

Chromogenic *in situ* hybridization-detected hotspot *MYCN* amplification associates with Ki-67 expression and inversely with nestin expression in neuroblastomas

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Since neuroblastomas are intratumorally heterogeneous, the analysis of genetic and biologic features of randomly selected tumor specimen spots may lead to erroneous conclusions. Our purpose was therefore to construct an easily assessable and strictly defined strategy to unify the detection of various molecular markers in paraffin-embedded neuroblastoma samples. We selected tumor specimen spots of highest proliferation activity, that is, hotspots, for the analysis of *MYCN* amplification status and proliferation-associated molecular markers, such as nestin, which role in neuroblastoma specimens was evaluated for the first time. Using a chromogenic *in situ* hybridization (CISH) technique, we showed that patients with a *MYCN* copy number higher than six in anti-Ki-67-detected hotspots have significantly worse overall survival prognosis than patients with low *MYCN* copy numbers ($P=0.0006$). The chosen cutoff value of six was shown to dichotomize *MYCN*-amplified neuroblastomas at least as specifically as Southern blot hybridization, in which amplification was defined by a copy number of ≥ 10 . Interestingly, we also detected without difficulty *MYCN*-amplified neuroblastic cells in bone marrow samples using the CISH technique. The proliferation activity, assessed with an anti-Ki-67-based proliferation index, was significantly higher in *MYCN*-amplified than in nonamplified hotspots. The proliferation indices of the hotspots had also a significant correlation with the prognosis (International Classification) and histological type, whereas the proliferation accelerator Id2 did not associate with any of the mentioned parameters. The expression of nestin associated inversely with *MYCN* amplification ($P=0.018$), which challenges a previously suggested role of nestin in neuroblastomas. In summary, hotspot focusing provides a means of analyzing proliferation-associated markers in neuroblastomas, and together with the CISH detection of the *MYCN* copy number enables an easy and reliable examination of *MYCN* status in neuroblastomas.

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Neuroblastic tumors, that is, neuroblastomas, are the most frequent extracranial solid malignancies in young children, and account for 9% of all childhood cancers.¹ The most significant mutation in neuroblastomas is *MYC*-related oncogene (*MYCN*) ampli-

fication, which was first shown in a panel of neuroblastoma cell lines.² There is a correlation between *MYCN* amplification and specific histological appearance, as *MYCN*-amplified neuroblastomas lack neuroblastic differentiation.³ In addition, *MYCN*-amplified neuroblastomas have significantly higher proliferation rates than nonamplified tumors.⁴ *MYCN* amplification, with the overall prevalence of about 20–30% in neuroblastomas, is an independent molecular indicator almost invariably predicting an aggressive clinical behavior and poor prognosis, especially in localized disease.⁵

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Despite the lack of a universal method, many countries have begun to implement an analysis of *MYCN* amplification status in neuroblastomas. Thus, a strictly defined *MYCN* copy number detection method is needed.

The assessment of mitotic activity by the mitosis-karyorrhexis index (MKI) was adopted in the histopathological classification system of neuroblastomas in 1984.⁶ Despite the laboriousness and relative error-proneness of MKI evaluation, it has remained the most used method in estimating proliferation status of neuroblastomas. As neuroblastomas display intratumoral heterogeneity of biological variables including proliferation activity³ and *MYCN* copy number,⁷ the estimation of prognostic parameters is complicated. Therefore, a well-defined selection of a single spot, which would represent the tumor in the analysis of the proliferation activity, *MYCN* copy number and other prognostic parameters, might unify results and facilitate evaluations.

In the present study, we utilized the chromogenic *in situ* hybridization (CISH) technique⁸ in the assessment of *MYCN* amplification status in archival neuroblastoma tissue microarray of hotspots (Ø 2 mm), each representing the highest Ki-67 antigen-based proliferation index of a single tumor specimen. As the scrutiny of the *MYCN* copy number in paraffin-embedded bone marrow biopsies has been far from a routine procedure, we also tested the use of CISH in evaluating *MYCN* status of bone marrow-disseminated neuroblastic cells. Moreover, we examined the relationship between the indirect proliferation stimulant Id2 protein and *MYCN* status, as the focusing on hotspots might elucidate recent contradictory results.^{9–11} Finally, as very recent *in vitro* finding suggests that the intermediate filament nestin, which is expressed in some tumors originating from neuroectodermal lineages, regulates neuroblastoma cell proliferation possibly through *MYCN* protein,¹² we compared the nestin protein expression to *MYCN* amplification in neuroblastomas. In summary, our data combines new methodological approaches to the evaluation of *MYCN* status with new findings of proliferation-associated molecular markers in neuroblastomas.

