**PDGFRA gene rearrangements are frequent genetic events in PDGFRA-amplified glioblastomas**

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Gene rearrangement in the form of an intragenic deletion is the primary mechanism of oncogenic mutation of the epidermal growth factor receptor (EGFR) gene in gliomas. However, the incidence of platelet-derived growth factor receptor-α (PDGFRA) gene rearrangement in these tumors is unknown. We investigated the PDGFRA locus in PDGFRA-amplified gliomas and identified two rearrangements, including the first case of a gene fusion between kinase insert domain receptor (KDR) (VEGFR2) and the PDGFRA gene, and six cases of PDGFRA D8,9, an intragenic deletion rearrangement. The PDGFRA D8,9 mutant was common, being present in 40% of the glioblastoma multiformes (GBMs) with PDGFRA amplification. Tumors with these two types of PDGFRA rearrangement displayed histologic features of oligodendroglioma, and the gene products of both rearrangements showed constitutively elevated tyrosine kinase activity and transforming potential that was reversed by PDGF blockade. These results suggest the possibility that these PDGFRA mutants behave as oncogenes in this subset of gliomas, and that the prevalence of such rearrangements may have been considerably underestimated.

[Keywords: Copy number alteration; glioma; PDGFRA gene rearrangement; receptor tyrosine kinase]

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Although the molecular mechanisms underlying gliomagenesis are rapidly being uncovered, gliomas remain nearly uniformly fatal with the standard of care that includes surgical resection followed by radiation therapy and temozolomide. Many therapeutic trials of signal transduction inhibitors have failed to improve the outcome of glioblastoma multiforme (GBM) patients. This may be the case in part because, although molecular targeted therapy is a rational strategy, GBM comprises a heterogeneous group of tumors, only a subset of which responds to any particular inhibitor. Such a possibility makes accurate subdivision of gliomas by signaling characteristics a critical step toward therapeutic success.

Recent analysis of a large number of gliomas has shown that they can be divided into three or four groups by genomic and transcriptomal measures (Phillips et al. 2006; Verhaak et al. 2010), and that these transcriptomal subgroups reflect the common signaling abnormalities found in these tumors (Brennan et al. 2009). Two of the largest subgroups of gliomas are the “classical” GBMs that predominantly harbor epidermal growth factor receptor (EGFR) amplification and rearrangement, and the “proneural” GBMs that are predominantly driven by platelet-derived growth factor (PDGF) signaling. Approximately one-third of the proneural/PDGF tumors show...
amplification of the PDGF receptor α (PDGFRA) locus, while the remainder shows overexpression of the PDGF ligand [Brennan et al. 2009]. EGFR and PDGFR inhibition using single agents has limited success in the treatment of unselected GBM patients; however, such an approach may be more effective in particular patients with gliomas harboring genomic activation of either the EGFR or PDGFRA loci [Wen et al. 2006].

Amplification of the EGFR gene is the most frequent genetic alteration of receptor tyrosine kinases [RTKs] found in GBMs (43%) and correlates with EGFR overexpression [The Cancer Genome Atlas Research Network 2008]. Among GBMs with EGFR overexpression, EGFR gene amplification is identified in 70%–90%, many of which additionally express various truncated mutant forms of EGFR, the most common of which is EGFRvIII (Ohgaki and Kleihues 2007). EGFRvIII is generated by an intrachromosomal rearrangement leading to a 267-base-pair [bp] in-frame deletion of exons 2–7 in the extracellular domain, leading to ligand-independent constitutive activation [Sugawa et al. 1990, Wong et al. 1992, Ohgaki and Kleihues 2007]. This particular mutant form is found in 50%–60% of GBMs with an amplification of the EGFR gene, and is not found in GBMs without amplification or normal tissue [Gan et al. 2009].

