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Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification

(oncogenes/*in situ* hybridization/chromosomes)

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ABSTRACT **Primary malignant gliomas from 63 patients were analyzed to determine the relationship between amplification of the gene encoding the epidermal growth factor receptor (EGFR) and expression of the corresponding mRNA. Twenty-four tumors were found to have amplified the *EGFR* gene and amplification of other genes occurred in three additional tumors. Hybridization with synthetic RNA probes was used to quantitate mRNA levels *in situ*. All 24 tumors with amplification of the *EGFR* gene had high levels of expression of this gene, while none of the 39 tumors without amplification had increased levels. This shows that, in human gliomas, large increases in the expression of the *EGFR* gene are invariably associated with alterations in gene structure.**

In vitro experiments have shown that greatly increased expression of some protooncogenes can lead to neoplastic transformation (1–3). In naturally occurring tumors, increases in gene expression have been postulated to occur through two kinds of mechanism (4–6). One class of mechanism involves structural changes within or surrounding the expressed gene, either through DNA amplification (7, 8) or rearrangement (9). The other class of mechanism includes changes in DNA-binding proteins (10) or DNA methylation (11) in the absence of structural alterations of the expressed gene. In several human tumors, increased expression of protooncogenes apparently takes place in the absence of genetic changes at the protooncogene locus (see, e.g., refs. 12–15) and these increases have been suggested to play an active role in tumor formation. The presence of genomic alterations of a protooncogene in a tumor provides strong evidence for involvement of the protooncogene in formation of the tumor. However, in the absence of such structural alterations, it is difficult to know whether increased expression of a protooncogene is *causally* related to the tumorigenic process or simply *reflects* the abnormal growth status or unusual microenvironment present in tumors.

The epidermal growth factor receptor (EGFR) is a protooncogene that has been extensively studied (reviewed in ref. 16); it represents the cellular homologue of the viral oncogene *erbB* (17). Malignant gliomas, which often amplify and express the gene for the EGFR (18), provide an attractive system to evaluate the mechanisms underlying increased protooncogene expression in tumors. Through examination of a large number of tumors with *in situ* techniques, we have found that large increases in expression of this protooncogene occur if and only if the *EGFR* gene itself is altered.

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METHODS

Selection of Tumor Samples and Preparation of DNA. The tumor samples used were either embedded in paraffin blocks or freshly frozen biopsy samples. Areas of tumor were selected that had little stromal infiltration or inflammation. DNA was purified by the method of Goelz *et al.* (19) and quantitated by a diphenylamine assay.

Analysis of Amplification by Hybridization. For DNA extracted from paraffin-embedded sections, 2 μg samples were slot blotted onto a nitrocellulose membrane using a Minifold II apparatus (Schleicher & Schuell) (20). For DNA purified from frozen blocks of tissue, 1.5- to 4-μg samples were cleaved with *Eco*RI, separated by electrophoresis through a 1% agarose gel, and blotted on nitrocellulose. Prehybridization, hybridization, and washing conditions were as described (19). The EGFR probe used was the 1.6-kilobase *Eco*RI fragment of pE7 (21), a cDNA clone of EGFR mRNA generously provided by G. Merlino and I. Pastan (National Institutes of Health). Filters were rehybridized sequentially with three other probes: a 1.0-kb *Eco*RI/*Bam*HI fragment of pNB-1 (22), containing part of the second exon of the N-*myc* (human, *NMYC*) gene; a 1.6-kb *Sst* I fragment of pHSR-1, containing the second exon of c-*myc* (human, *MYC*) (23); a 1.55-kb *Pst* I insert of pKK36P1, containing *gli* sequences from chromosome 12 (24); and a 5.0-kb *Eco*RI insert of pAW101, containing sequences from chromosome 14 (25).

