Receptor Tyrosine Kinase Genes Amplified in Glioblastoma Exhibit a Mutual Exclusivity in Variable Proportions Reflective of Individual Tumor Heterogeneity

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Abstract

Intratumoral heterogeneity in human solid tumors represents a major barrier for the development of effective molecular treatment strategies, as treatment efficacies will reflect the molecular variegation in individual tumors. In glioblastoma, the generation of composite genomic profiles from bulk tumor samples has allowed one to map the genomic amplifications of putative genetic drivers and to prioritize therapeutic targeting strategies aimed at eradicating the tumor burden. Notably, amplification of multiple receptor tyrosine kinases (RTK) within a single tumor specimen obtained from patients is frequently observed. In this study, use of a detailed multicolor FISH mapping procedure in pathologic specimens revealed a mutual exclusivity of gene amplification in the majority of glioblastoma tumors examined. In particular, the two most commonly amplified RTK genes, EGFR and PDGFRα, were found to be present in variable proportions across the tumors, with one or the other gene predominating in certain areas of the same specimen. Our findings have profound implications for designing efficacious therapeutic regimens, as it remains unclear that how the cells with different gene amplification events contribute to disease propagation or the response to molecular targeted therapies. Cancer Res; 72(7); 1614-20. ©2012 AACR.

Introduction

Cancer heterogeneity presents a major barrier for the development of effective molecular treatment strategies aimed at combating the disease. Intratumoral heterogeneity has long been recognized and cataloged by clinicopathologic classification systems; however, the imprecision of this approach in accurately predicting an individual patient’s outcome has been found to be underpinned by the presence of biologically distinct subtypes identified by molecular profiling (1) within these otherwise similar entities.

Further complicating matters is the appreciation that there also exists considerable heterogeneity within the cells of an individual's tumor mass (2). Debates as to whether these distinct subpopulations arise through clonal evolution (3) or via propagation of tumor-initiating cells with stem-like qualities (4) are alighting on the idea that the two concepts may be interlinked (5, 6), and the cellular and genetic context determines which may predominate.

Within glioblastoma, this intratumoral heterogeneity may refer to the concurrent observation of multiple cellular morphologies (7), differential patterns of vascular proliferation (8), and discrete transcript and/or protein expression patterns (9, 10), as well as genetic variegation (11, 12). Despite the long-recognized "multiforme" nature of glioblastoma specimens, and a recent in-depth cataloging of the molecular alterations driving the disease (13, 14), a thorough mapping of molecular events across individual tumors at a single-cell level has been lacking.

DNA copy number profiling has shown that multiple receptor tyrosine kinase (RTK) amplifications may frequently be found in the same glioblastoma specimens (13, 14). Although tumor clonality would imply that these events would be present in all neoplastic cells, we and others have previously noted through detailed fluorescent and chromogenic in situ hybridization (FISH/CISH) experiments on pathologic specimens that not all cells harbor individual amplification events (15, 16).

Materials and Methods

Patient samples

Three hundred and sixty-two formalin-fixed, paraffin-embedded (FFPE) high-grade glioma samples were retrieved after Research Ethics Committee approval from 342 consecutive patients diagnosed within the last 5 years from the...
archives of King’s College Hospital, with diagnosis confirmed by re-review (S. Popov and S. Al-Sarraj). The age of the patients was 26 to 83 years (median, 58 years) and comprised 61%-39% males/females. Clinical follow-up was available for 329 patients, with a median survival of 6.5 months (range, 2 days–5.6 years).

**FISH**

BAC clones were purchased from BACPAC Resources Center and FISH-mapped onto metaphase slides to ensure specificity (EGFR:RP11-433C10/RP11-381G5; centromere 7:RP11-714F8/RP11-714E10/RP11-717E9; PDGFRA: RP11-58C6/RP11-819D11; centromere 4:RP11-317G22/ RP11-191S2). Probes were labeled with either biotin or DIG using the BioPrime DNA Labeling System (Invitrogen), hybridized overnight at 37°C to FFPE sections, and scanned on the Ariol System (Leica Microsystems) at ×20 using filters for DAPI/Cy3/FITC.

**FISH scoring**

On the tissue microarray sections, hybridization was carried out using differentially labeling of one gene and one centromeric probe (EGFR-Cy3/cent7-FITC; PDGFRA-Cy3/cent4-FITC). Each core was screened for cells with 10 or more gene copies, or 5 to 9 gene copies with a gene:centromere ratio of more than 2. Any core with at least one such cell was considered amplified. At least 50 cells per core were screened. For whole sections, each topographically distinct region of the tissue was numbered, and further subdivided into every tenth frame from the Ariol scan for assessment. FISH was carried out using differentially labeled gene probes for EGFR (fluorescein isothiocyanate; FITC) and PDGFRA (Cy3). Within each subdivision of the tissue, more than 100 cells were scored for (i) EGFR amplification, normal copy number of PDGFRA; (ii) PDGFRA amplification, normal copy of EGFR; (iii) amplification of both EGFR and PDGFRA; or (iv) normal copies of both EGFR and PDGFRA (Supplementary Fig. S1). Any cell with 5 or more gene copies was considered amplified. Normal copy number was classed as 1–4 signals per nucleus.

