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Immunohistochemical Detection of High-affinity Nerve Growth Factor Receptor in Neuroblastoma

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High levels of mRNA (as assessed by northern blot) for the high-affinity nerve growth factor receptor (p140^{TRK}) are predictive of favourable outcome in neuroblastoma. The feasibility of determining p140^{trk} on frozen sections using a recently developed monoclonal antibody was evaluated, and immunohistochemical findings were compared to those obtained from northern blot analysis. Primary tumour samples from 28 untreated patients were quick frozen and an indirect immunofluorescence assay was performed on 4-μm acetone-fixed cryostat sections. 9 cases were positive with immunohistochemistry, and these were among the 15 cases also positive by northern blot. None of the cases negative by northern blot were positive with immunohistochemistry. The concordance rate was 79% ($P < 0.03$), with a sensitivity of 60% and a specificity of 100%. Immunohistochemistry can thus be rather reliable for assessing p140^{trk} expression, even when only very small amounts of tissue are available, such as with needle biopsy.

Key words: neuroblastoma, nerve growth factor, nerve growth factor receptors, immunohistochemistry
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INTRODUCTION

NEUROTROPHINS are a family of related molecules that promote neuronal survival and differentiation in the central and peripheral nervous system [1]. These include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) [1]. Neurotrophin binding to the cell surface is mediated by two types of transmembrane glycoproteins, the p75^{LNGFR} receptor and the members of the TRK family of tyrosine kinase receptors [2–4]. The p75^{LNGFR} receptor (MW 75 kDa) exhibits low-affinity binding ($K_d \sim 10^{-9}$ M) to all four neurotrophins and contains no apparent catalytic activity [1–4]. Its function may be limited to the recruitment and concentration of neurotrophins, thus facilitating their presentation to the *TRK* receptors [1–4]. The *TRK* receptors are encoded by the *TRK* genes (*TRK* or *TRKA*, *TRKB*, *TRKC*) and bind more specifically with high-affinity ($K_d = 10^{-11}$ M), each to a certain neurotrophin: p140^{trk} (MW 140 kDa) to NGF, p145^{trkB} to BDNF and NT-4/5, and p145^{trkC} to NT-3 [1–4]. Although some conflicting findings still need to be explained, current evidence supports the primary role of *TRK* products in the effective signal transduction through the autophosphorylation of tyrosine residues within the receptor's cytoplasmic domain, this in turn activating intracellular signalling proteins [1–4].

NGF, the first of the neurotrophins to be discovered, has been shown to be essential for survival and differentiation of neural crest-derived peripheral sympathetic and sensory neurons, as well as cholinergic neurons in the central nervous system [5]. Neuroblastoma, a malignant childhood tumour arising from the neural crest-derived sympathetic nervous system, exhibits a remarkable clinical heterogeneity ranging from a favourable outcome due to spontaneous regression or complete remission following low-dose therapy, to a poor outcome due to progressive disease, despite intensive treatment [6]. It has previously been suggested that the interaction between NGF and neuroblastoma cells could be a critical factor in modulating tumour growth [7]. Multiple defects in the expression of p75^{LNGFR} mRNA and responsiveness to NGF have been reported in neuroblastoma cell lines [8]. Subsequent studies on human tumour samples demonstrated that high levels of p140^{trk} and p75^{LNGFR} mRNAs were associated with young age at diagnosis, and favourable clinical stage and outcome [9–11].

However, the evaluation of NGF receptor expression by northern blot requires adequate amounts of tissue, usually available only with open surgical biopsy. Moreover, although reverse transcriptase polymerase chain reaction (RT-PCR) is highly sensitive, its routine clinical use is expensive, time-consuming and sometimes technically difficult.

In order to overcome these limitations, the feasibility of using immunohistochemistry in detecting NGF receptors was assessed in neuroblastoma. The p140^{trk} receptor was evaluated because its prognostic significance is greater than that of p75^{LNGFR} [9–11]. Furthermore, immunohistochemical findings were compared with those obtained by northern blot used as a standard of validity.

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PATIENTS AND METHODS

Patients and sample handling

Twenty-eight children with histological diagnosis of neuroblastoma were included in this study. They were staged according to the Evans' criteria [12] as follows: 3 at stage I, 5 at stage II, 6 at stage III, 8 at stage IV, and 6 at stage IV. Primary tumour tissue was obtained at surgery before any treatment, divided into aliquots for both molecular and immunohistochemical analysis, and quick frozen at −70°C.

Northern blot analysis

Total RNA was extracted, blotted on to nitrocellulose membranes, hybridised to a 2.7 kb *Eco*RI fragment from a human *TRK* cDNA clone, and measured as previously described [11].