***In Situ* Hybridization.** Tissue sections of 6 μm thickness were cut from paraffin blocks and baked at 60°C for 1 hr on gelatin-subbed slides. The protocol used was adapted from Cox *et al.* (26). Sections were soaked in 2× standard saline citrate (SSC; 1× = 0.15 M sodium chloride/0.015 M sodium citrate/1 mM Tris Cl, pH 7.5) for 20 min, digested with proteinase K (3 μg/ml, predigested for 30 min at 37°C; Bethesda Research Laboratories) in 2 mM $CaCl_2$/20 mM Tris Cl, pH 7.5, and then acetylated using 0.025% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature. The slides were washed with 2× SSC, and hybridization was carried out in 45% formamide/10% dextran/2× SSC/10 mM dithiothreitol containing yeast RNA at 1 mg/ml, sheared salmon sperm DNA at 1 mg/ml, bovine serum albumin at 2 mg/ml, and radioactive probe at a final concentration of 10^5 dpm/μl. After overnight hybridization at 50°C, the slides were washed with three changes of 50% formamide/2× SSC for 1 hr at 54°C, then rinsed with 2× SSC at room temperature and treated with RNaseA (8 μg/ml; Sigma) in 2× SSC at 37°C for 0.5 hr. Slides were soaked for an additional hour in 50% formamide/2× SSC at 50°C, rinsed with 2× SSC, dehydrated in graded ethanol, and autoradio-

Abbreviation: EGFR, epidermal growth factor receptor.

Table 1. Clinical characteristics of glioma patients

	Tumors with amplification (n = 27)	Tumors without amplification (n = 36)
Patient age (mean ± SEM), yr	55.5 ± 2.1	53.0 ± 3.4
Male sex, %	56	53
Gene amplified		
EGFR	24 (89)	NA
gli	1 (4)	NA
N-*myc*	2 (7)	NA
Tumor classification		
Glioblastoma multiforme	23 (86)*	24 (67)
Anaplastic astrocytoma	2 (7)†	4 (11)
Gliosarcoma	2 (7)†	3 (8)
Oligodendroglioma	0	1 (3)
Anaplastic mixed glioma	0	3 (8)
Giant cell glioblastoma	0	1 (3)

NA, not applicable. Numbers in parentheses represent % total.
**EGFR* amplified in 20, *gli* in 1, and N-*myc* in 2.
†*EGFR* amplified in both.

graphed. For rRNA hybridization, slides were treated similarly except that dextran was omitted from the hybridization solution, and hybridization was for 5 hr.

The probes used for *in situ* hybridization were a 1.6-kb *Eco*RI fragment from pE7 (EGFR cDNA probe; ref. 21) and a 1.3-kb *Bam*HI fragment from pA4, which contains a genomic fragment of the 28S ribosomal gene (ref. 27; kindly provided by R. Schmickel, University of Pennsylvania). Fragments were subcloned into pGEM3 (Promega Biotec, Madison, WI) by standard methods. ^{32}P-labeled probes were generated using either SP6 or T7 RNA polymerase and [^{32}P]CTP (811 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) according to conditions specified by the manufacturer. Specific activities for the EGFR sense and antisense probes were 6×10^8 dpm/μg; the specific activity of the rRNA probe was 7.3×10^7 dpm/μg. ^{3}H-labeled probes were prepared as above except that the probes were labeled in the presence of [^{3}H]CTP and [^{3}H]UTP to a specific activity of 9.7×10^7 dpm/μg.

