

# Amplification of the *c-myc* Gene in Human Medulloblastoma Cell Lines and Xenografts<sup>1</sup>

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## ABSTRACT

Cultured cell lines and xenografts derived from 7 human medulloblastomas were evaluated for amplification of the *c-myc*, *N-myc*, epidermal growth factor receptor, and *gli* genes by Southern blot analysis. Karyotypes of the original biopsies and early passaged cells demonstrated double minute chromosomes in 4 of the 7 cases. All 7 samples (3 cell lines and 4 xenografts) from the 4 tumors with double minute chromosomes contained amplification of the *c-myc* gene. Cell lines and xenografts derived from the 3 biopsies without double minute chromosomes failed to demonstrate amplification of the 4 genes which were tested, but a rearrangement of the *c-myc* gene occurred in 1 of the 3 tumors. These observations demonstrate that the *c-myc* gene is often amplified and/or rearranged in human medulloblastomas and suggest that amplification of this gene provides a growth advantage for medulloblastoma cells *in vitro* and *in vivo*.

## INTRODUCTION

The incidence of gene amplification and the spectrum of genes which are amplified varies markedly among human tumor types. Tumors of neurogenic origin, in particular, have a relatively high incidence of gene amplification. For example, 30–40% of malignant human gliomas contain amplification of the EGFR<sup>3</sup> gene, and the *N-myc* gene is amplified in approximately 40% of neuroblastomas (1–3). Gliomas often lose EGFR gene amplification during their establishment in culture (4). Glioma xenografts, however, maintain amplification of the EGFR gene during passage in athymic mice (5). Neuroblastomas present a different situation in that the majority of cultured neuroblastoma-derived cell lines contain amplification of the *N-myc* gene suggesting that *N-myc* gene amplification increases the probability that a neuroblastoma can be established *in vitro* (6).

In addition to providing insight into the pathogenesis of human tumors, the presence of gene amplification sometimes has useful clinical correlates. For example, patients whose neuroblastomas contain amplification of the *N-myc* gene tend to have a more advanced stage of tumor at diagnosis and more rapid clinical course than patients without this characteristic (3, 7); amplification of the EGFR gene in human gliomas is seen almost exclusively in high grade tumors (8); similarly *neu* gene amplification appears to preferentially occur in more aggressive forms of breast tumors (9).

Approximately 10–20% of human medulloblastomas contain DMs, thus providing cytogenetic evidence for gene amplification (10). Identification of the amplified gene, however, has been limited to one example of amplification of the *c-myc* gene

in the D-341 Med medulloblastoma cell line and 2 cases of amplification of the *N-myc* gene in biopsied cerebellar medulloblastomas, one of which contained gangliocytic differentiation (11, 12). In the present study, cultured cell lines and xenografts derived from 7 medulloblastomas were evaluated for amplification of the EGFR, *N-myc*, *c-myc* and *gli* genes. All xenografts and cell lines from the 4 tumors with DMs contained amplification of the *c-myc* gene; specimens without DMs failed to demonstrate amplification of the 4 genes which were tested. These observations suggest that amplification of the *c-myc* gene imparts a growth advantage to medulloblastoma cells *in vitro* and *in vivo*.

## MATERIALS AND METHODS

**Medulloblastoma Biopsies.** Freshly resected biopsies from 7 human medulloblastomas which were established *in vitro* or which grew progressively in athymic mice were subjected to chromosomal and molecular analyses. These tumors originated in 5 boys and 2 girls ranging from 17 months to 12 years of age (Table 1). D-283 Med-bx was obtained from a peritoneal metastasis of a cerebellar medulloblastoma while the remaining biopsies were from the initial resections of cerebellar medulloblastomas.

**Xenograft Establishment and Transplantation.** Male or female athymic BALB/*c-nu/nu* mice, 3–4 weeks of age, were given single cell suspensions of tumor cells prepared from the resected tumor tissue as above into the right cerebral hemisphere ( $1-3 \times 10^5$  cells/mouse) or s.c. into the right flank ( $1-5 \times 10^6$  cells/mouse). Resulting xenografts were serially transplanted into additional mice as described previously (13).