Immunohistochemical analysis

The anti-p140^{trk} monoclonal antibody (mAb) H10 was raised by immunising Balb/c mice with NIH 3T3 cells transfected with human full length trk [13]. This mAb recognises with high affinity (2.2×10^{11} M^{-1}) a tunicamycin-sensitive epitope of the p140^{trk} extracellular domain (Figure 1) and does not display detectable cross-reactivity with *TRKB* gene product (data not shown). 4-μm-thick acetone-fixed cryostat sections from each sample were evaluated by indirect immunofluorescence, under standard conditions, using a fluorescein isothiocyanate-labelled $F(ab)_2$ rabbit anti-mouse IgG antiserum (Organon Teknika-Cappel, Turnhout, Belgium). As controls, sections incubated without the primary antibody were used.

Statistical analysis

The correlation between northern blot and immunohistochemistry was evaluated by the McNemar test.

RESULTS

Northern blot revealed detectable levels of p140^{TRK} mRNA in 15 of the 28 cases. These were also assumed to be positive for p140^{trk} protein, although there is no certainty that, in all these cases, p140^{trk} was actually translated. Nevertheless, since previous studies addressed their attention to the prognostic significance associated with p140^{trk} mRNA without evaluating p140^{trk} protein, these 15 cases were considered as positive in order to compare the findings obtained by immunohistochemistry to those obtained by northern blot.

With immunohistochemistry, nine of 28 cases were scored positive. In general, the immunostaining was weak and outlined the cell boundaries (Figure 2). While five cases displayed a homogeneous reactivity, the remaining four samples stained with variable intensity. When compared with northern blot findings, nine were true positives (i.e. positive both with northern and immunohistochemistry) and six were false negatives (i.e. positive with northern but negative with immunohistochemistry), but no false positives (i.e. negative with northern and positive with immunohistochemistry) were detected. The concordance rate was thus 79% (22 of 28 cases) ($P < 0.03$), the sensitivity 60% (9 of 15 cases), and the specificity 100% (13 of 13 cases).

