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Analysis of DNA Ploidy and Proliferative Activity in Relation to Histology and *N-myc* Amplification in Neuroblastoma

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Diploid DNA content, advanced stage, unfavorable histology, and* N-myc *amplification are all associated with aggressive disease and poor prognosis in childhood neuroblastoma. DNA diploidy is associated with advanced stage and unfavorable histology, but the relationships among ploidy,* N-myc *amplification, and proliferative activity are not known. To determine if DNA diploidy is associated with* N-myc *amplification, we studied 29 neuroblastomas with flow cytometric analysis and Southern blot analysis. Clinical and histologic features were also evaluated. Sixty percent of the* N-myc*-amplified tumors were diploid, compared to 26% of the neuroblastomas, which lacked* N-myc *amplification (P = 0.11). In our analysis of proliferative activity and* N-myc *amplification, a higher mean percentage of cells in S phase was seen in the* N-myc*-amplified tumors (13.4%) than in the unamplified tumors (10%), but again the result was not statistically significant (P = 0.14). Significant associations were seen between unfavorable histology and DNA diploidy (P = 0.05), and between unfavorable histology and high proliferative activity (P = 0.007). Our data suggest that biologic factors other than* N-myc *amplification play a role in determining the aggressiveness of at least some diploid neuroblastomas. (Am J Pathol 1990, 136:1043–1052)

Many flow cytometric (FCM) studies of tumor cell DNA content and cell-cycle distribution in a variety of tumors have reported an association of poor prognosis with a high percentage of cells in the S phase of cell cycle, and abnormal or aneuploid DNA content.[1–5] Although the sensitivity of DNA FCM limits resolution of karyotypic abnormalities to additions or deletions at the level of approximately two to three average size chromosomes,[3] an advantage of FCM for such determinations stems from the capacity to perform DNA ploidy measurements on interphase cells from fresh or paraffin-embedded tissue without requiring chromosome analysis. In contrast to most neoplasms, in childhood acute lymphoblastic leukemia[6,7] and neuroblastoma[8–11] DNA aneuploidy has been reported to be associated with improved survival and a better response to therapy. Patients with DNA diploid neuroblastomas, especially those with a high percentage of cells in S phase, have more aggressive disease and a poor prognosis.[9]

Others[9–11] have reported that in neuroblastoma DNA aneuploidy parallels several favorable prognostic indicators, including favorable clinical stage (I, II, and IVS) and histologic evidence of Schwann cell and ganglion cell differentiation. Neuroblastomas with diploid DNA content are associated with advanced stage of disease (III and IV) and unfavorable histology. However there has been only one study reported in abstract form examining the relationship between ploidy and *N-myc* amplification, another important prognostic factor.[12] The two DNA diploid tumors examined in that study were *N-myc* amplified while the 16 tumors exhibiting DNA aneuploidy had varying *N-myc* gene dosages from 0.5 to 2.0 times the normal level.[12]

N-myc amplification is seen more commonly in tumors from advanced-stage disease[13] and in tumors with rapid

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progression.[14] The progression-free survival rate at 18 months for patients whose tumors have 1, 3 to 10, and more than 10 copies of *N-myc* is reported to be 70%, 30%, and 5%, respectively ($P < 0.0001$).[14] These data suggest that genomic amplification of *N-myc* plays a key role in determining the aggressiveness of neuroblastomas. If *N-myc* amplification and diploidy are associated, similar biologic events may contribute to the malignant behavior of these prognostically unfavorable tumors. No correlation would imply that biologic factors not related to *N-myc* amplification play a role in determining the aggressive behavior of diploid neuroblastomas.

To determine if DNA diploidy and *N-myc* amplification are associated, we studied 29 neuroblastomas with FCM and Southern blot analysis. We also analyzed the relationship between these two prognostic factors and cell cycle. In addition, the histopathology of the tumors was evaluated according to the criteria of Shimada and coworkers[15] and the relationships between these prognostic factors and clinical outcome were examined.

Materials and Methods

Tumor Samples

The neuroblastomas studied were from patients diagnosed at Children's Memorial Hospital, the University of Illinois, or the Wyler Children's Hospital in Chicago from October 1982 to April 1988 who had either fresh or frozen tumor available for *N-myc* analysis and paraffin-embedded tumor tissue available for FCM analysis. Their charts were reviewed and pertinent clinical data are summarized in Table 1. All tumors studied were from primary lesions.

