Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 435–443, 1995
Elsevier Science Ltd
Printed in Great Britain
0959–8049/95 $9.50+0.00

0959-8049(95)00025-9

Biochemical Evidence for a Mature Phenotype in Morphologically Poorly Differentiated Neuroblastomas with a Favourable Outcome

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Neuroblastoma is an embryonal tumour of the sympathetic nervous system with marked heterogeneity in terms of histological maturity and clinical course. A previous study revealed that high tumour levels of the csrc protein, particularly its neuronal isoform ($pp60^{csrcN}$), correlated with favourable outcome. To test whether this feature reflects a higher degree of neuronal maturation in these tumours, an extended series (47 consecutive neuroblastomas and 10 ganglioneuromas) were analysed for levels of csrc protein isoforms, neuron-specific enolase, and synaptophysin. Immunoblotting and radioimmunoassay techniques were employed. The results were compared with conventional histological signs of neuronal maturation. High $pp60^{csrcN}$ levels were specific for prognostically favourable neuroblastomas and correlated with high neuronal marker levels. However, signs of histological maturation correlated poorly with these parameters. It is therefore concluded that low stage tumours are highly differentiated in biochemical terms despite their frequently immature histology. Furthermore, the clinical usefulness of these biochemical parameters as prognostic markers was compared with established parameters in a multivariate analysis. Stage 4 disease, *MYCN* amplification, and age above 18 months at diagnosis was the most powerful combination of variables found for predicting a poor outcome. As expected, none of the neuronal differentiation markers investigated could add to the prediction of aggressive disease when compared with this model. However, high expression of $pp60^{csrcN}$ appeared to be useful in predicting long-term survival in high stage infant neuroblastoma.

Key words: neuroblastoma, prognosis, csrc, *MYCN* amplification, tumour site, age, stage, histological grade, NSE, synaptophysin
Eur J Cancer, Vol. 31A, No. 4, pp. 435–443, 1995

INTRODUCTION

NEUROBLASTOMA IS a childhood tumour originating from various parts of the sympathetic nervous system [1]. From the earliest reports on this tumour it was evident that the tumour cells are arrested at different stages of embryonal development [2]. Clinically, neuroblastoma can be divided into two major forms occurring with a similar frequency. One highly malignant form usually displays an extensive spread at diagnosis and usually occurs after 2 years of age in the adrenal area [3], whereas the other is prognostically favourable, primarily diagnosed during infancy, and has a restricted local growth, usually with tumour localisations indicating an origin from the sympathetic trunk or prevertebral abdominal parts of the sympathetic nervous system [4, 5]. *MYCN* gene amplification is a highly significant marker for a poor prognosis independently of these clinical parameters [6]. Although the degree of histological maturation also correlates with prognosis [5, 7, 8], it is a less significant prognostic parameter in infantile tumours since an undifferentiated histology, in spite of a favourable patient outcome, is common in this age group [5].

In a previous study on tumour specimens from children with neuroblastoma [9], we found a correlation between expression of the neuronal variant of the csrc protein, $pp60^{csrcN}$ [10–12], and prognosis. In general, infant tumours expressed high levels of $pp60^{csrcN}$ compared with the fibroblast form, $pp60^{csrc}$, while malignant neuroblastomas, most of which affect older children, expressed comparatively more of the fibroblast form. Furthermore, $pp60^{csrcN}$ was only detected in neuroblastomas and retinoblastomas and not in any of the non-neuronal childhood tumours studied. Thus, $pp60^{csrcN}$ appeared to be a useful diagnostic marker for neuroblastoma and a positive prognostic marker for infant neuroblastomas.

The purpose of this investigation was to confirm the previous clinical findings concerning $pp60^{csrcN}$ expression in neuroblastoma in a larger series and to test whether high levels of this protein reflect the stage of neuronal differentiation of the tumour. We have therefore extended our previous study by analysing additional tumours in this respect, and by determining the tumour levels of neuron-specific enolase (NSE) and synaptophysin, two established markers for neuronal and neuroendoc-

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rine differentiation [13–20]. The levels of these proteins have also been compared with conventional morphological signs of neuronal maturation in neuroblastoma [7]. The prognostic value of these biochemical markers has been compared with that of established prognostic parameters in a multivariate analysis.

MATERIALS AND METHODS

Tumour material and cell lines

Snap-frozen surgical specimens from primary tumours were obtained from 47 infants and children with neuroblastoma and 10 children with ganglioneuroma. INSS criteria [21] were used for clinical classification. Most high stage tumours had been subjected to chemotherapy prior to surgery. The specimens were collected during the autumn of 1986 to the end of 1991, and represent a consecutive series of tumour samples from all medical centres in Sweden that provide surgical treatment of neuroblastoma. A detailed clinical presentation of cases 32–112 is provided in a previous report [22]. The human neuroblastoma cell line SH-SY5Y (kindly provided by Dr June Biedler, Sloan Kettering Institute, New York, U.S.A.), used as a reference, was routinely grown as previously described [9].

