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Recent Studies on the *ret* Proto-oncogene

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I. Introduction

The *ret* proto-oncogene was first found as an activated form by NIH3T3 cell transfection assay(1). In its activated forms, the region 5' to the kinase domain of the *ret* proto-oncogene was shown to be replaced by an unrelated sequence to form fusion proteins. However, these activations were proved to have occurred during transfection assay and no rearrangements have been found so far in the original tumors(1, 2). The *ret* proto-oncogene has been found to be highly expressed in a very few specific types of tumors, and to be expressed at low levels in normal tissues. It has been predicted to encode receptor tyrosine kinases (RTK) for an unidentified ligand. Although its biological function is not known yet, there is increasing circumstantial evidence suggesting its relation to the development of neuroblastoma. In this article, our findings on the structures of the *ret* proto-oncogene and its activated forms are described and its biological function and significance in clinical medicine are discussed.

II. Structure of *ret* proto-oncogene products

1. Characteristics of the ret *proto-oncogene product as a receptor tyrosine kinase*

So far we have found human *ret* proto-oncogene mRNA in malignant cell lines, and we have cloned cDNAs of the *ret* proto-oncogene from Nagai, a human neuroblastoma cell line, which expressed a high level of mRNA(3). As described later, the *ret* proto-oncogene expresses 5 species of mRNA. By extensive studies on the nucleotide sequences of these mRNAs, two kinds of proteins were found to be encoded from these 5 species of mRNA. Their nucleotide sequences of coding regions and the structures of these two proteins are shown in

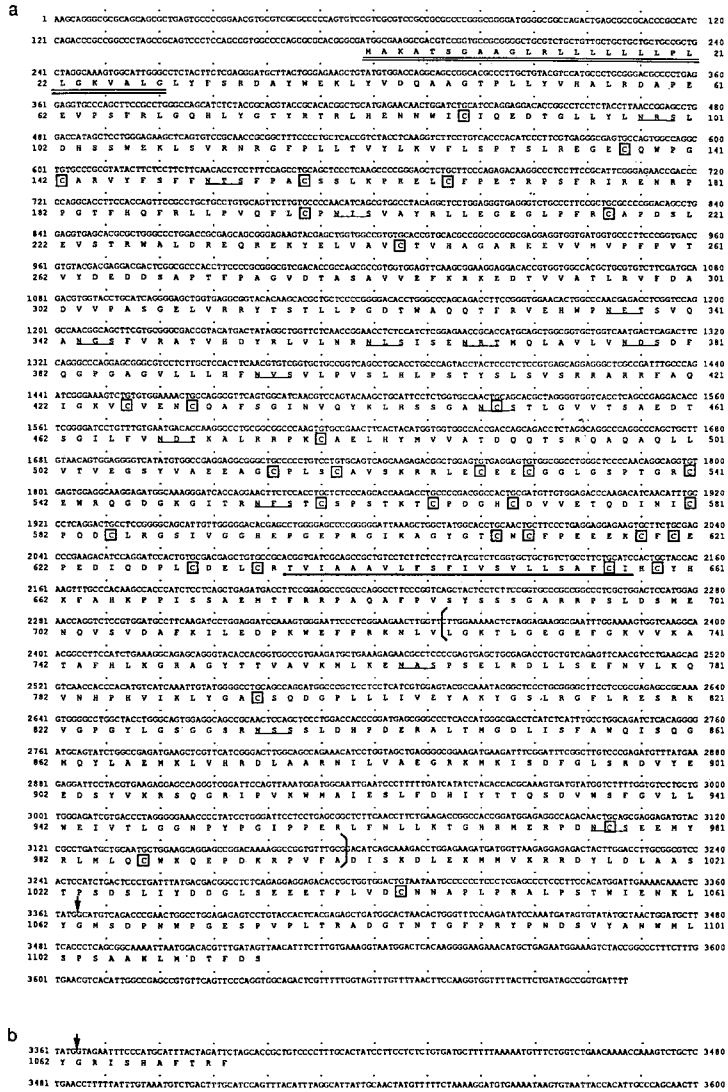

Fig. 1 Nucleotide and deduced amino acid sequences of the *ret* proto-oncogene cDNA clones (3).

a. Nucleotide sequence of cDNA clones that corresponds to the 1114 amino acid protein. The putative signal peptide is underlined with double lines and the transmembrane domain is underlined with a bold line. The consensus sequences for N-linked glycosylation are underlined with a single thin line. Cysteine residues are boxed. The region corresponding to the kinase domain is shown in brackets.

b. Nucleotide sequence of the *ret* proto-oncogene cDNA clones that corresponds to the 1072 amino acid protein. The 3′ region differing from the sequence shown in a is indicated by arrows.

