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Original Paper

Coexpression of mRNA for the Full-Length Neurotrophin Receptor trk-C and trk-A in Favourable Neuroblastoma

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Neuroblastoma, a childhood tumour of the sympathetic nervous system, may sometimes regress spontaneously in infants, or progress to a poor clinical outcome despite intensive therapy. Neuroblastomas express neurotrophin receptors and high levels of mRNA for trk-A correlates with favourable outcome, whereas trk-B mRNA is expressed by more unfavourable tumours. Using a sensitive RNase protection assay, mRNA expression for the neurotrophin receptor trk-C was investigated in 50 tumour samples from 45 children at different stages including metastatic and relapsing tumour tissue, out of which 22 were also investigated for trk-A mRNA. Thirty-seven of 43 primary tumours (86%) showed trk-C mRNA with more than 300-fold difference between the highest and the lowest values. A higher trk-C index (trk-C mRNA/*GAPDH* mRNA) was associated with favourable features such as younger age ($P = 0.009$–0.003), favourable tumour stage (1, 2 or 4S; $P < 0.001$) and favourable prognosis ($P = 0.044$). Better survival probability was shown in children with intermediate or high trk-C index compared with patients with low or undetectable levels ($P = 0.031$). All localised tumours co-expressed mRNA for trk-A and trk-C receptors. RT–PCR analysis detected mRNA encoding the cytoplasmic trk-C tyrosine kinase region only in favourable neuroblastomas. We conclude that favourable neuroblastoma may express the full-length trk-C receptor while unfavourable tumours, especially those with *MYCN* amplification, seem to either express no trk-C or truncated trk-C receptors with unknown biological function. Trk-C and possibly its preferred ligand NT-3 may be involved in the biology of favourable neuroblastomas showing apoptosis or differentiation. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, neurotrophin, differentiation, apoptosis, RNase protection, tyrosine kinase receptor, prognosis, ganglioneuroma, *MYCN* oncogene
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INTRODUCTION

NEUROBLASTOMA, AN embryonal malignant tumour of the sympathetic nervous system, is the most common paediatric extracranial tumour, mainly diagnosed during infancy or early childhood. Neuroblastoma shows a remarkable heterogeneity in both biological and clinical behaviour, ranging from spontaneous regression or complete remission after minimal therapy in one subset, to unfavourable outcome due to aggressive tumour growth in spite of intensive multimodal therapy in another [1]. Neuroblastoma tumours are classified into five stages 1 to 4 and 4S [2]. Unfavourable tumours are mostly diagnosed at metastatic stage 4 in children over one year of age, whereas widespread tumours stage 4S in infants

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often show spontaneous regression or differentiation. Aggressive neuroblastomas with poor prognosis may often show deletions for distal chromosome 1p [3, 4], amplification of the *MYC*N oncogene [5, 6], absence of trk-A mRNA expression [7, 8] and diploid or tetraploid DNA content [9, 10].

Neurotrophins and their receptors are critical for the differentiation and survival of neural cells during development [11–13]. The tyrosine kinase receptors trk-A, trk-B, and trk-C are necessary for high-affinity binding and full biological effect of the neurotrophins (nerve growth factor [NGF], brain derived neurotrophic factor [BDNF], neurotrophin-3 [NT-3] and NT-4/5) [12, 13]. NGF and NT-3 are the preferred ligands for trk-A and trk-C, respectively. In addition, all neurotrophins bind with similar affinity to the low-affinity receptor $p75^{LNGFR}$ [13, 14]. However, $p75^{LNGFR}$ was recently shown to be able to act independently as a receptor and proposed to be involved in programmed cell death in the nervous system [15, 16].

