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Biomarkers, Genomics, Proteomics, and Gene Regulation

2p24 Gain Region Harboring *MYCN* Gene Compared with *MYCN* Amplified and Nonamplified Neuroblastoma

Biological and Clinical Characteristics

Marta Jeison,* Shifra Ash,† Gili Halevy-Berko,* Jacques Mardoukh,* Drorit Luria,‡ Smadar Avigad,†§ Galina Feinberg-Gorenshtein,§ Yacov Goshen,† Gabriel Hertzel,¶ Joseph Kapelushnik,‖ Ayelet Ben Barak,** Dina Attias,†† Ran Steinberg,‡‡ Jerry Stein,† Batia Stark,*† and Isaac Yaniv†§

From the Cancer Cytogenetic Laboratory, the Pediatric Hematology Oncology Department,† the Flow Cytometry Laboratory of the Felsenstein Medical Research Center,‡ the Molecular Oncology Laboratory of the Felsenstein Medical Research Center,§ and the Department of Pediatric Surgery,‡‡ of the Schneider Children's Medical Center of Israel, Petah Tikva; the Sackler School of Medicine, Tel Aviv University, Tel Aviv; the Pediatric Oncology Unit,¶ Haemek Medical Center, Afula; the Pediatric Day Care Unit,‖ Soroka University Medical Center, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva; the Department of Pediatric Hematology Oncology,** Meyer Children's Hospital, Rambam Health Care Campus, Haifa; the Division of Pediatric Hematology/Oncology,†† Bnei Zion Medical Center, Bruce Rappoport Faculty of Medicine, Technion Institute, Haifa, Israel*

Although the role of *MYCN* amplification in neuroblastoma is well established, the biological and clinical characteristics of the 2p gain region harboring the *MYCN* gene remain unclear. The aim of this study was to compare the biological and clinical characteristics of these tumors with *MYCN* amplified and nonamplified neuroblastoma and to determine their impact on disease outcome. Samples from 177 patients were analyzed by fluorescence *in situ* hybridization, including *MYCN*, 1p, 17q, and 11q regions; 2p gain was identified in 25 patients, *MYCN* amplification in 31, and no amplification in 121 patients. Patients with 2p gain had a significantly worse 5-year event-free survival rate than patients with no *MYCN* amplified ($P < 0.001$), and an intermediate 5-year overall survival rate difference existed between the *MYCN* amplified tumors ($P = 0.025$) and nonamplified ($P = 0.003$) groups. All of the 2p gain samples were associated with segmental and/or numerical alterations in the other tested regions. The presence of segmental alterations with or without *MYCN* amplification was recently found to be the strongest predictor of relapse in a multivariate analysis. The results of the present study suggest that the determination of *MYCN* gene copy number relative to chromosome 2, when evaluating *MYCN* status at diagnosis, may help to reveal the underlying genetic pattern of these tumors and better understand their clinical behavior. ***(Am J Pathol 2010, 176:2616–2625; DOI: 10.2353/ajpath.2010.090624)***

Neuroblastoma is an embryonal tumor of childhood originating from cells of the sympathetic neuroectodermal system. Its clinical behavior varies widely, from spontaneous regression in infants or maturation to benign ganglioneuroma in older patients, to a disseminated aggressive chemoresistant tumor with a fatal outcome. A large spectrum of heterogeneous genetic changes underlies this biological diversity.[1,2]

The clinical behavior of human neuroblastoma tumors may be predicted by the analysis of a panel of prognostic factors: patient age at diagnosis,[3] disease stage,[4] tumor histopathology (favorable/unfavorable),[5] DNA index,[6,7] and *MYCN* status.[8,9] In addition, controversial studies have indicated that 1p deletion[10–12] and 17q gain are also factors of poor prognosis.[13–16] Deletions in chromo-

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Address reprint requests to Marta Jeison, Ph.D., Ca-Cytogenetic Lab, Pediatric Hematology Oncology Department, Schneider Children's Medical Center of Israel. Kaplan St. 14, 49202 Petah Tikva, Israel. E-mail: martaj@clalit.org.il.

some arms 3p and 11q have been defined as poor prognostic markers in localized and 4s neuroblastoma.[17] A deletion of the 11q region is also associated with a poor outcome, particularly in Neuroblastoma without *MYCN* amplification.[18,19] Lastowska et al (2007)[20] reported that the expression of genes localized in a novel region on 4p correlated with the DNA copy number and survival. According to the last report of the International Neuroblastoma Risk Group, regression analysis of the data base confirmed the prognostic significance of *MYCN* amplification, deletions on 11q, and ploidy, in addition to age and disease stage.[21] In patients without *MYCN* amplification, a genomic profile characterized by any segmental alteration (partial chromosome gain and loss) adds prognostic information to these clinical markers.[22] In a recent study, multivariate analysis, taking into account the overall genetic profile of NB tumors, in addition to the well known genetic and clinical markers with prognostic significance, the presence of segmental alterations with or without *MYCN* amplification was found to be the strongest predictor of relapse.[23] A novel marker, telomere length, has recently been suggested to be added to the conventional prognostic indicators.[24]

MYCN is a cellular proto-oncogene of the myc family of transcription factors that maps to the short arm of chromosome 2 (band 2p24).[25] *MYCN* amplification occurs in 20% to 30% of primary neuroblastoma. During amplification, genetic material from the chromosomal region is transposed to extrachromosomal double minutes or to intrachromosomal homogeneously staining regions. The tumorigenic potential of this process is most probably a consequence of an increase in gene dose, as mutations within the gene have not been detected.[26]

