PDGFRA Gain in Low-Grade Diffuse Gliomas

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Abstract
Glioblastomas with a proneural expression signature are characterized by frequent IDH1 mutations (i.e. genetic hallmarks of secondary glioblastomas) and PDGFRA (platelet-derived growth factor receptor-α) amplification. Mutations in IDH1/2 are frequent and early genetic events in diffuse astrocytomas (World Health Organization grade II), precursor to secondary glioblastomas, but little is known about the role and timing of PDGFRA amplification in these tumors. We assessed PDGFRA gain in 342 low-grade diffuse gliomas by quantitative polymerase chain reaction. Gain in PDGFRA was detected in 27 (16.3%) of 166 diffuse astrocytomas, significantly more frequent than in oligodendrogliomas (3 [2.6%] of 115, p < 0.0001). Analyses using previously published data from our laboratory showed an inverse correlation between PDGFRA gain and IDH1/2 mutations (p = 0.018) or 1p/19q loss (p < 0.0001). The vast majority of diffuse astrocytomas showed IDH1/2 mutations and/or PDGFRA gain (154 [93%] of 166). Mean survival of diffuse astrocytoma patients with PDGFRA gain was 8.8 ± 1.6 years, similar to that with IDH1/2 mutations (7.8 ± 0.5 years) or TP53 mutations (7.6 ± 0.6 years) but significantly longer than those with MET gain (4.4 ± 0.7 years). Dual-color fluorescence in situ hybridization in 6 diffuse astrocytomas with PDGFRA/MET co-gain identified by quantitative polymerase chain reaction revealed that PDGFRA and MET were typically amplified in different tumor cell populations. Tumor cells with coamplification were also focally observed, suggesting intratumoral heterogeneity, even in diffuse astrocytomas.

Key Words: Diffuse astrocytoma, IDH1/2 mutations, MET gain, PDGFRA gain.

INTRODUCTION
The vast majority (~90%) of glioblastomas (World Health Organization [WHO] grade IV) develop without clinical or histologic evidence of a less malignant precursor lesion (primary glioblastomas). In contrast, secondary glioblastomas develop through progression from diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III) (1–3). Although primary and secondary glioblastomas are largely indistinguishable histologically, they significantly differ in their genetic and epigenetic profiles, with the most reliable genetic marker of secondary glioblastomas being IDH1 mutations (4–6).

Glioblastomas may also be classified on the basis of cDNA expression profiles, such as proneural, neural, classic, mesenchymal, proliferative subgroups (7–9). There are certain overlaps between genetic and expression profiles in glioblastomas. IDH1 mutations and PDGFRA amplification are typical for glioblastomas with proneural signature (9); most glioblastomas with IDH1 mutations (11/12, 92%) show a proneural signature, the majority with wild-type PDGFRA, while approximately 30% of glioblastomas with a proneural signature had IDH1 mutations (9).

Diffuse astrocytomas are also characterized by frequent IDH1/2 mutations (10) and a proneural signature (11). However, little is known about PDGFRA amplification in low-grade diffuse gliomas. One immunohistochemical study showed PDGFRA expression in 24 (69%) of 35 of diffuse astrocytomas, although high-level amplification was not detectable by quantitative polymerase chain reaction (PCR) (12). Martinho et al (13) reported PDGFRA gain (>5 copies) in 5 of 10 diffuse astrocytomas using quantitative PCR; immunohistochemistry revealed PDGFRA expression (>5% scores) in 15 (46%) of 33 of diffuse astrocytomas; there was a significant association between PDGFRA amplification and expression in diffuse astrocytomas (13).