Fig. 1. The nucleotide sequence of two of four species of *ret* proto-oncogene cDNA analyzed, cloned from human neuroblastoma cell line, Nagai, encodes the same peptide as that from human monocytic leukemia, THP-1, from which only one species of cDNA was analyzed(4). The *ret* proto-oncogene in Nagai may also have the same structure as the bona fide *ret* proto-oncogene, at least in the coding region.

The primary structure of the *ret* proto-oncogene protein has a common architecture of a receptor tyrosine kinase (RTK)(3) which permits signal transmission: an amino terminal signal peptide, an extracellular ligand binding domain in the amino terminal half of the molecule followed by a hydrophobic sequence and a tyrosine specific kinase domain, which is in the cytoplasmic carboxy terminal portion of the the molecule (Fig. 2).

The extracellular domain of the *ret* proto-oncogene protein has 12 consensus sequences for N-glycosylation. There is a cysteine-rich cluster in a region close to the transmembrane portion as in most RTKs. However, the nucleotide sequence of the extracellular domain shows no homology with other RTKs. Takahashi *et al.* reported that the *ret* proto-oncogene has two transmembrane domains as the sevenless protein(4, 5) in contrast to our suggestion that there is a single hydrophobic region for a transmembrane domain(3). This difference is probably due to the method of analysis of the secondary structure deduced from

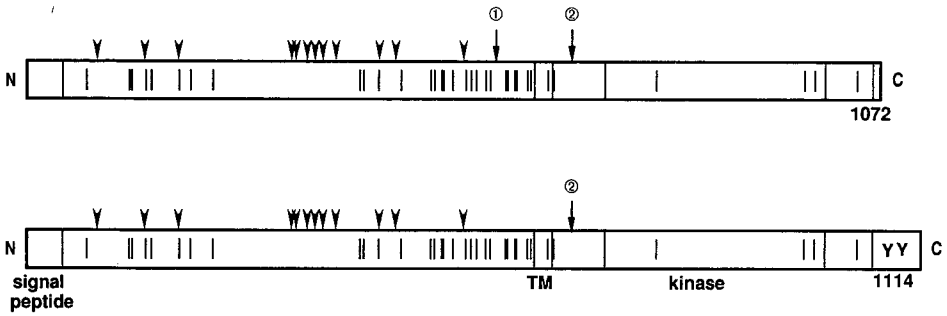

Fig. 2 Structure of *ret* proto-oncogene products deduced from nucleotide sequences of *ret* proto-oncogene cDNAs(3).

The upper scheme is for the *ret* proto-oncogene product consisting of 1072 amino acids, and the lower one is for that consisting of 1114 amino acids. The C-terminal 9 amino acids of the 1072 amino acid protein are different from the C-terminal 51 amino acids of the 1114 amino acid protein which contains two tyrosine residues indicated as Y. The positions of the signal peptide, transmembrane domain (TM) and kinase domain are shown. Arrowheads indicate possible N-linked glycosylation sites and vertical bars indicate cysteine residues. The recombination points of *ret* and *ret*-II are indicated as ① and ②, respectively. 3′ portion of the *ret* oncogene was cloned only 1072 ammino acid type.

the putative primary amino acid sequence. This problem may be elucidated by studies using an antibody against the *ret* proto-oncogene product. The cytoplasmic tyrosine kinase domain displays an extensive sequence-homology in members of the protein tyrosine kinase family, and the tyrosine kinase domain of the *ret* proto-oncogene also has 40-50% homology with those of other RTKs.

The RTK family was classified into three distinct groups by Yarden *et. al.*(6) based on a detailed comparison of the established RTK primary sequences. Subclass I RTKs are monomeric receptors with two repeats of cysteine rich sequences within their extracellular domains [ex. EGFR(7), HER2/neu(8, 9, 10)]. Subclass II RTKs have two subunits (α and β) connected by disulfide bonds [insulin receptor(11, 12), IGF I receptor(13)]. Subclass III proteins have no cysteine rich clusters, but strictly conserve 10 cysteine residues. The catalytic domains of subclass III are interrupted by a long (77-107 residues) hydrophilic insertion sequence [PDGF receptor(14), CSF-1 receptor(15, 16)]. The *ret* proto-oncogene protein has a cysteine-rich cluster in the extracellular domain with a short interrupting sequence (25 residues) in the catalytic domain. Thus, it appears to belong to a new subclass of the tyrosine kinase gene family.

