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Amplification of the N-*myc* Gene in Untreated Human Neuroblastomas[1]

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=Abstract= Gene alteration and abnormal expression of cellular oncogenes has been implicated in the causation of various types of malignant disease. Gene amplification is thought to be a potent device for increasing the amount of normal oncogene product and has been reported in a variety of human tumors. We have now examined the amplification of a c-*myc* related sequence called N-*myc* in several neuroblastoma tissues donated by patients of clinical stages IV and in an established tumor cell lines. Though N-*myc* amplification occurred as a common event in these patients, the degree of amplification was found to be quite different among them so that it ranged from several fold of amplification to more than 100 fold of amplification. Considering their clinical stages, it seemed that the degree of N-*myc* amplification has some correlation with the advanced stages of the disease.

Key words: ***N-myc, Gene amplification, Neuroblastoma***

INTRODUCTION

Cellular oncogenes are a class of genes whose aberrant expression or function may be implicated in the causation of various types of malignant disease. Such aberration can occur when regulation of a single gene is faulty, when multiple copies of a gene are formed (genomic amplification), or when mutations results in an oncogenic product.

Gene amplification is thought to be a potent device for increasing the amount of normal oncogene product. For example, amplificaton 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 (Alitalo *et al.* 1983; Little *et al.* 1983; Collins 1982; Dalla-Favera *et al.* 1982).

Recently amplification of N-*myc*, a celluar gene related to the c-*myc* proto-oncogene, has been frequently reported in a restricted set of tumors, most notably human neurobalstomas (Schwab *et al.* 1983; Kohl *et al.* 1983; Michistch *et al.* 1984). The finding that N-*myc* amplication is a common event in untreated human neuroblastomas and furthermore is highly correlated with advanced stages of the disease —50% occurrance in patients of stage III & IV-can be suggestive that N-*myc* may have a causal role in the genesis of the disease and at least have some clinical prognostic significance (Seeger *et al.* 1985).

To elucidate the frequency and degree of N-*myc* amplification in neuroblastomas we have examined five primary untreated neuroblastomas with the established cell lines and compared the estimated degree of amplification with the prognosis of the diesease.

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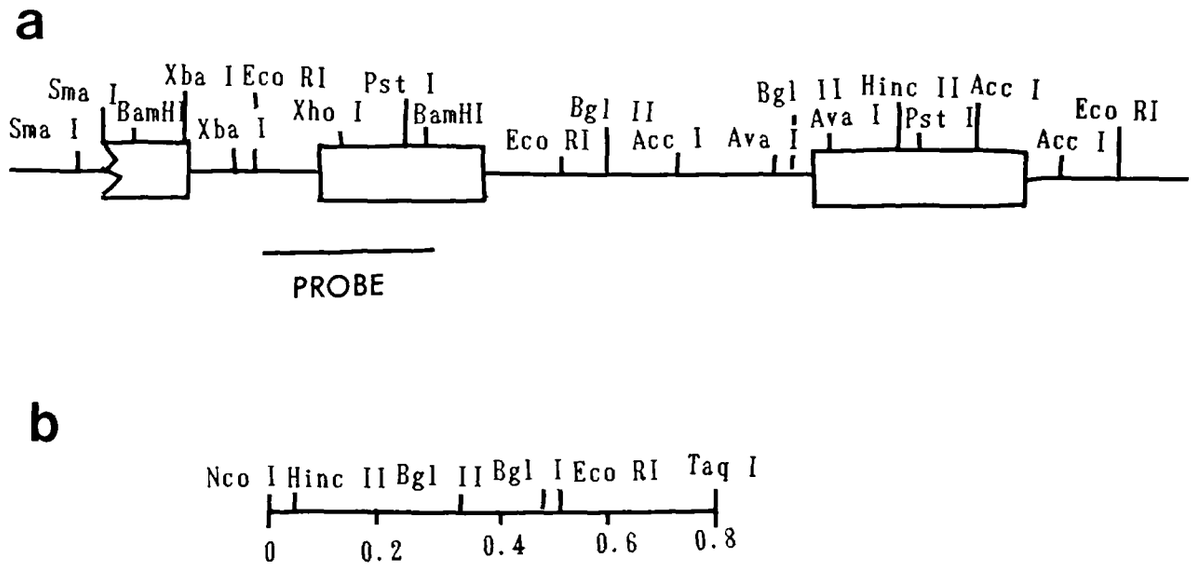

