Does MYCN Amplification Manifested as Homogeneously Staining Regions at Diagnosis Predict a Worse Outcome in Children with Neuroblastoma? A Children's Oncology Group Study

Lisa A. Moreau,1 Patrick McGrady,2 Wendy B. London,2 Hiroyuki Shimada,3 Susan L. Cohn,4 John M. Maris,5 Lisa Diller,6 A. Thomas Look,1,6 and Rani E. George1,6

Abstract

Purpose: MYCN amplification in neuroblastoma tumor cells is manifested primarily as double minutes (dmins), whereas in cell lines it often appears in the form of homogeneously staining regions (HSR), suggesting that HSRs are associated with a more aggressive tumor phenotype and worse clinical outcome. The aim of this study was to determine whether children with neuroblastoma in which MYCN oncogene amplification is manifested as HSRs at diagnosis have a worse prognosis than those whose tumors exhibit dmins.

Experimental Design: A retrospective analysis of primary neuroblastomas analyzed for MYCN amplification by the Children's Oncology Group between 1993 and 2004 was done. Tumors with MYCN amplification were defined as having dmins, HSRs, or both (dmins + HSRs), and associations with currently used risk group stratification variables and patient outcome were assessed.

Results: Of the 4,102 tumor samples analyzed, 800 (19.5%) had MYCN amplification. Among the 677 tumors for which the pattern of amplification was known, 629 (92.9%) had dmins, 40 (5.9%) had HSRs, and 8 (0.1%) had dmins + HSRs. Although MYCN amplification is associated with older age, higher stage, and unfavorable histology, whether the amplification occurred as dmins or HSRs did not significantly affect these risk factors. There were no differences in the event-free survival (EFS) or overall survival in patients with MYCN amplification manifested as either dmins or HSRs (5-year EFS, 35 ± 3% versus 38 ± 15%; P = 0.59). Although the eight patients with dmins + HSRs fared worse than either of the individual subgroups (EFS, 18 ± 16% versus 35 ± 3% for dmins and 38 ± 15% for HSRs), these differences were not significant.

Conclusions: MYCN amplification in any form (HSRs or dmins) is associated with a poor outcome.

DNA amplification is a mechanism by which a cancer cell acquires multiple copies of part of its genome, enabling it to overexpress oncoproteins that confer a selective growth advantage or to acquire resistance to chemotherapeutic agents (1, 2).

Gene amplification is most often manifested as paired extrachromosomal self-replicating double-minute or homogeneously staining bodies (dmins), which are acentric and atelomeric (3), or as uniformly staining, linearly integrated chromosomal segments called homogeneously staining regions (HSR; ref. 4). dmins are commonly seen in primary tumors, whereas HSRs tend to predominate in cultured neoplastic cells (5, 6). HSRs are thought to be formed by integration of multimerized sequences of dmins into chromosomes (7, 8). They are considered stable forms of amplification because they provide a faithful mechanism by which the extra copies of the gene can be passed onto the daughter cells. dmins, on the other hand, are considered highly unstable, as they tend to be lost over time by micronucleation or by uneven distribution into daughter cells (2, 9). The transition from dmins to HSRs is thought to occur through stepwise selection that confers on cells a selective growth advantage, enabling them to survive in culture through several passages. It has been suggested, therefore, that only cells able to generate stable HSRs will ultimately survive in vitro (10).

The MYCN gene was the first oncogene found to be amplified in solid tumors and has emerged as the only consistently amplified gene in neuroblastoma (11). MYCN amplification occurs in ~20% of primary neuroblastomas and is highly correlated with aggressive disease, early chemoresistance, and
a poor prognosis (12). Hence, it is one of the variables used by the Children’s Oncology Group to assign patients with newly diagnosed neuroblastoma to a high-risk group requiring intensive multimodality therapy (13). Similar to other amplified genes, MYCN amplicons are present on dmins (14) and HSRs (15), with the former typically seen in primary tumors and the latter in established human neuroblastoma-derived cell lines (16). It is generally thought that the MYCN gene initially becomes amplified as extrachromosomal dmins possibly persisting in this form and then, in a subset of cases, becomes linearly integrated into a chromosome to form HSRs (17). The clinical significance of the different cytogenetic mechanisms underlying MYCN amplification has not been analyzed, but most investigators have assumed that HSRs are associated with a worse prognosis. Here, we define MYCN amplification as HSRs, dmins, or both (HSRs + dmins) and ask whether these categories are associated with currently accepted variables of risk group stratification and with clinical outcome.

