Distinct Phenotypic Differences Associated with Differential Amplification of Receptor Tyrosine Kinase Genes at 4q12 in Glioblastoma

Anna Burford1,2, Suzanne E. Little1,2, Alexa Jury1,2, Sergey Popov1,2, Ross Laxton3, Lawrence Doey3, Safa Al-Sarraj3, Juliane M. Jürgensmeier4, Chris Jones1,2*

1 Divisions of Molecular Pathology, The Institute of Cancer Research, Sutton, United Kingdom, 2 Cancer Therapeutics, The Institute of Cancer Research, Sutton, United Kingdom, 3 Neuropathology, King’s College Hospital, London, United Kingdom, 4 AstraZeneca, Alderley Park, Macclesfield, United Kingdom

Abstract

Gene amplification at chromosome 4q12 is a common alteration in human high grade gliomas including glioblastoma, a CNS tumour with consistently poor prognosis. This locus harbours the known oncogenes encoding the receptor tyrosine kinases PDGFRA, KIT, and VEGFR2. These receptors are potential targets for novel therapeutic intervention in these diseases, with expression noted in tumour cells and/or associated vasculature. Despite this, a detailed assessment of their relative contributions to different high grade glioma histologies and the underlying heterogeneity within glioblastoma has been lacking. We studied 342 primary high grade gliomas for individual gene amplification using specific FISH probes, as well as receptor expression in the tumour and endothelial cells by immunohistochemistry, and correlated our findings with specific tumour cell morphological types and patterns of vasculature. We identified amplicons which encompassed PDGFRA only, PDGFRA/KIT, and PDGFRA/KIT/VEGFR2, with distinct phenotypic correlates. Within glioblastoma specimens, PDGFRA amplification alone was linked to oligodendrogial, small cell and sarcomatous tumour cell morphologies, and rare MGMT promoter methylation. A younger age at diagnosis and better clinical outcome in glioblastoma patients is only seen when PDGFRA and KIT are co-amplified. IDH1 mutation was only found when all three genes are amplified; this is a subgroup which also harbours extensive MGMT promoter methylation. Whilst PDGFRA amplification was tightly linked to tumour expression of the receptor, this was not the case for KIT or VEGFR2. Thus we have identified differential patterns of gene amplification and expression of RTKs at the 4q12 locus to be associated with specific phenotypes which may reflect their distinct underlying mechanisms.

Introduction

Molecular profiling of glioblastoma has recently uncovered a catalogue of DNA copy number changes [1,2], gene expression [1,3,4] and DNA methylation patterns, and mutations [1,2] which appear to drive subgroups of the disease. Amongst this data, gene amplification at chromosome 4q12 has been reported in 74/463 (16%) cases assessed by array-based comparative genomic hybridisation (aCGH) [5], and is linked to a subclass of tumours associated with a younger age at diagnosis, Proneural gene expression signature, IDH1 mutation, and a CpG island methylator phenotype [4,6].

Within the 4q12 amplicon reside genes encoding the receptor tyrosine kinases (RTKs) and drug targets PDGFRA, KIT, and KDR (VEGFR2). Bulk profiling of tumour specimens suggests that only PDGFRA is common to 4q12 amplified cases, with 39% of these harbouring PDGFRA amplification alone, 23% including PDGFRA and KIT, and 38% encompassing all three RTKs [5]. Recent studies using multi-colour fluorescent in situ hybridisation (FISH) on archival pathology specimens has demonstrated the marked genetic heterogeneity of glioblastoma specimens in relation to RTK gene amplification [5,7,8]. Individual cells, or small foci of cells may harbour increased DNA copy number of a gene that is not found amplified in the majority of cells, and would not be detected by bulk tumour aCGH [7]. Thus there may be instances in which isolated cells/foci may harbour KIT and/or VEGFR2 amplification in the absence of copy number gain of PDGFRA, as has been implied by previous FISH studies of all three genes separately [9].

The specific RTKs involved at 4q12 have translational interest for several reasons. Receptor expression of PDGFRA and KIT has been found in numerous malignancies, particularly sarcomas such as GIST (gastro-intestinal stromal tumour), against which small molecule inhibitors have demonstrated clinical response [10,11]. In fact selectivity between these class III RTKs can be difficult to achieve, so many of these targeted inhibitors act on both receptors [12–14]. In glioblastoma, amplification/overexpression of PDGFRA has provided the rationale for clinical studies, with disappointing results to date [15–17]. The role of KIT expression...
in glioblastoma, and thus the necessity of targeting the receptor clinically, is less clear.