RESULTS

Tumors from 63 patients with malignant gliomas were studied. The age, sex, and histologic diagnosis of the patients are summarized in Table 1. These tumors usually arise from astrocytes and are the most common neoplasms of the central nervous system (28). For 32 of the patients, DNA was purified from paraffin-embedded tumor samples (19, 20) and analyzed by slot-blot analysis using a probe for the *EGFR* gene (21). *EGFR* amplification (more than eight copies of the *EGFR* gene per cell) was evident in 10 of the 32 tumors (examples in Fig. 1*A*). Rehybridization of filters to probes representing sequences from different chromosomes was used as a technical control to quantitate the hybridization signals (Fig. 1*B*). For an additional 31 patients, DNA was prepared from freshly frozen tumor tissue. For these tumors, DNA amplification was assessed by Southern blotting of DNA digested with the restriction endonuclease *Eco*RI. Examples of the results of hybridization with the EGFR cDNA probe are shown in Fig. 1*C*. Fragments of 8.0, 6.8, 5.8, 2.5, 2.0, 1.8, 1.5, and 1.2 kb were identified, in agreement with previous results using this probe (29). Of the 31 tumor specimens examined by this technique, 14 showed significant amplification (greater than eight copies of the *EGFR* gene per cell). Quantitation was assessed, as in the case of the slot-blot results, by rehybridization of the filters to control probes (Fig. 1*D*). Interestingly, 6 of the 14 tumors exhibiting gene amplification in this assay also exhibited rearrangements of the gene as shown by the absence of normal bands or the presence of abnormally migrating bands (examples in Fig. 1*C*). Rearrangements are commonly found in gene amplification units (7, 8), including those containing the *EGFR* gene (18, 29–31). In six cases where different biopsies from the same patient were studied (see Table 1), the amplification was either present or absent concordantly in each lesion. In 16 tumors, we noted small increases in number of the *EGFR* gene (three to six copies per cell); such small increases are usually due to chromosome duplication rather than true gene amplification (see ref. 20).

Incidentally, during the course of this study we found that the N-*myc* gene was amplified in two of the 63 tumors and the

