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Original Paper

Expression and Function of Trk-C in Favourable Human Neuroblastomas

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Human neuroblastomas express the neurotrophin receptors trk-A and trk-B. Favourable outcome is associated with expression of trk-A, while unfavourable, *MYCN* amplified tumours express trk-B. In this study we examined the expression of trk-C in primary neuroblastoma tumour-derived cell lines. We found by Northern blot analysis that trk-C mRNA is expressed in 14 of 55 (25%) primary tumours. Trk-C was expressed in significantly more lower stage tumours (stage 1, 2, 4S) than higher stage tumours (stage 3, 4, $P < 0.04$). The expression of trk-C was correlated positively with survival and negatively correlated with *MYCN* amplification. We also studied the function of trk-C in transfected cell lines and found that NT-3 promotes both cell survival and differentiation. Our results suggest that trk-C is involved in the biology of favourable neuroblastomas. © **1997 Published by Elsevier Science Ltd.**

Key words: neuroblastoma, trk-A, trk-B, trk-C, NT-3
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INTRODUCTION

NEUROBLASTOMAS ARE tumours of neural crest origin and have been found to express the trk family of tyrosine kinase receptors for neurotrophins. The expression of different members of the Trk family has been found to correlate with clinical features and outcome. Favourable, lower stage tumours express high levels of trk-A, with expression significantly correlated with favourable outcome and survival [1–5]. In contrast, advanced-stage tumours with *MYCN* amplification primarily express full-length trk-B as well as the receptor's primary ligand, brain-derived neurotrophic factor (BDNF) [6]. These observations suggest that favourable neuroblastomas express trk-A, with activation leading to differentiation or neurotrophin deprivation leading to apoptosis, while unfavourable neuroblastomas have an autocrine loop between trk-B and BDNF, leading to cell survival and proliferation.

In this study we examined the expression and function of trk-C in neuroblastomas. We found by Northern blot analysis that trk-C is expressed significantly more in favourable, lower stage (1, 2, 4S) tumours compared to unfavourable, higher stage (3, 4) tumours. Trk-C expression was not detected by Northern blot analysis in neuroblastoma cell lines. We also examined the function of trk-C by transfecting the neuroblastoma cell line SH-SY5Y with a human trk-C cDNA. We found that trk-C transfected SH-SY5Y cells respond to NT-3 by undergoing differentiation and they can survive and grow in serum-free media in the presence of NT-3. Our results suggest that trk-C plays a role in the biological behaviour of favourable neuroblastomas.

PATIENTS AND METHODS

Patients

Tumour specimens were obtained from 55 neuroblastoma patients who were diagnosed in Japan and the United States from 1982 to 1991. These have been described in detail elsewhere and were treated on either Japanese protocols or Pediatric Oncology Group protocols [1, 2]. Patients were classified according to the criteria of Evans and colleagues [7] with 15 stage 1 tumours, 7 stage 2 tumours, 8 stage 4S tumours, 11 stage 3 tumours and 14 stage 4 tumours. *MYCN* amplification was found in 2 of the stage 4S tumours, 2 of the stage 3 tumours and 4 of the stage 4 tumours.

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Isolation of human trk-C cDNAs

We screened a human infant hippocampus cDNA library (Stratagene) in order to obtain human trk-C probes. Using a rat trk-C probe that encodes a portion of the extracellular domain (pJDM836, a gift of Dr Jeffrey Milbrandt, Washington University School of Medicine, Missouri, U.S.A.), a 690 bp probe (trk-C-EC) was obtained (corresponding to nucleotides 531–1221 of rat trk-C [8] and nucleotides 635–1325 of human trk-C [9]). Using a mouse trk-B probe to the tyrosine kinase domain (a gift of Dr K. Horigome, Washington University School of Medicine, Missouri, U.S.A.), a 969 bp probe (trk-C-TK) to the tyrosine kinase domain was obtained (corresponding to nucleotides 1578–2541 of rat trk-C [8] and nucleotides 1684–2653 of human trk-C [9]).

