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Original Paper

The Current Contribution of Molecular Factors to Risk Estimation in Neuroblastoma Patients

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The association of molecular characteristics with prognosis has been reported, but not their relationship with each other and their impact in the context of known clinical risk factors. In this study, data of 1249 consecutive intent-to-treat-neuroblastoma patients with more than 1 year follow-up were examined by multivariate analysis using loglinear and Cox proportional hazard regression models on a stage-related basis (stages 1–3: 600, 4S: 116, 4: 533). In a first step, risk factors were identified from 18 selected clinical variables, and risk groups defined. The second step investigated whether molecular characteristics (*MYC*N, LOH 1p, del 1p, CD44, N-*ras*, NGF-R, bcl-2, APO-1 (CD95)) contributed additional prognostic information to the model. The loglinear model demonstrated several interactions between clinical factors. By the Cox regression model, seven independent clinical risk factors were found for stages 1–3, seven for stage 4 and two for stage 4S. By subsequent introduction of all molecular variables, *MYC*N amplification only added significant prognostic information to the clinical factors in localised and stage 4 neuroblastoma. The models allowed the definition of risk groups for stages 1–3 patients by age ($e^{\beta} = 5.09$) and *MYC*N ($e^{\beta} = 4.26$), for stage 4 by *MYC*N ($e^{\beta} = 2.78$) and number of symptoms ($e^{\beta} = 2.44$) and for stage 4S by platelet count ($e^{\beta} = 3.91$) and general condition ($e^{\beta} = 2.99$). Molecular factors and in particular *MYC*N contribute significantly to risk estimation. In conjunction with clinical factors, they are powerful tools to define risk groups in neuroblastoma.

Key words: *MYC*N amplification, CD44, LOH 1p, N-*ras*, NGF-R, APO-1 (CD95), bcl-2, DNA ploidy, risk factors, neuroblastoma
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INTRODUCTION

THE GREEK word πρόγνωσις (prognosis) means 'early knowledge'. In neuroblastoma, early knowledge of patients' outcome may be obtained from biological characteristics of the tumour, such as loss of chromosome 1 material [1–3], DNA ploidy [4, 5], *MYC*N amplification [6–8] and expression of CD44 [9, 10], MRP [11], trk-A [12, 13] or NGF-R [14], Ha-*ras* [15, 16] and other factors. From a clinical point of view, the problem with these studies is their focus on one or two variables. The association between clinical risk factors [17, 18] is also likely to apply to molecular characteristics, but this has rarely been investigated and then with only a restricted number of covariates [9, 19]. A comparison of the investigations is also hampered partly by differently chosen cutpoints of continuous variables, partly by different mergers of classes for categorical factors and finally by different numbers of available data, which may lead even to apparently contradictory results (e.g. [1] versus [3], [7] versus [8]). To our knowledge, multivariate evaluation of the most important molecular and clinical factors has not been reported thus far.

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Multivariate evaluation according to the Cox proportional hazards regression model [20] is critically dependent on the number of cases with complete data sets and on the number of events. Although the amount of molecular data is considerable in our series, the number of complete cases with all molecular characteristics is limited due to their consecutive introduction into the diagnostic workup during the late 1980s and early 1990s. Therefore, we chose a three-step procedure.

(1) Investigation of non-random association of possible prognostic factors (hierarchic loglinear model, partial association [21]).
(2) Multivariate evaluation of clinical, non-randomly associated risk factors (Cox proportional hazards model [20]), definition of clinical risk groups.
(3) Introduction of molecular risk factors with respect to additional independent prognostic information, definition of risk groups.

Because of the well-known major impact of stage of disease, all analyses were performed on a stage-related basis.

PATIENTS AND METHODS

Patients' characteristics

1270 consecutive patients (1 July 1979 to 1 January 1994) of the cooperative German neuroblastoma trials NB79, 82, 85 and 90 with histological diagnosis of neuroblastoma were eligible for the study. 21 children whose parents refused the therapy were excluded from analysis, resulting in 1249 intent-to-treat patients (including children with protocol violations) in the analysed cohort.

