in clonal MYCN-transfectants of the SK-N-SH neuroblastoma cell line. *Int J Cancer*, in press.
17. Tanaka K, Apella E, Jay G. Developmental activation of the H-2K gene is correlated with an increase in DNA methylation. *Cell* 1983, 35, 457–465.
18. Engler P, Haasch D, Pinkert CA, *et al.* A strain-specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci. *Cell* 1991, 65, 939–947.
19. Li E, Beard C, Jaenisch R. Role for DNA methylation in genomic imprinting. *Nature* 1993, 366, 362–365.
20. Rainier S, Johnson LA, Dobry CJ, *et al.* Relaxation of imprinted genes in human cancer. *Nature* 1993, 362, 747–749.
21. Schleiermacher G, Peter M, Michon J, *et al.* Two distinct deleted regions on the short arm of chromosome 1 in neuroblastoma. *Genes Chrom Cancer* 1994, 10, 275–281.
22. Takeda O, Homma C, Maseki N, *et al.* There may be two tumour suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. *Genes Chrom Cancer* 1994, 10, 30–39.
23. Caron H. Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavourable prognostic markers in neuroblastoma. *Med Ped Oncol*, in press.
24. Ellmeier W, Aguzzi A, Kleiner E, *et al.* Mutually exclusive expression of a helix-loop-helix gene and *MYCN* in human neuroblastomas and in normal development. *EMBO J* 1992, 7, 2563–2571.
25. Lathi JM, Valentine M, Xiang J, *et al.* Alterations in the PITSLRE protein kinase gene complex on chromosome 1p36 in childhood neuroblastoma. *Nature Genet* 1994, 7, 370–375.
26. Enomoto H, Osaki T, Takahashi E, *et al.* Identification of human DAN gene, mapping to the putative neuroblastoma tumour suppressor locus. *Oncogene* 1994, 9, 2785–2791.
27. Ozaki T, Sakiyama S. Tumour-suppressive activity of NO3 gene product in v-src transformed rat 3Y1 fibroblasts. *Cancer Res* 1994, 54, 646–648.

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Comparison of DNA Aneuploidy, Chromosome 1 Abnormalities, *MYCN* Amplification and CD44 Expression as Prognostic Factors in Neuroblastoma

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A comparison of the prognostic impact of five molecular variables in a large series was made, including tests of their nonrandom association and multivariate analysis. Molecular data were available for 377 patients and *MYCN* amplification, cytogenetic chromosome 1p deletion, loss of chromosome 1p heterozygosity, DNA ploidy and CD44 expression were investigated. Their interdependence and influence on event-free survival was tested uni- and multivariately using Pearson's χ^2-test, Kaplan–Meier estimates, log rank tests and the Cox's regression model. *MYCN* amplification was present in 18% (58/322) of cases and predicted poorer prognosis in localised ($P < 0.001$), metastatic ($P = 0.002$) and even 4S ($P = 0.040$) disease. CD44 expression was found in 86% (127/148) of cases, and was a marker for favourable outcome in patients with neuroblastoma stages 1–3 ($P = 0.003$) and 4 ($P = 0.017$). Chromosome 1p deletion was cytogenetically detected in 51% (28/55), and indicated reduced event-free survival in localised neuroblastoma ($P = 0.020$). DNA ploidy and loss of heterozygosity on chromosome 1p were of less prognostic value. Most factors of prognostic significance were associated with each other. By multivariate analysis, *MYCN* was selected as the only relevant factor. Risk estimation of high discriminating power is, therefore, possible for patients with localised and metastatic neuroblastoma using stage and *MYCN*.

Key words: *MYCN* amplification, chromosome 1, DNA ploidy, CD44, lactate dehydrogenase, prognostic factors, multivariate analysis
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INTRODUCTION

THE GOAL of investigations into neuroblastoma at a molecular level is to understand better the biological diversity of the disease. In the last 10 years, several factors have been described including deletions of the short arm of chromosome 1 (del 1p) [1–3], amplification of the oncogene *MYCN* [4–6], DNA ploidy [7–9], overexpression or absence of the *Ha-* and *NRAS* gene [10, 11], CD44 receptor [12, 13], multidrug resistance gene (*PGY*, previously MDR) [14, 15], the low affinity nerve growth factor receptor and *TRKA* gene [16, 17]. Since the presence

(*MYCN*, del 1p, euploid DNA, MDR) or absence (*NRAS*, *TRKA*, CD44) appears to be an indication of poor prognosis, these factors gained much interest for the clinic and relevance for individual patients. Most of these factors are stage dependent and, therefore, probably not independent of each other. While the interrelationship of various "clinical" factors (such as age and stages, ferritin and lactate dehydrogenase (LDH) levels, surgical resectability, white blood count) has been investigated [18, 19], this has not been done for the molecular factors. Therefore, we analysed selected factors for their mutual relationship in order to define their relative prognostic impact.

