Neuroblastoma in a transgenic mouse carrying a metallothionein/ret fusion gene

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Summary We have recently succeeded in producing transgenic mice carrying a hybrid gene consisting of mouse metallothionein promoter-enhancer and the ret oncogene (MT/ret). (Iwamoto et al., 1991b). A retroperitoneal tumour developed in one of 17 MT/ret transgenic founder mice. Histological analysis revealed that the tumour consisted of undifferentiated neuroblasts and differentiated ganglion cells, the latter of which were strongly positive for neuron specific enolase. Expression of the ret transgene was observed at high levels in RNA from the tumour, but not in those of other normal tissues. In addition, a 100kDa ret protein was detected in the cell lysate of the tumour. Taken together with our previous data, these results suggest a possible role for the ret oncogene in the proliferation of neural crest cells.

The ret proto-oncogene encodes a receptor type tyroine kinase (Takahashi et al., 1988a, 1989; Tahira et al., 1990) and is frequently expressed in human neuroblastoma, pheochromocytoma and thyroid medullary carcinoma that originate from neural crest cells (Takahashi & Cooper, 1987; Nagao et al., 1990; Ikeda et al., 1990; Santoro et al., 1990; Takahashi et al., 1991). Although a ligand for the ret proto-oncogene product has not been identified, this finding suggests that the ret proto-oncogene might play a role in the differentiation or proliferation of neural crest cells.

The ret oncogene is a hybrid gene of the ret proto-oncogene and a 'zinc finger'-containing gene (c-fos) (Takahashi et al., 1985; Takahashi & Cooper, 1987; Takahashi et al., 1988b). This hybrid gene was generated by DNA rearrangement which occurred during the transfection assay. We recently reported the establishment of transgenic mice carrying the ret oncogene driven by a mouse metallothionein regulatory element (Iwamoto et al., 1991b). We obtained 17 founder mice, four of which unexpectedly showed severe pigmentations in their whole skin, resulting from proliferation of melanin-producing cells. Melanocytic tumours developed in three of the four mice with the pigmented skin. Northern blot and in situ hybridisation experiments indicated that the ret transgene was expressed preferentially in melanin-producing cells. These results indicated that the MT/ret gene affected the proliferation or differentiation of part of the neural crest cells in our transgenic mice. In addition to these mice, we found the development of a retroperitoneal tumour in one MT/ret founder mouse (Iwamoto et al., 1991b). In the present study, we report the characterisation of this tumour that was histologically diagnosed as a neuroblastoma.

Material and methods

Mice

The methods to produce the MT/ret transgenic mice were described previously (Iwamoto et al., 1991b).

Antibody

A polyclonal antibody was developed against the tyrosine kinase domain of the Ret protein (Takahashi et al., 1991). Briefly, a fragment of the ret cDNA was inserted into the pET expression vector and the resulting recombinant plasmid was transformed into E. coli BL2(DE3) strain carrying a single copy of the gene for T7 RNA polymerase under control of the lacUV5 promoter. The Ret protein was induced with isopropyl-β-D-thiogalactopyranoside (IPTG). The protein was then subjected to SDS-polyacrylamide gels and recovered by electroelution. Rabbits were immunised five times subcutaneously with 500μg of the protein in Freund's adjuvant. Anti-phosphotyrosine (PTYR) antibody was purified by affinity chromatography from the sera of the rabbits immunised with v-abl-encoded bacterial protein (Hamaguchi et al., 1988).

Immunohistochemistry

Paraffin sections of neuroblastoma were stained with anti-neuron specific enolase antibody by a peroxidase anti-peroxidase (PAP) method (Wajjwalku et al., 1991).

Northern hybridisation

Total cellular RNA (15μg) was isolated by a single step method (Chomczynski & Sacchi, 1987), separated using agarose formaldehyde gels and transferred to nylon membranes (Amerham, UK). The probe used for this analysis is a PstI-PstI fragment of the kinase domain of the ret oncogene (Iwamoto et al., 1990, 1991a). Prehybridisation, hybridisation and washes were performed as described previously (Iwamoto et al., 1990).

Western blotting

Total cell lysates were prepared from tissues of MT/ret transgenic mice (Iwamoto et al., 1991b) as described previously (Takahashi et al., 1991). The lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Schleicher & Schuell, Germany) or polyvinylidene difluoride (PVDF) (Nihon Millipore Kogyo K.K., Yonezawa, Japan) membranes. Reaction with anti-Ret antibody was performed by the avidin-biotin complex immunoperoxidase method. Colour development was performed using the POD immunostain set (Wako Pure Chemical Ind., Ltd., Osaka, Japan). In the case where the anti-PTYR antibody was used as the first antibody, the membranes were probed with 125I-protein A (ICN, Irvine, CA, USA).