Materials and methods

Human Tissue and Tumor Classification

A total of 37 archival paraffin-embedded neuroblastomas of years 1967–2001 were obtained from the Turku University Central Hospital and the Tampere University Hospital, Finland. There were 34 primary tumors and three metastases included in the analysis series. Primary tumors of three patients with metastases were not available. One primary tumor was intracranial.¹³ Two primary tumor samples were cytotoxically pretreated before surgery.

All the tumors were immunohistochemically stained and microscopically re-evaluated by two experienced pathologists. For histological typing and grading (excluding the intracranial tumor and two cytotoxically pretreated tumors), the International Neuroblastoma Pathology Classification (International Classification) was applied.¹⁴ The 34 classified tumors (31 primary tumors and three metastases) comprised 31 neuroblastomas (14 undifferentiated, eight poorly differentiated, and nine differentiating), two ganglioneuroblastomas (one nodular, one intermixed) and one ganglioneuroma. Of these 34 cases, nine (26%) were with favorable, and 25 (74%) with unfavorable histology. Follow-up of the patients was conducted by review of outpatient medical records. The clinical stage of 22 cases could not be evaluated according to the International Neuroblastoma Staging System (INSS),¹⁵ because the tumors were diagnosed before 1993, and therefore the clinical evaluation of dissemination status was inadequate.

A total of 19 paraffin-embedded bone marrow samples from six patients were available for the CISH analysis, but the original histopathological analysis of the samples revealed only two certain and one uncertain bone marrow involvements. Therefore, we selected these three samples, which were from the same patient at different time points, for the CISH analysis. The patient was diagnosed in 2001. The primary tumor was undifferentiated neuroblastoma (stage 4) with unfavorable histology.

Hotspot Selection with MIB-1 Antibody

Tumor cell proliferation was determined with the monoclonal antibody MIB-1 against the Ki-67 protein (clone MIB-1, 1:110 dilution, DakoCytomation, Denmark). Briefly, 5 µm-thick sections were cut from paraffin-embedded specimens, mounted on slides and dried overnight at 37°C. Immunoreactivity was restored by microwave pretreatment (2 × 7 min, 850 W) in Tris-EDTA (pH 9.0). The primary antibody was incubated on the sections for 25 min at room temperature, and immunostaining was performed in an automated immunostainer (TechMate™ 500 Plus, DakoCytomation, Denmark) using the biotin–streptavidin–peroxidase procedure with diaminobenzidine (DAB) as the chromogen (ChemMate Envision Detection Kit, DakoCytomation, Denmark). Counterstaining was carried out using 0.4% ethyl green in acetate buffer for 15 min. The whole tumor specimen was evaluated for the number of MIB-1-stained cells with transmitted light microscopy (Zeiss), and hotspots, which were areas expressing quantitatively the highest number of immunopositive nuclei, were analyzed with a computer-assisted image analysis as described.¹⁶ The proliferation index of the hotspots was the percentage expressing the ratio of brown and green nuclei in at least 1000 cells (mean 1945 cells,

median 1796 cells) and at least 20 microscopic fields ($\times 400$ magnification).

Hotspot Tumor Tissue Microarray

Tissue microarrays of neuroblastomas were constructed from a total of 37 formalin-fixed, paraffin-embedded blocks. The hotspots were determined as described above. After marking the hotspot on each slide, the donor block was positioned for sampling based on a visual alignment with the corresponding hotspot. The block surface was punched with a tissue arrayer (Beecher Instruments, USA), and the cylindrical tissue column (\varnothing 2 mm) was transferred to a corresponding receiver pore of the recipient paraffin block with defined array coordinates. After the block construction was completed, 5 μm -thick sections of the resulting tumor tissue microarray block were cut with a microtome.

