pathogenetic step. In particular, amplification may prevent the physiologic c-*myc* or c-*myb* suppression that in myeloid cells appears to correlate with the switch from a proliferative to a differentiative program (*15*).

Finally, the finding of alternative c-*myb* or c-*myc* oncogene amplification in two phenotypically similar tumors suggests that tumor subtypes that are indistinguishable by current morphological or biochemical techniques may be characterized by different genetic markers and pathogenetic pathways. This form of genetic heterogeneity could be further explored in relation to variability in other parameters; for example, differences in drug sensitivities or in the properties of malignant cells in vitro, which sometimes occur in phenotypically similar tumors.

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Amplification of N-*myc* in Untreated Human Neuroblastomas Correlates with Advanced Disease Stage

Abstract. *A domain of DNA designated N-*myc *is amplified 20- to 140-fold in human neuroblastoma cell lines but not in cell lines from other tumor types. N-*myc *has now been found to be amplified in neuroblastoma tissue from 24 of 63 untreated patients (38 percent). The extent of amplification appears to be bimodal, with amplification of 100- to 300-fold in 12 cases and 3- to 10-fold in 10 others. Amplification was found in 0 of 15 patients with stage 1 or 2 disease, whereas 24 of 48 cases (50 percent) with stage 3 or 4 had evidence of N-*myc *amplification. These data indicate that N-*myc *amplification is a common event in untreated human neuroblastomas. Furthermore, N-*myc *amplification is highly correlated with advanced stages of disease (*$P < 0.001$*) and with the ability to grow in vitro as an established cell line, both of which are associated with a poor prognosis.*

Aberrant expression of cellular oncogenes has been implicated in the causation of various types of malignant disease. Oncogenes appear to be activated from their normal quiescent or regulated state by (i) increased expression of a single gene, (ii) gene amplification, and (iii) gene alteration resulting in a product with increased oncogenic potential. Some of these events have cytogenetic correlates. For example, the increased expression of c-*myc* in Burkitt lymphoma is frequently associated with a specific 8;14 translocation (*1*). Instances of oncogene activation by gene amplification may be manifested cytogenetically by the presence of homogeneously staining regions (HSR's) on specific chromosomes, or by extrachromosomal double minutes (DM's) (*2*). As expected, a cytogenetic correlate of gene alteration has not been observed, but this appears to be the most common mechanism for activation of oncogenes of the *ras* family (*3*).

Amplification of the oncogene c-*myc* has been demonstrated in several human tumor cell lines, including the colon tumor COLO-320, the lung tumor NCI-417, and the promyelocytic leukemia cell line HL-60 (*2*, *4*). Recently we have identified amplification of a c-*myc*-related sequence called N-*myc* in eight of nine human neuroblastoma cell lines, but not in HSR-bearing cell lines derived from other tumor types (*5*). In addition, we have mapped the normal single-copy locus of N-*myc* to the short arm of chromosome 2, bands 2p23 or 24 (*6*). These findings are in agreement with other recent reports (*7*). Although we demonstrated one case of amplification of N-*myc* in neuroblastoma tissue from an untreated patient (*5*), our previous study was confined almost exclusively to the analysis of established cell lines.

We have now examined 63 primary untreated neuroblastomas for N-*myc* amplification. Our result shows that amplification of this sequence is a common event in vivo, and that it is associated with advanced stages of disease.

Neuroblastoma tissue was obtained from the primary tumors of 63 untreated patients undergoing diagnostic surgery; it was frozen until the time of analysis. Most of the samples were obtained from member institutions of the Children's Cancer Study Group (CCSG), and addi-

tional samples were obtained at the coauthors' institutions. Patients from whom tumor tissue was obtained were grouped according to the clinical staging system of Evans *et al.* (*8*). Stage 1 indicates a tumor that is confined to the organ or structure of origin. Stage 2 tumors extend in continuity beyond the organ or structure of origin but do not cross the midline; regional ipsilateral lymph nodes may be involved. Stage 3 tumors extend beyond the midline and may involve regional lymph nodes bilaterally. Stage 4 tumors have distant metastases involving distant lymph nodes, or hematogenous dissemination to organs, tissues, bone, or bone marrow.