Results and discussion

One founder male (designated 301) of the MT/ret transgenic mice developed a tumour on the back at 3 months of age. Since the tumour grew rapidly, the animal was sacrificed at 3.5 months of age. The tumour occupied the right ret-
roperitoneum and involved the right kidney and the adrenal gland (data now shown).

Histologically, the tumour consisted of undifferentiated neuroblasts (Figure 1a) and differentiated ganglion cells (Figure 1b). The former were small round cells with round nuclei and scanty cytoplasm and the latter were large cells with large nuclei and basophilic cytoplasm. The tumour was also characterised by an eosinophilic fibrillar matrix that corresponded to nerve fibers (Figure 1a). Histochemical analysis indicated that the ganglion cells were strongly positive for neuron specific enolase, while neuroblasts were weakly stained (Figure 1c). On the other hand, neither of these cells were reactive with anti S-100 antibody (data not shown). Thus the tumour was diagnosed as a neuroblastoma or ganglioneuroblastoma.

We analysed the transgene expression in RNAs from several tissues of the transgenic mouse. As shown in Figure 2, a 4.5kb transcript of the transgene was detected in the tumour, using a 3' ret cDNA probe corresponding to the tyrosine kinase domain (Figure 2). A 5' ret cDNA probe corresponding to the rfp sequence also detected the same 4.5kb transcript (data not shown), indicating that this transcript was derived from the transgene. In other tissues, its expression was weak or undetectable.

Our recent study (Taniguchi et al., 1992) demonstrated that the Ret proteins were expressed as 100kDa and 96kDa glycoproteins in melanocytic tumours which developed in MT/ret transgenic mice (Figure 3). These proteins were not

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**Figure 1** Histopathology of a retroperitoneal tumour. The tumour consisted of undifferentiated neuroblasts a, and differentiated ganglion cells b, (Hematoxylin and eosin staining). Asterisks indicate eosinophilic fibrillar matrix. c, Histochemical analysis of the tumour by anti-neuron specific enolase antibody. The ganglion cells were strongly stained. Magnification × 270.

**Figure 2** Northern blot analysis of the transgene. Total cellular RNAs (15 μg) isolated from various tissues of the transgenic mouse (301) were applied to each lane. The blot was hybridised with a 0.55kb PstI-PstI ret cDNA probe containing part of the tyrosine kinase domain (Iwamoto et al., 1990, 1991a). The positions of 28S and 18S ribosomal RNA are indicated.
detected in the lysates from normal liver and kidney of a transgenic mouse with melanocytic tumours. The same 100 kDa Ret protein was also observed in the cell lysate of the neuroblastoma, while the presence of a 96 kDa Ret protein was unclear (Figure 3). Rather, the anti-Ret antibody recognised a broad band of 93 to 100 kDa in the lysate of the neuroblastoma, suggesting that part of the Ret proteins might have been degraded.

To examine phosphotyrosine (PTyr)-containing proteins in the neuroblastoma, the lysate was reacted with an anti-PTyr antibody. As shown in Figure 4, a 100 kDa band was detected in the neuroblastoma as well as in the melanocytic tumour. On the other hand, this 100 kDa phosphorylated band was absent in the lysates from normal liver and kidney of an MT/ret transgenic mouse. Since the electrophoretic mobility of this band was consistent with the 100 kDa Ret protein, it is possible that the 100 kDa phosphorylated band represented the Ret protein. In addition, the level of tyrosine phosphorylation in neuroblastoma cells seemed to be lower than that in melanocytic tumour cells (Figure 4).

Although several kinds of transgenic mice carrying oncogenes driven by a metallothionein regulatory unit have been produced (Messing et al., 1985; Ruther et al., 1987; Heisterkamp et al., 1990), there have been no reports of development of neuroblastoma. In addition, there was no spontaneous development of neuroblastoma in the MT/ret transgenic mice. It is known that the ret proto-oncogene is expressed at high levels in human neuroblastomas (Takahashi & Cooper, 1987; Ikeda et al., 1990; Nagao et al., 1990; Takahashi et al., 1991). Thus, it is interesting that neuroblastoma developed in an MT/ret transgenic mouse. Since the Ret proteins are present as membrane-bound glycoproteins like the proto-Ret proteins (Taniguchi et al., 1992), it is possible that both of them have similar functions in prolifera-

tion of neuroblasts. The fact that four of 17 MT/ret transgenic founder mice displayed disorders of melanoblasts which also originate from the neural crest cells (Iwamoto et al., 1991b) suggested that the MT/ret transgene is expressed preferentially in these cell types. The sequence present in the MT/ret fusion gene may be responsible for this unique expression pattern in our transgenic mice. Analysis by in situ hybridisation is necessary to elucidate the precise expression pattern of the ret oncogene during embryogenesis of the MT/ret transgenic mice.

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