Chromogenic *In Situ* Hybridization

MYCN amplification status in neuroblastomas was determined by CISH analysis with a digoxigenin-labeled probe complementary to the *MYCN* gene (Spot-Light N-Myc Probe, Zymed, South San Francisco, CA, USA) as described.¹⁷ Briefly, hotspot tumor tissue microarray slides and three bone marrow samples were hybridized after deparaffination, denaturation and dehydration with 10 μl of probe cocktail (2 μl of digoxigenin-labeled *MYCN* probe, 1 μl of 9.9 $\mu\text{g}/\mu\text{l}$ human placental DNA, and 1 μl of 1 $\mu\text{g}/\mu\text{l}$ DNA (Roche Molecular Biochemicals, Mannheim, Germany), and 6 μl of master mix (Rummukainen *et al*, 2001)). Hybridization was carried out after codenaturation of the probe mixture in a humid chamber at 37°C for 16–24 h. After hybridization, the slides were washed, and the *MYCN* probe detected as described.¹⁷ Microscopy was performed after light counterstaining with hematoxylin with a transmitted light microscope (Zeiss). In every hotspot tumor tissue microarray sample, 100 nonoverlapping tumor cell nuclei were randomly scored to determine the number of *MYCN* signals. A tumor sample was considered to be *MYCN* amplified, when 6.00 or more nuclear signals per cell in an average of 100 nuclei were seen, or when tumor nuclei showed large clustered signals. No adjustment for potential hyperploidy was made.

Immunostaining of Id2 and Nestin

Hotspot tumor tissue microarray slides were used for conventional immunostainings. After the slides were deparaffinized, microwave pretreatment (2 \times 5 min, 750 W) in 0.01 mol/l citrate buffer (pH 6.0) was used for all antigen retrievals. Rabbit anti-human nestin IgG antibody at a concentration of 1 $\mu\text{g}/\text{ml}$ (Immuno-Biological Laboratories, Japan),

and rabbit anti-Id2 polyclonal antibody at a concentration of 2–4 $\mu\text{g}/\text{ml}$ (Zymed Laboratories, San Francisco, CA, USA) were used as primary antibodies. All antibody incubations were carried out overnight at 4°C. In immunohistochemistry, antibody detection was carried out using the anti-rabbit HRP polymer (PowerVision; ImmunoVision Technologies, Daly City, CA, USA). Immunoreaction was visualised with 3-aminoethyl-carbazole (AEC) for 10 min (ready-to-use, LabVision, Fremont, CA, USA), or DAB as chromogen. Slides were slightly counterstained with hematoxylin, and mounted with Faramount (DakoCytomation). In immunofluorescence, the Alexa Fluor 594 chicken anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA) was used, and slides were mounted with Immu-Mount (Shandon, USA).

Statistical Analyses

Differences between two groups in categorical data were analyzed by means of the Pearson's χ^2 test. Overall survival analysis was computed by means of the Kaplan–Meier method and the difference between the curves was compared with the log-rank test. Differences of mean values between multiple groups were analyzed by using the one-way Anova test. All statistical analyses were performed with SPSS 12.0 for Windows, and *P*-values of <0.05 were considered statistically significant.

Results

The CISH Technique in the Analysis of *MYCN* Amplification Status

CISH analysis of all 37 neuroblastoma specimens in hotspot tumor tissue microarrays was successfully performed, but two specimens (*MYCN* non-amplified) were excluded from statistical analyses on account of cytotoxic pretreatment before biopsy. Eight (23%) neuroblastomas out of 35 were shown to contain *MYCN* amplification with CISH, and seven of these positive samples were primary tumors. In general, the *MYCN* unadjusted copy number varied between 1.85 and strongly hybridized clusters, which were not countable. The highest countable copy number was 5.55. One tumor with the *MYCN* copy number of 3.01 (Figure 1a) and another with strongly hybridized clusters (Figure 1b) have been chosen to demonstrate the CISH results. Interestingly, the presence of *MYCN*-amplified tumor cells in two out of three paraffin-embedded bone marrow samples from a single patient with metastatic neuroblastoma was detected with CISH without difficulty (Figures 1c and d). These two samples were the same, which showed clear bone marrow involvement in the original histopathological analysis. In the *MYCN* nonamplified sample, there was no clear evidence of meta-

