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Prognostic importance of DNA flow cytometrical, histopathological and immunohistochemical parameters in neuroblastomas

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Summary. In 42 tumour samples of human neuroblastoma, histological classification by differentiation (Shimada) was significantly correlated with strong positivity for neuron-specific enolase (NSE) and inversely correlated with rosette formation. Most ganglioneuroblastomas were positive for S-100 protein and reacted strongly with NSE antibody. Histological signs of high proliferative activity included intermediate or high mitosis-karyorrhexis index, necrosis and lack of calcification, which were significantly correlated with each other. Flow cytometric DNA analysis demonstrated that 88% of the tumour samples had DNA aneuploid stem lines. High S phase fraction (≥ 0.20) was significantly correlated with necrosis and lack of calcification. Univariate analysis of prognosis for 26 patients whose tumour samples were obtained before adjuvant treatment showed that five factors were significantly related to a better outcome: early stage of the disease (stages I, II, IV–S), S phase fraction <0.20, favourable Shimada histology, positivity for S-100 protein, and strong positivity for NSE. In multivariate analysis, only S phase fraction *or* stage of disease remained significantly associated with prognosis. DNA index did not correlate with prognosis in this study.

Key words: Immunohistochemical markers – Flow cytometry – Ploidy – S phase fraction – Shimada classification

Introduction

Neuroblastoma, a malignant embryonal tumour arising from the sympathetic nervous system, comprises about 7% of all childhood neoplasms (Brown et al. 1989). With progress in basic science several new prognostic indicators have emerged (Carlsen 1991 b), but clinical stage and age at diagnosis remain the most important prognostic factors (Breslow and McCann 1971; Carlsen et al. 1986; Coldman et al. 1980; Evans et al. 1987; Thomas et al. 1984; Wilson and Draper 1974). It is also generally recognized that the prognosis is better for differentiated than for undifferentiated tumours; some authors have, however, found the histology of the tumour to be an independent prognostic factor (Evans et al. 1987; Sandstedt et al. 1983; Thomas et al. 1984; Wilson and Draper 1974). Some of this discrepancy may be due to the absence of a uniform terminology (Beckwith and Martin 1968; Gitlow et al. 1973; Horn et al. 1956; Hughes et al. 1974; Mäkinen 1972; Sandstedt et al. 1983) and the fact that the differentiation may vary in different parts of the tumour (Dargeon 1962; Horn et al. 1956; Mäkinen 1972). In 1984, Shimada et al. proposed a histopathological grading system, which classifies the tumours into favourable and unfavourable subgroups depending on the age of the patient and the histology. Immunohistochemical markers of differentiation, such as S-100 protein (Aoyama et al. 1990; Carlei et al. 1984; Shimada et al. 1985) and neuron-specific enolase (NSE) (Carlei et al. 1984) have also been suggested to afford prognostic information. Cytogenetic studies of modal chromosome number have associated triploid karyotypes with early stage disease and favourable prognosis, whereas pseudo- or near-diploidy is associated with advanced stage, unfavourable age and poor prognosis (Carlsen et al. 1991; Hayashi et al. 1989; Kaneko et al. 1987). Similarly, DNA aneuploidy detected by flow cytometry has been associated with favourable outcome, whereas DNA diploidy of the tumour stem cells has adverse prognostic significance (Bourhis et al. 1991; Brenner et al. 1989; Gansler et al. 1986; Look et al. 1984, 1991; Oppedal et al. 1988; Taylor and Locker 1990; Taylor et al. 1988). High proliferative activity, which is associated with poor prognosis (Bourhis et al. 1991; Cohn et al. 1990; Gansler et al. 1986; Look et al. 1984; Shimada et al. 1984; Slavc et al. 1990; Suzuki et al. 1989), is suggested to be more common in DNA diploid than DNA aneuploid tumours.

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The aims of the present study were to assess the prognostic importance of tumour histology, immunohistochemical markers of differentiation, and the DNA distributional parameters obtained by flow cytometry, and to correlate these variables to other prognostic factors in neuroblastomas.