**Table 1. Frequency of EGFR and PDGFRA amplification in high-grade gliomas**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>WHO grade</th>
<th>Frequency</th>
<th>P, log-rank</th>
<th>Frequency</th>
<th>P, log-rank</th>
<th>Frequency</th>
<th>P, log-rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>—</td>
<td>112/294(38%)</td>
<td>0.106</td>
<td>49/239(20%)</td>
<td>0.0051*</td>
<td>18/226(8%)</td>
<td>0.0133*</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>IV</td>
<td>106/240(44%)</td>
<td>0.634</td>
<td>41/191(21%)</td>
<td>0.0018a</td>
<td>17/180(8%)</td>
<td>0.11</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>III</td>
<td>2/13(15%)</td>
<td>0.589</td>
<td>2/11(18%)</td>
<td>0.70</td>
<td>0/9(0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Anaplastic</td>
<td>III</td>
<td>4/41(10%)</td>
<td>0.892</td>
<td>6/37(16%)</td>
<td>0.152</td>
<td>1/36(3%)</td>
<td>0.336</td>
</tr>
<tr>
<td>oligodendroglioma</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

NOTE: Frequency of amplifications observed by FISH in an initial tissue microarray screen of 342 high-grade glioma patients. Tumors are taken as a whole, as well as subdivided by histology, and the log-rank test applied to determine univariate prognostic significance (*, P < 0.05).

Abbreviation: NA, not applicable.

**Statistical analysis**

All statistical tests were conducted in R2.11.0. Correlations between FISH cell counts and categorical variables were conducted using ANOVA. Detection of foci showing independent patterns of amplification was carried out by t test. All tests were two-tailed, with a confidence interval of 95%. P values of less than 0.05 were considered statistically significant.

**Results and Discussion**

To explore the concept of intratumoral genetic heterogeneity more thoroughly, tissue microarrays comprising 342 high-grade glioma samples were screened by FISH for cells amplified for genes encoding either EGFR or PDGFRA. Evidence of high-level DNA copy number gain of the gene in as few as a single cell of the tissue microarray core was taken as evidence of amplification. Within assessable cores, we identified 112 of 294 (38%) cases to harbor EGFR amplification (106 of 240, 44% glioblastoma; 2 of 13, 15% anaplastic astrocytoma; 4 of 41, 10% anaplastic oligodendroglioma) and 49 of 239 (20%) to harbor PDGFRA amplification (41 of 191, 21% glioblastoma; 2 of 11, 18% anaplastic astrocytoma; 6 of 37, 16% anaplastic oligodendroglioma; Table 1). While the EGFR data were similar to literature reports, the frequency of PDGFRA amplification was approximately twice that from molecular profiling data (13, 14) and is itself likely an underestimate due to the restricted nature of tumor sampling afforded by using tissue microarrays. Thus, it appears that the frequencies of certain abnormalities may be significantly underestimated by focusing only on the composite genetic landscapes afforded by bulk tumor profiling.

A total of 18 cases (8%) contained both gene amplifications. Seventeen of these were glioblastoma, with a single case of anaplastic oligodendroglioma. We used 2-color FISH with differential labeling of probes specific to EGFR and PDGFRA on whole sections from these samples and assessed the relative copy numbers per cell of each gene. We observed a high degree of variability in gene copy number of both genes in individual cells across the entire pathologic specimen. Co-amplification was observed as either cosegregation of double minutes containing both genes (Fig. 1A), or in one case, a homogeneously
staining region (HSR) of EGFR amplification alongside PDGFRA double minutes (Fig. 1B). To our surprise, however, we also discovered a high degree of mutual exclusivity of gene amplification within adjacent glioblastoma cells (Fig 1C). Such instances were rigorously examined for both fluorescent dyes, such that scored cells were only included where both probes had worked—that is, at least a normal number of copies was observed per cell.

To quantify the extent to which these patterns were reflected across the tumor, we carefully mapped the relative copy numbers of EGFR and PDGFRA in cells from throughout the pathology specimen of all dual amplified glioblastoma cases. By counting signals in 41,997 cells from 190 distinct loci across 17 samples, we noted a startling heterogeneity of cases. By counting signals in 41,997 cells from 190 distinct cases were not only isolated, distinct cell populations harboring PDGFRA amplification (Fig. 2A, RMH5698) but also restricted foci within the same specimen where PDGFRA-amplified cells made up more than half of the tumor population (Fig. 2B, RMH5724).

This blueprint was the norm, rather than the exception, across these EGFR-predominant samples, with 10 of 13 (77%) cases harboring topographical regions in which PDGFRA-amplified cells, and represented a significantly enriched subpopulation of the tumor (Supplementary Table S1 and Fig. S2). We observed that these PDGFRA-restricted cells tended to be present close to endothelial cells (Fig. 3A). Of note, 64 of 71 (90%) of foci in which PDGFRA-amplified cells were found to comprise more than 10% of the total number of cells were areas of increased vascularity (Fig. 3B). In contrast, poorly vascularized areas were largely the domain of EGFR-amplified tumor cells, with 84% of such defined regions harboring EGFR amplification alone (vs. 59% cells in more highly vascularized regions; P < 0.001, t test). Thus, although the prevalence of specific RTK gene amplifications was strongly associated with the extent of vascularization in the tumor, it was not absolutely predictive.