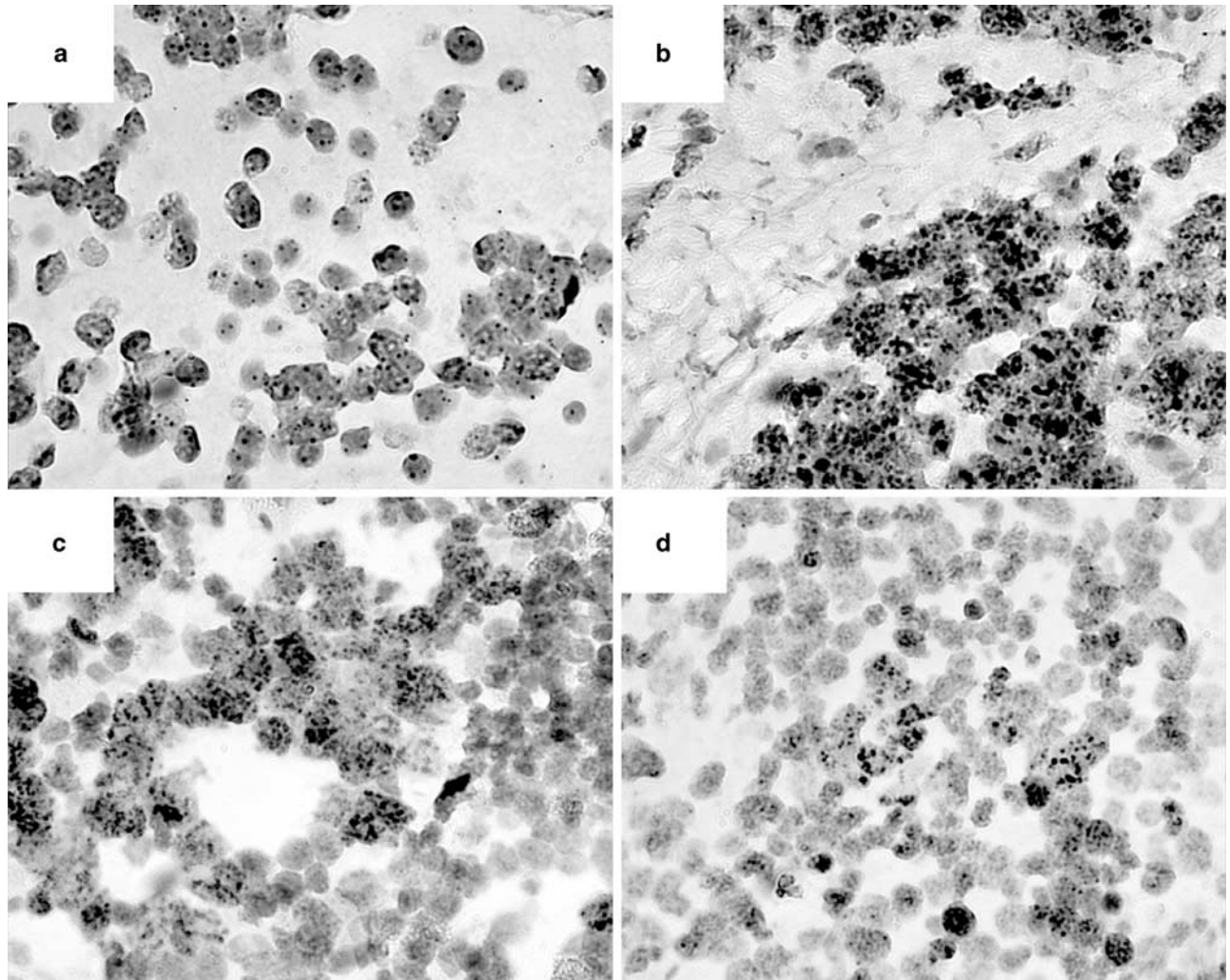


Figure 1 Chromogenic *in situ* hybridization (CISH) with the *MYCN* probe. (a) Poorly differentiated neuroblastoma with the *MYCN* copy number of 3.01. (b) Undifferentiated neuroblastoma with *MYCN* clusters. (c) Metastatic *MYCN*-amplified neuroblastic cells of undifferentiated neuroblastoma in bone marrow before, and (d) after cytotoxic treatment.

static disease in the original histopathological analysis.

MYCN amplification (*MYCN* copy number ≥ 6.00) had strong prognostic significance ($P=0.0006$), as shown in an overall survival plot in Figure 2a. The *MYCN* copy number was between 3.00 and 5.99 in seven tumors, which did not differ prognostically from 20 nonamplified tumors (Figure 2b). As the *MYCN* copy numbers varied from 1.85 to uncountable clusters, the use of continuous variables in statistical analysis was not applicable. *MYCN* amplification status in 16 out of 35 tumors was measured by Southern blot hybridization at the time of diagnosis. Southern blot analysis detected *MYCN* amplification (≥ 10 copies) in three out of the 16 tumors (19%), and all *MYCN*-amplified tumors had strongly hybridized clusters in CISH analysis. All 13 nonamplified neuroblastomas had *MYCN* copy numbers below 6.00 in CISH analysis.