To avoid the differences between the Evans' and the INSS staging system, localised neuroblastoma (stages I–III and 1–3, respectively) were grouped together, with 600 patients in that group, 116 patients with stage 4S and 533 with stage 4. The incidence of stages (1–3 (I–III) versus 4S (IVS) versus 4 (IV)) was stable during the years (e.g. no increase of stage 4). The minimum follow-up period was 1 year. The median age at diagnosis was 19 months for all patients (range <1–25.5 months) and 2.4 months for stage 4S (range <1–11 months), 13 months for stages 1–3 (<1–25.3 months) and 32.4 months for stage 4 (<1–25.5 years). The event-free survival rate after more than 12 years was 49% for all patients, 76% for stages 1–3, 68% for stage 4S and 17% for stage 4. The overall survival rate at 12 years for all patients was 54%, 82% for stage 1–3, 78% for stage 4S and 18% for stage 4. The primary tumour was localised in abdominal midline (29%), left adrenal (26%), right adrenal (23%), in thoracic (15%), cervical (4%), combined (2%) sites and was not found in 2%. At diagnosis, metastases were found in bone marrow (stage 4: 80%; stage 4S: 42%), bone (stage 4: 61%), liver (stage 4: 17%; stage 4S: 80%), distant lymph nodes (stage 4: 17%), intracranial (stage 4: 8%), skin (stage 4: 2%, stage 4S: 16%). All these characteristics are in agreement with published data of other series.

Investigated variables

Clinical parameters. The 125 cooperating hospitals reported approximately 100 clinical variables on prepared case report forms. With respect to earlier investigations [17, 18] and clinical experience (trial NB90), the 18 most predictive and practical variables were selected. These were 'age at diagnosis', 'general condition', 'number of symptoms', 'primary site', 'metastatic site' '> or $\leq$ 5% tumour cells in bone marrow', 'therapeutic trial', 'haemoglobin', 'leucocyte count', 'platelet count', 'relative lymphocytes count', 'serum ferritin', 'serum lactatedehydrogenase (LDH)', 'urinary catecholamine metabolites', 'histological grade (Hughes) (untreated cases only)', 'tumour infiltration over midline (INSS)', 'resectability of the primary' (best result of several approaches). For definition and cutpoints, please see [18].

Molecular parameters. Molecular data were available from 577 patients (untreated and treated at sampling). Since a possible influence of the therapy cannot be excluded (in particular for the expression of proteins) we considered only the samples before treatment to be eligible for the study. This resulted in a reduction of the number of patients to 408. *MYCN* amplification was determined by Southern blot technique using the 1.0 kb insert of the plasmid Nb-1 [3, 14]. Detection of four or more copies was defined as amplified. Cytogenetic chromosome 1p deletion was detected by classical metaphase cytogenetics with Giemsa banding [1]. Absence of the region 1p36 $\rightarrow$ ter was considered as a 1p deletion. Loss of chromosome 1p heterozygosity (LOH) was performed by PCR (polymerase chain reaction) analysis (VNTR–PCR) of loci *D1S76* and *D1S80*. LOH was defined as loss of one of the alleles in the tumour sample [3, 22]. DNA ploidy was investigated by interphase cytogenetics using the pUC 1.77 probe. Evidence of more than two signals per nucleus in more than 70% of nuclei (approximately 1000 nuclei counted) was defined as polysomy.

Immunostaining was performed on unfixed snap frozen tumour tissue using the antibodies 2C5 (Biermann GmbH, Germany) for CD44, MAB 124 (Boehringer, Mannheim, F.R.G.) for BCL-2, 8211 (Boehringer, Mannheim, Germany) for gp75 NGF-R, F155 (Santa Cruz Biotec, U.S.A.) for N-*ras* p21, and MAB APO-1 (gift of Dr Debatin, Heidelberg, Germany) for CD95. Expression was defined as positive staining (+/++/+++) in more than 20% of tumour cells, except for bcl-2 (in more than 70%).