Materials and methods

From 1970 to 1980 46 patients under the age of 15 years were treated for neuroblastoma in the State University Hospital, Rigshospitalet. The overall survival rate was 18 of 46 (39%) (Carlsen et al. 1981). Eleven of these patients were excluded from the present study because the histological material was insufficient for analysis. The excluded patients consisted of 1 of 6 stage I, 3 of 10 stage II, 1 of 8 stage III and 6 of 21 stage IV cases. Forty-two formalin-fixed paraffin-embedded tissue blocks of sufficient size were available from the remaining 35 patients. The tumour specimens were collected before treatment in 19 cases, both before and after adjuvant treatment in 7 cases, and after treatment in 9 cases (Tables 1, 2). Because stage, age and treatment given were the only independent prognostic variables in a greater sample size of patients including the present population (Carlsen et al. 1986), and because all but 3 stage I–II patients have received chemotherapy, only stage and age were considered clinical prognostic variables in the present study.

Sections stained with haematoxylin and eosin were graded according to Shimada et al. (1984). The patient's age at diagnosis is included in the Shimada Classification (1984) to allow categorization into favourable or unfavourable histological subgroups (Carlsen 1991b; Gansler et al. 1986; O'Neill et al. 1985; Shimada et al. 1985). Each case was evaluated on a scale of − to +++ in the following features: rosette formation, necrosis and calcification. Sections were also immunohistochemically stained for S-100 protein (Dakopatts, Copenhagen), NSE (Dakopatts) and glial fibrillary acidic protein (GFAP) (Dakopatts) (Carlei et al. 1984; Molenaar et al. 1990) using peroxidase-antiperoxidase techniques. Sections were scored as: − (no positive cells); + (a few positive cells); ++ (easily recognizable numbers of positive cells); or +++ (numerous positive cells). The slides were graded and scored without knowledge of other data (Tables 1, 2).

The staining method used for flow cytometric DNA analysis of paraffin-embedded material (Ornvold et al. 1990) was a modification of the method of Schutte et al. (1985). From each tumour, two paraffin sections were stained and analysed independently. Nuclei prepared and stained in the same way from paraffin-embedded normal breast tissue were used for quality control, and showed a single stem line as indicated by a symmetrical and narrow histogram peak together with minimum debris and clumping and satisfactory yield of nuclei. DNA histograms were deconvoluted by maximum likelihood using a model described elsewhere (Christensen et al. 1978; Vindeløv and Christensen 1990). The proportion of nuclei, G0/G1 peak mean and the phase fractions G0/G1, S and G2+M of each subpopulation were estimated. The S phase fractions were calculated using a uniform distribution. Debris was estimated by a truncated exponential function. The DNA index was calculated as the ratio of the estimated G0/G1 peak mean to the first G0/G1 peak mean, correcting for non-linearity between G2+M and G0/G1 peak fluorescence (Vindeløv and Christensen 1990). This definition implicitly defines the first peak as diploid.

Table 1. Patient characteristics of 26 patients whose tumour specimens were obtained before treatment