Biochemical and morphological characterisation of the tumour material

For immunoblots, small pieces (approximately 2 mm^3) of tumour specimens were homogenised in 300 ml of 10 mM Tris–HCl buffer pH 7.2 containing 0.16 M NaCl, 1% (w/v) Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and supplemented with 1 mM EDTA and 1 mM EGTA. DNAse I (2 ml of 1 mg/ml) was added to each sample prior to homogenisation. The homogenates were cleared in an Eppendorff centrifuge for 10 min and the supernatants were recovered. For $pp60^{src}$ immunoblots, 0.3 mg of total protein was used, as determined by a modified Lowry procedure [23]. For synaptophysin immunoblots, 0.5 mg of total protein was used. The material was subjected to SDS-PAGE under reducing conditions according to Laemmli [24] using either a 7.5% gel (for $pp60^{src}$) or a 10% gel (for synaptophysin), thereafter the separated proteins were blotted on to nitrocellulose filters electrophoretically [9]. Monoclonal antibodies used were the MAb-327 anti$pp60^{src}$ antibody (kindly provided by Dr Joan Brugge, ARIAD Pharmaceuticals, Inc., Cambridge, Massachusetts, U.S.A.), and the antisynaptophysin antibody SY 38, (Dakopatts, Glostrup, Denmark), both diluted 1 : 100 in PBS containing 1% gelatin. The proteins, $pp60^{csrc}$, $pp60^{csrcN}$ and synaptophysin were identified by their molecular weights. Quantification of the immunoreactive protein levels, using ^{125}I-labelled protein A, in the blots was performed as previously described [9]. The content of $pp60^{src}$ and synaptophysin in the neuroblastoma cell line, SH-SY5Y, was used to standardise between different experiments. For synaptophysin, the values obtained, after excising the radioactive bands and measuring the radioactivity for the different tumours, were expressed as a ratio of the value of the tumour and that of SH-SY5Y [9]. The value for SH-SY5Y was set as 1.0 (Tables 1a and b). For $pp60^{src}$, the tumours were divided into three groups where those with similar levels as SH-SY5Y were denoted '++', those with lower levels were denoted '+', and those with values just above background were denoted 'bg' [9]. For determination of the proportions of $pp60^{csrc}$ and $pp60^{csrcN}$ in each tumour specimen, semiquantification was performed visually, since the two proteins could not be excised and counted separately due to poor electrophoretic separation [9].

NSE was measured with a radioimmunoassay (Pharmacia AB, Uppsala, Sweden) based on an antiserum and a technique described by Påhlman and associates [25].

The neuroblastoma material was morphologically classified according to the criteria of Beckwith and Martin [7], i.e. the tumours were classified according to their content of tumour cells with histological signs of maturation. These signs were: nuclear and cytoplasmic enlargement with well-defined cell borders and increased cytoplasmic eosinophilia, cell processes and presence of nerve sheath cells (indicated in Figure 1a–d). Four categories were defined with the following content of maturing cells. I: >50%, II: 5–50%, III: <5%, IV: 0%.

Statistical analyses

In a univariate analysis, the prognostic significance of individual variables was evaluated by Kaplan–Meier estimates and Log-Rank tests. Cox regression models were employed in estimating the relative risk for progressive disease with the possibility of evaluating several variables simultaneously. The independent capacity of variables in predicting outcome was evaluated in a multivariate analysis, where they were tested in pairs, triplets and quadruplets. The predictive impact of single and combinations of variables was determined by likelihood ratio tests [26].

RESULTS

Clinical characterisation of the tumour material

General prognostic characterisation of neuroblastomas. Of the neuroblastomas, 24 were curable and none of these children had remaining evidence of disease after 12 to 79 months follow-up (median: 42 months). The remaining 23 tumours were highly aggressive, and all children affected died from progressive disease 1 month to 8 years after diagnosis (median: 11 months). The tumours could, therefore, be divided into two distinct prognostic categories (Tables 1a and b).

Age-dependent characterisation. Prognosis correlated with age at diagnosis, with the most significant limit found at 18 months (tested against 12 and 24 months). Most children in the younger age group had a favourable outcome (19/23), with a follow-up time ranging between 22 and 79 months. These prognostically favourable tumours usually displayed local growth (stages1–3) at diagnosis (16/19; Table 1a). Long-term survival was also seen in some infants with metastatic stage 4 (1/4) and 4S (2/3) disease (Tables 1a and b). The origin of prognostically favourable tumours in this age group was mostly non-adrenal (13/19). All (4/4) prognostically unfavourable infantile tumours were, however, localised to the suprarenal area, possibly indicating an adrenal medullary origin. Amplification of the *MYCN* gene was present in all infant stage 4 tumours (4/4), and correlated with aggressive disease when 10-fold or higher (3/3; Table 1b).

In contrast, the outcome for children diagnosed at older ages (>18 months) was generally poor (19/24; Tables 1a and 1b). An adrenal tumour site (18/24) and a more than 10-fold *MYCN* amplification (6/24) was more common in this age group. The 5 favourable tumours of this age group showed varying clinical characteristics (Table 1a). Survivors have been followed for 12–53 months after diagnosis and survival times in lethal cases range from 3 months to 8 years (median time 17 months).

Expression of $pp60^{csrc}$ isoforms and neuronal markers

In accordance with our previous report [9], this extended clinical material also showed $pp60^{csrcN}/pp60^{csrc}$ ratios generally

