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, since 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 (RTKs) within a single tumor specimen obtained from patients is frequently observed. In this study, use of a detailed multi-colour FISH mapping procedure in pathological 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 PDGFRA, 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, since it remains unclear how the cells with different gene amplification events contribute to disease propagation or the response to molecular targeted therapies.
Introduction

Cancer heterogeneity presents a major barrier for the development of effective treatment strategies aimed at combating the disease. Intertumoral heterogeneity has long been recognised and catalogued by clinicopathological 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 tumour mass (2). Debates as to whether these distinct subpopulations arise through clonal evolution (3) or via propagation of tumour-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), discrete transcript and/or protein expression patterns (9, 10), as well as genetic variegation (11, 12). Despite the long-appreciated ‘multiforme’ nature of glioblastoma specimens, and a recent in-depth cataloguing of the molecular alterations driving the disease (13, 14), a thorough mapping of molecular events across individual tumours at a single cell level has been lacking.

DNA copy number profiling has demonstrated that multiple RTK amplifications may frequently be found in the same glioblastoma specimens (13, 14). Although tumour clonality would imply that these events would be present in all neoplastic cells, we
and others have previously noted through detailed fluorescent and chromogenic \textit{in situ} hybridisation (FISH/CISH) experiments on pathological specimens that not all cells harbour individual amplification events (15, 16).
Materials and Methods

Patient samples

362 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 (SP, SA-S). The age of the patients was 26–83 years (median 58yrs), 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–5.6yrs).

Fluorescent in situ hybridisation (FISH)

BAC clones were purchased from BACPAC Resources Center (CHORI, CA, USA) and FISH-mapped onto metaphase slides to ensure specificity (\textit{EGFR}:RP11-433C10/RP11-381G5; centromere 7:RP11-714F8/RP11-714E10/RP11-717E9; \textit{PDGFRA}:RP11-58C6/RP11-819D11; centromere 4:RP11-317G22/RP11-191S2). Probes were labelled with either biotin or DIG using the BioPrime® DNA Labeling System (Invitrogen, Paisley, UK), hybridised overnight at 37°C to FFPE sections, and scanned on the Ariol System (Leica Microsystems, Wetzlar, Germany) at x20 using filters for DAPI/Cy3/FITC.

FISH scoring

On the tissue microarray sections, hybridisation was carried out using differential labelling of one gene and one centromeric probe (\textit{EGFR}-Cy3/cent7-FITC; \textit{PDGFRA}-Cy3/cent4-FITC). Each core was screened for cells with ≥10 gene copies, or 5-9 gene copies with a gene:centromere ratio >2. Any core with at least one such cell was considered amplified. At least 50 cells/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 labelled gene probes for EGFR (FITC) and PDGFRA (Cy3). Within each subdivision of the tissue >100 cells were scored for a) EGFR amplification, normal copy number of PDGFRA; b) PDGFRA amplification, normal copy of EGFR; c) amplification of both EGFR and PDGFRA; or d) normal copies of both EGFR and PDGFRA (Supplementary Figure S1). Any cell ≥5 gene copies was considered amplified. Normal copy number was classed as 1-4 signals per nucleus.

**Statistical analysis**

All statistical tests were performed in R2.11.0. Correlations between FISH cell counts and categorical variables were performed 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

In order 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 TMA core was taken as evidence of amplification. Within assessable cores, we identified 112/294 (38%) cases to harbour EGFR amplification (106/240, 44% glioblastoma; 2/13, 15% anaplastic astrocytoma; 4/41, 10% anaplastic oligodendroglioma) and 49/239 (20%) to harbour PDGFRA amplification (41/191, 21% glioblastoma; 2/11, 18% anaplastic astrocytoma; 6/37, 16% anaplastic oligodendroglioma) (Table 1). Whilst 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 tumour 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 tumour profiling.

