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Detection of *MYCN* Gene Amplification in Neuroblastoma by Fluorescence *In Situ* Hybridization: A Pediatric Oncology Group Study[1]

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Abstract

To assess the utility of fluorescence *in situ* hybridization (FISH) for analysis of *MYCN* gene amplification in neuroblastoma, we compared this assay with Southern blot analysis using tumor specimens collected from 232 patients with presenting characteristics typical of this disease. The FISH technique identified *MYCN* amplification in 47 cases, compared with 39 by Southern blotting, thus increasing the total number of positive cases by 21%. The major cause of discordancy was a low fraction of tumor cells (≤30% replacement) in clinical specimens, which prevented an accurate estimate of *MYCN* copy number by Southern blotting. With FISH, by contrast, it was possible to analyze multiple interphase nuclei of tumor cells, regardless of the proportion of normal peripheral blood, bone marrow, or stromal cells in clinical samples. Thus, FISH could be performed accurately with very small numbers of tumor cells from touch preparations of needle biopsies. Moreover, this procedure allowed us to discern the heterogeneous pattern of *MYCN* amplification that is characteristic of neuroblastoma. We conclude that FISH improves the detection of *MYCN* gene amplification in childhood neuroblastomas in a clinical setting, thus facilitating therapeutic decisions based on the presence or absence of this prognostically important biologic marker. *Neoplasia* (2001) **3,** 105–109.

Keywords: neuroblastoma, fluorescence *in situ* hybridization, *MYCN* gene, gene amplification, double minute chromatin bodies.

Introduction

Amplification of the *MYCN* proto-oncogene is recognized as an independent prognostic factor in neuroblastoma, identifying children with rapidly progressive disease who respond poorly to conventional therapy [1,2]. Increased *MYCN* copy numbers are found in less than 5% of patients with low-stage neuroblastoma, as well as stage IV-S tumors, compared with 30% to 40% of those with advanced disease [3,4]. When *MYCN* amplification occurs, it is almost always present at the time of diagnosis, and thus appears to be an intrinsic property of a subset of highly aggressive tumors that are invariably fatal unless effective alternative treatment can be found [5]. *MYCN* gene amplification may occur as either intrachromosomal homogeneously staining regions (HSRs) or as genetically unstable extrachromosomal double minutes (DMs) [6–8].

Southern blot analysis has been the standard method for detecting amplified *MYCN* genes in tumor specimens; however, its usefulness is limited by a number of technical factors. One cannot, for example, obtain reliable measurements with small quantities of DNA, and false-negative results are common when the tissue sample contains an admixture of tumor cells and normal peripheral blood leukocytes, stromal elements, or other nonmalignant elements [9]. Thus, the incidence of *MYCN* gene amplification may have been underestimated in previous studies based on Southern blot analyses. Nor do Southern assays allow one to discern the cytogenetic basis of *MYCN* amplification, whether DMs or HSRs.

To compare these two approaches, we used both fluorescence *in situ* hybridization (FISH) and Southern blot analysis to detect *MYCN* gene amplification in neuroblastoma samples collected from patients enrolled in therapeutic, as well as nontherapeutic, studies of the Pediatric Oncology Group (POG). The intent was to test the concordance of these two methods applied to tumor samples from the same patients. The results reported here indicate that the FISH assay is the method of choice for detecting *MYCN* amplification, currently the most reliable marker of resistant disease warranting prompt intensification of treatment.

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Materials and Methods

Tumor Tissue and Patient Information

Fresh tumor specimens or bone marrow samples taken from 642 neuroblastoma patients enrolled on therapeutic or nontherapeutic protocols of the POG, between January 1991 and April 1994, met all criteria for analysis. The FISH assay was performed at St. Jude Children's Research Hospital (Memphis, TN) and the Southern blot analysis at Washington University (St. Louis, MO). Wright-stained smears of aliquots of each tumor cell suspension were analyzed for the percentage of tumor cells. Specimen accrual was limited to patients whose diagnosis of neuroblastoma was based on histologic examination of tumor tissue or of involved bone marrow. Clinicopathologic tumor staging was based on standard POG criteria [10]. Selected clinical and laboratory data (e.g., age at diagnosis, sex, tumor site, and ploidy) were retrieved from the POG statistical center.

