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Clinical Value of N-myc Oncogene Amplification in 52 Patients with Neuroblastoma Included in Recent Therapeutic Protocols

V. COMBARET,* Q. WANG,† M.C. FAVROT,* P. THIESSE,* I. PHILIP,* E. BOUFFET,* C. BAILLY,* R. BOUVIER, F. CHAUVIN,* J.M. ZUCKER,§ J.L. BERNARD,‖ G. LENOIR† and T. PHILIP*

**Centre Léon Bérard, Pediatric and Bone Marrow Transplant Unit, Departments of Radiodiagnosis and Surgery, 28 rue Laënnec, Lyon, France (LAK Study Group), †International Agency for Research on Cancer, 150 cours Albert Thomas, Lyon, France, ‡Hôpital Edouard Herriot, Lab. of Histology, Place d'Arsonval, Lyon, France, §Institut Curie, Pediatric Oncology Dept., 26 rue d'Ulm, Paris, France (LAK Study Group) and ‖Hôpital de la Timone, Pediatric Oncology Unit, Bd Jean Moulin, Marseille, France (LAK Study Group).*

Abstract—*Southern blot analysis of neuroblastoma (NB) cell DNA from 52 patients (58 samples) allowed the detection of an N-myc amplification on three of the 13 BM samples and three of 13 tumor samples at diagnosis, on two of 17 tumor samples taken after induction therapy, on three of seven BM samples and two of the five local tumor samples taken after relapse. N-myc was amplified in two of the 15 patients with stage I to III NB and in 10 of the 36 patients with stage IV neuroblastoma over 1 year of age.*

Conclusions from the analysis are as follows: first tumor samples obtained on previously treated patients are histologially modified and detection of N-myc amplification is not accurate; consequently N-myc amplification must be defined at diagnosis. Surgical biopsies of the primary tumors can, however, be delayed and malignant cells obtained by ultrasound-guided punctures or analyzed on bone marrow samples if they represent more than 50% of the total population. Second, any attempt to define the prognostic value must be performed on a group of patients treated with a new aggressive protocol of chemotherapy; it will require a multiparametric analysis including, in particular, results of an extensive clinical staging at diagnosis and histological criteria.

INTRODUCTION

In 1983, Schwab *et al.* [1] demonstrated that eight out of nine neuroblastoma (NB) cell lines were bearing an amplified sequence related to the cellular oncogene c-myc. This second member of the family of myc-related genes is now known as N-myc (for NB derived). In 1984, Brodeur *et al.* [2] confirmed that N-myc was amplified in about one third of primary NB from untreated patients; they subsequently showed that the number of N-myc copies was homogeneous within an individual patient, independent of the anatomical sites of the tumor and without significant variation from diagnosis to relapse [3]. The presence of more than 10 copies of N-myc in the tumor was associated with a rapid tumor progression [2, 4–6]. This biological parameter could be fundamental for the clinician if it can be used as an independent prognostic factor to better define the group of high risk patients and the therapeutic strategy. If so, it becomes important to evaluate the N-myc oncogene copy number routinely for each patient as soon as possible during the course of the disease.

There are, however, practical and technical limitations to a systematic DNA analysis of neuroblastoma. In practice, clinical and biological criteria (i.e. catecholamine secretion) are usually considered as sufficient for diagnosis in Europe, without requirement of histological control of the primary tumor. This tumor is thus removed surgically only after induction therapy; surgery is then curative and permits the evaluation of the response to initial chemotherapy. Technically, N-myc amplification is quantified by southern blot analysis performed on the global cell population although tumor samples are often very heterogeneous, with immature neuroblasts and more differentiated neuroectodermal malignant cells, and normal fibroblastic or lymphocytic stroma cell reaction. The distribution in the tumor of these different components is, of course, modified by chemotherapy.

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Author for correspondence and reprints: T. Philip, Pediatric and BM transplant unit, Centre Léon Bérard, 28 rue Laënnec, 69373 Lyon Cedex 08, France.
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The aim of our study, then, was to define the quality of the samples required for a quantification of N-myc copies by southern blot analysis. In particular, the oncogene was analyzed on ultrasound-guided punctures of the primary tumor at diagnosis and relapse, on pathological bone marrow (BM) samples at diagnosis and relapse and on tumors taken after first-line induction therapy. The quality of the samples and the percentage of malignant cells were evaluated by cytohistology and immunology, malignant cells being defined by neuroectodermal antigen expression [7]. Histological features of neuroectodermal malignant cells and expression of HLAABC class I antigen were both used as criteria for evaluating the degree of maturation and the modifications induced by therapy [8]. Tumor samples from 52 patients were analyzed and the results of the N-myc analysis were correlated with the outcome in individual patients.