**Materials and Methods**

**Patient characteristics.** Between July 1993 and December 2004, 4,102 neuroblastoma samples were sent from institutions within the United States, Australia, and Canada to the Children’s Oncology Group Neuroblastoma Cytogenetics Reference Laboratory for analysis of MYCN gene status and DNA ploidy. Tumor staging was based on the International Neuroblastoma Staging System (18), and patients were stratified according to other known prognostic factors, such as age, stage, Shimada histology, and tumor cell ploidy (13). All patients were enrolled in biological studies sponsored by the Pediatric Oncology Group (9047) or the Children’s Oncology Group (ANBL00B1). Although patients were treated on several different regimens, the vast majority underwent some form of intensive induction chemotherapy followed by consolidation. The study reported here was conducted with parental informed consent, and institutional review board guidelines were followed for the procurement of each sample.

**Analysis of MYCN amplification.** Fluorescence in situ hybridization, done on diagnostic samples as described previously, was used to determine MYCN amplification status (19). The probe was a cosmid clone from the MYCN genomic locus or a commercially available labeled MYCN probe (Vysis, Inc., Des Plaines, IL). Briefly, touch prep slides were prepared from fresh or frozen tumor specimens or bone marrow aspirates having >30% tumor at time of initial diagnosis. The slides were fixed in Carnoy’s fixative, air dried, and dehydrated through a series of alcohol washes. The slides were then placed in 2X SSC at 37°C and again dehydrated through alcohol washes. The MYCN probe and slides were then processed according to the manufacturer’s directions (Vysis). Slides were incubated overnight, counterstained with 4’,6-diamidino-2-phenylindole, and analyzed under a fluorescence Zeiss microscope (Carl Zeiss, Inc., Berlin, Germany).

MYCN copy number was obtained by counting the number of signals present in 100 interphase nuclei. Tumors with cells containing ≥10 copies of MYCN or HSRs that hybridized to the MYCN probe were scored as amplified. In amplified cells having dmins, accurate scoring was not possible because of the coalescing of the fluorescent signals. dmins appeared as several signals throughout the nucleus, whereas HSRs were represented by a cohesive domain of attached signals (Fig. 1).

**DNA ploidy analysis.** DNA index or tumor cell ploidy was determined on fresh or frozen tumor samples or bone marrow aspirates by flow cytometry as described previously (20). A tumor stem line was considered to have a DNA content indistinguishable from that of normal diploid cells (DNA index of 1.0) if the percentage of cells in the diploid G0-G1 peak of the DNA histogram was at least 30% greater than the percentage of normal blood leukocytes. Hyperdiploid stem lines were those with a DNA index >1.0. For
patients with multiple tumor stem lines, analysis of the effect of ploidy on prognosis was based on the DNA index of the lowest ploidy stem line.

Statistical analysis. Tests of association were done using a two-sided Fisher’s exact test for each of the risk factors versus MYCN amplification category. Patients with dmins + HSRs were excluded from the tests of association as were patients with unknown values for a given risk factor. Event-free survival (EFS) and overall survival (OS) curves were generated by the methods of Kaplan and Meier (21), with SE calculated as per Peto (22). EFS and OS rates are presented as the rate ± SE. The time to an event was calculated as the time from study enrollment until the time of first occurrence of relapse, progressive disease, secondary malignancy or death, or the time to last contact with the patient if no event occurred. Death was the event for the OS analysis. Ps < 0.05 were considered statistically significant.