In the present study, we screened for PDGFRA gain in 166 low-grade diffuse astrocytomas, as well as in 61 oligoastrocytomas and 115 oligodendrogliomas, and correlated these data with other genetic data (IDH1 mutations, TP53 mutations, 1p/19q loss, MET gain) that were previously reported from our laboratory (10, 14, 15). Furthermore, FISH analysis in 6 diffuse astrocytomas with PDGFRA/MET co-gain was carried out to assess whether PDGFRA and MET are co-amplified in single cells or these alterations are observed in different cell populations, since recently several studies in glioblastomas showed amplification of different receptor tyrosine kinase genes (RTKs; PDGFRA, MET, EGFR) in different tumor cells within single tumors (16, 17).
MATERIALS AND METHODS

Tumor Samples

We analyzed a total of 342 low-grade diffuse WHO grade II gliomas (166 diffuse astrocytomas, 61 oligoastrocytomas, and 115 oligodendrogliomas) from our previous studies (10). These samples were obtained from the Department of Neuropathology, University Hospital Zurich, Switzerland; the Edinger Institute (Neurological Institute) University Hospital Frankfurt, Frankfurt, Germany; the Institute of Neuropathology and Department of Neurosurgery, University Hospital Muenster, Muenster, Germany; the Departments of Neuropathology and Neurosurgery, University Hospital Essen, Essen, Germany; the Department of Pathology, Gunma University, Gunma, Japan; the Institute of Neuroscience, Bordeaux, France; and the Department of Neurosurgery, University Hospital Bern, Bern, Switzerland. The study was approved by the International Agency for Research on Cancer Ethics Committee. All brain tumor specimens were obtained at the time of first surgical resection.

Quantitative PCR

DNA was extracted from formalin-fixed, paraffin-embedded histologic sections as previously reported (2). To screen for gain of the PDGFRA gene, quantitative PCR was performed, as previously reported (14). Briefly, quantitative PCR was performed using an iCycler (BioRad) in a 20-μL reaction mixture composed of 10 μL 2× iQ SYBR Green Supermix, 2 μL H2O, 1.6 μL template DNA (~20 ng/μL), and primers. The sequence of primers was as follows: 5’-GCT GTT TCT GTT GAC TTT GAA C3’ (sense) and 5’-AAA CCA GGA ACT CAG AGA GGA-3’ (antisense) for exon 2 of the PDGFRA (PCR product, 125 bp), 5’-TCA GCT ACA GAT GCC TTG ATC C-3’ (sense) and 5’-GCC AAA GTC ACA GAT CTT CAC AAT-3’ (antisense) for exon 18 of the PDGFRA (PCR product, 124 bp), and 5’-GTG CAC CTG ACT CCT GAG GAG A-3’ (sense) and 5’-CCT TGA TAC CAA CCT GCC CAG-3’ (antisense) for the β-globin (PCR product, 102 bp) (13, 18, 19).

DNA was first denatured at 95°C for 12 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, and annealed at 55°C for 20 seconds, with extension at 72°C for 45 seconds. The final extension step was at 72°C for 2 minutes. Each plate contained measurements for the target and the reference genes. Each reaction was performed in triplicate. We calculated the PCR cycle number (Ct) value, ΔCt (Ct [PDGFRA]-Ct [β-globin]), ΔΔCt (ΔCt [tumor]-ΔCt [normal]) values, and the relative copy number (2-ΔΔCt), as previously reported (20). For normalization, DNA was extracted from paraffin-embedded sections of 20 samples from various human tissues including brain. A tolerance interval with a confidence interval of 95% was determined from the SD of normal DNA, as reported (20). We considered that the presence of PDGFRA gain was assessed when PCR fragments of both PDGFRA exon 2 and exon 18 showed positive results, that is, showed a copy number more than 2.879 (Fig. 1).

