

Histopathology (International Neuroblastoma Pathology Classification) and MYCN Status in Patients with Peripheral Neuroblastic Tumors

A Report from the Children's Cancer Group

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BACKGROUND. The International Neuroblastoma Pathology Classification (International Classification), which was established in 1999, is significant prognostically and is relevant biologically for the evaluation and analysis of patients with neuroblastic tumors (NTs). MYCN amplification is a known molecular marker for aggressive progression of NTs. These have been used together as important prognostic factors to define risk groups for patient stratification and protocol assignment.

METHODS. A total of 628 NTs (535 neuroblastomas [NBs]); 21 ganglioneuroblastoma, intermixed [GNBi]; 9 ganglioneuromas [GN]; and 63 ganglioneuroblastoma, nodular [GNBn]) from the Children's Cancer Group studies were evaluated histologically (favorable histology [FH] tumors vs. unfavorable histology [UH] tumors) according to the International Classification and were tested molecularly for MYCN status (amplified vs. nonamplified). Four tumor subsets (FH-nonamplified, FH-amplified, UH-nonamplified, and UH-amplified) were defined by histopathology and MYCN status, and their prognostic effects were analyzed. Detailed analysis between morphologic indicators (grade of neuroblastic differentiation and mitosis-karyorrhexis index [MKI]) and MYCN status was done by using tumors in the NB category.

RESULTS. There were 339 FH-nonamplified tumors (5-year event free survival [EFS], 92.1%); 8 FH-amplified tumors (EFS, 37.5%); 172 UH-nonamplified tumors (EFS, 40.9%); and 109 UH-amplified tumors (EFS, 15.0%). The prognostic effects on patients with tumors in the four subsets were independent from the factors of patient age and disease stage ($P < 0.0001$). MYCN amplification was seen almost exclusively in tumors of the NB category, and no patients with tumors in either the GNBi category or in the GN category and only two patients with tumors in the GNBn category had amplified MYCN. Among the patients with tumors in the NB category, patients with FH-nonamplified tumors (309 patients) had an excellent prognosis, and patients with UH-amplified tumors (107 patients) had the poorest clinical outcome in any age group. The prognosis for children with UH-nonamplified tumors (111 patients) was poor when they were diagnosed at age > 1.5 years. It was also noted that patients with UH-amplified tumors (median age, 2.14 years) were diagnosed at a significantly younger age compared with the patients with UH-nonamplified tumors (median age, 3.55 years). Histologically, MYCN-amplified tumors lacked neuroblastic differentiation regardless of the age of patients. MYCN amplification also was linked generally to increased mitotic and karyorrhectic activities. However, MKI classes in patients with MYCN-amplified tumors varied significantly, depending on the age at diagnosis, and younger patients had higher MKI classes.

CONCLUSIONS. The combination of histopathologic evaluation and MYCN status distinguishes four clinical and biologic tumor subsets in patients with NTs. MYCN

amplification seems to be the powerful driving force for preventing cellular differentiation regardless of patient age and for increasing mitotic and karyorrhectic activities in an age dependent manner. *Cancer* 2001;92:2699-708.

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Recent advances in clinical and basic research have revealed that peripheral neuroblastic tumors (NTs), including neuroblastoma (NB), ganglioneuroblastoma (GNB), and ganglioneuroma (GN), are biologically heterogeneous, and their individual molecular properties are closely related to unique clinical behaviors, such as involution,¹⁻⁴ spontaneous regression,⁵⁻¹⁰ maturation,¹¹⁻¹³ and aggressive progression.¹⁴ MYCN amplification is a well-established molecular indicator for predicting aggressive clinical behavior in NTs.¹⁵⁻¹⁸ It also has been reported that there is a significant correlation between the molecular event of MYCN amplification and the morphologic manifestation in NTs: Neuroblastic cells in those tumors with amplified MYCN frequently have characteristic features, such as limited or no cellular differentiation and increased mitotic and karyorrhectic activities.¹⁹⁻²³

In 1999, the International Neuroblastoma Pathology Classification (International Classification) was established^{24,25} by adopting and slightly modifying the original Shimada classification²⁶ to unify the international terminology and the criteria for prognostic evaluation based on the morphologic features of tumors. The International Classification also was designed to offer a tool to better understand the biology of NTs by comparing their molecular properties and morphologic features. This is the first study to test the biologic relevance of the International Classification in a thorough analysis of histopathology and MYCN status in a large series of NTs from the Children's Cancer Group (CCG) studies.

According to the International Classification, two morphologic indicators, i.e., grade of neuroblastic differentiation and mitosis-karyorrhexis index (MKI), were recorded for tumors in the NB category. Three grades of neuroblastic differentiation (undifferentiated, poorly differentiated, differentiating), as described in the accompanying paper,²⁷ had significantly different prognostic effects according to the age of the patients: an excellent prognosis for patient with *poorly differentiated tumors at age < 1.5 years* and *differentiating tumors at age < 5 years* and a poor prognosis for patients with *undifferentiated tumors at in any age*, *poorly differentiated tumors at age ≥ 1.5 years*, and

differentiating tumors at age ≥ 5 years. Three MKI classes (low, intermediate, and high) also had significantly different prognostic effects according to the age of the patients: an excellent prognosis for patients with *low MKI at age < 5 years* and *intermediate MKI at age < 1.5 years* and a poor prognosis in patients with *low MKI at age ≥ 5 years*, *intermediate MKI at age ≥ 1.5 years*, and *high MKI in any age*. Thus, the correlation between these morphologic indicators and MYCN status was analyzed with and without the age factor with cut-off ages at 1.5 years and 5.0 years of age.