By contrast, VEGFR2 does not tend to be expressed in cancer cells, but is extensively expressed within the tumour endothelium, and is the major transducer of VEGF signals, resulting in increased angiogenesis through endothelial cell survival, proliferation and migration [18,19]. The critical role of VEGF/VEGFR2 in tumour angiogenesis has been demonstrated in both animal studies and clinical trials in numerous human malignancies, including glioblastoma [20–22]. PDGFRα and KIT too, may be expressed in the glioblastoma-associated vasculature, although they have been less extensively studied. Consistent with a potential role in angiogenesis, KIT is found on circulating endothelial precursor cells and human umbilical vein endothelial cells (HUVEC) [23,24], and its ligand stem cell factor (SCF) has been implicated as both an autocrine and paracrine growth factor for gliomas. In comparison, the role of PDGFRα has been much less appreciated [25,26], although there is evidence to suggest that signalling through the receptor plays an important role in regulating VEGF-driven tumour angiogenesis particularly when tumour cells are deficient in VEGF production.

The aim of this study was to assess the relative contributions of PDGFRα, KIT and VEGFR2 to high grade glioma tumour biology, in terms of gene amplification and expression in the tumour cells and associated vasculature. Given the heterogeneity of clinical glioblastoma specimens, we further correlated these data with tumour cell morphological subtypes, specific patterns of vascular proliferation and known genetic prognostic markers such as IDH1 mutation [27] and MGMT promoter methylation [28]. We identified the unique observation that specific patterns of gene amplification at 4q12 were associated with distinct phenotypes in glioblastoma specimens.

### Materials and Methods

#### Patient samples

A series of 362 formalin-fixed, paraffin-embedded (FFPE) samples were retrieved after approval from Wandsworth Research Ethics Committee from 342 consecutive patients diagnosed with glioblastoma multiforme (WHO grade IV, n = 276), anaplastic astrocytoma (WHO grade III, n = 17) or anaplastic oligodendroglioma (WHO grade III, n = 49) within the last 5 years from the archives of King’s College Hospital, London, with diagnosis confirmed by re-review (SP, SA-S). All the samples enrolled in the present study were unlinked and unidentified from their donors. Due the retrospective nature of the study, no written informed consent from patients was obtained, with the exception of the UK samples obtained after 2006, where all patients signed a written informed consent. Following the UK Human Tissue Act approved in that year. Other grade III/IV glioma subtypes such as mixed oligoastrocytomas were not included in the present study. The age range of the patients was 26–83 years (median 58 years), and comprised 61% males to 39% females. Clinical follow-up was available for 329 patients, with a median survival of 6.5 months (range 2 days –5.6 years).

A detailed morphological assessment of the 276 GBM samples was also carried out and the predominant cell types recorded as either as fibrillary (composed of pleomorphic cells with scant or more prominent pink cytoplasm; some cells can demonstrate cytoplasmic extensions); gemistocytic (>20% cells having abundant, glassy cytoplasm, eccentric nuclei); giant cell (composed primarily of bizarre, pleomorphic, often multinucleated cells); small cell (monomorphic round to oval nuclei, uniform in size with bland chromatin but with high mitotic activity; occasional clear perinuclear haloes, branching capillaries and microcalcifications can be seen); oligodendrogial (cytologic monotypy with rounded, bland nuclei surrounded by perinuclear haloes; in some cases branching; "chicken wire"-like capillaries); or sarcomatous (consisting of fascicles of spindle cells, sometimes with collagen depositions; stellate cells in a myxoid stroma can be seen in some cases).

#### Tissue microarray construction

1 mm diameter cores from each case were taken and constructed in four TMAs containing approximately 90–100 samples each. In addition, a series of control tissues were included, comprising normal human brain, normal mouse brain, placenta, colon and lung. A further H&E was taken of each TMA and re-reviewed to ensure the presence of tumour cells.