In contrast to the common and well-known activating mutations of EGFR, reports of activating rearrangements of PDGFRA in GBM are only sporadic. There is one report of an in-frame deletion of the Ig-like domain [PDGFRA8,9 mutant], and a second with the C-terminal end of PDGFRA [Kumabe et al. 1992; Rand et al. 2005]. Additionally, deep sequencing analysis of GBM has also found several point mutations of the Ig-like domain [Verhaak et al. 2010]. Although PDGFRA amplification is less common in gliomas than EGFR amplification, PDGFRA gene amplification is found in 11% of GBMs, making it the second most frequent RTK gene amplified in this family of tumors [The Cancer Genome Atlas Research Network 2008]. The question remains as to whether activating rearrangements are as frequent at the PDGFRA locus of PDGFRA-amplified gliomas as they are at the EGFR locus of EGFR-amplified gliomas.

In addition to the intragenic rearrangements described above, the process of gene fusion can also create constitutively active signaling proteins. Historically, most gene fusions were identified in hematological disorders and pediatric sarcomas, and generally were thought to be rare in most solid tumors. However, recent technical advances in array-based analysis have allowed increased cytogenetic characterization of complex rearrangements in solid tumors, as exemplified by the identification of the TMPRSS2-ETS fusion in prostate cancer [Tomlins et al. 2005]. Therefore, it is possible that the underrepresentation of gene fusions in solid tumors may be due in part to technical issues rather than pathogenetic differences between them and hematological cancers [Mitelman et al. 2004, 2007; Kumar-Sinha et al. 2006]. The only glioma-associated gene fusion reported thus far was a fusion between FIG and ROS identified in the U118MG glioblastoma cell line, and BRAF and RAF1 gene-associated gene fusions reported recently in childhood pilocytic astrocytomas (Charest et al. 2003; Jones et al. 2008, 2009). To date, a fusion gene has not been identified in adult GBM tumor samples.

In this study, we screened for PDGFRA gene rearrangements in glioma samples, including intragenic deletions and unbalanced fusions. First, based on high-resolution array-based comparative genomic hybridization [aCGH] data, we identified several patterns of copy number alterations [CNAs] involving the PDGFRA gene in glioma samples, including the first example of a gene fusion in a human glioblastoma patient sample. This gene fusion, presumably arising from a small paracentric inversion with segmental amplification on chromosome 4q, results in the fusion of the 5′ segment of the kinase insert domain receptor [KDR] gene with the 3′ segment of the PDGFRA gene, generating a KDR-PDGFRA (KP) fusion transcript. We then screened for the PDGFRA8,9 deletion mutation in gliomas and identified six tumors with this mutation. Of note, all tumors with either the PDGFRA8,9 or the KP fusion occurred in GBMs with PDGFRA gene amplification. Subsequently, we performed a functional analysis of KP and PDGFRA8,9 proteins in NIH3T3 fibroblasts and demonstrated that both PDGFRA mutants are transforming oncogenes. The frequency of PDGFRA8,9 in our series of PDGFRA-amplified GBM was 40%, nearly similar to the frequency of EGFRvIII in EGFR-amplified GBM.