A total of 18 cases (8%) contained both gene amplifications. Seventeen of these were glioblastoma, with a single case of anaplastic oligodendroglioma. We utilised two-colour FISH with differential labelling 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 pathological specimen. Co-amplification was observed as either co-segregation of double minutes containing both genes (Figure 1A), or in one case, a homogenously staining region (HSR) of EGFR amplification alongside PDGFRA double minutes (Figure 1B). To our surprise, however, we also discovered a high degree of mutual exclusivity of gene
amplification within adjacent glioblastoma cells (Figure 1C). Such instances were rigorously examined for both fluorescent dyes, such that scored cells were only included where both probes had worked – i.e. at least a normal number of copies was observed per cell.

To quantify the extent to which these patterns were reflected across the tumour, 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 amplification patterns both across and within tumour specimens, and a considerably higher than anticipated degree of mutual exclusivity of gene amplification (Supplementary Table S1). Across our entire dataset, we observed that approximately three-quarters of cells harboured amplification of only one of the genes, with the other demonstrating normal copy number. Considered another way, in only one case did the single-cell ‘co-amplification’ model represent more than 15% of the sample as a whole, in all the others the vast majority of the cells harboured only EGFR or PDGFRA amplification (Supplementary Table S1).

Although some cases (RMH6416, RMH6674) demonstrated a relatively uniform admixture of differential DNA copy number across the sample, most showed significantly distinct frequency patterns in restricted topographical components of the tumour (Supplementary Figure S2). Within an individual sample, cells harbouring one, both, or neither amplification could be found in highly variable frequencies, either presenting as a mosaic of genetically distinct cells, or forming foci where one event would strongly predominate. Across the specimen as a whole, EGFR amplification predominated in 13/17 (76%) samples, and there were regions in which this event was present in >99% cells. Despite this, within these cases were not only isolated, distinct cell populations harbouring PDGFRA amplification (Figure 2A–
RMH5698), but also restricted foci within the same specimen where PDGFRA-amplified cells made up more than half of the tumour population (Figure 2B–RMH5724).

This blueprint was the norm, rather than the exception, across these EGFR-predominant samples, with 10/13 (77%) cases harbouring topographical regions in which PDGFRA-amplified cells, and represented a significantly enriched subpopulation of the tumour (Supplementary Table S1, Supplementary Figure S2). We observed that these PDGFRA-restricted cells tended to be present close to endothelial cells (Figure 3A). Of note, 64/71 (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 (Figure 3B). By contrast, poorly vascularised areas were largely the domain of EGFR-amplified tumour cells, with 84% of such-defined regions harbouring EGFR amplification alone (versus 59% cells in more highly vascularised regions, p<0.001, t-test). Thus, although the prevalence of specific RTK gene amplifications was strongly associated with the extent of vascularisation in the tumour, it was not absolutely predictive.

As well as assessing the level of vascularity, we also determined the morphological tumour 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 (Figure 3C), as has previously been suggested (17) (89% EGFR amplification alone in giant cell foci versus 62% in other regions, p<0.001, t-test; 88% EGFR amplification alone in small cell foci versus 62% in other regions, p<0.001, t-test). All high frequency PDGFRA-amplified cells were found in areas of fibrillary histology (Figure 3D) (36/36, 100% foci with >25% PDGFRA-amplified cells alone; 41/45, 91% foci with >20% PDGFRA-amplified cells alone). This contrasts sharply with the recent correlations.
reported between PDGFRA abnormalities and an oligodendroglial phenotype (18), though in these tumours, 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 glioblastoma cells marked by differential gene amplification, and these authors noted in particular that each subpopulation was derived from a single precursor, was actively dividing, with the specific amplification driving protein expression (19). The relative contributions of cell populations harbouring one or other gene amplification to disease progression, and the implications for novel treatment strategies, remains to be elucidated.

Little is also known regarding to what extent this heterogeneity is reflected at the level of tumour initiating cells, as has recently been demonstrated in acute lymphoblastic leukaemia (20). Here, it was apparent that leukaemia 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 demonstrated in solid tumours, though if present may be associated also with a diversity of functional properties and drug or irradiation sensitivity. Unravelling the possible functional heterogeneity in glioblastoma would seem essential given the endemic failure of both chemotherapeutic and targeted treatment strategies.