**Cell Lines.** Permanent cultured cell lines D-283 Med-C, D-341 Med-C, D-384 Med-C, and D-425 Med-C were derived at Duke Medical Center by mechanical dissociation of resected tumor tissue and plating into Richter's (14) improved minimal essential zinc option medium (Grand Island Biological Co., Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 584 mg glutamine/liter and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere (11, 14). D-283 Med-C and D-341 Med-C have been described in earlier communications (11, 15); the remaining 2 lines have not been reported previously. All 4 lines grow in suspension. The cell lines were tested (with negative results) every 10th passage for mycoplasma contamination by testing the ability of the conditioned medium to convert deoxyadenosine to adenine (in the presence of an inhibitor of adenosine deaminase) and thymidine to thymine (16).

**Chromosomal Analysis.** Biopsies and xenografts were aseptically dissected free of necrosis and normal tissue, were finely minced, dissociated by incubation for 12–24 h in collagenase, and plated in zinc option medium containing 10% fetal calf serum. Permanent cell lines were passaged and cultured until they achieved exponential growth. Cells in all types of preparations were arrested in mitosis by treatment with Colcemid, and G-banded chromosomal spreads were prepared (17).

**Analysis for Gene Amplification by Hybridization.** DNA from medulloblastoma biopsies was prepared from frozen blocks from which cryostat sections had been examined histologically. All blocks contained a high proportion of tumor cells with minimal stromal contamination. DNA was purified from biopsies, xenografts and frozen pellets of cultured cells using methods detailed previously (18). Five  $\mu$ g of DNA

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<sup>3</sup> The abbreviations used are: EGFR, epidermal growth factor receptor; DMs, double minute chromosomes; Bx, biopsy; C, cultured cell line, X, xenograft.

Table 1 Characteristics of medulloblastoma cell lines and xenografts

Case	Age/sex/ location	Karyotype <sup>a</sup>	Gene amplification/rearrangement <sup>b</sup>		
			Biopsy	Culture	Xenograft
D-283 Med	6 yr/M/Met <sup>c</sup>	47,XY,+11,i(17q),der(8)t(8;?) (q13;?),der(20)t(5;20)(q11;q13)	None	None	None
D-306 Med	12 yr/F/CB	46,XX,der(20)t(13;20)(q13;q13)	None	NA <sup>d</sup>	None
D-341 Med	3 yr/M/CB	49,XY,+6,+8,+18,-22,-del(1)(p13), i(17q),+DMs	c-myc	c-myc	c-myc
D-382 Med	4 yr/F/CB	52,X,-X,+4,+5,+6,+7,+7,+14,+18, i(17q)+DMs	None	NA	c-myc
D-384 Med	17 mo/M/CB	46,XY,-8,i(17q),+der(8)t(1;8) (p11;q24),+DMs	c-myc	c-myc	c-myc
D-386 Med	7 yr/M/CB	53,XY,(+),+4,+6,+8,+12,+17, +18,+20	None	NA	None
D-425 Med	5 yr/M/CB	46,XY,i(17q),del(10)(q22),+DMs	None	c-myc	c-myc

<sup>a</sup> Stemline karyotype of the original tumor biopsy in 24-, 72-, and 96-h cultured preparations for D-283 Med-Bx, 144 h in culture for D-306 Med-Bx, 10 *in vitro* passages for D-341 Med-Bx, 4 *in vitro* passages for D-382 Med-Bx, 18 *in vitro* passages for D-384 Med-Bx, 72 h in culture for D-386 Med-Bx, and 24 h in culture for D-425 Med-Bx.

<sup>b</sup> Samples were evaluated for amplification of the *c-myc*, *N-myc*, *EGFR*, and *GLI* gene by Southern blot analysis. Amplification was defined as >4-fold increase in gene copy number. Blots of cultured and xenograft samples were rehybridized with *N-myc*, *EGFR*, and *gli* gene probes.

<sup>c</sup> CB, cerebellum; Met, peritoneal metastasis; NA, not available.

was cleaved with *EcoRI*, separated by electrophoresis through a 1% agarose gel, blotted onto a nylon membrane, and hybridized with a *c-myc* probe labeled with [<sup>32</sup>P]dCTP via the random primer method. Washing of filters and autoradiography was as described before (18). The *c-myc* probe was a 1.6-kilobase *SstI* fragment of pHSR-1 containing the second exon of *c-myc* (19). Filters were rehybridized sequentially with 3 other probes: a 1.0-kilobase *EcoRI/BamHI* fragment of pNB-1 containing part of the second exon of the *N-myc* gene (20), a 1.6-kilobase *EcoRI* fragment of pE7, a complementary DNA clone of *EGFR* mRNA generously provided by Drs. G. Merlino and I. Pastan (21), and a 1.55-kilobase *PstI* insert of pKK 36P1, containing *gli* sequences (22). Greater than 4-fold increase in gene copy number was considered to represent gene amplification.