Statistical procedures

The statistical work was performed on a personal computer using the package BMDP Release 7 (1992) with program 1L for Kaplan–Meier estimates, log-rank test (α set at 0.05), program 4F for the loglinear model and program 2L for multivariate analysis. For the Cox proportional hazard model [20], the stepwise regression technique was used. The regression parameters (β) were estimated by the maximum partial likelihood ratio method. Both stepwise regression procedures were applied by forward and backward selection at $\alpha = 0.05$ to introduce a covariate to or to remove it from the model. The proportionality assumption was verified for all possible combinations of the covariates. The codes of covariates were chosen as positive numbers to allow uniform interpretation of the regression coefficients. The results of the Cox models were very similar for event-free survival and overall survival.

The relationships among selected categorical variables were modelled by hierarchical loglinear models including effects up to the third order. With respect to the numbers per statistical cell, Fisher's exact or Pearson's χ^2-test were used to test the independence of categorical data in two-way tables. In these tables, a P value of <0.05 was interpreted as significant [21].

In case of interaction between two or more variables, further selection was performed according to the clinical

practicability [1] and balanced statistical distribution [2] of the variable (e.g. LDH preferred before ferritin). Thus, 10 non-associated clinical variables were included in the Cox model for stages 1–3, 12 for stage 4 and 12 for stage 4S. To the clinical risk factors identified by multivariate analysis, each of the molecular variables was stepwise included to see whether or not it would contribute additional prognostic information. The relevant molecular and clinical factors were used to define risk groups.

RESULTS

Stages 1–3 *(localised neuroblastoma)*

The loglinear model demonstrated various partial associations e.g. between LDH versus ferritin ($P < 0.0001$), LDH versus age-related lymphocyte count ($P = 0.001$), LDH versus haemoglobin ($P = 0.0002$), ferretin versus lymphocyte count ($P = 0.004$), ferritin versus haemoglobin ($P = 0.003$) and LDH versus ferritin versus number of symptoms ($P = 0.04$). Thus, LDH was chosen for the Cox model instead of ferritin, haemoglobin and lymphocyte count. 39.6% of LDH values were elevated ($n = 470$). Other variables included in the clinical Cox model were age at diagnosis ($n = 600$, 51.6% > 1 year), general condition ($n = 578$, 24.9% more than slightly reduced), number of symptoms ($n = 600$, 69.7% > no sympton), primary site ($n = 600$, 37.7% adrenal), trial ($n = 600$, NB79 13.2%, NB82–85 50.3%, NB90 36.5%), leucocyte count ($n = 580$, 10.7% decreased for age), histological grade ($n = 531$, 35.2% Hughes grade 3), infiltration over midline ($n = 521$, 41.4% yes) and resectability of the primary tumour ($n = 593$, 29.8% biopsy or subtotal removal).

Table 1 demonstrates the parameters which appeared independently informative for prognosis. Since seven variables are impractical in a clinical setting for risk estimation, risk groups were created using two or three variables and amalgamating those with similar prognosis (non-different at $\alpha = 0.05$). Figure 1 shows Kaplan–Meier estimates with 'LDH' and 'age' as simple variables. Similar groups were obtained with other combinations.

Table 1. *Multivariate clinical risk factors for event-free survival in* 341 *patients with neuroblastoma stages* 1–3 *(*71 *events)*

Factor	β/SE(β)	exp(β)	Unfavourable
Infiltration over midline	2.71	2.40	yes
Site of primary tumour	2.50	1.88	adrenal
Histological grading	2.33	1.81	grade 3 (Hughes)
Resectability of primary tumour	2.19	1.74	biopsy, subtotal removal
Age at diagnosis	2.11	1.90	>1 year
LDH	2.00	1.73	elevated
Number of symptoms	1.99	2.29	>0