Southern Blot Analysis

High-molecular weight DNA was isolated from fresh or frozen neuroblastoma by phenol extraction and ethanol precipitation, as previously described.[16] Five micrograms of DNA from each sample were digested with the restriction enzyme EcoRI (Bethesda Research Laboratories, Gaithersburg, MD), separated by size on an 0.8% agarose gel by electrophoresis, and transferred to a single nylon membrane (Amersham, Arlington Heights, IL) using standard techniques.[17,18] The membrane was hybridized with a nick-translated 1.0-kilobase (Kb) insert from the plasmid Nb-1 containing the second exon of the human *N-myc* gene.[19] Autoradiography at −70°C was performed and *N-myc* amplification was quantified by a laser scanning densitometer.

To demonstrate that equal amounts of DNA were transferred, the nylon membrane was stripped and rehybridized with a nick-translated 2.0-Kb insert from the plasmid pA-1, which contains a complete DNA copy of the chicken B-actin gene.[20]

Tissue Deparaffinization and Tissue Dissociation

Paraffin-embedded tissue was deparaffinized and dissociated using a modification of the method of Hedley et al,[21] as detailed previously.[1,22] After morphologic evaluation, three 50-μm thick sections from each paraffin block were cut and tumor was dissected from nontumor tissue using a blunt needle and scalpel. The tissue was deparaffinized, rehydrated, and incubated in a 0.5% pepsin solution for 30 minutes at 37°C. The pepsin proteolysis was next stopped by the addition of pepstatin A (0.51 μg/ml), the digested material was filtered, and the filtrate was resuspended in Hanks' balanced salt solution plus 10 mmol/l (millimolar) of 4 -(2-hydroxyethyl)-1-piperazine-ethanesulforic acid, and stored at 4°C until DNA staining and FCM analysis were performed.

DNA Staining and FCM Analysis

After tissue dissociation, nuclei were stained with propidium iodide (PI) using a concentration of 50 μg/ml after RNase digestion (180 units/ml × 20 minutes at 37°C), as detailed previously.[1,22] The fluorescence of PI-stained cells was measured on an EPICS 752 flow cytometer (Coulter Corp., Hialeah, FL) equipped with a 5-watt argon ion laser. Fluorescent microspheres (Lot 5325; Coulter) were used to aid instrument alignment and to calibrate instrument performance during each run).

Immediately before FCM analysis, the nuclear suspensions were filtered through a 37-μm nylon monofilament mesh and microscopically examined to assure that a minimum of 95% of single nuclei (as opposed to nuclear aggregates) were obtained. Propidium iodide-stained nuclei were excited at 488 nm, 500 mW, and fluorescence was measured through a 488 ± 15 nm laser-blocking filter. A minimum of 2.5×10^4 nuclei were evaluated from each specimen. The coefficient of variation of the G_0/G_1 population of each sample ranged from 2.8 to 5.9, with a mean of 4.3 in this study. Two specimens were excluded from subsequent analysis on the basis of demonstrating a broad coefficient of variation (ie, more than 6%). All samples were electronically gated on peak *versus* integrated fluorescence signals to minimize retention of cell aggregates in the analyzed DNA distributions. All FCM analyses

Table 1. *Summary of Clinical Data, Pathology, Ploidy, and* N-MYC *Gene Amplification*

Pt	Stage‡	Age (months)	Path§	%S	Ploidy (DNA index)	*N-MYC*	Progressive disease*	Disease-free interval (months)
1	III	41	U (f)	20.7	D (1.0)	1	Yes	6.2
2	IV	33	U (f)	17.8	D (1.0)	~20	No	38.7
3	III	19	U (f)	17.1	D (1.0)	~8	Yes	9.7
4	II	8	U (f)	15.3	D (1.0)	~25	Yes	14.5
5	IV	48	U (d)	14.9	D (1.0)	1	Yes	11.7
6	IV	7	U (f)	14.1	D (1.0)	~50	Yes	8.5
7	IV	52	U (f)	13.4	D (1.0)	1	Yes	16.0
8	IV	12	U (f)	9.0	D (1.0)	~40	Yes	27.4
9	II	25	F (a)	3.7	D (1.0)	1	No	28.2
10	III	84	U (f)	1.2	D (1.0)	~20	No	52.5
11	I	180	U (c)	1.1	D (1.0)	1	No	36.7
12	III	37	U (f)	23.3	A (2.04)	~25	Yes	23.3
13	IV	9	U (f)	18.6	A (1.78)	1	†	—
14	IV	10	U (f)	18.1	A (1.62)	~40	Yes	9.1
15	III	60	U (f)	17.1	A (2.0)	1	No	17.0
16	IV	25	U (f)	12.5	A (1.19) (1.96)	1	Yes	19.7
17	III	10	F (c)	11.6	A (1.35)	1	No	17.0
18	III	35	U (c)	10.9	A (1.98)	~10	Yes	39.2
19	II	5	F (d)	9.9	A (1.25)	1	No	17.4
20	IV	8	ND	9.7	A (1.57)	1	†	—
21	II	144	U (f)	9.4	A (1.19) (2.18)	1	Yes	16.0
22	II	2	F (d)	9.2	A (1.23)	1	No	23.4
23	III	21	U (e)	8.9	A (1.69)	1	No	54.9
24	II	11	F (c)	8.8	A (1.58)	1	No	17.0
25	II	13	F (d)	8.3	A (1.39) (1.69)	1	No	60.0
26	IVS	6	F (d)	7.6	A (1.18)	~65	Yes	12.5
27	II	17	F (d)	5.3	A (1.12)	1	No	26.2
28	II	17	U (b)	5.1	A (1.38)	1	No	46.9
29	IV	60	F (a)	2.0	A (1.16)	1	Yes	68.0