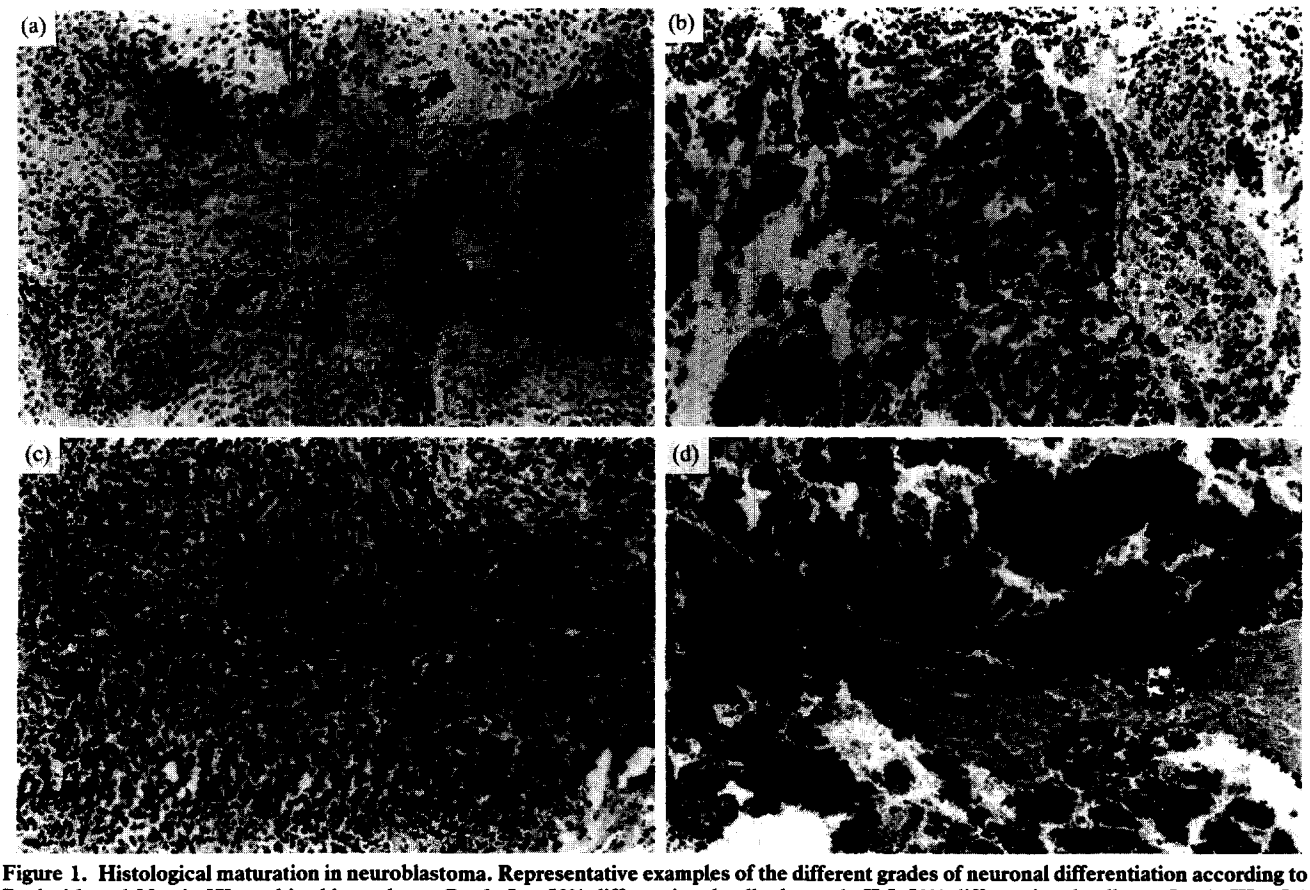

Figure 1. Histological maturation in neuroblastoma. Representative examples of the different grades of neuronal differentiation according to Beckwith and Martin [7] used in this study. a: Grade I > 50% differentiated cells; b: grade II 5–50% differentiated cells; c: Grade III <5% differentiated cells; d: Grade IV no signs of differentiation.

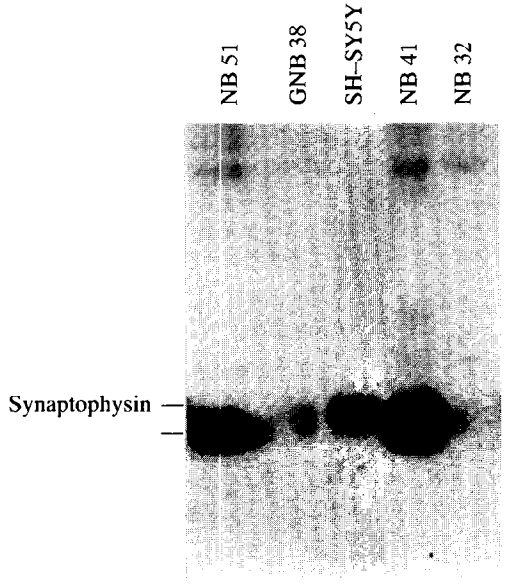

Figure 2. Analysis of the expression of synaptophysin in human neuroblastomas and ganglioneuroblastomas, using an immunoblot technique. The human neuroblastoma cell line SH-SY5Y was used as a quantitative standard. Lysates from tumour biopsies, identified by numbers (abscissa), were prepared as described in Materials and Methods, and subjected to SDS-PAGE. After electrophoresis, the proteins were blotted on to nitrocellulose filter followed by sequential incubation with an antisynaptophysin antibody, rabbit antimouse immunoglobulin antibodies and ^{125}I-labelled protein A. 0.5 mg of total protein was used in each lane. NB, neuroblastoma; GNB, ganglioneuroblastoma.

higher than 1 in the infant cases, and lower than 1 in the non-infant cases (Table 1). In this series additional trends were seen, although based on few observations. The aggressive stage 4 infant tumours appear to have a low $pp60^{csrcN}/pp60^{csrc}$ ratio (3/3) similar to that found in stage 4 tumours of children diagnosed at older ages, whereas the 4S tumours (3/3) and one (1/1) favourable infant stage 4 tumour had high ratios like other favourable infant tumours (Table 1a).