Significance of Hotspot Proliferation Indices

The proliferation indices of hotspots were measured with the MIB-1 antibody against Ki-67, and the values ranged from 2.9 to 63.2, with a mean value of 28.5 ± 17.9 , among 34 histopathologically classified tumors (International Classification). A statistically significant ($P=0.040$) association between proliferation index and the International Classification was found, as especially undifferentiated neuroblastomas had higher proliferation indices (Table 1). In addition, nine tumors with favorable histology according to the International Classification had a mean proliferation index 18.5 ± 15.9 , whereas 25 prognostically unfavorable tumors had a higher mean proliferation index 32.1 ± 17.4 ($P=0.048$). In contrast, an association between proliferation index and stages was not significant ($P=0.589$) among those 15 tumors, which were staged according to INSS¹⁵ (Table 1). However, an

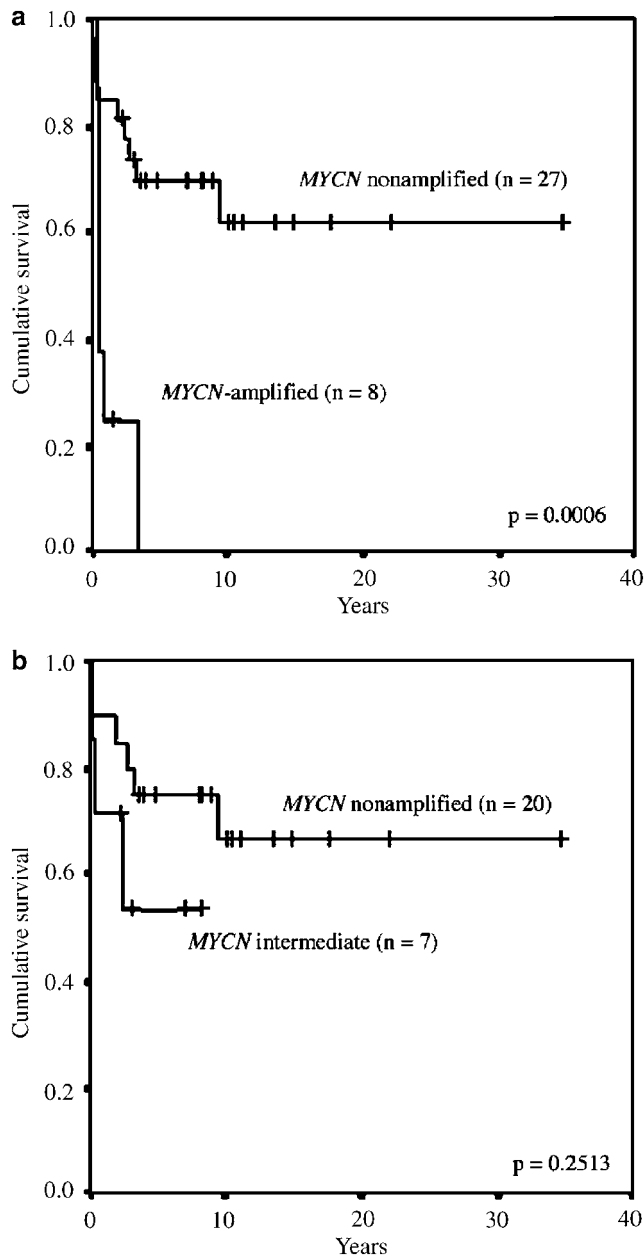


Figure 2 (a) Overall survival of MYCN-amplified vs nonamplified patients, and (b) survival of patients with intermediate (3.00–5.99) vs low MYCN copy numbers.

obvious association between higher proliferation indices and stages was discovered, as stage 1 and 2 neuroblastomas (four tumors) had a mean proliferation index 21.4 ± 12.4 and stage 4 neuroblastomas (11 tumors) had a mean proliferation index 33.1 ± 19.8 ($P=0.294$). There were no stage 3 neuroblastomas. Furthermore, MYCN-amplified tumors had higher mean proliferation index ($P=0.047$) than MYCN single copy tumors (Table 1). Two cytotoxicity pretreated primary tumors (MYCN single copy) were excluded from the analysis. In summary, the neuroblastoma growth fraction (proliferation index) in hotspots, assessed