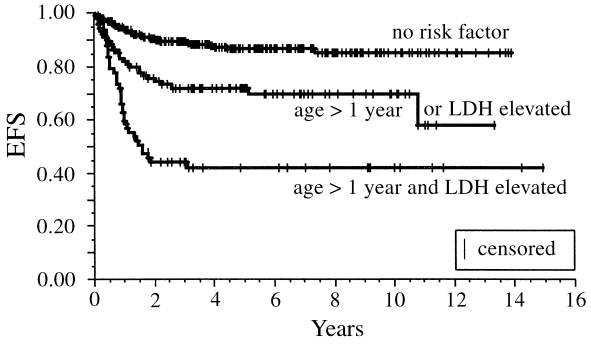

Figure 1. Kaplan–Meier estimates for event free survival (EFS) by clinical risk factors 'age' and 'LDH' in 470 patients with stages 1–3 neuroblastoma. No risk factor, $n = 156$ (12 events, 92% censored): 0.85 ± 0.03; age >1 year or LDH elevated, $n = 190$ (36 events, 81% censored): 0.59 ± 0.10; age >1 year and LDH elevated, $n = 124$ (52 events, 58% censored): 0.42 ± 0.04.

10.1% of patients with localised tumour showed *MYC*N amplification ($n = 149$), 21.3% CD44 expression ($n = 122$), 13.6% LOH 1p ($n = 44$), 37% 1p deletion ($n = 27$), 26.8% DNA disomy ($n = 82$), 61.2% N-*ras* ($n = 49$), 42.6% NGF-R ($n = 61$), 51.2% bcl-2 ($n = 84$) and 55.4% APO-1 ($n = 56$) expression. By univariate estimation (Kaplan–Meier), only *MYC*N (log-rank test $P < 0.001$) and CD44 ($P = 0.003$) were prognostically informative. *MYC*N and CD44 were associated (Fisher's exact test $P < 0.0001$).

The multivariate Cox model selected, from the clinical and molecular variables, *MYC*N and age as risk factors (Table 2). Two prognostically highly different risk groups emerged (Figure 2). The discrimination for survival was equally efficient (0–1 risk factor: Survival 0.92 ± 0.03, 2 risk factors: 0.29 ± 0.14). A similar good estimation resulted from the risk factors LDH and *MYC*N (data not shown).

Stage 4

Partial associations were found between the variables LDH versus ferritin ($P = 0.015$), LDH versus haemoglobin ($P = 0.0001$), ferritin versus platelet count ($P = 0.03$), ferritin versus haemoglobin ($P < 0.0001$), platelets versus leucocytes ($P < 0.0001$). The following parameters were investigated by the Cox model: age ($n = 533$, 77.1% > 18 months), general condition ($n = 522$, 54.2% severely ill), number of symptoms ($n = 533$, 74.5% > 1), primary site ($n = 533$, 57.2% adrenal), metastatic site ($n = 533$, 39.4% bone), tumour cells in bone marrow ($n = 533$, 43.2% > 5%), trial ($n = 533$, NB79 16.3%, NB82–85 47.5%, NB90 36.2%), leucocytes ($n = 525$, 20% decreased), platelets ($n = 522$, 15.1% decreased), LDH ($n = 462$, 87.7% elevated), histological grade ($n = 419$, 47.7%

Table 2. *Multivariate risk factors (clinical and molecular) in* 149 *patients with neuroblastoma stages* 1–3 *(*29 *events)*

Factor	β/SE(β)	exp(β)	Unfavourable
*MYC*N	2.53	4.26	amplified
Age at diagnosis	2.06	5.09	>1 year