FISH

MYCN gene amplification was investigated by simultaneous interphase and metaphase FISH analysis of single-cell suspensions from tumor specimens, as described previously [9]. Hybridization studies were performed with a cosmid probe (from the *MYCN* genomic locus on chromosome 2) that had been nick-translated with digoxigenin-dUTP (Boehringer Mannheim). The labeled probe was combined with human C_ot1 DNA and allowed to hybridize overnight at 37°C to fixed tumor cells in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in a solution containing fluorescein-conjugated antidigoxigenin antibodies. Following signal detection, the slides were counterstained with propidium iodide and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Probe detection for two-color experiments included Texas red avidin and counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI).

Fluorescence microscopy was performed with a Zeiss microscope equipped with either fluorescein filter sets or a three-color filter set for FITC, Texas red, and DAPI; observers had no knowledge of the Southern blot results. Each sample was analyzed to determine the origin of the amplification unit (extrachromosomal DMs or intrachromosomal HSRs) and the proportion of cells with amplified *MYCN* genes. The *MYCN* copy number was scored as either amplified or unamplified. Two-color FISH was performed with a biotin-labeled chromosome 2 centromere-specific probe, in addition to the *MYCN* probe, whenever a modal copy number of 5 to 12 was detected to distinguish gene amplification from chromosome 2 aneusomy.

Southern Blot Analysis for MYCN Gene Amplification

High-molecular-weight DNA was isolated from tumor samples, digested with restriction endonuclease *Eco*RI, fractionated on agarose gels, transferred to nylon filters, and hybridized to a ^{32}P-labeled *MYCN* probe, as previously described [1]. Washed filters were exposed for 12 to 48 hours to Kodak XAR-5 film at −80°C with an intensifying screen. Signal intensity was quantitated directly and by serial dilution from autoradiograms with a laser densitometer (LKB Model 2222), as previously described [1]. The gene was considered to be amplified if the copy number was >3 per haploid genome and unamplified if it was <3.

Results

Heterogeneity of MYCN Gene Amplification

MYCN gene amplification was detected in 105 of 642 neuroblastoma samples examined by FISH analysis. Figure 1 illustrates the typical findings in tumors with single-copy (panel A) or amplified (panels B and C) *MYCN* genes. Different forms of *MYCN* amplification within tumor cells from a single clinical sample are shown in Figure 2*A*, where amplification is present as either DMs or HSRs in interphase

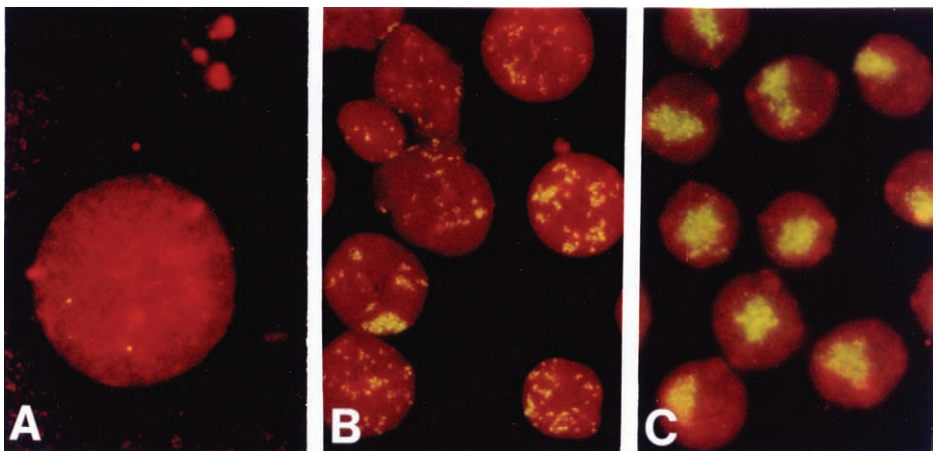

Figure 1. *Typical findings obtained by FISH analysis of MYCN genes in neuroblastoma: (A) unamplified; (B) amplified as DMs; and (C) amplified as HSRs.*

