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Prognostic Significance of the Proliferative Activity in Neuroblastoma

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The prognostic significance of the immunohistochemically assessed growth fraction in neuroblastomas was determined in relation to tumor grade and tumor stage. A total of 101 cases of neuroblastoma were examined with the monoclonal antibodies PC10 against proliferating cell nuclear antigen (PCNA) and Ki-S5 against the Ki-67 protein. Patients were followed for a mean time of 4.8 years. Expression of both PC10 and Ki-S5 was found to be significantly linked to tumor grade and tumor stage. Prognostically favorable stage IVs was associated with low PCNA and Ki-S5 levels. For ganglioneuroblastoma, significant differences were found between the diffuse and the composite type. In univariate analysis of stage III and IV tumors, Ki-S5 and PCNA scores were significantly correlated with disease-free survival (P < 0.0015), allowing definition of a subset of cases with favorable outcome. As to Shimada's group with poor prognosis, significant differences in the clinical course were found for low and high Ki-S5 scores (P = 0.036) but not for PCNA. In multivariate analysis, only patient age, Shimada's grade, and Ki-S5 scores achieved prognostic significance. We conclude that proliferation marker Ki-S5 may provide substantial prognostic information and might become a useful adjunct for predicting the clinical courses of neuroblastoma. (Am J Pathol 1997, 150:133–145)

Pronounced biological heterogeneity, resulting in notoriously unpredictable clinical developments, is a particularly intriguing aspect of neuroblastoma. Besides rapid fatal spread of the disease, the vagaries of its biological behavior encompass spontaneous involution and terminal differentiation to ganglioneuroma. As current therapeutic strategies are essentially dependent on the predicted tumor biology, there is a strong craving for reliable prognostic indicators.[1] Among these, Evans' stage,[2] the patient's age at the time of diagnosis,[2–4] and the histopathological tumor grade have been thoroughly investigated and gained general acceptance. At least four different grading systems have been proposed,[5–8] each of which has its strengths and limitations; nonetheless, they share a common denominator, ie, the focus on histological signs of differentiation. Indeed, in contrast to most malignancies of adulthood and adolescence, the hallmark of which is an escalating dedifferentiation of the tumor cells, neuroblastoma retains the ability of maturation, which appears to be age linked and is considered to be decisive of the clinical course.[1,6,9,10] Accordingly, in addition to histological features of neuronal differentiation[9,11,12] and the presence of Schwann cells and a stromal component,[13,14] phenotypic markers such as S-100 protein,[15] synaptophysin, neuron-specific enolase, protein gene product 9.5, different chromogranins, neurofilaments, vasoactive intestinal peptide, and Leu7 antigen are likely to predict a favorable course,[10,16] whereas high levels of ferritin seem to indicate a more aggressive biological behavior.[15,17] Maturation of neuroblastoma cells is likely to be at least partly dependent on the expression of the high-affinity nerve growth factor receptor ($p140^{trk}$/TRKA),[18] and correspondingly, high *TRKA* expression is associated with a better prognosis.[19] DNA analysis, although providing no diagnostic information, has been reported to improve the prognostic accuracy of the aforementioned parameters, triploidy or aneuploidy being associated with a lower percentage of cells in S through M phase and a more favorable clinical outcome.[19–21]

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Molecular cytogenetics has revealed unique features of neuroblastoma. Paired extrachromosomal punctual chromatin bodies, so-called double minutes, although being a frequent feature of neoplastic cells, were first observed in neuroblastomas.[22,23] It is assumed that they can integrate into chromosomes to form homogeneously staining regions, which in turn may break down to form double minutes.[24] In neuroblastoma, these homogeneously staining regions represent sites of *MYCN* amplification.[25–27] Although high levels of its protein product are regularly and specifically found in immature neural cells,[28] amplification of the *MYCN* oncogene has emerged as one of the most significant predictors of poor prognosis.[19,29–31] By dimerizing with max, myc-N acquires DNA-binding capacity and might be involved in the transactivation of proliferation-related or tumor-promoting genes.[32,33]

On the other hand, allelic losses, partial deletions, or unbalanced translocations involving the region of chromosome 1 distal to p32 are found in a large number of cases and are considered to be indicative of adverse prognosis.[14,30,34–36] Interestingly, in contrast to many other types of malignancy,[37] this trait of genetic instability appears not to be associated with an impaired p53 function.[38–40] Rather, evidence is accumulating that the 1p36 locus itself may harbor one or more yet unidentified tumor suppressor genes.[36,41] As *MYCN* amplification is invariably associated with chromosome 1p alterations and often preceded by these, it seems likely that at least one of the 1p36 gene products is involved in the regulation of the *MYCN* gene.[41]

However, despite the promising disclosures of genetics, a number of clinically unfavorable tumors escape detection by these methods.[19] This is why we have addressed the hitherto rather neglected issue of proliferation by immunohistochemical methods using monoclonal antibodies to two different allegedly proliferation-related antigens. One, PC10,[42] recognizes proliferating cell nuclear antigen (PCNA), an auxiliary compound of DNA polymerase-δ.[43,44] PCNA was the first proliferation marker detectable in paraffin-embedded specimens; however, the significance of its distribution in fixed material remains debatable.[45] Recently, we have generated the monoclonal antibody Ki-S5[46,47] directed against a formalin-resistant epitope of the Ki-67 antigen,[48,49]the expression of which is considered to define the growth fraction in cell populations and tissues.[50] By means of this antibody, we will show that the proliferative activity correlates with tumor grade and tumor stage and that it provides a novel prognostic indicator offering the advantage of an easy assessability and a good reproducibility in morphologically unaltered material.