For several years, we have been characterizing neuroblastoma tumors by fluorescence *in situ* hybridization (FISH), paying special attention to the *MYCN* copy number, following the recommendations of the European Neuroblastoma Quality Assessment (ENQUA) group.[27] FISH makes it possible to rapidly and sensitively detect single-copy DNA sequences in metaphase and interphase nuclei. Additional advantages of this method include reduced tissue requirement and high versatility in determining the nature of the amplification unit (double minutes or homogeneously staining regions). Furthermore, because the copy number can be determined at the single-cell level, FISH has the capability to demonstrate intercellular heterogeneity in *MYCN* amplification within a given tumor cell population.[28,29] The International Neuroblastoma Risk Group Biology Committee defined 2p gain as equal number of *MYCN* and 2p signals, exceeding 2q signals in mostly one or two copies. A balanced ratio between the *MYCN* specific signals and the signal numbers of the reference probe on the chromosome 2q arm defines a *MYCN* nonamplified tumor (*MYCN* normal status). An over fourfold increase in the *MYCN* signal number relative to chromosome 2q indicates a *MYCN* amplified tumor.[30] Although the association of *MYCN* amplification with poor prognosis is well established, the biological and clinical characteristics of 2p/*MYCN* gain have not been clearly defined. To the best of our knowledge, only three publications to date have addressed these findings and their possible influence on the disease outcome, but no definitive conclusions were reached.[31–33] In addition, several case reports have been published on the management of tumors with low-level gain in the *MYCN* region.[33,34]

The aim of the present retrospective study was to investigate the cytogenetic and clinical characteristics of patients with neuroblastoma showing 2p/*MYCN* gain and to compare the findings with *MYCN*-amplified neuroblastoma and neuroblastoma with a balanced number of *MYCN* gene copies, and explore a possible impact on outcome.

Materials and Methods

Patients

Samples from 177 patients with neuroblastoma who were diagnosed and treated in seven centers in Israel between 1993 and 2007 were analyzed at the Cancer Cytogenetic Laboratory of the Pediatric Hemato/Oncology Department of Schneider Children's Medical Center of Israel. One hundred fifty-two samples were derived from tumors and 25 from involved bone marrow; 169 were analyzed at diagnosis and eight after relapse. The study group included 102 male patients and 75 female patients of median age 2.9 years at diagnosis (range, 0 to 17 years). Fifty-three patients were under 1 year old, and 124 were over 1 year old. Stage was assigned according to the International Neuroblastoma Staging System[4] as follows: stage 1, 26 patients (15%); stage 2, 19 (10%); stage 3, 37 (21%); stage 4, 82 (47%); and stage 4s, 13 (7%). Median follow-up was 80 months (range, 0.23 to 200 months). Three patients were lost during follow-up and were included only in the analysis of biological characterization.

Treatment consisted of local protocols including surgery, intensive chemotherapy, and myeloablative therapy with stem cell rescue for high risk patients. Forty-nine high risk patients are included in the Société Internationale d'Oncologie Pédiatrique Europe Neuroblastoma, High Risk Neuroblastoma 1 (SIOPEN HRNBL1) study.

Fluorescence in Situ *Hybridization*

FISH was performed on cytogenetic slides or touch imprints from fresh tumor tissue. Interphase tumor nuclei were screened for *MYCN* copy number in the whole cohort with a *MYCN* DNA (2p24) probe (Oncor, Gaithersburg, MD; Vysis, Downers Grove, IL), together with a reference gene such as *LAF* (2q11) or centromere of chromosome 2 (Qbiogene, Illkirch, France; Kreatech, Amsterdam, the Netherlands; Vysis). The status of three additional genetic parameters was also analyzed: in 174 samples 1p region, with CEP 1 (satellite II/III) band 1q12 for the centromeric region of chromosome 1 (Vysis) and midisatellite *D1Z2*, for the 1p36 locus (Oncor/Qbiogene, Kreatech); in 155 samples 11q region, with CEP 11 (α-satellite, *D11Z1*) combined with *MLL* (11q23.3; ONCOR, Vysis); and in 169 samples 17q region, with CEP17 (α-satellite, *D17Z1*) for centromere 17 or band 17p11.1-q11.1 locus (Vysis) combined with 17q probe