A curious observation linked to the mutual exclusivity of receptor tyrosine kinase gene amplifications that we report here is the presence across many of the tumours, 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
carefully assessed to contain normal gene copy number, and are confirmed histologically as glioblastoma, rather than non-tumorigenic, cells. They were strongly associated with oligodendrogial and, particularly, sarcomatous morphological types, perhaps representing a further, unrecognised 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 intratumoural heterogeneity underlying the disease.
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LEGENDS FOR FIGURES

Figure 1 – Amplification of EGFR and PDGFRA in glioblastoma. (A) Co-amplification pattern, whereby amplified signals for both EGFR (FITC/green) and PDGFRA (Cy3/red) co-segregate as double minutes in the same cells (RMH6417). Original magnification ×1000. (B) HSR pattern, with signals for EGFR presenting as homogenously staining regions (arrow) in the same cells as PDGFRA double minutes (RMH6674). Original magnification ×1000. (C) Mutually exclusive pattern, with amplification of EGFR and PDGFRA as double minutes in distinct cells, with a normal copy number (arrows) of the unamplified gene per nucleus (RMH6388). Original magnification ×1000.

Figure 2 – Topographical differences in amplification patterns of EGFR and PDGFRA in glioblastoma. Whole tissue section mapping of RMH5698 (A) and RMH5724 (B). Left, H&E staining assessed for predominant morphological types of tumour cells in distinct regions (insets). Original magnification ×40 (insets, original magnification ×630). Vascularity (grey boxes) was assessed by CD31 immunostaining. Right, dual-colour FISH for EGFR (FITC/green) and PDGFRA (Cy3/red) in the same topographical areas, assessed for differential patterns of amplification of the two genes. Original magnification ×40 (insets, original magnification ×1000). Distinct topographical locations displayed significantly different amplification patterns from the tumour bulk as a whole (*p<0.001, Chi-square test).

Figure 3 – Pathological correlates of topographically-restricted EGFR/PDGFR amplification. (A) Dual-colour FISH for EGFR (FITC/green) and PDGFRA (Cy3/red)
in glioblastoma sample RMH6388, demonstrating a clustering of PDGFRA (only)-amplified cells in the proximity of tumour endothelial cells (H&E). Original magnification ×1000. (B) Boxplots of extent of tumour 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-colour FISH for EGFR (FITC/green) and PDGFRA (Cy3/red) in glioblastoma sample RMH6905, highlighting an association of EGFR (only)-amplified cells with giant cell and small cell morphology. Original magnification ×1000. (D) Boxplots of tumour cell morphological type versus amplification pattern across the 17 glioblastoma cases studied in detail.
REFERENCES


Table 1 – Frequency of EGFR and PDGFRA amplification in high grade gliomas. (A) Frequency of amplifications observed by FISH in an initial tissue microarray screen of 342 high grade glioma patients. Tumours 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).
Figure 2

A

RMH 5698
H&E

2, 3a-d: Gemistocytic
3b: Fibrillary: small cell
1b: Fibrillary

Vascularity: low  medium  high

RMH 5698
FISH: EGFR and PDGFRA

1a. EGFR: EGFR amp 96.7%
PDGFRA amp 0.0%
EGFR + PDGFRA amp 0.0%
neither amp 3.3%

1b. EGFR: EGFR amp 94.8%
PDGFRA amp 1.2%
EGFR + PDGFRA amp 0.0%
neither amp 3.9%

1c. EGFR: EGFR amp 98.0%
PDGFRA amp 0.0%
EGFR + PDGFRA amp 0.0%
neither amp 2.0%

B

RMH 5724
H&E

2a, b: Fibillary: giant cell; small cell; gemistocytic
1: Fibillary

Vascularity: low  medium  high

RMH 5724
FISH: EGFR and PDGFRA

1a. EGFR: EGFR amp 85.5%
PDGFRA amp 3.3%
EGFR + PDGFRA amp 1.4%
neither amp 10.0%

1b. EGFR: EGFR amp 90.9%
PDGFRA amp 3.6%
EGFR + PDGFRA amp 5.5%
neither amp 0.0%

1c. EGFR: EGFR amp 93.7%
PDGFRA amp 0.0%
EGFR + PDGFRA amp 3.8%
neither amp 2.5%
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