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Selection Pressures of *TP53* Mutation and Microenvironmental Location Influence *Epidermal Growth Factor Receptor* Gene Amplification in Human Glioblastomas

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ABSTRACT

Epidermal growth factor receptor (*EGFR*) gene amplification occurs in glioblastomas as so-called double minutes. Because double minutes are extrachromosomal fragments, selection pressures must operate to maintain high *EGFR* copy number over multiple cell divisions. In analyses of glioblastoma lysates, *EGFR* amplification has been observed almost exclusively in glioblastomas harboring wild-type *TP53* genes, which raises the alternative hypotheses that *TP53* mutation either prevents amplification or selects against maintenance of *EGFR*-amplified cells. To address these possibilities at the cellular level, we studied 14 glioblastomas for *TP53* mutation and *EGFR* gene amplification status, using fluorescence *in situ* hybridization (FISH) for the latter. Remarkably, four of the six cases with *TP53* mutation had isolated *EGFR*-amplified cells in different regions, demonstrating that *EGFR* amplification occurs frequently at the cellular level in *TP53*-mutant glioblastomas. Thus, *TP53* mutation does not prevent *EGFR* amplification but does not facilitate selection of *EGFR*-amplified cells. Of the eight cases without *TP53* mutation, five had widespread *EGFR* amplification. In four of these five cases, multiple regions of the tumor were available for examination; FISH demonstrated a gradation of *EGFR* amplification, with highly amplified cells, primarily at the invading edges rather than the relatively solid tumor centers, suggesting that EGFR overexpression, when selected for *in vivo*, may be related to tumor invasion.

INTRODUCTION

Clinicopathological and genetic data suggest that glioblastoma may encompass a variety of different biological entities (1). Many decades ago, Scherer divided glioblastomas into primary and secondary tumors, based on histological and clinical features, with primary glioblastomas occurring in a clinically *de novo* fashion and secondary glioblastomas arising from pre-existing, lower-grade astrocytomas (2). In the last decade, molecular genetic investigations have revealed that specific genetic alterations occur preferentially in some types of glioblastomas: amplification of the *EGFR*[2] gene in primary and small cell glioblastomas and mutations of the *TP53* gene in secondary and giant cell glioblastomas (3–5). Such findings argue that tumor genotype is a powerful determinant of tumor phenotype, and are supported by recent findings demonstrating that common genetic alterations dictate phenotype more strongly than tumor cell of origin (6).

The two hallmark genetic events that seem to demarcate clinicopathological subtypes of glioblastoma, *EGFR* gene amplification and *TP53* mutation, have been considered almost mutually exclusive. On the basis of a series of Southern blot and PCR studies of glioblastoma, the co-occurrence of *EGFR* gene amplification and *TP53* mutation appeared to be extremely rare (4, 7). At a biological level, this near exclusivity has not been explained, and coexpression of aberrant p53 and EGFR has been observed in other human tumors, typically in higher-grade carcinomas (8, 9). During the multistep tumorigenesis of *TP53*-mutant glioblastomas, it is not known whether amplification of the *EGFR* gene does not take place, or if *EGFR* amplification occurs but is not selected for in the presence of *TP53*-mutant cells.

EGFR gene amplification occurs in glioblastomas as extrachromosomal, double minute fragments that, to maintain consistently high copy numbers, must be repeatedly selected for during cell division. Evidence for such selection pressure is found when *EGFR*-amplified glioblastomas are passaged *in vitro*, because these cells rapidly lose their additional, extrachromosomal copies of *EGFR* (10). This indicates that the local environment is necessary for the maintained selection of *EGFR* amplification and raises the possibility that the heterogeneous intratumoral environment may affect regional variability of *EGFR* copy number even within a single glioblastoma.