The two most important prognostic variables for patients with neuroblastoma are clinical stage and age at diagnosis. Patients with disease stage 1 or 2 have a good prognosis, with 75 to 90 percent 2-year disease-free survival, whereas patients with stage 3 or 4 have a poor prognosis (10 to 30 percent 2-year disease-free survival) (*9*). Patient age at diagnosis is the only known factor with prognostic significance that is independent of clinical stage. Patients less than 1 year of age at the time of diagnosis do substantially better than older patients with the same disease stage, especially stages 3 and 4 (60 percent as compared to 10 percent 2-year disease-free survival, respectively) (*9*).

Of the 63 patients whose tumors were examined, five had stage 1, ten had stage 2, 23 had stage 3, and 25 had stage 4 disease, a distribution similar to that expected from earlier studies (*9*). Of the 62 patients whose age at diagnosis was known, 17 were less than 1 year old, and 45 were older than 1 year at diagnosis.

DNA was prepared from about 100 mg (wet weight) of each tumor (*10*). The DNA was quantified by fluorometric assay with ethidium bromide dimer (Fluka) (*11*), with confirmation by agarose gel analysis. Samples (4 μg) of DNA from each tumor were digested to completion with Eco RI, subjected to electrophoresis on 0.8 percent agarose gels, and transferred to nitrocellulose filters (*12*). Samples (4 μg) of human leukocyte DNA were used as standards for single-copy intensity. Also, 4 μg of DNA from the neuroblastoma cell line NGP (*5*), which was known to have about 140-fold amplification of N-*myc*, was used as a positive control. Filters were baked and hybridized with the isotopically labeled plasmid probe pNB-1, which contains a 1.0-kb Bam HI–Hind III fragment from the 5′ end of the N-*myc* sequence (*5*). The plasmid was labeled with [α-^{32}P]dCTP (deoxycytidine triphosphate) by nick translation (*13*) to a specific activity of 2×10^8 cpm/μg. Filters were hybridized at 68°C for 14 to 18 hours, washed with 0.1× SSC (standard saline citrate), 0.1 percent SDS (sodium dodecyl sulfate) at 56°C and exposed for 18 to 24 hours at −70°C with an intensifying screen. A hybridization signal was not considered amplified unless it appeared to have at least three times the intensity produced by an equal amount of control DNA in at least three separate experiments. The extent of amplification was quantified by serial dilution of the DNA samples to obtain a hybridization signal of about single-copy intensity (for example, 1:100 dilution which yielded single-copy intensity would indicate about 100-fold amplification). Determinations based on visual inspection were confirmed by densitometric analysis.

In 24 of the 63 tumors (38 percent) amplification, ranging from 3- to 300-fold, was easily detected (Table 1). Although the range of N-*myc* amplification was broad, the distribution appeared to be bimodal. Ten cases had consistently detectable amplification of three- to ten-fold, while 12 others showed an N-*myc* amplification of 100- to 300-fold; the remaining two cases showed about 20- and 40-fold amplification, respectively. The results of hybridization of the N-*myc* probe to equal amounts of DNA from 55 of the 63 neuroblastomas are shown in Fig. 1. The variation in the intensity of bands in cases with apparently unamplified N-*myc* can be attributed to variation in DNA degradation in the tumor samples. This may have led to an underestimation of the number of cases with low-level amplification.

We analyzed our data on N-*myc* amplification to determine whether or not there was any correlation with disease stage (Table 2). None of the 15 tumors from patients with stage 1 or 2 disease had N-*myc* amplification. In contrast, 24 of 48 patients (50 percent) with stage 3 or 4 disease had tumors with readily detectable amplification. Thus, the correlation of N-*myc* amplification with advanced disease stage was statistically significant ($\chi^2 = 10.09$, $P < 0.001$) (*14*). The distri-

Table 1. Patient age (in months) and clinical stage of 63 untreated patients with neuroblastoma and determination of N-*myc* copy number in their tumor DNA's by DNA hybridization. ID, identification.