Patient no.	Age (months)	Stage	Histopathology		Histological features			Immunohistochemistry			DNA flow cytometry		Clinical outcome (months)
			Grade	Class	Ros	Necros	Calc	S-100	NSE	GFAP	Number of Stem lines	Highest S phase	
1	6	I	6	Fav	+++	−	−	+	+++	−	3	0.05	S (>192)
2	6	I	5	Fav	−	−	+	−	+++	−	2	0.11	S (>144)
3	96	I	9	Fav	−	−	+	++	++	−	3	0.16*	S (>144)
4	41	I	7	Unf	−	−	+	+++	+++	−	2	0.12	S (>120)
5	6	I	6	Fav	−	−	−	−	+++	−	3	0.22	S** (Homicide)
6	6	II	3	Fav	+	−	++	ND	+++	ND	2	0.08	S(>120)
7	36	II	6	Fav	−	−	+++	+	+++	−	2	0.03*	S (>192)
8	17	II	6	Fav	−	+	−	ND	+++	ND	2	0.24	M (7)
9	13	II	2	Fav	+	+	+	+	+	−	2	0.15*	S (>168)
10	18	II	9	Fav	−	−	+	+	+++	+	3	0.09*	S (>132)
11	3	II	3	Fav	+++	+	+	+	++	−	2	0.09	S (>120)
12	20	II	8	Fav	−	−	−	ND	ND	ND	1	0.03*	S (>120)
13	20	III	6	Fav	−	−	+	+	+	−	2	0.07*	S (>216)
14	16	III	6	Fav	−	−	+	−	+++	−	2	0.08*	M (5)
15	31	III	2	Unf	+	+	−	−	++	−	2	0.26	M (11)
16	48	III	2	Unf	+	++	−	−	+	−	3	0.25	M (2)
17	34	III	2	Unf	−	−	−	−	++	−	2	0.41	M (21)
18	5	IV-S	3	Fav	++	−	−	−	+++	−	2	0.04	S (>144)
19	0	IV	3	Fav	+	−	++	+	+	−	3	0.10	M (0)
20	6	IV	3	Fav	++	−	+	−	++	−	2	0.38*	M (3)
21	20	IV	6	Fav	−	−	−	−	+	−	3	0.33	M (0)
22	20	IV	5	Unf	−	+++	−	−	++	−	1	0.29*	M (3)
23	37	IV	5	Unf	−	+	+	−	++	−	3	0.21*	M (17)
24	49	IV	3	Unf	−	−	−	−	−	−	2	0.28	M (23)
25	31	IV	3	Unf	+++	+	+	−	+	−	3	0.48	M (2)
26	0	IV	2	Fav	+++	−	−	−	+	−	1	0.12*	M (0)

* S phase fraction of first G0/G1 peak, representing the stemline with lowest DNA content; ** the patient died by homicide free of disease and is recorded as being alive in the analysis of prognostic factors; Class, Shimada classification; Ros, rosette formation; Necros, necrosis; Calc, calcification; S, long-term survivor; M, dead; ND, not done; Fav, favourable histology; Unf, unfavourable histology

Table 2. Patient characteristics of 16 patients whose tumour specimens were obtained after treatment

Patient no.	Age (months)	Stage	Histopathology		Histologic features			Immunohistochemistry			DNA flow cytometry		Clinical outcome (months)
			Grade	Class	Ros	Necros	Calc	S-100	NSE	GFAP	Number of Stem lines	Highest S phase	
Responding tumours													
13	20	III*	8	Fav	−	−	−	+	+++	−	2	0.15**	S (>216)
14	16	III*	9	Fav	−	−	−	−	+++	−	3	0.11**	M (5)
15	31	III*	6	Fav	++	−	+++	+	+++	−	2	0.19	M (11)
18	5	IV-S*	7	Unf	−	−	+++	−	++	−	2	0.08**	S (>144)
27	15	III	9	Fav	−	−	−	+	++	−	2	0.11**	S (>168)
28	10	IV	6	Fav	+	−	+	−	+++	−	3	0.44**	S (>180)
29	17	IV	3	Unf	−	−	+	−	++	−	1	0.09**	S (>180)
30	44	IV	4	Unf	−	−	−	−	+++	−	3	0.31	M (5)
31	101	IV	9	Fav	−	−	−	++	++	−	1	0.27**	M (10)
32	10	IV	7	Unf	+	+++	+++	−	+++	−	3	0.41**	M (1)
Progressing tumours													
8	17	II*	2	Unf	++	+	−	−	++	−	2	0.31**	M (7)
17	34	III*	2	Unf	−	+	−	ND	ND	ND	2	0.25	M (21)
20	6	IV*	6	Fav	+	−	+	−	+++	−	2	0.22	M (3)
33	35	III	5	Unf	−	−	−	−	++	−	2	0.29**	M (16)
34	35	IV	2	Unf	+	++	−	+	++	−	2	0.29	M (6)
35	23	IV	5	Unf	−	−	−	−	+++	−	2	0.23	M (3)

* Tumour tissue retrieved both before and after chemotherapeutic treatment; ** S phase fraction of first G0/G1 peak, representing the stem line with lowest DNA content; for other abbreviations, see Table 1

The median coefficient of variation (CV) was 5%. The flow cytometric analysis was made without knowledge of other data (Tables 1, 2).

