)$^+$ RNAs from 21

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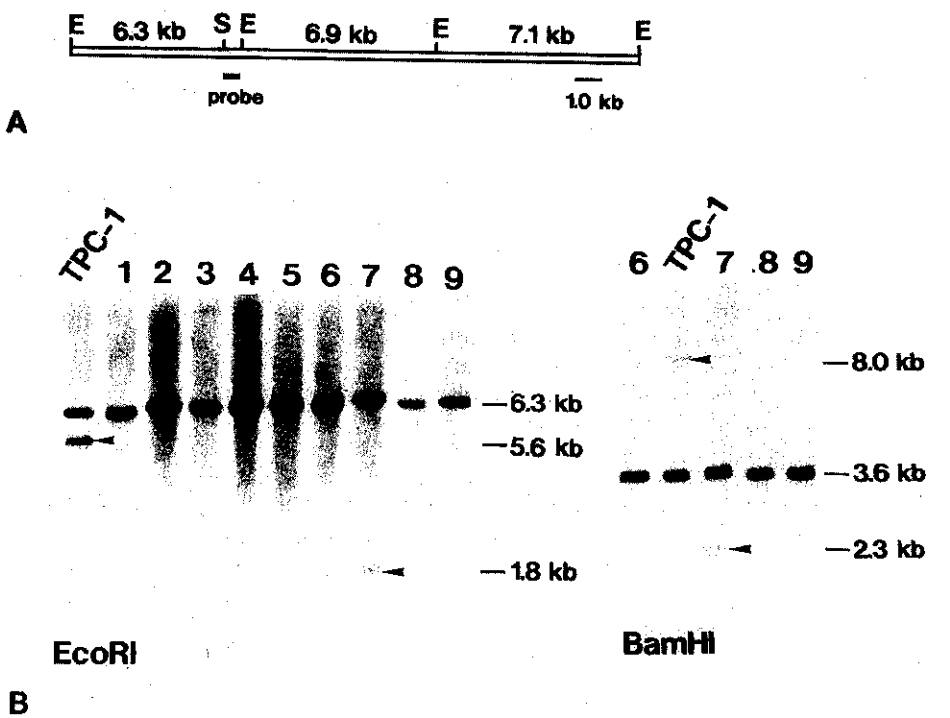

Fig. 1. Detection of rearrangement of the *ret* proto-oncogene in thyroid papillary carcinomas. (A) Partial restriction map of the *ret* proto-oncogene. A 0.6 kb *Sal*I-*Eco*RI fragment was used as a probe for Southern blotting. Restriction enzyme sites are E, *Eco*RI; S, *Sal*I. (B) Hybridization of DNAs from thyroid carcinomas and TPC-1 cell line with the *ret* probe. DNAs (5 μg) were digested with *Eco*RI or *Bam*HI and analyzed by Southern blotting. Numbers above the blots indicate the patient number. Arrows indicate rearranged fragments of the *ret* proto-oncogene.

carcinomas, including the rearrangement-positive tumor from patient 7 and 6 adenomas. Although 2 μg of poly(A)$^+$ RNAs was used for Northern blotting, mRNAs of the rearranged *ret* gene and the *ret* proto-oncogene were undetectable in them (data not shown). Thus, we examined the expression of the rearranged *ret* gene by a reverse transcriptase-polymerase chain reaction (RT-PCR) method. Two primers, one corresponding to the H4 sequence and the other to the kinase domain of the *ret* proto-oncogene, were used as described previously.[14] Amplification was carried out using 0.5 μg of poly(A)$^+$ RNAs and aliquots of PCR products were applied to polyacrylamide gels (Fig. 2). An expected band of 204 bp was amplified only from RNAs of TPC-1 cells and of the tumor of patient 7. In addition, the 204 bp product was detected with a 450 bp *Nco*I *ret* cDNA fragment,[5] which includes part of the amplified sequence, by Southern blotting (Fig. 2). The quality of cDNAs synthesized from tumor RNAs was confirmed by amplification of an *rfp* sequence known to be highly expressed in tumor cells[15] (data not shown). These results indicated that the H4 gene was also involved in the *ret* rearrangement detected in patient 7.