Synaptophysin is a protein present in synaptic vesicles and hormone storing granular vesicles. Its expression levels in neuroblastoma specimens were analysed by immunoblot technique (Figure 2). Synaptophysin was detected in the majority of tested tumours (40/46). Its ratio to the control (SH-SY5Y cells) ranged from 0.13 to 3.7 (Tables 1a and b). An abundant expression (ratio >1; arbitrary limit) of synaptophysin was frequently found in tumours diagnosed before 18 months of age (18/23). Among the tumours of older children, all tested specimens from the prognostically favourable tumours had high levels (4/4), whereas only a few of the progressing tumours had levels above 1 (5/19), with a marked over representation in extra-adrenal tumours (3/4 thoracic tumours versus 2/15 adrenal tumours) (Table 1b). With one exception (case 44; Table 1a), all tumours found to express high levels of $pp60^{csrcN}$ also expressed high levels of synaptophysin (17/18).

The NSE levels in tumour homogenates were studied using a radioimmunoassay. The values obtained ranged from 0.02 to 1.52 μg/mg protein (Tables 1a and b). We could again find a correlation between clinical parameters, such as age of the patient, outcome, and site of tumour versus expression of the

Table 1a. pp60$^{c\text{-}src}$, pp60$^{c\text{-}srcN}$, synaptophysin, and NSE in neuroblastoma specimens from tumours with favourable outcome

Patient number	Age at diagnosis	Stage	Site of origin	*MYCN* copies†	pp60^{src}*	src-protein level	NSE μg/mg	Synaptophysin‡	Grade§
				Age at diagnosis ≤ 18 months					
44	4 m	2b	adr	1	N > c	++	0.20	0.71	II
45	0 m	2a	th	1	c = N	++	0.11	0.75	II
51	3 m	2b	th	1	N > c	++	0.55	2.9	IV
58	0 m	2a	th	1	N > c	++	0.33	1.6	I
60	2 m	1	pelv	1	N > c	++	0.35	1.2	III
66	4 m	1	th	1	c > N	++	1.45	3.5	I
78	12 m	3	abd	1	c = N	++	0.77	2.4	II
84	3 m	3	abd	1	N > c	++	0.10	3.7	III
85	1 m	3	th	1	N > c	++	0.72	2.8	III
87	18 m	2a	th	1	N > c	++	0.72	2.5	I
88	16 m	1	th	1	N > c	++	0.84	1.4	nt
103	0 m	1	adr	1	N > c	++	0.30	2.9	III
104	2 m	3	th	1	N > c	++	1.14	2.7	II
106	10 m	4	adr	6–8	N > c	++	1.20	3.2	III
118	1 m	1	pelv	1	N > c	++	1.52	3.5	III
125	0 m	4S	adr	1	N > c	++	0.02	3.2	IV
128	6 m	3	pelv	1	N > c	++	0.73	1.5	II
133	12 m	2a	adr	1	c = N	++	nd	1.4	III
137	0 m	4S	adr	1	N > c	++	0.52	3.2	I
				Age at diagnosis >18 months					
61	8 y	2b	adr	1	c > N	++	0.79	3.1	nt
110	5 y	4	adr	1	ne	++	0.91	2.5	III
111	3.5 y	4	abd	1	c = N	+	1.08	1.1	II
127	8.5 y	2b	adr	1	N > c	++	1.29	3.1	I
138	2.5 y	2b	pelv	1	c = N	+	0.44	nt	II

Table 1b. pp60$^{c\text{-}src}$, pp60$^{c\text{-}srcN}$, synaptophysin and NSE in neuroblastoma specimens from tumours with unfavourable outcome

Patient number	Age at diagnosis	Stage	Site of origin	*MYCN* copies*	pp60^{src}†	src-protein level	NSE μg/mg	Synaptophysin‡	Grade§
				Age at diagnosis ≤18 months					
39	0 m	4S	adr	1	N > c	++	0.42	2.6	IV
52	5 m	4	adr	10	c > N	+	nd[7]	0.81	IV
55	10 m	4	adr	40	c > N	+	0.40	1.0	II
123	11 m	4	adr	70	c > N	++	0.06	nd	IV
				Age at diagnosis >18 months					
32	6 y	4	adr	1	c = N	++	0.03	0.13	IV
38	23 m	4	adr	1	c	bg	0.13	0.43	nt
41	6.5 y	4	th	1	c = N	++	1.12	3.5	I
48	4.5 y	4	adr	40	nd	nd	nd	nd	III
49	7 y	4	adr	1	c	bg	0.02	0.45	II
56	6 y	4	adr	1	c	bg	0.31	0.53	I
68	3.5 y	4	adr	70	c > N	+	0.02	0.59	III
69	5 y	3	th	1	c = N	+	0.94	3.1	IV
89	6 y	3	adr	1	c > N	+	0.06	nd	nt
95	26 m	4	adr	50	c = N	++	0.12	0.63	IV
107	24 m	4	adr	1	c > N	++	0.08	0.55	I
112	3.5 y	4	adr	1	c > N	+	0.07	nd	I
114	6 y	4	adr	1	ne	++	1.0	2.8	nt
121	6 y	2a	th	1	c > N	++	0.13	1.5	nt
126	11 y	4	adr	40	nd	nd	0.05	0.72	IV
130	4 y	4	th	1	c > N	+	0.08	nd	IV
131	3 y	3	adr	1	nd	nd	nd	nd	nt
135	21 m	3	adr	50	N > c	++	0.50	1.6	III
136	4 y	3	adr	60	c > N	+	0.33	0.35	IV

nt, not tested; ne, not evaluable; nd, not detectable; bg, positive signal just above background; abd, abdomen; th, thoracic; pelv, pelvic; adr, adrenal; m, months; y, years.