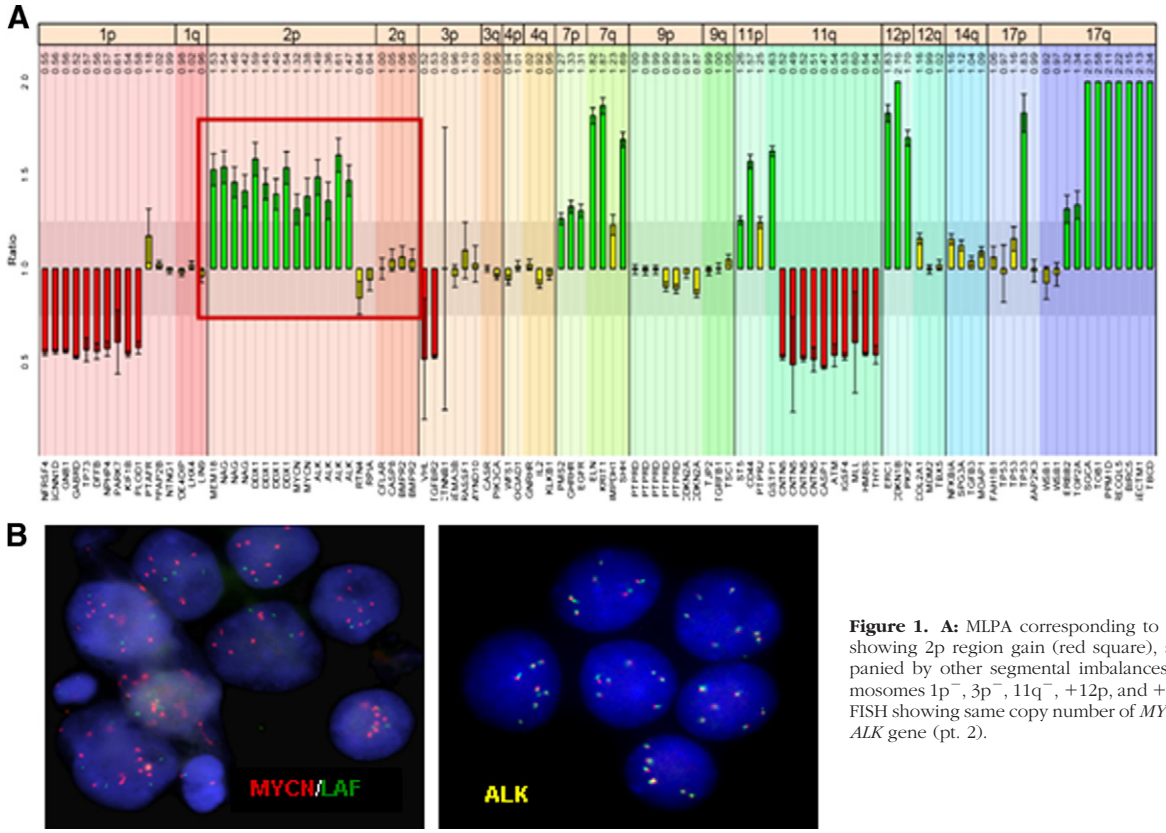

Figure 1. A: MLPA corresponding to pt. 171 showing 2p region gain (red square), accompanied by other segmental imbalances: chromosomes $1p^{-}$, $3p^{-}$, $11q^{-}$, +12p, and +17q. **B:** FISH showing same copy number of *MYCN* and *ALK* gene (pt. 2).

(17q25-qter; Oncor) or telomere 17q (Vysis); two dual-color probes: *LIS1/RARA*, which hybridize to band 17p13.3 and 17q21.1, respectively (Vysis); and *p53/mpo* (17p13, 17q23) (Kreatech). FISH was performed according to the manufacturer's instructions with slight modifications, as previously described.[35] An average of 200 nuclei was counted for each sample.

In cases where the relevant abnormal populations were detected only in low percentages, we performed sequential FISH analysis with different probes on the same cells for up to three consecutive hybridizations per slide. Using this strategy, we were able to confirm the tumor character of the cell population, taking advantage of the FISH technique's efficiency in detecting gene aberrations at the cellular level.

Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) was performed only in the *MYCN* gain cases to identify whether the region gained included adjacent genes in addition to the *MYCN* gene. With this technique, partial gains or losses in 10 different chromosomes could be identified in one experiment.[36,37] We used the neuroblastoma-specific SALSA MLPA probe, which consists of three kits P251/P252/P253 (MRC-Holland, Amsterdam, the Netherlands). The P251 probe mix contains 38 probes for chromosomes 1, 3, and 11; P252 probe mix contains 36 probes for chromosomes 2 and 17; and the P253 probe mix contains 32 probes for chromosomes 4, 7, 9, 12, and 14. Region 2p is covered by 16 clones from seven different genes: *TMEM18, NAG, DDX1, MYCN, ALK, RTN4,* and *RPIA*. Each of these three probe mixes contains five control fragments in chromosomal regions rarely altered in NB. Each probe is composed of two adjacent oligonucleotides containing the target-specific sequence, a universal PCR primer, and in one of them, a stuffer sequence of unique length for each one. The procedure was performed as described by the manufacturer. Briefly, 50 to 100 ng DNA in 5 μl TE-buffer was denaturated for 5 minutes at 98°C and subsequently cooled down to 25°C. After the addition of 1.5 μl probe mix and 1.5 μl buffer, the sample was denaturated for 1 minute at 95°C, and the probes were allowed to hybridize for 16 hours at 60°C. Ligation was performed for 15 minutes at 54°C after the addition of Ligase -65mix, and then heat inactivated at 98°C for 5 minutes. The ligation products were PCR amplified by the addition of 10 μl of this ligation mixture to 40 μl PCR mixture provided by the supplier. In each set of MLPA experiments, we included at least three normal control DNA samples for data processing. PCR was performed in a T3000 Thermocycler (Whatman, Biometra) for 35 cycles. Samples were analyzed by an ABI 3100 DNA analyzer (Applied Biosystems, Foster City, CA) using the Genescan software 3.7. The results were saved as an Excel file (Microsoft Corporation, Redmond, WA, United States of America) and imported for graphical visualization to the MLPAVizard software, specially developed for the neuroblastoma kit and kindly provided by Professor Ambros and Austrian Research Centers GmbH, Vienna, Austria. In cases when MLPA could not be performed, we used *ALK* gene probe on 2p23 (Vysis) to confirm an enlarged region of gain on 2p containing extra copies of *MYCN* gene.