2. Two types of ret *proto-oncogene products generated by alternative splicing*

Five species of mRNA of the *ret* proto-oncogene were detected by a probe of the *ret* proto-oncogene kinase domain (Fig. 3). In human cell lines these species were estimated to be about 7.0 kb, 6.0 kb, 4.6 kb, 4.5 kb and 3.9 kb in size(3, 17).

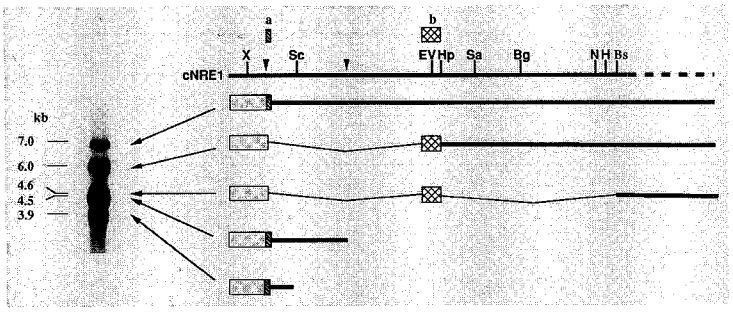

Fig. 3 Alternative splicing in the 3′ portion of 5 species of *ret* proto-oncogene mRNA(3).
The restriction map of the 3′ portion of *ret* proto-oncogene genomic DNA is shown (cNRE1). Box a shows the genomic region encoding the C-terminal 9 amino acids of the 1072 amino acid protein, and box b shows that encoding the C-terminal 51 amino acids of the 1114 amino acid protein. The 3′ portions of 7.0 kb, 6.0 kb, 4.6 kb, 4.5 kb and 3.9 kb mRNA are generated by alternative splicing and alternative polyadenylation. The consensus sequence of poly (A) additional sites are indicated by arrows. 7.0 kb, 6.0 kb, 4.6 kb, 4.5 kb and 3.9 kb mRNA of the *ret* proto-oncogene expressed in neuroblastoma, SK-N-SH, are shown in the left part.

Detailed analysis of the *ret* proto-oncogene cDNA from Nagai cell line revealed that these 5 species of mRNA encoded two kinds of protein products which have different carboxy terminal structures(3) ; one consists of 1114 amino acids, and the other of 1072 amino acids. The 51 carboxy terminal amino acids of the 1114 amino acid protein are replaced by different 9 amino acids in the 1072 amino acid protein. Comparison of the structures of genomic DNA and cDNA of the *ret* proto-oncogene indicated that this carboxy terminal difference is generated by alternative splicing and polyadenylation. As shown in Fig. 3, the protein of 1114 amino acids would be encoded from 6.0 kb and 4.6 kb mRNA of the *ret* proto-oncogene, whereas the 1072 amino acid protein would be encoded from 7.0 kb, 4.5 kb and 3.9 kb mRNA. Although 7.0 kb mRNA hybridized with the two fragments that are specific to the 9 and the 51 carboxy terminal amino acids respectively, it would encode the protein of 1072 amino acids because there is a stop codon before the sequence coding 51 carboxy terminal amino acids (data not shown). It remains to be elucidated whether these two protein molecules have different roles in signal transduction.

Coding sequence of *ret* proto-oncogene cDNA cloned from Nagai encodes the 1114 amino acid protein was the same as that from THP-1 cloned by Takahashi *et al.*(4).

