Virchows Archiv (1994) 424:563–568

ORIGINAL ARTICLE

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Frequent expression of 75 kDa nerve growth factor receptor and phosphotyrosine in human peripheral nerve tumours: an immunohistochemical study on paraffin-embedded tissues

Received: 15 November 1993 / Accepted: 28 March 1994

Abstract One hundred and three benign, and 10 malignant peripheral nerve tumours were examined immunohistochemically for expression of 75 kDa nerve growth factor receptor (NGFR). In benign tumours NGFR was demonstrated at 61% in neurinoma, 71% in neurofibroma, 93% in neurofibromatosis and 90% in traumatic neuroma. Malignant neurogenic tumours were 100% positive for NGFR. Phosphotyrosine-immunoreactivity was detected in 76% of NGFR-positive tumours but the frequency of immunostained tumour cells was low. These results suggest that both benign and malignant peripheral nerve tumours express 75 kDa NGFR. The receptor seems to serve as growth signal transduction of the tumour cells in terms of phosphorylation of the tyrosine residue of the receptor or the target protein of the NGFR protein tyrosine kinase.

Key words Nerve growth factor receptor
Phosphotyrosine · Peripheral nerve tumours
Immunohistochemistry

Introduction

Tumours derived from peripheral nerve tissues, especially when malignant, are far less frequent than tumours originating from epithelial cells and haematopoietic/lymphoid cells. Investigation of growth factor(s) or growth factor receptor(s) has not been performed systematically on these tumours because of the limitation of number of cases available and requirement for fresh tumour samples. With the advent of a monoclonal antibody against nerve growth factor receptor (NGFR) which reacts with cryostat section and/or formalin-fixed, paraffin-embedded antigen [23] a number of these tumours can be used for research on receptor expression. In fact, several reports on the expression of NGFR in human tumours or normal tissues have been documented indicating preferential expression in peripheral nervous system, neural crest tumours and normal epithelial, mesenchymal and lymphoid tissues [4, 21, 23]. NGFR expression in malignant tumours generally parallels its normal distribution [4], but also cultured human cell lines derived from three germ layers express NGFR [29]. Little is know about the precise expression and distribution of NGFR in peripheral nerve tumours and we therefore undertook a study to determine the expression pattern of the NGFR in these tumours, since the monoclonal antibody used reacts with paraffin-embedded tissues. As the NGFR has tyrosine kinase activity [10, 12, 14, 24], phosphotyrosine-immunoreactivity was also investigated with the use of a monoclonal antibody to phosphorylated tyrosine residue.

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Materials and methods

Formalin-fixed, paraffin-embedded materials obtained from surgical specimens of benign and malignant peripheral nerve tumours were employed in this study. Spinal cord tumours including 1 glioma, 10 meningiomas and 4 ependymomas were also used for reference. Diagnosis of each disease was established on routine paraffin-embedded sections according to the classification of WHO in 1969 [6] with or without special staining.

The monoclonal antibody against 75 kDa low affinity NGFR of Man (Boehringer-Mannheim, Mannheim, Germany) monoclonal antibody against phosphotyrosine (PT) (Transformation Res., Framingham, Mass., USA) and monoclonal S-100 protein (S-100β) antibody [28] were used. The isotype of the three monoclonal antibodies is IgG_1. The working titre was 0.1 μg/ml IgG_1 for the NGFR antibody, 1: 200 for the PT antibody and 1: 100 for the S-100 protein antibody, respectively. Biotinylated rabbit antimouse immunoglobulins, reactive with all mouse isotypes for the monoclonal antibody and a streptoavidin-peroxidase complex served as detection systems for the antibodies and were provided by Nichirei (Tokyo, Japan). We obtained 3,3'-diaminobenzidine tetrahydrochloride from Dojindo Lab. (Kumamoto, Japan), sodium azide from Wako Chemicals (Osaka, Japan) and skimmed milk from Yukijirushi (Tokyo, Japan).