Table 1 Distribution of a mean proliferation index according to grouping by the histological type, stage and MYCN status of neuroblastomas

International classification (INPC)	Proliferation index (mean)	N	s.d.
Undifferentiated neuroblastoma	38.3	14	18.3
Poorly differentiated neuroblastoma	20.6	8	14.7
Differentiating neuroblastoma	21.9	9	11.5
Ganglioneuroblastoma	35	2	25.2
Ganglioneuroma	3.7	1	—
<i>International neuroblastoma-staging system (INSS)</i>			
Stage 1	21.7	3	15.2
Stage 2	20.5	1	—
Stage 3	—	—	—
Stage 4	33.1	11	19.8
<i>MYCN amplification</i>			
MYCN amplified	38.9	8	18.9
MYCN nonamplified	24.6	27	16.6

by using the monoclonal MIB-1 antibody, has a significant association with histological type, histopathological prognosis and MYCN amplification in neuroblastomas.

Id2 and Nestin Expression

Id2 antibody gave very faint and unreliable immunohistochemical staining results. Therefore, we used a fluorescent secondary antibody and confocal microscopy to detect Id2-positive signals. Id2 expression was detected in seven (22%) out of 32 neuroblastomas, when staining in more than 50% of the tumor cells was considered a positive case. Five samples could not be interpreted due to detached samples and unreproducible staining patterns. In our series, Id2 expression did not associate with INPC, INSS, proliferation indices, histopathological prognosis, or outcome. None of the seven MYCN-amplified tumors expressed Id2 protein. Interestingly, six out of seven Id2-expressing neuroblastomas expressed nestin ($P=0.051$).

Nestin immunohistochemical staining results were interpreted without difficulty, and detectable levels of nestin expression were observed in 19 (54%) of 35 neuroblastomas, when staining in more than 50% of the tumor cells was considered a positive case. Two samples could not be interpreted due to detached samples. The immunostaining pattern in all positive cells was cytoplasmic (Figure 3). Most interestingly, only one out of seven MYCN-amplified tumors showed nestin expression in hotspot analysis ($P=0.018$). Nestin expression did not correlate with INPC, INSS, proliferation indices, histopathological prognosis, or outcome.

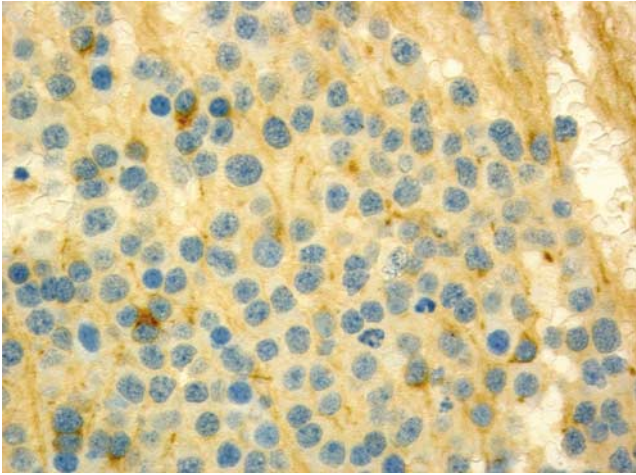


Figure 3 Cytoplasmic nestin expression in poorly differentiated neuroblastoma. DAB staining.

Discussion

The poor availability of fluorescence microscopes in clinical diagnostics has been one of the major obstacles for the widespread use of the fluorescence *in situ* hybridization technique,¹⁸ which has been most often used to detect *MYCN* amplification of archival neuroblastoma samples. We have formulated a new easily assessable strategy to assess this only prognostically significant example of oncogene activation in neuroblastomas. CISH-detected *MYCN* amplification status of the hotspots showed that the strictly defined selection of a single focus of a whole tumor sample appears to be representative. This is indicated by the observation that the results of CISH and Southern blot hybridization analysis of *MYCN* amplification did not differ from each other, and the fact that the proportion of CISH-detected *MYCN*-amplified tumors (23%) is in accordance with the common view of the frequency of *MYCN* amplification. Also, the *MYCN* copy numbers more than 5.55 (cutoff value 6.00) in the hotspots led to a significantly diminished survival. Similar to our previous CISH studies concerning a *HER-2/neu* copy number in human breast cancers,¹⁹ it seems that the measurement of ploidy is not essential prior to the *MYCN* gene copy number assessment. The unadjusted analysis with a cutoff value of 6.00 may be appropriate at least when intermitotic cells compose the majority of the tumor sample. However, the cutoff value of 6.00 excludes an intermediate increase of the *MYCN* copy number (between 3.00 and 5.99 copies per nucleus), which may reflect the initiation of *MYCN* amplification. The intermediate increase of the *MYCN* copy number was easily detected with CISH, but these tumors did not differ prognostically from nonamplified tumors in overall survival (Figure 2b). Previously, an intermediate increase in the *MYCN* copy number in a single focus (\varnothing 2 mm)