PATIENTS AND METHODS

Patients characteristics

For 377 patients of the co-operative German neuroblastoma trials NB 85 and NB 90, molecular data were available and could be included in the analysis. This represents 44% of the total trial population. The criteria for diagnosis and staging adhered to the international neuroblastoma staging system (INSS) [20]. 15% (n = 55) were classified as stage 1 patients, 9% (n = 33) stage 2, 26% (n = 97) stage 3, 42% (n = 160) stage 4 and 8% (n = 32) stage 4S. There were no differences between the study group and the total group regarding the known clinical risk factors (LDH, resectability, histological grade, leucopenia, age, general condition) and outcome (event-free survival (EFS) and survival (S)), i.e. the analysed group was representative of the total population. EFS was 0.49 ± 0.03 for all patients 5 years after diagnosis, 0.66 ± 0.05 for patients with stages 1–3, 0.25 ± 0.06 for stage 4 and 0.66 ± 0.09 for stage 4S. The corresponding survival figures were 0.59 + 0.03 (all), 0.78 ± 0.04 (stages 1–3), 0.33 ± 0.05 (stage 4), 0.91 ± 0.05 (stage 4S).

Molecular factors

Deletion of the distal part of the short arm of chromosome 1 was investigated by routine Giemsa banding technique [3]. Absence of the region 1p 36 —▶ter on chromosome 1 was considered as 1p deletion (del 1p). The investigation on loss of chromosome 1p heterozygosity was performed by polymerase chain reaction (PCR) (variable number of tandem repeats CuNTR) of loci *D1S76*, *D1S80*) [21]. Loss of heterozygosity (LOH 1p) was defined as loss of one of the alleles in the tumour sample. DNA ploidy experiments were carried out via interphase cytogenetics using the pUC 1.77 probe [22]. Aneuploidy was defined as the presence of more than two signals per nucleus. Approximately 1000 nuclei per tumour sample were evaluated. *MYCN* amplification was estimated via a Southern blot technique using the pNB-1 probe [23], and defined as present if four or more copies were found.

CD44 expression was determined by immunocytochemistry on frozen sections of tumour tissue [13]. Positive staining with monoclonal antibody 25-32, recognising the CD44 standard, was considered as indicative of the presence of CD44.

Statistical procedures

The statistical analysis was performed on an IBM personal computer model PS/2 with BMDP version 1990 as software. Kaplan–Meier estimates were utilised for univariate analysis comparing the curves by log rank (Mantel–Cox) tests at α = 0.05. Nonrandom correlation of univariately identified risk factors was tested by Pearson's χ^2-test for contingency tables. For multivariate analysis, the Cox's proportional hazards regression model [24] was applied.

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RESULTS

Table 1 demonstrates that DNA aneuploidy as estimated by *in situ* hybridisation techniques was found in 60% of cases. Stage 4 patients showed less (31%) aneuploidy than the good risk stages 1–3 (73%) and 4S (60%). The presence of aneuploidy did not predict a different outcome (EFS, S) compared with the group with euploid tumours. Neither in the total nor in stage-related groups did DNA ploidy appear as a variable of prognostic impact.

Cytogenetic detection of chromosome 1p deletion was absent in all stage 4S cases, and was present in 38% of localised, and 85% of metastatic disease. The overall incidence for all stages was 51%. The presence of del 1p was predictive of poor outcome in stages 1–3 neuroblastoma (EFS all stages with versus without deletion: 0.25 ± 0.08 versus 0.81 ± 0.04, $P < 0.001$; stages 1–3: 0.45 ± 0.15 versus 0.83 ± 0.09, $P = 0.020$; stage 4: only 3 patients in the group without deletion, $P = 0.30$).

Loss of heterozygosity on chromosome 1p (loci *D1S76* and/or *D1S80*) was found less often (12%) compared with cytogenetic observation. The incidence of losses did not vary with stage (stages 1–3: 11%, 4S: 17%, 4: 13%). There was a trend for worse survival with LOH 1p in the total group (EFS with versus without LOH: 0.42 ± 0.14 versus 0.37 ± 0.20, $P = 0.10$). For subgroup analysis, the number of the patients was too small (Table 1).