Results

Identification of a novel fusion RTK, KP, in a glioma surgical specimen

While only a subset of gene fusions arising from interchromosomal or intrachromosomal rearrangements are likely to be associated with an alteration in copy number, we hypothesize that these rare events can be identified in a general screen by detecting intragenic transitions in copy number. First, high-resolution aCGH was used to screen for potential rearrangements involving tyrosine kinase [TK] genes in glioma samples. An oligonucleotide microarray was designed to densely cover 89 TK genes for aCGH hybridization. Cases showing intragenic copy number variation between the 5′ and 3′ ends of TK genes were studied further using a second, custom-designed, high-density CGH array to fine-map the intragenic breakpoints, which eventually guided the identification of the novel gene rearrangements involving these genes. With the initial screening of 10 glioma tumor samples and 20 cell lines, four gene fusions were identified. These included the known fusion FIG-ROS characterized in the U118MG cell line and serving as a proof of principle for the method [data not shown], and a novel fusion, KP, that was isolated from a patient sample and was investigated further in this study. The aCGH screen identified a complex amplicon within 4q12, spanning the PDGFRA and KDR genes. Both the PDGFRA and KDR genes are localized on chromosome 4q12 and are transcribed in opposite directions [Fig. 1A,B]. The profile suggested amplification of chromosomal
regions preserving the 5′ end of KDR (excluding the transmembrane and kinase domains) and the kinase domain and 3′ portion of PDGFRA [Fig. 1B]. Based on the pattern of copy number changes across this region, we hypothesized that an intrachromosomal rearrangement may have resulted in a gene fusion between KDR and PDGFRA. We reviewed all of the possible exon combinations between KDR and PDGFRA and eliminated those that would produce out-of-frame fusion transcripts. Then, a panel of RT–PCR assays was designed to identify the potential fusion transcript in RNA extracted from this tumor. RT–PCR with different gene-specific primers confirmed a fusion transcript in the patient specimen (Fig. 1C). Subsequent sequencing analysis indicated that the breakpoint fell within exon 10 of the PDGFRA gene and intron 13 of the KDR gene, which functions as an in-frame cryptic exon, resulting in an in-frame fusion [Fig. 1B, D]. A partial sequence of the KP transcript around the fusion point is shown in Figure 1D, along with the predicted amino acid sequence. Examination of the fusion junction revealed an 8-bp sequence overlap between intron 13 of KDR and exon 10 of PDGFRA [AACATCAT], suggesting microhomology-mediated nonhomologous end-joining as a possible mechanism [Fig. 1D; Campbell et al. 2008]. Because the KDR gene is on the minus strand of chromosome 4, the creation of this fusion appears to have involved an intrachromosomal inversion. The PDGFRA copy number transition determined by aCGH aligns with the sequence-based breakpoint within the 60-bp resolution of the platform. The KDR transition is resolved to a 190-bp region of repeat sequence in intron 13 that also harbors the cryptic exon determined by sequencing (Supplemental Fig. S1). The predicted fusion protein consists of an extracellular domain containing the first to sixth Ig-like domain of KDR and the disrupted fifth Ig-like domain of PDGFRA, followed by the intact transmembrane domain and TK

Figure 1. Identification of the novel KP fusion in a glioblastoma tumor. [A] A complex amplicon on chromosome 4 is profiled by high-resolution aCGH [median probe interval, 53 bp]. Relative amplification of the 3′ portion of PDGFRA and the 5′ portion of the KDR locus was identified, suggesting possible gene fusion. [B] Schematic diagrams of KDR and PDGFRA partial gene structures as well as the formation of the KP fusion transcript, and of the predicted KP, KDR, PDGFRA, and PDGFRAK5,6 mutant proteins. Because the KDR gene is on the minus strand of chromosome 4, the creation of this fusion appears to have involved an intrachromosomal inversion. [Ig-like domain] Immunoglobulin-like domain; [S] signal sequence; [TM] transmembrane domain; [JM] juxtamembrane domain; [C] cryptic exon from KDR intron 13. [C] RT–PCR detection of the KP fusion. The presence of the KP fusion was revealed by RT–PCR using different combinations of KDR forward primers and PDGFRA reverse primers. [M] Molecular marker φX174/HaeIII (Invitrogen); [K9 and K13] forward primers in KDR exons 9 and 13, respectively; [P7, P9, and P12] reverse primers in PDGFRA exons 7, 9, and 12. [D] Partial sequence of the KP fusion transcript. Sequencing of RT–PCR products of K13/P12 and K9/P12 identified the sequence around the fusion point of the KP transcript. The transcript is an in-frame fusion of KDR exon 13 [red] to PDGFRA exon 10 [yellow] with an intervening cryptic exon from KDR intron 13 [blue]. The green area indicates 8 bases of overlap sequence between intron 13 of KDR and exon 10 of PDGFRA. The translated amino acid sequence is shown below the transcript sequence.
domain of PDGFRA [Fig. 1B]. Subsequently, we screened for the KP fusion by RT–PCR in an additional 205 glioma samples (total, 215 samples), but the KP fusion was not identified in those tumors [data not shown].