Fig. 1. a. Restriction map of the human N-*myc* locus and representation of the N-*myc* specific sequence in N-*myc* probe chosen.
b. Actin gene probe (chicken) in this study.

MATERIALS AND METHODS

Tumors and cell lines

The neuroblastoma tumors were obtained as fresh tissues at surgery from untreated patients; N1 is a tumor from 2-year-old male patient; N2 is a tumor from 3-year-old male patient; N3 is a tumor from 1-year-old male patient; N4 is a tumor from 4-year old male patient; N5 is a tumor from 2-year-old female patient. Neuroblastma cell line IMR 32 was used as positive control for N-*myc* amplification. Lymphoblastoma cell line was used as negative control of N-*myc* amplication.

Assays for amplified restriction fragments

DNA was purified from tumors or cell lines using proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. DNA (20 μg, quantitated by using Hoechst dye) was separately cleaved with the restriction endonuclease *Hind* III and *Eco*RI (5 units/μg of DNA; New England Biolab.) following the conditions recommended by the supplier. The cleaved DNA was separated by electrophoresis through 0.9% agarose gel. The DNA was then depurinated in the gel by brief washing in 5 × TAE (1 × TAE = 20 mM Tris-HCl, pH 7.5/0.5 mM EDTA/10 mM Acetate) and the DNA was denatured by alkaline treatment (0.2 M NaOH/0.5 M NaCl; 30 min). Subsequently the DNA was renatured in a solution of 20 × TAE (two 10 min. treatments). After brief equilibration with 1 × TAE, the DNA was transfered to Zeta-probe membrane (Bio-Rad) using Bio-Rad transblot apparatus. For quantitative analysis of N-*myc* amplification the equivalent amount (10 μg) of DNA cleaved with the restriction endonuclease *Eco* Ri was serially diluted and blotted to nitrocellulose filter using the manifold apparatus (Type II) of Schleicher and Schuell. We used cloned *Eco*RI-*Bam*HI fragment near exon II region as authentic N-*myc* probe (Fig. 1a). We also used Actin gene probe, regarding its hybridization signal as reference in our estimation of N-*myc* amplifcation (Fig. 1b). The N-*myc* probe (0.5 μg) was labeled with 100 μCi of [α-P^{32}]dCTP(3,000 Ci/M, NEN) using nick translation kit (Bethesda Research Lab.). The Actin probe (0.2 μg) was labeled with 100 μCi of [α-P^{32}] dCTP(3,000 Ci/M, NEN) using random primer extension kit(Boehringer Mannheim Biochemical). For molecular hybridization we employed conditions of high stringency (50% formamide, 0.75 M NaCl, 0.075M Na-citrate, 42°C) under which there is no cross-hybridization between c-*myc* and N-*myc*. The filter of dot blots first hybridized with Actin gene probe was washed and reused in second hybridization with N-*myc* probe. A hybridization signal was not considered amplified unless it had at least three times the