* Gene copies per haploid genome. † N, pp60$^{c\text{-}srcN}$, c, pp60$^{c\text{-}src}$; c alone means only pp60$^{c\text{-}src}$ was detectable.

Patient numbers 32–103, data taken from [9]; patient numbers 32–112, data taken from [22]. ‡Expressed as a ratio between the value obtained for the tumour and that of the neuroblastoma cell line SH-SY5Y. §I > 50, II = 5–50, III < 5, IV = 0% differentiating elements (according to Beckwith and Martin [7]).

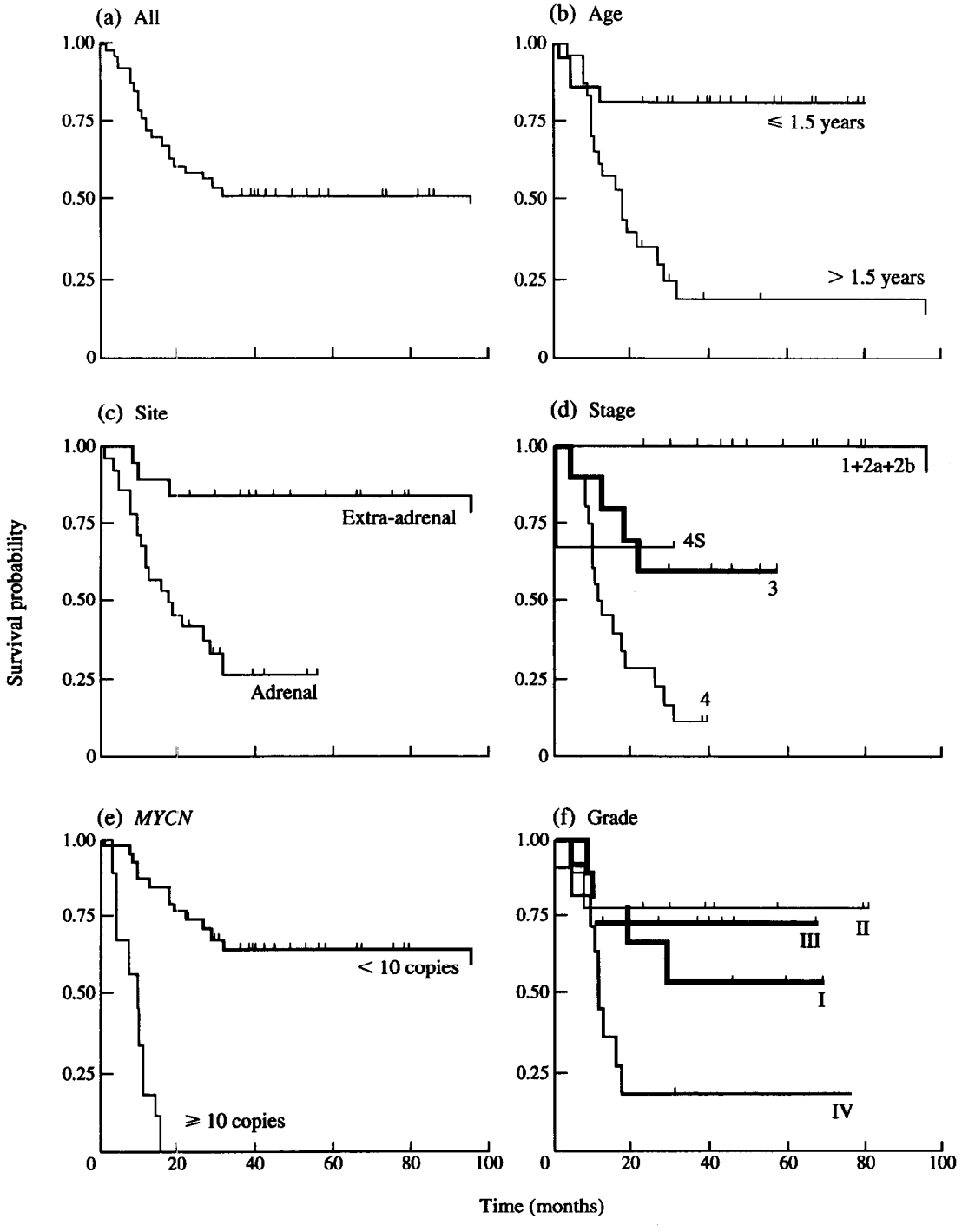

Figure 3. Probability of survival in neuroblastoma according to Kaplan–Meier estimates for the total material (a); age at diagnosis (b); site of tumour origin (c); disease stage (d) *MYCN* gene amplification (e) and histological grade (f). Number of patients included for each parameter and statistical significances of the variations found are shown in Table 2.

neuronal marker NSE. A covariation with synaptophysin levels was evident in view of the fact that only 3 tumours were discordant for these parameters when separating the values into high or low (NSE cut-off level 0.40 μg/mg protein, synaptophysin ratio 1; arbitrary limits).

Of the ganglioneuromas, 10 were examined for the expression of synaptophysin and NSE. All tumours had low or undetectable levels of these neuronal and neuroendocrine markers (data not shown). Furthermore, only one of these tumours expressed $pp60^{c\text{-}srcN}$, which was present at low levels (data not shown).