NGF, the first discovered member of the neurotrophin family, is essential for the differentiation and survival of sympathetic neurons and it has been suggested that NGF may be important for differentiation and regression of tumours of neural crest origin [7, 11]. NGF can induce morphological differentiation in certain primary cultures of neuroblastoma that express trk-A, whereas NGF deprivation results in cell death [8]. Trk-A mRNA is co-expressed with LNGFR mRNA encoding $p75^{LNGFR}$ in favourable neuroblastomas prone to differentiation, regression or favourable response to therapy [7, 8]. Aggressive *MYC*N amplified neuroblastomas and certain cell lines may co-express mRNAs for the full-length trk-B receptor and the ligand BDNF, while more favourable tumours express truncated trk-B receptors [17, 18].

Recently, we reported that trk-C mRNA expression could be detected in most neuroblastomas with an association to favourable clinical stage and prognosis [19]. In the present study, we have extended our investigations to a larger clinical sample in which higher trk-C expression correlates with favourable clinical factors as favourable stage and young age. Children with higher trk-C expression had a more favourable prognosis in this extended group, with similar significance as trk-A in a subset of tumours analysed for both trk-A and trk-C. Localised neuroblastomas seemed to co-express trk-A receptors and full-length trk-C receptors, whereas *MYC*N amplified tumours either lacked both these receptors or expressed truncated trk-C receptors with as yet unknown biological function.

MATERIAL AND METHODS

Patient material and sample handling

Forty-five children with neuroblastoma (pathological diagnosis of neuroblastoma or ganglioneuroblastoma) with primary tumour tissue available for analysis were included in the study (Table 1). All children were diagnosed and staged according to International Neuroblastoma Staging System (INSS) criteria including tumours from all five clinical stages (1, 2, 3, 4, 4S) [2]. Nine children died during follow-up, 0.5–56 months from diagnosis. The remaining 36 children have been followed for 9–96 months until last follow-up at 1 June 1996. Forty-three children are included for analysis of survival probability (67.3% in the whole group). Patient no. 18 was excluded because of a composite ganglioneuroblastoma with morphologically disparate parts analysed and patient 29 was also excluded because tumour tissue was initially obtained after pre-operative chemotherapy. Tumour tissue from all patients was surgically resected and frozen at –70°C until analysis. Multiple samples (metastases, relapses and one composite tumour) were analysed from 5 patients. *MYC*N amplification was analysed in all tumours with methods and results as previously reported [4, 7].

Statistical calculations

The Wilcoxon–Mann–Whitney rank sum test was used for comparison of two independent samples. The survival probability (± SE) was calculated using the Kaplan–Meier method and compared using the Mantel–Haenszel log-rank test [20, 21].

RNA preparation

Total RNA was extracted from tissues by homogenisation in 4 mol/l guanidine isothiocyanate and centrifuging through a CsCl cushion [22] or by acid phenol–chloroform extraction [23].

RNase protection analysis

Previously, Northern blot analysis showed that the level of trk-C mRNA expression was below the detection limit in most neuroblastoma samples. Hence, a more sensitive RNase protection analysis was developed [19]. For this assay, a DNA fragment was cloned using degenerate primers as previously described [19]. This clone is identical to the sequence encoding the transmembrane region of htrk-C [24]. In short, ^{32}P-α labelled riboprobes were generated from linearised plasmid using T3 or T7 RNA polymerase (Promega, Madison, Wisconsin, U.S.A.). Equal amounts of total RNA (5 μg) were analysed by the RNase protection assay according to the manufacturer's instructions (Ambion, Austin, Texas, U.S.A.). To control differences in RNA loading, a glyceraldehyde-3-posphate dehydrogenase (hGAPDH) probe was used.

Gels were exposed to X-ray films at −70°C and later scanned in an image analyser (Leica, Cambridge, U.K.). The trk-C index was calculated as the ratio of the optical densities of the trk-C band to the GAPDH band, and it was used as an estimate of trk-C mRNA expression.