Northern blot analysis

Total RNA was extracted from primary tumour samples [10], resolved on 1% agarose–formaldehyde gels and transferred by blotting to a nylon membrane (Hybond N+, Amersham). Hybridisation was performed as previously described [11]. To quantitate trk-C expression, images of autoradiographs were obtained from a Cohu CCD camera, background-subtracted and band intensity determined using a densitometric analysis program (NIH Image 1.54). After normalisation to *GAPDH*, trk-C mRNA expression was expressed as arbitrary density units (d.u.), and converted to the following scale: 0 (undetectable); +1 (< 150 d.u.); +2 (150–250 d.u.); +3 (251–350); 4+ (> 350 d.u.).

RT–PCR

Trk-C expression was examined by RT–PCR, using primers that flank an alternative splicing site at position 2288, where an insert of 42 bp has been described [9, 12–14]. The sense and antisense primers were: 5′-AATGCTCCACATTGCCAGTC-3′ (position 2119–2138 of human *trk-C* [9]), and 5′-TGAAGATCTCCCAGAGGAT-3′ (position 2388–2407), respectively. Total RNA (1 μg) was reverse transcribed using the antisense primer and Avian Myeloblastosis Virus reverse transcriptase (Promega). The product was directly amplified for 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final extension of 72°C for 7 min, and then visualised on a 6% Visigel (Stratagene).

Transfection of trk-C

Trk-C was cloned into the retroviral vector pLNCX, and transfection of SH-SY5Y cells was performed as previously described [15]. Clones were selected by resistance to G418, with serial dilution to obtain individual clones. Cells were maintained in RPMI 1640, 10% fetal bovine serum and G418 at 0.5 mg/ml.

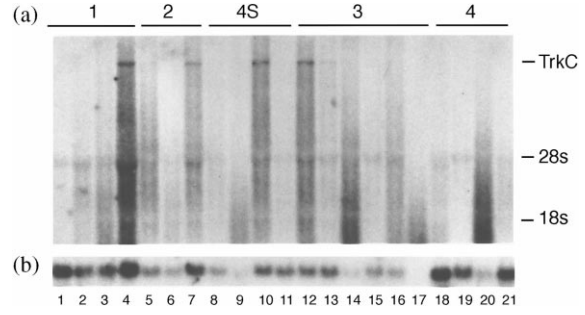

Figure 1. Northern blot analysis of trk-C mRNA expression in primary neuroblastomas. (a) Northern blot using an extracellular probe for trk-C (trk-C-EC). Tumours were staged by the criteria of Evans and colleagues [7] and are listed above (a). To control for the amount and integrity of mRNA, the same filter was stripped and rehybridised for GAPDH, and is shown in (b).

RESULTS

Northern blot analysis of trk-C expression in primary neuroblastomas and cell lines

Using a probe to the extracellular domain (probe trk-C-EC) of human trk-C, we were able to detect a 14 kb transcript (Figure 1) in 14 of 55 (25%) primary tumours. We were also able to detect a similar size transcript with the tyrosine kinase (trk-C-TK) probe (data not shown), indicating that the 14 kb transcript encodes the full-length trk-C protein. Smaller mRNA species were not detected, indicating that neuroblastomas do not express truncated forms of trk-C protein which lack the tyrosine kinase domain.

Trk-C expression was quantified by densitometry, normalised to *GAPDH* and converted to a scale with 0 (undetectable), +1 (low), +2 (moderate) and +3 to +4 (high) levels of expression (Table 1). Trk-C was expressed in significantly more favourable, lower stage (1, 2, 4S) tumours [11 of 30 (37%)] versus unfavourable, higher stage (3, 4) tumours [3 of 25 (12%)], with a $\chi^2 = 4.4$, $P < 0.04$. Amplification of *MYC*N was inversely correlated with trk-C expression, with low-level expression (+1) found in only one stage 4S tumour (1 of 8 *MYC*N amplified tumours).