## RESULTS

**Chromosomal Analyses.** Karyotypes were obtained on short-term cultured preparations of original biopsies D-283Med-Bx, D-306Med-Bx, D-286Med-Bx, and D-425Med-Bx and on early passage cultured cells from D-341Med-Bx, D-382Med-Bx, and D-384Med-Bx. All 7 samples had abnormal stemlines including 5 near-diploid and 2 hyperdiploid (greater than 50 chromosomes) tumors (Table 1). The most frequent structural abnormalities were i(17q) in 5 tumors and DMs in 4 cases. D-382 Med and D425 Med contained 10–20 tiny DMs in 10 and 20–30% of cells, respectively. D-384 Med and D-341 Med showed numerous DMs in all cells.

Xenografts were established from tumors both with and without DMs. All xenografts were similar histologically, composed of sheets of small anaplastic cells similar to those seen in the original biopsies. In each case, a stemline karyotype closely related or identical to the one seen originally was maintained through *in vivo* passage. Serially transplantable D-283 Med-X, D-306 Med-X, D-341 Med-X, D-384 Med-X, and D-425 Med-X xenografts have been maintained. D-382 Med-X and D-386 Med-X are presently in the second *in vivo* passage.

D-283 Med-C, D-341 Med-C, D-384 Med-C, and D-425 Med-C were established *in vitro* from the corresponding biopsies. All 4 lines grew in suspension as populations of small anaplastic cells. Each line maintained the same karyotype as seen originally including the presence of numerous DMs in 3 of the 4 lines.

**Gene Amplification.** Biopsies D-341 Med-Bx and D-384 Med-Bx contained a 4–5-fold increase in *c-myc* gene copy number while the remaining biopsies including D-382 Med-Bx and D-425 Med-Bx did not show increased gene copy number (Fig. 1). A 15-kilobase fragment in addition to the normal 13-

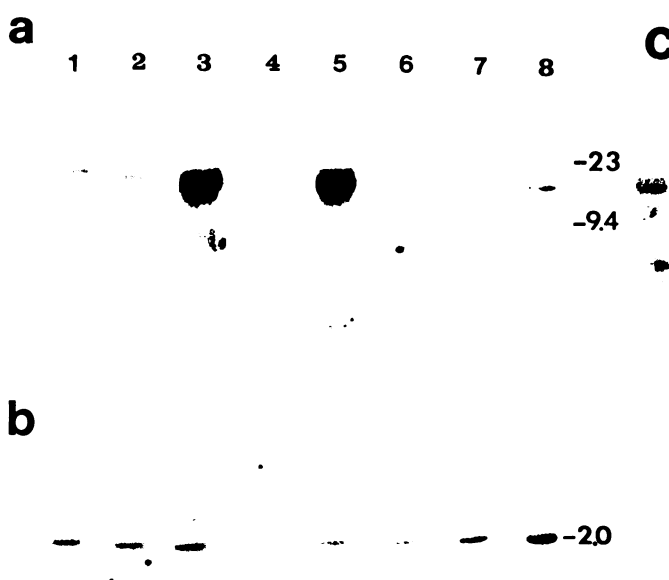


Fig. 1. In a, 5  $\mu$ g DNA from the medulloblastoma-derived cell lines and xenografts were cleaved with *EcoRI*, separated on a 1% agarose gel by electrophoresis, transferred to a nylon membrane, and hybridized with the *c-myc* probe. Samples from D-341 Med-C (Lane 4), D-341 Med-X (Lane 5), D-382 Med-X (Lane 6), D-384 Med-C (Lane 7), D-384 Med-X (Lane 8), D-425 Med-C (Lane 10), and D-425 Med-X (Lane 11) showed amplification and rearrangement of the *c-myc* gene, while increased copy numbers were not seen in D-283-C (Lane 1), D-283 Med-X (Lane 2), D-306-X (Lane 3), and D-386-X (Lane 9). Lane 12 contains 5  $\mu$ g of DNA from normal human lymphocytes. Both D-283 Med-C and D-283 Med-X showed a more slowly migrating band in addition to the normal 13-kilobase band. In b, the filter shown in a was rehybridized with a probe for the *N-myc* gene and showed that no sample contained increased copy numbers or rearrangements of this gene.

kilobase form was present in D-283 Med-Bx, suggesting that the gene was rearranged but not amplified (see below).

