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Increased N-*myc* Expression following Progressive Growth of Human Neuroblastoma

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ABSTRACT

Many human neuroblastoma tumors and cells in culture contain amplified N-*myc* DNA and this is associated with tumor stage. We have analyzed pairs of neuroblastoma cell cultures derived from two patients at the time of diagnosis and after tumor progression following the initiation of therapy. Cell cultures derived after progression have increased expression of N-*myc* RNA. In one pair this increase is associated with increased N-*myc* DNA amplification; in the other, amplification decreases and activation of N-*myc* is most likely the result of a regulatory change. Analysis of the pattern of DNA amplification in these cell cultures demonstrates additional changes that might be associated with tumor progression.

INTRODUCTION

N-*myc*, a cellular protooncogene (1) with homology to the c-*myc* protooncogene, is amplified and overexpressed in most cell lines derived from human neuroblastoma (2–4). It is also sometimes amplified in another tumor with neuronal characteristics, small cell carcinoma of the lung (5), and can be expressed at high levels in human retinoblastoma (6). It seems to be only rarely expressed in normal tissues and other types of tumors (7). Additional evidence that N-*myc* may be important in the pathophysiology of neuroblastoma includes the finding that although DNA isolated from tumors of patients with localized disease contains a single copy of the N-*myc* DNA sequence per haploid genome, approximately 50% of tumor specimens from patients with advanced stage neuroblastoma had amplified N-*myc* DNA (8). Less than 20% of patients with advanced stage neuroblastoma at presentation survive (9), although patients with lower stage disease have a very good prognosis. For such advanced stage patients, the degree of N-*myc* DNA amplification at presentation has also been associated with poor prognosis (10).

Using *in situ* hybridization analysis to examine tissue from a neuroblastoma tumor specimen for N-*myc* RNA, Schwab *et al.* (11) have found marked heterogeneity in N-*myc* expression among tumor cells, with expression appearing to vary inversely with the degree of cellular morphological differentiation. Thiele *et al.* (12) recently reported that the *in vitro* induction of neuroblastoma differentiation was associated with a rapid, marked decrease in the level of N-*myc* RNA. These findings are of particular interest since morphologically undifferentiated tumors may have a poorer prognosis than those with histological evidence of cellular differentiation (13, 14).

Whether higher stage or more undifferentiated tumors represent disease progression from less aggressive tumors or are unique diseases is unclear. Clinical tumor progression results when a tumor that has shrunk or remained stable in response to therapy begins to increase in size or to spread despite treatment. This phenomenon may result from the selection of genetic variants that express more fit or aggressive phenotypes in the host. To determine whether N-*myc* expression was important for such progressive tumor growth, we evaluated paired tumor cell cultures isolated from each of two patients with neuroblastoma at the time of their initial clinical presentation and during progressive tumor growth after the initiation of therapy. In both instances we found that N-*myc* mRNA expression was elevated in cell lines derived after tumor progression and an analysis of the structure of the amplified cellular DNA sequences revealed changes after tumor progression.

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MATERIALS AND METHODS

Cells. The human neuroblastoma cell lines, SK-N-BE(1), SK-N-BE(2), SMS-KCN, and SMS-KCNR have been previously described (15, 16). They were maintained in RPM1 1640 medium supplemented with 15% (vol/vol) fetal bovine serum.

Nucleic Acid Blotting and Analysis. High molecular weight DNA and total cytoplasmic RNA were isolated from logarithmically growing cells as previously described (12, 17). For analysis of RNA, 10 μg of total cytoplasmic RNA were electrophoresed through a 1% formaldehyde-agarose gel, transferred to nitrocellulose filters by Northern blotting, hybridized in 50% formamide to *in vitro* ^{32}P-labeled DNA probes, and stringently washed as described (12). DNAs were digested with restriction enzyme *Hind*III (Boeringer Mannheim) according to the supplier's recommendations. Ten μg of restricted DNA were electrophoresed through a 1% agarose gel, transferred to nitrocellulose filters by Southern blotting, hybridized, and washed as above (11, 17). DNA plasmid probes included: N-*myc* probe, pNb-1 (4), β-actin probe, pA1 (18), and c-*myc* exon 3 probe (19). Each was labeled *in vitro* to a specific activity of DNA ($1–2 \times 10^8$ cpm/μg) as previously described (12). Blots were reused after elution of the probe in 18 mM NaCl, 1 mM NaH_2PO_4, pH 7.4, 0.1 mM EDTA, and 0.1% sodium dodecyl sulfate at 90°C for 10 min.