Table 1. FISH, DNA Index, Survival, and Outcome of the 25 Patients with 2p/*MYCN* Gain

	CEP2/*MYCN** or *LAF/MYCN*		CEP1/1p†		CEP11/11q‡		17p/17q§				
Pt. no.	Copy no.¶	%	Copy no.	%	Copy no.	%	Copy no.	%	DNA index	Survival (mo.)	Status
2	4/7	30	4/2	30	4/2	27	4/7	20	1.93	16	D
	5/7	20									
16	3/4	95	3/2	58	2/2	99	4/4	17	1.41	LFU‖	—
25	4/7	80	4/3	55	4/2	72	4/5	80	1.94	19	D
41	2/4	30	3/3	18	3/3	34	3/3	14	1	122	A
	3/5										
	4/6										
47	4/6	81	4/3	55	ND	—	4/6–7	27	2	126	A
	6/9										
58	2/4	15	3/2	43	3/3	35	4/4	15	1.31	117	A
	2/5	33									
94	3/5	20	4/2	36	4/2	36	3/8	14	2.03	49	D
	4/5	20					4/6	15			
96	3/6	22	4/4	50	3/2	48	5/7	43	1.92	32	D
118	2/3	74	3/2	59	2/1	81	2/3–4	8	1	61	A
							3/4–5	21			
124	4/5	98	3/3	50	5/2	80	2/4	33	1.67	110	D
132	2/3	99	2/2	80	2/1	92	2/4	100	1	23	D
133	4/6	80	3/3	30	3/3	25	2/5	58	2.06	37	D
			4/4	53	4/4	61	3/6	25			
140	2/3	30	2/2	98	2/1	34	2/5	30	1	49	D
146	2/3	23	3/3	14	3/1	40	2/3	25	1.92	45	D
	3/6	22	4/4	18	4/2	40	2/6	35			
	4/6	44					3/7–8	35			
148	2/4	23	3/3	43	3/3	76	4/4	49	1.53	30	D
	3/4	65									
157	2/3	42	2/2	80	2/1	40	ND	—	ND	39	D
159	2/4	80	2/2	92	3/2	80	2/9–15	15	1.35	39	D
							3/5–9	43			
							4/10	22			
161	2/3	29	2/2	95	2/2	80	2/3	35	1	34	D
							2/4	15			
164	4/5–7	76	4/4	59	3/2	59	2/amp	21	2.07	21	D
					4/2	13	3/amp	68			
166	2/3	30	2/2	93	2/2	95	2/amp	82	1.16	28	A
168	3/4	24	3/3	10	3/1	19	4/4	20	1.24	26	A
171	2/3	52	2/1	67	3/1–2	70	2/5–7	90	1.09	22	A
179	1/2	66	2/1	58	4/4	24	3/4	30	1	11	A
	2/4	25					5/7	19			
186	2/3	80	2/2	99	2/1	85	2/5–6	81	1	4	A
189	3/5	80	3/3	29	2/2	25	4/5	80	1.46	3	A
			4/4	14	3/3	45					

D, dead; A, alive; amp, amplification.
**MYCN* probe with centromere chromosome 2 or *LAF* gene as reference.
†1p36 gene region with centromere of chromosome 1 as reference.
‡*MLL* gene for 11q region and centromere of chromosome 11 as reference.
§17q21, 17q23 (*mpo*) or 17q25 for 17q region with centromere of chromosome 17 or 17p13(*p53*) as reference.
¶Number of signals from reference gene (centromere or locus specific on p or q arm of the respective chromosome)/number of signals of the studied parameter: *MYCN* (2p),1p, 11q, and 17q.
‖Lost from follow up.

Flow Cytometry

The DNA index in 169 tumor samples was determined by flow cytometry with propidium-iodide; Modifit software (Beckton Dickinson, Heidelberg, Germany) was used for analysis. The cut-offs for ploidy were defined as follows: near-diploidy, 1 to 1.29; near-triploidy, 1.3 to 1.79; and near-tetraploidy, 1.8 to 2.19.

Quantitative Real Time PCR

Total RNA was extracted from 38 primary tumors by using Tri-reagent (MRC, Cincinnati, OH). One microgram of RNA was reverse transcribed by using the ImProm Reverse Transcription system (Promega Corporation, Madison, WI), according to the manufacturer's recommended procedure. The cDNAs were cleaned by DNA Clean and Concentrator TM-5 Kit (Zymo Research Corporation, Orange, CA). cDNA concentrations were evaluated by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

From each sample, 20 ng was used for quantitative real time PCR. We measured *MYCN* and *GAPDH* (reference gene) levels by using the LightCycler (Roche Diagnostics GmbH, Manheim, Germany). For *MYCN* gene we used Platinum SYBR Green qPCR Supermix UDG and for the

Table 2. Clinical and Genetic Data According to *MYCN* Status

Variables	2p/*MYCN* gain* 25 (14)	*MYCN* amplified† 31 (18)	*MYCN* balanced‡ 121 (68)	*P*
Male	12 (48)	21 (68)	69 (57)	NS
Age, median, yr	3.9	2.7	2.8	NS
Age, yr				
<1	3 (12)	1(3)	49 (40)	
>1	22 (88)	30 (97)	72 (60)	<0.001
Stage				
1, 2, 4s	2 (8)	0 (0)	56 (46)	
3, 4	23 (92)	31 (100)	65 (54)	<0.001
DNA index				
Near 2n/4n	20/24 (83)	27/31 (87)	77/114 (68)	0.02
Near 3n	4/24 (17)	4/31 (13)	37/114 (32)	0.04
Del 1p	9/25 (36)	18/30 (60)	29/119 (24)	<0.001
Del 11q	16/24 (67)	6/27 (22)	24/104 (23)	<0.001
Gain 17q	19/24 (79)	20/27 (74)	33/105 (31)	<0.001

Values given as *n* (%) unless stated otherwise. The cohort was divided into three groups according to *MYCN* status; χ^2 test and analysis of variance were used to compare significance of differences in variables between groups.