MATERIALS AND METHODS

A total of 911 patients with NTs were enrolled onto the clinical trials of the CCG (studies CCG-3881 and CCG-3891) from August 1, 1991, to August 1, 1995. Treatment protocols of CCG-3881 and CCG-3891 depended on risk groups,^{13,28-31} which were defined by stage, age, and tumor biology (MYCN status,^{15,16,32,33} histopathology according to the original Shimada classification system,²⁶ and serum ferritin level³⁴). Low-risk and intermediate-risk patients (all patients with Stage I disease, patients with Stage II and III disease at age < 1 years; patients with Stage II disease at age ≥ 1 year MYCN-nonamplified tumors; patients with Stage III disease at age > 1 year with MYCN-nonamplified tumors, favorable histology, and low ferritin levels; and patients with Stage IV disease at age < 1 year with MYCN-nonamplified tumors) were treated on the CCG-3881 protocol. High-risk patients (all other combinations) were treated aggressively with or without bone marrow transplantation on the CCG-3891 protocol. Appropriate informed consent procedures were followed, and consent was obtained from parents or guardians.

Among these 911 patients, 628 tumors were available for both histopathologic and MYCN analysis. Hematoxylin and eosin-stained sections of tumors (1-38 slides per patient; median, 5 slides) were classified according to the International Classification, and snap-frozen tissues were tested for MYCN status. These slides were selected based on the availability and evaluability for histopathology review and MYCN testing and did not necessarily represent all the clini-

cal cases of NTs from the CCG-3881 and CCG-3891 studies. Of 283 tumors that were excluded from this study, 118 had histopathology data only, 97 had MYCN data only, and 68 had neither histopathology nor MYCN data. Those excluded tumors were mainly from patients with Stage IV disease (61.1%) and commonly were diagnosed at age > 1 year (68.6%). The clinical and biologic characteristics of the available tumors in this study were analyzed in part in our previous publications.^{13,28–31}

Histopathologic Evaluation

All 628 tumors were first classified into four categories: neuroblastoma (NB; Schwannian stroma-poor); ganglioneuroblastoma, intermixed (GNBi; Schwannian stroma-rich); ganglioneuroma (GN; Schwannian stroma-dominant); and ganglioneuroblastoma, nodular (GNBn; composite, Schwannian stroma-rich/stroma-dominant and stroma-poor). Tumors in the NB category were divided further into three subtypes—undifferentiated, poorly differentiated, and differentiating—based on the grade of neuroblastic differentiation. Three MKI classes (low, < 2% or < 100 of 5000 mitotic and karyorrhectic cells; intermediate, 2–4% or 100–200 of 5000 mitotic and karyorrhectic cells; and high, > 4% or > 200 per 5000 mitotic and karyorrhectic cells) also were distinguished in the tumors of the NB category. There were two subsets in the GN category: maturing and mature. Based on the age-linked framework of the Shimada system, two prognostic groups, favorable histology (FH) and unfavorable histology (UH), were distinguished (as described in our previous papers²⁵ and in the accompanying paper²⁷).

MYCN Test

From 1989 to 1993, MYCN gene amplification was determined by Southern analysis of gene copy number^{15,16} and, after 1993, by the pattern of MYCN protein expression by immunoperoxidase stain³² combined with a semiquantitative polymerase chain reaction technique for MYCN gene copy number. These studies were performed in the centralized CCG neuroblastoma reference laboratory,³³ and those tumors with > 10 copies of MYCN were classified into an amplified group.^{15,16}

MYCN Status and Histopathology

Analysis of all NTs (NB, GNBi, GN, and GNBn)

Based on the combined histopathologic evaluation (FH or UH) and MYCN status (nonamplified or amplified), all 628 tumors were classified into four subsets: FH-nonamplified, FH-amplified, UH-nonamplified, and UH-amplified. The prognostic significance of

these four subsets were compared for patients with disease in all clinical stages and in individual stages according to the Evans system³⁵ and analyzed with treatment protocol.

Analyses of tumors in the NB category

Further analyses of the clinically and biologically significant correlation between MYCN status and morphologic indicators (grade of neuroblastic differentiation and MKI) for evaluating tumors in the NB category were performed with and without the age factor (age cut-off values at 1.5 years and 5.0 years at the time of diagnosis according to the International Classification). Those tumors in the GNBi and the GN categories were excluded from the analyses, because they were predominantly comprised of Schwannian stroma cells rather than neuroblastic cells. Tumors in the GNBn category, comprised of biologically different clones,^{24–26,36,37} also were excluded from the analysis because of difficulty in identifying tissue area (neuroblastomatous or Schwannian stromal) of sampling for the MYCN test.