Results

There were 4,102 clinical patient samples available for study between March 1993 and December 2004. Of these, 800 (19.5%) showed MYCN amplification. The type of MYCN amplification and complete clinical information were known in 677 cases, and this subgroup comprised the study cohort. The majority of patients were ages >18 months and had stage 4 disease (Table 1). Diploid predominated in patients with MYCN-amplified tumors as did unfavorable histology.

MYCN amplification was manifested as dmins in 629 (92.9%) of the 677 tumors and as HSRs in 40 (5.9%; Fig. 1A and B). Eight tumors (0.1%) displayed dmins + HSRs (Fig. 1C). With the possible exception of a trend toward more frequent occurrence of HSRs in hyperdiploid tumors (dmins or HSRs) to the four common risk factors in Table 1. Diploid predominated in patients with MYCN-amplified tumors as did unfavorable histology. Table 1 shows the relationship of MYCN amplification category (dmins or HSRs) to the four common risk factors in neuroblastoma (age, stage, ploidy, and Shimada histology). With the possible exception of a trend toward more frequent occurrence of HSRs in hyperdiploid tumors (P = 0.08), neither dmins nor HSRs showed any statistically important associations with these prognostic features. Surprisingly, dmins rather than HSRs predominated in the tumors of older patients with advanced-stage or metastatic disease.

The median follow-up time in patients alive without an event was 2.6 years (range, 1 day to 11.4 years). Of the 677 patients with evaluable tumors, 646 had sufficient outcome data for survival analyses. The overall 5-year EFS and OS rates of patients with MYCN-amplified neuroblastomas were 35 ± 3% and 38 ± 3%, respectively. Children whose tumors had dmins had a 5-year EFS rate of 35 ± 3% compared with 38 ± 15% in those whose tumors had HSRs (P = 0.59; Table 2). Although the eight patients with dmins + HSRs fared worse than either of the individual subgroups (EFS, 18 ± 16% versus 35 ± 3% for dmins and 38 ± 15% for HSRs), these differences failed to attain statistical significance (Table 2; Fig. 2). It should be noted, however, that all patients whose tumors harbored dmins + HSRs either relapsed or developed progressive disease within a year after treatment was begun (Fig. 2), suggesting an especially aggressive form of MYCN-amplified neuroblastoma.

Discussion

Because HSRs are likely selected during cell culture in neuroblastoma cell lines and their development is associated with the acquisition of growth advantages and resistance to chemotherapy, we hypothesized that primary neuroblastomas with HSRs would behave more aggressively than those with dmins. We therefore examined the clinical outcome of a large group of patients whose tumors had been analyzed for MYCN status. Comparisons of EFS and OS by tumor phenotype (MYCN/HSRs versus MYCN/dmins) did not reveal any significant difference in outcome between these subgroups. It should be emphasized, however, that the 5-year EFS and OS rates of patients with MYCN-amplified neuroblastomas, 35% and 38%, respectively, are higher than previously reported results (12). This probably reflects a general overall improvement in the outcome of patients with MYCN-amplified disease secondary to the institution of high-dose chemotherapy with stem cell rescue (23). Finally, from currently available evidence, we would have expected HSRs to predominate in the tumors of older children with advanced-stage or metastatic disease; however, dmins were identified more often in this subgroup. The trend for occurrence of dmins in diploid than in hyperdiploid tumors was also surprising. The lower survival rates in patients whose tumors contained HSRs + dmins are interesting and warrant further study in larger numbers of patients.