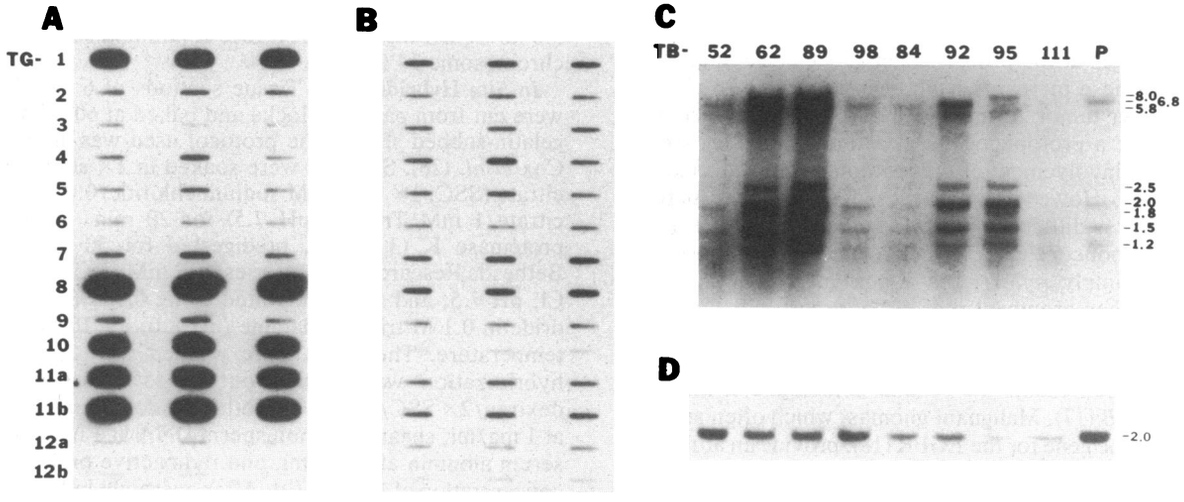

FIG. 1. Amplification of the *EGFR* gene in malignant gliomas. (*A*) DNA extracted from paraffin-embedded tissue samples was slot blotted onto a nitrocellulose membrane and hybridized with an EGFR cDNA probe. Samples TG-1, -8, -10, and -11 show amplification of the *EGFR* gene. Samples TG-11a and -b and samples TG-12a and -b were derived from different portions of the tumors of two patients. (*B*) The filter in *A* was rehybridized with an N-*myc* probe. (*C*) DNA extracted from frozen tumor specimens was cleaved with the endonuclease *Eco*RI, separated by electrophoresis, transferred to a nitrocellulose membrane, and hybridized with the *EGFR* probe. The amount of DNA loaded in each well varied due to availability of the DNA. Tumors TB-62, -89, -92, and -95 show amplification of the *EGFR* gene. Sample TB-92 also shows a deletion of the 1.8-kb *EGFR* gene fragment. Sample TB-95 shows a deletion of the 6.8- and 5.8-kb fragments and a rearrangement producing a 6.2-kb fragment. The numbers to the right are the sizes (kb) of the *Eco*RI fragments from normal DNA samples that hybridize with this probe. Lane P: DNA from a normal placenta. (*D*) The filter in *C* was hybridized with an N-*myc* probe.

gli gene (24) was amplified in one tumor (Table 1). The c-*myc* gene was not amplified in any of the 63 tumors, although c-*myc* amplification has been noted in one previously studied glioma cell line (32).

We measured expression of the *EGFR* mRNA using *in situ* hybridization with synthetic RNA probes (26). Examples of the results obtained with the tumors containing amplified *EGFR* genes are shown in Fig. 2*A*. All 24 tumors with *EGFR* amplification exhibited significant degrees of hybridization and the hybridization patterns paralleled the distribution of tumor cells within the sections. For example, sections of tumors TB-48 and TG-29 were composed of large areas of tumor tissue adjacent to equally large areas of normal brain or inflammatory tissue; the *EGFR* probe hybridized to the tumor tissue but not to the normal tissue. Sections from tumors TB-8, TG-1, TB-95, and TG-27 were composed of clusters of tumor cells enmeshed within normal brain or necrotic tissue. Cluster sizes varied considerably, but all clusters hybridized efficiently to the *EGFR* probe, even those containing as few as 20 cells.

Several controls showed that the hybridization was specific for the *EGFR* mRNA. First, adjacent normal brain did not hybridize to any significant extent (see tumors TB-48 and TG-29 in Fig. 2*A*). Second, when an adjacent section was hybridized under the same conditions with an *EGFR* sense (coding strand) probe, no hybridization was seen in tumor tissue, indicating that no nonspecific binding to RNA or DNA occurred under these conditions (data not shown). Finally, a probe for ribosomal RNA was used to show that the differences between normal and tumor tissue did not simply reflect increased cellular density (Fig. 2).

The level of hybridization to tumor tissues showing amplification was at least 10 times the level of hybridization found in normal brain tissue with the antisense *EGFR* probe or in tumor tissue with the sense EGFR probe, as determined by various exposure times of the autoradiographs. This hybridization was striking when compared to the samples from tumors without gene amplification—none of the 39 tumors without *EGFR* gene amplification exhibited hybridization signals higher than the background level of normal brain tissue (examples in Fig. 2*B*). rRNA hybridization, the control for RNA integrity and total RNA content, was similar in tumors with and without amplification (Fig. 2).

The *in situ* experiments described above were performed with a ^{32}P-labeled probe. To determine the distribution of mRNA at a finer level, a ^{3}H-labeled probe was used. The overall pattern of labeling matched that found with the ^{32}P-labeled probe. The distribution of grains was exclusively over tumor tissue and only background labeling was found for normal brain tissue (Fig. 3). Background labeling with the *EGFR* sense probe to five specimens of tumor or normal tissue averaged 2.3 ± 0.6 grains per cell. Hybridization of the *EGFR* antisense probe to normal brain or to tumors without *EGFR* amplification was no higher than that with the sense probe. In contrast, average hybridization signals were 34 ± 8 grains per cell in tumors with *EGFR* amplification. A small, but significant, proportion of cells (5–15%) from tumors with *EGFR* amplification had only background labeling. Since