Deparaffinized sections, cut at 5 μm thick, were treated with methanol-hydrogen peroxide for 20 min to block endogenous peroxidase activity. They were then soaked in 5% skimmed milk solution in phosphate buffered saline (PBS) (pH 7.4, 0.05 M) for

30 min to avoid non-specific adsorption of immunoglobulins. After staining with the primary antibodies overnight at 4° C, the slides were incubated with biotinylated secondary antibodies and streptoavidin peroxidase complex for 30 min at room temperature, respectively. All incubation steps were done in a humid chamber and followed by triple rinsing with PBS. Using 3,3'-diaminobenzidine as the chromogen at concentration 20 mg/dl in 0.05 M TRIS-HCl buffer, pH 7.2, containing 0.01% hydrogen peroxide for 10 min, the peroxidase reaction product caused an intense brown precipitate. In this step, 65 mg/dl sodium azide was added to block endogenous peroxidase activity again [26] a necessary step as there is not enough capacity to block enzyme activity when coupled with streptavidin, because of higher content of the peroxidase in the reagent. The sections were rinsed in tap water, counterstained with 1% methyl green, and mounted with new MX mounting reagent from Matsunami Glass (Osaka, Japan).

Negative control samples were constructed by omitting the primary antibodies or staining with non-immune mouse IgG: no staining was observed. In addition, the specimens contained peripheral nerve tissues served as an internal control showing positivity to NGFR and S-100β protein. As a positive control for phosphotyrosine, rat phaechromocytoma cell line PC12 established by Green and Tischler [8] was treated with 100 ng/ml NGF (2.5 s NGF, Sigma, St. Louis, Mo., USA), for 1, 3 and 5 days, since PC12 expresses NGFR [25] and NGF induces tyrosine phosphorylation of 130 kDa protein in PC12 [20]. After treatment, the cell line was fixed in 3.5% formaldehyde in PBS for 5 min followed by methanol at –20° C for 5 min. The fixed cells were immunostained by the method described above.

Immunostaining was evaluated as follows: a *focal* distribution of positive cells describes scattered and/or localized occurrence of immunolabelled cells at a frequency lower than 20%, and *diffuse* distribution implies wide-spread positively immunostained cells at a frequency higher than 20%.

Results

The results are summarized in Table 1. Benign peripheral nerve tumours or tumour-like condition (traumatic neu-