In-Gel Renaturation. In-gel renaturation analysis was performed essentially as described by Roninson (20). Fifteen μg of *Hind*III restricted cellular DNA (driver) was mixed with 0.1–0.2 μg of the same restricted DNA (tracer) which had been ^{32}P-end-labeled with T4 DNA polymerase to a specific activity of $2–3 \times 10^7$ cpm/μg DNA. This mixture was electrophoresed through a 1% agarose gel at 75 V for 18 h. The gel was denatured with 0.5 M NaOH-0.6 M NaCl, and then neutralized with and incubated in 0.9 M NaCl-50 mM NaH_2PO_4, pH 7.4-5 mM EDTA containing 50% formamide at 45°C for 2 h to allow renaturation of amplified DNA sequences in the gel matrix. The gel was then equilibrated with S_1-nuclease buffer (50 mM sodium acetate, pH 4.6-0.2 M NaCl-1 mM $ZnSO_4$) and incubated with S_1 nuclease, 1000 units/ml (Sigma) for 2 h at 37°C. This cycle was repeated and the gel was then washed 6 times in 0.54 M NaCl, 30 mM NaH_2PO_4, pH 7.4, 3 mM EDTA, and 0.1% sodium dodecyl sulfate at 37°C, dried, and autoradiographed.

RESULTS

Human Neuroblastoma Cell Lines Derived during Patients' Clinical Course. The data in Table 1 describe pairs of neuroblastoma cell lines derived from two patients at different times during their disease. Cell lines BE(1) and KCN were isolated from patients before either had received therapy. Subsequently, both patients were treated with combination chemotherapy and one received radiotherapy. In both cases this therapy caused

Table 1 *Neuroblastoma cell lines derived from patients with progressive disease*[a]

Cell cultures	Source	Interval therapy	Passage[b]	Karyotype[c]
SK-N-BE(1) (pretherapy)	Bone marrow	Adriamycin, cytoxan, radiation	12	DMs, del 1p
SK-N-BE(2)	Bone marrow		15	Homogeneous staining regions, del 1p
SMS-KCN (pretherapy)	Adrenal primary	Adriamycin, cytoxan	16	DMs, del 1p
SMS-KCNR	Bone marrow		8	DMs, del 1p

[a] The derivation of these cell cultures has been previously described (15, 16).
[b] Passage at which cells were harvested for DNA and RNA isolation.
[c] Karyotype analysis of all four near diploid cell lines has demonstrated considerable chromosomal similarity within each set. Each cell line pair shares two distinctive marker chromosomes indicative of a common cell of origin.

arrest of their disease, but in neither patient was clinical disease ever eradicated. In both patients tumor began to regrow after 2–3 months of therapy; at this point, after clinical tumor progression, cell lines BE(2) and KCNR were isolated.

Each of the cell cultures derived prior to therapy had DMs,[1] cytogenetic evidence of gene amplification (21–23). While DMs are also identifiable in KCNR, one of the cell cultures isolated after the initiation of therapy, and other posttherapy cell culture, BE(2), no longer contained detectable DMs but had acquired homogeneously staining regions, another cytogenetic feature associated with DNA amplification (23). In this and other neuroblastoma cell lines these structures have been shown to contain multiple copies of N-*myc* (2–4, 24, 25).

N-*myc* mRNA Expression and DNA Amplification before and after Tumor Progression. We examined the level of N-*myc* RNA expression in these cell cultures by Northern blot analysis of total cytoplasmic RNA (Fig. 1*A inset a*). In both pairs of cell cultures, N-*myc* expression was increased approximately 4-fold in the posttherapy cultures, BE(2) and KCNR, compared to the corresponding pretherapy cultures BE(1) and KCN. The same results were obtained when polyadenylated RNA was examined (data not shown). Rehybridization of the blot with ^{32}P-labeled actin DNA demonstrated that each lane contained an equivalent amount of RNA (Fig. 1*A inset b*). The amount of N-*myc* DNA in these cell cultures and in KCL, an EBV-transformed lymphoblastoid cell culture derived from the same patient as KCN and KCNR, was examined by Southern blot analysis of *Hind*III restricted cellular DNA (Fig. 1*B inset a*). The amount of N-*myc* DNA in BE(2) was increased approximately 4-fold above that detected in BE(1), from 45–170 copies. In contrast, N-*myc* DNA detected in KCNR decreased about 8-fold compared to that detected in the corresponding pretherapy culture, KCN, from 380 to approximately 50 copies. The amount of N-*myc* DNA detected in KCL with a longer exposure of the autoradiogram is compatible with the presence of a single copy of N-*myc* and confirms other data indicating that amplification of N-*myc* is tumor specific and not present in the germ line (2) (Fig. 1*B, insets a* and *b, lane 5*.) DNA content was normalized by rehybridization of the blot with ^{32}P-labeled c-*myc* DNA (Fig. 1*Bb*).