#### Immunohistochemistry

Each TMA was serially sectioned at 5 µm and immunohistochemistry was performed. Slides were dewaxed in xylene and rehydrated through a descending ethanol series. The rabbit EnVision™+ HRP System, (DAKO, Carpinteria, CA, USA) was used for PDGFRα (D1E1E) and VEGFR2 (55B1) (Cell Signalling, MA, USA). Antigen retrieval was performed by boiling the slides for 15 min in 10 mM citrate buffer pH6 in a coplin jar and cooled on the bench for 20 min, or for 30 min in DAKO Target Retrieval Solution pH9, respectively. Each section was blocked with DAKO Protein block for 10 min at room temp and incubated with primary antibody for 1 hour at room temp (PDGFRα 1:100) or overnight at 4°C (VEGFR2 1:100). The Universal R.T.U. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for CD117/c-kit (A4502) (DAKO). After blocking, each section was incubated with primary antibody for 1 hour at room temp (1:250). Staining was completed with a 5 minute incubation with DAKO 3,3-diaminobenzidine (DAB)+ substrate-chromogen and counterstained with Mayer’s Haematoxylin (Sigma-Aldrich, Poole, UK).

#### Fluorescence in situ hybridisation

FISH analysis was carried out on FFPE sections as previously described [7]. Briefly, BAC clones were purchased from BACPAC Resources Center (Children’s Hospital Oakland Research Institute, Oakland, CA, USA) and FISH-mapped onto metaphase slides to ensure specificity. BAC clones used were: PDGFRα – RP11-58C6 and RP11-819D11; KIT – RP11-452H23 and RP11-586A2; VEGFR2 – CTD2169F23, RP11-1125E19 and RP11-643K20; centromere 4 – RP11-317G22 and RP11-191S2 (Figure 1). BAC DNA was amplified from 10ng starting material using the Illustra™ GenomiPhi™ V2 DNA amplification kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer’s instructions. Probes were labelled with either biotin or DIG using the BioPrime™ DNA Labeling System (Invitrogen, Paisley, UK). TMAs were stripped of paraffin using xylene and rehydrated through a descending ethanol series. The rabbit EnVision™+ HRP System, (DAKO, Carpinteria, CA, USA) was used for PDGFRα (D1E1E) and VEGFR2 (55B1) (Cell Signalling, MA, USA). Antigen retrieval was performed by boiling the slides for 15 min in 10 mM citrate buffer pH6 in a coplin jar and cooled on the bench for 20 min, or for 30 min in DAKO Target Retrieval Solution pH9, respectively. Each section was blocked with DAKO Protein block for 10 min at room temp and incubated with primary antibody for 1 hour at room temp (PDGFRα 1:100) or overnight at 4°C (VEGFR2 1:100). The Universal R.T.U. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for CD117/c-kit (A4502) (DAKO). After blocking, each section was incubated with primary antibody for 1 hour at room temp (1:250). Staining was completed with a 5 minute incubation with DAKO 3,3’-diaminobenzidine (DAB)+ substrate-chromogen and counterstained with Mayer’s Haematoxylin (Sigma-Aldrich, Poole, UK).
more gene copies and with a ratio of gene:centromere of 10:2. Any core with at least one such cell was considered amplified. At least 50 cells per core were screened. All reactions were also assessed on normal brain control cores.

Genetic analysis

DNA was extracted from paraffin-embedded formalin-fixed tissue using the QIAamp DNA Micro Kit (Qiagen, Crawley, UK). Primers for the 129bp IDH1 fragment were designed and PCR carried out according to the method in Hartmann et al. [29]. The PCR product was sequenced and the IDH1R132H mutation analysed using Mutation Surveyor (SoftGenetics, Pennsylvania, USA) and manually with 4Peaks (Mekentosj, Aalsmeer, The Netherlands). MGMT promoter methylation was assessed by bisulfite conversion of the FFPE-extracted DNA using the Qiagen EpiTect Bisulfite Kit and Methylation-Specific PCR (MSP) using standard primers as previously described [30].

Statistical analysis

All statistical tests were performed in R2.11.0 (www.r-project.org). Correlations between categorical variables were analysed by Fishers exact test, and between continuous variable by Students t-test. Cumulative survival probabilities were calculated using the Kaplan-Meier method with differences between survival rates analysed with the log-rank test. Important prognostic information (including Karnofsky performance score) was not available for all cases in this retrospective study, so multivariate analysis was not able to be performed. All tests were two-tailed, with a confidence interval of 95%. P values of less than 0.05 were considered statistically significant.