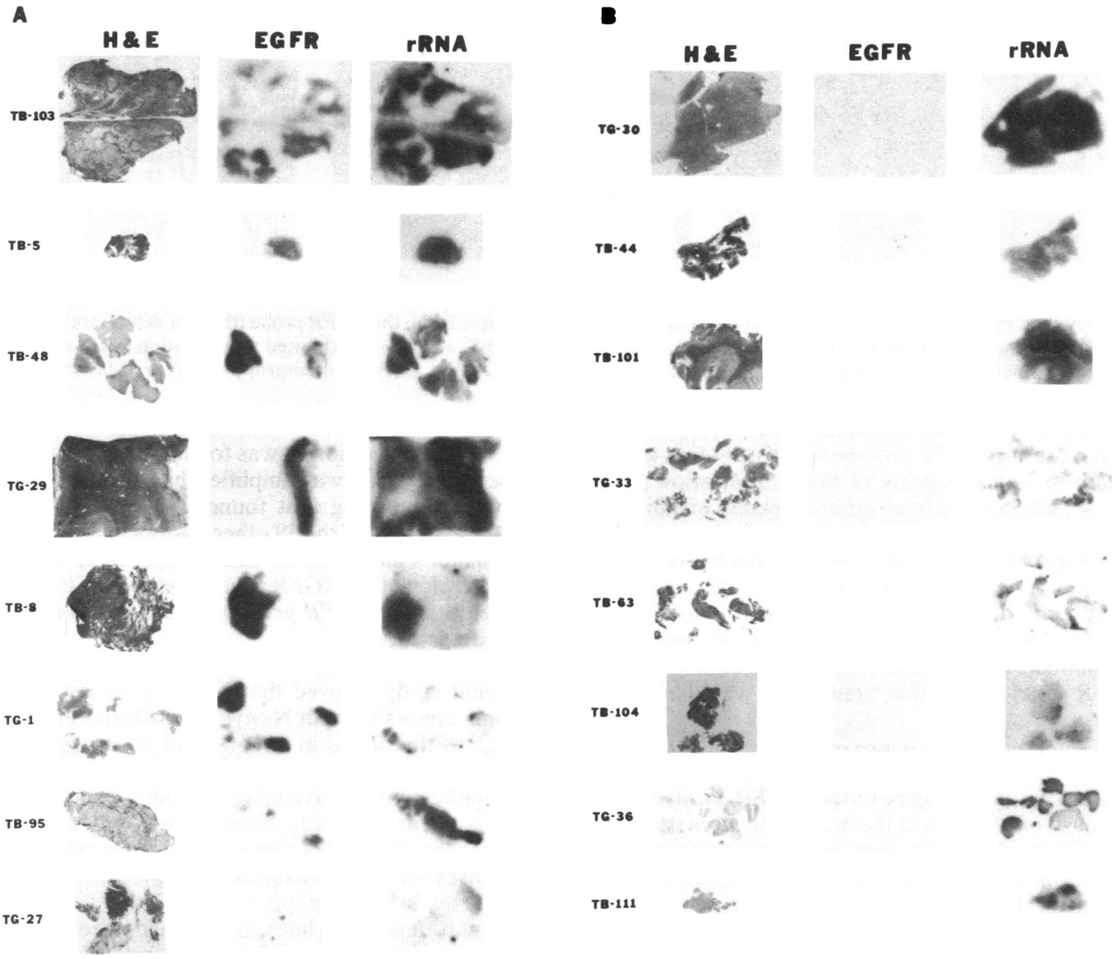

FIG. 2. Distribution of *EGFR* mRNA in tumor samples visualized by *in situ* hybridization. Adjacent tissue sections were stained with hematoxylin and eosin (H & E), or hybridized with a ^{32}P-labeled probe to the *EGFR* mRNA (EGFR), or hybridized with a ^{32}P-labeled probe to the 28S ribosomal subunit (rRNA). (*A*) Tumors with amplification of the *EGFR* gene. (*B*) Tumors without amplification.