Statistical Methods

All prognostic analyses for event free survival (EFS) and overall survival (OS) from the time of study entry were done by using the Kaplan–Meier method.³⁸ The log-rank statistic was used to compare the EFS and OS probabilities of the individual prognostic groups and subsets. The Pearson chi-square method was used to analyze correlations between morphologic indicators and MYCN status for tumors in the NB category. Age distribution of the patients for different subsets was compared by testing the median age between all pairwise combinations using the Mann–Whitney test. If not specified, the EFS and OS rates listed in the text represent calculated rates at 5 years from study entry.

Because the International Classification (histopathology), MYCN status, and clinical staging are well established and are known independent prognostic variables, the Cox proportional hazards regression method³⁹ was used for multivariate analyses. The estimated relative risk (RR) and 95% confidential interval (95%CI) for each variable (histopathology, MYCN status, and stage) are summarized.

RESULTS

All NTs (NB, GNBi, GN, and GNBn)

Among 628 tumors, there were 535 tumors in the NB category (420 MYCN nonamplified tumors and 115 MYCN amplified tumors), 21 tumors in the GNBi category (21 nonamplified tumors and 0 amplified tumors), 9 tumors in the GN category (9 nonamplified

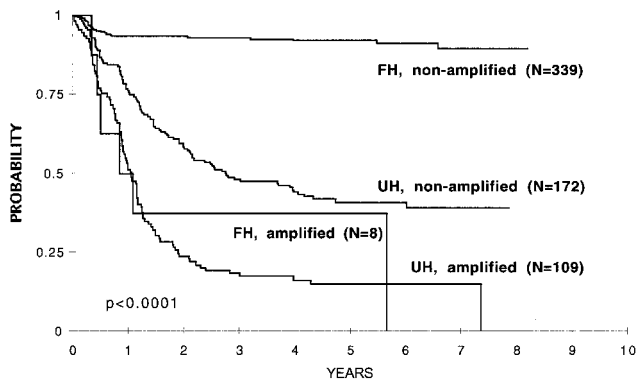


FIGURE 1. Event free survival for patients in four subsets defined by histopathology and MYCN status (all neuroblastic tumors). FH, nonamplified: favorable histology and nonamplified MYCN; UH, nonamplified: unfavorable histology and nonamplified MYCN; FH, amplified: favorable histology and amplified MYCN; UH, amplified: unfavorable histology and amplified MYCN. For the numbers of the patients at risk in each year, see Table 2.

tumors and 0 amplified tumors), and 63 tumors in the GNBn category (61 nonamplified tumors and 2 amplified tumors). According to the International Classification, there were 347 FH tumors and 281 UH tumors. The vast majority of tumors in the FH group (339 of 347 tumors; 97.7%) had nonamplified MYCN, and MYCN-amplified tumors were almost always (109 of 117 tumors; 93.2%) classified into the UH group. Among UH tumors, there were more tumors with nonamplified MYCN (172 of 281 tumors; 61.2%) than tumors with amplified MYCN (109 of 281 tumors; 38.8%). Figure 1 shows EFS curves for the patients in the four different subsets based on the combination of histopathologic evaluation and MYCN status of their tumors.

Table 1 shows patient distribution by clinical stage for the four different subsets. Among children with nonadvanced stage disease (Stage I and II; all treated on the CCG-3881 protocol in this series), 1) patients with FH-nonamplified tumors (Stage I, $n = 64$ patients; Stage II, $n = 112$ patients) had a better prognosis than patients with UH-nonamplified tumor (Stage I, $n = 8$ patients; Stage II, $n = 19$ patients; Stage I patients: $P < 0.0001$ for EFS, $P < 0.0001$ for OS; Stage II patients: $P = 0.0716$ for EFS, $P = 0.0051$ for OS); 2) one patient with an FH-amplified tumor had disease progression within 1 year but is alive 3 years after diagnosis; and 3) one patient with an UH-amplified tumor died 2.3 years after diagnosis.

All infants with Stage III disease ($n = 48$ patients; treated on the CCG-3881 protocol) are alive irrespective of histopathology and MYCN status. Among children with Stage III disease who were diagnosed after age 1 year, 1) all patients with FH-nonamplified tu-

mors ($n = 34$ patients; 31 patients who were treated on the CCG-3881 protocol and 3 patients who were treated on the CCG-3891 protocol due to elevated ferritin levels) are alive; 2) the outcome of patients with UH-nonamplified tumors ($n = 33$ patients; treated on the CCG-3891 protocol) was good, but their prognostic rate was still significantly lower than that in patients with FH-nonamplified tumors (EFS, $P = 0.01$; OS, $P = 0.01$); 3) patients with UH-amplified tumors ($n = 18$ patients; treated on the CCG-3891 protocol) had a very poor outcome (EFS, $P < 0.0001$; OS, $P < 0.0001$); and 4) one patient with an FH-amplified tumor died within 1 year after diagnosis.