Results

Amplification of receptor tyrosine kinase genes at 4q12 confers a poor prognosis in glioblastoma

We used specific FISH probes independently designed to recognise PDGFRA, KIT and VEGFR2 genes (Figure 1) to determine the relative frequencies of gene amplification at chromosome 4q12 in our high grade glioma series. Taken together, amplification of any gene or combination of genes at this locus was found in 63/283 (22.2%) cases, including 6/37 (16.2%) anaplastic oligodendroglioma, 3/13 (23.1%) anaplastic astrocytoma, and 54/233 (23.2%) glioblastoma. Although not statistically significant, there appears to be a trend towards the presence of the amplicon being associated with poor clinical outcome in glioblastoma (p = 0.054, log-rank test, overall survival, data not shown).

Taken individually, we observed PDGFRA amplification in 56/236 (23.7%) cases (6/37, 16.2% anaplastic oligodendroglioma; 2/11, 18.1% anaplastic astrocytoma; 48/188, 25.5% glioblastoma); KIT amplification in 49/235 (20.9%) cases (4/30, 13.3% anaplastic oligodendroglioma; 3/11, 27.3% anaplastic astrocytoma; 42/194, 21.6% glioblastoma); and VEGFR2 amplification in 26/190 (13.7%) cases (2/27, 7.4% anaplastic oligodendroglioma; 1/9, 11.1% anaplastic astrocytoma; 25/154, 14.9% glioblastoma).

We next assessed these markers specifically in glioblastoma specimens subclassified according to their predominant morphological features (representative FISH images showing amplification of each gene are shown in Figure 2A). Although there is a trend towards PDGFRA amplification being more common in three of the six morphological subgroups of glioblastoma, these numbers do not reach statistical significance (predominantly small cell (3/13, 23.1%, p = 0.086, Fishers exact test), oligodendroglial (11/43, 25.6%, p = 0.061, Fishers exact test), and sarcomatous (4/13, 30.8%, p = 0.116, Fishers exact test) morphology (Figure 2B)) compared to KIT-amplified (1/15 small cell, 6.7%; 7/40 oligodendroglial, 17.5%; 4/17 sarcomatous, 23.5%) or VEGFR2-amplified (1/9 small cell, 11.1%; 4/37 oligodendroglial, 10.8%; 1/10 sarcomatous, 10.0%) cases. All three genes were individually associated with poor clinical outcome when amplified (Figure 2C) (p = 0.0235, p = 0.0005 and p = 0.0019, respectively, log-rank test, overall survival).

Independent 4q12 gene amplification patterns have phenotypic correlates

We next examined the distinct patterns of amplification we observed for each of the three 4q12 genes in our cohort. Where assessable in the same sample, we observed amplification of all three genes in 24/154 (15.6%) glioblastoma cases (24/41, 58.5% of 4q12-amplified cases), PDGFRA and KIT in 8/154 (5.2%) (8/41, 19.5% 4q12-amplified) and PDGFRA only in 9/154 (5.8%) (9/41, 21.9% 4q12-amplified), (Figure 3A). We did not observe KIT and/or VEGFR2 amplification in the absence of PDGFRA amplification in any of our cases.

Tumours with different patterns of 4q12 gene amplification appeared to mark distinct clinicopathological subgroups of glioblastoma. Cases with both PDGFRA and KIT amplification had a younger age of diagnosis (median = 53.7 yrs) compared with other amplified tumours (median = 60.3 yrs, p = 0.046, t test) (Figure 3B). This is despite the relative clinical outcomes, with tumours amplified for two or three genes doing significantly worse
than those amplified for PDGFRA alone or harbouring normal copy number (p = 0.0159, log-rank test) (Figure 3C).

Assessable grade III tumours all were found to have all three genes amplified, as was the single case of glioblastoma with IDH1 mutation (Table 1). MGMT promoter methylation was significantly less frequent in tumours with all three genes amplified (6/19, 31.6%) than the cohort as a whole (154/247, 62.3%, p = 0.026, Fishers exact test); by contrast tumours amplified for PDGFRA alone had significantly more MGMT promoter methylation (7/9, 77.8%, p = 0.042, Fishers exact test). Those tumours with concurrent PDGFRA and KIT amplification fell in between these figures (3/7, 42.9%) (Table 1).