Table 1. *Tumor and Patient Characteristics*

Characteristics	Number of cases	Treated patients
Hughes' grade		
1d	23	10 (43.5%)
1c	8	3 (37.5%)
2	37	6 (16.2%)
3	34	4 (11.8%)
Evans' stage		
I	15	0 (0%)
II	12	0 (0%)
III	37	13 (35.1%)
IV	30	10 (33.3%)
IVs	7	0 (0%)
Total	101	23 (22.7%)

Quantitative distribution of the cases is presented in relation to histopathological grade and clinical stage with the corresponding fraction of patients having received previous chemotherapy.

Materials and Methods

Neuroblastoma Samples

A total of 101 cases of neuroblastoma from the archival material of the Kiel Pediatric Tumor Registry were available for this study. The patients' age at diagnosis ranged from <1 to 191 months (median, 30.6). Clinical staging was performed accordingly to Evans' criteria; stage I and II were grouped and accounted for 27 cases, stages III and IV for 37 and 30 cases, respectively, and 7 patients were staged IVs. A total of 23 patients (22.7%) had received cytotoxic therapy before biopsy.

Two different histopathological grading systems were applied for classification. According to Hughes' system, there were 31 grade 1, 37 grade 2, and 33 grade 3 tumors. By Shimada's grading, 45 cases with good prognosis and 33 with poor prognosis were distinguished after exclusion of the pretreated patients. All cases were re-evaluated for diagnosis and classification by three independent pathologists; additionally, grade 1 tumors (ganglioneuroblastomas, GNBLs) were further subclassified into 8 cases of the composite (1c) and 23 cases of the diffuse (1d) type, as suggested by Stout[51] and subsequently established by our group.[52] This distinction is based on differences in growth patterns: diffuse GNBL is characterized by a scatter of neuroblasts and ganglion cells through a fibrous stroma, whereas composite GNBL is defined as a mature ganglioneuroma containing nodular aggregates of undifferentiated neuroblasts. A survey of the grading and staging data is presented in Table 1.

The patients were followed up to 11.3 years (mean, 4.8 years; median, 4.9 years). The clinical records were reviewed for data concerning relapse-free intervals and overall survival by the German Neuroblastoma Study Group, F. Berthold, Cologne.

Immunohistochemistry

Sections (3 to 5 μm) from formalin-fixed, paraffin-embedded material were cut, mounted on 3-aminopropyl-triethoxysilane-coated slides and air dried overnight at 37°C. Subsequently, they were dewaxed in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersion of the sections in 1 to 3% (v/v) hydrogen peroxide in methanol/phosphate-buffered saline for 10 minutes. As described elsewhere,[53] primary antibody PC10 (Dako Immunochemicals, Hamburg, Germany) was incubated overnight at a dilution of 1:25. Ki-S5 immunostaining was performed as previously described.[47] Briefly, the sections were immersed in 0.1 mol/L citrate buffer, pH 6.0, and heated in a microwave oven (Toshiba) in two successive steps (15 minutes at 750 W and 10 minutes at 250 W) for antigen retrieval.[54] After washing in phosphate-buffered saline, lyophilized cell culture supernatant of the antibody Ki-S5, diluted 1:20, was incubated on the slides for 30 minutes. The immunoreactions were enhanced with the streptavidin-biotin complex, and the sections were briefly counterstained with Mayer's hematoxylin.

Evaluation of Immunoreactive Scores

Nuclear staining by PC10 and Ki-S5 was assessed by two independent observers using a standard light microscope (Axioplan, Zeiss, Jena, Germany). The slides were scanned at low magnification to determine the tissue areas that were most evenly and abundantly labeled. The immunoreactive scores were then evaluated quantitatively by counting a minimum of 1000 tumor cells at high power (×300). Comparison of the results showed an inter-observer agreement of at least 95%. PCNA and Ki-S5 indices are expressed as a percentage of the counted tumor cells (determined to the nearest percent).

Statistics

Statistical analysis was performed using the dbase version IV and BMDP 6.0 software on a PC 486/33-MHz computer to calculate mean values, ranges, and standard deviations of the immunoreactive scores. Correlations were determined using the Cox proportional hazards model, and statistical significance was verified by the Mann-Whitney and Kruskal-Wallis tests. Cumulative survival was calculated by Kaplan-Meier analysis on the basis of a cut-off value of 25% positive cells. Cox analysis was further used to determine significance levels for independent prognostic factors in a multivariate model including patient age at diagnosis, Hughes and Shimada grade, clinical stage, and Ki-S5 and PCNA immunoreactive scores.

Results

Immunoreactive Scores

The reaction was nuclear with both antibodies; all cells exhibiting an unequivocal nuclear labeling were scored as positive. In positive cells, PCNA yielded a homogeneous staining of the nucleoplasm; occasional diffuse cytoplasmic reactivity with the antibody PC10 was regarded as a nonspecific background reaction and neglected. Ki-S5 immunostaining of cycling cells was characterized by a uniformly strong labeling of interphase chromatin, mitotic chromosomes, and nucleoli. Thus, atypical mitoses could readily be distinguished from karyorrhectic figures (Figure 1a). Poorly differentiated tumors displayed an even distribution of intensely labeled nuclei in the majority of the cells (Figure 1b); the staining pattern was diffuse or patchy in more mature (grade 2) tumors (Figure 1c) and regional in GNBLs of the composite type (Figure 1d), whereas in diffuse GNBLs, scattered cells identified as primitive neuroblasts were highlighted by Ki-S5 immunostaining (not shown). The results are summarized in Tables 2 and 3.