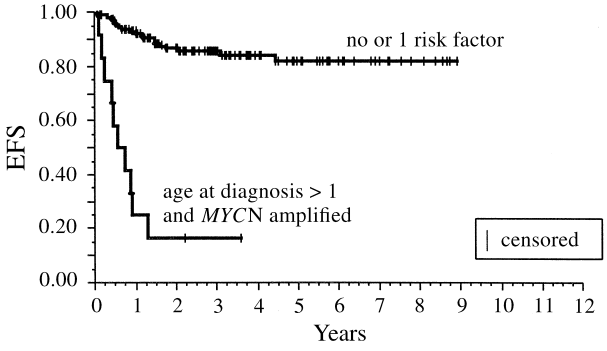

Figure 2. Kaplan–Meier estimates for EFS clinical ('age') and molecular ('*MYC*N') risk factors in 149 patients with stages 1–3 neuroblastoma. No or one risk factor, $n = 137$ (19 events, 86% censored): 0.82 ± 0.04, two risk factors, $n = 12$ (10 events, 17% censored): 0.17 ± 0.11.

grade 3) and resectability of the primary ($n = 482$, 43.2% biopsy or subtotal removal).

In Table 3, the seven independent prognostic factors for stage 4 are listed. From those, the parameters LDH and platelets were chosen to define risk groups. Due to amalgamation three risk groups resulted. They may be described schematically:

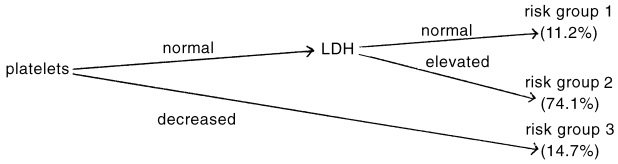

Figure 3 demonstrates the different outcome of the three clinical risk groups. The survival rates more than 8 years after diagnosis were 0.58 ± 0.08 (group 1), 0.19 ± 0.03 (group 2) and 0.03 ± 0.03 (group 3).

34.3% of cases with stage 4 neuroblastoma had *MYCN* amplification ($n = 102$), 80% 1p deletion ($n = 15$), 44.4% LOH 1p ($n = 27$), 56.7% DNA disomy ($n = 30$), 63.4% CD44 ($n = 71$), 84.6% N-*ras* ($n = 26$), 65.6% NGF-R ($n = 32$), 43.5% bcl-2 ($n = 62$) and 71.0% APO-1 ($n = 31$) expression. The Kaplan–Meier curves demonstrated univariate influence on prognosis only for *MYCN* ($P = 0.0002$) and a trend for LOH 1p ($P = 0.07$). In stage 4, *MYCN* amplification was associated with LOH 1p (Fisher's exact test $P = 0.01$), but not with CD44 ($P = 0.17$). The low numbers of data for some parameters may underestimate the discriminative power of these variables.

Table 3. Multivariate clinical risk factors for EFS in 351 patients with neuroblastoma stage 4 (254 events)

Factor	β/SE(β)	exp(β)	Unfavourable
Trial	3.74	1.47	NB79 < (NB82–85) < NB90
Histological grading	3.69	1.60	grade 3 (Hughes)
Resectability of the primary tumour	3.38	1.55	biopsy/subtotal removal
LDH	2.88	1.83	elevated
Number of symptoms	2.83	1.54	>1
Platelets	2.05	1.46	decreased
Tumour cells in bone marrow	2.03	1.31	>5%

MYCN retained its prognostic significance in the multivariate model (Table 4). Presence of more than one symptom was of similar importance for prognosis. The symptoms (asked for by the report forms) included visible/palpable swelling of the primary and/or metastases, fever, reduced general condition, weight loss/no weight gain, pain, ataxia/opsomyoclonus, treatment-resistant diarrhoea, transverse myelopathy (incomplete/complete), Horner's complex and others. With the two factors, three risk groups emerged (Figure 4). Discrimination of the risk groups was equally good for survival (no RF: 0.57 ± 0.12, one RF: 0.34 ± 0.07, two RF: 0).

