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

Yoshifumi Okada, Edward E. Hurwitiz, John M. Esposito, Melissa A. Brower, Catherine L. Nutt, and David N. Louis

The Molecular Neuro-Oncology Laboratory and Molecular Pathology Laboratory, Department of Pathology and Neurosurgical Service, Massachusetts General Hospital and Harvard Medical School, 149 13th St., Charlestown, Massachusetts 02129

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 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 EGFR 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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1 To whom requests for reprints should be addressed, at Molecular Pathology Laboratory, CNT7, Massachusetts General Hospital, 149 13th Street, Charlestown, MA 02129. Phone: (617) 726-5600; Fax: (617) 726-5079; E-mail: DLOUIS@PARTNERS.ORG.

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

3 Internet address: http://genomics.rorschpark.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. 1A–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. 1F). 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 passed 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-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

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<th>Case</th>
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*++ = numerous cells with amplification; periphery = amplified cells more numerous at probable edge of lesion; +, isolated = rare, scattered cells with amplification.
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 sampled 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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