Trk-C kinase domain mRNA detection by RT–PCR

To analyse expression of the trk-C kinase region by RT–PCR, we used primers for the kinase domain of trk-C [19]. The primers were expected to give two bands of different sizes, 350 bp and 390 bp, corresponding to different splice variants [24]. Primers for *hGAPDH* were included in each sample. RT–PCR was performed on total RNA from tumours using a GENE-AMP (Perkin-Elmer Roche Molecular System Inc., Branchburg, New Jersey, U.S.A.) RT–PCR kit. The reverse transcription step was performed at 50°C for 3 min followed by 70°C for 14 min. The PCR steps were run with 3 1-min rounds of annealing at 50°C followed by 39 1-min rounds at 55°C. Elongation was performed for 1 min at 60°C. The PCR products were separated on a 3% agarose gel. Only two bands were observed, a 190 bp band corresponding to *hGAPDH* and another one of approximately 360 bp. This band was recently subcloned and sequenced showing identity with the human trk-C tyrosine kinase region without insertions [19, 24].

Table 1. *Patient data and trk-C expression*

Patient number	Stage[a]	Age (months)	Outcome	Follow-up (months)	Trk-A[f]	*MYCN*[g]	Trk-C index[h]
1	4S	0	NED[b]	36+	+	< 3	3
2	4S	0	NED	58+	+	< 3	0.42
3	4S	5	NED	44+	n.a.	< 3	0.025
4	4S	5	NED	43+	+	< 3	0.17
5	4S	7	NED	11+	n.a.	< 3	0.64
6	4S	7	DOD[c]	10–	n.a.	< 3	1.4
7	1	0	NED	62+	+	< 3	0.93
8	1	1	Dead[d]	0.5	+	< 3	0.59
9	1	1	NED	9+	n.a.	< 3	0.07
10	1	1	NED	40+	n.a.	< 3	0.62
11	1	9	NED	39+	+	< 3	0.67
12	1	12	NED	46+	+	< 3	0.023
13	1	12	NED	10+	n.a.	< 3	1.7
14	1	17	NED	37+	+	< 3	1.5
15	1	29	NED	20+	n.a.	< 3	0
16A	1	30	NED	22+	n.a.	< 3	0
16B[i]	1 (rel)	30	NED	22+	n.a.	< 3	0.04
17	1	122	NED	18+	n.a.	< 3	0.08
18A[j]	1	144	AWD[e]	11+	n.a.	> 10	0
18B[j]	1	144	AWD	11+	n.a.	> 10	3.4
19	2	0	NED	96+	n.a.	< 3	1.3
20	2	1	NED	15+	n.a.	< 3	1.14
21	2A	9	NED	15	n.a.	< 3	0
22	2A	11	NED	9+	n.a.	< 3	0.28
23	2B	14	NED	11+	n.a.	< 3	0.02
24	2A	30	NED	15+	n.a.	< 3	0.05
25	2B	31	NED	47+	+	< 3	0.12
26	2A	33	NED	29+	+	< 3	0.045
27	2A	60	NED	21+	n.a.	< 3	0.15
28A[k]	2	102	NED	59+	+	< 3	3.2
28B[k]	2	102	NED	59+	+	< 3	0
29	3	0	NED	17+	n.a.	< 3	0
30	3	0	Dead	0.5	+	< 3	0
31	3	2	NED	28+	–	< 3	0.25
32	3	6	NED	57+	+	< 3	0.08
33	3	7	NED	42+	–	< 3	0.34
34	3	10	NED	37+	+	< 3	0.09
35	3	11	NED	41+	+	< 3	0
36	3	80	AWD	11+	n.a.	< 3	0
37	4	4	AWD	9+	n.a.	< 3	0
38	4	10	DOD	10	–	> 10	0.32
39	4	15	AWD	11+	n.a.	>3	0.16
40	4	16	AWD	26+	n.a.	< 3	0
41A	4	19	DOD	4	–	> 10	0.17
41B[l]	4 (res)	19	DOD	4	–	> 10	0
42	4	30	DOD	11	–	< 3	0
43	4	31	DOD	56	n.a.	< 3	0.0053
44	4	50	DOD	6	–	> 10	0
45A	4	137	DOD	8	–	> 10	0.01
45B[m]	4 (met)	137	DOD	8	–	> 10	0

[a]According to International Neuroblastoma Staging System [2]. [b]NED = no evidence of disease. [c]DOD = dead of disease. [d]Dead = dead from toxic or postsurgical complications. [e]AWD = alive with disease. [f]Trk-A mRNA expression by Northern blot analysis [7]. [g]*MYCN* gene copy number per haploid genome by Southern blot analysis [6]. [h]Trk-C index = trk-C mRNA/*GAPDH* mRNA. [i]Relapsed tumour tissue at primary site. [j]Composite ganglioneuroblastoma. [k]Relapsed metastatic tissue. [l]Residual tumour tissue after chemotherapy. [m]Metastatic tissue. n.a. = not analysed.

