RAPID COMMUNICATION Jpn. J. Cancer Res. 81, 309–312, April 1990

Expression of *ret* Proto-oncogene in Human Neuroblastomas

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We examined the expression of *ret* proto-oncogene (proto-*ret*) in surgically resected human neuroblastomas. Slot blot RNA hybridization revealed that all 29 neuroblastomas examined expressed the proto-*ret*, the relative intensity of the hybridization ranging from 1 to 48. No correlation was found between the level of expression of proto-*ret* and the clinical stage. The level of expression was also not correlated with N-*myc* amplification, the patient's age or the histological type of the tumor. Based on the previous finding that proto-*ret* expression is very rarely detected in tumor cell lines other than those of neuroblastoma, proto-*ret* expression was suggested to be a characteristic of neuroblastomas, and possibly to be involved in the genesis of neuroblastomas.

Key words: Neuroblastoma — Expression of *ret* proto-oncogene — Clinical stage — N-*myc* amplification

Neuroblastoma is one of the most malignant tumors in children. Generally, the prognosis of patients with neuroblastoma is poor. A good prognosis is expected only in cases where surgical removal is done at a clinically early stage, except for rare cases with a special category of tumors with stage IV-S.[1] The prognosis in cases associated with N-*myc* amplification is known to be poor.[2,3] N-*myc* amplification is found mainly in advanced cases, suggesting that this gene alteration has a role in tumor progression. Cytogenetically, aberration of chromosome 1p was detected in about 70% of a series of neuroblastomas examined.[4] There are also recent reports of loss of heterozygosity of chromosome 14q in about 50%,[5] and of that of 1p in about 28%[6] of two series of cases. These data suggest that several alterations of genes, possibly including suppressor genes, are required for development and progression of neuroblastomas.

We have observed a marked increase in the incidence of expression of the *ret* proto-oncogene (proto-*ret*) in human neuroblastoma cell lines.[7] Proto-*ret* has been predicted to encode two isoforms of a receptor type of tyrosine kinase,[8,9] and activated forms produced by rearrangements were shown to have transforming activity in NIH 3T3 cells.[8,10]

Proto-*ret* was found to be highly expressed in all 11 neuroblastoma cell lines studied.[7] Detailed analysis of cDNAs obtained from a neuroblastoma cell line, Nagai, revealed that there is no genetic alteration in the proto-*ret* coding region.[9] On the other hand, our studies[7,8] and those of others[12] on 50 cell lines of 23 different types of tumors indicated that proto-*ret* expression was not specifically associated with any other type of tumor than neuroblastoma. Proto-*ret* expression was also barely detected in normal tissues of adult rats.[13] These findings suggested that proto-*ret* expression might be closely related with the genesis of neuroblastomas. However, there was a possibility that proto-*ret* was expressed only in cultured cell lines. Therefore, in this work we examined surgically resected specimens of neuroblastomas to determine whether proto-*ret* was expressed in neuroblastomas *in vivo*.

The neuroblastomas examined had been resected for the purpose of treatment from 29 patients at the Hospital of Kyushu University Medical School or affiliated hospitals between 1982 and 1988. The patients ranged from 4 months to 11 years old (average: 2 years and 6 months); 13 patients were under 1 year old. The diagnosis of neuroblastoma was based on pathological findings, and catecholamine secretion. Histologically, 9 cases were classified as ganglioneuroblastomas and 20 as neuroblastomas. Specimens of all tumors were taken before any radio- or chemotherapy, and were promptly frozen. As controls, 3 human epidermoid carcinoma cell lines, KB3-1, its subline KB-Cl[14] and HC7-5/VCR, which is a subline of HC7-5, a cell line established from head and neck cancer,[15] were also examined.