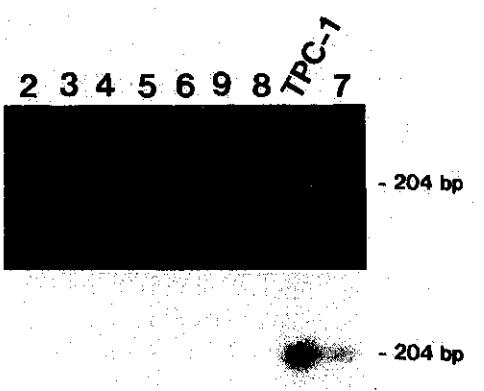

Fig. 2. Detection of PTC/*ret*^{TPC} transcripts by the RT-PCR method. (above) Poly(A)$^+$ RNAs (0.5 μg) were used for cDNA synthesis. A PCR product (204 bp) was amplified using the primers corresponding to the 5′ H4 sequence and the 3′ *ret* kinase sequence as described previously[14] and stained with ethidium bromide. Numbers above each lane indicate the patient number. (bottom) The PCR product was hybridized with a 450 bp *Nco*I *ret* cDNA fragment[5] which includes part of the amplified sequence.

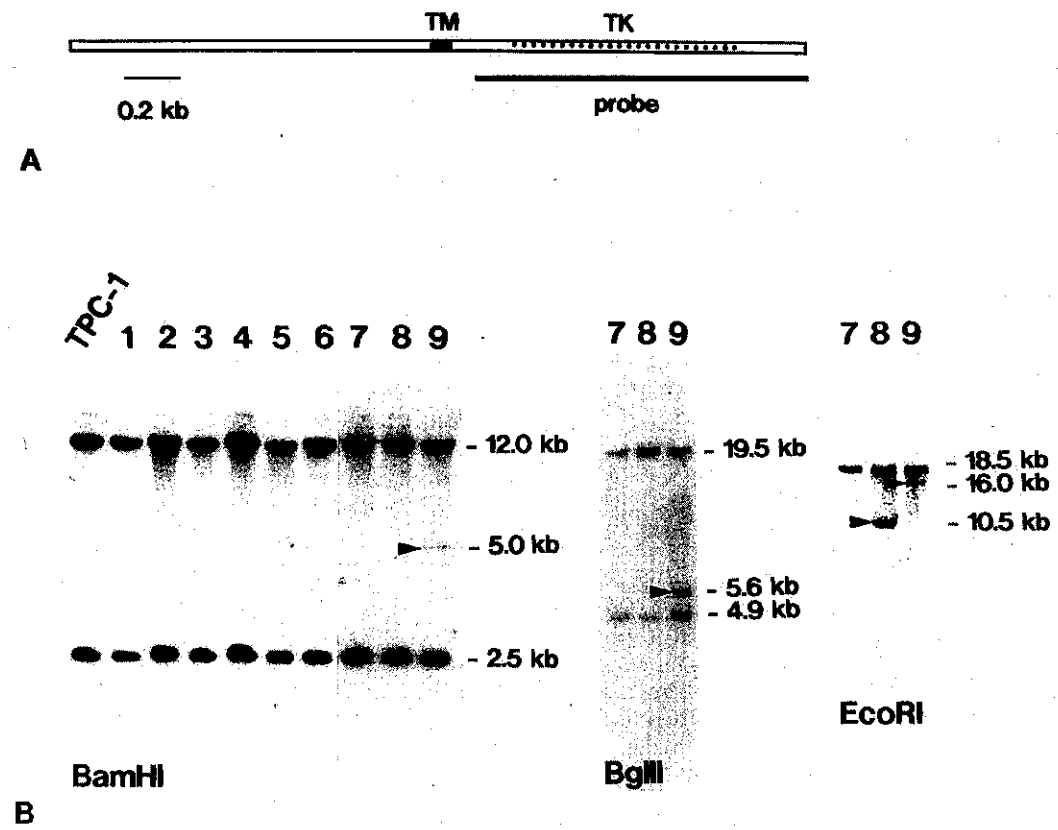

Fig. 3. Rearrangement of the *trk* proto-oncogene in thyroid papillary carcinomas. (A) Schematic illustration of the *trk* proto-oncogene cDNA. A 1.2 kb *Bal*I-*Eco*RI fragment[16] was used as a probe for Southern blotting. TM, transmembrane domain; TK, kinase domain. (B) *Bam*HI-, *Bgl*II- and *Eco*RI-digested DNAs (5 μg) were hybridized with the *trk* cDNA probe. Arrows indicate rearranged fragments of the *trk* proto-oncogene.

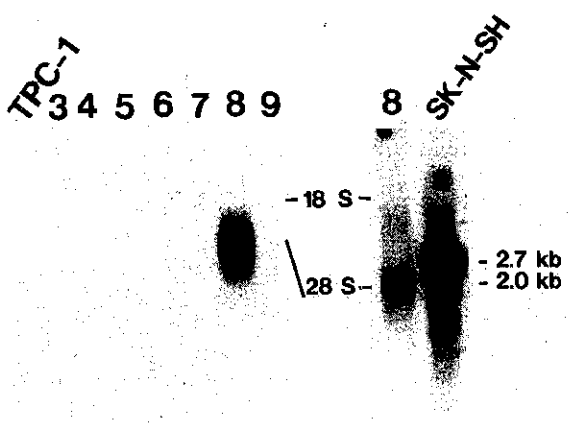

Fig. 4. Northern blot analysis of thyroid tumor RNAs with the *trk* cDNA probe. Poly(A)$^+$ RNAs (2 μg) from thyroid tumors and SK-N-SH neuroblastoma cells were hybridized with the 1.2 kb *Bal*I-*Eco*RI *trk* cDNA fragment (Fig. 3A). 2.0 kb and 2.7 kb transcripts were detected in RNAs of the tumor of patient 8 and SK-N-SH cells, respectively.

Similarly, we examined the rearrangement of the *trk* proto-oncogene, using a 1.2 kb *Bal*I-*Eco*RI *trk* cDNA fragment (Fig. 3A).[16] As a result, its rearrangement was found in two thyroid carcinomas from Japanese patients. In patient 9, 5.0 kb, 5.6 kb and 16.0 kb rearranged fragments were detected in *Bam*HI-, *Bgl*II- and *Eco*RI-digested DNAs, respectively (Fig. 3B). In patient 8, a 10.5 kb rearranged fragment was found only in *Eco*RI-digested DNA. This result suggests that the rearrangement point in the tumor of patient 8 might be present upstream of the *Bam*HI and *Bgl*II sites and downstream of the *Eco*RI site although we do not know the exact genomic restriction map of *trk* proto-oncogene.

To confirm whether these additional bands represented rearranged bands, we examined the expression of the *trk* sequence by Northern blotting. As shown in Figure 4, a 2.0 kb transcript of *trk* was highly expressed in papillary carcinoma from patient 8. On the other hand, its expression was undetectable in other mRNAs from 26 thyroid tumors including the tumor of patient 9. The size of *trk*