Among infants with Stage IV disease, 1) all patients with nonamplified tumors ($n = 39$ patients; 36 patients with FH tumors and 3 patients with UH tumors; treated on the CCG-3881 protocol) are alive; 2) patients with UH-amplified tumors had a significantly poor prognosis ($n = 12$ patients; treated on the CCG-3891 protocol; EFS, $P < 0.0001$; OS, $P < 0.0001$); and 3) 1 of 2 patients with FH-amplified tumors died 1 year after diagnosis. Among children with Stage IV disease who were diagnosed after age 1 year (all treated on the CCG-3891 protocol), 1) patients with FH-nonamplified tumors ($n = 7$ patients) had a good outcome, and their prognostic rate was significantly higher than that in patients with UH tumors ($n = 183$ patients; 106 patients with nonamplified tumors and 77 patients with amplified tumors; EFS, $P < 0.0001$; OS, $P < 0.0001$); 2) although patients with UH tumors generally had a poor outcome, there was a significant difference in EFS ($P < 0.0001$) and OS ($P < 0.0001$) between children with UH-nonamplified tumors and those with UH-amplified tumors; and 3) 2 of 3 patients with FH-amplified tumors died within 1 year after diagnosis. All children with Stage IVS disease ($n = 43$ patients; treated on the CCG-3881 protocol) had FH-nonamplified tumors and showed a good clinical outcome.

Multivariate analyses were performed on models for EFS and OS. The EFS model included histopathology (RR, 4.989; 95% CI, 3.283–7.583; $P < 0.0001$), MYCN status (RR, 2.168; 95% CI, 1.630–2.884; $P < 0.0001$), and stage (RR, 12.051; 95% CI, 5.875–24.719; $P < 0.0001$); and the OS model included histopathology (RR, 12.051; 95% CI, 5.875–24.719; $P < 0.0001$), MYCN status (RR, 2.570; 95% CI, 1.874–3.526; $P < 0.0001$), and stage (RR, 3.078; 95% CI, 1.983–4.778; $P < 0.0001$). Individual variables (histopathology, MYCN status, and stage) remained significant prognostic factors conditional on the other two variables.

Further analysis was done for comparing the prognostic effects of the four different subsets; i.e., FH-nonamplified, FH-amplified, UH-nonamplified,

TABLE 1
Distribution and Survival Rates of 628 Patients with Neuroblastic Tumors by Histopathology, MYCN Status, and Clinical Stage

Stage	FH (<i>n</i> = 347 patients)						UH (<i>n</i> = 281 patients)						Total
	Nonamplified			Amplified			Nonamplified			Amplified			
	No.	EFS	OS	No.	EFS	OS	No.	EFS	OS	No.	EFS	OS	
I	64	93.5	100.0	1 ^a	—	—	8	75.0 ^b	87.5 ^b	1	0.0	0.0	74
II	112	86.2	99.1	0	—	—	19	62.2	80.9	0	—	—	131
III													
Age < 1 yr	43	100.0	100.0	1	100.0 ^c	100.0 ^c	3	66.7	100.0	1	100.0	100.0	48
Age ≥ 1 yr	34 ^d	100.0	100.0	1 ^e	0.0	0.0	33 ^e	81.8	81.8	18 ^e	27.8	33.3	86
IV													
Age < 1 yr	36	100.0	100.0	2 ^e	50.0	50.0	3	100.0	100.0	12 ^e	16.7	16.7	53
Age ≥ 1 yr	7 ^e	85.7	85.7	3 ^e	33.3	33.3	106 ^e	21.4	34.2	77 ^e	10.2	19.3	193
IVS	43	84.6	97.6	0	—	—	0	—	—	0	—	—	43
Total	339	92.1	99.1	8	37.5	50.0	172	40.9	51.1	109	15.0	22.5	628

FH: favorable histology (according to the International Neuroblastoma Pathology Classification system); UH: unfavorable histology; nonamplified: nonamplified MYCN status; amplified: amplified MYCN status; EFS: 5-year event free survival (%); OS: 5-year overall survival (%).

^a This patient had tumor progression but is alive 3 years after diagnosis.

^b Three-year EFS and OS (%).

^c Four-year EFS and OS (%).

^d This number includes three patients who were treated on Children's Cancer Group protocol 3891 (CCG-3891) due to elevated ferritin levels.

^e These patients were treated on the CCG-3891 protocol (all other patients were treated on the CCG-3881 protocol).

and UH-amplified tumors, stratified according to age (< 1 year vs. ≥ 1 year at the time of diagnosis), and this resulted in a stratified *P* value < 0.0001 for both EFS and OS. Similar analysis showed that the prognostic effects of the four different subsets also were independent from disease stage, with a stratified *P* value < 0.0001 for both EFS and OS.