**Differential receptor expression and DNA copy number**

In addition to mapping the different DNA copy number patterns of 4q12-amplified genes, we also investigated the protein expression of the receptors themselves in the tumour cells (Figure 4A). PDGFRA was expressed in 196/341 (57.5%) cases

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**Figure 2. Amplification of 4q12 genes in glioblastoma.** (A) Representative FISH images showing individual amplification of PDGFRA, KIT, and VEGFR2 in glioblastoma samples RMH6862, RMH6427 and RMH6881, respectively. Original magnification x1000. (B) Pie charts showing the relative proportions of the predominant cellular morphology in glioblastoma samples for cases with PDGFRA, KIT, and VEGFR2 amplification. Fibrillary, purple; gemistocytic, blue; giant cell, green; small cell, light green; oligodendroglial, yellow; and sarcomatous, orange. (C) Kaplan-Meier curves for the effect of amplification of PDGFRA, KIT, and VEGFR2 on overall survival in glioblastoma patients. P values are given using the log-rank test for overall survival. doi:10.1371/journal.pone.0071777.g002
typic conclusions being drawn. Of note, only PDGFRA overexpression in the tumour cells precluded robust expression of VEGFR2 was only observed in 2/23 (8.7%) cases (8/41, 19.5% anaplastic oligodendroglioma; 2/15, 13.3% anaplastic astrocytoma; 69/246, 28.0% glioblastoma); and VEGFR2 expressed in 5/341 (1.5%) cases (0/48, 0% anaplastic oligodendroglioma; 0/16, 0% anaplastic astrocytoma; 5/257, 1.9% glioblastoma). KIT expression was often restricted to isolated tumour cells, whilst VEGFR2 frequently gave a granulated cytoplasmic staining in addition to decoration of the cell membranes.

As well as distinct patterns of protein expression (Table 1), expression of PDGFRA is associated with gene amplification, with 46/52 (88.5%) amplified cases showing receptor expression, compared to 90/169 (53.3%) normal copy cases (p < 0.0001, Fishers exact test). There was also a significant correlation between KIT amplification and protein expression, but only in cases where all three genes were amplified (11/25, 44% KIT-amplified, 14/140, 10% normal copy number, p = 0.0001, Fishers exact test) and not where KIT was amplified with only PDGFRA in the absence of VEGFR2 (1/8, 12.5%, p = 0.396, Fishers exact test). Tumour expression of VEGFR2 was only observed in 2/23 (8.7%) cases with corresponding gene amplification.

Correlating with these differences where phenotypic distinctions associated with tumour cell expression of the three receptors. PDGFRA positive glioblastoma cases showed a trend towards IDH1 mutation (7/8, 87.5% IDH1 mutant tumours positive for PDGFRA vs 135/263, 51.3% IDH1 wild-type, p = 0.068, Fishers exact test), and exhibited a similar distribution of predominant glioblastoma morphologies as for PDGFRA gene amplification (Figure 4B). KIT receptor overexpressing tumours showed a rather different spectrum of morphological correlates, with expression significantly enriched in oligodendroglial tumours (14/37, 37.8% KIT positive cases oligodendrogial vs 50/233 negative, 21.3%, p = 0.0136, Fishers exact test). Intriguingly, KIT receptor expression was also associated with an increased incidence of MGMT promoter methylation (19/32, 59.4% vs 34/268, 12.7%, p = 0.011, log-rank test). The few cases found to express the VEGFR2 receptor in the tumour cells precluded robust phenotypic conclusions being drawn. Of note, only PDGFRA overexpression conferred a worse overall survival in glioblastoma patients (p = 0.028, log-rank test) (Figures 4C).

Loss of vascular receptor expression is a negative prognostic indicator in glioblastoma

As all three RTKs have been implicated in glioblastoma angiogenesis, finally we also investigated their expression in the tumour vasculature (Figure 5A). PDGFRA positive vessels were found in 170/324 (52.5%) cases (15/44, 34.1% anaplastic oligodendroglioma; 5/18, 27.8% anaplastic astrocytoma; 150/262, 57.3% glioblastoma); KIT positive vessels in 79/302 (26.2%) cases (0/41, 19.5% anaplastic oligodendroglioma; 2/15, 13.3% anaplastic astrocytoma; 69/246, 28.0% glioblastoma); and VEGFR2 positive vessels in 306/341 (89.7%) cases (41/49, 83.7% anaplastic oligodendroglioma; 12/17, 70.6% anaplastic astrocytoma; 253/275, 92% glioblastoma). There were no correlations of endothelial cell positivity and gene amplification or tumour cell expression for any of the receptors (Table S1).

