Role of nm23 in neuroblastoma. From genetic aberrations to pathways abnormalities
Godfried, M.B.

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Chapter 5

MYCN and nm23-H1 protein expression are closely correlated and predict an adverse outcome in neuroblastoma.

(submitted)
MYCN and nm23-H1 protein expression are closely correlated and predict an adverse outcome in neuroblastoma

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Keywords: neuroblastoma, nm23-H1, nm23-H2, MYCN, chromosome 17q gain, immunohistochemistry, prognosis.
Abstract

**Purpose:** As we previously showed that the 17q genes nm23-H1 and -H2 are up-regulated by the MYCN oncogene in neuroblastoma, we analyzed the relationship between the protein levels of MYCN, nm23-H1, -H2 and gain of chromosome 17q in a neuroblastoma series and assessed whether there is a prognostic value for each of these proteins. **Methods:** Tissue micro arrays with 113 neuroblastomas were constructed. Protein levels were determined by immunohistochemical staining. **Results:** Nm23-H1 was expressed in 77 (70%) of 110 analyzed cases and nm23-H2 was expressed in 85 (79%) of 108 cases. Both were co-expressed in 71 (67%) of the 106 evaluable cases. MYCN was expressed in 39 of 108 (36%) cases. In the 36 of 38 evaluable cases with MYCN expression we also demonstrated nm23-H1 and/or nm23-H2 expression. Chromosome 17q gain was present in 33 out of 75 (44%) tumor samples. We found nm23-H1 and H2 expression in 26 out of the 29 (90%) evaluable cases with chromosome 17q gain. Added together, we found MYCN expression and/or chromosome 17q gain in 64% of the nm23-H1 and/or H2 expressing neuroblastoma samples. In this cohort we found that nm23-H1 and MYCN protein expression are significant predictors of a poor prognosis ($p<0.01$). MYCN protein expression identified a larger group of patients with a poor prognosis than MYCN amplification.

**Conclusion:** The close correlation in vivo between nm23 expression and the presence of MYCN expression and/or gain of chromosome 17q supports the previously described transcriptional up-regulation of the nm23-H1 and H2 genes by MYCN and gain of chromosome 17q. MYCN protein expression is predictive of a poor outcome in neuroblastoma patients.
Introduction

Neuroblastoma is a malignancy of childhood which arises from cells of the sympathetic neuronal lineage, which are neural-crest derived. The tumor has a wide range of clinical behavior. Clinical outcome is strongly correlated with tumor stage and age at the time of diagnosis. Infants under the age of 1 year and children older than 1 year of age and with localized disease (INSS 1-2B) have a good prognosis after surgical resection alone. However, children older than 1 year of age with widely disseminated disease (INSS 3-4) have a poor prognosis regardless of intense treatments of any type. Specific genetic aberrations in neuroblastoma are associated with a poor prognosis.

Amplification of the MYCN oncogene is the best studied biological prognostic factor, and is associated with advanced stage disease and rapid tumor progression. Other predictors of poor outcome include loss of chromosome 1p and gain of chromosome 17q.

In a previous report we demonstrated that the two genes nm23-H1 and H2 are both induced by MYCN. In addition, we showed that both genes are located at the edge of the minimal region of gain of chromosome 17q. Nm23-H1 and H2 encode for nucleoside diphosphate kinases NDPK-A and B, respectively, and these proteins are both involved in nucleoside triphosphate production. This gene family consisting of 8 members was discovered on the basis of the reduced nm23-H1 expression in a comparison of highly metastatic and less metastatic mouse melanoma cell lines. The biological role of nm23-H1 and H2 in cancer is still a matter of debate. Although nm23-H1 mutations have been reported in neuroblastoma, this could not be confirmed in any other study and the in vivo relevance remains unclear. In vertebrates no other mutations have been described. For certain cancer types, high nm23-H1 expression is associated with a decreased metastatic potential, e.g. in breast cancer and melanoma. However, in neuroblastoma and non-Hodgkin lymphoma, high nm23-H1 expression is correlated with an unfavorable prognosis. Nm23-H2 showed less significant correlations with progression than nm23-H1.