Trk-C expression was also associated with overall survival. There was a significant difference in survival between patients which had moderate- or high-level trk-C expression (+2 to +4) versus those with undetectable or low-level trk-C expression (0 to +1), with $\chi^2 = 4.6$, $P < 0.04$. All 10 patients whose tumours expressed moderate or high levels of trk-C are alive, with a median follow-up time of 41 months. In contrast, 15 of 45 patients (33%) with undetectable or low levels of trk-C expression have died.

We also examined trk-C expression in a panel of neuroblastoma cell lines: SMS-KAN, SMS-KCN, NGP, NMB, SH-SY5Y, CHP-134, LA-N-5, NBL-S. All cell lines except SH-SY5Y and NBL-S have *MYC*N amplification. We were unable to detect trk-C expression in any of the eight cell lines by Northern blot analysis using either the trk-C-EC or trk-C-TK probes.

RT–PCR analysis of trk-C *expression in primary neuroblastomas and cell lines*

In humans, a splice variant of trk-C has been described in which there is a 42 bp insert in the tyroskine kinase domain

Table 1. *Northern blot analysis of trk-C mRNA expression in* 55 *primary neuroblastomas*

Trk-C expression	Stage 1, 2, 4S	Stage 3, 4	Total
Undetectable (0)	19	22	41
Low (+1)	3	1	4
Med (+2)	3	1	4
High (+3, +4)	5	1	6
Total	30	25	55

Trk-C expression was quantified by densitometry, normalised to GAPDH, and converted to a scale with 0 (undetectable), +1 (low), +2 (moderate) and +3 to +4 (high) level of expression. Trk-C was expressed in significantly more favourable, lower stage (1, 2, 4S) tumours [11 of 30 (37%)] versus unfavourable, higher stage (3, 4) tumours [3 of 25 (12%)], with a $\chi^2 = 4.4$, $P < 0.04$.

[9, 16]. To examine the expression of this splice variant, we used RT–PCR with primers that flanked the alternative splice site and performed RT–PCR on a subset of primary tumours and cell lines. We found expression of only a 288 bp product, which corresponds to the non-splice variant, but not the 330 bp product, in both primary tumours and cell lines. Our RT–PCR results also demonstrated that *trk-C* is expressed in primary tumours and cell lines in which there was no detectable expression of *trk-C* by Northern blot analysis. In 5 primary tumours in which we could not detect trk-C by Northern blot analysis, there was a 288 bp band detected by RT–PCR. In addition, the cell lines LAN-6, CHP-134 and SMS-KAN, but not SH-SY5Y, were found to have expression of *trk-C* by RT–PCR. Overall, 11 of 11 primary tumours and 3 of 4 cell lines had trk-C expression by RT–PCR.

Lack of NT-3 expression in primary neuroblastomas and cell lines

NT-3 binds primarily to trk-C and induces phosphorylation of the receptor [13]. We examined expression of NT-3 in both primary tumours and cell lines by both Northern blot analysis and RT-PCR. We were unable to detect NT-3 expression in either primary tumours or cell lines by either method.

Trk-C *transfection of SH-SY5Y cells*

To examine the function of *trk-C* in neuroblastoma, we transfected the cell line SH-SY5Y with a retroviral vector pLNCX-trk-C. Clones were selected by serial dilution and resistance to the antibiotic G418. Northern blot analysis demonstrated that several clones (SH-SY5Y-TC1, -TC4, -TC7, -TC8) expressed moderate to high levels of *trk-C*. To determine if *trk-C* could promote survival, SH-SY5Y-TC1 cells were placed in serum-free medium. We found that the addition of NT-3 promoted a proliferative response with a doubling of cell number at 4 days. In addition, NT-3 also induced differentiation with long neurites produced.