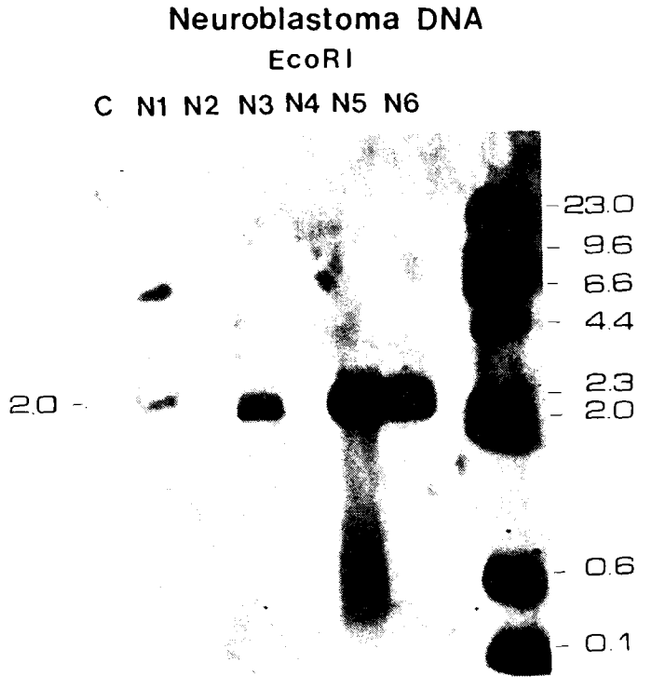

Fig. 2. Identification of amplifed N-*myc* DNA digested by *Eco*RI in human neuroblastomas (N1-N5) and control DNAs(L1 N6). The DNA on Zeta-probe membrane was hybridized with N-*myc* probe (1.6×10 cpm/μg) and exposed for 23hrs at -70°C with an intensifying screen.

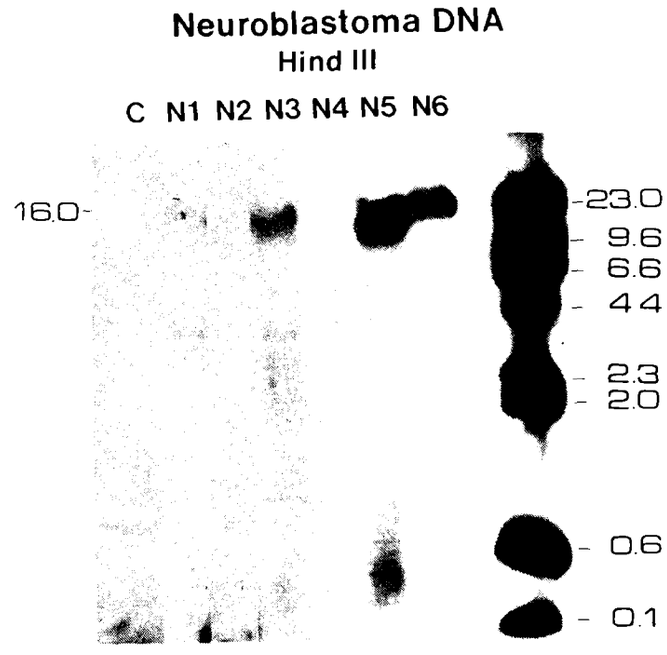

Fig. 3. Identification of amplified N-*myc* DNA digested by *Hind* III in human neuroblastomas (N1-N5) and control DNAs (L1, N6). The DNA on Zeta-probe memberane was hybridized with N-*myc* probe (1.6×10 cpm/μg) and exposed for 23 hrs at -70°C with an intensifying screen.

intensity of an equal amount of control DNA. Quantitative determinations based on visual inspection of autoradiograms were confirmed by densitometric analysis.