Stage 4S

Partial associations were detected for platelets versus LDH ($P = 0.056$), platelets versus general condition ($P = 0.036$) and leucocytes versus general condition ($P = 0.066$ (trend)). The parameters investigated by multivariate analysis were age at diagnosis ($n = 116$, 33.6% ≤ 1 month), general condition ($n = 114$, 15.8% critically ill), number of symptoms ($n = 116$, 40.0% > 1), primary site ($n = 116$, 68.1% adrenal), metastatic site ($n = 116$, 80.2% liver), tumour cells in bone marrow ($n = 116$, 9.5% > 5%), trial ($n = 116$, NB79 16.4%, NB82–85 48.3%, NB90 35.3%), leucocytes ($n = 115$, 13.9% increased), platelets ($n = 114$, 14.0% decreased), LDH ($n = 92$, 47.8% elevated), histological grade ($n = 88$, 61.4% Hughes grade 3) and resectability of the primary tumour ($n = 98$, biopsy/subtotal 56.1%).

Table 5 delineates the factors 'platelets' and 'general condition' as prognostically significant. Figure 5 shows the EFS curves for the three described risk groups. For survival, the factor 'general condition' was sufficient enough to discriminate the good (0.87 ± 0.04, $n = 96$) from the poor (0.29 ± 0.14, $n = 18$) risk group.

Too few molecular data were available in stage 4S patients to allow their inclusion in a meaningful Cox analysis.

Table 4. Multivariate risk factors (clinical und molecular) in 87 cases with neuroblastoma stage 4 (56 events)

Factor	β/SE(β)	exp(β)	Unfavourable
MYCN	3.63	2.78	amplified
Number of symptoms	2.74	2.44	>1

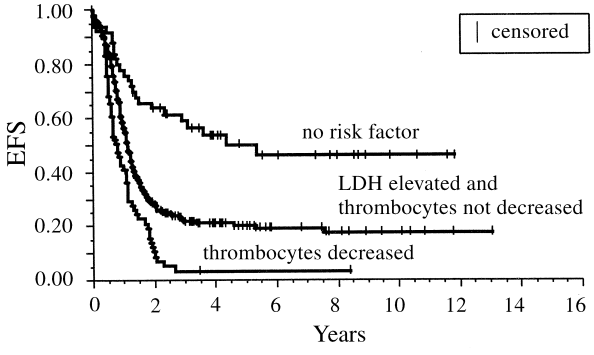

Figure 3. Kaplan–Meier estimates for EFS by clinical risk factors 'platelets' and 'LDH' in 455 patients with neuroblastoma stage 4. No risk factor, $n = 51$ (24 events, 63% censored): 0.47 ± 0.08; LDH elevated, platelets normal, $n = 337$ (249 events, 31% censored): 0.18 ± 0.03; platelets decreased, $n = 67$ (61 events, 9% censored): 0.04 ± 0.02.

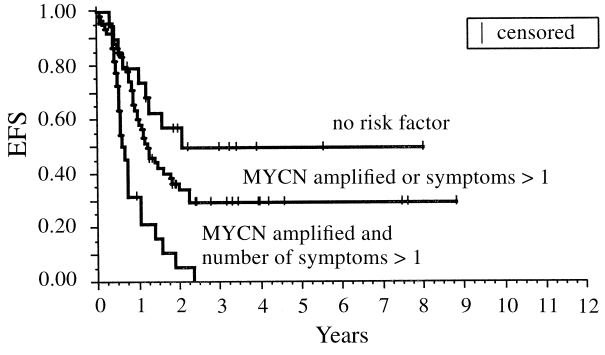

Figure 4. Kaplan–Meier estimates for EFS by clinical ('>1 symptom') and molecular ('*MYCN*') risk factors in 102 patients with stage 4 neuroblastoma. No risk factor, $n = 19$ (9 events, 53% censored): 0.50 ± 0.12; one risk factor, $n = 61$ (39 events, 36% censored): 0.30 ± 0.06; two risk factors, $n = 22$ (21 events, 5% censored): 0.