\textit{PDGFRA}^{8, 9} is a frequent gene rearrangement in PDGFRA-amplified GBM

\textit{PDGFRA}^{8, 9} is formed by an in-frame deletion of 243 bp in exons 8 and 9 of the extracellular portion [Fig. 1B; Kuma\-be et al. 1992]. This type of mutation is reminiscent of the \textit{EGF}rVIII mutant that is frequently observed in GBM. Given that \textit{PDGFRA}^{8, 9} is a known transforming oncogene due to ligand-independent receptor activation, it is important to determine the frequency of this rearrangement in gliomas [Clarke and Dirks 2003]. We initially screened for the \textit{PDGFRA}^{8, 9} mutant with RT–PCR analysis in 212 glioma samples from the KP fusion screening. Six cases of \textit{PDGFRA}^{8, 9} transcripts were identified in those tumors [Fig. 2A; Supplemental Fig. S2]. Interestingly, although five positive tumors expressed both wild-type and deletion mutant [DM] transcripts, one tumor (MSK543) expressed only the DM transcript, possibly suggesting a small homzygous deletion of this locus [Fig. 2A].

In gliomas, amplification of the \textit{EGF}r gene intimately correlates with not only EGFR overexpression, but also \textit{EGF}r gene rearrangement [Ekstrand et al. 1991; Mellinghoff et al. 2005]. Therefore, we sought to determine the incidence of \textit{PDGFRA}^{8, 9} mutation in PDGFRA-amplified gliomas. PDGFRA gene status was determined by quantitative PCR (q-PCR) analysis in the 87 tumors with significant PDGFRA mRNA expression [Supplemental Fig. S2]. Gene amplification was identified in 15 out of the 87 cases [Fig. 2B,C]. Interestingly, all amplifications were detected in GBMs, and all \textit{PDGFRA}^{8, 9} mutants were in these amplified tumors [six out of 15 amplified GBMs; 40%]. Amplification of the PDGFRA gene in these six positive samples was further confirmed with aCGH analysis [data not shown]. These results suggest that \textit{PDGFRA}^{8, 9} is a recurrent gene rearrangement in PDGFRA-amplified GBM.

**Tumors with either PDGFRA gene rearrangement present characteristic features of oligodendrogliaoma**

Aberrant PDGF signaling induces gliomas with clear oligodendroglioma features in mouse models [Uhrbom et al. 1998; Dai et al. 2001]. Therefore, we examined the immunohistochemistry of GBMs with the PDGFRA gene rearrangement to see if robust PDGF signaling from the mutant receptor correlates with characteristic morphology of oligodendrogliaoma, recognized as GBM with oligodendroglial component [GBMO]. Tumors with the PDGFRA gene rearrangement [MSK111, KB6, MSK538, and MSK572] demonstrated histological features of oligodendrogliaoma to various degrees, as supported by the presence of round nuclei, perinuclear halos, mingemistocytes accompanied by prominent PDGFRA, and Olig2 immunonegativity, suggesting PDGF signaling [Fig. 3]. As expected, these tumors also showed regions of astrocytic features with diverse level of GFAP expression. In particular, the tumor with the KP fusion expressed higher levels of GFAP relative to tumors with the \textit{PDGFRA}^{8, 9} mutation. For comparison, to further investigate whether PDGFRA expression correlates with histological features of oligodendrogliaoma, we examined the immunohistochemistry of GBMs showing various levels of the PDGFRA transcript. Two samples [MSK261 and KTS2] were found to have the PDGFRA transcript but not PDGFRA gene amplification, and KTS5 had no PDGFRA transcript [Fig. 2A; Supplemental Figs. S2, S3]. MSK261 exhibited typical gemistocytic astrocytomas with strong GFAP immunonegativity, while PDGFRA and Olig2 immunonegativity were detected in only subsets of cells. KTS2 had strong GFAP immunonegativity with slight PDGFRA immunonegativity and showed mainly astrocytic features, although few cells with oligodendrocytic features such as perinuclear halos were intermingled. KTS5 had no PDGFRA immunonegativity but a high level of GFAP immunonegativity, showing typical fibrillar astrocytic features. In contrast, MSK352x with PDGFRA amplification included histological features of high-grade oligodendroglioma as well as tumors with the mutant receptor [Fig. 3]. Oligodendrogliaoma [MSK486] showed obvious PDGFRA and Olig2 immunonegativity [Supplemental Fig. S3].