Overall immunoreactivity of both antibodies ranged from 0 to 80%, with mean values of 22.1 ± 20.4% for Ki-S5 and 17.0 ± 18.2% for PCNA. A scatterplot diagram (Figure 2) shows a linear correlation (r = 0.768) of PCNA and Ki-S5 scores. This must nevertheless be regarded with caution, as in 26 cases (25.7%) the differences between the respective labeling scores exceeded a ratio of 2 to 1.

With both antibodies, no significant differences between the immunoreactivity scores were found in stage I to III tumors. When these were grouped and compared with stages IV and IVs, however, the values for both antibodies achieved statistical significance (P = 0.0366 for PCNA and P = 0.0064 for Ki-S5 in the total number of cases; P = 0.0043 for PCNA and P = 0.0009 for Ki-S5 when only cases without previous therapy were considered). Interestingly, stage IVs presented with considerably lower PCNA scores and proliferation rates than both stage III and stage IV (Table 2).

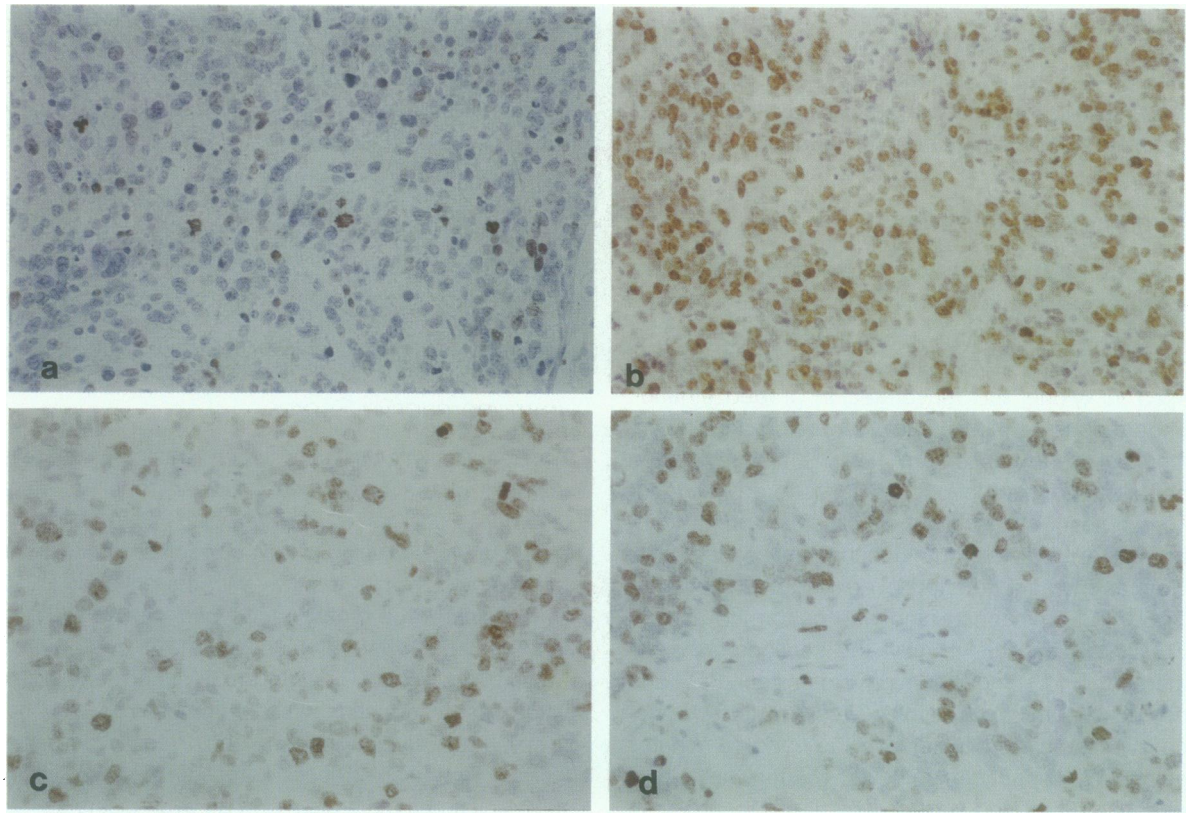

Figure 1. a: *Neuroblastoma with high mitosis-karyorrhexis index. Note the similarity of the mitotic (brown labeling) and apoptotic or karyorrhectic figures (blue staining).* **b:** *Poorly differentiated (Hughes' grade 3) neuroblastoma. At least 80% of the tumor cells exhibit a strong nuclear reaction with Ki-S5.* **c:** *Nuclear Ki-S5 positivity is typically diffuse or focally concentrated in Hughes' grade 2 neuroblastomas.* **d:** *GNBL of the composite type; areas of dense immunolabeling* (**top**) *alternate with sparsely labeled regions. Antibody Ki-S5; streptavidin-biotin technique; hematoxylin counterstain; magnification, ×350.*

A highly significant correlation was found between the Hughes histopathological tumor grade and Ki-S5 as well as PCNA expression levels (considering the totality of cases, $P < 0.00001$ for Ki-S5 and $P = 0.0115$ for PCNA). When pretreated patients were excluded, only Ki-S5 scores amounted to statistical significance ($P = 0.0097$), whereas PCNA values reflected a mere trend ($P = 0.085$). Moreover, a clear-cut separation between G1d and G1c tumors was seen with both antibodies ($P = 0.027$ for Ki-S5 and $P = 0.013$ for PCNA), the immunoreactive scores of grade 1c equaling or even outranging those of grade 2. When stratified on Shimada's grading, both the growth fraction (Ki-S5, $P < 0.00001$) and PCNA expression levels ($P = 0.0003$) showed significant differences (Tables 3 and 4). Comparison of medians by the overall median test yielded nearly equivalent results.