Patient		Clinical stage*	N-*myc* copy number†	Patient		Clinical stage*	N-*myc* copy number†
ID	Age			ID	Age		
1	66	4	5 to 6	33	47	3	15 to 20
2	1	3	1	34	18	4	250
3	11	3	1	35	10	4	6 to 8
4	56	3	100	36	32	3	1
5	72	4	1	37	57	3	1
6	6	1	1	38	58	2	1
7	26	4	1	39	6	2	1
8	22	3	150	40	22	3	1
9	8	2	1	41	13	2	1
10	6	2	1	42	3	2	1
11	7	3	1	43	11	4	100
12	1	1	1	44	59	4	1
13	11	3	200	45	21	4	150
14	8	2	1	46	24	1	1
15	?	3	1	47	20	4	1
16	30	1	1	48	30	4	1
17	132	4	1	49	40	4	1
18	26	4	1	50	53	4	5 to 6
19	15	3	1	51	16	4	200
20	14	3	200	52	120	3	8 to 10
21	52	3	1	53	24	3	150
22	17	3	3 to 4	54	72	4	1
23	76	3	1	55	5	2	1
24	6	1	1	AR	13	4	100
25	54	3	1	SH_1	60	4	1
26	12+	4	300	JT	24	4	30 to 40
27	22	3	5 to 6	KM	60	2	1
28	75	4	1	SH_2	30	3	8 to 10
29	33	4	1	LG	72	4	3 to 4
30	43	3	250	GK	54	4	5 to 6
31	6	3	1	CM	3	2	1
32	34	4	3 to 4				

*There were no stage 4*S* patients included in this study. †The estimation of the extent of N-*myc* amplification was based on (i) several separate experiments in which the samples were arranged in different orders and (ii) serial dilution as well as densitometric analysis of hybridization signal produced by the N-*myc* probe.

bution and the degree of amplification were almost identical in patients with stage 3 and stage 4 disease. Half the cases in both groups had amplification and both showed a bimodal distribution; most of the patients exhibited either less than tenfold or more than a 100-fold amplification.

Only 3 of 17 patients less than 1 year of age had N-*myc* amplification compared to 21 of 45 patients more than 1 year of age at diagnosis. While this suggested a trend, it was not statistically significant ($\chi^2 = 3.24$, $P > 0.05$). This trend could reflect the greater likelihood that infants in our series had lower stage disease, since infants with advanced disease were just as likely to have N-*myc* amplification. Three of seven infants with stage 3 or 4 had N-*myc* amplification compared to 21 of 40 older children ($\chi^2 = 0.004$, not significant).

Recently, we provided evidence that N-*myc* is amplified in eight of nine neuroblastoma cell lines regardless of the cytogenetic form of the amplified DNA (DM's compared to HSR) or of the chromosomal location of the HSR (*5*). Furthermore, we have mapped the amplified N-*myc* sequences to HSR's on three different chromosomes in three neuroblastoma cell lines by in situ hybridization, and we have mapped the normal single-copy locus of N-*myc* to 2p23 or 24 (*6*). We have now demonstrated that N-*myc* amplification is common in primary untreated tumors and that it is associated exclusively with disease stages 3 and 4 at diagnosis, which correlates with a poor prognosis.

One striking observation is that amplification of N-*myc* is present in virtually all neuroblastoma cell lines (*5, 7*) but in less than half of primary untreated tumors. Two possible explanations are (i) tumor cells with preexisting amplification of N-*myc* may adapt particularly well to growth in vitro or (ii) adaptation to continuous growth as a cell line may promote amplification of this sequence in vitro. The former hypothesis is supported by the finding that virtually all neuroblastoma cell lines have been established from patients with advanced disease, and amplification of N-*myc* appears to be exclusive to these patients. Indeed, patients whose tumor cells can be grown in vitro as a cell line are considered to have a poor chance of surviving (*15*). Taken together, these observations support the clinical association of N-*myc* amplification with advanced disease and a poor prognosis.