For the comparison of DNA histograms with clinical outcome and other prognostic indicators, the DNA distribution from each patient was characterized by the maximum of the estimated S phase fractions and the pattern of DNA indices of the estimated stem lines. DNA indices of non-diploid stem lines were grouped in the intervals <1.4, 1.4–1.6, 1.6–1.8, 1.8–2.2 and >2.2. Only stem lines comprising more than 10% of the distribution are included. Repeated measurements (from different paraffin sections) did not indicate marked differences due to the flow cytometric methodology.

The chi-square test and the four-fold table test were used for tests of homogeneity. A significance level of 5% was chosen. Logistic regression was used to evaluate the prognostic significance of age, stage, histopathological classification, immunohistochemical features and cell cycle analysis (Breslow and McCann 1971; Carlsen et al. 1986). For reasons discussed below, only the tumour samples retrieved before treatment were included in the study of prognostic factors (Table 1) (Anderson et al. 1985; Biedler et al. 1988; Carlsen 1991a, b).

Results

The data on patient characteristics, histopathology, immunohistochemistry, DNA ploidy and cell proliferation are summarized in Tables 1 and 2, and their univariate prognostic significance for the 26 patients, whose tumour samples were obtained before treatment, in Table 3.

DNA fluorescence histograms were obtained from all 42 tumour samples. In 37 tumours (88%), DNA aneuploidy was indicated by the presence of more than one stem line. Of these, 24 tumours (57%) had one additional stem line and 13 tumours (31%) had two additional stem lines. In the remaining 5 tumours (12%), only a single stem line was present, classified as DNA diploid; however, DNA aneuploidy cannot be excluded, because the technique does not include comparison with internal reference cells. In 7 cases, samples were retrieved from the primary tumour both before and after adjuvant treatment. In 1 case (patient 14) an additional stem line

Table 3. Results from univariate analysis of 10-year survival[a] in 26 neuroblastoma patients

	Degrees of freedom	*P*
Age at diagnosis (0–11 months versus 12 months or more)	1	0.41
Stage of disease (I, II, IV-S versus III and IV)	1	<0.001
Shimada classification (favourable histology versus unfavourable histology)	1	0.03
Positivity for S-100 protein	1	0.003
Strong (+++) positivity for NSE	1	0.03
Number of stem lines (1, 2 or more)	2	0.69
DNA index		
(<1.4 versus others,	1	0.41
1.4–1.6 versus others,	1	0.10
1.6–1.8 versus others,	1	0.38
1.8–2.2 versus others,	1	1.00
>2.2 versus others)	1	0.59
S phase fraction (<0.2 versus ≥0.2)	1	0.003

[a] A patient who died by homicide free of disease is recorded as being alive in the analysis of prognostic factors
NSE, Neuron-specific enolase