Survival Analysis

Forty-five patients (44.6%) suffered a relapse during the observation time, and eight of these (17.8%) died

Table 2. *Ki-S5 and PCNA Scores Stratified on Evans' Stage*

Stage	All cases		Untreated patients	
	Ki-S5	PCNA	Ki-S5	PCNA
I	18.0 ± 22.3 (9.5)	8.9 ± 14.7 (5.0)	18.0 ± 22.3 (9.5)	8.9 ± 14.7 (5.0)
II	19.5 ± 20.0 (13.5)	11.6 ± 11.7 (6.0)	19.5 ± 20.0 (13.5)	11.6 ± 11.7 (6.0)
III	19.1 ± 19.5 (15.0)	15.2 ± 15.4 (10.0)	22.4 ± 16.8 (20.0)	16.9 ± 16.8 (14.5)
I + II + III	18.9 ± 19.9 (11.0)	12.9 ± 14.6 (7.0)	20.4 ± 19.0 (15.5)	13.0 ± 15.1 (6.0)
IV	31.4 ± 20.8 (29.5)	25.7 ± 21.9 (22.5)	36.5 ± 19.5 (30.0)	30.6 ± 22.3 (29.0)
IVs	10.8 ± 7.8 (10.0)	16.0 ± 22.3 (9.5)	10.8 ± 7.8 (10.0)	16.0 ± 22.3 (9.5)

Results are presented as mean values and standard deviations (median in parentheses). The values for the collective without cytostatic treatment before biopsy were calculated separately.

Table 3. *Mean Ki-S5 and PCNA Scores with Standard Deviations Stratified on Hughes' Grading*

Grade	All cases		Untreated patients	
	Ki-S5	PCNA	Ki-S5	PCNA
1	12.2 ± 17.1 (3.0)	10.2 ± 14.7 (5.0)	15.3 ± 19.4 (6.5)	12.0 ± 17.9 (3.0)
2	20.9 ± 18.9 (12.0)	18.7 ± 15.7 (19.0)	22.5 ± 19.5 (15.5)	18.7 ± 16.4 (16.5)
3	32.3 ± 20.7 (29.5)	20.7 ± 22.1 (14.0)	29.8 ± 19.4 (24.5)	20.5 ± 22.2 (14.0)
1d	9.2 ± 16.6 (2.0)	6.3 ± 10.6 (3.0)	11.6 ± 20.4 (3.5)	7.1 ± 14.1 (2.5)
1c	21.4 ± 16.5 (26.5)	24.6 ± 19.5 (11.0)	26.6 ± 12.5 (26.5)	31.7 ± 20.2 (37.0)

Median values are in parentheses. A separate calculation was done for the patients not having received cytostatic treatment before biopsy.

of the disease. Before Kaplan-Meier analysis, the influence of the variables PCNA and Ki-S5 was estimated using Cox's multiple regression model, and a cut-off level of 25% labeled cells was selected for the subsequent calculations to optimize statistical significance. In univariate analysis of the totality of cases, the most significant predictors of cumulative disease-free survival were the Ki-S5 labeling index ($P < 0.00001$) and Shimada's grade ($P = 0.00001$) followed, in that order, by the PCNA labeling index ($P = 0.00002$), the patient age at diagnosis ($P = 0.00013$), tumor stage (stages I, II, and IVs *versus* stages III and IV; $P = 0.0002$), and the Hughes grade ($P = 0.0156$). Ki-S5 and PCNA scores also remained significant with respect to the clinical outcome when pretreated patients were excluded ($P < 0.00001$ and $P = 0.00016$, respectively).

As the high prognostic significance of Ki-S5 and PCNA scores was likely to depend on the substantial percentage of cases with other favorable prognostic factors, such as low stage (I, II,or IVs, 33.7%), age 1 year or less (39.6%), or good prognosis according to Shimada's grading (60%), the cases were broken down by these parameters for further analysis. In patients aged >1 year, both Ki-S5 and PCNA labeling indices were significantly linked to the clinical outcome ($P = 0.00009$ and 0.00053, respectively),

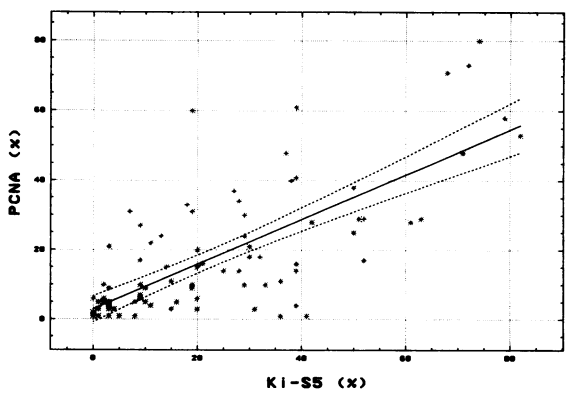

Figure 2. *Scatterplot diagram illustrating the distribution of Ki-S5 and PCNA labeling indices in the total number of cases. Although there is a linear correlation, individual values vary widely.*

