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Short Communication

MYCN Gene Amplification

Identification of Cell Populations Containing Double Minutes and Homogeneously Staining Regions in Neuroblastoma Tumors

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Neuroblastoma is the second most common solid tumor occurring in children. Amplification of the MYCN oncogene is associated with poor prognosis. To identify neuroblastoma tumors with MYCN amplification, we studied the number of copies of MYCN in interphase cells by fluorescence *in situ* hybridization in 20 neuroblastoma patients. MYCN amplification appeared in 7 tumor specimens. Interphase and metaphase studies showed a tumor cell population with both forms of amplification, double minutes and homogeneously staining regions, in two patients. These patients showed a smaller tumor cell subpopulation with the presence of more than one homogeneously staining region, suggesting that gene amplification was undergoing karyotype evolution. *(Am J Pathol 1999, 155:1439–1443)*

Neuroblastoma (NB) is a pediatric solid tumor arising from the postganglionic sympathetic nervous system and is a leading cause of death in infants below 1 year of age. One of its most remarkable features is a striking clinical heterogeneity. Characterization of genetic alterations in NB have been helpful in predicting clinical outcome and stratifying therapy for the various genetic changes. MYCN amplification has proven to be an independent prognostic factor for identifying rapid tumor progression and predicting a very poor prognosis irrespective of age and clinical stage.[1–3] The MYCN gene is a cellular proto-oncogene of the MYC family of transcription factors. MYCN maps to the short arm of chromosome 2 at band 2p24.[4] Although its role in oncogenesis is thought to involve extrachromosomal elements called double minutes (DMs), it may also be integrated as reiterated amplicons within a chromosomal site as a homogeneously staining region (HSR). Amplicons in NBs range from 350 to 2000 kb with a consensus commonly amplified domain defined as 130 kb.[5] Although the precise details of the amplification mechanism are poorly understood, it is thought that a large region of genomic DNA, including the entire MYCN gene, becomes amplified initially as extrachromosomal DMs,[6–8] possibly persisting in this form. However, occasionally extrachromosomal DMs become linearly integrated into a chromosome, forming one HSR by a mechanism such as unequal sister chromatid exchange.[9] Brodeur[9] suggests that MYCN amplification is an intrinsic biological property of a subset of aggressive NBs and that tumors without amplification at diagnosis rarely, if ever, develop this abnormality subsequently. It is

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Table 1. Clinical and Biological Features of 20 Neuroblastoma Cases

Patient/ tumor	Tumor specimens/sex	Age at diagnosis (months)	Primary tumor	Stage system (INSS)	Shimada classification	MYCN amplification	Treatment			Status	Follow-up (months)
							Surgery	CT	XRT		
1/NB	TF/M	21	adrenal	3	U	+	n.d.	+	+	DOD	12
2/NB	TF/F	13	adrenal	1	F	−	+	n.d.	n.d.	DF	38
3/NB	TF/F	9	paraspinal	3	F	−	n.d.	+	n.d.	DF	36
4/GNB	TF*/F	43	mediastinum	3	n.d.	−	+	+	n.d.	DF	38
5/NB	TF/F	30	adrenal	3	U	+	+	+	+	DOD	17
6/NB	TF/M	35	adrenal	3	U	+	+	+	+	DOD	13
7/NB	TF*/M	11	cervical	4	n.d.	−	+	+	n.d.	DF	31
8/NB	BM/M	12	adrenal	4	n.d.	+	n.d.	+	n.d.	DOD	12
9/NB	TF/M	5	paraspinal	3	F	−	+	+	n.d.	DF	17
10/NB	TF/M	10	adrenal	2	F	−	+	+	n.d.	DOI	5
11/NB	TF*/M	15	adrenal	3	F	−	+	+	n.d.	DF	36
12/GN	TF/F	26	paraspinal	2	n.d.	−	+	n.d.	n.d.	DF	13
13/NB	TF/M	84	adrenal	3	n.d.	−	n.d.	+	n.d.	T	13
14/NB	TF/M	12	adrenal	3	n.d.	+	n.d.	+	n.d.	DOD	7
15/NB	TF/F	39	adrenal	3	U	+	+	+	n.d.	LF	7
16/NB	BM/M	30	adrenal	4	n.d.	−	n.d.	+	n.d.	DF	11
17/NB	BM/M	4	adrenal	4	n.d.	+	n.d.	+	+	DOD	5
18/GNB	TF/M	36	mediastinum	1	n.d.	−	+	n.d.	n.d.	DF	9
19/GNB	TF/F	82	mediastinum	1	n.d.	−	+	n.d.	n.d.	DF	5
20/NB	TF/F	24	adrenal	3	F	−	+	+	n.d.	DF	7

NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma; TF, tumor fragment obtained at diagnosis; BM, bone marrow; M, male; F, female; n.d., not done; F, favorable; U, unfavorable; CT, chemotherapy; XRT, radiation therapy; DOD, died of disease; DF, disease-free; DOI, died of infection; LF, lost follow-up; T, in treatment.
*Sample was obtained after diagnosis.

generally accepted that amplification of the MYCN oncogene is relevant to prognosis,[10,11] and most current treatment protocols require examination of MYCN amplification in NB tumors before treatment begins.[12] Previous studies using fluorescence *in situ* hybridization (FISH) analysis of MYCN have demonstrated its utility for NB tumors.[13–17] FISH directly reveals MYCN copy number on a per-cell basis and also shows whether amplification is present as HSR or DMs. The observation of tumors containing both cytological forms of gene amplification may indicate either a transition from DMs to HSRs or independent generation of both types of structures, with subsequent cell selection favoring the predominance of one form.

Materials and Methods

Twenty tumor samples from patients with clinical NB diagnosis were studied. Patient data are summarized in Table 1. Neuroblastoma tumors were classified according to the International Staging System (INSS).[18] The histopathological evaluation was done by Shimada classification.[19] Among the 20 samples, 17 were obtained at diagnosis and 3 after chemotherapy. Samples were obtained by biopsy or surgery from the primary tumor in 17 cases, and in 3 cases from metastatic bone marrow aspirates. Malignant cell percentage in the bone marrow aspirates exceeded 50%. For FISH analysis the suspensions were performed from tumor tissue by mechanical and enzymatic disaggregation, and direct preparation for analysis of tumor or bone marrow cells was done using modifications of techniques described.[20,21] We used a MYCN DNA probe, digoxigenin-labeled; detection was obtained with fluorescein-conjugated sheep antibodies to digoxigenin (Oncor, Gaithersburg, MD), followed by counterstaining in propidium iodide solution containing antifade. We analyzed the cells in a Zeiss fluorescence photomicroscope equipped with fluorescein filter. A minimum of 100 interphase nuclei was scored per sample except for case 3, where study of only 22 nuclei was possible. Copy number was determined by counting and averaging the number of fluorescence signals per

Table 2. Copy Number of DMs per Cell, Interphase Nuclei with HSRs, and Nuclei with Both Structures

Patient no.	DMs	HSR	DMs + 1 HSR	DMs + 2 HSRs	DMs + 5 HSRs	Total no. of cells analyzed
1	93	5	4	1	1	104
5	100	0	0	0	0	100
6	96	4	0	0	0	100
8	94	2	4	0	0	100
14	100	0	0	0	0	100
15	100	0	0	0	0	100
17	100	0	0	0	0	100

DMs, double minutes; HSR, homogeneously staining region.