* Follow-up 6 to 68 months; mean 26.6 months; median 23.3 months.
† Toxic death.
‡ Evans' stage.[23]
§ Shimada classification.[15]
a) Stroma rich: absent nodules.
b) Stroma rich: nodules present.
c) Stroma poor: differentiating: lower mitotic karyorrhectic index (MKI).
d) Stroma poor: undifferentiated: lower MKI.
e) Stroma poor: differentiating: higher MKI.
f) Stroma poor: undifferentiated: higher MKI.
ND: not done.
U: Unfavorable.
F: Favorable.
A: Aneuploid.
D: Diploid.

were performed blindly (ie, without knowledge of *N-myc* amplification data).

Cell-cycle and Aneuploidy Analysis

DNA histogram analysis was performed on a Model 50 personal computer (International Business Machines Corp., Boca Raton, FL) using Cytologic software (Coulter). The algorithm used for cell-cycle analysis was based on a simple rectangle model, as detailed previously.[2] This software includes an algorithm for subcellular debris subtraction based on an exponential function fit to the left of the G_0/G_1 cells and to the right of the G_2M cells, which was used for each DNA histogram.[1] Cell proliferation in these studies is expressed in terms of the total percentage of S-phase cells.

DNA aneuploidy was considered detectable only when two distinct G_0/G_1 peaks were evident. The DNA index was calculated by dividing the mean fluorescence channel number of the aneuploid G_0/G_1 peak by the mean channel number of the diploid G_0/G_1 peak.

Histologic Classification

One of us (FG-C) reviewed the histology of all 29 primary tumors and classified the neuroblastomas as favorable or unfavorable according to the criteria of Shimada et al.[15]

Table 2. *Association of Prognostic Indicator with Clinical Outcome*

Prognostic indicator	Patient	Progressive disease (%)	*P* value
>10% S	13	10/13 (77%)	0.021
<10% S	14	4/14 (29%)	
>3 copies *N-myc*	10	8/10 (80%)	0.046
<3 copies *N-myc* (all ages)	17	6/17 (35%)	
Diploid	11	7/11 (64%)	0.44
Aneuploid (less than 18 months)	16	7/16 (44%)	
Diploid	3	3/3 (100%)	0.046
Aneuploid	9	2/9 (22%)	
Unfavorable histology	18	12/18 (67%)	
Favorable histology	9	2/9 (22%)	0.046

This classification was done without knowledge of the number of copies of *N-myc* or ploidy.

Statistical Analysis

Associations between categorical variables were assessed using Fisher's exact test and the Chi square. Mean percentage of S-phase cells was compared between subgroups using the unpaired Student's *t*-test. All statistical tests were two tailed. Associations were considered statistically significant if $P \leq 0.05$.

Results

Clinical Variables

Of the 29 patients studied, 18 (62%) presented with advanced-stage disease (Evans' stage III and IV)[23] and 11 (38%) were less than 12 months of age. Five patients were 5 years or older at diagnosis. Of the 18 patients with advanced disease, 10 developed progressive disease and two died from treatment-related events. Three of eleven patients with favorable stages of neuroblastoma (I, II, IVS) developed progressive disease. The median follow-up of the patients was 23.3 months, with a range of 6 to 68 months (Table 1).

Associations Between Progressive Disease and Ploidy, Percentage of S-phase Cells, Histology, and N-myc Amplification

Table 2 summarizes the associations between clinical outcome and the cytometric, histologic, and molecular variables analyzed. Representative examples of each variable are illustrated in Figure 1. High proliferative activity (more than 10% S-phase cells; $P = 0.021$), *N-myc* amplification ($P = 0.046$), and unfavorable histology ($P = 0.046$) were significantly associated with progressive disease. In our series, there was not a statistically significant association between DNA aneuploidy and disease-free survival when all patients were evaluated. However, when only those patients who were younger than 18 months were considered in the analysis, clinical outcome for patients with DNA aneuploid *versus* diploid tumors reached statistical significance ($P = 0.046$). All three patients (100%) who were less than 18 months old with DNA diploid tumors developed progressive disease compared to two of nine patients (22%) with aneuploid tumors.