MATERIALS AND METHODS

Patients and collection of the samples (*Table* 1)

Fifty-eight tumor samples from 52 patients were obtained from institutes belonging to the LMCE neuroblastoma study group in France. Patients were classified according to the clinical staging system of Evans *et al.* [9] with a particular subgroup for patients below 1 year of age. Children were treated as previously described [10–12]. Samples were taken either at diagnosis, relapse or after induction therapy. BM aspirates and ultrasound-guided punctures of the primary tumor were harvested on heparin-free medium. Granulocytes and red cells were eliminated by Ficoll separation [13]. Half the cells were frozen immediately in liquid nitrogen and kept at −70°C for molecular biology examination; the remaining cells were used for conventional cytology and immunological analysis (see below). Tumor samples (from the primary sites, or from lymph node metastases in cases Nos 2 and 8 in Table 2) were taken surgically and divided into three parts, judged to be representative of the same lesion by immediate examination of a frozen section; one part was reserved for histological analysis, one for immunological analysis and the third one was frozen at −70°C for molecular biology.

Cytohistological analysis

In standard techniques, tumors were classified as typical neuroblastoma when cells were undifferentiated, as ganglioneuroblastoma when cells were predominantly mature with the persistence of clumps of undifferentiated neuroblasts, and as ganglioneuroma when tumor samples were well differentiated. The stroma was described as poor or rich. Rich stroma represented at least 50% of the tumor with both DNA material in the nucleus and large cytoplasmic expansion. The degree of necrosis in

Table 1. *Analysis of the N-myc oncogene in* 58 *samples from* 52 *patients*

Stage of disease	Patients below 1 year of age*	Stage II	Stage III	Stage IV	Total
Number of patients (N-myc amplification)	3 (0)	1 (0)	12 (2)	36 (10)	52 (12)
Number of samples† (N-myc amplification)	3 (0)	1 (0)	14 (2)	40 (11)	58 (13)
BM samples					
diagnosis				13 (3)	
relapse‡			1 (1)	6 (2)	20 (6)
US guided punctures					
diagnosis§			2 (0)	3 (1)	7 (1)
relapse			2 (0)		
Surgical biopsies					
diagnosis‖	2 (0)	1 (0)	2 (0)	3 (2)	
post-induction therapy	1 (0)		6 (1)	10 (1)	31 (6)
relapse			1 (0)	5 (2)	

*One patient was neonatal stage I NB and two patients were stage IVS; one of these two relapsed.

†In six patients malignant cells were analyzed twice. In one patient neuroblastoma cells in the BM were analyzed twice (6 months interval) with N-myc amplification respectively at 30 and 100 copies. In one patient amplification at 100 copies was detectable on malignant cells in the BM at diagnosis but was not detected in the tumor biopsy taken after surgery; the samples were very necrotic and most cells had differentiated. In the other four patients N-myc amplification was not detected in any sample taken at diagnosis and post-induction therapy.

‡Patient with stage III NB relapsed with NB cells in the BM.

§One patient had pleural effusion; puncture of the effusion and analysis are recorded in this section.

‖In two patients the analysis was performed on metastatic lymph nodes (patients 2 and 8 in Table 2).

the tumor was quantified; samples with more than 90% necrosis were eliminated.

Immunological analysis

Samples were analyzed as previously described [13–15], either by a double immunofluorescence method on cells in suspension (BM samples on ultrasound-guided punctures) or by an alkaline phosphatase immunostaining of frozen sections of the tumor. UJ13A and SL-11.14, two monoclonal antibodies (MoAbs) which recognize neuroectodermal antigens [7, 16], were expressed on more than 90% of tumor samples, regardless of the degree of maturation. In double immunofluorescence analyses, an anti-panleucocyte MoAb (CD45, from G. Janossy) used in combination with these two neuroectodermal markers recognized normal hematopoietic cells. Anti-monomorphic HLA.ABC MoAB (F423E10 from Biosys, France) was expressed on normal cells and on the most mature neuroectodermal malignant cells whereas typical neuroblasts lacked class I membrane antigen.