whereas in the younger age group, the Ki-S5 score alone achieved statistical significance ($P = 0.0138$). When the survival estimates were calculated with respect to Evans' stage, analysis of stages I, II, and IVs yielded little consistent information, as 90% of the patients survived without assessable recurrence during a mean observation time of 6.1 years (median, 6.12). Nevertheless, these groups taken together, all cases with disease progression, exhibited Ki-S5 scores above 25%, whereas the disease-free survivors consistently had lower proliferation rates ($P = 0.0005$). By contrast, PCNA values were not correlated to the outcome in these stages ($P = 0.325$). In stage III and IV patients (excluding IVs), low immunoreactivity levels (<25%) of Ki-S5 and PCNA were significantly associated with improved patient survival ($P = 0.00031$ for Ki-S5 and $P = 0.00048$ for PCNA; Figure 3, a and b). When these cases were broken down by age (≤1 *versus* >1 year), Ki-S5 labeling indices were prognostically relevant in both groups ($P = 0.038$ and 0.0018, respectively), whereas PCNA values achieved statistical significance only in patients aged over 1 year at diagnosis ($P = 0.0021$). Exclusion of the pretreated patients did not substantially alter the significance levels. However, when only patients with poor prognosis (according to Shimada's grading system) were examined, Ki-S5 scores alone were predictive of the clinical outcome ($P = 0.0301$), whereas PCNA was not significant ($P = 0.27$; Figure 4, a and b).

In multivariate analysis including patient age at diagnosis, Hughes' grade, clinical stage, Shima-

Table 4. *Mean Values and Standard Deviations of Ki-S5 and PCNA Labeling Indices Stratified on Shimada's Grade*

Shimada's grade	Ki-S5	PCNA
Good prognosis	15.2 ± 15.2 (10.0)	11.6 ± 15.2 (5.0)
Poor prognosis	37.2 ± 19.4 (32.0)	27.5 ± 21.6 (24.0)

Median values are shown in parentheses.

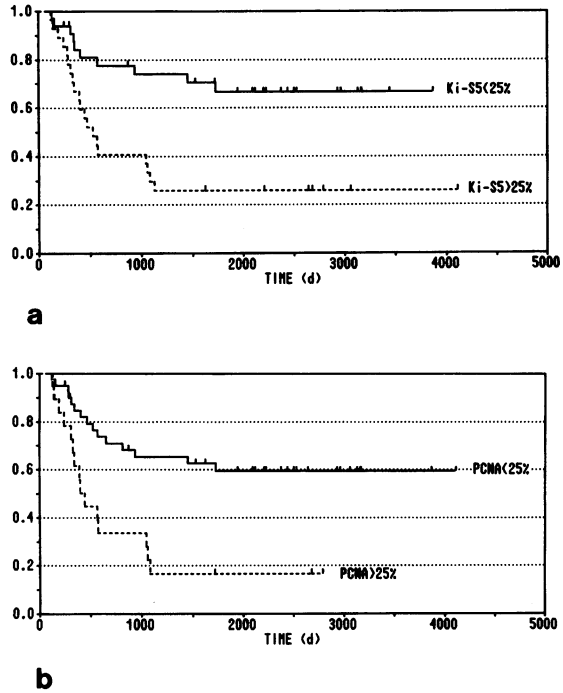

Figure 3. a: *Kaplan-Meier analysis of the cumulative disease-free survival in stage III and IV tumors. Survival is significantly improved in patients with a Ki-S5 labeling index <25%.* b: *Analysis of PCNA values below and above 25% yields similar results.*

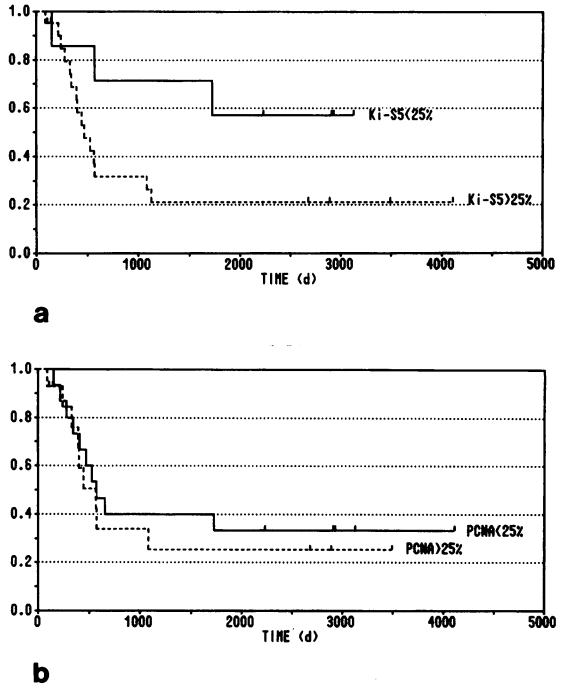

Figure 4. a: *Kaplan-Meier analysis of the cumulative disease-free survival in the patient collective with poor prognosis by Shimada's grading. Ki-S5 levels below 25% are significantly associated with a better outcome.* b: *Corresponding PCNA values show no significant correlation with the clinical course.*

da's classification, Ki-S5, and PCNA, age >1 year emerged as the most significant predictor of unfavorable outcome ($P < 0.00001$), followed by a Ki-S5 labeling index >25% ($P = 0.003$) and Shimada's grade ($P = 0.0192$). Hughes' grade, clinical stage, and PCNA scores were not significant. When patient age was disregarded, Shimada's grade achieved the highest prognostic value ($P = 0.0029$), followed by the Ki-S5 score ($P = 0.0114$), whereas the other covariates did not amount to significance. Conversely, when Shimada's grade was removed from the model, the only relevant predictors of poor prognosis were high Ki-S5 scores ($P < 0.00001$) and age over 1 year ($P = 0.0011$).