The number of cases analyzed was not sufficient for a multivariate analysis. However, the incidence of progression was 17%, 33%, and 83%, respectively for patients with 0, 1 to 2, or 3 to 4 unfavorable histologic, cytometric, or molecular indicators ($P = 0.0112$) (Table 3).

Associations Among Ploidy, Percentage of S-phase Cells, Histology, and N-myc Amplification

Associations among the favorable prognostic variables and the poor prognostic factors analyzed are summarized in Table 4. Genomic amplification (more than three copies) of *N-myc* occurred in 10 of the 29 tumors examined (34%). Six of the ten (60%) amplified tumors had diploid DNA content. There were 19 tumors that lacked *N-myc* amplification and 5 (26%) were diploid by FCM analysis ($P = 0.11$). Thus a trend relating DNA diploidy to *N-myc* amplification was seen but the association was not statistically significant. The percentage of cells in S phase varied from 1.1 to 20.7 (mean, 10.0) in the *N-myc*–unamplified tumors and from 1.2 to 23.3 (mean, 13.4) in the amplified neuroblastomas ($P = 0.14$) (Figure 2). When more than 10% of the cells examined were in the S phase of cell cycle, the tumor was considered highly proliferative. Seven of the ten (70%) *N-myc*–amplified neuroblastomas met this criteria for highly proliferative tumors, whereas 7 of 19 (36%) *N-myc*–unamplified tumors had more than 10% S-phase cells ($P = 0.13$). Thus trends associating

Table 3. *Liklihood of Disease Progression for Patients with 0, 1 to 2, or 3 to 4 Unfavorable Histologic, Cytometric, or Molecular Indicators*

Unfavorable indicator #	No. of patients	Progressive disease (%)
0	6	1/6 (17)
1 or 2	9	3/9 (33)
3 or 4	12	10/12 (83)

$P = 0.0112$ (Chi-square test).

Table 4. *Association of Prognostic Factors*

Favorable	No. of patients	% S < 10	% Aneuploid	% *N-myc* < 3	% FH
FH*	9	89	89	89	—
N-myc < 3	19	64	74	—	44
Aneuploid	18	61	—	78	47
% S < 10	15	—	73	80	57
Unfavorable	**No. of patients**	**% S > 10**	**% Diploid**	**% *N-myc* > 3**	**UH**
UH†	19	68	53	47	—
N-myc > 3	10	70	60	—	90
Diploid	11	64	—	55	91
% S > 10	14	—	50	50	93

* FH: Favorable histology.
† UH: Unfavorable histology.

proliferative activity and *N-myc* amplification were present but statistical significance was not reached. Trends were also seen between unfavorable histology and *N-myc* amplification. Nine of ten (90%) *N-myc*–amplified tumors were classified with unfavorable histology compared to 10 of 18 (56%) evaluable unamplified neuroblastomas ($P = 0.10$).

Eleven of twenty-nine tumors had a single G_0/G_1 peak with a DNA index of 1.0 and were considered diploid by FCM analysis. DNA aneuploidy was detected in 18 samples, with the DNA indices ranging from 1.12 to 2.04. In three samples two aneuploid peaks were detected. Ten of eleven (91%) of the DNA diploid tumors had unfavorable histology compared to 9 of 17 (53%) evaluable aneuploid tumors ($P = 0.05$). Thus a statistically significant association was detected between DNA diploidy and unfavorable histology. In addition unfavorable histology was also significantly associated with high proliferative activity. Thirteen of fourteen tumors (93%) with more than 10% S-phase cells had unfavorable histologic characteristics compared to 6 of 14 tumors (43%) with less than 10% S-phase cells ($P = 0.007$).

Discussion

Neuroblastomas with diploid DNA content are associated with more aggressive disease than DNA aneuploid tumors.[8–11] Because *N-myc* amplification is thought to contribute to the malignant nature of neuroblastoma,[14] a strong correlation between *N-myc* amplification and diploid DNA content would imply that multiple copies of this oncogene may play an important role in determining the aggressive clinical behavior of DNA diploid neuroblastoma. We examined 29 neuroblastomas by Southern blot and FCM analysis to determine if DNA diploidy is associated with *N-myc* amplification. Other prognostic factors, including the percentage of cells in the S phase of cell cycle and histology were also evaluated. A summary of the clinical data, histopathologic classification of the tumors according to the criteria of Shimada and coworkers,[15] DNA ploidy analysis, and oncogene *N-myc* copy number are shown in Table 1. Representative examples of favorable and unfavorable neuroblastoma histopathology, DNA histograms, and Southern blot analysis for *N-myc* copy number are shown in Figure 1.