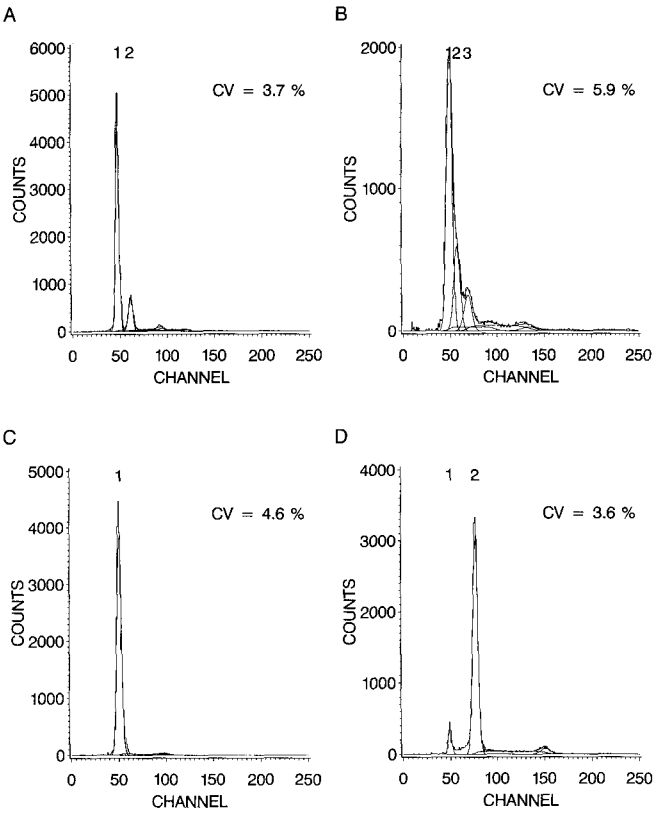

Fig. 1A–D. Four DNA distributions are shown. The step curve indicates the observed histogram and the smooth curves indicate the fitted distribution with G0/G1, S and G2+M components. S phases of stem lines representing 20% or less of the total population are not considered reliable and therefore not included in the further analysis. The debris has been subtracted from the histograms. The coefficient of variation (*CV*) of the first G0/G1 peak is indicated in each case. **A** A DNA distribution sampled from a tumour prior to adjuvant treatment (patient 14). The G0/G1 peak means of two stem lines are indicated by 1 and 2. **B** A DNA distribution sampled from the same tumour after adjuvant treatment (patient 14). There is an additional subpopulation compared to **A**. The G0/G1 peak means are indicated by 1, 2 and 3. The additional subpopulation has a DNA index of 1.16 (G0/G1 peak mean 2) and may be an artefact of the preparation due to cellular autolysis. **C** A DNA distribution sampled from a tumour after treatment (patient 29). There is only one peak which is assumed to be diploid. The G0/G1 peak mean is indicated by 1. **D** A DNA distribution sampled from a tumour prior to adjuvant treatment (patient 2). The S phase of the first stem line could not be estimated. The G0/G1 peak means are indicated by 1 and 2

was found after treatment that might be artefactual (Alanen et al. 1989).

The dominating stem line was in most cases the one with the highest S phase fraction (Fig. 1a–d). In 21 tumours, the highest S phase fraction was found in the stem line with the lowest DNA index (left sample peak), and the S phase fraction was relatively high (≥ 0.2) in 8 of these tumours. High S phase fractions (≥ 0.2) were found in 13 of the other 21 tumours (Tables 1, 2).

No major differences were obvious between the data sets of treated and untreated tumours, when the samples were classified according to histological grade (degree of maturation) (Shimada et al. 1984) and then examined with respect to immunohistochemical staining for S-100 protein and NSE, histological features of rosette formation, necrosis and calcification, and flow cytometric S phase fraction ≥ 0.2 (Tables 1, 2).

Only one tumour (a ganglioneuroblastoma) was positive for GFAP, whereas all but one of the tumour samples were positive for NSE; however, strong positivity (+++) was observed in about half of the samples, and this score was used for comparison with degree of maturation. Most ganglioneuroblastomas (Shimada grades 7+8+9) were positive for S-100 protein and had strong positivity for NSE. Among neuroblastomas, differentiating histology (Shimàda grades 4+5+6), as opposed to undifferentiated histology (Shimada grades 2+3), was positively correlated with strong NSE positivity ($P=0.01$) and inversely with rosette formation ($P=0.02$). No correlation between histological degree of maturation of neuroblastomas and positivity for S-100 protein was found.

Amongst neuroblastomas, intermediate or high mitosis-karyorrhexis index (MKI) ($\geq 100/5{,}000$ cells) (Shimada grades 2+4+5), as opposed to low MKI ($<100/5{,}000$ cells) (Shimada grades 3+6), was positively correlated with necrosis ($P=0.05$) and inversely with calcification ($P=0.05$). S phase fraction ≥ 0.2 was also inversely correlated with calcification ($P=0.02$), but not significantly correlated with necrosis or intermediate/high MKI. However, if ganglioneuroblastomas are included in the analysis, high S phase fraction (≥ 0.2) was also correlated with necrosis ($P=0.02$). The number of stem lines as assessed by flow cytometry was not correlated with necrosis or any other parameter of differentiation or proliferation. It is noticeable that 8 of 10 ganglioneuroblastomas had one or two additional stem lines (Tables 1, 2).

Only the tumour samples retrieved before treatment were included in the study of prognostic factors (26 patients) (Table 1) for reasons discussed below. Logistic regression analysis showed that neither age at diagnosis, number of tumour stem lines, nor DNA index intervals were significant for survival (Table 3).

Clinical stage was a highly significant prognostic factor with more than 90% long-term survival for the patients in stages I, II and IV-S compared with less than 10% long-term survival for patients in stages III–IV.