D-341 Med-C, D-341 Med-X, D-382 Med-X, D-384 Med-C, D-384 Med-X, D-425 Med-C and D-425 Med-X contained 10–30 copies of the *c-myc* gene (Fig. 2a). Additional and usually lower molecular weight bands were seen in all samples with gene amplification. Additional bands were also seen when *HindIII* or *PstI* was used to digest the samples. No bands were seen when the blots were rehybridized with vector sequences alone (data not shown). D-283 Med-C did not show an increased gene copy number. D-283 Med-X, however, showed a slightly elevated copy number of the *c-myc* gene in comparison to the other genes tested. We did not consider this to be definitive evidence for gene amplification. For both D-283 Med-C and D-283 Med-X there was a more slowly migrating band

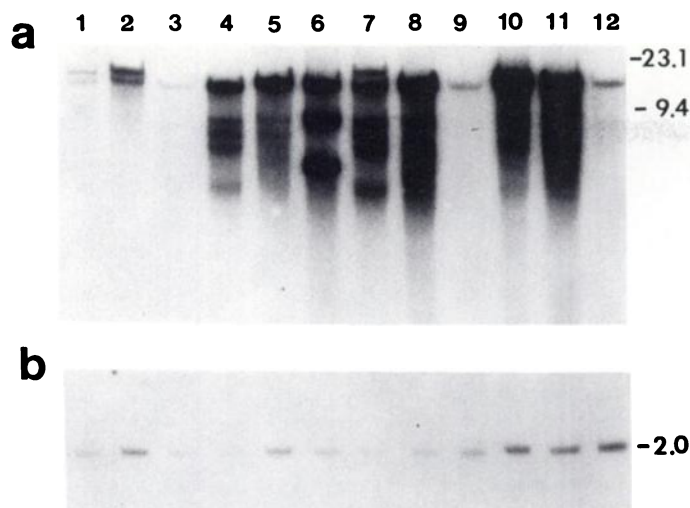


Fig. 2. In *a*, 5  $\mu$ g of DNA from the 7 original medulloblastoma biopsies was hybridized with the *c-myc* probe as described in Fig. 1*a*. D-341 Med-Bx (Lane 5) and D-384 Med-Bx (Lane 8) contained amplification of the *c-myc* gene, while D-283 Med-Bx (Lanes 1 and 2), D-306 Med-Bx (Lanes 3 and 4), D-382 Med-Bx (Lanes 6 and 7), D-386 Med-Bx (Lanes 9 and 10), D-425 Med-Bx (Lane 11), and normal human lymphocyte DNA (Lane 12) showed equivalent gene copy numbers. A slowly migrating band similar to the one seen in D-283 Med-C and D-283 Med-X (Fig. 1*a*) was also seen in the D-283 Med-Bx. In *b*, the filter shown in *a* was hybridized with the *N-myc* probe showing equivalent gene copy numbers in all samples.

similar to the one seen in D-283 Med-Bx. New (low molecular weight) bands were also observed when *Hind*III or *Pst*I was used instead of *Eco*RI (data not shown). The remaining samples did not show increased gene copy number or rearrangements. Rehybridization of these filters with probes for the *N-myc* (Fig. 2*b*), *EGFR*, and *gli* genes showed hybridization intensities which were no higher than those observed with DNA from normal lymphocytes (lane 12) and no rearrangements were detected.

## DISCUSSION

The *c-myc* gene is expressed in a variety of normal adult and fetal tissues as well as in many neoplastic tissues. In Burkitt's lymphoma, this gene is activated by translocations which bring it into the vicinity of immunoglobulin gene loci (23). There are, in addition, sporadic reports of activation of this gene by amplification in breast carcinomas, in small cell lung carcinomas and cell lines established from them, and in the HL60 acute promyelocytic leukemia cell line, the SF-188 cell line derived from a cerebral glioblastoma in an 8-year-old boy, COLO 320 derived from a neuroendocrine carcinoma of the colon, and the D-341 Med medulloblastoma cell line (11, 19, 24–27). The present demonstration of amplification of the *c-myc* gene in cell lines and xenografts derived from 3 additional medulloblastomas establishes that this gene is frequently amplified in this tumor type. Combining this series with cases reported previously (10), approximately 20% of medulloblastomas contain DMs and all cell lines, and xenografts derived from DM-bearing tumors contained amplification of the *c-myc* gene. Since these cases were selected for evaluation based on availability of sufficient tissue for karyotypic analysis, this series may be biased in favor of the larger, more rapidly growing tumors. Nevertheless, this study taken together with the 2 medulloblastomas with *N-myc* gene amplification reported by Rouah *et al.* (12) demonstrates that members of the *myc* gene family are amplified in a substantial proportion of medulloblastomas.