RNA was extracted from tissues and cells and hybridized essentially by a reported method.[16] For detecting the signal of proto-*ret*, an *Eco*RI-*Nco*I 0.4 kb fragment was used as a probe.[11,12] In a previous study we

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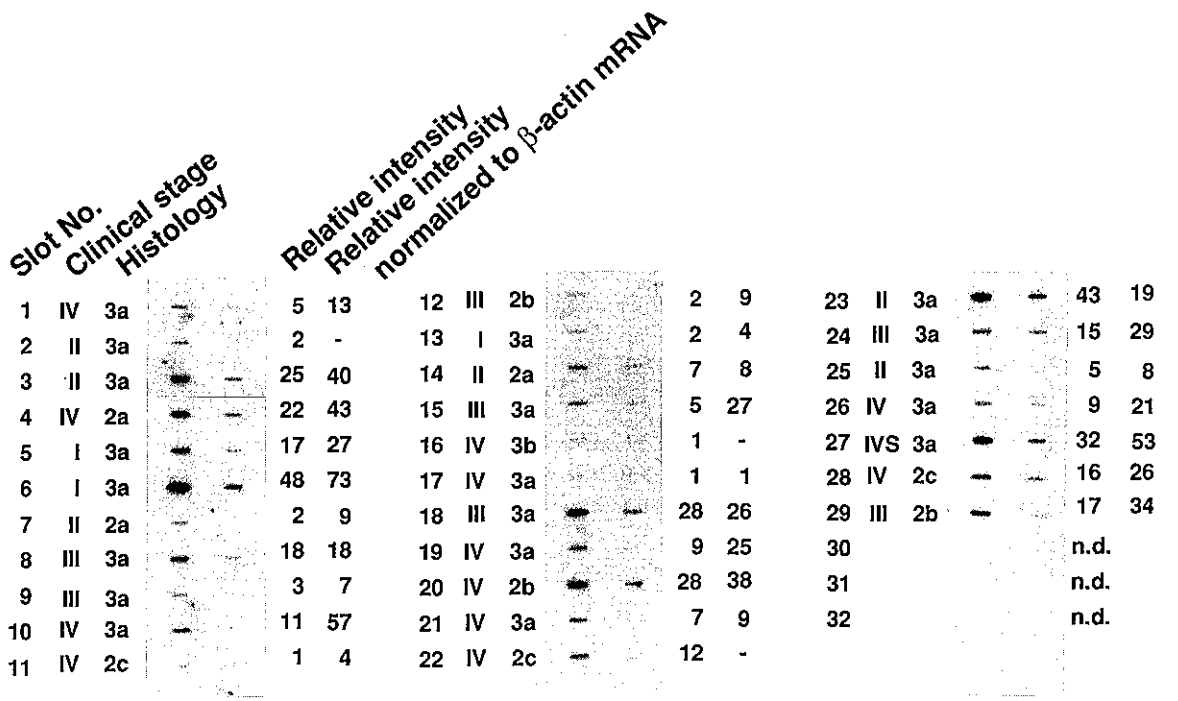

Fig. 1. Slot blot analysis for detecting proto-*ret* expression in 29 original biopsied neuroblastomas. Samples of 8 μg (left lane) and 2 μg (right lane) of RNA were used. RNA was blotted onto a nitrocellulose filter after heat denaturation. Then the filter was incubated in a solution of 50% deionized formamide, 5×SSC, 5×Denhardt's solution, 0.1% SDS, and 100 μg/ml of salmon sperm DNA at 42°C for 18 h. After hybridization, the filter was washed twice with a solution of 2×SSC and 0.1% SDS, and four times with 0.1×SSC and 0.1% SDS at room temperature. As controls, 3 human cell lines were used (slot No. 30–32). For detecting the proto-*ret* and β-actin transcripts, an *Eco*RI-*Nco*I 0.4 kb fragment[9, 11] and an H106 human genomic clone[21] were used as probes, respectively. The absence of cross hybridization with rRNA under the conditions used in this experiment is evident from the negative results with three cell lines (slot No. 30–32). Expression of β-actin mRNA was detected in these three cell lines (data not shown). The intensity of the signal was measured with a video densitometer. The weakest signal for proto-*ret* (slot No. 17) was assigned the value of unity. The relative intensities of signals (without and with normalization to β-actin transcript), clinical stages, and histological types (Histology) are also shown. n.d.: not detected. —: datum not available. Histological type: 2, ganglioneuroblastoma; a, well differentiated type; b, composite type; c, poorly differentiated type: 3, neuroblastoma; a, rosette-fibrillary type; b, round cell type.