N-myc amplification analysis

Tumor samples were minced and suspended in NET solution (NaCl 150 mM/EDTA 10 mM/ Tris–HCl (pH 7) 10 mM). SDS (1%), RNase (100 μg/ml) and proteinase K (100 μg/ml) were added. The mixture was incubated for 12 h at 37°C. The DNAs were then extracted by phenol/chloroform and precipitated with ethanol. Five to 10 μg DNA from each sample were completely digested with restriction enzyme EcoRI and separated by agarose gel electrophoresis (1%). DNA fragments were denatured and transferred to a nylon membrane (gene screen plus) as described [17].

The probe N-myc (pNb-1) was a gift from J. Minna (NCI). For each experiment, 50 ng plasmid was ^{32}P-labelled by 'Multiprimer labelling system' [18] to a specific activity about 10^9 cpm/μg. Hybridization was performed using 50% formamide, 50 mM sodium phosphate (pH 7), 1 M NaCl, 1% SDS and ^{32}P-labelled N-myc probe, for 12 h at 42°C. After hybridization, the filters were washed twice 5 min at room temperature in 200 ml 2 × ssc, and twice 30 min at 65°C in 200 ml 2 × ssc/1% SDS and were then exposed for autoradiography.

Restriction enzyme-digested tumor DNAs were compared with lymphocyte DNA in the same agarose gels. The DNA quantities of each lane on gel were controlled by rehybridizing or hybridizing simultaneously with other myc genes (c-myc and L-myc). Amplified gene copy number was measured by serial dilutions of DNA to obtain a hybridization signal of two-copy intensity (e.g. a 100-fold amplification is indicated if a 1:100 dilution achieves two-copies intensity).

RESULTS

Genes of the N-myc family have been proved to have functional analogy. Consequently, the three genes c-myc, L-myc and N-myc have been analyzed in neuroblastoma samples. c-myc and L-myc amplification was not detected in any sample. N-myc amplification was detectable in 13 tumor samples from 12 of the 52 patients, as summarized in Table 1. The amplification of N-myc was analyzed in 20 contaminated BM samples from 19 patients and detected in six. In all analyzed samples malignant cells were typically immature, described as undifferentiated in cytology and HLA.ABC negative in immunology. Double immunofluorescence analysis of the cell suspensions permitted the accurate quantification of NB cells within the normal BM. Thirty to 100 N-myc copies were detectable in five samples which contained 60–80% neuroblasts (two were obtained from the same patient at 6 month intervals). In the sixth sample, three N-myc copies were detectable although the BM contained 15% malignant cells; given the fact that the 85% of normal cells expressed one single copy of N-myc, NB cells theoretically expressed more than 10 copies in this last sample. A single N-myc copy could be detected in 14 BM samples; they contained 20–80% malignant cells; therefore any N-myc amplification equal or superior to 10 copies would have been detected, whereas a lower amplification could have been masked.

Malignant cells from the primary tumor were obtained by ultrasound-guided punctures of the primary tumor in six cases and from a pleural effusion in one. The number of harvested cells was sufficient to permit cytological, immunological and southern blot analyses. One hundred copies of N-myc were detected in one of the ultrasound-guided punctures of the primary tumor. Interestingly the punctures were not hemorrhagic and contamination by stroma cells was minimal; therefore, all samples contained at least 70% malignant cells.

N-myc was analyzed in the surgical biopsies of 29 primary tumors and in two lymph node metastases (Nos 2 and 8) from 27 patients (Table 2). N-myc amplification was detected in two of the eight samples taken at diagnosis, in two of the six samples taken at relapse, but only in two of the 17 samples taken after chemotherapy. Of note, N-myc amplification was detected in two of the three very necrotic samples taken after chemotherapy (Nos 10 and 16). In contrast, sample No. 19 was negative although an amplification of 100 copies was detected for the same patient in a pathological BM sample analyzed at diagnosis. Three of the positive samples were neuroblastoma, the other three being ganglioneuroblastoma. To summarize, N-myc amplification was detected in three of the 13 BM samples and three of the 13 tumor samples at