Amplification of *c-myc* gene was demonstrated in 2 of the 4 biopsies from which the cell lines and xenografts with amplification of this gene were derived. In the remaining 2 cases (D-382 Med-Bx and D-425 Med-Bx) increased copy numbers of the *c-myc* gene were not seen. There are several potential explanations for this observation. The possibility that DNA samples from these 2 tumors contained nonneoplastic tissue rather than representing solid tumor tissue was largely excluded by histologic examination of the same blocks. The demonstration of unique karyotypes for the cell lines and xenografts derived from the individual biopsies excluded cross-contamination. Thus, the most likely explanations are that (a) only a small proportion of cells in the D-382 Med-Bx and D-425 Med-Bx samples contained amplification of the *c-myc* gene, and the cells had a selective growth advantage *in vitro*; (b) gene amplification occurred in the majority of neoplastic cells within the biopsy tissue but at a relatively low level; *in vitro* culture selected for gradually increasing levels of *c-myc* gene amplification, just as methotrexate can select for cells with gradually increasing levels of *DHFR* gene amplification (28); and (c) amplification did not exist at all in the biopsy tissue but was an *in vitro* phenomenon. We believe that the first of these three explanations is more likely, since DMs were observed in the earliest karyotypes made from both D-382 Med-Bx and D-425 Med-Bx and the proportion of metaphase spreads with DMs was much lower in these initial karyotypes than in those generated from the cell lines. Application of *in situ* hybridization or immunohistochemical assays with *myc*-specific probes or antibodies could possibly answer this question in the future. In either case, our observations suggest that the amplification of the *c-myc* gene provides a selective advantage for medulloblastoma cells when propagated *in vitro* or through serial transplantation in athymic mice.

Another difference between the behavior of the *c-myc* gene in medulloblastoma biopsies and cells propagated *in vitro* and *in vivo* from the corresponding tumors is that abnormally migrating amplified bands were seen in D-341 Med C and X and D-384 Med-C and X while the corresponding biopsies contained *c-myc* gene amplification without rearrangement. The possibility that the abnormal bands were due to cleavage at *Eco*RI\* sites was excluded by the demonstration of abnormally migrating bands following *Hind*III and *Pst*I digestion. One explanation for this observation is that cells with rearrangements of the amplified *c-myc* gene may represent only a minor proportion of cells in the original tumors but these populations may be selected through propagation *in vitro* and in athymic mice. Alternatively, the gene alterations may have been acquired during progressive rounds of amplification in their new environments.

Other neurogenic tumors which often contain amplified genes include malignant adult gliomas, in which approximately 1 of 3 of the tumors contain amplification of the *EGFR* gene, and neuroblastomas, in which about 40% of primary tumors contain amplification of the *N-myc* gene (1–3). It is interesting that the only reported case of *c-myc* gene amplification in a glioma occurred in a child, raising the possibility that amplification of this gene may be associated with childhood malignant brain tumors in general rather than being associated with medulloblastoma alone (27).

All medulloblastoma-derived cell lines established in our laboratory grow in suspension as do the majority of neuroblastoma cell lines. Three of the 4 medulloblastoma lines contain amplification of the *c-myc* gene and this gene is rearranged, although not amplified, in the remaining cell line. These find-

ings suggest that alterations in this gene may be necessary for medulloblastomas to establish in culture, just as *N-myc* gene amplification appears to increase the efficiency of neuroblastoma growth *in vitro*.

This demonstration that patients whose neuroblastomas contain amplification of the *N-myc* gene have later stage and more rapidly progressive tumors than patients without this characteristic (3,17) suggests that amplification of the *myc* genes can alter the growth properties of neuroectodermal cells *in vivo* as well as *in vitro*. Examination of a large series of patients with medulloblastoma for the presence of *c-myc* or *N-myc* gene amplification will be necessary to determine whether a similar relationship exists for this tumor type.

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