Another interesting feature of this amplification is that the distribution of copy number in the affected cases was bimodal; 22 of 24 cases had either low (three- to tenfold) or high (100- to 300-fold) amplification. One possible explanation is that amplification proceeds in two or more steps: an initial increase in copy number by severalfold may be followed by another round of amplification which increases the copy number by one or two orders of magnitude. Alternatively, tumors with "low level" amplification may represent a mixed population of cells in which a minor subpopulation has high-level amplification.

Table 2. Correlation of N-*myc* amplification with disease stage in 63 untreated patients with neuroblastoma. Two-by-two chi-squared analysis of the correlation between N-*myc* amplification (present compared to absent) and disease stage (combined stages 1 and 2 compared to combined 3 and 4) was highly significant; ($\chi^2 = 10.09$, $P < 0.001$) (*14*).

Stage	Patients (No.) with tumors having copy numbers of N-*myc* from				Total
	1	2 to 10	11 to 100	> 100	
1	5	0	0	0	5
2	10	0	0	0	10
3	12	4	1	6	23
4	12	6	1	6	25

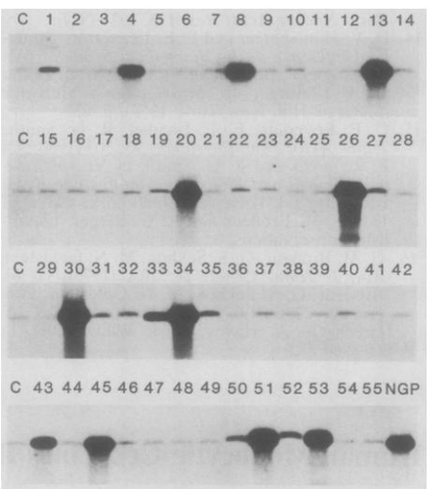

Fig. 1. Autoradiograms of DNA from 55 untreated human neuroblastomas hybridized with N-*myc* probe. Tumor samples were assigned numbers 1 to 55 in the order received. These numbers correspond to the first 55 cases listed in Table 1. The first lane in each row contains normal leukocyte DNA as a measure of single-copy intensity ("C"), and the last lane in the last row contains DNA from neuroblastoma cell line NGP, which has about 140-fold amplification of N-*myc* (*5*). Samples (4 μg) of Eco RI–digested DNA were placed on the 60 lanes, and in all cases a 2.0-kb band of variable intensity was detected. Additional bands of larger or smaller size (lanes 1, 13, 26, and 53) were not consistently seen. Low-level amplification (three- to tenfold) of N-*myc* was consistently seen in at least three separate experiments for neuroblastomas 1, 22, 27, 32, 35, 50, and 52. However, neuroblastomas 19 and 31 appeared to have bands of similar intensity on these autoradiograms, suggesting low-level amplification, but this was not confirmed by subsequent experiments.

Although our series of 63 patients includes a majority with stage 3 and stage 4 disease, the prevalence of N-*myc* amplification (38 percent) appears to be higher than the frequency with which DM's or HSR's have been observed in primary neuroblastomas (*16*). On the basis of previous estimates, the limits of resolution of DNA with the light microscope may be on the order of 1×10^6 to 5×10^6 base pairs (*17*). If this is true, some neuroblastomas may have DM's which are too small to be detected. Indeed, given the typically suboptimal quality of metaphase preparations obtained from primary tumor tissue, small HSR's or abnormally banding regions (ABR's) (*18*) might escape detection even if they exceed the usual size threshold. Thus, the higher frequency of N-*myc* amplification compared to the frequency of observable DM's, HSR's, or ABR's probably reflects the greater sensitivity of the DNA-hybridization assay.