of neuroblastomas did not seem to lead to diminished survival.²⁰ It should be noted that the chromosome two copy number may distort the unadjusted CISH analysis of intermediate *MYCN* copy numbers. In order to estimate the role of low-level *MYCN* copy numbers in survival prognostics, further studies of the unadjusted CISH analysis are needed.

In consequence of intratumoral heterogeneity in neuroblastomas, an overall estimation of various tumor progression-associated parameters in one randomly selected spot of a tumor may lead to deceptive conclusions. In account of an idea that malignant changes in geno- and phenotypic level associate with accelerated proliferation, we selected the spot with highest proliferation index for further analyses. As *MYCN*-amplified tumors had higher proliferation indices (38.39 ± 18.90 vs 24.63 ± 16.64 , $P = 0.047$), it is tempting to speculate that genetic aberrations are associated with higher proliferation rates. In a previous study of 87 neuroblastomas, it was also shown that Southern blot hybridization detected *MYCN*-amplified neuroblastomas (22%) were associated with disease progression and higher proliferation indices (36.4 ± 24.4 vs 18.7 ± 16.9 in tumors without *MYCN* amplification, $P = 0.0034$).²¹ In our study, proliferation indices of the hotspots also correlated significantly with other prognostic variables, such as histological subtype and INPC prognosis. Larger studies are needed to determine possible subgroups of proliferation indices, which could contribute to the prognostic histopathological analysis.

It has been reported by Lasorella *et al* that a high correlation exists between *MYCN* and Id2 expression in immunohistochemical analysis of primary neuroblastomas. They also showed that the expression of Id2 strongly predicts poor outcome.⁹ In our study, the expression of the indirect proliferation stimulant Id2 protein did not associate with *MYCN* amplification, proliferation index or outcome in hotspot analysis. Furthermore, Id2 expression did not associate with histological type despite the assumption, that Id2 causes a differentiation arrest. Our results support the previous findings, which have shown that *ID2* mRNA expression is not associated with *MYCN* mRNA expression levels or *MYCN* amplification in primary neuroblastomas.^{10,11} Therefore, Id2 expression does not seem to serve as an independent prognostic marker in neuroblastomas.

Recent *in vitro* findings suggest that nestin expression in neuroblastic cells might correlate with *MYCN* amplification, and that nestin is present in the nucleus of *MYCN*-amplified neuroblastic cells.¹² According to our *in vivo* findings, the nestin expression does not correlate with *MYCN* amplification, INPC, INSS, proliferation indices, histopathological prognosis, or outcome. Furthermore, the nestin expression pattern was shown to be cytoplasmic in immunohistochemical analysis.

Thus, nestin appears to have a less important role in neuroblastomas, as might have been expected.

In summary, the implementation of CISH in the evaluation of *MYCN* copy number in neuroblastomas allows pathologists to detect this significant prognostic factor without difficulty. As disseminated *MYCN*-amplified tumor cells can also be detected with CISH in bone marrow samples, an efficient detection of *MYCN*-positive residual disease as well as determination of response to treatment is possible. In addition, the hotspot approach describes the strict selection criteria for tumor spots of heterogeneous neuroblastomas. For researchers, the presented hotspot CISH methodology may serve as a basis for a uniform *MYCN* assessment protocol, which allows the comparison of the results of different groups. The methodology enables a re-evaluation of archival neuroblastoma specimens in tumor banks, and together with the tumor tissue microarray method a high-throughput analysis can be carried out. The hotspots seem to be prognostically representative, at least in the analysis of *MYCN* status and proliferation indices, and therefore the idea might be applicable to general use in studies of intratumorally heterogeneous tumors.

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