III. Activated forms of the *ret* proto-oncogene, *ret* and *ret*-II

Two types of activated forms, *ret* and *ret*-II, have so far been found in NIH3T3 cell transformants. *ret* and *ret*-II were derived from the DNA of a human T cell lymphoma(1) and a human sigmoid colon cancer(2). *ret*(18) and *ret*-II(19) are activated by DNA rearrangements, resulting in the replacement of the 5' half of the *ret* proto-oncogene by different and unrelated sequences. The recombination points are indicated in Fig. 2. (① and ② show the recombination points of *ret* and *ret*-II, respectively.) The recombination point of *ret* is upstream of the membrane spanning portion of the *ret* proto-oncogene(4), whereas that of *ret*-II is downstream of this portion(19). The replaced sequence of *ret*-II has no sequence encoding a hydrophobic amino acid stretch for a putative transmembrane domain(19), indicating that the fusion protein encoded by *ret*-II has no transmembrane portion, while that by *ret* does have this portion. These rearrangements found in *ret*(1) and *ret*-II(2) did not exist in the original tumor DNA, and might have occurred during transfection processes.

Two kinds of products differing in their carboxy termini were deduced from the *ret*-II oncogene(19). These different carboxy termini were shown to be generated by alternative splicing(19) as found in the case of the *ret* proto-oncogene(3).

Table 1 *Transforming activities of pRSVretII-p 51 and pRSVretII-p 9*

DNA	No. of foci/μg DNA	
	Exp. 1	Exp. 2
Salmon sperm	0	0
pRSVneo	0	0
pRSVretII-p 51	107	99
pRSVretII-p 9	112	133
a1-1*	6	3

For expressing cDNAs of *ret*II, pRSVretII-p 51 and pRSVretII-p 9 were constructed. Carboxy terminal sequence of pRSVretII-p 51 was 51 amino acids type, while that of pRSVretII-p 9 was 9 amino acids type. Each plasmid DNA was transfected into NIH3T3 cells with salmon sperm DNA. The number of foci were counted 2-3 weeks after transfection(19).

* DNA extracted from a1-1, a transformant of NIH3T3 cells containing activated human c-Ha-ras(34).

We examined the influence of these different carboxy termini on the transforming activity of *ret*-II. The two kinds of *ret*-II cDNAs were ligated with RSV LTR as a promoter. As shown in Table 1, the two *ret*-II cDNAs had almost the same transforming activity on NIH3T3 cells.

The carboxy terminal sequence of the *ret* oncogene reported(18) was the same as that of our 9 amino acid type cDNA of the *ret* proto-oncogene and *ret*-II oncogene. By a chimera experiment, Takahashi *et al.* showed that the 51 amino acid type cDNA down-regulated the transforming activity of the *ret* oncogene(4), suggesting that the tyrosine residues in the carboxy terminus (Y in Fig. 1) regulate the transforming activity, as detected in *src* and c-*fms* oncogenes. This difference between the transforming activities of *ret* and *ret*-II might be due to the difference in their amino terminal structures.

IV. Expression of the *ret* proto-oncogene in normal rat tissue

In rat tissues 4 major species of mRNA, 7.0, 6.0, 4.5 and 3.9 kb were so far detected(17).

The levels of expression of the *ret* proto-oncogene are very low in adult rat tissues and could be detected only after 10 days exposure to X-ray film using poly $(A)^+$ RNA (Fig. 4-a). Relatively strong signals were observed in samples of the brain, thymus and testis. The lung, heart, spleen and small intestine also expressed detectable levels of *ret* proto-oncogene transcripts. The bone marrow, liver and kidney expressed undetectable levels of mRNA, although they showed