Our working hypothesis was based on several lines of evidence: the human leukemia cell line HL-60 harbors MYC amplification in the form of dmins during early passages that are then replaced by HSRs during continuous long-term culture (6). In HL-60 cells treated with DMSO, granulocyte differentiation is induced in cells with either dmins or HSRs. However, in those with HSRs, there was no apoptosis following

<table>
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<th>Risk factor</th>
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<th>Higher risk</th>
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<tr>
<td>Age</td>
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<td>≥1 year</td>
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Table 2. Log-rank test comparison of survival curves by MYCN amplification category (n = 644 with sufficient follow-up data)

<table>
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<tr>
<th>Comparison</th>
<th>n</th>
<th>5-year EFS ± SE</th>
<th>P</th>
<th>5-year OS ± SE</th>
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<tr>
<td>dmins</td>
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<td>35 ± 3%</td>
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<td>37</td>
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<td>dmins vs dmins + HSRs</td>
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<td></td>
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<tr>
<td>dmins</td>
<td>601</td>
<td>35 ± 3%</td>
<td>0.07</td>
<td>38 ± 3%</td>
<td>0.08</td>
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<tr>
<td>HSRs</td>
<td>8</td>
<td>18 ± 16%</td>
<td>17 ± 16%</td>
<td></td>
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<tr>
<td>HSRs vs dmins + HSRs</td>
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<td></td>
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<tr>
<td>dmins</td>
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<td>38 ± 15%</td>
<td>0.23</td>
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<td>8</td>
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<td>17 ± 16%</td>
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differentiation, and after withdrawal of the drug, the cells reverted to the undifferentiated state. By contrast, in those containing dmins, there was a substantial decrease in the level of MYC expression (6). This implies that cells with MYC amplification manifesting as HSRs are not as susceptible to differentiating agents and may have developed additional mechanisms of resistance. Secondly, it has been shown that dmins can be eliminated (by incorporation within micronuclei) and tumorigenicity can be abrogated in drug-resistant lines by low concentrations of hydroxyurea, but this is not possible with HSRs (9, 24). Finally, MYCN amplification in primary neuroblastoma tumors shows a strong statistical association with gain of chromosome segment 17q21-qter, itself an adverse prognostic factor (25), and chromosome 17q is a preferential site for integration of MYCN HSRs (26, 27).

It is evident from this study that tumors with HSRs are not “more aggressive” than those with dmins. Our results therefore illustrate that findings in cell lines do not necessarily mirror in vivo tumor pathology. Why, then, are dmins predominant in tumors and HSRs in cell lines? It is possible that the generation of HSRs over dmins is only a compensatory mechanism to ensure cell survival in vitro and does not by itself contribute to increased tumorigenicity. It has been shown that dmins harboring amplified MYCN can be spontaneously eliminated as micronuclei in both cell lines and tumors (28, 29). Thus, the mere fact that dmins are more vulnerable to elimination may be reason enough for the switch from dmins to HSRs.

It is also possible that the predominance of either dmins or HSRs is simply dependent on the cell type as well as the surrounding milieu (10, 30). Methotrexate-resistant murine cell lines, for example, usually maintain dihydrofolate reductase ampiclons in dmins, whereas Chinese and Syrian hamster cells maintain dihydrofolate reductase and other ampiclons in HSRs; however, they both have similar propensities to confer drug resistance. Thus, the neuroblastoma cell line environment may select for the presence of HSRs, whereas tumors are better able to accommodate dmins.

Another explanation could be that HSRs occur at a later step in the evolution of amplified sequences and that the longer the clone has evolved, the greater the possibility that HSRs will be the predominant form. The mechanism of MYCN amplification proposed by Amler and Schwab (15) involves unscheduled DNA replication, recombination, and formation of extrachromosomal DNA (dmins) followed by integration into a chromosome and subsequent in situ multiplication forming HSRs (15, 31, 32). Evidence to support this theory comes from studies in which both MYCN and MDM2 amplification were initially detected as dmins in the tumor sample and early passage cells but as HSRs in later passages. Thus, it may be that dmins are formed early in neuroblastoma development only to be replaced by HSRs in later stages of clonal evolution and that most neuroblastomas come to medical attention before HSRs have had a chance to develop.

Acknowledgments

We thank Susan Rowe, Nicholas Campisi, and Elizabeth Ronan for excellent technical assistance.

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