RESULTS

Trk-C mRNA expression in neuroblastoma tumours

Using a sensitive RNase protection assay, human trk-C mRNA expression could be detected in the majority of neuroblastoma tumour samples (Figure 1a). For quantitative comparisons, a trk-C index was obtained as described in Materials and Methods.

The expression of trk-C mRNA could be detected in 37 of 43 (86%) analysed primary tumours with a highly variable trk-C index ranging from 0.0053 to 3.4 (Figure 2 and Table 1). Trk-C mRNA could be detected in most neuroblastomas at favourable stage (1, 2 and 4S). The highest trk-C index was observed in samples from stage 1, 2 and 4S tumours (nos. 1, 18B and 28). In contrast, the lowest levels

were observed in tumours of stage 3 and 4, although significant expression of trk-C was detected in a few tumours at advanced stages as well (5/17 with trk-C index > 0.1; nos. 31, 33, 38, 39 and 41A). Few tumours at favourable stages expressed relatively low levels of trk-C (nos. 3, 9 and 23) and some did not express any trk-C at all (15, 16A and 21).

Five samples obtained at relapse, a metastatic site or as residual tumour mass after preoperative chemotherapy showed undetectable trk-C (4/5) or a low level of expression (Figure 2 and Table 1). One *MYCN* amplified composite ganglioneuroblastoma of localised stage 1 showed a different trk-C index in morphologically different parts of the tumour: 3.4 and 0, respectively (nos. 18A and 18B, Table 1).

Correlation between trk-C and stage, age and prognosis

A significantly higher trk-C index was found in tumours of a favourable stage 1, 2 and 4S ($n = 27$, 0.28: 0.05–1.03), median: lower-upper quartile) compared with advanced regional and metastatic stage 3 and 4 tumours ($n = 16$, 0.01: 0–0.16, $P < 0.001$). Younger children, under 18 months at diagnosis, showed a higher trk-C index ($n = 29$, 0.28, 0.02–0.80) than older children ($n = 14$, 0.03, 0–0.12, $P = 0.004$). Also using cut-off levels at 12 and 24 months, respectively, showed a significantly higher trk-C index in younger children ($P = 0.009$ and 0.003, respectively). Children with favourable outcome (alive without evidence of disease followed for more than one year) had a higher trk-C index than those dying from tumour progression ($P = 0.044$). The survival probability for children with a low trk-C index (≤ 0.001) was significantly worse (65.6% ± 14% and 0% at 3 and 6 years, respectively) compared with those with a higher trk-C index (86.6% ± 6.2%, $P = 0.031$, Figure 3).