Sixty percent of the *N-myc*–amplified tumors we analyzed had DNA diploid content, compared to 26% of the neuroblastomas that lacked *N-myc* amplification ($P = 0.11$). In our analysis of proliferative activity and *N-myc* amplification, 70% of the *N-myc*–amplified tumors had more than 10% S-phase cells compared to 36% of the *N-myc*–unamplified tumors ($P = 0.13$) (Figure 2). Thus trends relating *N-myc* amplification with DNA diploidy and high proliferative activity were seen but statistical significance was not reached. Statistically significant associations were seen between unfavorable histology and DNA diploid content ($P = 0.05$) and between unfavorable histology and more than 10% S-phase cells ($P = 0.007$).

Although only 29 patients were evaluated in this study, many of the clinical characteristics of this group of patients parallel what has been reported in larger series.[23–25] Sixty-two percent of the patients presented with advanced disease (stages III and IV), 51% were younger than 2 years old at diagnosis, and 94% were younger than 10 years old. Thirty-seven percent of the patients with advanced-stage disease have remained disease free with a median follow-up of 28 months, which is somewhat better than what has been reported.[26] However, some of the patients in this study have received high-dose chemotherapy with total-body irradiation followed by bone marrow rescue, which has been shown to result in long-term survival in some patients previously considered incurable.[27] Age at diagnosis is another well-described prognostic factor for neuroblastoma and infants less than 12 months of age have improved survival.[24,25] Of the 11 infants less