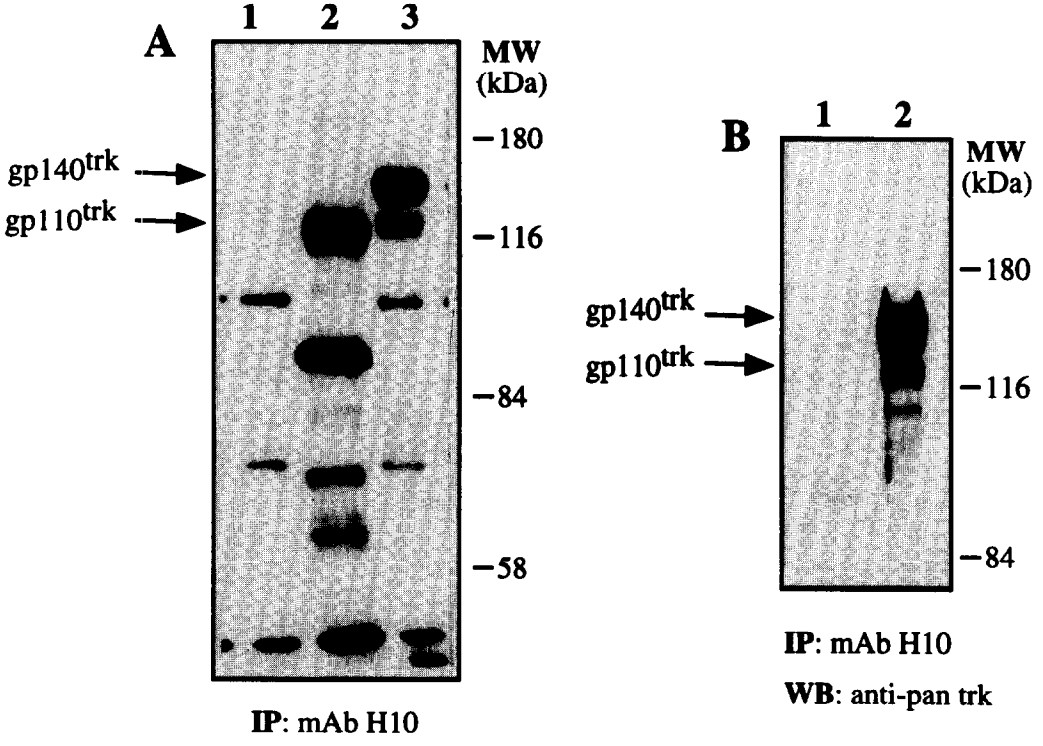

Figure 1. (a) NP40-solubilised [^{35}S]methionine-labelled cell proteins were immunoprecipitated with mAb H10 recognising human p140^{trk} (gp140^{trk} in the figure). Immunoprecipitates derived from untransfected Balb/c 3T3 cells (lane 1), insect cells (SF9) infected with recombinant baculoviruses coding for human TRK (lane 2), and Balb/c 3T3 cells transfected with human *TRK* (lane 3), were resolved on 7.5% SDS-polyacrylamide gel and visualised by autoradiography. (b) Cell extracts of two human leukaemia cell lines (lane 1: AML1; lane 2: K562) were immunoprecipitated with mAb H10, blotted on to a nitrocellulose filter, and probed with an anti-pan TRK polyclonal antibody (Biotechnology, Santa Cruz, California, U.S.A.). Immunocomplexes were visualised by the enhanced chemiluminescence (ECL) detection system (Amersham International, Amersham, England). IP, immunoprecipitation; WB, Western blot.

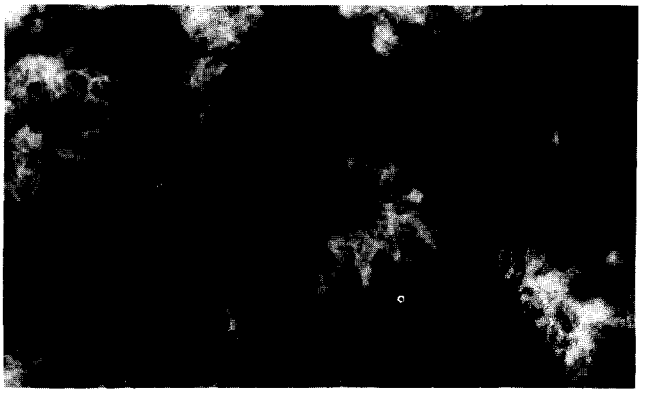

Figure 2. Immunohistochemical reactivity of p140^{trk} in neuroblastoma. The mAb H10 outlines cell boundaries with variable intensity (X500).

DISCUSSION

Previous studies have reported that p140^{trk} mRNA levels are related to prognosis in neuroblastoma, with high levels associated with favourable outcome [9–11]. Univariate analysis has also shown this to be true for p75^{LNGFR} mRNA, although to a lesser extent [10, 11]. Moreover, the coexpression of both high and low affinity NGF receptors has been associated with a subset of neuroblastoma having an excellent outcome [11].

In oncology, it is becoming increasingly evident that the evaluation of biological parameters is offering the possibility of identifying subgroups of patients who can benefit by tailored treatment [14]. In neuroblastoma, this is already so with *MYCN* oncogene copy number and cellular DNA content. More recently, it has been proposed that the combined analysis of all these factors, including NGF receptors, can further improve prognostic accuracy [11, 15]. For this purpose, algorithms have been formulated that require the measurement of these multiple parameters [11, 15]. However, in routine clinical use, this measurement is contingent on the amount of tissue available. To overcome this difficulty, our group has been involved in developing "micromethods" that can assess these features even on very small tumour samples or isolated tumour cells [16].

In this study, immunohistochemistry seemed reliable in detecting p140^{trk} expression. This was also demonstrated in another report, where a polyclonal antibody was used that recognised the cytoplasmic domain of not only p140^{trk}, but also of p145^{trkB} and p145^{trkC} [17]. As far as the high specificity of H10 antibody in this series was concerned, it is likely that it was overestimated since comparison of the amino acid sequence of p140^{trk}, p145^{trkB} and p145^{trkC} indicated strong homologies in both their extracellular and cytoplasmic domains (approximately 50% versus 80%, respectively) [1, 3, 18], and cross-reactions may thus occur among these. Since H10 antibody recognises an epitope on the extracellular domain where the homology is lower, its specificity should then be expected to be rather high. However, until simultaneous northern blot analysis for p145^{trkb} and p145^{trkc} mRNAs can be performed, this specificity still needs to be confirmed.

Concerning the lower sensitivity of the immunohistochemistry when compared with that of northern blot, there is also the possibility that the low density of p140^{trk} receptors on the neuron cell surface [4] could adversely affect their immunohistochemical detection. However, this low sensitivity may not be critical when assessing prognosis in neuroblastoma patients since only intermediate to high levels of p140^{trk} mRNA have been related to a favourable outcome, while tumours with both low or undetectable levels behave the same, that is, do poorly [9, 10]. Thus, in a clinical setting, immunohistochemistry could allow for the identification of that tumour subset with significant levels of p140^{trk} mRNA, the only group with favourable outcome. Nevertheless, for investigational purposes we are working at present on improving the sensitivity of this assay using pools of monoclonal antibodies recognising different epitopes of the p140^{trk} extracellular domain.

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