Histological maturation in neuroblastoma tumour sections

Beckwith and Martin [7] developed a staging system for neuroblastoma based on tumour morphology. Since the aim of this study was to compare different criteria for biochemical and morphological differentiation, and their respective usefulness in

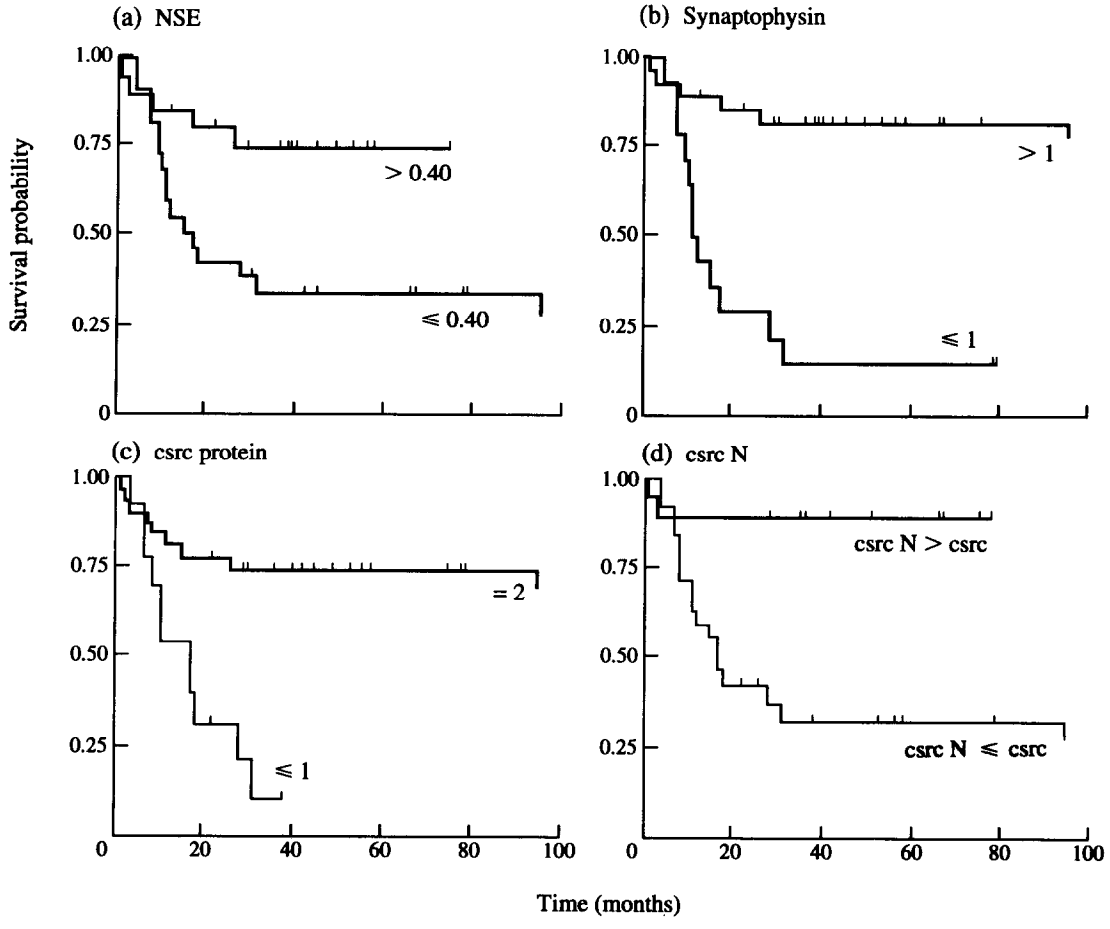

Figure 4. Probability of survival in neuroblastoma according to Kaplan–Meier estimates for the neuronal markers investigated, as follows: NSE (a); synaptophysin (b); total c-src protein levels (c); and dominance of the neuronal isoform $pp60^{csrcN}$ (d). Number of patients included for each parameter and statistical significances of the variations found are shown in Table 2.

evaluating outcome, the tumour material was classified according to grade of histological maturation, which resulted in equal-sized groups (grade I: 9, II: 9, III: 11 and IV: 11 tumours; Tables 1a and b). Histological evidence for neuronal maturation was, however, a poor prognostic indicator, in contrast to the levels of csrc proteins and neuronal differentiation markers. A definitive prognostic trend was seen only with grade IV tumours, since few (2/22) favourable tumours had this completely undifferentiated histology compared with the number found in the group of aggressive tumours (9/18).

Statistical analyses

Univariate anlaysis. Kaplan–Meier estimates for survival of the total neuroblastoma material and with respect to clinical and morphological variables and *MYCN* gene amplification are shown in Figure 3. The corresponding results for c-src protein isoform expression and the neuronal markers investigated are shown in Figure 4. Table 2 shows the statistical estimates for these results giving the relative risk for fatal disease for each variable. Significantly increased risk for progressing disease was found for the following variables: disease stage 3 and stage 4, respectively, compared with stages 1–2b, age over 18 months at diagnosis (also tested for 12 and 24 months, but showing less significance); adrenal site compared with extra-adrenal sites; 10-fold or higher *MYCN* gene amplification; $pp60^{csrcN}/pp60^{csrc}$ ratio ≤ 1; total csrc protein low-relative to control levels (+, bg, nd); synaptophysin, relative level ≤ 1.0 and NSE levels ≤ 0.40 μg/mg protein (Table 2). A 10-fold or higher *MYCN* gene amplification was a particularly specific indicator for a rapid demise (0% survival; 9 cases; Figure 3e and Table 2), whereas children with stage 1–2b tumours (Figure 3d and Table 2), and a high $pp60^{csrcN}/pp60^{csrc}$ ratio (Fig. 4d and Table 2) showed particularly high survival rates (93 and 89%, respectively). Although tumours with a totally undifferentiated histology (grade IV) were associated with a poor outcome, the ratio of differentiating cells in remaining tumours showed no prognostic significance (Fig. 3f). In statistical terms, however, the prognostic significance of a grade IV morphology was evident only when comparing it with the grade I+II group of tumours ($P = 0.02$), but not when comparing it with grade I tumours alone. The most appropriate comparison would, however, be grade (I+II) versus grade (III+IV) as these criteria are used in the widely accepted classification system suggested by Shimada and associates [5], and as the demarcation between tumours with lack of differentiating cells (grade IV) and those with <5% differentiating cells (grade III) was found to be the most imprecise aspect of the classification system. When looking at the total series (and thus disregarding the age factor used in the Shimada classification system), the difference in outcome between these two groups was not significant (Table 2). It can therefore be concluded that traditional morphological evidence for tumour maturation is a poor marker for prognosis.