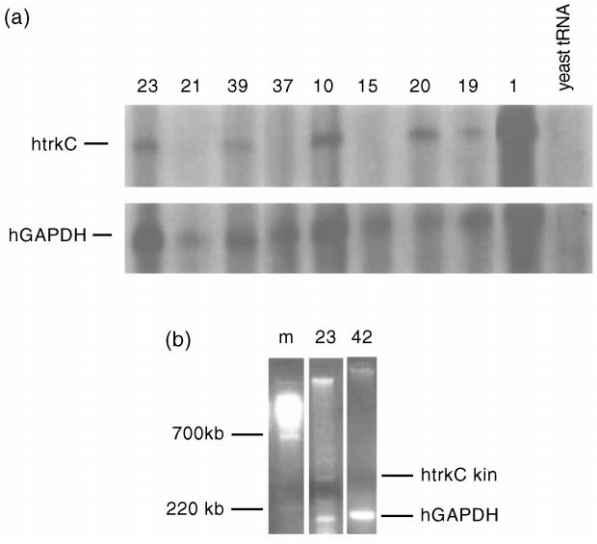

Figure 1. (a) RNase protection analysis of trk-C mRNA in total neuroblastoma RNAs. Yeast RNA was used as a negative control. Protected bands corresponding to human trk-C and human *GAPDH* are indicated. Patient numbers are indicated. (b) RT–PCR analysis of trk-C tyrosine kinase mRNA in total RNA from neuroblastoma tumours. Molecular weight marker (pBs KS+/HpaII) denoted by m. A 190 bp long fragment corresponding to *hGAPDH* was seen in all lanes. Sample no. 23, a stage 2B tumour with favourable outcome, showed an approximately 360 bp long amplified fragment encoding the htrk-C kinase region. In sample no. 42, an advanced stage 4 tumour with unfavourable outcome, no trk-C product could be amplified.

The subset of 11 children with trk-C expression higher than that of the cerebral cortex (index ≥ 0.6) showed the best outcome with all but one surviving (90.9% survival probability).

Correlation between trk-C, trk-A and MYCN amplification

Six out of 44 primary neuroblastomas were amplified for the *MYCN* oncogene. One (no. 18) showed a different trk-C index in different parts of the tumour. The remaining 5 tended to have lower trk-C indices ($P = 0.15$).

A subset of 22 tumours were analysed for trk-C mRNA using the RNase protection assay and trk-A mRNA using Northern blot analysis as previously described [7]. All localised and stage 4S tumours showed co-expression of trk-A and trk-C mRNA (Table 1). However, there was only a non-significant trend towards a higher trk-C index in trk-A-positive tumours. In this subset of neuroblastoma patients, both detectable trk-A mRNA and higher trk-C index were associated with favourable outcome with similar levels of significance (Figure 4).

Full-length trk-C expression in neuroblastoma

Different spliced variants of the full-length human trk-C have been described, depending on the presence or absence of small insertions in the tyrosine kinase domain [25, 26]. These insertions are absent in the functional form of the

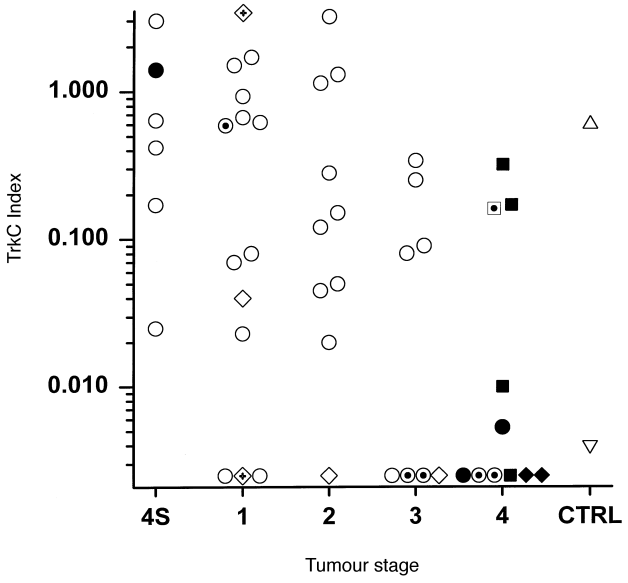

Figure 2. Summary of trk-C mRNA expression in primary neuroblastoma tumours of different clinical stages (4S, 1, 2, 3 and 4, respectively, according to INSS, Brodeur and associates [2]). The trk-C index (trk-C mRNA/*GAPDH* mRNA, in logarithmic scale) was higher in favourable stages (4S, 1 and 2) compared with advanced stages (3 and 4, $P < 0.001$). The trk-C index was higher in tumours from children with favourable outcome (open symbols) than from children who died from tumour progression (solid symbols, $P = 0.044$). ○, ● = primary neuroblastomas, □, ■ = primary neuroblastomas amplified for *MYCN*; ◇, ◆ = relapsed or metastatic tumour tissue or residual tumour after chemotherapy; ⟐ = two samples from morphologically different parts of a *MYCN* amplified composite ganglioneuroblastoma; ●, ■, ◆ = children dead from tumour progression; ⊙, ⊡ = children dead from toxic or post-surgical complications, or alive with disease under treatment. △ = control human cortex, ▽ = control SH-SY5Y neuroblastoma cells.