All 4 patients with ganglioneuroblastoma (nos. 3, 4, 10 and 12) survived in contrast to 8 of 22 patients (36%) with neuroblastoma. However, one of the neuroblastoma patients (patient 5) died by homicide free of disease, and is recorded in the statistical analysis as being alive. The grading of neuroblastoma into undifferentiated (Shimada grades 2+3) and differentiating (Shimada grades 5+6) histology did not yield prognostic information (Table 1). The immunohistochemical markers of differentiation, positivity for S-100 protein and strong (+++) positivity for NSE, were both significantly associated with a better prognosis (Table 3). The histological markers of high proliferative activity (i.e. + necrosis, – calcification, intermediate MKI) were all non-significantly associated with a worse prognosis (Table 1). The survival was significantly better for the patients with

favourable histology according to the Shimada classification (Shimada et al. 1984) (Table 3).

DNA ploidy was not a significant prognostic factor in our study; however, it is notable that of 4 patients with a triploid or near triploid stem line (DNA indices 1.4–1.6), 3 are long-term survivors (nos. 1, 3 and 10) and 1 died free of disease (patient 5); the tumour samples had, however, additional stem lines. In contrast. high proliferative activity, as reflected in an S phase fraction ≥ 0.2 is a highly significant prognostic factor (Table 3).

Multivariate analysis of the same data showed that number of stem lines, DNA index and age at diagnosis were not significant in any statistical model. S phase fraction was a stronger prognostic indicator ($P=0.004$) than Shimada classification and the immunohistochemical markers of differentiation, NSE and S-100 protein, which were not significant when combined with S phase fraction. S phase fraction and clinical stage could not be included in the same model due to the high correlation between them. However, similar results were obtained when stage replaced S phase fraction in the model.

Discussion

Histological evidence is the pivotal criterion for the diagnosis of neuroblastoma. The tumour may show the full range of neuronal differentiation from an undifferentiated small, round, blue-cell neoplasm to a tumour that has full ganglionic differentiation. Various grading systems have been used to evaluate histological differentiation at the light microscopic level, either by quantitative and semi-quantitative methods based on the proportion of various differentiated elements (Beckwith and Martin 1968; Gitlow et al. 1973; Molenaar et al. 1990; Sandstedt et al. 1983; Shimada et al. 1984) or by qualitative systems based on the presence or absence of any such elements (Carlei et al. 1984; Horn et al. 1956; Hughes et al. 1974; Mäkinen 1972; Tsuda et al. 1987). We have used the grading system of Shimada et al. (1984), and the correlation we found between histological degree of maturation and the patterns of positivity for the neural markers, NSE and S-100 protein, is in accordance with other studies (Aoyama et al. 1990; Carlei et al. 1984; Shimada et al. 1985). NSE is found in differentiated neuroblasts and, in particular, mature ganglion cells (Carlei et al. 1984). S-100 protein is found in differentiated neuroblasts (Carlei et al. 1984), in Schwann cell precursors and mature Schwann cells, but not in mature ganglion cells (Aoyama et al. 1990; Shimada et al. 1985). A very high level of differentiation is indicated by the presence of GFAP-positive Schwann cells (Molenaar et al. 1990); however, only 1 of 9 ganglioneuroblastomas was positively stained in our study. Several authors have also tried to correlate the histological picture with prognosis, and our study supports the view that histological features associated with differentiation and proliferative activity may yield prognostic information.