To verify that increased PDGFRA signaling was associated with characteristic morphologies of oligodendrogliaoma, we compared the histological features of 56 tumors with PDGFRA mRNA expression [Supplemental Table S1]. Features of oligodendrogliaoma were identified in six out of 45 tumors without PDGFRA amplification, and in six out of 11 tumors with PDGFRA amplification [Fisher’s exact test, two-sided: \( P = 0.0075 \), comparing the no amplification group with the amplification group], suggesting that excessive PDGFRA signaling correlates with the histological features of oligodendrogliaoma.

**The KP fusion is a constitutively phosphorylated TK similar to the PDGFRA^{8, 9} mutant**

Given that the PDGFRA^{8, 9} protein is autophosphorylated in a ligand-independent manner [Clarke and Dirks 2003], we examined whether the KP fusion behaves as a constitutively active TK as well. We generated retroviral expression vectors encoding the KP fusion and PDGFRA^{8, 9}. These constructs were used individually to infect NIH3T3 mouse fibroblasts, allowing a comparison of the TK activity between the KP fusion and PDGFRA^{8, 9}. In these cells, under serum-deprived conditions, both the KP fusion and PDGFRA^{8, 9} proteins were autophosphorylated on tyrosine residues and associated with the activation of downstream MAPK and PI3K signaling pathways, as detected by phosphorylated Erk and phosphorylated Akt, respectively [Fig. 4A]. In contrast, the activity of these signaling components in cells expressing vector alone, a kinase-inactive mutant of the KP fusion [K831R], wild-type PDGFRA, and wild-type KDR were low [Fig. 4A; Supplemental Fig. S4A].

To further examine whether the KP fusion protein has an intrinsic TK activity, we performed an in vitro kinase assay. As shown in Figure 4B, the KP fusion is capable of autophosphorylation under serum-deprived conditions.
In contrast, the tyrosine phosphorylation of the K831R mutant was undetectable under these conditions. The KP fusion construct used in this experimental setting contains a c-myc or HA epitope in the C-terminal portion of the protein (see below). To exclude the possibility that these epitopes affect the kinase activity of the protein, we analyzed the level of phosphotyrosine using a KP fusion construct without an epitope. Both constructs had similar elevations of phosphotyrosine of the KP fusion, activation of the MAPK and PI3K signaling pathways, and transformed cell morphology (see below; data not shown).

The KP fusion construct used in this experimental setting contains a c-myc or HA epitope in the C-terminal portion of the protein (see below). To exclude the possibility that these epitopes affect the kinase activity of the protein, we analyzed the level of phosphotyrosine using a KP fusion construct without an epitope. Both constructs had similar elevations of phosphotyrosine of the KP fusion, activation of the MAPK and PI3K signaling pathways, and transformed cell morphology (see below; data not shown).

The KP fusion has an intact kinase domain of PDGFRA. Therefore, to investigate whether the KP fusion behaves as a constitutively active PDGFRA, we compared the KP fusion-induced activation of the MAPK and PI3K pathways under serum-deprived conditions with WT-PDGFRA and WT-KDR after cognate ligand stimulation. As shown in Figure 2.