*Extra copies of *MYCN* gene up to fourfold relative to chromosome 2 (reference gene: centromere 2 and/or *LAF*).

†More than fourfold *MYCN* signals relative to chromosome 2.

‡Balanced number of *MYCN* and reference gene (centromere 2 and/or *LAF*).

GAPDH gene, we used Taqman probe and Platinum Quantitative PCR Supermix (Invitrogen, Paisley, Scotland, UK). To minimize the probability of contamination real time RT-PCR reactions were performed in the CleanSpot PCR/UV station (Coy Laboratory Products, Grass Lake, MI). Primer sequences for *MYCN* were as follows: forward, 5′-AGCCCTGCTTCTACCCGGAC-3′; reverse, 5′-GCTGGGTCACGGAGATGCTG-3′. Primer sequences for *GAPDH* gene and probe were as follows: forward, 5′-CAACAGCCTCAAGATCATCAGC-3′; reverse, 5′- CTCATGACCACAGTCCATGCCA-3′; *GAPDH*-probe: 5′-(FAM) CCTGGCCAAGGTCATCCATGACAAC (TAMRA)-3′. All samples were performed in duplicates. *MYCN* gene expression of each tumor was categorized as low or high by dichotomizing around the median PCR ratio obtained.

Statistical Analysis

We used the χ^2 test and analysis of variance to compare the significance of differences in variables between groups. Kaplan-Meier estimates for event-free survival (EFS) and overall survival (OS) were calculated and compared by log-rank test. A *P* value of <0.05 was considered statistically significant. A stepwise Cox regression model was applied, incorporating the genetic parameters *MYCN* (in the three categories: gain, balanced, and amplified), 1q, 11q, and 17q regions. Relapse and death from disease were counted as events. Death resulting from toxicity was not counted as an event, but censored for the OS analysis. The eight patients analyzed after relapse were not taken in account for survival analysis. Three patients lost from follow up were included for biological characterization only.

Results

Extra copies of the *MYCN* gene were identified in 25 (14%) patients, *MYCN* amplified in 31 (18%) patients, and *MYCN* balanced in 121 (68%) of the 177 patients with neuroblastoma included in the study. Twenty of 25 cases of the 2p/*MYCN* gain samples had one or two extra gene copies of *MYCN* relative to chromosome 2, and five patients had three extra copies. Ten of these samples were also tested by MLPA, which confirmed an enlarged region of gain including genes adjacent to the *MYCN* gene, proximal: *RTN4, ALK,* and distal: *DDX1, NAG,* and *TMEM18* (Figure 1A). The smallest region of gain was represented by *ALK*, *MYCN,* and *DDX1* genes. FISH with *ALK* probe on 2p23 was performed in the 15 cases when DNA was not available for MLPA analysis. In all of these instances, an identical copy number for *MYCN* and *ALK* genes was detected, confirming the co-gain of these two genes in our series (Figure 1B). Table 1 shows that 23 of the 25 2p/*MYCN* gain samples harbored at least one additional segmental aberration in eight cases, 2 in 10 cases, three in five cases, and, in two of the samples, none of the three regions, 1p, 11q, and 17q were found to be aberrant.

Table 2 summarizes the clinical and genetic data according to *MYCN* status. *MYCN* balanced tumors occurred more often in younger patients (<12 months), whereas 2p/*MYCN* gain and *MYCN* amplified tumors were seen in older patients (>12 months; $P < 0.001$). Advanced disease (stages 3 to 4) predominated in the 2p/*MYCN* gain group (92%) and the *MYCN* amplified group (100%) compared with the *MYCN* balanced group in which clinical stage was similarly distributed: 46% of patients with stage 1, 2, and 4s, and 54% with stages 3 and 4 ($P < 0.001$). DNA index was obtained from 169 patients. Near diplo/tetrapolid and near triploid tumors were almost equally distributed within 2p/*MYCN* gain and *MYCN* amplified groups, but significantly different from the *MYCN* balanced group ($P = 0.02$ and $P = 0.04$, respectively). The 2p/*MYCN* gain group was characterized by a significantly higher rate of segmental loss of the 11q region (67%) compared with the *MYCN* amplified group (22%) and the *MYCN* balanced group (23%; $P < 0.001$). The rate of 17q region gain was similar in the 2p/*MYCN* gain and *MYCN* amplified groups (79% and 74%, respectively) and significantly lower in the *MYCN*

balanced group (31%; $P < 0.001$). Deletions in the 1p region were more frequent in the *MYCN* amplified group ($P < 0.001$). By MLPA, the most frequent segmental aberration found was +17q in nine cases, followed by $11q^-$ in seven cases, $3p^-$ in four cases, $1p^-$ in three cases, +7q in two cases, and $9p^-$ in one case. Numerical alterations were as follows: +7 in three cases, +17 in one case, and −3 in one case.