Table 2. Analysis of N-myc amplification in 31 surgical biopsies

Patients stage of disease	Clinical status at collection	Histological and immunological features*	Percentage of malignant cells (immature cells)	N-myc copies
1: st I (<1 year)	Diagnosis	NB, poor stroma	>90% (90%)	1
2: st IVS (<1 year)	Diagnosis	NB, poor stroma	>90% (90%)	1
3: st II	Diagnosis	Ganglio NB, rich stroma, HLA.ABC+	50% (5%)	1
4: st III	Diagnosis	Ganglio NB, rich stroma, HLA.ABC+	40% (10%)	1
5: st III	Diagnosis	Ganglio NB, rich stroma, HLA.ABC+	25% (5%)	1
6: st IV	Diagnosis	NB, poor stroma	>90% (90%)	1
7: st IV	Diagnosis	NB, poor stroma	>90% (90%)	10
8: st IV	Diagnosis	NB, residual normal lymphoid cells	30% (30%)	2
9: st IVS (<1 year)	Post-induction therapy	Ganglio NB, rich stroma	10% (5)	1
10: st III	Post-induction therapy	Ganglio NB, rich stroma, necrosis	10% (5%)	30
11: st III	Post-induction therapy	Ganglio NB, rich stroma, HLA.ABC+	40% (10%)	1
12: st III	Post-induction therapy	Ganglio NB, rich stroma	30% (10%)	1
13: st III	Post-induction therapy	Ganglio NB, rich stroma	30% (5%)	1
14: st III	Post-induction therapy	NB, poor stroma, HLA.ABC+	90% (90%)	1
15: st III†	Post-induction therapy	Ganglio NB, rich stroma	30% (10%)	1
16: st IV	Post-induction therapy	Ganglio NB, rich stroma, necrosis	15% (10%)	50
17: st IV‡	Post-induction therapy	Ganglio NB, rich stroma, HLA.ABC+	60% (20%)	1
18: st IV‡	Post-induction therapy	NB, rich stroma	70% (30%)	1
19: st IV‡	Post-induction therapy	Ganglio NB, rich stroma, necrosis	10% (5%)	1
20: st IV	Post-induction therapy	Ganglio NB, rich stroma, HLA.ABC+	50% (10%)	1
21: st IV	Post-induction therapy	Ganglio NB, rich stroma	60% (20%)	1
22: st IV	Post-induction therapy	Ganglio NB, rich stroma, HLA.ABC+	30% (10%)	1
23: st IV	Post-induction therapy	Ganglio NB, rich stroma	50% (20%)	1
24: st IV	Post-induction therapy	Ganglio NB, rich stroma	30% (10%)	1
25: st IV	Post-induction therapy	Ganglio NB, rich stroma	50% (20%)	1
26: st III†	Relapse	Ganglio NB, poor stroma, HLA.ABC+	70% (30%)	1
27: st IV	Relapse	Ganglio NB, rich stroma HLA.ABC+	60% (30%)	1
28: st IV	Relapse	NB, poor stroma, HLA.ABC−	>90% (90%)	1
29: st IV	Relapse	NB, poor stroma, HLA.ABC−	>90% (90%)	50
30: st IV	Relapse (autopsy)	Ganglio NB, rich stroma	20% (10%)	2
31: st IV	Relapse (autopsy)	NB, poor stroma, HLA.ABC−	80% (80%)	1

NB: neuroblastoma.

*When not specified, samples were HLA.ABC negative.

†Samples 15 and 26 were taken from the same patients in post-induction therapy and relapse; ultrasound-guided puncture of the primary tumor at relapse also expressed a single N-myc copy.

‡Samples from patients 17 and 18 were analyzed on ultrasound-guided puncture or BM and negative. In contrast, patient 19 had 100 copies in BM cell samples taken at diagnosis.

diagnosis, in two of the 17 tumor samples taken after induction therapy, in three of the seven BM samples and two of the six local tumor samples taken after relapse.

One of the major issues for the interpretation of the data was the quantification of stroma cells as well as the immature and mature components of the tumor. Stage III NB samples and stage IV NB samples taken after induction therapy or even after relapse in a few cases were described as ganglioneuroblastoma; the malignant population did not exceed 50% of the sample; part of these cells had histological features typical of mature cells and, in a few cases, expressed HLA.ABC class I antigen.

N-myc amplification was detected in 10 of the 36 patients with stage IV NB over 1 year of age (28%), without significant difference if one considers only patients analyzed at diagnosis (31%). Disease-free survival is not significantly different between stage IV patients with or without N-myc amplification in tumors, but the number of patients in each group is low and their follow up is too short. N-myc amplification was detected in two of the 12 patients with stage III NB; one of the two died of infectious disease during induction therapy; the second relapsed after conventional therapy and was resistant to salvage therapy; subsequently, a large contamination of the BM did not permit the harvesting of the BM for an autograft and the patient progressed under immunotherapy with interleukin 2 and lymphokine-activated killer cells. Among the other 10 stage III NB patients with non-amplified tumor cells, eight are disease-free survivors at 22 months (3–39 months) but two relapsed after conventional therapy and entered a protocol of high dose chemotherapy and autologous BM transplan-

tation. The stage II NB patient is alive, disease-free, at 30 months. Finally, of the three patients below 1 year of age without detectable N-myc amplification, one had a neonatal stage I NB and is alive, disease-free, at 30 months after a complete surgical resection; one patient is stage IVS NB with only 3 months follow up under conventional induction therapy; the last patient relapsed twice, once after conventional therapy and once after autologous BM transplantation.