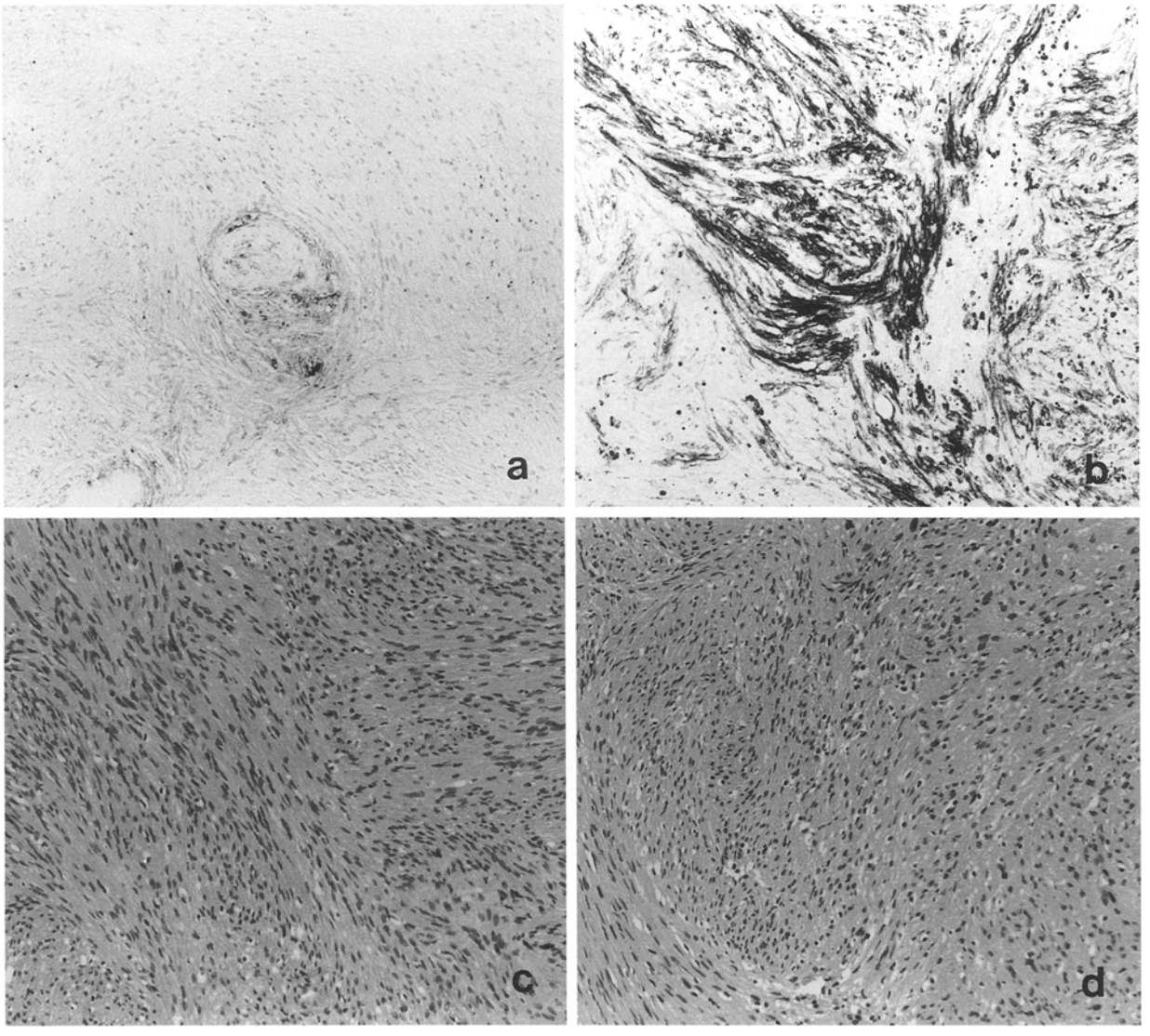

Fig. 1 Nerve growth factor receptor (NGFR) immunostaining reveals a few labelled cells in the first, primary acoustic neurinoma (**a,** × 120) and numerous positive tumour cells in the second, recurrent acoustic neurinoma (**b,** × 120), while histological features of both tumours seem alike each other (**c, d:** haematoxylin and eosin, × 120)

Table 1 Immunohistochemical detection of 75 kDa NGFR of human peripheral nerve tumours

Tumour	Frequency	(%)	Distribution	(n)
Benign				
neurinoma	39/64	(60.9)	diffuse	(7)
			focal	(32)
neurofibroma	10/14	(71.4)	diffuse	(3)
			focal	(7)
neurofibromatosis	14/15	(93.3)	diffuse	(1)
			focal	(13)
traumatic neuroma	9/10	(90)	diffuse	(5)
			focal	(4)
Malignant				
malignant neurinoma[a]	10/10	(100)	diffuse	(5)
			focal	(5)
Total	82/113(73)			