showed that this proto-*ret* cDNA probe did not cross-hybridize with any other members of the tyrosine kinase family under the conditions used in experiments.[10, 13, 17] For detecting N-*myc* amplification, Southern blot analysis was performed using an *Eco*RI-*Bam*HI 1.0 kb fragment[18] as a probe.

As shown in Fig. 1, samples of 8 μg of total RNA of all 29 neuroblastomas examined gave a signal of proto-*ret*, and 23 of these tumors gave a definite signal even with samples of 2 μg of RNA. In contrast, none of the 3 non-neuroblastoma tumor cell lines gave any signal of proto-*ret* (Fig. 1, slot No. 30–32). The intensities of the signals were measured with a video densitometer (Model 620, Bio-Rad). The lowest intensity of expression among the 29 samples was referred to as 1 (slot No. 17 in Fig. 1), and the signal intensities of the other samples were expressed as ratios to it (values do not necessarily represent ratios of amounts of proto-*ret* mRNA). We normalized the levels of proto-*ret* transcript to β-actin mRNA on 26 samples and expressed them as relative ratios to the sample of No. 17 (Fig. 1), although it is not known yet whether the levels of β-actin mRNA remain constant in various primary neuroblastomas. Figure 2 shows the relative expressions of proto-*ret* in the samples classified according to the clinical stage. No correlation between the level of proto-*ret* expression and the clinical stage is apparent. Of these 26 neuroblastomas, 5 were associated with N-*myc* amplification (manuscript in preparation). The number of cases was small, but among them there seemed to be no correlation between the level of proto-*ret* expression and N-*myc* amplification. These data suggest that expression of proto-*ret* is a characteristic of neuroblastomas that is detectable before alteration of the N-*myc* gene. The prognosis of patients of over 1

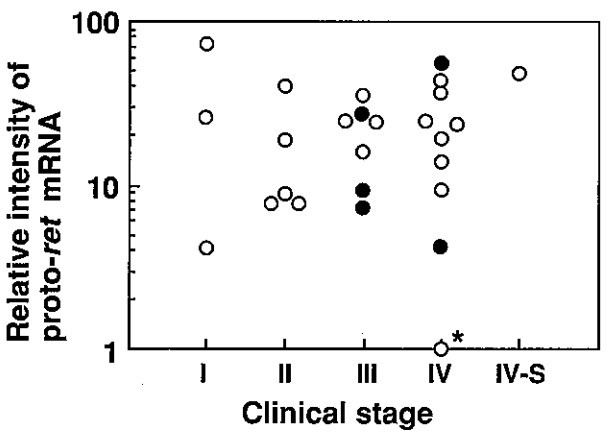

Fig. 2. Relative levels of expression of proto-*ret* at different clinical stages. The intensity of proto-*ret* expression normalized to β-actin mRNA is plotted according to the clinical stage of the patients. (*) indicates the case in which the intensity of proto-*ret* expression is defined as 1 (slot No. 17 in Fig. 1). Solid circles indicate cases associated with more than 10-fold amplification of N-*myc*.