DISCUSSION

In this series, the amplification of N-myc is detected in 30% stage IV NB patients when samples are analyzed at diagnosis or relapse but in only 10% patients when samples are taken after induction therapy. The percentage of stage IV NB samples with more than 10 N-myc copies is similar to that of previously published analyses but the percentage of samples with less than 10 copies is lower. In contrast only 16% stage III NB have the molecular abnormality, a very low incidence if compared to published data.

These results lead to various comments on the quality of the samples required for southern blot detection of N-myc amplification and on the prognostic value of the new parameter compared to already defined criteria. The detection of the amplification and, what is more important, its quantification by southern blot analysis largely depend upon the percentage of malignant cells in the sample since a single copy of N-myc is detectable in normal cells. It will then only be possible if the percentage of malignant cells in the sample is determined precisely. With such technical precautions, N-myc analysis can be performed from diagnosis without surgical biopsy of the primary tumor, either on pathological BM samples or on ultrasound-guided punctures. On pathological BM samples with more than 50% malignant cell infiltration, any amplification of more than two copies will be detectable whereas the analysis of samples with 10–50% malignant cells will only permit to detect an amplification of more than 10 copies. This last point could easily explain why we found very few samples with amplification inferior to 10, minor amplification being missed on BM samples with less than 50 malignant cells. A similar rule applies to ultrasound-guided punctures although this technique usually provides adequate samples of a rich malignant cell population and rare normal cells [19]. In contrast, negative results obtained on tumor samples taken in previously treated patients must be considered with caution and only positive results could safely be considered as valid. Indeed, variations of N-myc amplification have been reported to coincide with the expression of HLA.ABC and with the cell maturation in the rodent model [20]. Histological modifications induced by therapy, with both necrosis and maturation, could easily explain some negative results of the analysis.

To underline this point and as mentioned above, N-myc amplification was detected in only one of the 10 stage IV NB biopsies taken after surgery. Furthermore, contradictory results were obtained in one case between the analysis of tumor cells taken at diagnosis and after chemotherapy. Similarly, Brodeur *et al.* [3] found N-myc amplification in five surgical primary tumor biopsies at diagnosis, but only three of the five cases were positive when tumor cells were analyzed on second look surgical biopsies.

In stage III NB, the question is whether a very rich stroma could mask a low level of N-myc and whether, as suggested by Nakagawara *et al.* [5], N-myc amplification will be observed in the immature tumors described as neuroblastoma. Our series will confront this last point since all non-amplified stage III neuroblastoma except one were described as ganglioneuroblastoma. However, if such a correlation is confirmed, the prognostic significance of N-myc amplification will be very similar to that of the histological classification described by Shimada *et al.* [21]. The analysis of N-myc protein expression using immunohistological techniques might provide more information on the heterogeneity of partially differentiated tumor samples [22, 23]. The predominance of well differentiated neuroblastoma and the lack of N-myc amplification in our series of stage III NB is rather different from what was observed in earliest studies but can easily be explained by the date of patients' inclusion in the study. Indeed, the extensive staging of the disease at diagnosis (in particular a better detection of BM and bone metastases), as was performed very recently, permitting the identification of stage IV NB patients previously misclassified as stage III. The amplification of N-myc oncogene in primary NB tumors taken at diagnosis has been correlated to a worse prognosis, particularly when the number of copies exceeded 10. The amplification of the oncogene might not be the only factor correlated to progression; relapses observed in this study in stage III and IVS NB with a single N-myc copy and previously published analyses underline this point [24]. More important, the outcome of neuroblastoma has been largely modified by the use of aggressive chemotherapeutic protocols (i.e. chemotherapy and ABMT); the prognostic value of N-myc amplification must then be reevaluated, with a sufficient follow up, in a multiparametric analysis including in particular histological criteria [22], clinical staging with optimal methods of investigation and the new therapeutic protocols.

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