As well as assessing the level of vascularity, we also determined the morphologic tumor cell types which were present across the whole pathology sections. Although associated with all cell types, EGFR amplification was significantly linked with areas of glioblastoma containing large numbers of giant cells and/or small cells (Fig. 3C), as has previously been suggested (ref. 17; 89% EGFR amplification alone in giant cell foci vs. 62% in other regions, P < 0.001, t test; 88% EGFR amplification alone in small cell foci vs. 62% in other regions, P < 0.001, t test). All high-frequency PDGFRA-amplified cells were found in areas of fibillary histology (Fig. 3D; 36 of 36, 100% foci with >25% PDGFRA-amplified cells alone; 41 of 45, 91% foci with >20% PDGFRA-amplified cells alone). This contrasts sharply with the recent correlations reported between PDGFRA abnormalities and an oligodendroglial phenotype (18), although in these tumors, EGFR amplifications are not concurrently present.

These data are remarkably consistent with, and complimentary to, a recent study describing the same phenomenon in glioblastoma as "mosaic amplification" (19). Taken together, these studies are suggestive of distinct subpopulations of
Figure 2. Topographical differences in amplification patterns of EGFR and PDGFRA in glioblastoma. Whole tissue section mapping of RMH5698 (A) and RMH5724 (B). Left, hematoxylin and eosin (H&E) staining assessed for predominant morphologic types of tumor cells in distinct regions (insets). Original magnification, ×40 (insets, original magnification, ×630). Vascularity (gray boxes) was assessed by CD31 immunostaining. Right, dual-color FISH for EGFR (FITC/green) and PDGFRA (Cy3/red) in the same topographical areas, assessed for differential patterns of amplification of the 2 genes. Original magnification, ×40 (insets, original magnification, ×1,000). Distinct topographical locations displayed significantly different amplification patterns from the tumor bulk as a whole (*, P < 0.001; χ² test).
Figure 3. Pathologic correlates of topographically restricted EGFR/PDGFRα amplification. A, dual-color FISH for EGFR (FITC/green) and PDGFRα (Cy3/red) in glioblastoma sample RMH6388, showing a clustering of PDGFRα (only)-amplified cells in the proximity of tumor endothelial cells (hematoxylin and eosin; H&E). Original magnification, ×1,000. B, box plots of extent of tumor vascularity (low/moderate/high) versus amplification pattern across the 17 glioblastoma cases studied in detail. Whiskers represent the lower and upper quartiles of data. C, dual-color FISH for EGFR (FITC/green) and PDGFRα (Cy3/red) in glioblastoma sample RMH6905, highlighting an association of EGFR (only)-amplified cells with giant cell and small cell morphology. Original magnification, ×1,000. D, box plots of tumor cell morphologic type versus amplification pattern across the 17 glioblastoma cases studied in detail. Gemisto, gemistocytic; Sarcom, sarcomatous.
glioblastoma cells marked by differential gene amplification, and these authors noted in particular that each subpopulation was derived from a single precursor and was actively dividing, with the specific amplification driving protein expression (19). The relative contributions of cell populations harboring one or other gene amplification to disease progression, and the implications for novel treatment strategies, remain to be elucidated.

Little is also known about to what extent this heterogeneity is reflected at the level of tumor-initiating cells, as has recently been shown in acute lymphoblastic leukemia (20). Here, it was apparent that leukemia-propagating cells displayed a genetic variegation mirroring the subclonal patterns observed in the bulk cancer and that these varied in their regenerative capacity in vivo. This genetic diversity of a stem-like cell compartment has yet to be comparably shown in solid tumors, although if present may be associated also with a diversity of functional properties and drug or irradiation sensitivity. Unraveling the possible functional heterogeneity in glioblastoma would seem essential given the endemic failure of both chemotherapeutic and targeted treatment approaches.

A curious observation linked to the mutual exclusivity of RTK gene amplifications that we report here is the presence across many of the tumors, in one case (RMH6887) the majority of the specimen, of cells with neither genes amplified. These cells are not the product of a failed FISH assay, as they are strongly associated with oligodendroglial and, particularly, sarcomatous morphologic types, perhaps representing a further, unrecognized genetic alteration linked to these cells. It is tempting to speculate that the two distinct populations observed here in fact represent only the tip of the iceberg in terms of distinct, genetically restricted, subclonal populations of cells which make up the remarkable intratumoral heterogeneity underlying the disease.

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C. Jones: commercial research grant, AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: S.E. Little, S. Popov, D.A. Bax, J.M. Jurgensmeier, C. Jones
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Popov, A. Jury, D.A. Bax, S. McIndoo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.A. Bax, C. Jones
Study supervision: C. Jones

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References


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