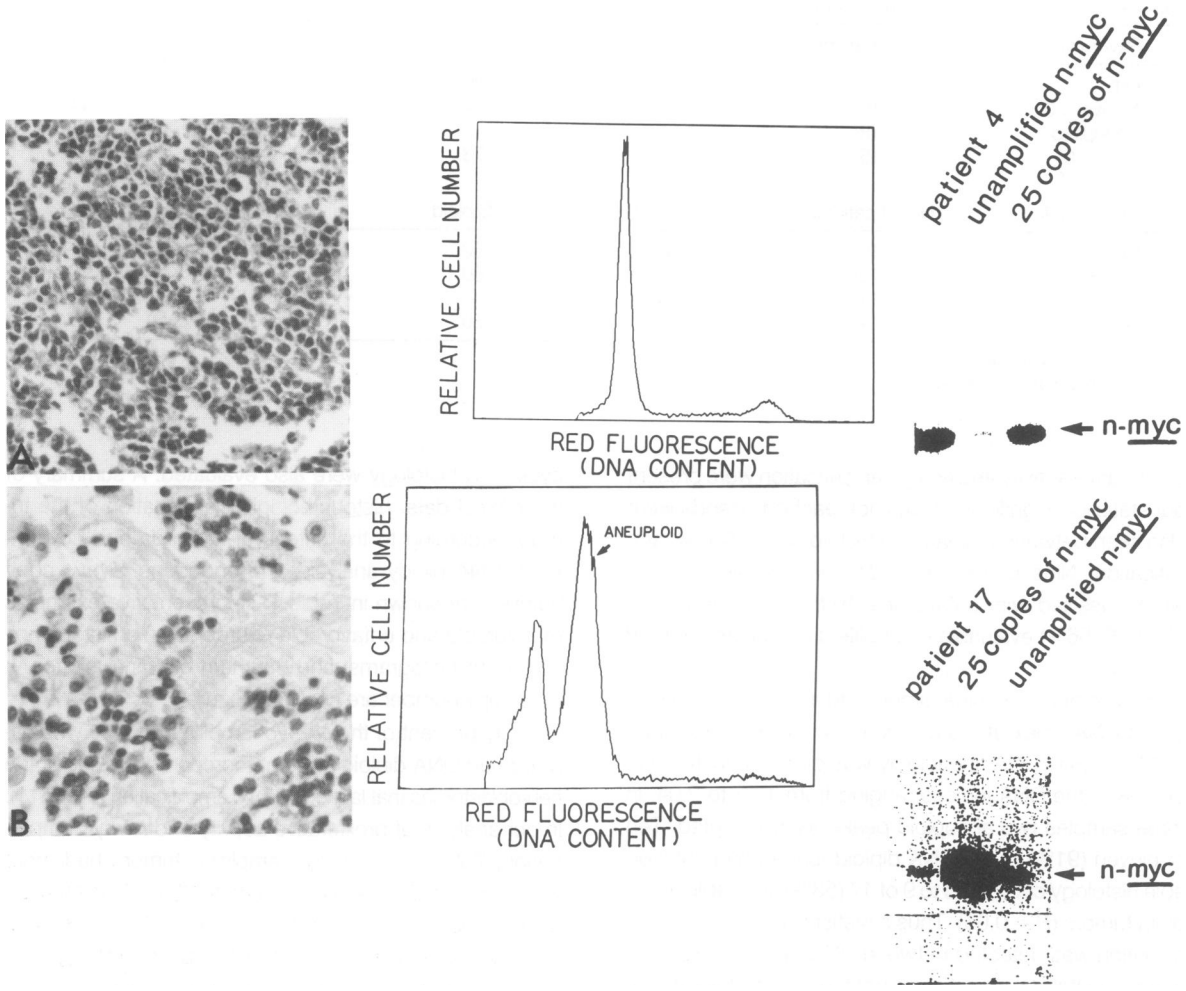

Figure 1. *Representative examples of unfavorable (top) and favorable (bottom) histology, ploidy, and* N-myc *copy number.* **A:** *The histology (unfavorable; magnification 25×), histogram (diploid), and southern blot analysis for* N-myc *copy number of the tumor from patient 4. Lane 1 of the southern blot contains DNA isolated from the primary neuroblastoma from patient 4 (approximately 25 copies). Lane 2 contains DNA isolated from the* N-myc *unamplified human neuroblastoma cell line SK-N-SH.*[32] *Lane 3 contains DNA isolated from the* N-myc *amplified neuroblastoma cell line IMR-5 (approximately 25 copies), which is a clone of IMR-32.*[32] **B:** *The histology (favorable; magnification ×25), histogram (aneuploid), and southern blot analysis for* N-myc *copy number of the tumor from patient 17. Lane 1 of the southern blot contains DNA isolated from the primary neuroblastoma from patient 17 (one* N-myc *copy). Lane 2 contains DNA isolated from the IMR-5 cell line. Lane 3 contains DNA isolated from the SK-N-SH cell line.*

than 1 year of age in this series two died from treatment-related complications and therefore cannot be evaluated. Four of the nine (44%) evaluable infants are currently in disease-free remission, which is a similar survival rate to what has been previously reported.[24] Thus the group of patients studied appears to be unbiased with clinical characteristics similar to much larger studies.

N-myc amplification was present in 34% of the tumors analyzed, which is similar in incidence to what was reported by Seeger and coworkers in a large Children's Cancer Study Group series.[14] Although most of the patients with advanced-stage disease received more aggressive treatment than the patients reported in the study by Seeger et al,[14] *N-myc* amplification remained prognostically significant. The presence of *N-myc* amplification was associated with unfavorable clinical outcome ($P = 0.046$) (Table 2). Unfavorable histology ($P = 0.046$) and high proliferative activity (more than 10% S-phase cells) ($P = 0.021$) were also associated with a significantly greater incidence of progressive disease. In our study, 62% of the tumors had aneuploid DNA content, which is similar in incidence to other reports.[9,11] DNA ploidy was prognostically important in children younger than 18 months old ($P = 0.046$), but ploidy was not of prognostic value when older children were included in the analysis ($P = 0.44$). Too few patients were studied to perform a multivariate analysis and therefore we could not determine which prognostic variables were independent of each other.

However, patients with 0, 1 to 2, or 3 to 4 unfavorable prognostic indicators had a 17%, 33%, and 83% incidence, respectively, of progressive disease ($P = 0.012$) (Table 3). Thus this data suggests that analyzing neuroblastomas for *N-myc* amplification, ploidy, proliferative activity, and histology may provide useful prognostic information.

Previous authors have found that patients with DNA aneuploid neuroblastomas have a significantly more favorable clinical course than those with DNA diploid tumors. Look and colleagues[8] found that patients younger than 12 months old with unresectable hyperdiploid tumors had a better response to chemotherapy than patients with DNA diploid neuroblastomas. A study by Oppedal and coworkers[10] demonstrated that tumor-cell ploidy provided additional prognostic information for patients younger than 1.5 years old. In their study, 11 patients who were less than 1.5 years old at diagnosis with DNA aneuploid tumors all survived, in contrast to only five of nine patients with diploid tumors in the same age group. Similar to our findings, most of the patients with aneuploid tumors and good clinical outcomes analyzed in the study by Oppedal et al[10] were younger than 18 months old. DNA ploidy was also examined in 38 cases of neuroblastoma from patients aged newborn to 74 months in a study by Gansler and colleagues.[9] Again favorable outcome was associated with an aneuploid stem line. Nine of twelve patients with DNA aneuploid tumors who remain disease free were younger than 18 months old. Three of the four patients in the Gansler study[9] with DNA aneuploid tumors who died were older than 18 months. In that study a low percentage of cells in the S and G_2/M phase of cell cycles also was correlated with good clinical outcome. Taylor et al,[11] in a large 10-year retrospective study of DNA analysis on 147 samples from 89 patients with neuroblastoma and ganglioneuroma using flow cytometry, demonstrated that the nuclear DNA content was a stable tumor marker. The DNA content remained the same when tumors were analyzed at different periods of time during the course of a patient's disease. This study confirmed that DNA aneuploidy was highly associated with survival ($P < 0.001$).[11] The number of patients younger than 18 months old with DNA aneuploid tumors is not mentioned. Therefore it is not possible to determine how many of the patients with DNA aneuploid tumors and good clinical outcome were in this age group.