[a]Four cases with von Recklinghausen's disease

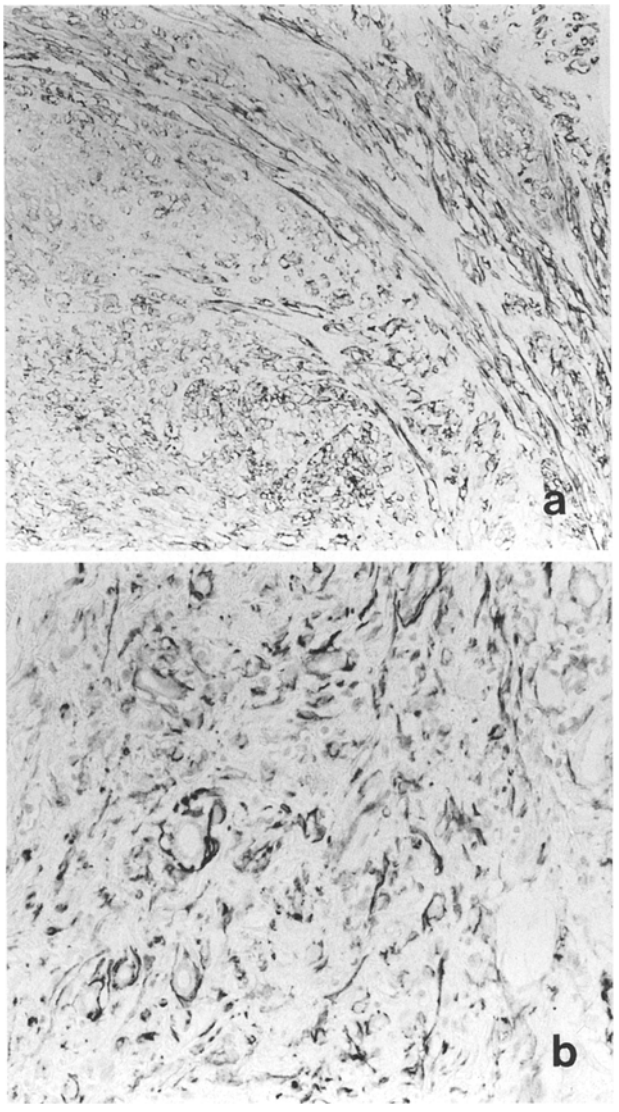

Fig. 2 NGFR immunostaining discloses numerous positive cells in malignant neurinoma (**a,** × 120) and malignant neurinoma occurred in von Recklinghausen's disease (**b,** × 200)

Table 2 Immunohistochemical detection of phosphotyrosine in NGFR-positive peripheral nerve tumours

Tumor	Phosphotyrosine	(n)	NGFR	(n)
Traumatic neuroma	5/5 diffuse	(4)	5/5 diffuse	(2)
	focal	(1)	focal	(3)
Neurinoma	4/6 focal	(4)	6/6 diffuse	(6)
Neurofibroma	3/3 focal	(3)	3/3 diffuse	(3)
Neurofibromatosis	3/4 focal	(3)	4/4 diffuse	(1)
			focal	(3)
Malignant neurinoma	4/7 diffuse	(2)	7/7 diffuse	(4)
	focal	(2)	focal	(3)
Total	19/25 (76%)		25/25 (100%)	

roma) showed positivity at high frequency: neurinoma 61%, neurofibroma 71%, neurofibromatosis of von Recklinghausen 93%, and traumatic neuroma 90%. The distribution pattern of immunostained cells was mainly diffuse in the traumatic neuroma, whereas it was mainly focal in neurinomas and neurofibromas/neurofibromatosis (Table 1). One case with acoustic neurinoma showed change in NGFR expression during patient's clinical course. The tumour from the first operation revealed very few immunolabelled cells (Fig. 1a) but recurrent tumour 3.5 years later disclosed diffuse expression of the NGFR in tumour cells (Fig. 1b) while the histology of the tumour did not exhibit any noticeable difference between the two (Fig. 1c, d). The malignant tumours derived from peripheral nerve expressed NGFR at 100% frequency (Table 1). The distribution pattern was diffuse in half of the cases of malignant neurogenic tumour and was mainly focal in the others. Representative cases are shown in Fig. 2. Malignant neurinoma (Fig. 2a) and malignant neurinoma with von Recklinghausen's disease (Fig. 2b) exhibited membranous or linear positivity of or in the tumour cells.

The results of PT-immunostaining are summarized in Table 2. Among 25 cases with expression of NGFR, 19 cases were positive for PT-immunoreactivity at 76% frequency. The distribution pattern of PT-positive cells was mainly focal and only 4 traumatic neuromas and 2 malignant neurinomas revealed a diffuse distribution (Fig. 3a–c). The reaction product localized in the cell membrane and/or in the cytoplasm. Serial sections indicated that NGFR positive cells were partly identical to PT-positive cells (Fig. 4a, b).