The oncogenic effect of the MYCN protein has been demonstrated in several studies. In vitro studies demonstrated alteration of proliferation and phenotype after inducing MYCN expression in neuroblastoma cell lines. Transgenic mice with ectopic MYCN expression in neuroectodermal cells developed neuroblastomas. The aggressive phenotype of neuroblastoma is thought to be a result of the high MYCN expression. However, in clinical trails the prognostic relevance of MYCN expression in patients remains unclear. In several studies a correlation was found between levels of
MYCN expression and poor outcome\(^{21-29}\). In contrast, other studies have shown that the MYCN mRNA and protein is not predictive of outcome\(^{17,28,30-35}\).

To extend the finding of the up-regulation of 17q genes nm23-H1 and nm23-H2 by MYCN overexpression in vitro, we measured the protein expression of the three mentioned genes in vivo.

To analyze co-regulation in the tumors we constructed a tissue micro array (TMA) containing tumor cores of neuroblastomas. In addition, we studied the prognostic value of nm23-H1, H2 and MYCN protein expression.

**Material and methods**

*Samples collection and clinical characteristics*

We selected NB from 113 patients from the period 1968-2000. Paraffin-embedded tumor specimen where collected from the Emma Kinderziekenhuis/Academic Medical Center, Pediatric Oncology Center of the Southern Netherlands, and the Sophia Kinderziekenhuis. Patients were staged accordingly to the International Neuroblastoma Staging System (INSS) by utilizing CT imaging techniques and radiinated-metaiodobenzyl-guanine (MIBG) scanning. All tumor samples were centrally reviewed by one pathologists. Stage 1, 2a and 2b tumors were treated by surgical resection, while stage 3 and 4 patients received different chemotherapeutical agents and regimens. Since 1990 stage 3 and 4 patients received \(^{131}\)IIBG incorporation as first line treatment. Stage 4s received treatment only when vital functions were endangered; in most cases this consisted of radiotherapy or a combination of low dose chemotherapy and corticosteroids.

Genetic analysis was performed on tumor samples containing more than 60% tumor cells.

*Tissue micro array*

A TMA was constructed from formalin-fixed, paraffin-embedded tumor specimens. Areas of pure tumor cells were pre-selected by localizing and demarking tumor cells in a hematoxylin and eosin stain section of the original tumor slide. Three 0.6-mm diameter punches were obtained by a TMA puncher (Beecher instruments, Silver Spring, MD, USA). The array contains triple punched 357 histospots of neuroblastomas from 113 patients. The TMA includes control tissue, normal kidney (4x), lymphomas (2x) and normal colon (2x). Sections of 5\(\mu\)m were cut from the microarray.
**Immunohistochemistry and scoring**

First standard hematoxylin and cosin stain was performed to check for tumor cell content of each histospot punch. Histospots containing <10% tumor were excluded from further analysis. Subsequently the arrays were stained for nm23-H1 protein (monoclonal antiNDP Kinase β, clone KM1103, Kamiya Biomedical Company, USA; 1:6400), nm23-H2 protein (monoclonal antiNDP Kinase α, clone KM1121, Kamiya Biomedical Company, USA; 1:1600) and NMYC protein (Ab-1, OP13L, monoclonal, Oncogene, USA; 1:5000). Briefly, TMA sections were deparaffinized with xylene rinses and then transferred through two changes of 100% alcohol. Antigen retrieval was performed by 15’ boiling of the slides with sodium citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked by a 30’ 2.5% hydrogen peroxidase buffer. Slides were rinsed 2’ in 1x PBS/0.01% Triton and 1xTriton. Primary antibody was applied for over night at 4°C temperature. Slides were rinsed in 1xPBS and primary antibody was detected using horseradish peroxidase-labeled, secondary antibody using PowerVision Kit (LabVision, USA).

Scoring of protein expression was performed by two pathologists independently using a binary scale (yes or no expression). Tumors were scored positive when more than 10% of the tumor cells were positively stained.

**Southern blot analysis**

Southern blot analysis was done according to standard procedures. Briefly, filters with TaqI digestions of 10 μg DNA tumor and corresponding constitutional DNA were hybridized with the following polymorphic VNTR probes, in distal to proximal order: D17S74/CMM86, D17S24RMU3 and D17S4/THH59. All probes are placed on chromosome 17q according to the CEPH map (www.cephb.fr). In case of an overrepresentation of 17q DNA an allelic imbalance is found between the tumor lane and the corresponding control lane. To detect MYCN amplification copy number mutual densiometric analysis was performed after co-hybridization of pNB1, MYCN exon 2 probe, together with a single copy probe pL2.3. Using Fosfor Imager (Molecular Dynamics) for quantification of MYCN copy number.
Statistics analysis