human trk-C receptor [24]. In order to evaluate the expression of these spliced variants, we analysed a subset of neuroblastoma tumours and used primers for RT–PCR that were designed to differentiate among these splice variants [19]. The amplified product, corresponding to the predicted trk-C kinase region without the insertions, could be detected in a subset of the tumours with favourable stage and outcome, but not in any of the unfavourable tumours tested (Figure 1b). This RT–PCR product corresponded in size to the functional variant without insertions and its identity with trk-C was proven after purification, subcloning and sequencing as recently reported [19, 24].

DISCUSSION

Neurotrophic factors and their receptors have been suggested to play significant roles in neuroblastoma tumour behaviour. The results in this study indicate that trk-C expression is associated with favourable tumours of lower stages in younger children. Furthermore, only favourable tumours express the functional tyrosine kinase domain of the receptor. In a subset analysed for both trk-A and trk-C, localised favourable tumours co-expressed these neurotrophic receptors with similar association and with good prognosis.

Expression of the NGF receptors trk-A and $p75^{LNGFR}$ has been shown to be a good prognostic marker of less agressive neuroblastoma tumours [7, 8, 27–29], whereas full-length trk-B receptors appear to be expressed in neuroblastoma tumours with poor prognosis [17, 18]. Nakagawara and associates reported that trk-C mRNA was not expressed in neuroblastomas using a rat probe [17]. However, RNase protection analysis allows a more sensitive detection of trk-C mRNA, and the cloning of a human trk-C DNA fragment allowed a specific analysis [19]. Using this approach, we found a significant association of higher trk-C expression with localised tumours, young age at diagnosis and favourable outcome as recently described and extended in the present study. However, trk-C mRNA expression was not significantly correlated with trk-A mRNA expression or absence of *MYCN* amplification. These results may in part be explained by the limited number of patients in the present study with an over-representation of favourable tumours. In addition, calculation of the trk-C index was based on expression of the transmembrane part of the receptor, whereas RT–PCR analysis showed that a subset of unfavourable neuroblastomas with detectable expression of transmembrane trk-C mRNA did not express the tyrosine kinase domain, implying non-functional truncated receptors.

During the course of our studies, Hoehner and associates presented data on the presence of different trk receptors in neuroblastoma tumours using a panel of specific antibodies [30]. Similar to investigators analysing mRNA expression, they found trk-A expression to be associated with good prognosis. They also found that more differentiated tumour cells stained most intensely for trk-C, although no significant correlation with stage or outcome was found. Similar to our results, Yamashiro and associates have reported trk-C to be expressed in a subset of favourable neuroblastomas using Northern Blot analysis [31]. They found a significant correlation between trk-C expression and favourable stage (1, 2

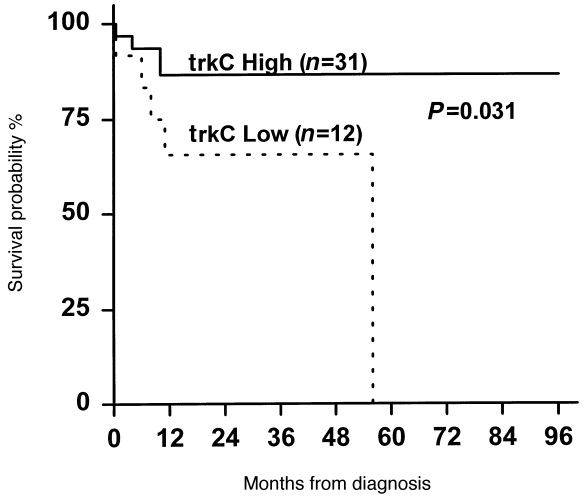

Figure 3. Survival probability according to Kaplan–Meier for 31 children with primary neuroblastoma tumours with intermediate or high trk-C mRNA expression, (solid line, survival probability 86.6% ± 6.2% at 3 and 6 years) compared with 12 children with very low or undetectable trk-C expression (broken line, survival probability 65.6% ± 14% and 0% at 3 and 6 years, respectively, $P = 0.031$, Mantel–Haenszel log-rank test).