In general, PT-immunoreactivity was found in a more limited distribution in the tumour tissues than NGFR. A case with malignant neurinoma on the basis of von Recklinghausen neurofibromatosis, however, exhibited dominant expression of PT-immunoreactivity compared with NGFR expression (Fig. 4c, d). None of spinal cord tumours exhibited NGFR immunoreactivity.

Discussion

In the present study, we confirm that NGFR is frequently expressed in benign and malignant tumours originating

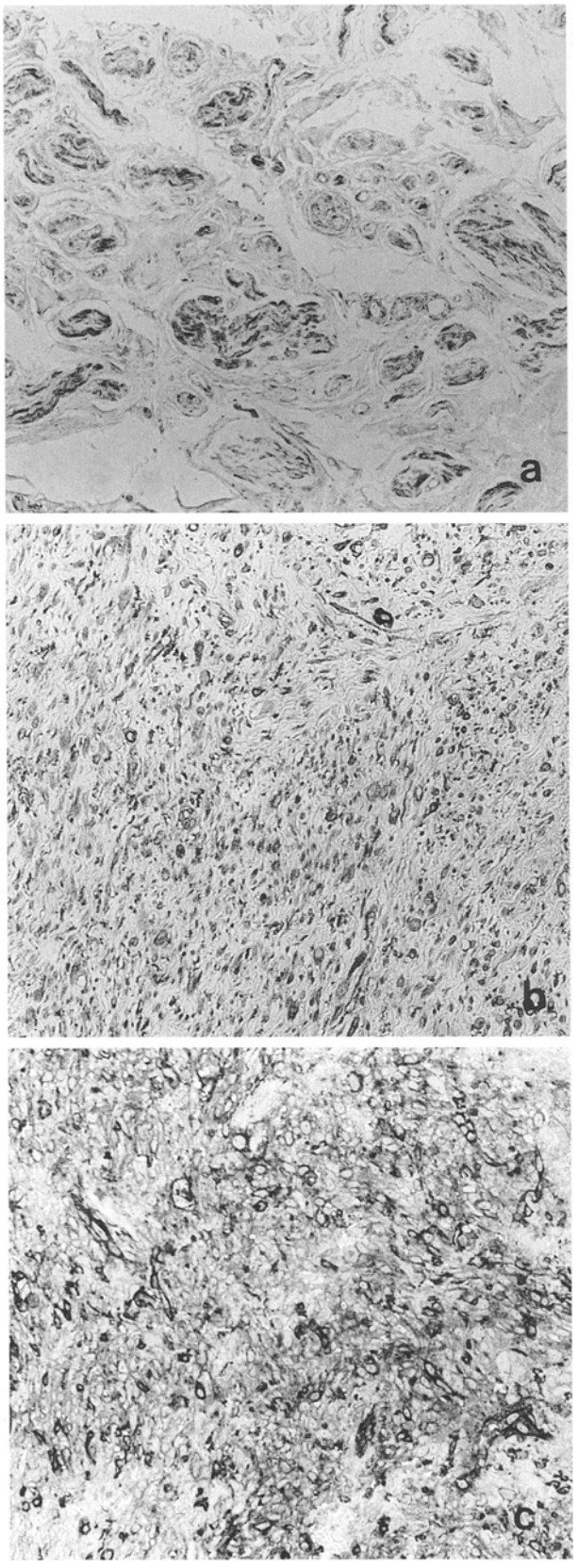

Fig. 3 PT-immunostaining labels traumatic neuroma (**a,** × 75), malignant neurinoma (**b,** × 150) and malignant neurinoma of von Recklinghausen disease (**c,** × 150)

from peripheral nerve, consistent with previous reports [4, 21].

Spinal cord tumours, however, did not show positivity for the NGFR despite their neuronal origin. Among peripheral nerve tumours a case of acoustic neurinoma revealed an interesting result; more intense expression of the NGFR in the recurrent tumour compared with the first tumour removed 3.5 years previously. This change in expression suggests acquisition of a growth advantage in the recurrence without noticeable change in histological features.