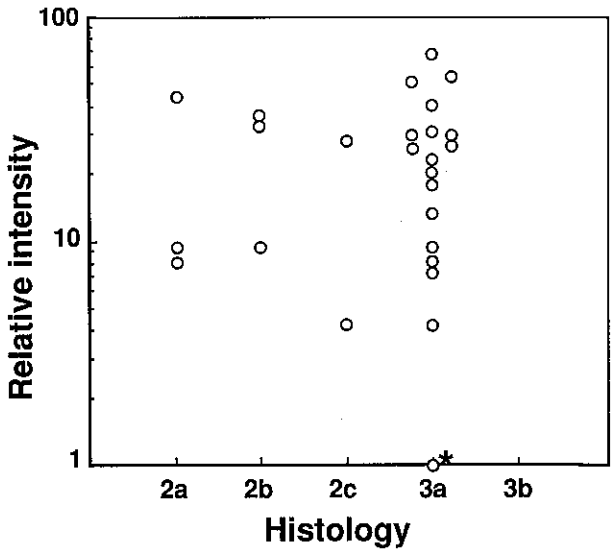

Fig. 3. Correlation between proto-*ret* expression normalized to β-actin mRNA and histological types. Histological types (2a–3b) are in order of well differentiated to poorly differentiated as described in the legend to Fig. 1.

year old is reported generally to be poor.[1] The expression levels of proto-*ret* of our patients of under and over 1 year old were not different (data not shown). The expression levels were also not correlated with the histological degrees of differentiation of the tumors (Fig. 3 and also refer to Fig. 1).

Based on these results, we speculate that overexpression of proto-*ret* is involved in the genesis of neuroblastomas, or that the expression is a characteristic of neuroblast cells.

Recently, human proto-*ret* was mapped to chromosome 10q11.2 near the possible locus responsible for multiple endocrine neoplasia 2A (MEN2A).[8] MEN2A induces multiple tumors such as medullary thyroid carcinoma and pheochromocytoma, which originate from the neural crest. It is interesting to note that neuroblastoma also originates from the neural crest. However, among the tumors originating from the neural crest, including melanomas, only neuroblastomas express proto-*ret*,[8] suggesting that proto-*ret* is related to the differentiation of precursor cells into neuroblasts, although it is possible that constitutively high expression of proto-*ret* is involved in malignant transformation.

Recently, proto-*ret* was found frequently to be activated by rearrangement in papillary thyroid tumors.[19] We also observed rearrangement of this gene in a cell line, TPC-1, of human papillary thyroid carcinoma, and found that the TPC-1 cells weakly expressed aberrant transcripts.[20] Thus, papillary thyroid carcinomas may frequently express aberrant transcripts.

F. Itoh and I. Ikeda are recipients of Research Resident Fellowships from the Foundation for Promotion of Cancer Research, Japan. This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Health and Welfare for the Comprehensive 10-year Strategy for Cancer Control, Japan.

(Received February 21, 1990/Accepted March 7, 1990)

REFERENCES

1) Breslow, N. and McCann, B. Statistical estimation of prognosis for children with neuroblastoma. *Cancer Res.*, **31**, 2098–2103 (1971).
2) Schwab, M., Ellison, J., Busch, M., Rosenau, W., Varmus, H. E. and Bishop, J. M. Enhanced expression of the human gene N-*myc* consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. *Proc. Natl. Acad. Sci. USA*, **81**, 4940–4944 (1984).
3) Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. Y. and Hammond, D. Association of multiple copies of the N-*myc* oncogene with rapid progression of neuroblastomas. *N. Engl. J. Med.*, **313**, 1111–1116 (1985).
4) Gilbert, F., Feder, M., Balaban, G., Brangman, D., Lurie,