Dual-Color FISH Analysis

Dual-color FISH analysis was performed on 5-μm-thick formalin-fixed, paraffin-embedded tissue sections as previously described (21, 22). Briefly, sections were deparaffinized, digested with pepsin, heat denatured, and allowed to hybridize with probe sets overnight 37°C in a humidified oven. A Spectrum Green–labeled home brew probe for PDGFRA (BAC clone RP11-231C18; CHORI BACPAC Resources Center, Oakland, CA) diluted 1:10 in DenHyb (Institut-Insitus, Albuquerque, NM) was paired with Spectrum Orange–labeled MET (BAC clone RP11-163C9). After washes to remove excess unbound probe, the nuclei were counterstained with 10 mL DAPI (Institut-Insitus), and slides were coverslipped. The fluorescent signals were enumerated under an Olympus BX41 fluorescent microscope with appropriate filters (Olympus, Melville, NY). For each hybridization, slides were scanned for regional variability with green and orange signals enumerated in nonoverlapping nuclei. Gene amplifications for PDGFRA and/or MET were defined by the presence of at least 12 probe signals in individual nonoverlapping nuclei.

Statistical Analysis

Statistical analysis was performed using the statistical software IBM SPSS Statistics for Windows, version 20.0 (IBM Corporation, Armonk, NY). The χ2 test and Fisher exact test were used to assess the association of clinical variables and genetic alterations. Survival was evaluated using the Kaplan-Meier method, and survival curves were compared using the logrank test. Overall survival was calculated from the date of surgery until death or the end of follow-up. Multivariate analysis was performed to test the potential influence of baseline characteristics on survival. The Cox proportional hazards model was used to assess the effect of different combinations of genetic alterations on patients’ survival after adjusting for age at diagnosis (<40 vs ≥40 years) and sex. Factors with no significant association with survival in the multivariate analysis (p > 0.05) were eliminated. The remaining factors in the multivariate proportional hazards model (p < 0.05) were considered to be independent predictors of survival.

RESULTS

Frequency of PDGFRA Gain in Low-Grade Diffuse Gliomas

Quantitative PCR revealed PDGFRA gain in 9.9% low-grade diffuse gliomas (Table; Fig. 1). PDGFRA gain was significantly more frequent in diffuse astrocytomas (16.3%) than in oligoastrocytomas (6.6%, p = 0.04) or oligodendrogliomas (2.6%, p < 0.0001; Table). There was no significant difference in histologic variants between diffuse astrocytomas with and diffuse astrocytomas without PDGFRA gain (fibrillary, 16% vs gemistocytic, 21%, p = 0.449). Most of the gains was low level (3–5 copies), whereas 5 cases (3 diffuse astrocytoma with IDH1/2 mutations plus TP53 mutations and 2 diffuse astrocytomas with IDH1/2 mutation only) showed a high-level gain (>5 copies).

PDGFRA Gain and Other Genetic Alterations in Low-Grade Diffuse Gliomas

We correlated PDGFRA gain with other genetic alterations in low-grade diffuse gliomas that were previously reported from our laboratory (10, 14, 15, 23). The vast majority of
Diffuse astrocytomas (154 [93%] of 166) carried PDGFRA gain and/or IDH1/2 mutations. PDGFRA gain was significantly more frequent in diffuse astrocytomas, oligodendrogliomas, and low-grade diffuse gliomas without IDH1/2 mutations (36.8% vs 13.6%, p = 0.018; 16.7% vs 1.0%, p = 0.028; 24.3% vs 8.3%, p = 0.006, respectively). PDGFRA gain was most frequent in gliomas that lacked any of the common genetic alterations (i.e. IDH1/2 mutations, TP53 mutations, and 1p/19q loss; 28.6%) (Table). PDGFRA gain was found in low-grade diffuse gliomas with TP53 mutation ± IDH1/2 mutations (16.3%) and those with IDH1/2 mutation only (10.5%) but was rare in tumors with 1p/19q loss ± IDH1/2 mutations (1.4%; Table). PDGFRA gain was mutually exclusive, with 1p/19q loss in all but 2 cases. In diffuse astrocytomas, PDGFRA gain positively correlated with MET gain (p = 0.016) and inversely correlated with 1p/19q loss (p = 0.005) and IDH1/2 mutations (p = 0.018).