There were few tumour phenotypic differences between receptor-positive and negative vessels, save for an increased proportion of oligodendrogial-predominant glioblastoma specimens with KIT positive endothelial cells (25/69, 36.2% vs 33/177, 18.6%, p = 0.0022, Fishers exact test) (Figure 5B). Despite the wide variation in vascular expression of the receptors (26–92%), for all three RTKs, a positive tumour vasculature conferred a better overall survival compared with cases whose vessels were negative (p = 0.008 PDGFRA, p = 0.0012 KIT, p = 0.015 VEGFR2, log-rank test) (Figure 5C). VEGFR2-negative vessels were uncommon (8% glioblastoma cases), however were found to have less glomeruloid proliferation (9/20, 45% vs 188/254, 74%, p = 0.00692, Fishers exact test), and a total absence of arcade-like structures (0/20, 0% vs 57/254, 22.4%, p = 0.0077, Fishers exact test).

Discussion

Recently we and others have established that the profound intratumoural heterogeneity of glioblastomas may extend to the molecular level in terms of gene amplification [5,7,8]. There appear to exist subclones within individual tumour specimens which harbour distinct oncogenic gene amplifications which may be mutually exclusive with each other [5,7,8]. For this reason we have chosen in the present study to use FISH to assess 4q12 gene amplification in glioblastoma and other high grade gliomas in
| RMH_No | PDGFRA_FISH | PDGFRA_IHC | KIT_FISH | KIT_IHC | VEGFR2_FISH | VEGFR2_IHC | Diagnosis                  | Age_years | IDH1 | MGMT          |
|--------|-------------|------------|----------|---------|-------------|------------|---------------------------|-----------|------|---------------|
| RMH6668 | Amplification | Positive  | Amplification | Positive | Amplification | Positive | Glioblastoma multiforme   | 55.96     | Negative | UNmethylated |
| RMH6596 | Amplification | Positive  | Amplification | Positive | Amplification | Positive | Glioblastoma multiforme   | 57.1      | Negative | Methylated   |
| RMH6881 | Amplification | Positive  | Amplification | Positive | Amplification | Negative | Glioblastoma multiforme   | 44.6      | IDH1 | Methylated   |
| RMH6357 | Amplification | Positive  | Amplification | Positive | Amplification | Negative | Glioblastoma multiforme   | 59.71     | Negative | NA           |
| RMH6010 | Amplification | Positive  | Amplification | Positive | Amplification | Negative | Glioblastoma multiforme   | 62.88     | Negative | NA           |
| RMH6862 | Amplification | Positive  | Amplification | Positive | Amplification | Negative | Glioblastoma multiforme   | 67.45     | Negative | Methylated   |
| RMH5697 | Amplification | Positive  | Amplification | Positive | Amplification | Negative | Glioblastoma multiforme   | 75.96     | Negative | UNmethylated |
| RMH5685 | Amplification | Positive  | Amplification | Positive | Amplification | Negative | Glioblastoma multiforme   | 76.49     | Negative | Methylated   |
| RMH6674 | Amplification | Positive  | Amplification | Positive | Amplification | Negative | Glioblastoma multiforme   | 78.13     | Negative | UNmethylated |
| RMH6440 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Anaplastic oligodendroglioma | 40.24     | IDH1 | Methylated   |
| RMH6400 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 49.87     | Negative | Methylated   |
| RMH6574 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 53.34     | Negative | UNmethylated |
| RMH6417 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 55.79     | Negative | UNmethylated |
| RMH6388 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 55.91     | Negative | UNmethylated |
| RMH6382 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 61.47     | Negative | UNmethylated |
| RMH6433 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 64.95     | Negative | UNmethylated |
| RMH5989 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 69.18     | Negative | UNmethylated |
| RMH6416 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 73.68     | Negative | Methylated   |
| RMH5981 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Anaplastic astrocytoma    | 75.25     | Negative | UNmethylated |
| RMH6892 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 76.04     | Negative | UNmethylated |
| RMH6926 | Amplification | Positive  | Amplification | Negative | Amplification | Negative | Glioblastoma multiforme   | 76.07     | Negative | UNmethylated |
| RMH_No  | PDGFRA_FISH | PDGFRA_IHC | KIT_FISH | KIT_IHC | VEGFR2_FISH | VEGFR2_IHC | Diagnosis     | Age_years | IDH1  | MGMT       |