The evaluation of intratumoral selection of *EGFR*-amplified cells requires *in situ* assessment of copy number relative to overall tumor genotype and to intratumoral location. To determine whether *EFGR* amplification occurs on an individual cellular basis in glioblastomas without frank *EGFR* gene amplification, we, therefore, investigated *EGFR* copy number using FISH in both *TP53*-normal and *TP53*-mutated glioblastomas. In addition, to evaluate whether *EGFR* gene amplification was more likely to occur in particular regions of glioblastomas, we examined the relative location and distribution of *EGFR*-amplified cells within individual tumors.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded surgical specimens of 14 archival glioblastomas were obtained from Massachusetts General Hospital, Boston, MA, after appropriate human studies approval. Cases were selected to favor larger resections that had areas of relatively solid tumor, which presumably represented the central regions of the neoplasm, as well as regions of less cellular cortical or white matter infiltration, which most likely represented the invasive edge of the lesion. For *TP53* mutation analysis, tumor DNA was extracted from microdissected sections. Exons 5–8 of the *TP53* gene were screened for mutation by single-strand conformation analysis and direct sequencing (11). For evaluation of *EGFR* gene status, dual-color FISH on paraffin sections was performed (12). To detect amplification, BAC 343B1 (*EGFR*, 7p12; Ref. 13) and RP11–170H15 (7q11.22; Research Genetics, www.resgen.com) were made into an *EGFR* probe and control probe, respectively, using the Nick Translation kit, SpectrumOrange and SpectrumGreen (Vysis, Downers Grove, IL). RP11–170H15 is a pericentromeric BAC clone, confirmed by both PCR and FISH.[3] FISH slides and corresponding H&E stained slides were evaluated in comparison. The entire area of each slide was examined, and the numbers of signals were counted in 100 cells for each available region (*i.e.*, highly cellular tumor center *versus* less cellular, invading tumor edge in cerebral cortex). The *EGFR* gene was scored as amplified in individual cells when the *EGFR*:control signal ratio was greater than 3 (14). A case was not considered as demonstrating general *EGFR* gene amplification, however, in the presence of only scattered cells with *EGFR* gene amplification (see "Results" and Table 1).

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[2] The abbreviations used are: EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization.

[3] Internet address: http://genomics.roswellpark.org/human/overview.html.

RESULTS

The data are catalogued in Table 1. Six of the 14 cases had *TP53* mutations. Of the eight cases lacking *TP53* mutations, five tumors had numerous cells with high copy number *EGFR* gene amplification (>100 copies; cases 4, 5, 6, 7, and 8), and the remaining three cases exhibited two or three copies in the vast majority of cells. Six cases showed three copies of the control signal, indicating trisomy of chromosome 7. Among the five glioblastomas with frank *EGFR* amplification, four cases had slides that sampled both the invading edge and the relatively solid central region of the tumor. A gradation of *EGFR* amplification in tumor cells was seen in all four cases: the invading edges had larger populations (>90%) of *EGFR*-amplified tumor cells than the relatively solid tumor centers (<20%; Fig. 1*A–E*). Of note, not all of the glioblastomas with EGFR amplification had a small cell phenotype.

In the six glioblastomas with *TP53* gene mutation, individual cells with *EGFR* amplification were observed in four cases (67%). In contrast to the glioblastomas without *TP53* mutations, however, *EGFR*-amplified cells were always isolated, accounting for considerably less than 1% of all of the tumor cells (Fig. 1*F*). Only one *TP53*-mutant case (case 14) had samples of both invading edge and invading central tumor; this case had four copies of the *EGFR* gene in both centrally located and peripheral cells, thus showing no gradation of gene amplification.

DISCUSSION

The present findings argue that intratumoral selection pressures influence the emergence of tumor cell populations with *EGFR* gene amplification. In addition to our findings, which are discussed below, evidence for such selection pressure in glioblastomas is provided by the long-standing observation that *EGFR* gene amplification is rapidly lost when these tumors are passaged *in vitro* (10); clearly, the growth advantage provided by *EGFR* overexpression *in vivo* is not present *in vitro*. We suggest that both the genetic and environmental milieu act *in vivo* to effect such selection.

Contrary to the results of prior studies of glioblastoma using approaches such as Southern blotting and PCR of tumor lysates, which suggested the near exclusivity of *EGFR* amplification and *TP53* mutation (4, 7), the present FISH studies demonstrate that *EGFR* amplification occurs in the majority of glioblastomas with *TP53* mutation. However, the cells with *EGFR* amplification remain isolated and few in number and, therefore, remain below the level of detection of tumor lysate-based approaches. Although *EGFR* gene amplification can occur as frequently in *TP53*-mutated tumors as in *TP53*-wild-type lesions, *EGFR* amplification does not appear to bestow a growth advantage to *TP53*-mutant glioblastoma cells. In other words, *TP53* mutation does not facilitate the selection of *EGFR*-amplified tumor cell populations. Recently, mutant p53 proteins have been shown to promote *CAD* gene amplification *in vitro* (15), bestowing drug resistance to cell lines. Although selection pressures governing drug resistance *in vitro* are likely different from those operative in glioblastoma growth *in vivo*, such data demonstrate that p53 inactivation, by encouraging genomic instability, may promote rather than prevent gene amplification. In light of these findings, it is perhaps not surprising that we have detected frequent but scattered *EGFR*-amplified cells in *TP53*-mutant tumors. The combined observations suggest that mutant p53, although enabling gene amplification, does not support the selection of cellular populations that require EGFR overexpression to promote growth.