RESULTS

As our expectation from the restriction map of N-*myc* gene (Fig. 1a) the authentic N-*myc* signal appeared in 2 kb EcoRI fragments and 16kb *Hind* III framgents (Fig. 2 & Fig. 3.) The DNAs of lymphoblastoma cell line, neuroblastoma patient N2 and N4 did not show any signal of N-*myc* in moderate-long term (72 hr) exposed autoradiograms (data not shown). By contrast the DNAs of neuroblastoma patient N1, N3, N5 and neuroblastoma cell line showed very strong signal of N-*myc* in autoradiograms. The relatively faint signals obtained in neurobalstoma patient N1 for 6.5 kb *Eco*RI fragments and 4 kb Hind III fragments and in neuroblastoma patient N3 for 4 kb *Hind* III fragments might be thought as signals of translocated N-*myc* fragments which occasionally follow gene amplification, though those sequences are to be cloned and further mapped. In these two southern blots only 3 cases of 5 neuroblastoma patients who were grouped into the same diagnostic evaluations, all clinical stage IV, showed strong evidence of N-*myc* amplification.

To confirm the amplification of N-*myc* more quantitatively we further examined the extent of amplification through dot blot analysis. (Fig. 4) We used the same amount of lymphobastoma cell line DNA and Actin gene probe as standards for single copy intensity and criteria for quantity correction, respectively. (on the assumption that there is no N-*myc* amplification reported in lymphoblastoma cell line DNA and that all kinds of cells have the same copy number of actin gene as house keeping gene). Comparing the hybridization signal with that of lymphoblastoma cell line DNA, the sample DNAs showed insignificant differences in hybridzation signal of Actin gene. This could be a decisive evidence that the intensity variation of N-*myc* signal produced were not due to the incorrect quantitation of individual DNA but due to the real difference in N-*myc* copy number among them. The 3 cases out of