Tumors in the NB Category

Analyses without the age factor

Among 535 tumors in the NB category, there were 317 FH tumors (309 nonamplified and 8 amplified) and 218 UH tumors (111 nonamplified and 107 amplified). Patients with FH-nonamplified tumors had excellent EFS (91.6%) and OS (99.0%) rates, and 199 of 309 patients (64.4%) presented with nonadvanced clinical stage disease (Stage I or II) or with Stage IVS disease. In contrast, patients with FH-amplified tumors had significantly lower EFS (37.5%) and OS (50.0%) rates, and the majority (7 of 8 patients; 87.5%) presented with advanced clinical stage disease (Stage III or IV; EFS, *P* < 0.0001; OS, *P* < 0.0001; stage distribution: *P* < 0.0001). Patients with UH-nonamplified tumors also presented frequently (104 of 111 patients; 93.7%) with advanced stage disease, and patients with UH-amplified tumors almost always presented (106 of 107 patients; 99.1%) with advanced clinical stage disease. The EFS (38.9%) and OS (47.1%) rates for patients with UH-nonamplified tumors were significantly lower

compared with the rates in patients with FH-nonamplified tumors (EFS, *P* < 0.0001; OS, *P* < 0.0001) but significantly better compared with the EFS (15.3%) and OS (23.0%) rates in patients with UH-amplified tumors (EFS, *P* < 0.0001; OS, *P* < 0.0001; see EFS curves in Fig. 2a).

The next step was to compare the morphologic indicators and MYCN status. Figure 3a shows that the vast majority of MYCN amplified tumors (108 of 115 tumors; 93.9%) in the NB category did not show neuroblastic differentiation and were classified into either the undifferentiated subtype or the poorly differentiated subtype. In contrast, 67 of 420 MYCN nonamplified tumors (16.0%) in this category were classified into the differentiating subtype with morphologic evidence of neuroblastic differentiation (*P* = 0.0068). Among the different MKI classes, a significant difference also was observed between MYCN amplified tumors (low MKI, 16 patients [13.9%]; intermediate MKI, 28 patients [24.3%]; high MKI, 71 patients [61.7%]) and nonamplified tumors (low MKI, 299 patients [71.2%]; intermediate MKI, 88 patients [20.9%]; high MKI, 33 patients [7.9%]), in which MYCN-amplified tumors had higher MKI classes (*P* < 0.0001).

Analyses with age as a factor according to the Shimada system

Age ranges and median ages at the time of diagnosis for patients in the four different subsets were as fol-

TABLE 2
Follow-Up Information for Patients with Neuroblastic Tumors According to Favorable or Unfavorable Histopathologic Classification and Amplified or Nonamplified MYCN Status: Numbers of the Patients at Risk at Year for Figure 1 and 2

Figure 1	Year								
	0	1	2	3	4	5	6	7	8
FH, nonamplified	339	308	298	280	224	156	85	32	2
UH, nonamplified	172	130	98	76	63	42	23	11	—
FH, amplified	8	4	3	3	3	2	—	—	—
UH, amplified	109	56	26	20	13	10	6	4	—

Figure 2	Year								
	0	1	2	3	4	5	6	7	8
All ages									
FH, nonamplified	309	280	274	257	205	142	80	31	2
UH, nonamplified	111	78	61	48	42	30	18	9	—
FH, amplified	8	4	3	3	3	2	—	—	—
UH, amplified	107	54	26	20	13	10	6	4	—
Age < 1.5 yr									
FH, nonamplified	275	250	244	229	180	125	71	28	2
UH, nonamplified	11	7	7	6	6	4	1	—	—
FH, amplified	8	4	3	3	3	2	—	—	—
UH, amplified	33	9	6	5	5	4	3	3	—
Age 1.5–5 yr									
FH, nonamplified	34	30	30	28	25	17	9	3	—
UH, nonamplified	77	56	42	33	29	22	15	9	—
UH, amplified	68	44	19	14	7	5	2	1	—
Age ≥ 5 yr									
UH, nonamplified	23	15	12	9	7	4	2	—	—
UH, amplified	6	1	1	1	1	1	1	—	—

FH: favorable histology (according to the International Neuroblastoma Pathology Classification system); UH: unfavorable histology; nonamplified: nonamplified MYCN status; amplified: amplified MYCN status.

lows: ages 0–4.65 years (median, 0.58 years) for patients with FH-nonamplified tumors; ages 0.01–1.49 years (median, 0.99 years) for patients with FH-amplified tumors; ages 0–12.57 years (median, 3.55 years) for patients with UH-nonamplified tumors; and ages 0.35–10.20 years (median, 2.14 years) for patients with UH-amplified tumors. It was noted that patients who had FH tumors, regardless of MYCN status, were diagnosed at a significantly younger age compared with patients who had UH tumors with or without MYCN amplification ($P < 0.0001$). Among the patients with UH tumors, it was also noted that tumors with amplified MYCN were diagnosed in significantly younger children compared with tumors with nonamplified MYCN ($P < 0.0001$). The incidence of MYCN amplification was the highest among patients ages 1.5–5.0 years (68 of 179 patients; 38.0%), whereas 41 of 327 tumors (12.5%) and 6 of 29 tumors (20.7%) had amplified MYCN in patients age < 1.5 years and in patients age ≥ 5 years, respectively ($P < 0.0001$).