Widespread *EGFR* gene amplification was found in five (62.5%) of eight *TP53*-wild-type glioblastomas. In these cases, cells with *EGFR* gene amplification were not uniformly distributed in the tumors. Cells with *EGFR* amplification were clustered at the less cellular, more infiltrative portions and were less conspicuous in the relatively solid tumor centers. Sauter *et al.* (14) also observed heterogeneity in *EGFR* copy number within single tumors, but the pattern of heterogeneity was not detailed. These authors suggested that the heterogeneity was related to the presence of different amplified clonal lines. However, various *EGFR* gene copy numbers does not necessarily imply multiple clones, because amplified *EGFR* genes are present as double-minute, extrachromosomal elements in glioblastoma (1). To maintain consistently high copy numbers across large numbers of cells, the extrachromosomal fragments must be repeatedly selected for during many cell divisions. We would, therefore, argue that such intratumoral heterogeneity reflects ongoing selection pressures.

Higher copy numbers of *EGFR* found at the probable periphery of the glioblastoma presumably provide a tumorigenic advantage for infiltrating the surrounding brain parenchyma. The reported inverse relationship between tumor cell dispersal and division, with migrating cells generally demonstrating lower proliferation indices than do solid tumor components (16), argues that up-regulation of *EGFR* at the edge of a glioblastoma is more likely related to invasion than to proliferation. Indeed, EGFR may play a role in the hallmark diffuse infiltration of glioblastomas into the adjacent brain. EGFR has been implicated in *in vitro* studies of glioma invasion (17–19). Furthermore, up-regulation of invasion-related transcripts has recently been reported in glioblastoma cell lines and postmortem glioblastomas expressing mutant EGFR (20, 21). Our observation of *EGFR* gene

Table 1 *Summary of TP53 mutation analysis and EGFR FISH analysis*

Case	*TP53* mutation	*EGFR* amplification pattern	Average *EGFR* number	Average control number	Mode of chromosome 7
1			2.35	2.31	2
2			3.07	2.92	3
3			3.23	3.18	3
4		+++[a]	>100	3.34	3
5		+++, periphery	>100	3.57	3
6		+++, periphery	>100	2.56	2
7		+++, periphery	>100	2.79	3
8		+++, periphery	>100	3.05	3
9	+		3.07	3.00	3
10	+		2.11	1.99	2
11	+	+, isolated	3.22	3.17	3
12	+	+, isolated	3.88	3.84	4
13	+	+, isolated	1.95	1.99	2
14	+	+, isolated	4.21	4.12	4

[a] +++ = numerous cells with amplification; periphery = amplified cells more numerous at probable edge of lesion; +, isolated = rare, scattered cells with amplification.