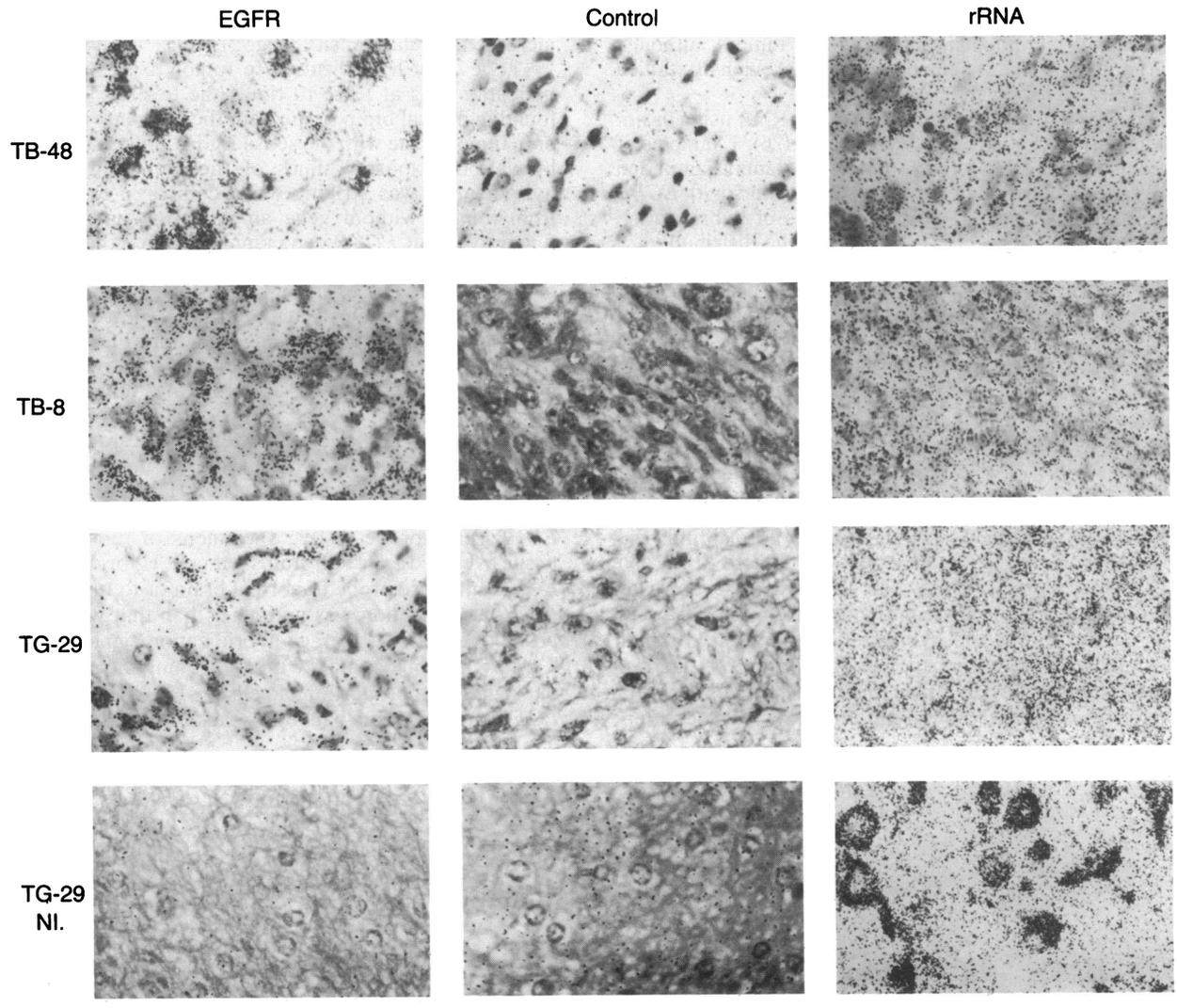

FIG. 3. Cellular distribution of the *EGFR* mRNA. Tumor sections were hybridized with ^{3}H-labeled RNA probes, washed and coated with NTB-2 emulsion, and stained with hematoxylin and eosin. Sections were hybridized with the *EGFR* probe (the anti-sense strand), a control probe (the *EGFR* sense strand), or the rRNA probe as indicated. Tumors TB-48, TB-8, and TG-29 exhibited amplification of the *EGFR* gene. TG-29 NI., sections of normal brain from sample TG-29. Exposures were for 28 days (the *EGFR* and control probes) or 7 days (the rRNA probe).

hybridization with the rRNA probe also showed a somewhat nonuniform pattern (Fig. 3), this uneven distribution may be partly due to technical aspects of the hybridization procedure, such as sectioning through different planes of individual cells. However, the uneven distribution of *EGFR* mRNA may also be partly due to the unstable nature of double minute chromosomes, which are the karyotypic manifestation of gene amplification (7, 8). Since such chromosomes segregate randomly, some tumor cells would be expected to have high numbers of them (with consequent high expression of the *EGFR* gene) while other tumor cells would have few.

DISCUSSION

Amplification of a specific gene (either *EGFR*, N-*myc*, or *gli*) was noted in 27 of 63 (43%) of the malignant gliomas studied. This finding is in close agreement with cytogenetic studies showing that approximately 50% of malignant gliomas exhibit double minute chromosomes (33, 34). The *EGFR* gene was amplified in 24 of 63 tumors, confirming and extending the data of Libermann *et al.*, (18) who found that *EGFR* amplification occurred in 4 of 10 malignant gliomas examined.

One of the primary objectives of this study was to determine whether increased EGFR expression occurred in the absence of detectable structural alterations of its gene. Using *in situ* hybridization, it was found that all 24 tumors in which the EGFR gene was amplified had levels of *EGFR* mRNA greatly exceeding that found in normal brain. In marked contrast, none of the 39 other tumors had comparable levels. A major conclusion of this study, then, is that increased expression of the *EGFR* gene is found only in tumors in which alterations of *EGFR* gene structure (i.e., amplification) can be detected.