D. K., Podolsky, R., Rinaldt, V., Vinikoor, N. and Weisband, J. Human neuroblastomas and abnormalities of chromosome 1 and 17. *Cancer Res.*, **44**, 5444–5449 (1984).
5) Suzuki, T., Yokota, J., Mugishima, H., Okabe, I., Ookuni, M., Sugimura, T. and Terada, M. Frequent loss of heterozygosity on chromosome 14q in neuroblastoma. *Cancer Res.*, **49**, 1095–1098 (1989).
6) Fong, C-T., Dracopoli, N. C., White, P. S., Merrill, P. T., Griffith, R. C., Housman, D. E. and Brodeur, G. M. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastoma: correlation with N-*myc* amplification. *Proc. Natl. Acad. Sci. USA*, **86**, 3753–3757 (1989).
7) Ikeda, I., Ishizaka, Y., Tahira, T., Suzuki, T., Sugimoto, T., Sugimura, T. and Nagao, M. Expression of *ret* oncogene in human neuroblastoma cell lines. *Proc. Jpn. Cancer Assoc., 47th Annu. Meet.*, 222 (1988).
8) Ishizaka, Y., Itoh, F., Tahira, T., Ikeda, I., Sugimura, T., Tucker, J., Fertitta, A., Carrano, A. V. and Nagao, M. Human *ret* proto-oncogene mapped to chromosome 10q-11.2. *Oncogene*, **4**, 1519–1521 (1989).
9) Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. and Hiai, H. Cloning and expression of the *ret* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene*, **3**, 571–578 (1988).
10) Tahira, T., Ishizaka, Y., Itoh, F., Sugimura, T. and Nagao, M. Characterization of *ret* proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. *Oncogene*, **5**, 97–102 (1990).
11) Ishizaka, Y., Ochiai, M., Tahira, T., Sugimura, T. and Nagao, M. Activation of the *ret*-II oncogene without a sequence encoding a transmembrane domain and transforming activity of two *ret*-II oncogene products differing in carboxy-termini due to alternative splicing. *Oncogene*, **4**, 789–794 (1989).
12) Takahashi, M. and Cooper, G. M. *ret* Transforming gene encodes a fusion protein homologous to tyrosine kinases. *Mol. Cell. Biol.*, **7**, 1378–1385 (1987).
13) Tahira, T., Ishizaka, Y., Sugimura, T. and Nagao, M. Expression of proto-*ret* mRNA in embryonic and adult rat tissues. *Biochem. Biophys. Res. Commun.*, **153**, 1290–1295 (1988).
14) Akiyama, S., Fojo, A., Hanover, J. A., Pastan, I. and Gottesman, M. M. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somatic Cell Mol. Genet.*, **11**, 117–126 (1985).
15) Komiyama, S., Matsui, K., Kudoh, S., Nogae, I., Kuratomi, Y., Sabui, Y., Asoh, K., Kohno, K. and Kuwano, M. Establishment of tumor cell lines from a patient with head and neck cancer and their different sensitivities to anti-cancer agents. *Cancer*, **63**, 675–681 (1989).
16) Maniatis, T., Fritsch, E. F. and Sambrook, J. "Molecular Cloning, A Laboratory Manual," p. 196 (1982). Cold Spring Harbor Laboratory Press, New York.
17) Ishizaka, Y., Tahira, T., Ochiai, M., Ikeda, I., Sugimura, T. and Nagao, M. Molecular cloning and characterization of human *ret*-II oncogene. *Oncog. Res.*, **3**, 193–197 (1988).
18) Schwab, M., Alitalo, K., Klempnauer, K-H., Varmus, H. E., Bishop, J. M., Gilbert, F., Brodeur, G., Goldstein, M. and Trent, J. Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. *Nature*, **305**, 245–248 (1983).
19) Bongarzone, I., Pierotti, M. A., Monzini, N., Mondellini, P., Manenti, G., Donghi, R., Pilotti, S., Grieco, M., Santoro, M., Fusco, A., Vecchio, G. and Della Porta, G. High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. *Oncogene*, **4**, 1457–1462 (1989).
20) Ishizaka, Y., Itoh, F., Tahira, T., Ikeda, I., Ogura, T., Sugimura, T. and Nagao, M. Presence of aberrant transcripts of *ret* proto-oncogene in a human papillary thyroid carcinoma cell line. *Jpn. J. Cancer Res.*, **80**, 1149–1152 (1989).
21) Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, K. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA*, **82**, 6133–6137 (1985).