Discussion

For clinicopathological use, methods designed to provide information pertaining to the prognosis of neoplastic diseases need to be easily performable, rapidly assessable, and well reproducible.[55] Considering the wide variety of tumors and the diversity of their biological behavior, there is no universal marker fulfilling these conditions. Rather, individual prognostic parameters have to be elaborated for each type of malignancy and cautiously validated by correlation with the clinical data.

For neuroblastoma, the capital importance of the clinical stage has been amply documented.[1–4] Next to it, the histopathological tumor grade has proved to be a reliable indicator of prognosis.[4,7,8,11] It may be profitably supplemented by phenotypic markers of maturation,[10,15] DNA measurements,[19–21,56] or nucleolar organizer regions counting.[57,58] Aneuploid tumors appear to carry a better prognosis than those with a diploid or tetraploid DNA content.[19,20,56] Also, aneuploidy was found associated with a lower fraction of cells in S through M phase,[20] and Huddart et al[21] documented a significantly improved 10-year survival in association with a low S phase fraction as determined by flow cytometry. A decreased number of nucleolar organizing regions with a concomitant increase in size was found associated with lower histopathological grades, high nerve growth factor receptor (TRKA) expression and a better 5-year survival;[57] also, in stage IVs patients, lymph node and distant metastases exhibited reduced nucleolar organizing region counts as compared with those in stage IV.[58] When correlated to survival, however, these data did not amount to statistical significance. Molecular cytogenetics is expected to yield more precise information but requires laborious tech-

niques and expensive equipment. Yet, even taken together, these methods fail to identify a certain number of cases with unexpectedly unfavorable clinical outcome.[19] Therefore, additional prognostic information is highly desirable.

It is now common wisdom that the intrinsic growth potential of a malignant disease accounts largely for its biological behavior. This is underscored by the constitutive role assigned to histological features of proliferation by two more recent grading systems for neuroblastoma.[6,8,11,59] The traditional method for morphological assessment of cell growth, the mitotic count, may be skewed by several systematic errors. These include technical variations, such as thickness of the section and heterogeneity of the tumor sample as well as the size of the tissue specimen and the time of fixation, which may influence the preservation of mitotic figures.[60] Moreover, the mitotic count being defined as the number of mitotic figures per 10 high-power fields, up to sevenfold variations may result from the size of the microscope objective.[61] Such deviations may be largely circumvented by application of the mitotic index (number of mitotic figures per total number of cells) instead of the mitotic count.[62] Also, scrupulous observation of protocol guidelines as set up by the Multicenter Mammary Carcinoma Project[63] may considerably improve the reproducibility of mitotic counts. However, particularly in neuroblastomas, confusion may arise due to the morphological similarity of mitotic, apoptotic, and karyorrhectic figures, as pointed out by Shimada et al.[59]

Whereas mitosis occupies only a very short period in the cell cycle, immunostaining with specific proliferation markers is considered to identify the totality of actually cycling cells. Some 10 years ago, Gerdes et al[48] described an antigen defined by the antibody Ki-67, which is exclusively expressed in the nuclei of cells that are in a proliferative state. However, retrospective studies using this antibody were considerably hampered by the fact that the epitope recognized by Ki-67 does not survive formalin fixation. This is why antibodies against PCNA[42] suitable for paraffin-embedded material were welcomed with great enthusiasm. In flow cytometric assays, the fraction expressing PCNA was found to overlap almost completely with the population of cells in S through M phase.[53] In malignant lymphomas, immunostaining with the antibody 19A2 displayed a Ki-67-like pattern.[64] By comparison of PCNA staining with BrdU uptake, however, Coltrera and Gown demonstrated substantial qualitative and quantitative differences between different cell lines up to mutual exclusion of the labeled cell fractions and thereby concluded that PCNA expression might define particular subsets of cells in mixed populations.[65] In addition, Hall and colleagues observed aberrant PCNA expression in normal tissues adjacent to foci of neoplastic growth[53] and later were able to demonstrate dramatic increases of PCNA levels in response to ultraviolet-induced DNA damage.[66,67] Indeed, PCNA functions as an auxiliary compound of DNA polymerase-δ and appears to be involved in repair as well as in replication,[68] which may, in addition to hypothetical autocrine loops,[53] account for its occasionally incongruent tissue distribution pattern. Therefore, PCNA immunoreactivity must be interpreted cautiously, which explains the increasing use of antibodies to the more specific Ki-67 antigen suitable for paraffin-embedded material, such as MIB1[69] or Ki-S5. In our experience, these two antibodies yield equivalent immunoreactive scores when compared on the same specimen. However, depending on the antibody concentration, MIB1 staining occasionally displays a nonspecific cytoplasmic cross-reactivity with epithelial cells, which we never observed with Ki-S5.