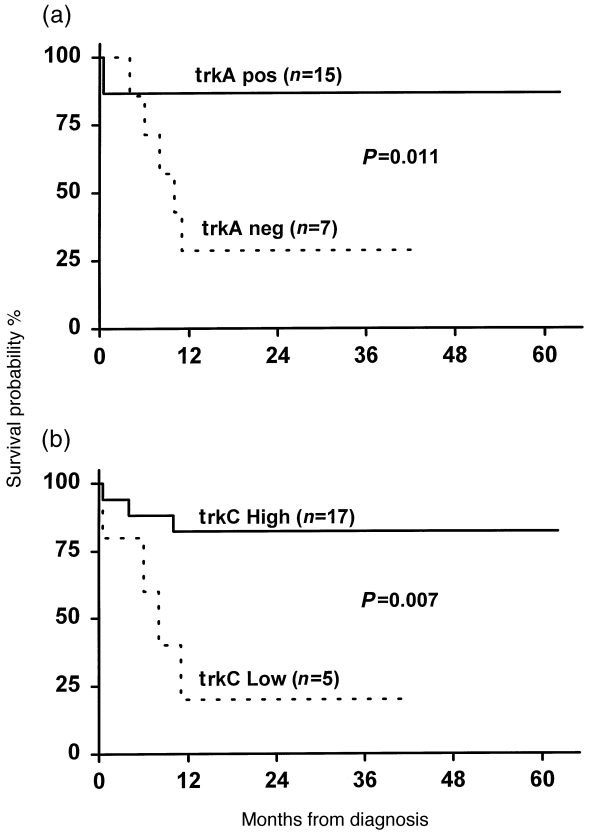

Figure 4. Survival probability according to Kaplan–Meier for 22 children with primary neuroblastomas analysed for both trk-A and trk-C mRNA. (a) Survival probability for 15 children with tumours expressing trk-A (solid line, 86.7% ± 8.8% at 3 years) compared with 7 children without detectable trk-A expression (28.6% ± 17.1%, $P = 0.011$, Mantel–Haenszel log-rank test). (b) Survival probability for 17 children with tumours with high or intermediate trk-C expression (solid line, 82.47% ± 9.2% at 3 years) compared with 5 children with low or undetectable trk-C expression (20% ± 17.9%, $P = 0.007$, Mantel–Haenszel log-rank test).

and 4S) as well as a better outcome. Interestingly, in paediatric medulloblastomas, high trk-C expression was reported to be correlated with good prognosis [32].

The present study suggests a functional role of full-length trk-C receptors in a subset of favourable neuroblastomas, although the functional significance of neutrophins and their receptors in neuroblastoma tumour biology remains to be fully defined. It may be suggested that the presence of trk-A and full-length trk-C receptors together with low-affinity $p75^{LNGFR}$ in tumours prone to apoptosis or differentiation may be dependent on the presence of the ligands NGF and NT-3, respectively. Furthermore, BDNF and full-length trk-B may define an autocrine loop promoting survival of aggressive neuroblastomas. It was recently suggested that neuroblastom differentiation was promoted by infiltrating normal Schwann cells [33]. These cells may, during normal circumstances, provide neurotrophic factors to neighbouring cells in a paracrine manner. It may therefore be suggested that maturation of favourable neuroblastomas may depend on the provision of neurotrophins by infiltrating normal Schwann cells that interact with tumour cells expressing functional neurotrophic receptors. The absence of respective ligands will result in regression due to apoptosis of tumour cells expressing neurotrophic receptors.

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