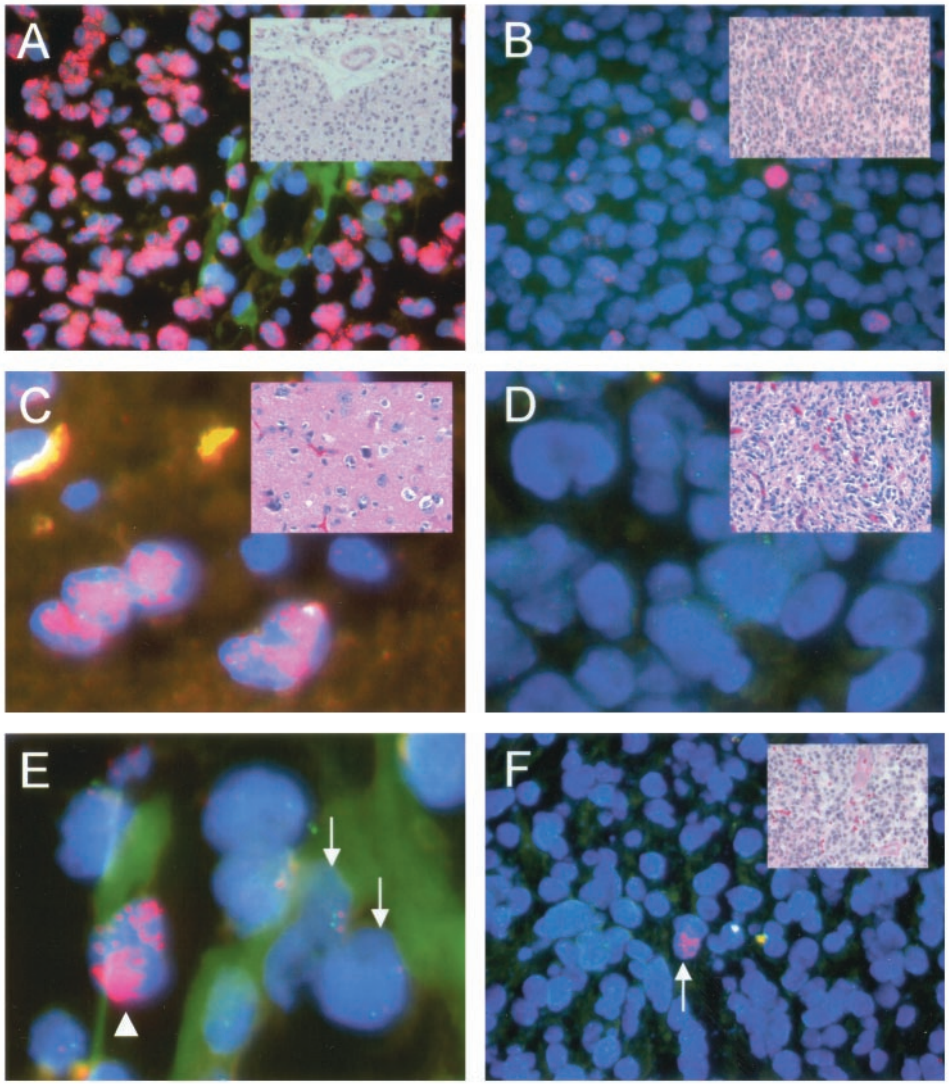

Fig. 1. *EGFR* FISH, with *corresponding H&E insets*. *A* and *B*, case 6, *TP53*-wild-type glioblastoma. The tumor cells have more than 100 copies of *EGFR* gene (*red signals*) in the peripheral region (*A*, subpial region), whereas less than 20% of the cells have amplification in the tumor center *B*. *C* and *D*, case 8, *TP53*-wild-type glioblastoma. Tumor cells at the invading edge (*C*, cerebral cortex) have more than 100 copies of *EGFR* gene, whereas the central region (*D*) has only three copies. *E*, enlargement of *A*. The *EGFR* probe discriminates between normal endothelial cells (*arrows*) and the tumor cell (*arrowhead*). *Two green signals* for the control probe are clearly visible in the endothelial cells. *F*, case 13, *TP53*-mutant glioblastoma. An isolated *EGFR*-amplified cell (*arrow*) is present.

amplification preferentially in the infiltrative portions of human gliomas suggests that EGFR has a similar role *in vivo*.

Intratumoral *EGFR* gene amplification heterogeneity also highlights potential pitfalls, as well as advantages, for molecular classification of glioblastomas. A glioblastoma could be misclassified as "lacking *EGFR* amplification" if only the central region of a tumor is sample for genomic assessment. On the other hand, in difficult cases of infiltrating malignant diffuse astrocytomas in which diagnostic criteria for glioblastoma are not met, molecular analysis for *EGFR* gene amplification may suggest a diagnosis of glioblastoma in the absence of definite necrosis or microvascular proliferation. In light of the growing number of reports investigating the clinical relevance of *EGFR* amplification (22, 23), the present results imply that intratumoral heterogeneity for *EGFR* gene status should be taken into account.

In summary, *EGFR* gene amplification occurs commonly in glioblastoma cells with *TP53* mutation, perhaps reflecting the propensity of mutant p53 to induce genomic instability and gene amplification. The previously observed exclusivity of *TP53* mutation and widespread *EGFR* amplification in glioblastoma cell lysates is most likely because *EGFR* gene amplification does not confer a growth advantage in *TP53*-mutant cells, raising interesting questions about how these pathways interact at a molecular level. In addition, *EGFR* amplification in *TP53*-normal glioblastomas can be heterogeneous and exhibit topological gradation, supporting a role for *EGFR* in glioblastoma cell invasion of the adjacent brain, and begging caution in the selection of tumor regions for molecular genetic analyses.

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