Despite the above considerations, a comparison of the reactivity patterns of PCNA and Ki-S5 in neuroblastomas remained interesting. Let us recall that the fraction of PCNA-expressing cells is identical to the growth fraction recognized by Ki-67 in some tumors, whereas only a partial overlap is found in others. On the other hand, cellular proliferation fails to provide prognostic information in some kinds of neoplasia,[70] which again might be supplied by other parameters. Lastly, a presently well established prognostic factor for neuroblastoma, amplification of the *MYCN* oncogene, was reported to be associated with increased PCNA levels[71] and correlated to the proliferative activity as determined by histone H3 expression.[72] It appears indeed that, as the counterpart of c-*myc* in tissues of the neural crest, mycN may act as a transcription factor for S-phase genes.[32]

In the absence of standard criteria for evaluation,[55] we opted for the method performed by McGurrin and colleagues on mammary carcinomas.[73] We are aware of the concomitant interpretive bias, the areas for evaluation being selected on the basis of the most dense immunolabeling. This scoring method, however, influences only the absolute values of the cell counts, whereas their interrelation remains constant. Accordingly, Wintzer and associates[74] demonstrated that the prognostic relevance of Ki-67 labeling is not dependent on the evaluation method but rather on the adequate selection of cut-off levels. In this context, it has to be emphasized

that the cut-off levels determined in this study represent relative values corresponding to the scoring method. Moreover, the assessment of immunoreactivity scores may be affected by several factors, notably, the quality of immunostaining and the experience of an observer in selecting the microscopic areas or in discriminating between immunoreactive and negative nuclei.[75] A confident inter-institutional reproducibility of results might therefore be warranted only by the use of computer-assisted analysis of immunoreactive scores.[75]

We found a linear correlation between PCNA and Ki-S5 indices ($r = 0.768$), which is statistically relevant but nonetheless misleading. In fact, this apparent overlap only conveys the meaning that the PCNA- and Ki-S5-positive populations are numerically comparable but fails to reflect the marked discrepancies in a high percentage of individual cases. This observation would be consistent with the hypothesis that different subpopulations of cells are characterized by the expression of these two antigens. When the antigen expression was stratified on the histopathological tumor grade, however, similar results were obtained with both antibodies, higher scores being significantly associated with poor differentiation. Both antibodies also distinguished clearly between GNBLs of the diffuse and the composite type,[51,52] the latter displaying consistently higher immunoreactive scores. PCNA levels in composite GNBLs (31%) even markedly outranged those of G2 and G3 tumors (18 and 20%, respectively). The prognostic importance of this subclassification of Hughes' grade 1, which was suggested by Stout almost 50 years ago, is presently well documented.[12,15] Indeed, diffuse GNBL appears to be engaged in a process of terminal differentiation, whereas the nodular clusters of immature cells in composite GNBL retain their malignant potential, which results in a more aggressive biological behavior.[76] The striking difference between the immunoreactive scores is in line with these observations and additionally emphasizes the usefulness of this subclassification.

In stage I to III tumors, mean Ki-S5 immunoreactivity scores were clustered around 19%, whereas stage progression was associated with a gradual, yet not significant, increase of mean PCNA values from 10 to 20%. However, a comparison of these stages with stage IV and IVs yielded significant differences. It seems noteworthy that stage IVs, which is defined by an extensive metastatic spread with exception of bone involvement,[2] was associated with considerably lower PCNA and Ki-S5 levels than stage IV, and even stage III. Interestingly, stage IVs not only carries a better prognosis in general but is known for its propensity for spontaneous regression.[4] Thus, low Ki-S5 and PCNA scores appear to reflect a tendency toward maturation in addition to a more benign biopotential. In this context, it has to be mentioned that among tumors with a single *MYCN* gene copy, neoplasms belonging to stage IV and IVs were shown to display equivalent PCNA levels,[71] which implies that PCNA scores may lose prognostic significance when the cases are stratified on N-myc amplification. We had the opportunity to make a similar observation. From another study including patients of our collective, information on *MYCN* amplification was available in 87 of our cases (data kindly supplied by Dr. I. Leuschner, Department of Pediatric Pathology, Kiel). Tumors carrying 10 or more copies of the *MYCN* gene (21.8%) were significantly associated with disease progression ($P = 0.0061$) and higher proliferation rates (36.4 ± 24.4% *versus* 18.7 ± 16.9% in tumors without *MYCN* amplification, $P = 0.0034$) and, to a lesser extent, with PCNA scores (26.6 ± 20.6% *versus* 15.1 ± 16.9%, $P = 0.015$). When we compared stage IV and IVs tumors containing a single *MYCN* copy, however, both groups displayed equivalent PCNA scores (23.4 and 23.8%, respectively), whereas mean Ki-S5 values were markedly different (25.8% in stage IV, 16.2% in stage IVs). In tumors without *MYCN* amplification, both Ki-S5 and PCNA yielded significant prognostic information ($P = 0.0001$ and $P = 0.0034$, respectively), whereas only low Ki-S5 was significantly predictive of a better prognosis in the remaining tumors ($P = 0.047$).

In univariate analysis of the totality of our cases, Ki-S5 was found the most significant predictor of disease-free survival, followed by Shimada's grade, PCNA scores, patient age, and Evans' stage. To test the predictive value of Ki-S5 and PCNA in prognostically different subgroups, the cases were broken down by patient age, stage, and Shimada's grade. Ki-S5 yielded significant prognostic information independently of the patient age, whereas PCNA levels were significant only in children aged over 1 year. In stages III and IV, we found a statistically significant correlation between low immunoreactivity scores and a prolonged disease-free interval ($P < 0.0015$). Grouping of stages I, II, and III yielded comparable results ($P = 0.00004$ for Ki-S5; $P = 0.00379$ for PCNA). A similar difference in the prognostic relevance of Ki-S5 and PCNA was also found when stage I, II, and IVs tumors were examined as one group. Considering the small number of patients with disease progression, however, the corresponding statistical analysis has to be interpreted with great