Does this observation apply to other tumors as well? A recent study showed that N-*myc* expression in retinoblastoma tumors without N-*myc* gene alterations is not nearly as high as that found in tumors with N-*myc* gene amplification (35). Although only one primary retinoblastoma tumor with amplification was available for comparison (35), this result is in accord with our observations on a much different protooncogene and tumor type. Conversely, apparent increases in protooncogene expression in the absence of changes in protooncogene DNA have been noted in some human tumor cell lines and primary neoplasms (12–15, 36, 37). In many cases, no normal cell of the same type or proliferative potential was available for study, so the significance of the increased expression was difficult to interpret. Indeed, recent reports suggest that the enhanced levels of

myc RNA in tumors without alterations of the *myc* gene may reflect the relatively increased expression of *myc* protooncogenes in stem cells or embryonal tissues (35, 38). In such tumors, it is notable that the expression of genes with no transforming potential, such as histone genes, is often increased to a similar degree as that found for protooncogenes, further complicating analysis (39).

Finally, one must ask why mechanisms other than those involving alterations of *EGFR* gene structure do not increase *EGFR* gene expression to high levels in malignant gliomas. Such mechanisms are clearly involved in controlling gene expression during normal cellular differentiation. However, there are only a few genes whose expression, if very high, can lead to neoplastic transformation (1–3). Expression of these genes may be tightly regulated by the organism specifically to preclude the very high levels of expression that might lead to lethal effects through tumorigenesis. Thus, mechanisms not involving structural alterations would account for control of the moderate levels of protooncogene expression found in both normal and neoplastic cells, but only genetic changes directly affecting the protooncogenes themselves would lead to the high levels required for tumorigenesis.

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