Although the evidence presented in this and other papers demonstrates a prognostic impact of DNA ploidy, it is important to recognize inherent limitations of this method. First DNA ploidy measurements are limited to the resolution of karyotypic abnormalities to additions or deletions at the level of approximately two to three chromosomes. Neuroblastoma tumors with diploid DNA content as determined by FCM may, therefore, contain double-minute chromatin bodies or chromosomes with structural abnormalities and homogenously staining regions. Second the ability to resolve DNA ploidy abnormalities is dependent on both the frequency and the relative ploidy of the aneuploid population. For example, while it may be possible to detect a triploid population comprising less than 5% of the total cells in a tumor, a near-diploid population (ie, DI = 1.1) comprising 10% of the cells in a tumor may not be resolvable. However, despite these limitations, FCM analysis is a rapid and accurate means of estimating the ploidy of malignant stem lines.

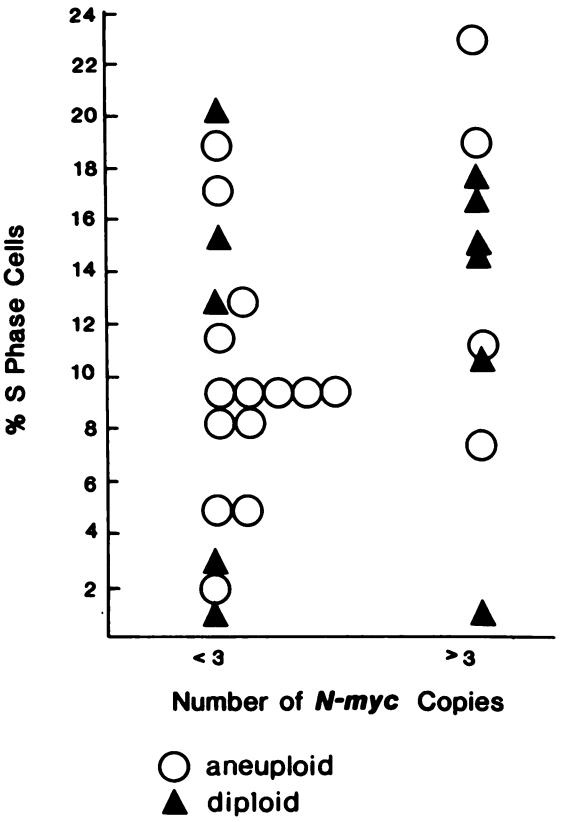

Figure 2. N-myc *gene amplification in relation to the percentage of cells in S phase of cell cycle and ploidy. The mean percentage of cells in S phase was 13.4% in the* N-myc*-amplified tumors and 10% in the unamplified tumors (*P = *0.14). Sixty percent of the* N-myc*-amplified tumors had diploid DNA content compared to 26% of the neuroblastomas lacking* N-myc *amplification (*P = *0.11). Of the seven highly proliferative tumors (> 10% S-phase cells) with DNA diploid content, four were* N-myc *amplified and three lacked* N-myc *amplification. Although trends were seen, significant associations among ploidy,* N-myc *amplification, and % S-phase cells were not present.*

Cytogenetic studies of neuroblastoma have confirmed that hyperdiploidy in the triploid range is associated with favorable prognostic factors and long-term disease-free survival in young children. However, these studies also have inherent methodologic limitations because only

~20% of primary tumors can be karyotyped. Nevertheless, Christiansen and Lampert[28] performed karyotype analysis on tumors from seven children who were younger than 1 year old with favorable stage of disease (I, II, and IVS). All seven patients remain disease free and all seven cases were characterized by hyperploidy in the triploid range, lack of 1p abnormality, and absence of *N-myc* amplification. Eighty-three percent of the tumors studied from patients with advanced-stage disease had chromosome 1p abnormalities. Diploid karyotypes were present in one half of the advanced-stage tumors and 33% of these tumors were also *N-myc* amplified.