caution. By contrast, in Shimada's poor prognosis group, only Ki-S5 scores were prognostically relevant ($P = 0.0301$), whereas the correlation with PCNA values did not achieve statistical significance ($P = 0.27$). In addition, we performed a multivariate analysis to assess the relative significance of the most likely predictors of clinical outcome, ie, patient age, Hughes' and Shimada' grade, clinical stage, and Ki-S5 as well as PCNA labeling indices. In this model, age over 1 year was found to be the most significant predictor of adverse prognosis ($P < 0.0001$), followed by a high Ki-S5 immunoreactive score (>25%, $P = 0.003$) and Shimada's grade ($P = 0.0192$). None of the other parameters achieved statistical significance. However, as the age at diagnosis enters Shimada's system as a grading criterion,[6] these two parameters cannot be regarded as independent covariates. Therefore, age and Shimada's grade were removed in turn from the model. In the first case, Shimada's grade achieved the highest statistical significance ($P = 0.0029$), and next to it, a high Ki-S5 labeling index was predictive of poor prognosis ($P = 0.0114$). Conversely, when Shimada's grade was disregarded, the Ki-S5 labeling index emerged as the most relevant prognostic factor ($P < 0.00001$). Of the other parameters, only the age at diagnosis was statistically significant ($P = 0.0011$).

Our results are in keeping with a previous investigation,[77] in which we demonstrated for arbitrarily chosen theoretical cut-off points of 5, 20, and 50% a statistically significant correlation of Ki-S5 scores with the overall survival in stage IV patients. Corresponding PCNA values were not found to be significant in this study. Neither different fixation times and formalin concentrations, which may affect the preservation of the PCNA protein,[78] nor its comparatively long half-life of approximately 20 hours[79,80] are likely to account for the heterogeneousness of the PC10 and Ki-S5 staining patterns. In contrast to our expectations based on the findings of other investigators,[79] mean and median PCNA counts were lower than Ki-S5 indices when evaluated separately for different tumor grades. Conversely, PCNA expression in composite GNBLs was not only higher than in G2 and G3 tumors but also markedly exceeded the corresponding Ki-S5 values. The only explanation we feel able to offer at the moment is that PCNA recognizes a subset of cells that, although associated with malignant features, are not necessarily involved in proliferation.

The Ki-67 protein is closely associated with chromatin and thickly coats mitotic chromosomes,[81] which are thus highlighted by Ki-S5 immunostaining. As pointed out above, this phenomenon enables a reliable discrimination between mitotic and karyorrhectic figures (Figure 1). By a similar approach using an antibody to PCNA and a DNA nick end-labeling technique, Gestblom and colleagues sought to subdivide the mitosis-karyorrhexis index and to determine the rates of proliferation and apoptosis in neuroblastoma.[82] They found that, independently of the number of PCNA-positive cells, high apoptosis levels correlated with a more favorable outcome and were of greater prognostic importance than PCNA labeling indices. High PCNA positivity alone was associated with a poorer prognosis, which was nevertheless not significant. This observation is consistent with our findings in tumors with a high mitosis-karyorrhexis index, ie, the poor prognosis grade according to Shimada's classification. Although the combined evaluation of proliferation and apoptosis is likely to provide more substantial information on the tumor biology than just one of these parameters,[83] an exact definition of the cell population recognized by an antibody appears to be of greatest importance.

In this regard, monoclonal antibody Ki-S5 appears to provide a reliable means for the assessment of the tumor growth fraction in neuroblastoma. This immunohistochemical approach has the advantage of being easily performable and readily assessable. The weight of the proliferative activity as a prognostic indicator for neuroblastoma is also reflected by the relevance of the S phase fraction as determined by flow cytometry.[21] The latter method, however, is far more laborious and has the shortcoming of being unsuccessful in nearly 20% of paraffin-embedded specimens[21] (also, P. Rudolph, U. Kellner, F. Collin, R. Parwaresch, and J. M. Coindre, manuscript in preparation). Nucleolar organizing regions provide a different kind of information in that they reflect the doubling time of a cycling tumor cell population.[84] It could therefore be of interest to correlate the quantity of cycling cells to the rapidity of proliferation for more complete information on tumor biology.[85]

To summarize our results, the growth fraction in neuroblastomas, as assessed by the monoclonal antibody Ki-S5, is significantly correlated with the histopathological grade according to two different grading systems and the cumulative disease-free survival as estimated by Kaplan-Meier analysis. It provides additional prognostic information after stratification on established criteria, such as patient age and tumor stage. Indeed, Ki-S5 scores allow recognition of a subset of patients with better prognosis even in advanced tumor stages and enable a subdivision of Shimada's poor prognosis grade with respect to the clinical outcome. PCNA is expressed

in a quantitatively similar but qualitatively different cell population, which may not be specifically cell cycle related and, so far, appears to be less relevant for prognosis. Accordingly, in multivariate analysis, the Ki-S5 labeling index emerged as one of the three most weighty prognostic indicators, whereas PCNA scores did not reach the level of statistical significance. A major concern regarding the present study, however, is the uneven distribution of pretreated cases in the different subgroups (Table 1), accounting for approximately one-third of the low grades (1b and 1c) and advanced stages. The reason may be a pharmacological induction of differentiation in otherwise higher histopathological grades and the need for preoperative chemotherapy in stages III and IV only. The concomitant bias may account for the comparatively low significance of the histopathological grade and the clinical stage in our study, and the results of the statistical analysis should therefore be interpreted with caution. Given these limitations, our results have to be considered as preliminary. Nevertheless, they characterize Ki-S5 as an indicator providing valuable information about the proliferative activity of neuroblastomas, which appears to be pertinent to the clinical course. Provided confirmation is obtained by additional studies on larger collectives, we suggest that the assessment of the growth fraction might merit inclusion in the panel of criteria used to determine prognosis and subsequent therapeutic strategies.

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