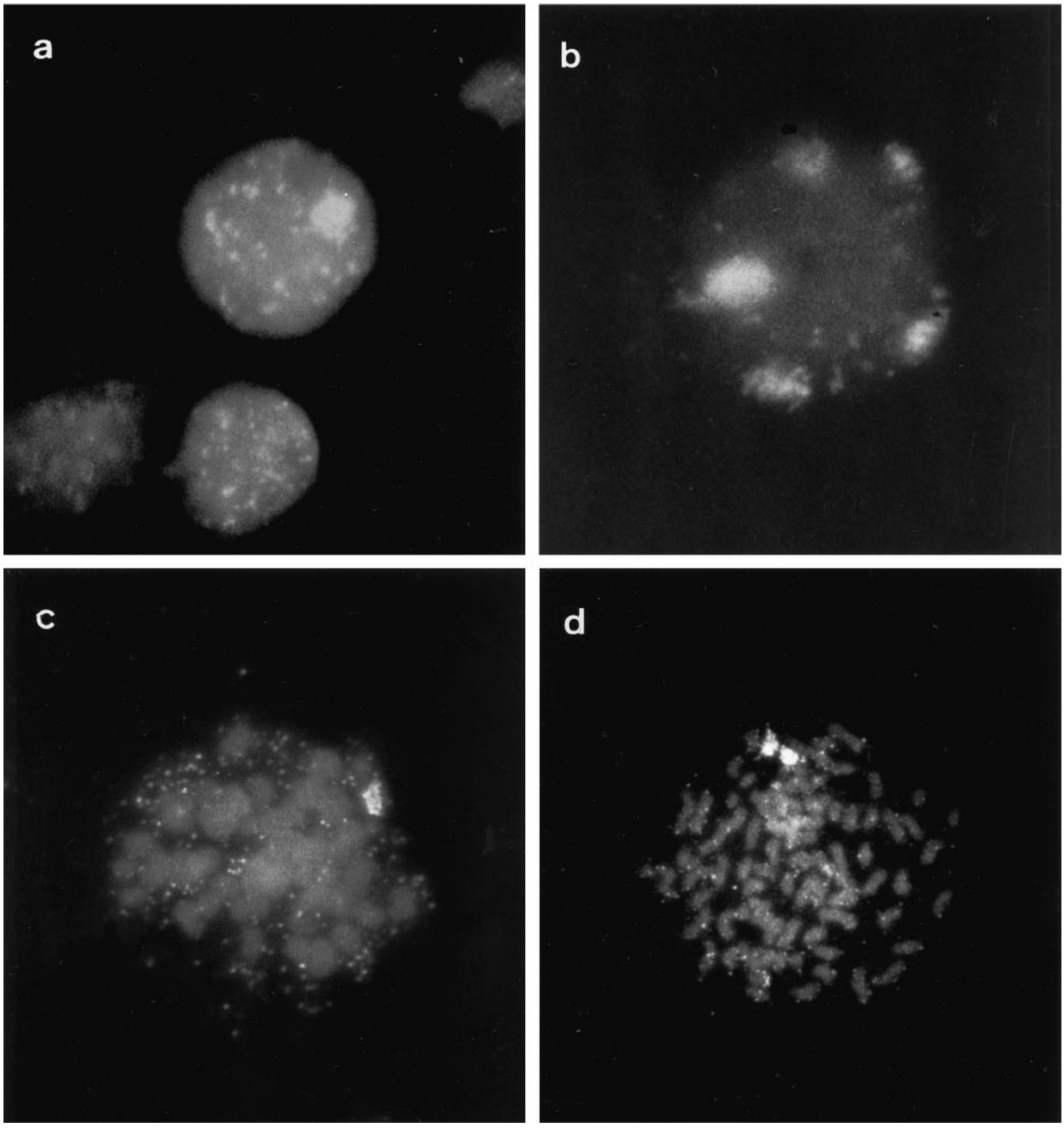

Figure 1. Fluorescent *in situ* hybridization with probe MYCN. **a:** Interphase nucleus with DMs and one HSR from case 1. **b:** Interphase nucleus with DMs and five HSRs from the same patient. **c:** Metaphase cell with DMs and one HSR from case 8. **d:** Metaphase cell from the same case with DMs and two HSRs.

interphase nucleus. In cells with high levels of gene amplification, accurate scoring was impossible, then we included these cells, therefore, in a class of more than 50 copies.[14] We only studied slides with hybridization efficiencies higher than 90%. MYCN amplification was considered as number of copies per nuclei exceeding 10.[10] We analyzed 2000 cells from case 8 to find metaphase nuclei with both cytological structures of gene amplification.