In-Gel Renaturation Analysis of Neuroblastoma DNA. Our results indicate considerable variation in the amount of N-*myc* DNA present in tumor cell cultures isolated at different times during the course of a neuroblastoma patient's illness. Since amplification of DNA sequences may be an important regulatory mechanism by which tumor cells respond to selective pressures, we utilized an in-gel renaturation technique (20) to characterize the amplified DNA sequences in these cell cultures. Bands which correspond to restriction fragments of amplified DNA are visualized by this technique. In these experiments, the sensitivity of detection is 50–60 copies/genome. The results of such an analysis are shown in Fig. 2. DNA from normal brain tissue (*lane 1*) and the KCL lymphoblastoid cell culture (*lane 2*) share a pattern with other normal tissues and many tumors (data not shown). DNA from most neuroblastoma tumor cell cultures displays many additional bands corresponding to amplified regions of DNA (data not shown). An evaluation of *Hind*III digested DNA from the cell cultures described in Table 1 is shown in Fig. 2, *lanes 3, 4, 7,* and *8*.

Despite the time and therapy separating the derivation of KCN and KCNR (Fig. 2, *lanes 3* and *4*) or BE(1) and BE(2) (Fig. 2, *lanes 8* and *7*), the band patterns of DNA from these

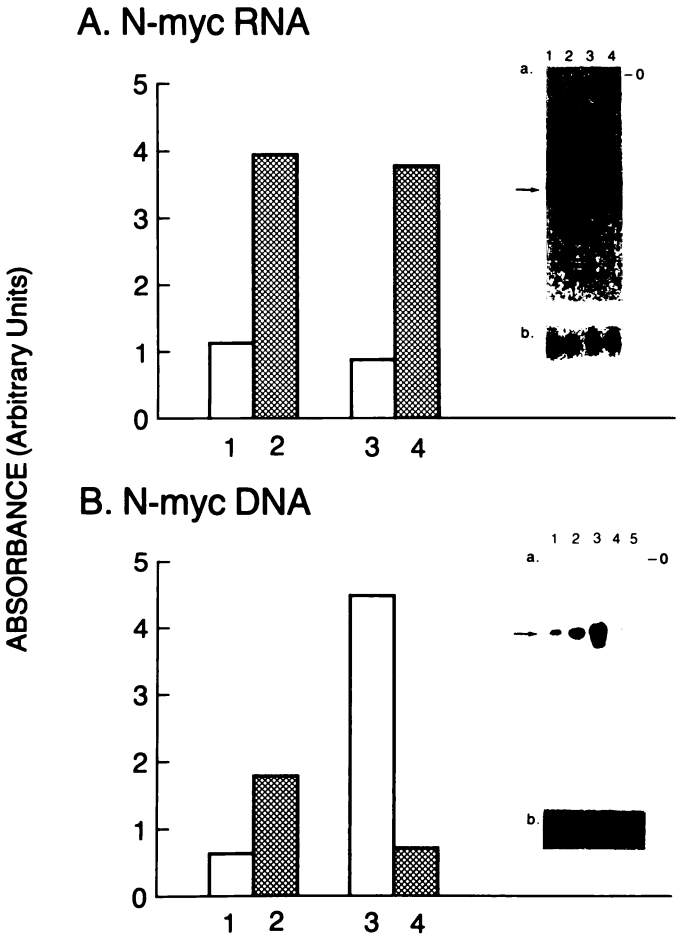

Fig. 1. N-*myc* mRNA expression and DNA amplification in tumor cell cultures. *A*, graphic representation of the relative levels of N-*myc* RNA expression in SK-N-BE(1) (*bar 1*), SK-N-BE(2) (*bar 2*), SMS-KCN (*bar 3*), SMS-KCNR (*bar 4*), as determined by densitometric scanning of the autoradiogram shown in A, *inset a*. Such analysis was performed on autoradiograms in which the band intensity was in a linear range as a function of time of exposure. The *numbered lanes* in *inset a* correspond to the *numbered bars* in the accompanying graph. β-actin expression is shown on the same blot in *A, inset b*. *B*, relative levels of N-*myc* DNA determined by densitometric scanning of a Southern blot (*B, inset a*). The cell lines examined in each of the *numbered lanes* and *bars* in *B* correspond to the cell lines examined in the identically labeled lanes in *A*. *Lane 5* in *B* contains DNA from KCL, an Epstein-Barr virus-transformed lymphocyte cell line. In *B, inset b*, the filter was stripped and hybridized to ^{32}P-labeled c-*myc* exon 3 DNA.