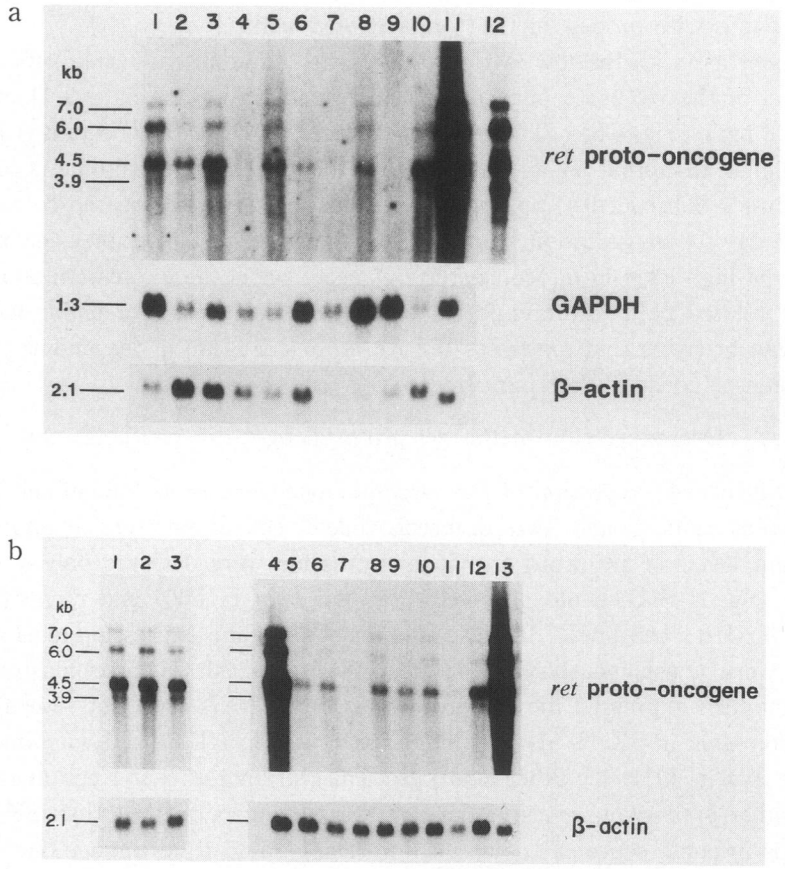

Fig. 4 Expression of the *ret* proto-oncogene in adult and embryonal rat tissues(17).

a. Expression of the *ret* proto-oncogene in adult rat tissues. RNA blots were hybridized with a ^{32}P labeled 0.4 kb EcoRI-NcoI fragment of *ret*-II cDNA which contained a portion of the *ret* proto-oncogene kinase domain. Southern blot hybridization using this probe under the same conditions as those used in RNA blot hybridization demonstrated that the intensity of a band detected in rat DNA was less than 20% of that detected in human DNA. Sources of samples were as follows: lane 1. brain; lane 2, spleen: lane 3, thymus; lane 4, bone marrow; lane 5, lung; lane 6, small intestine; lane 7, liver; lane 8, heart; lane 9, kidney; lane 10, testis; lane 11, SK-N-SH; lane 12, SK-N-SH (short time exposure)

b. Expression of the *ret* proto-oncogene during embryogenesis. Sources of samples were as follows: lane 1, conceptus on day 9; lane 2, conceptus on day 10; lanes 3 and 4, conceptus on day 11; lane 5, fetus on day 14; lane 6, placenta on day 14; lane 7, extra-embryonal membrane on day 14; lane 8, head of day 20 fetus; lane 9, chest of day 20 fetus: lane 10, abdomen to tail of day 20 fetus; lane 11, liver of day 20 fetus; lane 12, placenta on day 20; lane 13, SK-N-SH

definite bands with probes for GAPDH and/or β-actin.

In contrast with the low levels of expression in adult rat tissues, the expression level of the *ret* proto-oncogene in entire conceptuses on days 9-11 of gestation were high (Fig. 4-b). The level of expression in day 10 conceptus was 20-50 times higher than that in adult thymus as measured by densitometry of autoradiograms. Subsequently *ret* proto-oncogene expression decreased to the basal level by day 14 of gestation. Considering the low level of expression in adult tissues and high level in the conceptus, *ret* proto-oncogene expression seems likely to be related to the differentiation or growth of placental or embryonic cells. The organ or tissue that expresses the *ret* proto-oncogene in the middle stage of embryogenesis must be determined.

V. Overexpression of the *ret* proto-oncogene in neuroblastoma

The levels of expression of the *ret* proto-oncogene in 44 human cell lines of 19 different tumors and two different rodent cell lines were examined(19). Significant levels of *ret* proto-oncogene expression were detected only in neuroblastoma (SK-N-SH), acute promyelocytic leukemia (HL60) and monocytic leukemia (THP-1) cell lines. In our preliminary data, the expression level and frequency were conspicuously high in neuroblastoma: All 11 neuroblastoma cell lines examined expressed the *ret* proto-oncogene mRNA(data not shown). The expression level in SK-N-SH was higher than that in HL 60 and was almost the same as that in THP-1. Other neuroblastoma cell lines except 1 cell line showed much higher expression levels of the *ret* proto-oncogene mRNA than SK-N-SH, the levels in some neuroblastoma cell lines being 25-45 times higher than that in SK-N-SH. Thus the *ret* proto-oncogene is overexpressed specifically in neuroblastoma cells. Furthermore, the *ret* proto-oncogene was also found to be expressed in all 29 cases of surgical specimens of neuroblastoma examined, including those in clinical stage I and stage II(data not shown).