It is, however, obvious from the data in Table 2 that only tumour samples retrieved before adjuvant treatment can yield prognostic information. Six of 10 responding tumours had favourable histology compared with only 1 of 6 relapsing or progressing tumours (Anderson et al. 1985). Even in responding tumours, the Shimada classification did not yield prognostic information (survival favourable histology 3/6 versus survival unfavourable histology 2/4) (Table 2) (Biedler et al. 1988). Furthermore, in 7 cases, samples were retrieved from the primary tumour in the same patient both before and after adjuvant treatment. In all 4 responders (patients 13–15, 18), the tumour showed morphological maturation after treatment. However, in only 1 of 3 relapsing tumours (patients 8, 17, 20), the histological picture was slightly more "mature" than before treatment (Tables 1, 2). Most likely this "maturational" effect of treatment simply represents cytoreduction of the most undifferentiated cells in responding tumours (Biedler et al. 1988; Carlsen 1991a). It has been suggested that the degree of tumour necrosis following preoperative chemotherapy may be of prognostic value. Our study points out the association between necrosis and high proliferative activity, which may hamper the interpretation of necrosis in tumour specimens obtained at "delayed surgery" (Oppedal et al. 1988). Some authors have suggested that flow cytometric analysis of tumour specimens obtained after adjuvant treatment may be used in analysis of prognostic factors, because the DNA histograms appear stable (Oppedal et al. 1988; Taylor and Locker 1990; Taylor et al. 1988). We agree that the DNA histograms in the 7 pairs of tumour tissue retrieved both before and after adjuvant chemotherapy are similar; however, in 1 of these cases, an additional stem line was found after treatment. Furthermore, for stage I–II tumours only pretreatment samples will normally exist, because the survival rate is more than 80% with surgical treatment only (Matthay et al. 1989; Nitschke et al. 1988), whereas for stages III–IV pretreatment samples of primary tumour will be rather uncommon and post-treatment samples will be rather frequent. Thus, bias due to this fact is to be expected, and we cannot recommend that flow cytometric data from post-treatment samples be used for prognostic analysis until more data are available. Therefore, only the 26 patients whose tumour samples were obtained before treatment are included in the study of prognostic factors.

The prognostic significance of the histological parameters in our study was in accordance with the classical distinction between ganglioneuroblastoma and neuroblastoma, with the former having the better prognosis. However, the histological grading of neuroblastomas into undifferentiated or differentiating histology did not yield prognostic information in our study (Carlsen 1991b). Necrosis, lack of calcification and higher MKI showed a trend toward a poor prognosis (Sandstedt et al. 1983), but the association was not significant. The survival was significantly better for patients with favourable histology compared to the patients with unfavourable histology according to Shimada et al. (1984); however, the prognostic factor age is included in the classification (Carlsen 1991b; Oppedal et al. 1988). A significant association with a better prognosis was obtained

by strong positivity for NSE and/or positivity for S-100 protein (Aoyama et al. 1990; Biedler et al. 1988; Shimada et al. 1985). However, neither Shimada classification, nor the immunohistochemical marker pattern were independent prognostic indicators in this study in accordance with other studies (Haase et al. 1989; Nitschke et al. 1988; Oppedal et al. 1988).

The extent of the disease (stage) is the most significant independent prognostic variable in neuroblastomas, and the importance of stage was documented again in our study. Age at diagnosis is the next most significant variable in most studies (Breslow and McCann 1971; Carlsen et al. 1986; Coldman et al. 1980; Evans et al. 1987, 1990; Oppedal et al. 1988; Thomas et al. 1984; Wilson and Draper 1974). However, a previous analysis was unable to define age as an independent prognostic factor for our patient population (Carlsen et al. 1981).