Previous work has established that two types of nerve growth factor receptors, low and high affinity or fast and slow types, can be distinguished on the neurons of the peripheral nervous system [7, 27]. The high affinity receptor is composed of two kinds of low affinity receptor, 75 kDa and 140 kDa in molecular weight, respectively [10, 22]. The 140 kDa NGF receptor is *trk* proto-oncogene product with activity of tyrosine kinase [10, 12, 13, 14, 24]. More recently, however, it has been clarified that the *trk* proto-oncogene product itself exhibits properties characteristic of the high affinity NGFR [18].

We examined 75 kDa low affinity NGFR alone and thus it is not clear whether *trk* proto-oncogene is co-expressed in the tumours examined. In some human normal tissues only low-affinity NGFR is detected without co-expression of the high-affinity NGFR encoded by the *trk* proto-oncogene [17]. However, in human neuroblastomas over-expression of the *trk* oncogene has been reported especially in non-advanced stages [1, 2, 19]. Presence of PT-immunoreactivity in about three-quarters of cases of the NGFR-positive cases, however, strongly suggests that phosphorylation of tyrosine residue occurs in the *trk* gene product of the NGFR or protein(s) downstream in the signal transduction through the NGF-ligand system. The frequency of positive tumour cells for PT-immunoreactivity was lower than that of NGFR positive tumour cells and NGFR-expressing tumour cells are only partly identical to PT-immunostained cells. The reasons for this are not clear but the possible presence of protein tyrosine phosphatase in the tumour cells might be responsible since protein tyrosine phosphatases consist of several isozymes [15], found in various tissues and cells [3, 5, 9, 11, 30]. They are plentiful in brain [16], although their presence in the peripheral nervous system has not been described. A case with malignant neurinoma occurred in von Recklinghausen neurofibromatosis which expressed PT-immunoreactivity predominantly rather than NGFR. This may imply depressed or deficient activity of phosphotyrosine phosphatase or absence of NGFR phosphotyrosine kinase in the tumour tissue. The former possibility implies that inactivation or loss of phosphotyrosine phosphatase gene may have the effect of enhancing tyrosine phosphorylation of particular protein(s) [11]. In the latter case, other kinds of phosphotyrosine kinase than NGFR may contribute to the phosphorylation of tyrosine residues in this tumour.

To elucidate the precise mechanism of these findings further study is required including the determination of