MYCN amplification was seen in 18% of cases with a higher prevalence in patients with metastatic disease (stages 1–3: 13%, 4S: 14%, 4: 25%). The prognostic impact of amplified *MYCN* was high in localised neuroblastoma (Figure 1). In stage 4 (Figure 2) and stage 4S disease, *MYCN* amplification was still associated with poorer outcome, although the predictive strength was weaker (4S $MYCN^+$ versus $MYCN^-$: EFS 0.25 ± 0.22 versus 0.63 ± 0.13, $P = 0.040$).

CD44 expression could be demonstrated in 86% of cases with no major incidence variation throughout the stages, except for stage 4S (1–3: 84%, 4S: 100%, 4: 85%). In all groups, CD44 expression was associated with better EFS ($CD44^+$ versus

Table 1. Incidence of DNA aneuploidy, cytogenetic deletion of chromosome 1p (del 1p), loss of heterozygosity of chromosome 1, MYCN amplification and CD44 expression as a function of stage

Marker	Stages (INSS) 1–3	4S	4	All
DNA aneuploidy	68/93*	9/15	14/45	91/153
del 1p	11/29	0/6	17/20	28/55
LOH 1p	5/45	1/6	3/23	9/74
MYCN amplification	11/167	4/29	32/126	58/322
CD44 expression	61/73	14/14	52/61	127/148

*x/y, x = number of patients with present marker, y = number of patients tested for that marker.

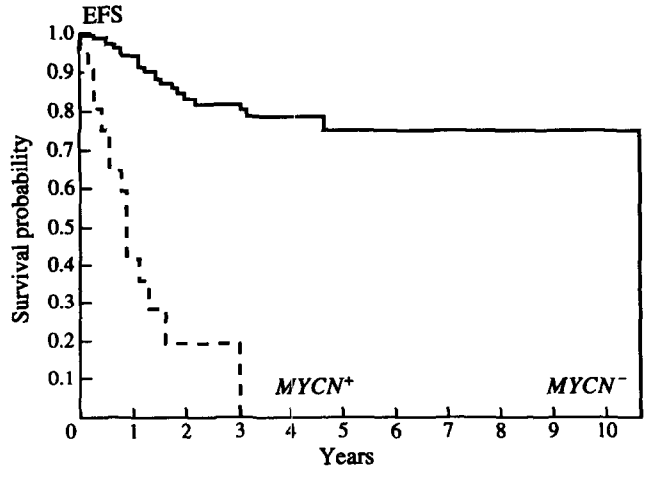

Figure 1. Event-free survival in 167 patients with neuroblastoma stages 1–3 by *MYCN* amplification. Log rank test $P < 0.001$. *MYCN*$^-$, 145 patients (22 events): 0; *MYCN*$^+$, 22 patients (15 events): 0.

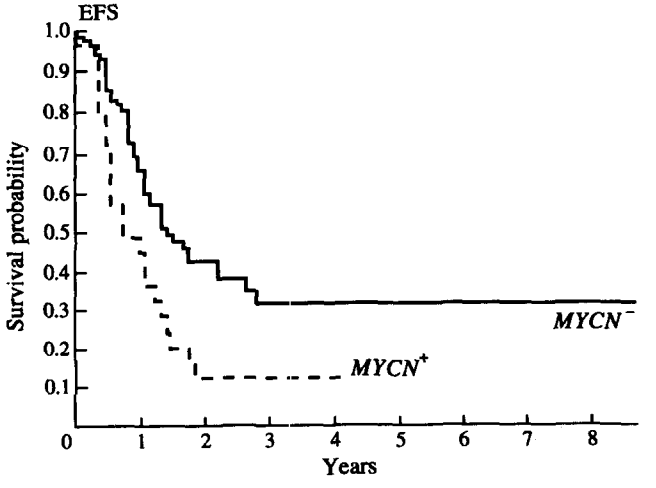

Figure 2. Event-free survival in 126 patients with neuroblastoma stage 4 by *MYCN* amplification. Log rank test $P = 0.002$. *MYCN*$^+$, 94 patients (48 events): 0.32 ± 0.06; *MYCN*$^-$, 32 patients (23 events): 0.12 ± 0.07.

CD44$^-$ all stages: 0.56 ± 0.06 versus 0, $P = 0.001$; stage 4S: only one group with CD44$^+$; stages 1–3: 0.72 ± 0.09 versus 0, $P = 0.003$; stage 4: 0.36 ± 0.10 versus 0, $P = 0.017$).