Table 2. Outcome in neuroblastoma related to clinical and biochemical findings

	Number	Poor outcome (%)	Hazard ratio	*P*-value
Age at diagnosis				
≤18 months	23	17	1.0	reference
>18 months	24	79	6.3	= 0.001
Disease stage*				
1–3	25	24	1.0	reference
4	19	84	9.8	<0.001
Tumour site				
non-adrenal	19	21	1.0	reference
adrenal	28	68	6.7	0.003
MYCN amplification (≥10 fold)				
not present	38	37	1.0	reference
present	9	100	12.2	<0.001
$pp60^{csrcN}/pp60^{csrc}$ ratio				
>1	18	11	1.0	reference
≤1	24	71	8.0	0.006
$pp60^{csrcN}$ + $pp60^{csrc}$ level				
high	31	29	1.0	reference
low	16	88	4.6	= 0.001
Synaptophysin				
high	27	22	1.0	reference
low	19	89	7.2	<0.001
NSE				
high	21	24	1.0	reference
low	26	69	3.3	0.02
Grade				
I+II	17	35	1.0	reference
III+IV	23	52	2.0	0.18

*Stage 4S tumours omitted (3 cases).

Multivariate analysis. The independent prognostic significance of all biochemical (synaptophysin, NSE, $pp60^{csrcN}/pp60^{csrc}$ ratio, total c-src proteins, *MYCN* amplification), clinical (age at diagnosis, disease stage, tumour site) and morphological (grade) variables were investigated in a multivariate analysis (Table 3). The same limits as described in the univariate analysis were used, with the exception of grade of maturation. To evaluate better the prognostic significance of a grade IV morphology, histological data were grouped into grades I+II, grade III, and grade IV.

Of the covarying neuronal markers NSE and synaptophysin, the latter was the best prognostic indicator and was therefore used for comparison with $pp60^{csrcN}$. Although both were accurate markers for outcome, synaptophysin was better and no independent prediction of a prognostically unfavourable disease was obtained by the $pp60^{csrcN}$ ratio. Unlike synaptophysin, however, $pp60^{csrcN}$ showed an age dependence. This covariance between $pp60^{csrcN}$ and synaptophysin indicates $pp60^{csrcN}$ to be another neuronal differentiation marker, at least in infant neuroblastoma. As illustrated in Table 3, *MYCN* gene amplification, but not the $pp60^{csrcN}$ ratio, added to the prognostic accuracy of synaptophysin combined with disease stage in a multivariate three-factor model. The prognostic significance of a more than 10-fold *MYCN* amplification was superior to all other parameters investigated. When comparing the clinical parameters age and site, a mutual independence in predicting outcome was evident, but neither contributed significantly when analysed together with disease stage. The best combination of parameters found in predicting a poor outcome was stage 4 disease together with an age over 18 months at diagnosis and a 10-fold or higher degree of *MYCN* amplification (Table 3).

DISCUSSION

Differentiation-related biochemical differences in the separate prognostic forms of neuroblastoma have previously been shown. Aggressive forms correlate with a more primitive pattern of catecholamine secretion [27] and with lower expression of neuronal and neuroendocrine markers e.g. NSE [19, 20], synaptophysin [13, 16], neurofilament proteins [28], and vasointestinal peptide [28]. Low expression levels of the high affinity NGF receptor gene (i.e c-trk proto-oncogene) have been found in aggressive neuroblastomas [29, 30], indicative of an immature sympathetic tumour phenotype in view of the neuronal maturational response elicited in fetal neuronal and neuroendocrine sympathetic cells upon NGF stimulation [31–33]. *MYCN* amplification in neuroblastoma does not appear, however, to correlate with degree of differentiation when studied in neuroblastoma cell lines [34]. Various histopathological signs, such as evidence of neuronal maturation, mitotic figures, karyorrhexis, calcifications, amount of stroma, and Schwann cell infiltration have also proved to be clinically useful for prognostic evaluation of the neuroblastoma patient [5, 7, 8, 35]. However, signs of neuronal maturation, as a single morphological factor, are not a reliable prognostic indicator, particularly in tumours diagnosed before the age of 18 months, which commonly display an undifferentiated histology despite a favourable outcome [5].