No gene alteration, such as rearrangement or amplification, of the *ret* proto-oncogene was detected in neuroblastoma cell lines by Southern blot hybridization using a full length *ret* proto-oncogene cDNA which encoded the 1114 amino acids protein.

N-myc gene amplification is reported to be a marker of poor prognosis of neuroblastoma(20, 21). NSE, an isozyme of a glycolytic enzyme, is highly expressed in some neuroendocrine tumors, such as neuroblastoma, small cell lung carcinoma and nonfunctioning pancreatic islet cell carcinoma(22, 23, 24), and an increase in the level of NSE protein is reported to be associated with neuronal differentiation(25). The correlation of *ret* proto-oncogene expression with N-myc amplification and the titer of neuron specific enolase (NSE) were examined

on the 11 cell lines. N-myc was also overexpressed in 10 of 11 neuroblastoma cell lines, and gene amplification was observed in all the cell lisnes that had overexpressed N-myc. But the level of expression of the *ret* proto-oncogene was not correlated with either the level of expression or amplification of the N-myc gene. Similarly there was not so strong correlation between the levels of NSE production and expression of the *ret* proto-oncogene.

The specific high expression of the *ret* proto-oncogene in neuroblastoma suggests that *ret* proto-oncogene expression should be a good marker in diagnosis of neuroblastomas and that antibodies to the *ret* proto-oncogene product could be used in treatment of neuroblastomas.

Because the overexpression in neuroblastomas was not associated with any structural alteration such as rearrangement or amplification, this overexpression might be due to changes of the cis-acting DNA sequences and/or transcription factors of the *ret* proto-oncogene. Recent study showed that consensus sequences for the binding site of SP1, a transcription factor(26), are clustered in the 5' flanking region of the *ret* proto-oncogene. Considering that expression of the *ret* proto-oncogene is restricted to neuroblastomas, it seems unlikely that only SP1 is involved in regulation of *ret* proto-oncogene expression in neuroblastomas: additional *cis*-acting DNA sequences and/or transcription factors may also be important for expression of the *ret* proto-oncogene acting in co-operation with SP1. Clarification of the mechanism of the regulation of transcription would provide a clue to the pathophysiology of neuroblastoma.

VI. Chromosome assignment of the *ret* proto-oncogene

The *ret* proto-oncogene was mapped on chromosome 10q11.2 by fluorescence *in situ* hybridization (Fig. 5) using two cosmid clones cret-1 and -2 which included the 5' part of *ret* proto-oncogene cDNA(27). This region is near the locus responsible for an inheritable disease, multiple endocrine neoplasia type 2A (MEN2A). MEN2A is inherited in an autosomal dominant fashion, and induces pheochromocytoma, medullary thyroid carcinoma and parathyroid hyperplasia or adenoma. The locus responsible for MEN2A is located near the interstitial retinol binding protein (IRBP) locus and the genetic marker D10S5(28, 29). IRBP is located on chromosome 10p11.2-q11.2 and D10S5 on 10q21.1. As restriction fragment length polymorphism is detectable with *ret* proto-oncogene cDNA, it would be a good genetic marker to search for MEN2A responsive gene.

Recently the PTC oncogene(30), which was detected by transfection assay in NIH3T3 cells with thyroid papillary carcinoma DNA, was also mapped near the MEN2A locus. PTC oncogene may also be a suitable marker in searching for the gene responsible for MEN2A.

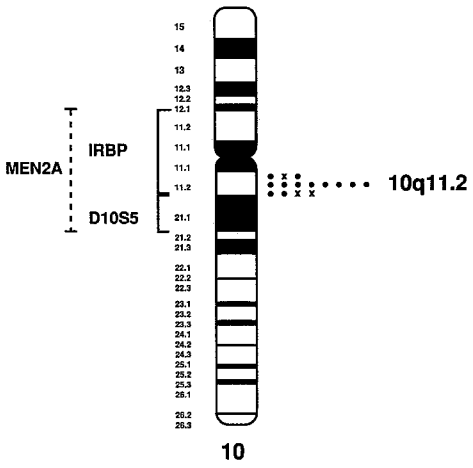

Fig. 5 An idiogram of chromosome 10 showing results of *in situ* hybridization with cosmid clones including the 5′ portion of *ret* proto-oncogene cDNA(27).