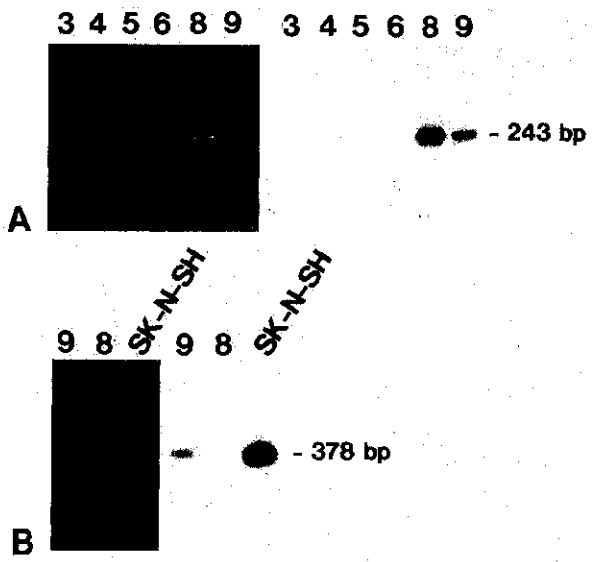

Fig. 5. PCR products for the *trk* sequences. Primers corresponding to the *trk* kinase domain (nucleotides 1462 to 1481 and 1685 to 1704)[16] (A) and to the *trk* receptor domain (nucleotides 907 to 924 and 1265 to 1284) (B) were used for amplification. (left panels) PCR products were detected by ethidium bromide staining. (right panels) The products were transferred to Gene Screen Plus filters and hybridized with the 1.2 kb *BalI-Eco*RI *trk* cDNA fragment (A) or a cDNA fragment corresponding to the *trk* receptor domain which contained the amplified sequence (B).

mRNA detected in the tumor of patient 8 was different from that (2.7 kb) in SK-N-SH neuroblastoma cells in which the *trk* proto-oncogene is expressed (Fig. 4). Thus it is likely that the *trk* mRNA in the tumor of patient 8 represented a rearranged message.

To investigate the *trk* expression by the RT-PCR method, we made two pairs of primers, one pair corresponding to the *trk* kinase domain (nucleotides 1462 to 1481 and 1685 to 1704)[16] and the other to the receptor domain (nucleotides 907 to 924 and 1265 to 1284). The primers for the kinase domain were able to amplify an expected product of 243 bp from RNAs of patients 8 and 9 but not from those of 25 other patients (Fig. 5A). On the other hand, when the primers for the receptor domain were used, a product of 378 bp was amplified from RNA of patient 9 but not from that of patient 8, although the level of its expression was very low (Fig. 5B). This product was also observed in SK-N-SH cells. Both 243 bp and 378 bp fragments of *trk* were detected by Southern blotting using the corresponding *trk* cDNA fragments as probes (right panels in Fig. 5A and 5B). These results indicated that the mRNA detected in the tumor of patient 8 did not include the *trk* receptor domain and represented a rearranged message. In the case of the tumor of patient 9, even when we used the primers (nucleotides 254 to 273 and 446 to 465) close to the amino-terminal end of the *trk* proto-oncogene cDNA, an expected PCR product was observed (data not shown). These results suggested that mRNA of non-rearranged *trk* proto-oncogene was present in the tumor RNA of patient 9 although it is still possible that both non-rearranged and rearranged mRNAs of *trk* were expressed in it.

We next examined whether the nonmuscle tropomyosin gene was involved in the *trk* rearrangements of patients 8 and 9. However, no PCR product was amplified (data not shown), using the primers corresponding to nucleotides 555 to 574 and 793 to 812 of the published cDNA sequence of the *trk* oncogene.[6] Thus, it is unlikely that the tropomyosin gene was involved in the *trk* rearrangements in patients 8 and 9.

It was reported that rearrangements of the *ret* and *trk* proto-oncogenes were detected in approximately 25% of thyroid papillary carcinomas from Italian patients, respectively.[7] In contrast, our data showed that their frequency was very low ($<6\%$) in Japanese patients. These results suggested that racial differences in the genetic background affect the frequency of rearrangement of these genes and that other oncogenes or tumor suppressor genes could be more important in the development of thyroid papillary carcinomas in Japanese.

Recently, Ishizaka *et al.*[14] detected the PTC/*ret*^{TPC} chimeric transcript in heterogenous populations of thyroid follicular adenomas and adenomatous goiters by the RT-PCR method. This contrasts with our present failure to detect the *ret* rearrangement and the PTC/*ret*^{TPC} transcript in follicular adenomas. It is possible that a small population of PTC/*ret*^{TPC} positive cells in follicular adenomas may have been undetectable under our experimental conditions. Screening a large number of patients will be necessary for evaluating the role of the PTC/*ret*^{TPC} oncogene in benign thyroid tumors.

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