Table 3. Cytogenetic Forms of Gene Amplification in Metaphase and Interphase Nuclei of Case 8

Nucleus	DMs	HSR	DMs + 1 HSR	DMs + 2 HSRs	Total of 2000 cells analyzed
Interphase	1974	9	11	0	1994
Metaphase	4	0	1	1	6
Total	1978	9	12	1	2000

DMs, double minutes; HSR, homogeneously staining region.

Results and Discussion

We studied twenty tumors, 17 at diagnosis and 3 after therapy. MYCN amplification was seen in 7 of 17 patients at diagnosis (41%), a frequency surpassing that shown by the data of other authors,[22] and resulting either from the number of tumors studied or their stage of progression. Six patients with MYCN amplification had progressive and fatal disease, despite intensive therapy (Table 1). All patients presenting MYCN amplification had stage 3 or 4 INSS classification.[18] Among the 13 tumor specimens without MYCN amplification, 5 were stage 1 or 2 (INSS) and 8 were stage 3 or 4 (INSS). Within follow-up time limits, just one patient (case 10, stage 2) died due to infection 5 months after diagnosis. Eleven are alive and disease-free, and one patient continues under therapy. Thus, MYCN amplification was correlated with rapid tumor progression and a poor outcome independent of tumor clinical stage or age of the patient at diagnosis.

Table 2 shows cytogenetic data of the tumors with MYCN amplification. We found the MYCN gene most frequently amplified episomally in DMs, as seen in previous reports.[14,23] Intrachromosomal amplification as HSR is rare in comparison to DMs in NB, and is more often observed *in vitro*[24,25] in cell lines rather than in primary tumors. In three tumor samples we saw beside DMs, cells with only one HSR (cases 1, 6, and 8); the other two (cases 1 and 8) showed both structures in the same cells (Figure 1a). NB cell lines have rarely been described as having two cell subpopulations, one with DMs and the other with HSR.[24,26] However, the same phenomenon was reported for other cell lines.[27–30] Although seldom observed in the same cell,[28,30] when present in the same cell population, they are mutually exclusive in individual cells.[26,29,31] We also observed cells with DMs and more than one HSR (Figure 1b). Presence of 2 or 3 HSRs per nucleus has been seen in NB cell lines,[4,32,33] but not in direct preparations from tumors. We believe that our observations correspond to what happens *in vivo* because we did not culture our tumor samples in any way, thus avoiding *in vitro* selection bias. In an *in vitro* transformed mouse salivary gland epithelial cell line, DMs were observed in 100% of cells at an early passage level.[29,34] After approximately 17 *in vitro* passages a subpopulation of cells devoid of DMs appeared, whereas in DM-negative cells one HSR was present.[29]

In case 8, we demonstrated in metaphase nuclei the presence of both structures in the same cell. The results of our analysis of 2000 nuclei from this case are shown in Table 3 and Figures 1c and 1d. MYCN amplification in these tumors (cases 1, 6, and 8) presented cells where both forms of amplification (DMs and HSR) are detectable by interphase FISH analyses. We have seen cells where HSR appears as a distinct domain of signals together with DMs as double-specked signals throughout the same nuclei. These observations confirm the putative mechanism of gene amplification which proposes extrachromosomal DMs reintegrating into another chromosomal site and amplifying by a mechanism such as unequal sister chromatid exchange, generating a HSR.[33,35] The occurrence of both cytological structures in the same cell, as observed, indicates that a transition may occur from DM to HSR bearers; the alternative hypothesis that both cytological forms exist, with cell selection eventually favoring HSR, is less likely. However, the gene amplification mechanism is not yet well understood. High levels of gene amplification in tumors of patients who died after intensive therapy may account for the presence of more than one integration site, and we are tempted to conclude that this contributed to the rapid cytogenetic evolution of gene amplification.

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