|---------|-------------|------------|----------|---------|-------------|------------|---------------|-----------|-------|------------|
| RMH6667 | Amplification | Positive  | Amplification | NA      | Amplification | Negative   | Glioblastoma multiforme | 55.98     | Negative | UNmethylated |
| RMH6872 | Amplification | Negative  | Amplification | Positive | Amplification | Negative   | Anaplastic oligodendroglioma | 50.17     | Negative | UNmethylated |
| RMH8889 | Amplification | NA        | Amplification | Negative | Amplification | Negative   | Glioblastoma multiforme | 51.83     | Negative | UNmethylated |
| RMH6927 | Amplification | Positive  | Amplification | Positive | Normal       | Negative   | Glioblastoma multiforme | 66.83     | Negative | Methylated   |
| RMH6006 | Amplification | Positive  | Amplification | Negative | Normal       | Negative   | Glioblastoma multiforme | 38.15     | Negative | Methylated   |
| RMH6905 | Amplification | Positive  | Amplification | Negative | Normal       | Negative   | Glioblastoma multiforme | 43.68     | Negative | UNmethylated |
| RMH6935 | Amplification | Positive  | Amplification | Negative | Normal       | Negative   | Glioblastoma multiforme | 45.36     | Negative | Methylated   |
| RMH6669 | Amplification | Positive  | Amplification | Negative | Normal       | Negative   | Glioblastoma multiforme | 53        | Negative | UNmethylated |
| RMH5726 | Amplification | Positive  | Amplification | Negative | Normal       | Negative   | Glioblastoma multiforme | 61.49     | Negative | UNmethylated |
| RMH5966 | Amplification | Positive  | Amplification | Negative | Normal       | Negative   | Glioblastoma multiforme | 79.65     | Negative | NA          |
| RMH5725 | Amplification | Negative  | Amplification | Negative | Normal       | Negative   | Glioblastoma multiforme | 54.47     | Negative | Methylated   |
| RMH6873 | Amplification | Positive  | Normal       | Positive | Normal       | Negative   | Glioblastoma multiforme | 65.5      | Negative | Methylated   |
| RMH6834 | Amplification | Positive  | Normal       | Negative | Normal       | Positive   | Glioblastoma multiforme | 41.97     | Negative | Methylated   |
| RMH6645 | Amplification | Positive  | Normal       | Negative | Normal       | Negative   | Glioblastoma multiforme | 52.15     | Negative | Methylated   |
| RMH5701 | Amplification | Positive  | Normal       | Negative | Normal       | Negative   | Glioblastoma multiforme | 54.66     | Negative | UNmethylated |
| RMH6887 | Amplification | Positive  | Normal       | Negative | Normal       | Negative   | Glioblastoma multiforme | 54        | Negative | UNmethylated |
| RMH6374 | Amplification | Positive  | Normal       | Negative | Normal       | Negative   | Glioblastoma multiforme | 72.35     | Negative | NA          |
| RMH5714 | Amplification | Negative  | Normal       | Negative | Normal       | Negative   | Glioblastoma multiforme | 61.25     | Negative | Methylated   |
| RMH5716 | Amplification | Negative  | Normal       | Negative | Normal       | Negative   | Glioblastoma multiforme | 79.08     | Negative | Methylated   |
| RMH6925 | Amplification | NA        | Normal       | Negative | Normal       | NA         | Glioblastoma multiforme | 54.91     | Negative | Methylated   |

DNA copy number status, tumour receptor expression, diagnosis, age, IDH1 mutation status and MGMT methylation status are provided. Grey boxes, protein expression in conjunction with gene amplification for a given receptor. NA = not available.
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order not to exclude any samples with only rare events that may be missed by bulk tumour profiling.

As we include some cases with only one cell with *bona fide* amplification in our tissue microarray screen, we have identified a higher frequency of 4q12-amplified cases than in previous reports [5,31], although these data are not directly comparable as the microarray- or PCR-based methods used bulk tumour profiling. In our hands, amplification of any of *PDGFRA*, *KIT* or *VEGFR2* is found in nearly a quarter of glioblastoma cases, with individual genes ranging from 14–25%, which may itself be an underestimate due to sampling. The mechanistic significance of isolated tumour cells harbouring distinct gene amplifications in a wider bulk population without these events is still unclear, although *in vitro* these subpopulations are dynamic under ligand or receptor inhibitor exposure [5], and may contribute to the clinical failure of single targeted inhibitors.