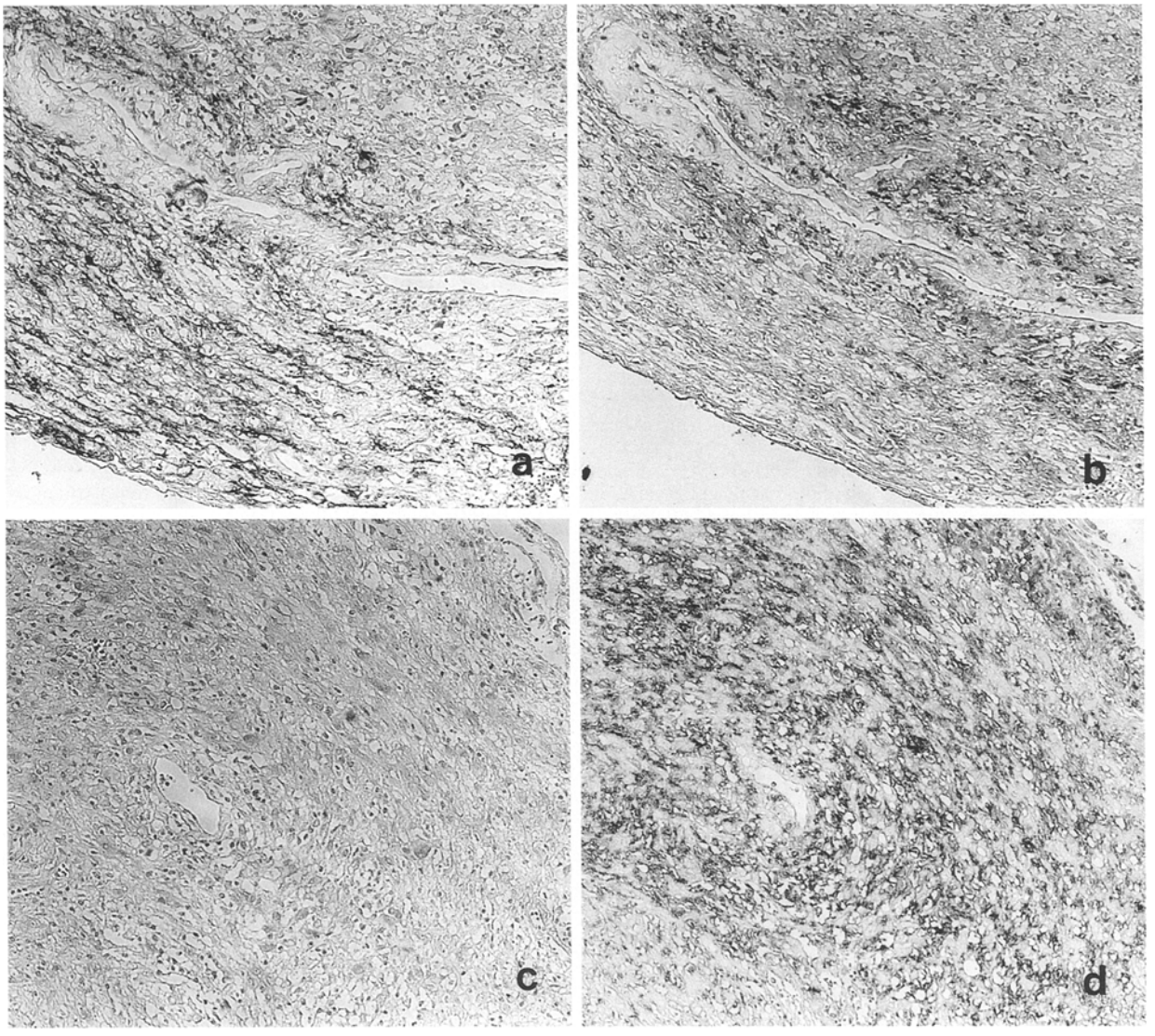

Fig. 4 Malignant neurinoma of von Recklinghausen's disease exhibits dominant NGFR expression (**a,** × 120) but focal PT-immunoreactivity (**b,** × 120). Both NGFR and PT immunolabellings seem to be identical in sone parts (**a, b;** serial section). In other parts, NGFR is absent (**c,** × 120) but PT is preferentially expressed (**d,** × 120; **c, d;** serial section)

the expression kinetics of the receptor and protein tyrosine phosphatase(s).

References

1. Borrello MG, Bongarzone I, Pierotti MA, Luksch R, Gasparini M, Collini P, Pilotti S, Rizzetti MG, Mondellini P, De Bernardi B, DiMartino D, Garaventa A, Brisigotti M, Tonini GP (1993) *trk* and *ret* proto-oncogene expression in human neuroblastoma specimens: high frequency of *trk* expression in non-advanced stages. Int J Cancer 54: 540–545
2. Brodeur GM (1993) TRK-A expression in neuroblastomas: a new prognostic marker with biological and clinical significance. J Natl Cancer Inst 85: 344–345
3. Chernoff J, Schievella AR, Jost CA, Erikson RL, Neel BG (1990) Cloning of a cDNA for a major human protein-tyrosine-phosphatase. Proc Natl Acad Sci USA 87: 2735–2739
4. Chesa PG, Rettig WJ, Thomson TM, Old LJ, Malamed MR (1988) Immunohistochemical analysis of nerve growth factor receptor expression in normal and malignant human tissues. J Histochem Cytochem 36: 383–389
5. Cool DE, Tonks NK, Charbonneau H, Walsh KA, Fischer EH, Krebs EG (1989) cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family. Proc Natl Acad Sci USA 86: 5257–5261
6. Enzinger FM, Lattes R, Torloni R (1969) Histological typing of soft tissue tumours. International histological classification of tumours. No. 3, World Health Organization, Geneva
7. Godfrey EW, Shooter EM (1986) Nerve growth factor receptors on chick embryo sympathetic ganglion cells: binding characteristics and development. J Neurosci 6: 2543–2550
8. Green LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of a rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73: 2424–2428
9. Gu M, York JD, Warshawsky I, Majerus PW (1991) Identification, cloning and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. Proc Natl Acad Sci USA 88: 5867–5871
10. Hempstead BL, Martin-Zanca D, Kapland DR, Parada LF,