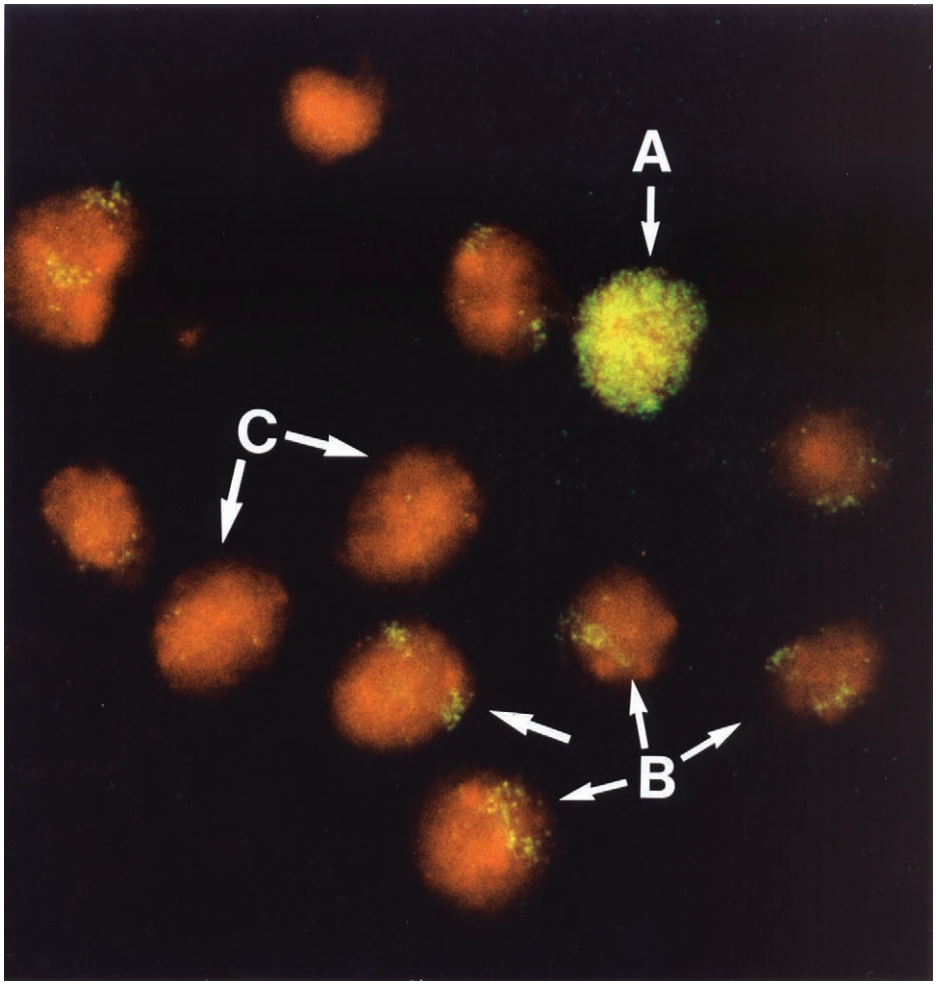

Figure 2. *Heterogeneity of MYCN copy number among tumor cells in a single diagnostic sample, including amplification as DMs (A); HSRs (B); or single no amplification (C).*

cells. Some of the cells have the normal number of *MYCN* copies. One of the advantages of using the FISH assay is depicted in Figure 3, which illustrates multiple copies of *MYCN* in a tumor with chromosome 2 aneusomy (tumor cell DNA index, 2.22). In this instance, the extra copies of *MYCN* did not arise from the formation of DMs or HSRs, but from increased copies of the entire chromosome [11].