Tumors in patients age < 1.5 years. Among patients age < 1.5 years (Figs. 2b, 3b), there were 286 nonamplified tumors and 41 amplified tumors. The vast majority of nonamplified tumors (275 tumors; 96.2%) were classified into the FH group. In contrast, 33 of 41 amplified tumors (80.5%) were classified into the UH group ($P < 0.0001$). The respective EFS and OS rates were 91.7% and 99.3% for patients with FH-nonamplified tumors, 37.5% and 50.0% for patients with FH-amplified tumors, 72.7% and 90.9% for patients with UH-nonamplified tumors, and 15.2% and 18.2% (4-year EFS and 4-year OS rates) for patients with UH-amplified tumors (see EFS curves in Fig. 2b). All UH tumors, regardless of MYCN status, had a high MKI among patients in this age group. A majority of both nonamplified (256 tumors; 89.5%) and amplified (36 tumors; 87.8%) tumors did not show morphologic evidence of neuroblastic differentiation. However, MKI classes were significantly different between the nonamplified and amplified tumors: 80.5% (33 tumors) of amplified tumors had high MKI, and only

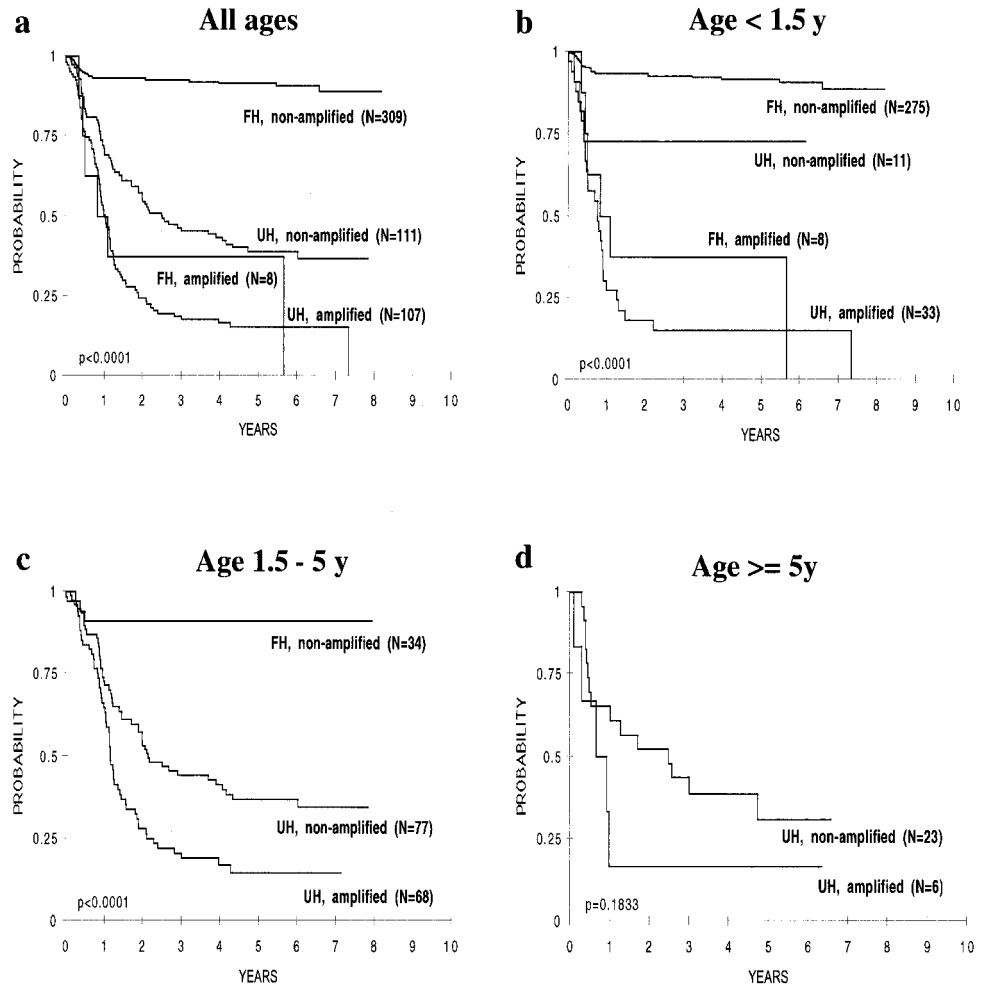


FIGURE 2. Event free survival for four prognostic patient groups defined by histopathology and MYCN status (tumors in the neuroblastoma category): (a) patients at all ages, (b) patients age < 1.5 years at the time of diagnosis, (c) patients ages 1.5–5.0 years at the time of diagnosis, and (d) patients age \geq 5 years at the time of diagnosis. FH, nonamplified: favorable histology and nonamplified MYCN; UH, nonamplified: unfavorable histology and nonamplified MYCN; FH, amplified: favorable histology and amplified MYCN; UH, amplified: unfavorable histology and amplified MYCN. The numbers of patients at risk in each year of survival are shown in Table 2.

3.8% (11 tumors) of nonamplified tumors had high MKI ($P < 0.0001$) (Fig. 3b).