The nonrandom correlation of the investigated variables is shown in Table 2. A strong association was found for *MYCN* amplification with chromosome 1 abnormalities (both methods). *MYCN* amplification was also correlated with the absence of CD44 expression and with euploid tumour DNA. The expected but missed correlation between the two chromosome 1 methods (del 1p, LOH 1p) may be due—at least in part—to the practically non-overlapping groups investigated. Additionally, vNTR-PCR of *D1S76* and *D1580* (1p36) is accompanied by a substantial false negative rate due to tumour DNA contamination with DNA from non-tumour cells and/or complex partial chromosome 1 formations in neuroblastoma.

Recently, we reported that serum LDH levels at diagnosis may be important "clinical" prognostic factors in localised [18] and in metastatic [19] neuroblastoma. Table 3 shows a nonrandom association between abnormal LDH levels and *MYCN* amplification as the most important "molecular" prognostic factor.

Table 2. Nonrandom correlation of molecular tumour markers in patients with neuroblastoma

Markers	*n*	*P* value
MYCN and del 1p	55	<0.001
MYCN and LOH 1p	74	<0.001
MYCN and DNA aneuploidy	141	<0.001
MYCN and CD44	127	<0.001
LOH 1p and DNA aneuploidy	39	0.17
LOH 1p and del 1p	3	—
LOH 1p and CD44	38	0.18
CD44 and DNA aneuploidy	85	0.95
CD44 and del 1p	44	0.14
del 1p and DNA aneuploidy	21	0.003

Table 3. Association of MYCN *amplification and elevated serum LDH levels in 258 cases with neuroblastoma*

LDH	Amplified *MYCN*	
	No	Yes
Normal ($n = 82$)	96%	4%
Abnormal ($n = 176$)	72%	28%

Pearson's χ^2-test: $P < 0.001$.

Owing to the limited number of patients with complete data sets (a prerequisite for the Cox model), we performed some multivariate analyses with two variables. The question was whether the second chosen factor would improve the predictability of the outcome (EFS). In all models, *MYCN* appeared as the most powerful factor. Neither in localised stages nor in metastatic cases (stage 4) did the addition of CD44 status, DNA ploidy, cytogenetic del 1p increase the prognostic power. Only LOH 1p data, in addition to *MYCN*, was helpful in stages 1–3 disease (Table 4), demonstrating a 36-fold increase of risk if *MYCN* amplification was present and an 8-fold increase of risk if LOH 1p was present. This was not observed for stage 4 neuroblastoma. For stage 4S, the number of cases was too small for meaningful multivariate anlaysis.

Table 4. Results of multivariate evaluation of risk factors for EFS in 45 patients with neuroblastoma stages 1–3

Factor	β	e^{β}	P^*
MYCN amplification	3.58	35.82	0.004
LOH chromosome 1p	2.10	8.22	0.058

*P, improvement χ^2 P value.

DISCUSSION

We report a possible prognostic influence of the molecular markers *MYCN* amplification (stages 1–3, 4, 4S, all stages), CD44 expression (stages 1–3, 4, all stages), cytogenetic 1p deletion (stages 1–3, all stages), and a marginal effect of LOH 1p (all stages). No predictive power could be demonstrated for DNA ploidy. This is in agreement with the literature for *MYCN* (see [5, 6]), del 1p [3], LOH 1p [21] and CD44 [12, 13], while earlier reports on DNA ploidy [7–9] as prognostic factor were not supported by our data. This may be due primarily to the different methods used (impulse cytophotometry versus interphase cytogenetics). This finding emphasises the necessity to refer to the methods if one compares different studies.

The univariately identified prognostic factors exhibited stage dependence (Table 1). Therefore, multivariate analysis (Cox) was carried out on a stage-related base. However, only loss of chromosome 1 material (LOH 1p) was helpful, in addition to *MYCN* amplification, for stages 1–3 cases to estimate outcome. In all other instances, *MYCN* alone was sufficient (stage 4, 4S) and knowledge of CD44, del 1p and DNA ploidy status did not improve the predictability.

Owing to the limited number of patients in this study, the proposed model should be considered preliminary and the usefulness confirmed by other groups. Furthermore, it certainly remains unclear whether the molecular factors are really the best prognostic indicators available. The correlation of *MYCN* with LDH indicates the necessity of further studies comparing molecular and clinical variables. Nonetheless, this first comparison and multivariate model with molecular factors reduces the essential variables to a maximum of two.