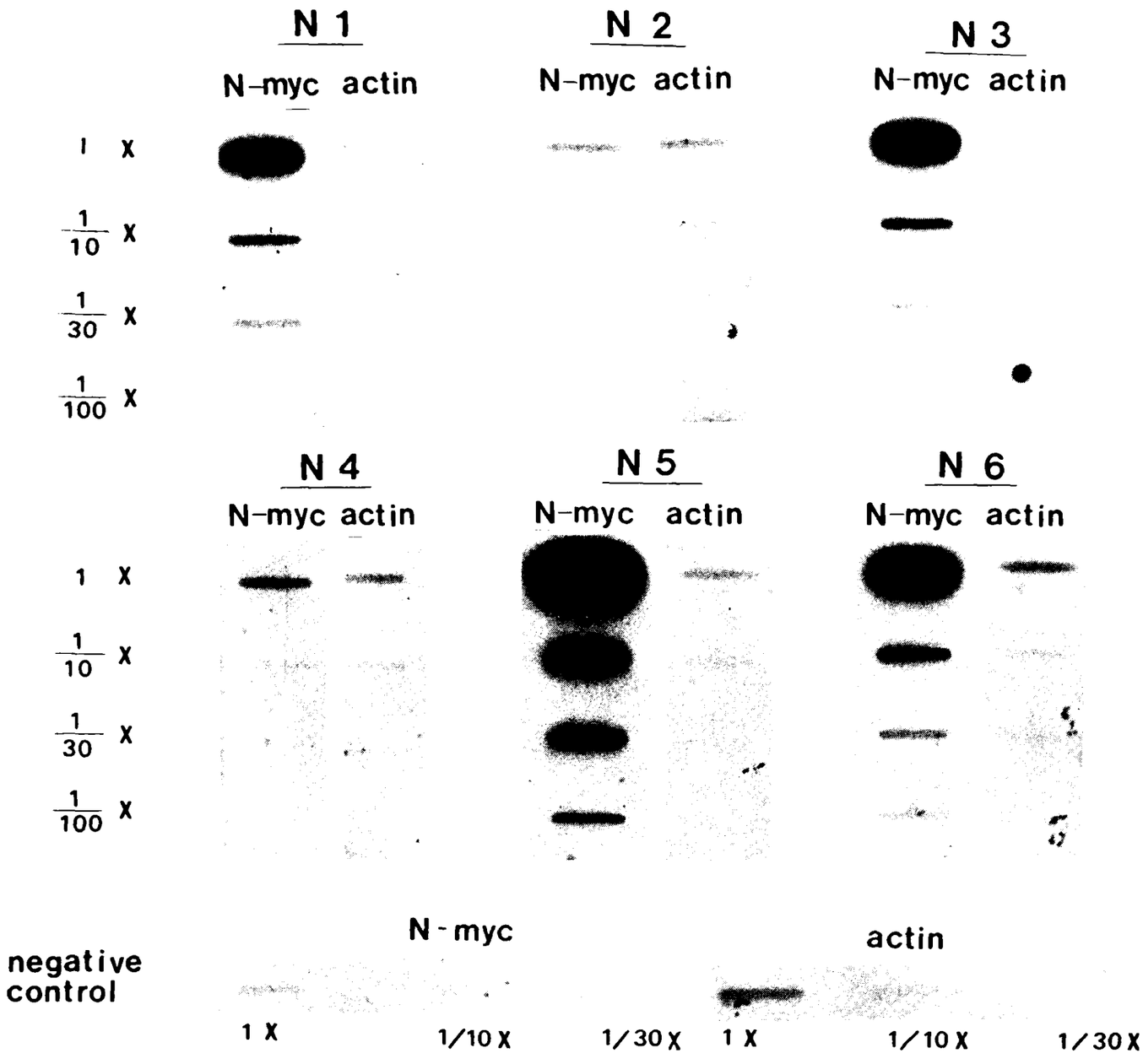

Fig. 4. Dot blot analysis for the estimation of the extent of N-*myc* amplification.

Table 1. Patient age and clinical stages of 5 untreated patients with neuroblastoma and determination of N-*myc* copy number in their tumor DNA by DNA hybridization

	Sample	age/sex	clinical stage	N-*myc* copy No. per genome
N 1	P.B.S.	2Y2M/M	IV	~30
N 2	M.J.Y	3Y6M/M	IV	2-3
N 3	H.S.W	1Y2M/M	IV	~30
N 4	J.J.W	4Y2M/M	IV	2-3
N 5	O.S.W	2Y2M/F	IV	> 100
N 6	Neuroblastoma cell line	—	—	~60
C	Lymphoblastoma cell line	—	—	1

5, neuroblastoma patient N1, N3, N5 and positive control DNA consistently showed amplified N-*myc* signal and the single-*myc* copy intensity were found in more than 30-fold dilutions. In the case of the remaining two patients N2 and N4 N-*myc* signals obtained were slightly (2-3 fold) stronger than that of lymphoblastoma DNA. The estimates of N-*myc* copy number in these sample DNAs obtained through densitometric analysis (Table 1).

DISCUSSION

Oncogene amplification has been identified in a variety of other tumors, in addition to neuroblastomas (Schwab *et al.* 1982; Kohl *et al.* 1983; Michistch *et al.* 1984; Bordeur *et al.* 1984); N-*myc* amplification in retinoblastomas (Lee *et al.* 1984); c-*myc* in acute promyelocytic leukemia (Collins *et al.* 1982; Dalla-Favera *et al.* 1982; Nowell *et al.* 1983), as well as carinomas of the colon (Alitalo *et al.* 1983), breast (Kozbor and Croce 1984), lung (Little *et al.* 1985), and stomach (Shibuya *et al.* 1985); c-*myc* in acute myeloblastic leukemia (Pellici *et al.* 1984) and adenocarcinoma of the colon (Alitalo *et al.* 1984); c-*abl* in chronic myelocytic leukemia (Collins and Groudine 1983; Selden *et al.* 1983).

Our current investigation of N-*myc* oncogene in 5 untreated primary neuroblastomas demonstrates a significant association between genomic amplification and rapid tumor progression. Although the clinical stages of the neubalstoma tumors were the same, all stage IV, their extents of N-*myc* gene amplification were very different among them and the prognoses varied accordingly. In 3 patients whose N-*myc* genes were amplified more than 30 fold, the neuroblastoma had very poor prognosis, eg., in the patient whose N-*myc* gene was amplified more than 100 fold the disease relapsed and metastasized within 6 months after surgery.