The median follow up of our cohort was 80 months, and relapse occurred in 60 of the 174 patients: 48 in stage 4, 11 in stage 3, and one in stage 4s. Five-year EFS was similar for the 2p/*MYC*N gain and *MYCN* amplified groups (40% ± 13% and 28% ± 9%, respectively; $P = 0.18$) and significantly higher in the *MYCN* balanced group (78% ± 4%; $P < 0.001$; Figure 2A). The 5-year OS was 51% ± 14% in the 2p/*MYCN* gain group, 85% ± 4% in the *MYCN* balanced group, and 34% ± 10% in the *MYCN* amplified group. The difference between the 2p/*MYCN* gain group and both the *MYCN* balanced group ($P = 0.003$) and *MYCN* amplified group were significant ($P = 0.025$; Figure 2B). EFS and OS of the 2p/*MYCN* gain group did not differ significantly whether associated or not with an additional single genetic marker alteration such as 11q loss (Figure 3, A and B), 1p del, or 17q gain. Tumors having 2p/*MYCN* gain without 17q gain showed a better, but not statistically significant, outcome than those with segmental 17q gain, probably due to the small size of the cohort (Table 3).

To investigate the possible association between *MYCN* copy number and expression, 38 of the nonamplified neuroblastoma patient samples were evaluated. The median PCR ratio value was 260. Low and high *MYCN* expression NB were compared between tumors with a balanced number of *MYCN* gene and with *MYCN* gene extra copies. The χ^2 test was applied to analyze this association and no statistically significant correlation was noted ($P = 0.637$; Table 4).

The stepwise Cox regression model was used, incorporating the three *MYCN* groups (gain, balanced, and amplified), 1q, 11q, and 17q. In the final stage, the remaining predictors of adverse outcome were *MYCN* amplification (hazards ratio: 3.2; $P = 0.006$) and 11q loss (hazards ratio: 3.7; $P < 0.001$).

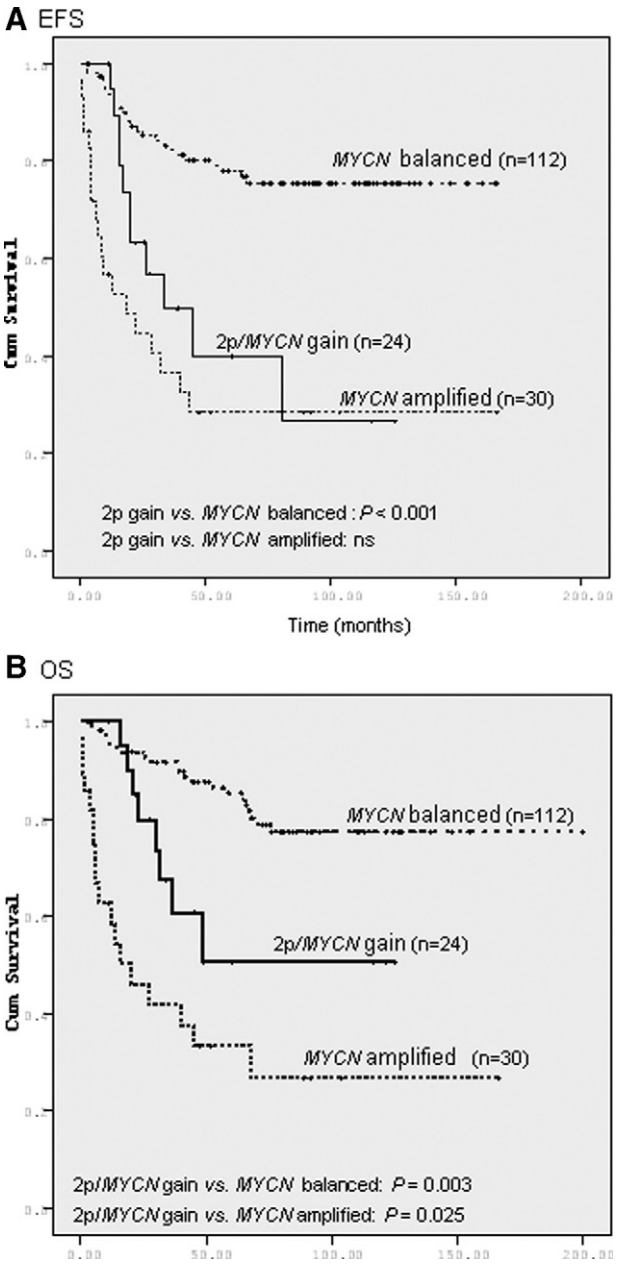

Figure 2. EFS and OS for the whole cohort according to *MYCN* status. **A:** 5-year EFS *MYCN* balanced: balanced number of *MYCN* and cep2/*LAF* genes: 78% ± 4%; 2p/*MYCN* gain: 40% ± 13%; and *MYCN* amplified: 29% ± 8%. **B:** 5-year OS *MYCN* balanced: 85% ± 4%; 2p gain: 51% ± 14%; and *MYCN* amplified: 34% ± 10%.

Discussion

Since FISH was performed on all NB samples, we were able to determine the *MYCN* status according to the established criteria.[30] *MYCN* amplification was detected in 18% of the tumors, which is lower than the 20% to 25% reported in the literature.[38,39] Tumors with 2p/*MYCN* gain accounted for 14% of the sample. Reported rates for the few additional copies of the *MYCN* gene range from 6% to 13%; most of the studied tumors had one to three extra copies.[31–33] In accordance with these studies, 20 of the 25 patients in this group had one or two extra copies of the *MYCN* gene, and in another five patients, three extra copies were found.