Chao MV (1991) High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. Nature 350: 678–683
11. Hunter T (1989) Protein-tyrosine phosphatases: the other side of the coin. Cell 56: 1013–1016
12. Kaplan DR, Martin-Zanca D, Parada LF (1991a) Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. Nature 350: 158–160
13. Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991b) The *trk* proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252: 554–558
14. Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991) *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell 65: 189–197
15. Krueger NX, Streuli M, Saito H (1990) Structural diversity and evolution of human receptor-like protein tyrosine phosphatase. EMBO J 9: 3241–3252
16. Lombroso PJ, Murdoch G, Lerner M (1991) Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. Proc Natl Acad Sci USA 88: 7242–7246
17. MacGrogan D, Saint-André J-P, Dicou E (1992) Expression of nerve growth factor and nerve growth factor receptor genes in human tissues and in prostatic adenocarcinoma cell lines. J Neurosci 59: 1381–1391
18. Meakin SO, Suter U, Drinkwater CC, Welcher AA, Shooter EM (1992) The rat *trk* protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc Natl Acad Sci USA 89: 2374–2378
19. Nakagawara A, Arima M, Azar CG, Scavarda NJ, Brodeur GM (1992) Inverse relationship between *trk* expression and N-*myc* amplification in human neuroblastomas. Cancer Res 52: 1364–1368
20. Ohmichi M, Decker SJ, Saltiel AR (1991) Nerve growth factor induces the association of a 130-kD phosphoprotein with its receptor in PC-12 pheochromocytoma cells. Cell Reg 2: 691–697
21. Perosio PM, Brooks JJ (1988) Expression of nerve growth factor receptor in paraffin-embedded soft tissue tumors. Am J Pathol 132: 152–160
22. Ragsdale C, Woodgett J (1991) trking neurotrophic receptors. Nature 350: 660–661
23. Ross AH, Grob P, Bothwell M, Elder DE, Ernst CS, Marano N, Ghrist BFD, Slemp CC, Herlyn M, Atkinson B, Koprowski H (1984) Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies. Proc Natl Acad Sci USA 81: 6681–6685
24. Ross AH (1991) Identificatin of tyrosine kinase *trk* as a nerve growth factor receptor. Cell Reg 2: 685–690
25. Schechter AL, Bothwell MA (1981) Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with differing cytoskeletal association. Cell 24: 867–874
26. Sternberger LA (1986) Immunohistochemistry 3rd edn, John Wiley, New York, p100
27. Sutter A, Riopelle RJ, Harris-Warrick RM, Shooter EM (1979) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J Biol Chem 254: 5972–5982
28. Suzuki T, Ajioka Y, Kogure S (1988) Monoclonal S-100 immunocytochemistry of pigmented naevi with tactile-like corpuscles. J Pathol 155: 121–126
29. Thomson TM, Rettig WJ, Chesa GP, Green SH, Carrato Mena CA, Old LJ (1988) Expression of human nerve growth factor receptors on cells derived from all three germ layers. Exp Cell Res 174: 533–539
30. Yang Q, Tonks NK (1991) Isolation of cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cytoskeletal-associated proteins band 4.1, ezrin, and tralin. Proc Natl Acad Sci USA 88: 5949–5953