FISH Analysis for PDGFRA and MET Gain

Dual-color FISH analysis was carried out in 6 diffuse astrocytomas with PDGFRA and MET co-gain detected by quantitative PCR. In all 6 cases, PDGFRA and MET amplifications were confirmed to be present in scattered neoplastic cells, likely explaining the lower level copy number gains found by a dose-averaging technique such as PCR. In 3 tumors, both PDGFRA and MET amplifications were detected in a similar fraction of neoplastic cells. Gains in PDGFRA and MET were observed mostly in separate cell populations (Fig. 2). In 1 tumor, MET gain was more prevalent, and in 2 tumors, PDGFRA gain was predominant (Fig. 2). Rare tumor cells with coamplification of PDGFRA and MET was also detected in all 6 tumors analyzed (Fig. 2).

Genetic Alterations and Survival of Diffuse Astrocytomas

The mean survival of diffuse astrocytoma patients with PDGFRA gain (8.8 ± 1.6 years) was similar to that in patients with IDH1/2 mutations (7.8 ± 0.5 years) and in patients with TP53 mutations (7.6 ± 0.6 years) but was significantly longer than those with MET gain (4.4 ± 0.7 years, p = 0.025; Fig. 3).

<table>
<thead>
<tr>
<th>Tumors With PDGFRA Gain</th>
<th>n</th>
<th>%</th>
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<tbody>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse astrocytoma</td>
<td>27/166</td>
<td>16.3*</td>
</tr>
<tr>
<td>Oligoastrocytoma</td>
<td>4/61</td>
<td>6.6</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>3/115</td>
<td>2.6</td>
</tr>
<tr>
<td>Genetic alterations</td>
<td></td>
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<tr>
<td>TP53 mutation ± IDH1/2</td>
<td>20/123</td>
<td>16.3</td>
</tr>
<tr>
<td>1p/19q loss ± IDH1/2</td>
<td>2/141</td>
<td>1.4</td>
</tr>
<tr>
<td>IDH1/2 mutation only</td>
<td>6/57</td>
<td>10.5</td>
</tr>
<tr>
<td>No alteration*</td>
<td>6/21</td>
<td>28.6</td>
</tr>
<tr>
<td>Total</td>
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*Significantly more frequent than in oligoastrocytomas (p = 0.84) and oligodendrogliomas (p < 0.001).

*No IDH1/2 mutation, TP53 mutation, or 1p/19q loss.
similar to those with PDGFRA gain only. After adjusting for age and sex, we established multivariate survival analysis for overall survival. The model was designed to consider each of several gene alterations independently. IDH1/2 mutations were found to be the only independent prognostic factor for overall survival (Cox regression hazards ratio, 0.025; 95% confidence interval, 0.001–0.498; p = 0.016).

DISCUSSION

Platelet-derived growth factor (PDGF), a major mitogen for connective tissue cells and glia, plays a number of critical roles in normal embryonic development, cellular differentiation, and response to tissue damage, as well as in pathologic processes, such as wound healing, inflammation, and neoplasms (24–27).

The PDGF is recognized by 2 types of cell surface receptors, that is, PDGFRα and PDGFRβ, which are cell-surface tyrosine kinase receptors for members of the PDGF family (24, 25).

Here, we assessed PDGFRA gain in a large number of low-grade diffuse gliomas with different histology and genetic alterations. PDGFRA gain was present in 16.3% of diffuse astrocytomas but rare (2.6%) in oligodendrogliomas. It was noted that 154/166 (93%) of diffuse astrocytomas carried IDH1/2 mutations and/or PDGFRA gain, indicating that the vast majority of diffuse astrocytomas carry genetic alterations typical for glioblastomas with proneural expression signature as early genetic events.

In most of the low-grade diffuse gliomas, the levels of PDGFRA gain detected were relatively low (3–5 copies).