Solid circles indicate a pair of fluorescent spots which were detected at the same location on both chromatids and crosses indicate a single spot detected on one chromatid. The regions detectable by interstitial retinol binding protein (IRBP) cDNA (10p11.2-q11.2) and D10S5 (10q21.1) are also shown.

VII. Possible role of the *ret* proto-oncogene

It is interesting that neuroblastomas and the tumors associated with MEN2A originate from neural crest cells. We examined the expression of *ret* proto-oncogene mRNA in tumors originating from the neural crest. No signal of *ret* proto-oncogene expression was, however, detected in a rat pheochromocytoma (PC-12) or rat medullary thyroid carcinoma. Neither was it detected in four human melanoma cell lines by northern blot analysis. (Fig. 6)

Melanocytes in the nevus sometimes show neuronal cell morphology(31). Moreover, one neuroblastoma cell line, SK-N-SH, sometimes differentiates melanoma-like cells morphologically and enzymatically(32). In preliminary studies, we found that the *ret* proto-oncogene is expressed only in the neuronal-like SK-N-SH cells. Based on these findings, we propose the hypothesis for the function of the *ret* proto-oncogene as shown in Fig. 7. At least two possibilities are considered for its biological functions. One is that the *ret* proto-oncogene is required for differentiation of precursor cells into neuroblasts, and the other is that constitutively high expression of the *ret* proto-oncogene induces neuroblasts to convert to malignant cells. It remains to be elucidated what biological role the ret proto-oncogene has and whether it is really involved in development of neuroblastoma or not.

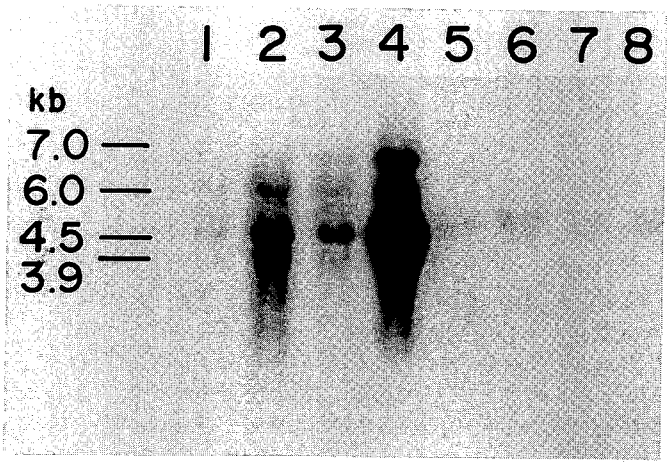

Fig. 6 Northern blot hybridization of four neuroblastoma cell lines and four human melanoma cell lines.

The *ret* proto-oncogene cDNA fragment which have kinase domain sequence was used as probe. Sources of samples: lanes 1-4, neuroblastoma cell lines (lane 1, SK-N-SH; lane 2, YT-nu; lane 3, TGW-1-nu; lane 4, NB-39-nu) lanes 5-8, human melanoma cell lines (lane 5, G336; lane 6, WMRG-MENG; lane 7, C32TG; lane 8, MeWo)

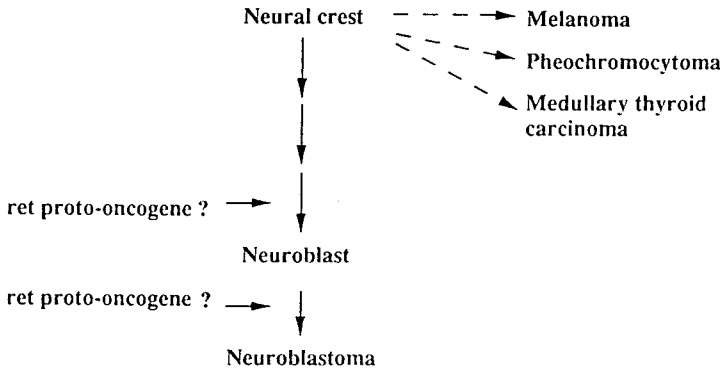

Fig. 7 Possible functions of the *ret* proto-oncogene. The *ret* proto-oncogene may be required for differentiation from precursor cells to neuroblasts, or may be closely related with development from original cells to neuroblastomas.

Acknowledgment

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