1. Brodeur GM, Green AA, Hayes FA, *et al.* Cytogenetic features of human neuroblastomas and cell lines. *Cancer Res* 1981, **41**, 4678–4686.
2. Gilbert F, Balaban G, Moorhead P, *et al.* Abnormalities of chromosome 1p in human neuroblastoma tumors and cell lines. *Cancer Genet Cytogenet* 1982, **7**, 33–42.
3. Christiansen H, Lampert F. Tumour karyotype discriminates between good and bad prognostic outcome in neuroblastoma. *Br J Cancer* 1988, **57**, 121–126.
4. Schwab M, Alitalo K, Klempnauer KH, *et al.* Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 1983, **305**, 245–248.
5. Brodeur GM, Seeger RC, Schwab M, *et al.* Amplification of *N-myc* in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984, **224**, 1121–1124.
6. Seeger RC, Brodeur GM, Sather H, *et al.* Association of multiple copies of the *MYCN* oncogene with rapid progression of neuroblastomas. *N Engl J Med* 1985, **313**, 1111–1116.
7. Oppedal BR, Storm-Mathisen I, Lie SO, *et al.* Prognostic factors in neuroblastoma. Clinical, histopathologic, and immunohistochemical features and DNA ploidy in relation to prognosis. *Cancer* 1988, **62**, 772–780.
8. Look AT, Hayes FA, Nitschke R, *et al.* Cellular DNA content as a predictor of response to chemotherapy in infants with unresectable neuroblastoma. *N Engl J Med* 1984, **311**, 231–235.
9. Cohn SL, Rademaker AW, Salwen HR, *et al.* Analysis of DNA ploidy and proliferate activity in relation to histology and *MYCN* amplification in neuroblastoma. *Am J Path* 1990, **136**, 1043–1052.
10. Tanaka T, Slamon DJ, Shimada H, *et al.* A significant association of HA-RAS P21 in neuroblastoma cells with patient prognosis. A retrospective study of 103 cases. *Cancer* 1991, **68**, 1296–1302.
11. Nakada K, Fujioka T, Kitagawa H, *et al.* Expressions of *MYCN* and *ras* oncogene products in neuroblastoma and their correlations with prognosis. *Jpn J Clin Oncol* 1993, **23**, 149–155.
12. Favrot MC, Combaret V, Lasset C. CD44—a new prognostic marker for neuroblastoma. *N Engl J Med* 1993, **329**, 1965.
13. Terpe HJ, Christiansen H, Berthold F, *et al.* Absence of CD44-standard in human neuroblastoma correlates with histological dedifferentiation, *MYCN* amplification and reduced survival probability. *Cell Death Different* 1994, **1**, 123–128.
14. Goldstein LJ, Fojo AT, Ueda K, *et al.* Expression of the multidrug resistance, MDR1, gene in neuroblastomas. *J Clin Oncol* 1990, **8**, 128–136.
15. Chan HSL, Haddad G, Thorner PS, *et al.* P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. *N Engl J Med* 1991, **325**, 1608–1614.
16. Kogner P, Barbany G, Dominici C, *et al.* Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. *Cancer Res* 1993, **53**, 2044–2050.
17. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, *et al.* Association between high levels of expression of the *trk* gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993, **328**, 847–854.
18. Berthold F, Trechow R, Utsch S, *et al.* Prognostic factors in metastatic neuroblastoma. A multivariate analysis of 182 cases. *Am J Pediat Hematol/Oncol* 1992, **14**, 207–215.
19. Berthold F, Kassenböhmer R, Zieschang J. Multivariate evaluation of prognostic factors in localized neuroblastoma. *Am J Pediat Hematol/Oncol* 1994, **16**, 107–115.
20. Brodeur GM, Pritchard J, Berthold F, *et al.* Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993, **11**, 1466–1477.
21. Christiansen H, Delattre O, Fuchs St, *et al.* Loss of the putative tumor suppressor-gene locus 1p36 as investigated by a PCR-assay and *MYCN* amplification in 48 neuroblastomas: results of the German Neuroblastoma Study Group. *Prog Clin Biol Res* 1994, **385**, 19–25.
22. Christiansen H, Schestag J, Christiansen NM, *et al.* Clinical impact of chromosome I aberrations in neuroblastoma: a metaphase and interphase cytogenetic study. *Genes Chrom Cancer* 1992, **5**, 141–149.
23. Christiansen NM, Christiansen H, Berthold F, *et al.* Transcriptional activity of *MYCN* and *ngf-r* in 50 primary human neuroblastomas as predictor for clinical outcome. *Int J Oncol* 1993, **3**, 853–857.
24. Cox DR. Regression models and life tables (with discussion). *J Roy Soc Statist* 1972, **34**, 187–220.

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