The PDGFRA\textsuperscript{D8,9} mutant is a recurrent gene rearrangement. (A) RT–PCR detection of the PDGFRA\textsuperscript{D8,9} mutant in glioma tumors. Identification of the PDGFRA\textsuperscript{D8,9} mutant was revealed by RT–PCR analysis with a PDGFRA-S3 and PDGFRA-A2 primer pair that covers an in-frame junction. The putative PCR products of the wild type and the PDGFRA\textsuperscript{D8,9} mutant are 885 and 642 bp, respectively. Molecular weight (M) is shown in the figure. Sequencing of RT–PCR products from the lower bands of MSK538, MSK543, MSK572, MSK573, KB6, KTS647, and KT648 revealed an in-frame deletion of 243 bp. PMXIG-PDGFRA and PDGFRA\textsuperscript{D8,9} plasmids were used as controls for wild-type PDGFRA [WT] and the PDGFRA\textsuperscript{D8,9} mutant [DM], respectively. KTS647 and KTS648 are samples from the same tumor taken from different regions during resection. Samples with the mutation are marked with an asterisk. (B) PDGFRA gene quantitation in GBM tumors. PDGFRA gene amplification was identified using q-PCR analysis in tumors showing high expression of the PDGFRA transcript (Supplemental Fig. S2). The Y-axis indicates average PDGFRA gene quantities relative to the reference sample and 18S gene. Average DNA quantities greater than four were defined as gene amplification (dotted line). (White bars) Normal lymphocytes; (Blue bars) GBMs; (red bars) GBMs with the PDGFRA\textsuperscript{D8,9} mutant; (yellow bars) grade 3 gliomas, including anaplastic astrocytoma, oligodendroglioma, and ependymoma; (green bars) grade 2 gliomas, including diffuse astrocytoma, oligodendroglioma, mixed oligoastrocytoma, and pilomyxoid astrocytoma; (magenta bars) grade 1 gliomas, including pilocytic astrocytoma. (C) Summary of PDGFRA\textsuperscript{D8,9} mutant screening. (TMs) Tumors.
in Figure 4, A and C, and Supplemental Figure S4A, the cells expressing the KP fusion (Fig. 4C, lane 3) exhibited ligand-independent elevated activation of the MAPK and PI3K pathways compared with either the mock controls (Fig. 4C, lane 1) or cells expressing the K831R mutant (Fig. 4C, lane 2). PDGF ligand stimulation of WT-PDGFRα activated both the MAPK and PI3K pathways, similar to KP fusion-expressing cells (Fig. 4C, lanes 3,4). In contrast, WT-KDR expression alone had no effect, and VEGF ligand stimulation of WT-KDR activated mainly the MAPK pathway in these cells (Fig. 4C, lane 5; Supplemental Fig. S5D).

Many kinases have some basal level of activity. NIH3T3 cells express endogenous PDGFRs but not KDR, leaving open the formal possibility that wild-type PDGFRs affect the kinase activity of the KP fusion (Supplemental Fig. S4B). Therefore, we investigated the level of phosphorytrosine and principal downstream signaling pathways of the KP fusion in Cos 7 cells under serum-deprived conditions, and then probed immunoblots with the indicated antibodies (as shown in Fig. 4E,F). The homodimer of the KP fusion was readily observed in both NIH3T3 and Cos 7 cells expressing the HA-tagged KP fusion construct together with the c-myc-tagged KP fusion (Fig. 4E,F, both panels, lane 2).

PDGFR can form a heterodimeric receptor complex between the α and β receptors (Rupp et al. 1994). In addition, PDGFRα and PDGFRβ can also form heterodimers with other RTK family members, as demonstrated for FGF-R1 and EGFR, respectively (Saito et al. 2001; Faraone et al. 2006). Therefore, we used immunoprecipitation/Western blot analysis to address the possibility that the KP fusion could form heterodimers with PDGFRα or KDR under serum-deprived conditions. The KP fusion formed heterodimers together with tyrosine phosphorylated forms of either PDGFRα or KDR (Fig. 4F, both panels, lanes 3,4). These results suggest that the KP fusion can form homodimers and heterodimers in a ligand-independent manner

Ligand binding to its cognate receptor leads to receptor dimerization and tyrosine autophosphorylation, followed by its activation. These phosphorylated tyrosine residues mediate the specific binding of cytoplasmic signaling proteins containing Src homology-2 (SH2) and protein tyrosine-binding (PTB) domains (Blume-Jensen and Hunter 2001). The above data indicate that the KP fusion behaves as a constitutively active TK in a ligand-independent manner, implying that the KP fusion might constitutively exist in a dimeric form.