Association between the scores of the histospots-stains and the different immunohistopathological and clinicopathological parameters were evaluated with the chi-square test. Event free survival (EFS) and overall survival (OS) were clinical endpoints in our analysis. Events were defined as death or recurrence of disease after achievement of complete remission and as disease progression during therapy. We estimated the 5 years survival for the subgroup defined by the above mentioned parameters from the calculated Kaplan-Meier curves. Prognostic significance of the parameters on EFS and OS was determined with the Mantel-Cox log-rank test. Analysis was performed with SPSS (SPSS for Windows, USA).
Results

Patient characteristics

The clinical and biological features of the study cohort is listed in table 1. Of the 113 cases there were 59 (52%) girls and 54 (48%) boys. At diagnosis thirty-eight (34%) of the patients were under the age of 1 year, 75 (66%) were 1 year or older. Forty-six (41%) had a favorable stage (INSS 1, 2, 4s) and 67 (59%) had an unfavorable stage (INSS 3, 4). Of the biological variables, 75 of the 113 cases were analyzed for chromosome 17q copy number, 42 (56%) cases had chromosome 17q gain and 33 (44%) had no chromosome 17q gain. MYCN copy number was assessed in 83 of the 113 cases, 71 (86%) were MYCN single copy and 12 (14%) had MYCN amplification.

Nm23-H1 and H2 expression in neuroblastoma

We measured the nm23-H1 and H2 expression by immunohistochemical (IHC) staining. Expression pattern of the nm23 proteins was predominantly found in the cytoplasm, whereas nm23-H2 also occurred in the nucleus (figure 1). This is in agreement with the literature. Of the 113 cases, 110 could be evaluated for nm23-H1 expression: 77 (70%) showed expression whereas 33 (30%) lacked nm23-H1 expression (table 1). Concerning nm23-H2, 107 cases were evaluable, 23 (21%) had no expression, while in 85 (79%) there was nm23-H2 expression. In 71 (67%) of the 106 available cases we found co-expression of both proteins. Nineteen (18%) cases lacked expression of any of the 2 proteins (table 2). There was a significant correlation (p=2.0x10^-10) of nm23-H1 and H2 co-expression. Overall, nm23-H1 and H2 are frequently expressed in neuroblastoma and are strongly co-expressed.

MYCN expression and MYCN copy number

MYCN protein expression was present in 39 out of 108 evaluable cases (36%). Of 83 samples, also MYCN copy number analysis had been performed of which 71 were evaluable for MYCN protein expression. Of the 11 samples with MYCN amplification, 10 also showed MYCN protein expression. In 27 out of 60 evaluable MYCN single copy cases we found MYCN expression. Thus MYCN amplification correlates closely with high protein expression, but also a large proportion of MYCN single copy cases have a high MYCN expression.
**NM23-H1 and H2 expression correlate with MYCN expression**

In 105 cases both MYCN expression and expression of the nm23 genes was scored (figure 2a). Of 38 cases expressing MYCN we demonstrated co-expression of nm23-H1 and/or H2 in 36 cases (95%). Co-expression of both nm23 genes was present in 30 cases (79%) or expression of either one of the nm23 genes in 6 cases (16%). In 2 cases with MYCN expression no nm23-H1 or H2 expression was present.

Seventeen cases showed no expression of nm23 genes nor MYCN. In 50 cases nm23-H1 and/or H2 were expressed without expression of MYCN.

This close correlation between MYCN protein expression and nm23 expression is in line with our in vitro observations that MYCN induces nm23-H1 and H2 expression by transcriptional up-regulation.

**NM23-H1 and H2 expression correlated to chromosome 17q**

In 75 cases we could reliably determine the 17q status. We found partial 17q gain in 29 (39%) out of 75 cases. Twenty-six out of 29 cases (90%) with 17q gain also showed nm23-H1 or H2 expression (figure 2b). In 23 of those cases both nm23 genes were expressed. This correlation supports the notion that 17q gain leads to up-regulation of the nm23-H1 and or H2 genes.