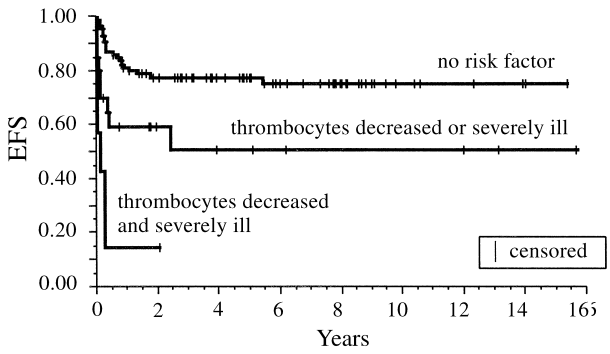

Figure 5. Kaplan–Meier estimates for EFS by clinical risk factors 'platelets' and 'general condition' in 113 patients with neuroblastoma stage 4S. No risk factor, $n = 86$ (20 events, 77% censored): 0.75 ± 0.05; one risk factor, $n = 20$ (9 events, 55% censored): 0.51 ± 0.12; two risk factors, $n = 7$ (6 events, 14% censored): 0.14 ± 0.13.

DISCUSSION

The extremely different course of neuroblastoma evolving from tumours with identical histological features may be predicted with good reliability by clinical parameters [17, 18, 23]. However, this approach relies basically on the characteristics of the phenotype which results from the interaction of the genotype, the time of the detection and supposedly environmental factors promoting or inhibiting tumour growth. Since the genotype is considered to have the major influence on the final outcome, genetic characteristics are utilised increasingly to predict prognosis. This appears to be of particular value in the rare cases with good clinical but poor molecular features [2, 4, 7, 20, 24, 25]. The current, very incomplete understanding of the important steps in neuroblastoma oncogenesis prevents molecular characteristics alone being used for clinically relevant decisions. This study attempted to define the value of the present molecular tumour markers in conjunction with established clinical factors by re-evaluating them in multivariate analyses in a large series of patients.

For localised neuroblastoma, '*MYCN* amplification' and 'age over 1 year at diagnosis' proved to be powerful prognostic factors which allowed discrimination of three risk groups. In stage 4 disease, '*MYCN* amplification' and 'presence of >1 symptom at diagnosis' indicated independently poor risk and were useful in describing three risk groups with EFS estimates between 0 and 50% (Figure 4). The available number of molecular data from infants with stage 4S disease was too small, but clinical risk groups on the basis of the independent factors 'general condition' and 'platelet count' could be described. In stages 1–3 and 4, *MYCN* demonstrated considerable prognostic power. This supports earlier reports describing the association with rapid progression [7, 25]. In contrast, Cohn and associates [8] did not find a correlation between poor prognosis and *MYCN* amplification in 6 out of 181 cases of localised neuroblastoma, which appears an unusually low incidence of *MYCN* amplification. Other authors reported an inverse correlation with good prognostic markers such as hyperdiploidy [4], CD44 [9, 10, 19], NGF-R [14], Ha-*ras* p 21 [15] and catecholamine metabolism [26]. The current controversy over whether *MYCN* [3, 7] or LOH 1p [1, 2, 27], represents the more informative progression marker is associated with their close correlation to each other [19] and with the dependence of statistical tests on the number of investigated cases. We cannot rule out the possibility that the emphasis on *MYCN* amplification in this study is promoted by the far higher number of available data on *MYCN* compared with LOH 1p.

Table 5. Multivariate clinical risk factors in 66 infants with neuroblastoma stage 4S (20 events)

Factor	β/SE(β)	exp(β)	Unfavourable
Platelets	3.07	3.91	decreased
General condition	2.56	2.99	critically ill

The selection of variables for multivariate analysis depends on the number of available data and on the estimated clinical usefulness of the factor. Therefore, the results presented here are only one of several possible interpretations of our data. None the less, this proposal is derived from a large number of unselected patients with a considerable amount of controlled clinical and molecular data. The study shows that molecular factors and in particular *MYCN* amplification contribute significantly to risk estimations. Although on its own still insufficient, *MYCN* proved to be very powerful in conjunction with clinical factors for defining reliable risk groups in neuroblastoma. Multinational efforts utilising stage, biochemical and molecular data in an even larger series of patients are currently in progress.

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