[1] The abbreviation used is: DMs, double minute bodies.

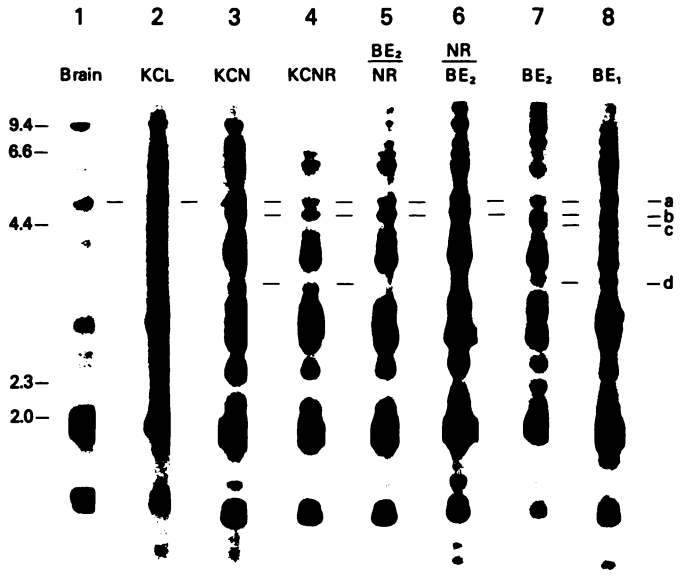

Fig. 2. In-gel renaturation analysis of neuroblastoma cellular DNA. In-gel renaturation was performed essentially as described by Roninson (20). Fifteen μg of *Hind*III restricted cellular DNA (driver) was mixed with 0.1–0.2 μg of restricted ^{32}P-end-labeled DNA (tracer DNA). In all *lanes* but *5* and *6*, tracer and driver DNAs are derived from the same tissue. *Lane 1*, normal adult brain; *lane 2*, SMS-KCL; *lane 3*, SMS-KCN; *lane 4*, SMS-KCNR; *lane 5*, tracer KCNR/driver BE(2); *lane 6*, tracer BE(2)/driver KCNR; *lane 7*, SK-N-BE(2); *lane 8*, SK-N-BE(1). Examples of bands with unique hybridization patterns are denoted *a–d* and discussed in text.

cultures are very similar to each other, suggesting extensive homology in the structure of their amplified DNA. This similarity in the genomes of BE(1) and BE(2), despite the apparent loss of DMs and acquisition of homogeneously staining regions, is important evidence supporting the possibility that these structures arise from one another (26) and is the first observation suggesting that this may occur *in vivo*. DNA bands observed in Fig. 2 may be considered to fall into several different classes. A few gel bands which comigrate in *lanes 3, 4, 7,* and *8* are also present in DNA from normal tissue (Fig. 2, *lane 1*) and an Epstein-Barr virus-transformed lymphocyte cell culture (Fig. 2, *lane 2*). An example of such a band is labeled *a* in Fig. 2. Although its identity is unknown, we have characterized other such bands as corresponding to mitochondrial DNA and DNA encoding the ribosomal RNA genes (data not shown).

However, most gel bands in lanes *3, 4, 7,* and *8* (Fig. 2) are present only in neuroblastoma tumor cell DNA. To confirm the DNA sequence similarity of comigrating bands in BE(2) and KCNR, we performed a "mixing" experiment in which a small amount of ^{32}P-labeled DNA (tracer) from one cell culture was added to an excess of unlabeled DNA (driver) from the other cell culture and evaluated by the in-gel renaturation technique. In such an experiment, detectable gel bands correspond to DNA sequences that are amplified in both cell cultures. The gel pattern observed when ^{32}P-labeled DNA from KCNR is mixed with unlabeled DNA from BE(2) is shown in *lane 5* of Fig. 2; a reciprocal analysis is shown in *lane 6* of Fig. 2. These cell cultures share many commonly amplified DNA sequences. Examples of bands with similar mobility which are not identical are labeled *d* (Fig. 2), and may represent amplified sequences peculiar to individual tumors. Another band, *c* (Fig. 2), is apparent only in the BE cell cultures (Fig. 2, *lanes 7* and *8*) and may also be a rearrangement unique to this tumor.