**Relationship between MYCN expression, chromosome 17q gain and nm23-H1/H2 expression**

In 67 cases we could evaluate the relationship between both MYCN expression and chromosome 17q gain and the nm23-H1 and H2 expression. Nm23-H1 and/or H2 was expressed in 53 out 67 (79%) cases. In 24 of 53 (45%) cases expressing nm23-H1 and/or H2, MYCN expression was present and in 25 out of those 53 (47%) cases chromosome 17q gain was demonstrated. In 34 of the 53 nm23 expressinf cases we found MYCN expression, chromosome 17q gain or a combination of both. This correlation suggest that in vivo approximatly 2/3 of the cases with up-regulation of nm23 genes can be explained by the concerted action of MYCN mediated up-regulation and gene copy number increase by chromosome 17q. In approximately 1/3 of the neuroblasomas with nm23 expression other mechanisms play a role.
**Univariate survival analysis**

To evaluate possible prognostic value of nm23-H1, H2, and MYCN protein expression we performed survival analysis. The estimated 5-years event free survival (EFS) and overall survival (OS) of the entire cohort were 45%±5% and 48%±5%, respectively. The mean follow up of the entire cohort was 52 months, the mean follow up for the patients without a clinical event was 95 months. For each factor with 2 categories the EFS was determined (table 1). There is a significant difference of 5-years EFS between infants and patients 1 year or older, 73%±7% and 31%±6%, respectively (p=0.003). Tumor stage was the strongest predictor of clinical outcome with a 5-years EFS of 83%±6% for favorable stage (INSS 1, 2, 4a) and 21%±5% for unfavorable stages (INSS 3, 4).

Of the genetic factors 17q gain was the strongest predictor of outcome. The 5-years EFS for patients with chromosome 17q gain is 20%±7% and for patients without chromosome 17q gain the 5-years EFS was 69%±8%. The 5-years EFS of the MYCN single copy cases was 54%±6%, for cases with MYCN amplification the 5-years EFS was 0%±15%.

We also analyzed the possible prognostic impact of the protein expression levels of MYCN, nm23-H1 and H2. The prognostic value of MYCN protein expression was significant (p=0.0034). Cases with MYCN expression showed a 5-years EFS of 27%±8%, while in the cases without MYCN expression the 5-years EFS is 57%±6% (table 1 and figure 3a).

Also nm23-H1 protein expression is a significant predictor of outcome. The 5-years EFS was worse for patients with nm23-H1 expression versus no expression, respectively 37%±6% and 70%±9% (p=0.0078) (figure 3b). For nm23-H2 expression we found no significant difference in outcome.

**Multivariate survival analysis**

Expression of nm23-H1, H2, MYCN and gain of chromosome 17q are correlated in neuroblastoma. Therefore, their individual prognostic values are interrelated with each other. To identify their independent prognostic power we performed pair wise a multivariate Cox regression analyses (table 3).

In pair wise analysis of MYCN expression and MYCN amplification it is shown that MYCN expression remains significantly correlated with prognosis whereas MYCN amplification is no longer significant. In the pair wise model the hazard ratio for MYCN expression is 2.8 (1.3-5.7; p=0.006) and for MYCN amplification the hazard ratio was 1.8 (0.8-4.2; p=0.166). This indicates
that MYCN expression identifies a larger group of bad prognosis patients than MYCN amplification. In the group of 60 MYCN single copy cases MYCN expression indeed identifies additional bad prognosis patients (n=). The 5-years EFS was 25%±10% for the MYCN single copy cases with MYCN expression and 66%±7% for MYCN single copy cases without MYCN expression (n=) (p=0.0129) (figure 3b). The prognostic value of MYCN protein expression was also determined in the subset of patients under 1 year of age (n=) or older than 1 year at diagnosis (n=) without MYCN amplification. MYCN protein was not significantly predictive of outcome in infants. The 5-years EFS of the infants with and without MYCN expression was 63%±17% and 88%±7%, respectively (p=0.12). In contrast to the results observed with infants, however, MYCN protein expression was highly predictive of outcome in children older than 1 year of age at diagnosis. Among these patients (n=), the 5-years EFS with and without MYCN protein expression was 9%±8% and 53%±10%, respectively (p=0.0027).

The pairwise comparison of the prognostic impact of nm23-H1 expression and chromosome 17q gain showed that 17q gain retains prognostic power, whereas nm23-H1 expression is no longer predictive of outcome (table 3). This might indicate that, besides the nm23 genes, also other genes play an important role in determining the biological effect of chromosome 17q gain.