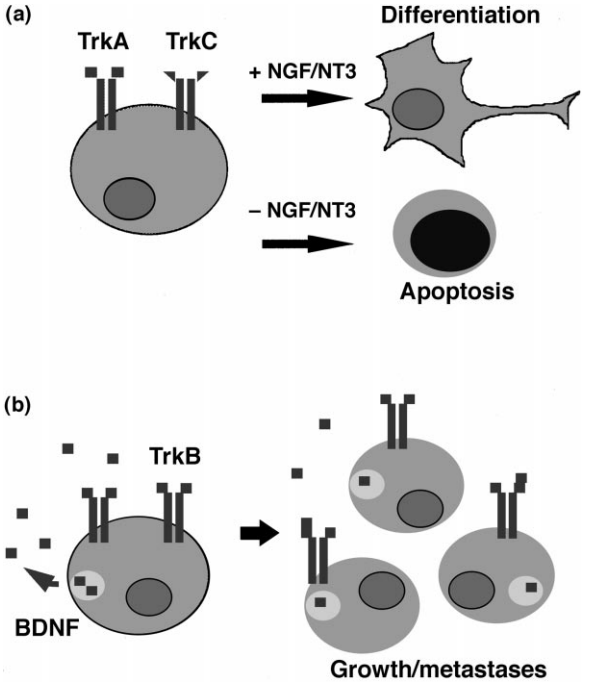

Figure 2. Function of trk receptors in favourable and unfavourable neuroblastomas. (a) Favourable neuroblastomas express trk-A and trk-C, with addition of NGF or NT-3 leading to differentiation, while the removal of NGF or NT-3 leads to apoptosis. (b) Unfavourable neuroblastomas express trk-B and BDNF allowing for autocrine stimulation of growth and ultimately metastases to occur.

DISCUSSION

In this study we found that 14 of 55 (25%) neuroblastomas express a 14 kb transcript, which was detected with both the extracellular and tyrosine kinase domain probes. We were unable to detect transcripts of other sizes which lack the tyrosine kinase domain [8, 12, 13]. In addition, the splice variant form of trk-C with a 14 amino acid insert was not expressed in neuroblastomas. This alternatively spliced form binds NT-3 but is unable to induce signal transduction [14, 16]. Therefore, our results indicate that neuroblastomas express the full-length, functional isoform of trk-C.

The exact function and role of trk-C in neuroblastoma remains to be elucidated. Our results with the trk-C transfected cell lines demonstrate that trk-C mediated signalling can induce neuronal differentiation, which might be one of the mechanisms by which a neurotrophin receptor could lead to a favourable outcome. However, trk-C-mediated signalling can also promote cell survival and growth in serum-free medium. We are currently examining the trk-C signal transduction pathway in order to understand how differentiation versus cell survival/growth might occur.

This study demonstrates that trk-C is associated with favourable, lower stage tumours and overall survival. These results are similar to our previous studies on the expression of trk-A in neuroblastomas [1, 2]. Indeed, if we compare the Northern blot analysis results of our current trk-C study and our previous trk-A studies, we find that, in the 14 tumours in which there was detectable trk-C expression, almost all expressed trk-A at high levels (+3 to +4). Therefore, our studies demonstrate that trk-C expression strongly correlates with trk-A expression. However, trk-C does not appear to be an independent prognostic marker, since the trk-C expressing tumours are a subset of the larger number of trk-A expressing tumours. Further studies will need to be conducted to determine if the expression of an additional neurotrophin receptor can confer additional favourable status.

In contrast, the tumours that expressed trk-C did not have detectable levels of trk-B [6]. Furthermore, only one *MYCN* amplified tumour had detectable trk-C expression by Northern blot analysis. These results, in conjunction with the results on trk-A, further support the concept that neuroblastomas can be divided into favourable and unfavourable types based on biological features such as neurotrophin receptor expression. Figure 2 is our current model of the role of neurotrophins and their receptors in neuroblastoma biology. Favourable tumours express trk-A with or without trk-C and do not express trk-B. The addition of NGF or NT-3 would promote differentiation while neurotrophin deprivation would lead to cell death. In contrast, unfavourable neuroblastomas express full-length trk-B, but express little or no trk-A or trk-C. In addition, unfavourable neuroblastomas express BDNF producing an autocrine loop of trk-B/BDNF, resulting in cell proliferation and metastases. Further work will be needed to confirm the validity of this model.

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