It is likely that many of the gel bands in *lanes 3, 4, 7,* and *8,* (Fig. 2) correspond to DNA sequences flanking N-*myc*; their amplification is of unknown physiological significance; however, we found at least one restriction fragment, band *b,* which was detectable in BE(2) (*lane 7*), but not in BE(1) (*lane 8*) and was also present in KCN (*lane 3*) and KCNR (*lane 4*) (all Fig. 2). Its presence in *lanes 5* and *6* (Fig. 2) confirms the identity of this band in BE(2) and KCNR. The identification of an amplified region of DNA in BE(2) that is also amplified in KCN and KCNR suggests that this region of DNA may include sequences important for tumor progression.

DISCUSSION

The results presented in this report demonstrate that N-*myc* expression is increased in cell lines derived from two patients whose tumors had undergone clinical progression. This is the first data correlating expression of an oncogene with this clinical parameter, and although limited by the small sample size, together with the association of N-*myc* with tumor stage, prognosis, and differentiation, is evidence for the close association of N-*myc* and aggressive clinical phenotypes in neuroblastoma. Our study also highlights the importance of measuring mRNA expression when correlating oncogene amplification with a biological phenotype, as we have shown that serial changes in DNA copy number are not necessarily directly proportional to changes in mRNA expression. To date, no studies examining the clinical implications of N-*myc* gene activation have examined expression.

Molecular analysis of human tumor tissue specimens is complicated by cellular heterogeneity of the tissues, areas of tissue necrosis after cytotoxic therapy, and difficulty in obtaining appropriate specimens for RNA isolation. It is difficult, therefore, to compare quantitive N-*myc* mRNA expression between different samples of tissue. For these reasons, we have performed experiments on cells grown in culture. We believe it unlikely that a significant change in the amplified DNA sequences of these cells has occurred since they were placed in culture because the N-*myc* content of neuroblastoma tumor cells as well as the pattern of DNA amplification observed by in-gel hybridization experiments is invariant over many cell generations. In particular, the N-*myc* DNA content of KCNR has not changed in over 100 cell generations in culture (data not shown) suggesting that our tissue culture conditions may not specifically select for altered N-*myc* expression.

At least three different patterns of clinical tumor evolution can be identified: (*a*) tumor relapse, in which the tumor is eradicated by therapy and then recurs months to years later; (*b*) cellular evolution, as in chronic myelogenous leukemia, in which disease that remains slowly growing or stable for many years abruptly begins to grow rapidly or spread widely; and (*c*) clinically progressive disease in which a tumor grows despite therapy. While little is known about the biological properties of tumor cells that mediate these phenomena in the setting of host resistance factors and therapy, it seems likely that these patterns of tumor evolution are caused by a variety of genetic mechanisms. For instance, one possible event leading to tumor relapse is the selection of variant cells with amplified or mutant chemotherapeutic drug resistance genes (27, 28). Mechanisms involved in clonal evolution or clinical progression of disease are more obscure, although drug resistance may play a role in these situations also. Blast crisis in a patient with chronic myelogenous leukemia was recently demonstrated to occur in association with the amplification of c-*myc* DNA in tumor cells (29).

We detected increased N-*myc* expression in cell lines isolated during progressive growth of tumor in patients with neuroblas-

toma. This increase in mRNA might be associated with an increase in the number of copies of the N-*myc* DNA as is seen in BE(2), or might be caused by alterations resulting in increased transcriptional efficiency of N-*myc*, as may be relevant in KCNR where the amount of N-*myc* RNA per DNA copy is approximately 30-fold greater than in KCN (Fig. 1). In the future, it will be interesting to examine cell cultures isolated from patients before and after clonal evolution or tumor relapse to determine whether N-*myc* is involved in these events as well.

Our identification of changes in the amplified DNA present at different points during the natural history of neuroblastoma (Figs. 1 and 2) provides important documentation of the genetic plasticity of this tumor. The generation of cell populations with genomic variation resulting in such changes as elevated levels of N-*myc* RNA might allow the selection of phenotypes important for tumor progression. In this regard, drugs which inhibit the expression of genetic variation by decreasing the rate of tumor growth or mutation may have an important role in the treatment of human cancer, independent of their cytocidal activity. Similarly, other therapeutic agents such as retinoic acid, which dramatically diminishes the expression of N-*myc* (12), may be important in altering the natural history of neuroblastoma, although such drugs alone may not be curative.

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