Previous studies have suggested that the *MYCN* oncogene is amplified by replication-excision or at a distant region (translocated) from the resident site of the gene.[39] Another, rarer, phenomenon is the *in situ* duplication of the *MYCN* gene at its own locus, 2p24, as shown in two cell lines.[40] However, the most frequent finding is the acquisition of a few additional copies of *MYCN* through the formation of an iso(2p), or as a result of unbalanced translocations of chromosome 2p, which harbors the *MYCN* gene, with different partner chromosomes.[41,42] Studies applying comparative genomic hybridization,[43,44] array-comparative genomic hybridization,[44–47] and single-nucleotide polymorphism studies[48] could demonstrate that extra copies of *MYCN* gene are often accompanied by a set of adjacent

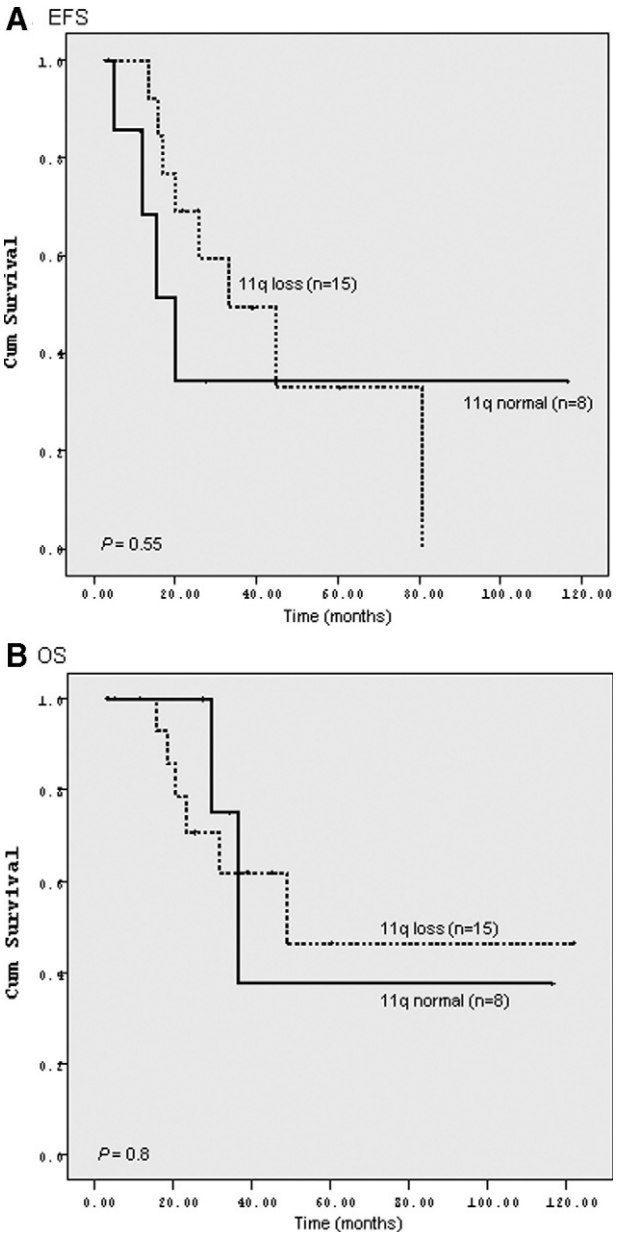

Figure 3. EFS and OS of 2p/*MYCN* gain group according to 11q status. **A:** 5-year EFS 11q normal: 34% ± 2%; 11q loss: 33% ± 17%. **B:** 5-year OS 11q normal: 38% ± 29%; 11 q loss: 46% ± 16%.

genes, thus supporting the suggestion that they may be the result of unbalanced translocations. In this study MLPA was performed mainly in the *MYCN* gain group only to confirm the partial gain of 2p region harboring *MYCN* gene. Accordingly, our MLPA results showed that all but one of the 10 tested samples had also a gain of *ALK*, *DDX1*, and *NAG* genes (Figure 1A), all of them localized adjacent to *MYCN*, defining a 2p gain.[30] Recently, in addition to gain or coamplification, somatic and germ line mutations of the *ALK* gene contributing to neuroblastoma oncogenesis have been reported in advanced neuroblastoma tumors correlating with a poor outcome.[49–51] It has been suggested that given that the down-regulation of *ALK* expression suppresses the proliferation of neuroblastoma cells, it may represent a therapeutic target for this disease. Furthermore,

Table 3. Five-year EFS and OS of 2p/*MYCN*-g Group in Relation to Additional Markers Changes

2p/*MYCN* gain	5-yr EFS	*P*	5-yr OS	*P*
1p				
no del* ($n = 15$)	55 ± 20	0.3 NS	54 ± 20	0.9 NS
del† ($n = 9$)	43 ± 15		55 ± 15	
11q				
no del ($n = 8$)	34 ± 20	0.6 NS	46 ± 16	0.8 NS
del ($n = 15$)	33 ± 17		38 ± 29	
17q				
no gain ($n = 5$)	60 ± 22	0.8 NS	67 ± 28	0.4 NS
gain ($n = 18$)	26 ± 4		42 ± 16	

No significant difference could be observed with or without additional of single marker alteration.
*no deleted.
†deleted.

ALK has been identified in one study as a major familial neuroblastoma predisposition gene.[52]

As in earlier reports, the 2p/*MYCN* gain group was strongly associated with 11q loss, and *MYCN* amplification was often accompanied by 1p deletion. In addition, we found that 76% of the 2p/*MYCN* gain group had advanced (stages 3 to 4) disease as noted by Spitz et al.[32]

Patients with 2p/*MYCN* gain had a poor 5-year EFS, similar to that of the *MYCN* amplified group and differing significantly from the *MYCN* balanced group (5-year EFS 2p/*MYCN* gain versus *MYCN* balanced, 40% ± 13% vs. 78% ± 4%, respectively; $P < 0.001$; Figure 2A), consistent with previous observations.[32] However, the 5-year OS of the 2p/*MYCN* gain group was intermediate, between that of the *MYCN* amplified group (51 ± 14% vs. 34% ± 10%, respectively; $P = 0.025$) and the *MYCN* balanced group (51% ± 14% vs. 85% ± 4%, respectively; $P = 0.003$; Figure 2B). Taken together, these data indicate that although both 2p/*MYCN* gain and *MYCN* amplified neuroblastoma point to a poor prognosis, the former progresses less rapidly.