**Figure 4. Expression of PDGFRA, KIT and VEGFR2 receptors in glioblastoma tumour cells.** (A) Representative images showing tumour cell positivity for PDGFRA, KIT, and VEGFR2 in glioblastoma samples RMH6374, RMH6357 and RMH6691, respectively. Original magnification ×400. (B) Pie charts showing the relative proportions of the predominant cellular morphology in glioblastoma samples for cases overexpressing PDGFRA, KIT, and VEGFR2. Fibrillary, purple; gemistocytic, blue; giant cell, green; small cell, light green; oligodendrogial, yellow; and sarcomatous, orange. (C) Kaplan-Meier curves for the effect of overexpression of PDGFRA, KIT, and VEGFR2 on overall survival in glioblastoma patients. P values are given using the log-rank test for overall survival.

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**PDGFRA** amplification has been linked with receptor overexpression, younger age at diagnosis, *IDH1* mutation, better clinical outcome and oligodendrogial morphology [4,6]. In this study, the link of DNA copy number to protein expression holds regardless of amplification pattern at 4q12, and the morphological correlates can also be extended to include small cell and sarcomatous tumour cell histologies. A younger age at diagnosis and better clinical outcome is only seen when **PDGFRA** and **KIT** are co-amplified.

4q12 amplification has been linked to a Proneural gene expression profile and IDH1 mutation [4,6]. The association with *IDH1* mutation in our series is only significant when all three genes are amplified. Given the relatively poor prognosis of 4q12 amplified cases in comparison with the *IDH1*/Proneural tumours, it is possible that these cases represent a distinct clinicopathological subgroup of these GBM. Of note, this is a subgroup which also harbours considerably less **MGMT** promoter methylation. By contrast, **MGMT** methylation is common in tumours amplified for **PDGFRA** alone.

**Figure 5. Vascular endothelial cell expression of PDGFRA, KIT and VEGFR2 receptors in glioblastoma.** (A) Representative images showing endothelial cell expression of PDGFRA, KIT, and VEGFR2 in glioblastoma samples RMH6401, RMH6657 and RMH6713, respectively. Original magnification ×400. (B) Pie charts showing the relative proportions of the predominant cellular morphology in glioblastoma samples for cases with vessels expressing **PDGFRA**, **KIT**, and **VEGFR2**. Fibrillary, purple; gemistocytic, blue; giant cell, green; small cell, light green; oligodendrogial, yellow; and sarcomatous, orange. (C) Kaplan-Meier curves for the effect of vascular expression of **PDGFRA**, **KIT**, and **VEGFR2** on overall survival in glioblastoma patients. P values are given using the log-rank test for overall survival.

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Although clearly frequently co-amplified, both KIT and VEGFR2 were less commonly overexpressed at the protein level in response to the increase in DNA copy number, in sharp contrast to PDGFRA. Despite this there were significant differences in glioblastoma phenotypes, with isolated KIT receptor-positive tumour cells corresponding with an increased oligodendrogial morphology and MGMT promoter methylation, an association not previously reported.

It is intriguing to note that in contrast to tumour expression, receptor positivity in the tumour vascular cells correlated with better clinical outcome for all three RTKs. For PDGFRA and KIT, this amounted to 52% and 26% of cases, respectively. Whilst such an association had not previously been reported for PDGFRA, KIT has been found expressed in tumour endothelial cells in 13/22 glioblastomas, with patients having moderate to strong endothelial cell KIT expression showing a favourable survival than those whose tumour vessels showed little or no expression [32,33]. More intriguingly, our study reveals a novel subset of cases (8%) to be negative for the otherwise ubiquitous vascular expression of VEGFR2, a cohort which also has a significantly worse outcome. Although there were no apparent biological correlates of these cases, it is an intriguing possibility that we may have identified a novel endothelial-marker negative poor prognosis subgroup of glioblastoma.

Supporting Information

Table S1 Full clinicopathological and molecular data for the sample set included in this study.

| Supporting Information |
|------------------------|
| **Author Contributions** |
| Designed and carried out the experiments: AB SEL JM CJ. Performed the experiments: AB AJ RL. Analyzed the data: AB SEL SP RL CJ. Contributed reagents/materials/analysis tools: LD SA-S. Wrote the paper: AB CJ. |

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