Discussion

In this study we analyzed the nm23-H1, H2 and MYCN protein expression in 113 neuroblastomas by using a tissue micro array. This protein expression analysis confirms our previous in vitro observations that nm23-H1 and H2 are also up-regulated in vivo by MYCN. We also showed that gain of chromosome 17q is associated with nm23-H1 and H2 expression, suggesting that 17q gain also up-regulates nm23-H1 and H2 expression. In this study, >60% of the cases with nm23-H1 and/or nm23-H2 expression have 17q gain, MYCN expression or a combination of both. This group may be larger as we have used Southern blot analysis to detect partial 17q gain. Molecular techniques can not detect 17q gain leading to a balanced allele distribution. The use of FISH to detect partial 17q gain might identified those additional cases with 17q gain. This still might leave a subset of neuroblastomas in which the nm23-H1 and/or H2 expression can not be explained by gain
of 17q or MYCN expression. In those cases another biological factor might be involved in inducing nm23-H1 and H2.

We found MYCN protein expression in 36% of our series. It has been reported that tumors that lack MYCN amplification can express the MYCN protein\textsuperscript{55,26}. Also, previous observations from aggressive neuroblastoma cell lines with high MYCN protein expression without amplification have been reported\textsuperscript{39,40}. The basis for MYCN protein expression in the absence of gene amplification is unclear.

Importantly we found that MYCN protein has a prognostic value, also in MYCN single copy neuroblastoma. MYCN expression did not appear to be predictive of poor outcome in infants, whereas in older children, expression of this oncoprotein retained significant prognostic power. This indicates that MYCN protein expression identifies a larger group of patients older than 1 year of age with a bad prognosis than MYCN amplification.

Several reports failed to demonstrate prognostic power of MYCN expression\textsuperscript{17,28,30-35,41}, whereas others did find a predictive value\textsuperscript{21-29,41}. One possible explanation for this discrepancy is that some reports measured MYCN expression of the mRNA level by using RT-PCR, which involves a amplification step that might lead to artefacts\textsuperscript{17,24}. Also tissue handling and quality of the tumor mRNA could influence the RT-PCR and Northern blot results\textsuperscript{21,27-33,35,41}. In addition, post-transcriptional regulation can also influence the cellular MYCN protein levels.

In 4 studies MYCN expression was studied at the protein level\textsuperscript{21,25,26,34}. All studies concluded a predictive value for MYCN protein expression except for one. Seeger et al\textsuperscript{34}, did not find a prognostic value for MYCN protein expression. The difference between these IHC studies findings may be due to the applied antibodies. In the 3 positive studies monoclonal antibodies specific for MYCN on formalin fixed section were used. Seeger et al. used a polyclonal anti-MYCN antibody for cryostat sections. This can lead to false positives since it has been demonstrated that also other members of the myc family are expressed in neuroblastoma\textsuperscript{35}. In our study we also relied on the monoclonal MYCN antibody on formalin fixed sections. Together the 4 studies using the monoclonal antibodies both showed additional prognostic value of MYCN expression in MYCN single copy cases. In total these studies encompass 83 patients. Despite the contradicting results of MYCN expression studies, we feel that the combined data on the MYCN protein expression are sufficiently encouraging to warrant evaluation in prospective clinical trials.
Acknowledgments
We kindly thank Dr. M. vd. Vijver, Netherlands Cancer Institute, for providing the TMA facilities, reviewing the TMA slides and the manuscript. This research was supported by grants from the Dutch Cancer Society (AMC97-1461) and the Stichting Kindergeneeskundig Kankeronderzoek.
**Protein staining**