Amplification of the N-*myc* sequence is relatively specific for neuroblastomas. N-*myc* amplification has been reported in a retinoblastoma cell line Y79 (*7*), but it was not found in HSR-bearing cell lines derived from carcinoma of the colon, breast, cervix, and lung or from melanomas (*5, 7*). Thus, the apparent specificity of N-*myc* amplification for neurogenic tissues, neuroblastomas, and probably retinoblastomas appears to be greater than the specificity of c-*myc* amplification, which has been found to be amplified in colon, lung, and hematopoietic cell lines (*4*).

DM's and HSR's are common findings in primary neuroblastomas and cell lines (*19*), although they are not exclusive to neuroblastomas. An additional karyotypic finding has been identified which is characteristic of human neuroblastoma cells, that is, deletion of the distal short arm of chromosome 1 (*16*). This abnormality has been identified in about 70 percent of both primary neuroblastomas and tumor-derived cell lines (*16*). Since N-*myc* maps to the short arm of chromosome 2 (*6*), and has been amplified as an HSR on the short arm of chromosome 1 in only one case [the IMR-32 cell line (*7*)], the relation between partial 1p monosomy and N-*myc* (if any) is not obvious. A neuroblastoma-derived transforming gene N-*ras* also has been mapped to the proximal short arm

of chromosome 1 (1p3200 → cen) (*20*). However, this appears to be proximal to the most common breakpoint of chromosome 1 in neuroblastomas (*16*), and no example of N-*ras* translocation in a human neuroblastoma has been identified thus far.

It is known that sequences from the short arm of chromosome 2 other than N-*myc* are amplified in neuroblastomas (*7*). However, the fact that N-*myc* is amplified may be particularly important since there is evidence that it may be a proto-oncogene. First, it bears partial homology with a known proto-oncogene, c-*myc*. Second, it appears to be a rather conserved sequence. We have used N-*myc* hybridization to analyze Eco RI-digested DNA from almost 100 different individuals and have not detected a single case with polymorphism. Furthermore, a band of different size but similar intensity was detected when this probe was hybridized under stringent conditions to DNA's from mouse, rat, and hamster, which suggests conservation through recent evolution (*6, 21*). Third, N-*myc* amplification is associated with increased levels of mRNA transcripts of N-*myc* in these tumors (*7, 22*). Our results demonstrate clinical prognostic significance of N-*myc* amplification, and they suggest an important role for this sequence in human neuroblastoma.

Note added in proof: In a recent report (*23*), ten retinoblastomas and a retinoblastoma cell line were studied for amplification and mRNA expression of N-*myc*. Genomic amplification of N-*myc* was identified in two of the ten tumors and in the Y79 cell line, but all 11 had increased levels of N-*myc*–related mRNA compared to control cultures. These results confirm the probable importance of N-*myc* in retinoblastoma and expand the tumors with which N-*myc* activation is associated to include both neuroblastoma and retinoblastoma.

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Human Monocytic Cell Lines Derived from Cord Leukocytes by Co-cultivation with Irradiated CM-S Cells

Abstract. *The CM-S cell line was established from the bone marrow of a child with congenital hypoplastic anemia and resembles its monocyte-macrophage lineage. Lethally x-irradiated CM-S cells from various passages and clones, representing different stages in the progression of the transformed growth phenotype, were tested for their ability to affect the survival and proliferation of normal human cord or adult blood leukocytes in co-culture. One clone, CM-SM, which is tumorigenic in athymic mice, consistently immortalized umbilical cord mononuclear cells but did not immortalize adult peripheral blood leukocytes. Six autonomous monocyte-like diploid cell lines were obtained and all were found to be of cord origin. Three lines were tumorigenic in athymic mice. Attempts to immortalize human leukocytes with cell-free supernatants from CM-S cells were unsuccessful.*

The autonomous cell line CM-S was derived from the bone marrow of a male child suffering from congenital hypoplastic anemia, a rare childhood defect of erythropoiesis (*1*). The cells grow in suspension culture, where most appear morphologically undifferentiated but express some of the enzyme and antigen markers characteristic of precursor monocytic and granulocytic cells (*2*). When incubated in culture with factors present in conditioned medium or with various chemical compounds, CM-S cells can undergo marked morphological and