Tumors in patients ages 1.5–5.0 years. Among patients ages 1.5–5.0 years (Figs. 2c, 3c), there were 111 nonamplified tumors and 68 amplified tumors. Nonamplified tumors were classified into either the FH group (34 tumors; 30.6%) or the UH group (77 tumors; 69.4%), whereas all amplified tumors were classified into the UH group ($P < 0.0001$). The respective EFS and OS rates were 91.2% and 97.0% for patients with FH-nonamplified tumors, 36.9% and 43.1% for patients with UH-nonamplified tumors, and 14.6% and 27.2% for patients with UH-amplified tumors (see EFS curves in Fig. 2c). All 34 FH-nonamplified tumors had an appearance of differentiating neuroblastoma with low MKI. The vast majority of both UH-nonamplified tumors (76 of 77 tumors; 98.7%) and UH-amplified tumors (67 of 68 tumors; 98.5%) did not show morphologic evidence of neuroblastic differentiation. However, the MKI classes between these two groups

were significantly different, and amplified tumors had higher MKI classes (UH-nonamplified tumors: low MKI in 36 tumors [46.8%], intermediate MKI in 23 tumors [29.9%], and high MKI in 18 tumors [23.4%]; UH-amplified tumors: low MKI in 8 tumors [11.8%], intermediate MKI in 22 tumors [32.4%], and high MKI in 38 tumors [55.9%]; $P < 0.0001$) (Fig. 3c).

Tumors in patients age \geq 5 years. Among patients age \geq 5 years (Figs. 2d, 3d), all tumors, including 23 nonamplified tumors and 6 amplified tumors, were classified into the UH group according to the International Classification. Figure 2d shows that there was no significant difference in prognosis between patients with MYCN nonamplified and amplified tumors. The respective EFS and OS rates were 30.9% and 41.7% for patients with UH-nonamplified tumors and 16.7% and 20.8% for patients with UH-amplified tumors (EFS, $P = 0.1833$; OS, $P = 0.1415$) (see EFS curves in Fig. 2d). It was noted that the vast majority of tumors in patients in this age group, regardless of MYCN status (21 tumors [91.3% of nonam-

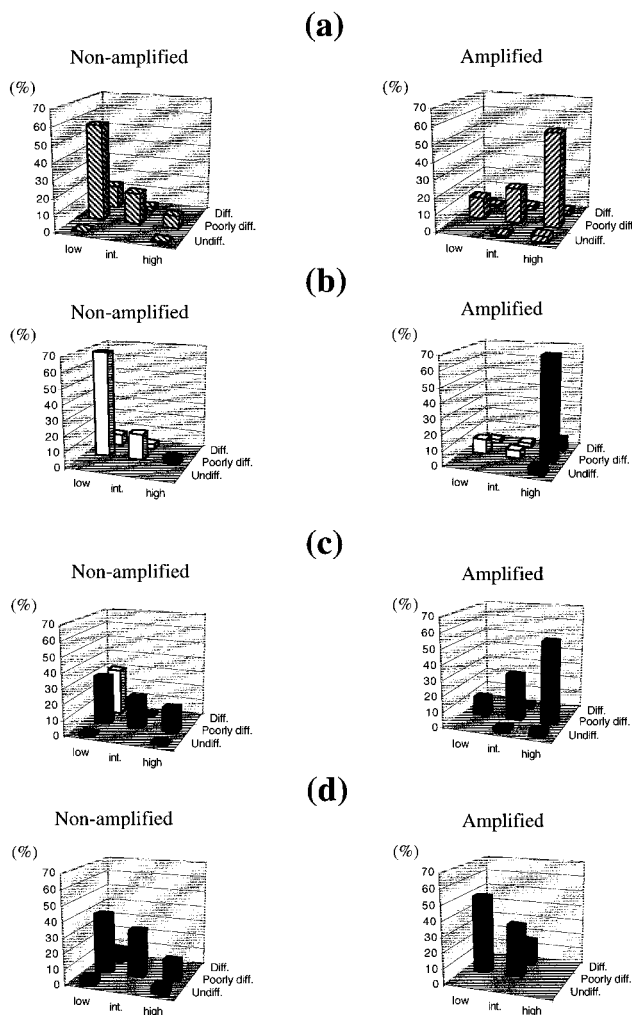


FIGURE 3. Correlations between morphologic indicators (grade of neuroblastic differentiation and mitosis-karyorrhexis index [MKI]) and MYCN status in tumors of the neuroblastoma category (MYCN nonamplified tumors [Non-amplified] and MYCN amplified tumors [Amplified]) with three grades of neuroblastic differentiation (differentiating [diff.], poorly differentiated [poorly diff.], and undifferentiated [undiff.]) and three MKI levels (low, intermediate [int.], and high) (a) in patients at all ages, (b) in patients age < 1.5 years at the time of diagnosis (c) in patients ages 1.5–5.0 years at the time of diagnosis, and (d) in patients age ≥ 5 years at the time of diagnosis. In panel a, the striped columns include tumors in both the favorable histology and unfavorable histology subgroups. White columns indicate favorable histology subgroups, and black columns indicate unfavorable histology subgroups in b–d.

plified tumors]; 5 tumors [83.3% of amplified tumors] did not show neuroblastic differentiation, and none of the amplified tumors and 17.4% (4 tumors) of the nonamplified tumors had a high MKI (Fig. 3d).