From the follow-up study the extent of N-*myc* amplification appears to be a good parameter in determining prognosis, even better than the existing parameter such as patient's age at diagnosis, regarding the fact that the patients N1, N3 and N5 whose prognosis of disease appeared more worse were rather younger than the patients N2 and N4 with better prognosis. thus, in these five neuroblastomas the correlation of N-*myc* amplification with advance of the disease appeared very significant and it is likely that the N-*myc* sequence has an important role in human neuroblastomas.

Four other clinically important prognostic factors have been identified recently that can be asessed at diagnosis:

i) Histopathological characteristics of the primary unterated tumor (Shimada *et al.* 1984).

ii) the amount of tumor-cell DNA as measured by flow cytometry (Look *et al.* 1984)

iii) the quantity of neuron-specific enolase in serum (Zeltzer *et al.* 1983 an 1985)

iv) measurement of serum ferritin (Hann *et al.* 1985).

The biological relationships between the N-*myc* oncogene and these variables, as well as prognostic value, remains to be determined.

Other proto-opncogenes have also been implicated in the pathogenesis of neuroblastoma. N-*ras,* a proto-oncogene related to v-*ras,* was first identified in the tumor cell line SK-N-SH by DNA transfection (Shimizu *et al.* 1983; Hall *et al.* 1983). Remarkably, to our knowledge, no other neurobalstoma cell line has been found to have such transforming activity. However, the identification of N-*ras* gene in neuroblastoma would be of great interest because it might be possible that N-*myc* and N-*ras* could serve complementary functions leading to oncogenic cellular tranformation (Land *et al.* 1983).

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= 국문초록 =

Neuroblastoma 암조직에서의 N-*myc* 암유전자 증폭

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세포성 암유전자는 종양유발 RNA 바이러스가 갖고있는 바이러스성 암유전자와 유사한 염기서열을 갖고있는 유전자로서 정상세포내에 존재한다. 이십여가지가 넘는 세포성 암유전자의 분자수준에서의 변화가 일어나면 정상세포는 암세포로 형질전환이 일어나게 된다. 암유전자에 일어나는 분자적 변화로는 점돌연변이, 유전자 증폭, 염색체 전위에 따른 위치이상등이 포함되며 이러한 변화로 인하여 정상상태의 세포성 암유전자는 활성화되게 된다. 본 연구는 활성화 기전중 암유전자 증폭과 증폭의 정도를 알아보기 위하여 클론된 N-*myc* 유전자 DNA를 탐식자로 사용하여 DNA 재결합법으 사용하여 측정비교하였다. Neuroblastoma 세포주인 IRM-32를 유전자 증폭의 양성 비교치로 인하여 1세부터 4세까지의 임상단계 제 4기의 진전된 환자 5명으로부터 얻은 Neuroblastoma조직에서 이를 조사하였다. 5예의 암조직 DNA을 두가지 제한효소 (*Eco*RI과 *Hind*III)로 처치한 후 전기영동과 southern blot을 시행하여 DNA 재결합 실험과 autoradiogram으로 N-*myc* 유전자의 증폭 여부를 확인하였다. 한편 증폭이 없는 actin 유전자를 이용하여 증폭의 정도를 dot blot 실험으로 측정하였다. 3예에서는 30배에서 100배이상 높은 증폭을 나타냈으며 나머지 2예에서는 2~3배의 낮은 증폭정도를 확인할 수 있었다. neuroblastoma의 말기 임상적 단계 (제 4기)에서 N-*myc* 암유전자의 유전자의 증폭이 서로 차이를 보이는 것은 암유전자의 증폭 정도 측정이 예후 판성에 이용될 수 있는 가능성을 시사해 준다.