![Dual-color FISH analysis in diffuse astrocytomas](image-url)

**FIGURE 2.** Dual-color FISH analysis in diffuse astrocytomas shows intratumoral heterogeneity of amplification of PDGFRA (green) and MET (red). (A) Amplifications of PDGFRA and MET were observed in separate cells in the same tumor. Amplifications of PDGFRA (B) or MET (C) were seen in individual diffuse astrocytoma cells within the same specimen. (D) Rare tumor cells displayed coamplification of PDGFRA and MET.
However, low-level gain may have significant biologic effects. For example, PDGFRA gain (~4 copies) was associated with PDGFRA overexpression in non–small cell lung cancer cells, and in a small fraction of squamous cell primary non–small cell lung cancers (28). Similarly, FISH and quantitative PCR revealed PDGFRA gain (2–6 copies) in 3 of 11 diffuse intrinsic pontine gliomas, and all of those cases showed PDGFRA overexpression (29). Gain in PDGFRA (3–5 signals per nucleus) detected by chromogenic in situ hybridization was observed in 47% of medulloblastomas and primitive neuroectodermal tumors and was significantly associated with poorer patient outcome (30).

It has been shown that diffuse astrocytoma patients with PDGFRA overexpression detected by immunohistochemistry had an unfavorable outcome (31). In contrast, in a study of 40 WHO grade II astrocytomas and oligoastrocytomas, Ribom et al (32) showed that PDGFRA overexpression, detected by immunohistochemistry, was associated with a favorable patient outcome. Another immunohistochemical study showed an association between PDGFRA expression and a longer survival in patients with WHO grade I-II astrocytomas (13). In the present study, survival of diffuse astrocytoma patients with PDGFRA gain was similar to that of those with IDH1/2 or TP53 mutations, which were significantly more favorable than those with MET gain (Fig. 3).

It has been shown that several RTKs are amplified in different tumor cell populations in brain tumors. Snuderl et al (16) carried out FISH for 3 RTKs (PDGFRA, MET, and EGFR) in 350 glioblastomas and found that 16 had more than 1 amplified RTK. Multiple RTKs were usually not present in the same tumor cell but were present in distinct intermingled subpopulations of tumors cells (16). Similarly, another study with dual-color FISH in 8 EGFR and PDGFRA–coamplified glioblastomas revealed distinct tumor cell subpopulations amplified for only 1 RTK (17). Paugh et al (33) reported focal amplifications of genes in the RTK-Ras-PI3K signaling pathway (PDGFRA and MET being most frequent) in 47% of diffuse intrinsic pontine gliomas. Again, some tumors contained amplification of more than 1 RTK, and there were cases of PDGFRA/MET coamplification within the same tumor cells, as well as an example of a heterogeneous tumor containing independent amplification of PDGFRA in 1 focal region and MET in a different area of the tumor (33).

In the present study, we similarly found intratumoral heterogeneity for PDGFRA/MET gain in 6 diffuse astrocytomas with PDGFRA and MET co-gain detected by quantitative PCR using dual-color FISH analysis. The PDGFRA and MET amplifications were typically present in different cell populations, although rare cells with PDGFRA and MET coamplification were also seen in all tumors analyzed. The presence

![Graph showing cumulative survival](image)

**FIGURE 3.** The mean survival of diffuse astrocytoma patients with PDGFRA gain (8.8 ± 1.6 years) was similar to those with IDH1/2 mutations (7.8 ± 0.5 years) or those with TP53 mutations (7.6 ± 0.6 years) but significantly longer than those with DMBT1 homozygous deletion (4.6 ± 1.0 years, p = 0.033; data not shown) (15) or those with MET gain (4.4 ± 0.7 years, p = 0.025).
of high-level amplification (>12 copies) in scattered cells likely explains the lower level gains (3–5 copies) detected by a dose-averaging technique such as PCR. Nonetheless, this cellular heterogeneity is rather unexpected because, in contrast to glioblastomas, which show remarkable genomic instability and intratumoral genetic heterogeneity, diffuse astrocytomas are generally considered more genetically stable. It remains to be shown whether and to what extent such intratumoral genetic heterogeneity in diffuse astrocytomas affects progression to secondary glioblastomas.

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