In contrast to the majority of DNA flow cytometric studies of neuroblastomas (Bourhis et al. 1991; Brenner et al. 1989; Gansler et al. 1986; Look et al. 1984, 1991; Oppedal et al. 1988; Taylor and Locker 1990; Taylor et al. 1988), this study was unable to define DNA ploidy as a prognostic factor. Neither the number of stem lines nor their DNA indices had prognostic significance (Cohn et al. 1990; Slavc et al. 1990; Suzuki et al. 1989). In the studies of Look et al. (1984, 1991), the analysis of the impact of ploidy on prognosis was based on the DNA index of lowest ploidy stem line for patients with multiple tumour stem lines, i.e. sometimes a diploid stem line. In most other studies, aneuploidy was defined as the presence of more than one G0/G1 peak. Most investigators conclude that DNA aneuploidy is more favourable than diploidy, at least for infants and young children (Cohn et al. 1990; Look et al. 1984, 1991; Oppedal et al. 1988). Cytogenetic analyses of screen-detected tumours and other early stage tumours with favourable prognosis (stages I, II, IV-S) have often shown near-triploid modal chromosome numbers without structural abnormalities (Carlsen et al. 1991; Hayashi et al. 1989; Kaneko et al. 1987; Look et al. 1991), and some DNA aneuploid tumours in infants and children younger than 2 years of age may have a near-triploid karyotype (Carlsen et al. 1991; Look et al. 1991). This suggestion has led some authors to define a triploid DNA content as DNA indices between 1.32 and 1.69 (Taylor and Locker 1990; Taylor et al. 1988), 1.33–1.74 (Oppedal et al. 1988), 1.25–1.68 (Bourhis et al. 1991), or 1.21–1.79 (Cohn et al. 1990) in their analysis. Nevertheless, the favourable impact of DNA aneuploidy on prognosis is in contradiction to the general concept of DNA aneuploidy as an unfavourable prognostic indicator in solid tumours. Due to the limits in resolution and calibration of flow cytometric histograms when derived from paraffin-embedded material without known internal reference standards, neuroblastomas classified as DNA diploid according to flow cytometry might very well be classified as genetically abnormal according to more sensitive methods. Abnormalities of chromosome 1p and the cytogenetic signs of N-*myc* amplification, double minutes or homogeneously staining regions, which are both associated with a very poor prognosis (Brodeur and Fong 1989; Carlsen et al. 1991; Hayashi et al. 1989; Kaneko et al. 1987; Look et al. 1991), involve insufficient amounts of DNA to alter the flow cytometric DNA index (Cohn et al. 1990; Look et al. 1991). Both abnormalities are more frequent in diploid than in hyperdiploid tumours (Bourhis et al. 1991; Brodeur and Fong 1989; Carlsen et al. 1991; Cohn et al. 1990; Hayashi et al. 1989; Look et al. 1991; Taylor and Locker 1990).

The proportion of neuroblastomas that was classified as DNA aneuploid in this study (88%) seems relatively high in comparison with other studies. This might be due to a more radical procedure for discriminating stem lines by histogram deconvolution. Tissue autolysis is known to cause false DNA aneuploid peaks (Alanen et al. 1989), but we did not find any correlation between necrosis and number of stem lines. Unfortunately the methods used for analysis of the flow cytometric data, that is to say, methods for discrimination and classification of stem lines and estimation of their DNA indices and S phase fractions, are not always clearly stated by the authors, and comparison of the results of different investigations may be difficult. Furthermore, post-treatment samples are included in several of the analyses (Bourhis et al. 1991; Oppedal et al. 1988; Taylor and Locker 1990; Taylor et al. 1988).

We found that S phase fraction is an independent prognostic indicator, high S phase fraction being unfavourable. The prognostic importance of S phase fraction, estimated by selection of the value of the highest S phase fraction of any stem line present in the particular tumour, has not, to our knowledge, been confirmed by other investigators. Most studies did not estimate the cell cycle fractions (Brenner et al. 1989; Look et al. 1991; Oppedal et al. 1988; Taylor and Locker 1990; Taylor et al. 1988). However, Look et al. (1984) found that S phase fraction was significantly higher for unresectable diploid tumours than for unresectable hyperdiploid tumours and speculated that the link between DNA diploid cases and poor prognosis could reside in the cell proliferative rate. Gansler et al. (1986) found that favourable outcome was associated with a DNA aneuploid stem line and a low percentage of cells in S+G2+M phases of the cell cycle, and that these features were associated with early stage and favourable histological pattern. Bourhis et al. (1991) estimated only the fraction of cells in S phase for diploid tumours; for these tumours high S phase fraction ($\geq 10\%$), N-*myc* amplification and poor prognosis were significantly correlated. Slavc et al. (1990) found that DNA index did not correlate with N-*myc* amplification or prognosis, whereas the fraction of cells in S+G2+M phases did appear to correlate with disease progression. Cohn et al. (1990) found that high S phase fraction ($> 10\%$), N-*myc* amplification and unfavourable Shimada classification were significantly associated with progressive disease. Suzuki et al. (1989) found that the survival in multivariate analysis was correlated with stage, fraction of cells in S+G2+M phases, tumour site and age of the patient. They found no correlation between prognosis and the DNA ploidy.

Thus, most studies where cell cycle fractions are estimated are in accordance with our finding of S phase fraction being an important prognostic indicator.

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