There is still some speculation as to whether low-level *MYCN* gene gain affects the clinical behavior of the tumor by increasing *MYCN* expression levels.[31,32] Some studies found no direct correlation between *MYCN* copy number and the level of *MYCN* expression,[53] or association of

Table 4. *MYCN* Gene Expression Values of 38 Nonamplified NB Patients

MYCN expression* $n = 38$		
	n (%)	*P*
Low (<260)†		
MYCN-b‡	17 (89)	
MYCN-g§	2 (11)	
		0.6 NS
High (>260)		
MYCN-b	15 (79)	
MYCN-g	4 (21)	

**MYCN* gene expression of each tumor was categorized as low or high by dichotomizing around the median PCR ratio.
†Median PCR ratio: 260.
‡Balanced number of *MYCN* gene and chromosome 2.
§Extra copies of *MYCN* gene up to 4-fold relative to chromosome 2, as defined by ENQUA.

the level of *MYCN* expression with any other prognostic factors.[54] In addition, genes proximal and distal to *MYCN* were found to be highly expressed when using comparative expressed sequence hybridization.[55] We, too, failed to detect any significant correlation between *MYCN* status and *MYCN* expression levels ($P = 0.637$; Table 4).

Segmental imbalance of the 2p24 region harboring extra copies of the *MYCN* gene is a relatively common event in advanced-stage neuroblastoma. A report of two cases of neuroblastoma without amplification at diagnosis but with low-level gain of the *MYCN* gene evolving into *MYCN* amplification at relapse have been described in the literature.[33–34] Valent et al[31] have shown simultaneous *MYCN* gain and amplification. The detailed study of these cases supplied information about several different mechanisms leading to increased *MYCN* copy number. However, the issues still being debated are whether gain is a necessary prerequisite for amplification, whether it is an independent event in NB, and whether it is a specific phenomenon of NB tumors or a general mechanism by which tumor cells can acquire selective growth advantage. As Valent et al[31] proposed, it is necessary to find more cases of primary NB with simultaneous gain and amplification to clarify the significance of this characteristic. In our series we detected only one case with simultaneous gain and amplification, and it was included in the *MYCN* amplified group. Spitz et al (2004)[32] suggested that owing to the close association of *MYCN* gain with 11q loss, and the inverse correlation between this alteration and *MYCN* amplification, the presence of extra copies of *MYCN* is probably an independent late event in advanced neuroblastoma not leading to *MYCN* amplification, and may not have a substantial prognostic influence. Indeed, the inverse correlation between 11q loss and *MYCN* amplification has already been reported as a frequent finding in high-stage neuroblastoma suggesting a poor prognosis.[18,56,57] To determine the possible influence of the strong association of 2p/*MYCN* gain with segmental 11q alterations on disease outcome, we performed a Kaplan-Meier analysis in the 2p/*MYCN* gain group, comparing patients with and without 11q loss. We found no statistical significance between subgroup differences in 5-year EFS (33% ±17% vs. 34% ± 20%, respectively; $P = 0.6$; Figure 3A) or 5-year OS (46% ± 16% vs. 38% ± 29%, respectively; $P = 0.8$; Figure 3B). These data suggest that poor outcome of 2p/*MYCN* gain group is apparently unrelated to the strong association with 11 q deletions. However, the small sample of patients did not allow for a statistically robust conclusion, and this assumption requires further confirmation in a larger study. We have previously identified a group of tumors (10 of which were included in the present cohort) with 11q segmental loss characterized by der(11)t(11,17) on SKY.[58,59] Although associated with a poor prognosis, these patients had a distinct course of slower progression than *MYCN*-amplified tumors. Nonrandom t(11;17) has previously been reported to be one of the mechanisms underlying 11q loss and 17q gain by tumors.[60,61] Only 3 of 10 tumors from our series had extra copies of *MYCN* gene.

In conclusion, our data show that neuroblastoma tumors characterized by 2p/*MYCN* gain region are associated with a low, though less rapidly progressive, EFS rate similar to that of neuroblastoma with *MYCN* amplification. This behavior does not seem to be influenced by 11q deletions, 5-year survival of patients with and without 11q deletions were similar within the 2p/*MYCN* gain group; neither does it seem to be related to the previously described group harboring 11q deletions through t(11;17) or other single studied segmental alterations such as 1p deletions or 17q gain (Table 3).

The evaluation of *MYCN* status by FISH is still the gold standard for neuroblastoma characterization. The use of a 2p specific probe in addition to 2q signals (optionally a centromeric probe), simultaneously, is recommended by the International Neuroblastoma Risk Group Biology Committee[30] to clarify the presence of a chromosome 2p gain versus a restricted *MYCN* gain. Following this recommendation, we could identify the 2p/*MYCN* gain group in our cohort, characterized by high frequency of cooccurrence of other segmental alterations and associated with a poor outcome. Altogether, these data suggest that the presence at diagnosis of extra copies of *MYCN* gene in nonamplified tumors may reveal an underlying genetic pattern of combined segmental and numerical alterations and subsequently help to understand the behavior of these tumors.

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