Kaneko et al[29] also reported that neuroblastomas with near-triploid chromosome number without evidence of chromosome 1p abnormalities are associated with favorable stage of disease and good outcome. Similar results have been described in Japanese studies on tumors analyzed from infants diagnosed by mass screening for vanillylmandelic acid in the urine. Hyperdiploid tumors with modal chromosome numbers ranging from 67 to 71 were again associated with long-term disease-free survival.[30] A study by Hayashi and colleagues[31] further confirmed that patients with hyperdiploid or near-triploid karyotypes were clinically distinct from those with diploid or hypotetraploid karyotypes. All but one of the patients with hyperdiploid or near-triploid karyotypes were younger than 1 year old, had localized disease, and were long-term disease-free survivors. In contrast, most of the patients with near-diploid or hypotetraploid karyotypes had advanced disease, were 1 year of age or older, and had a poor clinical outcome. All of the diploid or hypotetraploid tumors had several structural abnormalities, including marker chromosome 1. Five of eleven tumors with near-diploid karyotypes were also *N-myc* amplified. None of the hyperdiploid or near-triploid tumors had *N-myc* amplification. Thus an association between *N-myc* amplification and near-diploid karyotype was seen in this study.

Correlations of chromosome karyotype with FCM data have been hampered in neuroblastoma because of the difficulty of obtaining interpretable karyotypes from primary tumor material. However, if the tumors analyzed in our study are regrouped in an effort to compare the results with the cytogenetic studies, the FCM data closely correlate with the cytogenetic analysis. There were 11 patients in our study who had tumors with diploid DNA content (DI = 1.0), 3 patients with near-diploid tumors (DI = 1.1 to 1.2), and 5 patients with tumors with near-tetraploid DNA content (DI = 1.8 to 2.2). Of these 19 patients, 13 have developed progressive disease and 9 have *N-myc*–amplified tumors. There were 10 patients in our study with tumors with hyperdiploid or near-triploid DNA content (DI more than 1.2 but less than 1.8) and only one of these patients has developed progressive disease. Although this patient (patient 14) was treated with aggressive multiagent chemotherapy, she died 9 months after diagnosis of central nervous system metastasis. Her primary tumor was *N-myc* amplified and FCM analysis of the tumor revealed a population of cells with triploid DNA content (DI = 1.62). Despite this exception, when those patients with hyperdiploid or near-triploid tumors are compared to the remaining patients, statistically significant differences were seen between the numbers of patients with *N-myc*–amplified tumors (1 of 10 *vs.* 9 of 19; $P = 0.05$) in these two groups and the number of patients who developed progressive disease (1 of 10 *vs.* 13 of 19; $P = 0.002$). Thus patients with tumors with diploid, near-diploid, or near-tetraploid DNA content had a significantly higher incidence of *N-myc* amplification and a worse outcome than those with near-triploid DNA content.

Only one previous study examining the relationship between *N-myc* amplification and DNA ploidy has been reported. Taylor and coworkers[12] analyzed 18 neuroblastomas for DNA content and presence of *N-myc* amplification. Two tumors were diploid; both were *N-myc* amplified and rapidly fatal. The aneuploid tumors, however, had varying levels of *N-myc* copy number and the authors concluded that *N-myc* amplification and ploidy were independent factors.

Because patients with highly proliferative neuroblastomas and diploid DNA content have been reported to have more aggressive disease than those with DNA diploid tumors that are not actively cycling,[9] we also examined this small subset of tumors to determine if an association with *N-myc* amplification existed. There were seven patients with DNA diploid tumors and high proliferative activity (more than 10% S-phase cells). Three tumors lacked *N-myc* amplification and four were *N-myc* amplified. Six of these seven patients developed progressive disease. Thus this subset of patients had clinically aggressive disease whether *N-myc* amplification was present. Clearly activation of other oncogenes, inactivation of suppressor genes, or alternative mechanisms of *N-myc* activation must occur in these clinically aggressive unamplified diploid neuroblastomas. Others have shown that while amplification of *N-myc* is an important mechanism of *N-myc* activation, some unamplified neuroblastomas also express *N-myc* RNA and protein.[32–36] Although the clinical significance of *N-myc* expression remains controversial,[34–36] Grady-Leopardi and coworkers[35] reported a relationship between elevated levels of *N-myc* RNA expression and poor prognosis independent of gene amplification. Similar observations were made by Bartram and Berthold.[36] Whether clinically aggressive *N-myc*–unamplified diploid neuroblastomas express high levels of *N-myc* remains to be determined. However, because biologic factors not related to *N-myc* amplification appear to play a

role in determining the aggressive behavior in some DNA diploid tumors, a multivariate analysis of a large series of patients should be studied to determine if DNA diploidy and *N-myc* amplification are independent, clinically relevant prognostic factors.

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