In this investigation, the correlation between low tumour levels of NSE and synaptophysin and a poor prognosis were

Table 3. Relative risks (RR) of progressive disease in neuroblastoma according to selected multivariate models

Factor	RR	*P*-value
Model 1		
Stage		
1–3	1.0	reference
4	5.4	0.02
Synaptophysin		
high	1.0	reference
low	5.1	0.02
Model 2*		
Stage		
1–3	1.0	reference
4	3.9	0.06
Synaptophysin		
high	1.0	reference
low	3.6	0.08
$pp60^{csrcN}/pp60^{csrc}$ ratio		
>1	1.0	reference
≤1	3.5	0.3
Model 3†		
Stage		
1–3	1.0	reference
4	4.7	0.03
Synaptophysin		
high	1.0	reference
low	3.5	0.1
MYCN amplification (>10-fold)		
not present	1.0	reference
present	4.2	0.04
Model 4‡		
Stage		
1–3	1.0	reference
4	3.8	0.06
MYCN amplification (≥10-fold)		
not present	1.0	reference
present	7.4	0.02
Age at diagnosis		
≤18 months	1.0	reference
>18 months	4.8	0.05

*Non-significant difference in prediction of progressive disease between models 1 and 2; †Model 3 predicted progressive disease significantly better than model 1 ($P < 0.05$); ‡Model 4 predicted progressive disease significantly better than any other model.

confirmed. The previous finding of a positive correlation between high levels of csrc proteins, particularly $pp60^{csrcN}$, and a favourable outcome [9] was further confirmed in this extended material. A multivariate analysis showed that synaptophysin and $pp60^{csrcN}$ were covarying prognostic factors, particularly evident in the younger age group. It appears reasonable, therefore, to assume that high levels of $pp60^{csrcN}$ reflect an advanced stage of neuronal maturation. In contrast, the prognostic significance of *MYCN* gene amplification was statistically independent of these parameters, suggesting that the cellular effect of *MYCN* gene amplification in neuroblastoma is unrelated to tumour maturation.

Despite the high levels of csrc proteins, NSE, and synaptophysin in prognostically favourable infant tumours, many of these were morphologically immature, and therefore perhaps this biochemical evidence of an advanced neuronal phenotype reflects an early phase of ganglion cell differentiation during which morphological alterations are subtle or have not yet emerged. An alternative explanation for this discrepancy between morphological and biochemical differentiation in infant neuroblastoma would be that these tumours display neuroendocrine, as opposed to neuronal/ganglionic differentiation, and that such differentiation is accompanied by signs avoiding detection by the morphological criteria used. In support of this latter possibility, we have recently shown that *IGF2* expression is a marker for extra-adrenal neuroendocrine differentiation in the normal sympathetic nervous system during development, and in neuroblastoma *IGF2* is expressed in extra-adrenal tumours in morphologically similar cells within areas of tumour maturation. Subtle morphological characteristics of these cells were found, including an increase in nuclear size and brightness combined with a punctate pattern of nuclear basophilia [36].

From a clinical standpoint, an accurate prognostic evaluation of the individual patient at diagnosis is particularly urgent in neuroblastoma, in view of the considerable heterogeneity in clinical course and outcome in this disease. The introduction of clinical staging systems, based on evaluation of extent of disease at diagnosis, has provided an important guidance for the individual therapeutic strategy in most cases. Exceptions are found however, and in stage 3 disease, the prognostic significance is not as clear cut as in other disease stages (Figure 3d). The addition of age of the child at diagnosis is therefore important prognostic information, and a number of biochemical and genetic parameters can also be useful [1, 21]. Although the prognostically, most informative variable found in this study was clinical stage 4 disease, a 10-fold or greater *MYCN* gene amplification was the most specific, showing an association to poor outcome in all cases (9/9; Figure 3e). In the multivariate analysis, the addition of data on tumour levels of $pp60^{csrcN}$ did not add prognostic accuracy to a model including stage 4 disease, age over 18 months at diagnosis, and a 10-fold or higher *MYCN* gene amplification. However, this multivariate analysis tested only the specificity of a low $pp60^{csrcN}$ expression in predicting an unfavourable outcome, whereas the specificity of high $pp60^{csrcN}$ levels in predicting a favourable outcome was not tested. In the univariate analysis, however, a high $pp60^{csrcN}/pp60^{csrc}$ ratio was the most specific biochemical indicator for a favourable prognosis in this investigation (Figure 4). It was a particularly accurate positive prognostic indicator in the infant group with both high sensitivity (15/19) and specificity (15/16; Table 1a and b). The only infant with a tumour with high $pp60^{csrcN}/pp60^{csrc}$ ratio and a poor outcome had a stage 4S tumour and the cause of death was pulmonary compression rather than true tumour progression (case 39; Table 1b).

In summary, the present study indicates that a high level of $pp60^{csrcN}$ in neuroblastoma is a neuronal or neuroendocrine differentiation marker, present in prognostically favourable tumours. High level $pp60^{csrcN}$ is also correlated with an early age at diagnosis, extra-adrenal tumour sites, and 4S disease, but correlates poorly with conventional morphological signs of tumour maturation. In the statistical analysis, disease stage in combination with *MYCN* gene amplification and age at diagnosis provided the most accurate prognostic information with respect to unfavourable disease. However, high tumour levels of csrc proteins appear to be useful as positive prognostic signs in advanced infant neuroblastoma (see cases 84, 85,104, 106, 128).

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Acknowledgements—We are deeply grateful to Irja Johansson and Ulrika Larsson for skilful technical assistance and Per Kogner, Karolinska Hospital, Stockholm, and all others involved at East Hospital, Gothenburg and University Hospital, Lund for assistance with tumour material. This study was supported by grants from the Swedish Cancer Research Foundation, Childrens Cancer Foundation of Sweden, HKH Kronprinsessan Lovisas förening för barnasjukvård, Hans von Kantzows and Ollie och Elof Ericssons stiftelser, Lions Cancer Research Foundation at Uppsala University Hospital (to FH), Svenska Sällskapet för Medicinsk Forskning (to CB).