<table>
<thead>
<tr>
<th>HE</th>
<th>nm23-H1</th>
<th>nm23-H2</th>
<th>MYCN</th>
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<tbody>
<tr>
<td>N287</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
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<tr>
<td>N512</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 1.** Histospots of two neuroblastoma cases stained with hematoxylin and eosin (x160; middle and x400; tips of slides), left. Cases with (N287) expression and without (N512) expression of nm23-H1, H2 and MYCN protein.
Table 1. Characteristics of 113 neuroblastoma.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>N</th>
<th>113*</th>
<th>Syrs EFS* (SE)</th>
<th>p-value*</th>
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<tbody>
<tr>
<td>sex</td>
<td>girls</td>
<td>59</td>
<td>(52%)</td>
<td>42% (7%)</td>
<td>0.005</td>
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<tr>
<td></td>
<td>boys</td>
<td>54</td>
<td>(48%)</td>
<td>49% (7%)</td>
<td></td>
</tr>
<tr>
<td>age at diagnosis</td>
<td>&lt; 1 year</td>
<td>38</td>
<td>(34%)</td>
<td>73% (7%)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>&gt;= 1 year</td>
<td>75</td>
<td>(66%)</td>
<td>31% (16%)</td>
<td></td>
</tr>
<tr>
<td>Stage (INSS)</td>
<td>1, 2, 4s</td>
<td>46</td>
<td>(41%)</td>
<td>83% (6%)</td>
<td>0.00005</td>
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<tr>
<td></td>
<td>3, 4</td>
<td>67</td>
<td>(59%)</td>
<td>21% (5%)</td>
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<tr>
<td>chromosome 17q</td>
<td>no gain</td>
<td>42</td>
<td>(56%)</td>
<td>69% (8%)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>gain</td>
<td>33</td>
<td>(44%)</td>
<td>20% (7%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ne</td>
<td>38</td>
<td></td>
<td></td>
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<tr>
<td>MYCN (DNA)</td>
<td>single copy</td>
<td>71</td>
<td>(86%)</td>
<td>54% (6%)</td>
<td>0.0092</td>
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<tr>
<td></td>
<td>amplified</td>
<td>12</td>
<td>(14%)</td>
<td>0% (15%)</td>
<td></td>
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<tr>
<td></td>
<td>ne</td>
<td>30</td>
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<tr>
<td>MYCN expression</td>
<td>negative</td>
<td>69</td>
<td>(64%)</td>
<td>57% (6%)</td>
<td>0.9554</td>
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<tr>
<td></td>
<td>positive</td>
<td>39</td>
<td>(56%)</td>
<td>27% (8%)</td>
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<td></td>
<td>ne</td>
<td>5</td>
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<td>mm23-H1 expression</td>
<td>negative</td>
<td>33</td>
<td>(30%)</td>
<td>70% (9%)</td>
<td>0.9078</td>
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<td>positive</td>
<td>77</td>
<td>(70%)</td>
<td>37% (6%)</td>
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<td>ne</td>
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<td>mm23-H2 expression</td>
<td>negative</td>
<td>23</td>
<td>(21%)</td>
<td>43% (6%)</td>
<td>0.532</td>
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<tr>
<td></td>
<td>positive</td>
<td>85</td>
<td>(79%)</td>
<td>61% (10%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ne</td>
<td>5</td>
<td></td>
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* EFS: event free survival (standard error)
† HR: hazard ratio
### Table 2. Combined protein expression of nm23-H1 and H2

<table>
<thead>
<tr>
<th>nm23-H2</th>
<th>positive</th>
<th>negative</th>
<th>total</th>
</tr>
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<tbody>
<tr>
<td>nm23-H1 positive</td>
<td>71</td>
<td>12</td>
<td>83</td>
</tr>
<tr>
<td>negative</td>
<td>4</td>
<td>19</td>
<td>23</td>
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<tr>
<td>total</td>
<td>75</td>
<td>31</td>
<td>106</td>
</tr>
</tbody>
</table>

**Table 2.** Cross tab of 106 cases stained for nm23-H1 and or nm23-H2 proteins.
Figure 2. Venn diagrams of patients with expression of (a) nm23-H1 and/or nm23-H2 and/or MYCN proteins, (b) patients with expression of nm23-H1 and/or nm23-H2 and/or gain of chromosome 17q.
Figure 3. Kaplan-Meier analysis of EFS for patients with (a) nm23-H1 expression (p=0.0034), (b) MYCN expression (p=0.0078), (c) MYCN expression in the group of MYCN single copy patients (p=0.0129).
### Table 3. Multivariate survival analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR‡ (95% CI)</th>
<th>p-value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>pair1:</td>
<td></td>
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<tr>
<td>MYCN expression</td>
<td>2.8 (1.3-5.7)</td>
<td>0.006</td>
</tr>
<tr>
<td>MYCN(DNA)</td>
<td>1.8 (0.8-4.2)</td>
<td>0.166</td>
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<tr>
<td>pair2:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nm23-H1 expression</td>
<td>1.5 (0.7-3.3)</td>
<td>0.342</td>
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<tr>
<td>chromosome 17q</td>
<td>4.0 (1.9-8.3)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

#p-value of a test against the null hypothesis of a hazard ratio equalling 1.0.
‡ HR, Hazard ratio using Cox regression model

Table 3. Multivariate analysis.


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