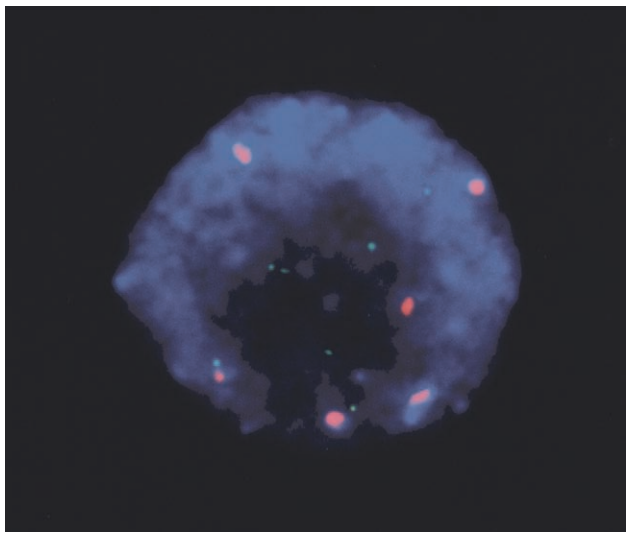

Figure 3. *Multiple copies of MYCN in a tumor with chromosome 2 aneusomy.*

Comparison of FISH and Southern Analysis

From January 1991 to June 1993, 232 neuroblastoma samples were successfully examined by both FISH and Southern blotting procedures. With the former assay, *MYCN* amplification was present in 47 of the samples, compared with 39 by Southern blotting, representing a 21% increase in the total number of positive tumors detected by FISH (Table 1). A single tumor with *MYCN* amplification by Southern blotting was scored as unamplified by FISH (patient 9).

Reasons for the discordant findings are suggested by the specimen characteristics reported in the table. Four of nine samples (from patients 1, 4, 7, and 8) consisted of tumor or bone marrow with a low percentage of malignant cells (9% to 30%), a situation conducive to false-negative measurements by the Southern method. With FISH, by contrast, it was possible to analyze multiple interphase nuclei rapidly, permitting the detection of clumps of tumor cells with

Table 1. Clinical Features of Discordant Cases.

Patient Number	Age (year)	Stage*	Percent Tumor Cells	Type of Sample		*MYCN* Status	
				FISH	Southern	FISH	Southern
1	2.3	D	30	Marrow	Marrow	Amplified	UA
2	3.7	D	66	Tumor	Marrow	Amplified	UA
3	1.0	C	88	Tumor	Tumor	Amplified	UA
4	4.5	D	9	Marrow	Marrow	Amplified	UA
5	1.1	D	80	Tumor	Tumor	Amplified	UA
6	1.2	A	50	Tumor	Tumor	Amplified	UA
7	2.0	D	10	Marrow	Marrow	Amplified	UA
8	2.2	D	10	Marrow	Marrow	Amplified	UA
9	1.1	D	81	Marrow	Tumor	NA†	Amplified

*By POG criteria [10].
†Unamplified.

amplified *MYCN* genes, regardless of their abundance in the sample (Figure 4). In two additional cases (2 and 3), the interphase tumor cells contained low levels of DMs. Apparently, the low *MYCN* copy levels in these tumor cell populations were below the limits of detection by Southern blotting. The same pattern of amplification was observed in case 4, which probably contributed (with the low tumor cell fraction) to a false-negative Southern result. The Southern blot finding in case 5 can be attributed to a degraded DNA sample that yielded a falsely low *MYCN* hybridization signal. Finally, the false-negative FISH classification of case 9 was due to a clerical error resulting in the analysis of a tumor-free marrow specimen. Thus, the FISH technique not only shows a high level of agreement with Southern blotting, but detects authentic gene amplification in a significant number of additional cases. A review of the 223 specimens with concordant FISH and Southern results revealed that all had more than 30% tumor cells, indicating that Southern blotting should be reserved for samples with intermediate to high fractions of tumor cells.

Discussion

Amplification of the *MYCN* oncogene identifies a high-risk subset of neuroblastoma patients who respond poorly to conventional therapy [2,12,14]. Hence, the ability to detect this biologic marker promptly and reliably is a prerequisite for sound clinical management. The data we have presented indicate that FISH offers distinct advantages over Southern blotting for the detection of *MYCN* amplification, in agreement with the recently reported study of Taylor *et al.* [15]. It can be readily performed on small tissue samples that may be only partially involved by tumor. Moreover, in contrast to Southern blotting, interphase FISH distinguishes a spectrum of *MYCN* changes that may have prognostic significance.