DISCUSSION

This is the first report presenting detailed analyses between MYCN status and morphologic features eval-

uated by using the International Classification.^{24,25} Four subsets were made by combining histopathology and MYCN status: FH-nonamplified, FH-amplified, UH-nonamplified, and UH-amplified. It was noted that the vast majority of tumors in the FH group had nonamplified MYCN status, whereas MYCN-amplified tumors were almost always classified into the UH group. Among UH tumors, however, there were more tumors with nonamplified MYCN than with amplified MYCN. The patients with tumors in the FH-nonamplified subset often presented with nonadvanced stages (Stage I and II disease) or Stage IVS disease and had an excellent prognosis. It was extremely rare to encounter patients with tumors in the FH-amplified subset. An excellent prognosis for patients with nonadvanced stage disease with tumors in this subset was reported previously by other investigators.⁴⁰ Of eight patients with tumors in the FH-amplified subset in this series, seven patients presented with advanced stage disease (Stages III and IV), and four patients died of disease. A majority of the patients with tumors in the UH-nonamplified subset also presented with advanced stage disease, and their EFS and OS rates generally were lower compared with the rates of patients with FH-nonamplified tumors. However, as reported previously, the prognosis of patients who are diagnosed after age 1 year with UH-nonamplified tumors in Stage III improved significantly in this series by aggressive treatment on the CCG-3891 protocol.¹³ Among the patients with tumors in the UH-amplified subset, the vast majority had advanced stage disease and had the poorest prognosis. Multivariate analyses demonstrated that each one of the variables (histopathology, MYCN status, and stage), after stratification by the other variables, remained prognostically significant in this series of patients. Furthermore, the prognostic effects of the four different subsets (FH-nonamplified, FH-amplified, UH-nonamplified, and UH-amplified) were independent from both patient age and disease stage.

MYCN amplification was seen almost exclusively in tumors of the NB category. Further analyses of the NB category clearly demonstrated a significant correlation between the molecular event and the morphologic manifestation: MYCN status was related closely to the two morphologic features, grade of neuroblastic differentiation and MKI class, in which age-linked prognostic effects were incorporated in the International Classification. The results of this study are summarized as follows:

- 1) Of the patients with FH tumors, the majority of which were MYCN-nonamplified, most were of the poorly differentiated subtype in patients who were

diagnosed at age < 1.5 years, and were exclusively of the differentiating subtype in patients who were diagnosed at ages 1.5–5.0 years. Patients with FH-nonamplified tumors had an excellent prognosis regardless of age.

- 2) The majority of patients with MYCN-amplified tumors (almost always classified into an UH group) did not have neuroblastic differentiation regardless of age. The majority of patients with MYCN-amplified tumors had a high MKI when they were diagnosed at age < 1.5 years and had either high or intermediate MKI when they were diagnosed at ages 1.5–5.0 years, whereas all patients with MYCN-amplified tumors, although they were small in number, had either intermediate or low MKI when they were diagnosed age > 5 years. Patients with UH-amplified tumors had the worst prognosis regardless of age.
- 3) The majority of patients with UH-nonamplified tumors also did not show neuroblastic differentiation regardless of age. Patients age < 1.5 years with UH-nonamplified tumors had a significantly better prognosis than patients age \geq 1.5 years of age with tumors in the same subset (EFS, $P = 0.0026$; OS, $P < 0.0009$). A poor prognosis for patients with UH-nonamplified tumors who were diagnosed after age 1.5 years was predicted only by histopathologic evaluation according to the International Classification.
- 4) When diagnosed after age 5 years, patients with tumors in the NB category all were classified into an UH subgroup histopathologically and had a poor prognosis regardless of MYCN status.
- 5) Patients with FH-amplified tumors were rare. All were diagnosed at age < 1.5 years and generally had a poor prognosis with advance clinical stages.

MYCN amplification is known to cause DNA instability and almost always is accompanied by the production of an excess amount of MYCN protein in neuroblastoma cells. Subsequent formation of an Myc-Max protein heterodimer in the tumor cell nucleus can prevent cellular differentiation, promote cellular proliferation (mitosis), and facilitate cellular death due to DNA instability (karyorrhexis).^{19,23,41–43} The current study strongly suggests that MYCN amplification can be a powerful driving force for preventing neuroblastic differentiation age independently and for increasing mitotic and karyorrhectic activities age dependently. Even though MYCN amplification was supposed to increase mitotic and karyorrhectic activities, those patients who were diagnosed at an older age did not have higher MKI classes in this series. Thus, it is speculated that MYCN amplified

tumors with higher MKI classes developed earlier than those with lower MKI classes. Further investigation between molecular properties, such as possible overexpression by a single copy of MYCN^{44,45} and prolongation of the half-life of MYCN protein,⁴⁶ as well as expression/amplification of oncogenes other than MYCN and their morphologic manifestations in NTs should be conducted. It is also speculated that other molecular mechanism(s), such as lower expression of neurotrophin receptors,^{47,48} may be responsible for a lack of neuroblastic differentiation in UH-nonamplified tumors.

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