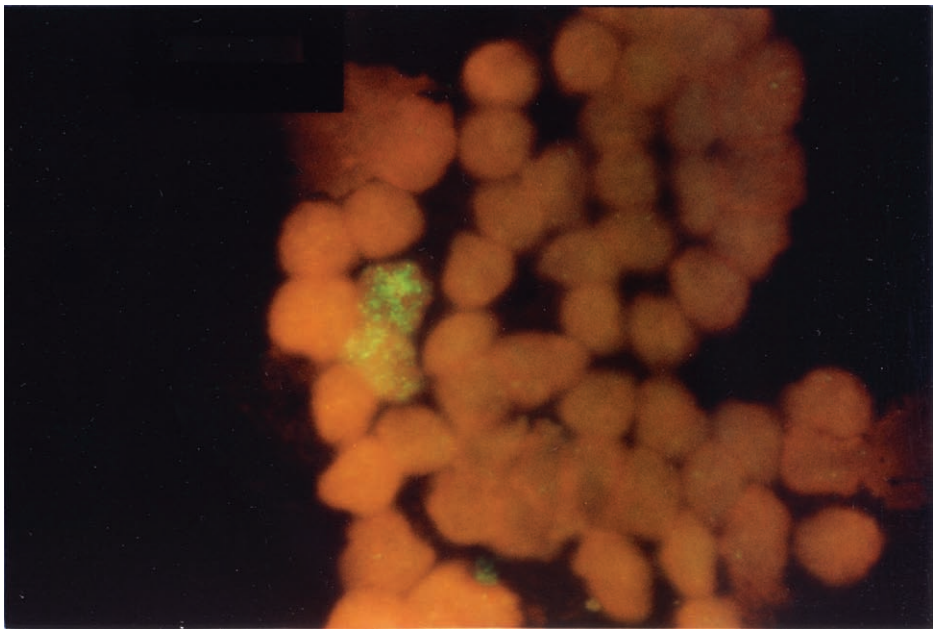

Figure 4. *Low percentage of amplified neuroblastoma cells in a discordant diagnostic bone marrow sample.*

Single-copy genes appear as two discrete fluorescence signals corresponding to each copy of the *MYCN* gene. Amplified sequences, whether HSRs or DMs, are clearly distinguished as multiple punctate or coalesced signals in interphase nuclei. In cases with chromosome 2 aneusomy, simultaneous cohybridization of interphase nuclei with a chromosome 2–specific alpha-satellite probe allows direct enumeration of the number of copies of chromosome 2 relative to *MYCN* [9]. Finally, the FISH procedure allows one to visualize cell-to-cell differences in the *MYCN* copy number due to the unequal segregation of DMs between daughter cells during mitosis, resulting in a heterogenous distribution of amplified sequences within a defined cell population [13].

Consistent with previous reports [7,14], we found that the *MYCN* oncogene was most frequently amplified as DMs. We did not observe HSRs in any of the discordant cases, although such regions were noted in two cases found to have amplified *MYCN* by both FISH and Southern blot analysis. The basis for this differential pattern of amplification is not known; however, experimental models of gene amplification suggest that it may reflect primary differences in the stage of tumor progression, with DMs representing an earlier event than the intrachromosomal integration of amplified sequences [9,13].

FISH has also shown promise for improving the detection of gene amplification in tumors other than neuroblastoma. Kallioneimi *et al.* [16] have demonstrated the utility of this method for recognizing both the level and spatial distribution of *ERBB2(HER-2/neu)* amplification in breast cancer cell lines and primary breast carcinomas [16]. *HER-2/neu* amplification in salivary gland carcinomas was characterized by Press *et al.* [17] using a method similar to ours. They showed that the *HER-2/neu* gene is amplified and overexpressed in approximately one third of these tumors and is independently associated with a poor prognosis. With increased attention being paid to the oncogenic role of human gene amplification, it will be important to consider FISH for assessment of the amplification pattern in clinical tumor samples and the detection of tumor cell subpopulations with strongly amplified genomic regions.

We conclude that the capacity of FISH to detect small populations of highly amplified neuroblasts within tissue or bone marrow specimens affords a clear advantage when one is evaluating clinical material for *MYCN* gene status. The improved rate of detection of amplified *MYCN* copies over Southern blot analysis could be expected to influence the selection of therapy and thus the overall survival of children with neuroblastoma.

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