To test this hypothesis, we performed immunoprecipitation analysis with NIH3T3 and 293 cells coexpressing HA-tagged KP fusion and c-myc-tagged KP fusion vector constructs under serum-deprived conditions, and then probed immunoblots with the indicated antibodies (as shown in Fig. 4E,F). The homodimer of the KP fusion was readily observed in both NIH3T3 and 293 cells coexpressing the c-myc-tagged KP fusion construct together with the HA-tagged KP fusion (Fig. 4E,F, both panels, lane 2).

PDGFR can form a heterodimeric receptor complex between the α and β receptors (Rupp et al. 1994). In addition, PDGFRα and PDGFRβ can also form heterodimers with other RTK family members, as demonstrated for FGF-R1 and EGFR, respectively (Saito et al. 2001; Faraone et al. 2006). Therefore, we used immunoprecipitation/Western blot analysis to address the possibility that the KP fusion could form heterodimers with PDGFRα or KDR under serum-deprived conditions. The KP fusion formed heterodimers together with tyrosine phosphorylated forms of either PDGFRα or KDR (Fig. 4F, both panels, lanes 3,4). These results suggest that the KP fusion can form homodimers and heterodimers in a ligand-independent manner.
fusion can allow receptor homodimerization and heterodimerization under conditions of overexpression, possibly leading to the activation of not only the KP fusion itself but also PDGFRα and KDR.

The PDGFRα<sup>Δ8, 9</sup> DM shows a predominantly cytoplasmic localization (Clarke and Dirks 2003). Therefore, we examined whether the KP fusion, as either a ligand-independent homodimer or a heterodimer with PDGFRα and KDR, is also predominantly localized to the cytoplasm. Subcellular localization was determined by immunocytochemistry following transient cotransfection of Cos 7 cells using the HA-tagged KP fusion construct with various c-myc-tagged receptor constructs under serum-deprived conditions. By itself, the KP fusion localized mainly to vesicles...
in the cytoplasm, especially concentrated around the perinuclear region [Supplemental Fig. S4C, panels b,d–f]. Both the HA-tagged KP fusion and c-myc-tagged KP fusion colocalized completely [Supplemental Fig. S4C, panels d–f]. Immunofluorescence analysis in serum-deprived conditions for WT-PDGFRA alone or WT-KDR alone revealed their cell surface localization in some cells along with uniform or punctate staining distributed in the cytoplasm and/or perinuclear region [Supplemental Fig. S4C, panels n,o]. When either WT-PDGFRA or WT-KDR was coexpressed with the KP fusion, the amount of wild-type receptors present on the cell surface was somewhat reduced and resulted in partial colocalization with the KP fusion in the cytoplasm [Supplemental Fig. S4C, panels j–l]. These results suggest that the KP fusion protein may to some degree trap PDGFRA and KDR in the cytoplasm, depending on expression levels, and may contribute to activation of these receptors in that subcellular location.

The KP fusion protein has ligand-independent transforming potential

Given that PDGFRA<sup>Δ8, 9</sup>,<sup>9</sup> is a transforming oncogene [Clarke and Dirks 2003], we determined whether the KP fusion may also have transforming potential. We found that NIH3T3 cells expressing the KP fusion were morphologically transformed, as demonstrated by spindle morphology, a loss of contact inhibition, and formation of foci in a pattern similar to the cells expressing the PDGFRA<sup>Δ8, 9</sup>,<sup>9</sup> mutant [Figs. 5A [panels b,c,f,g], 6B,D]. In contrast, the morphology of cells expressing the kinase-dead [K831R] mutant of KP was comparable with cells expressing vector alone [Fig. 5A, panels a,d,e,h]. Interestingly, the transformed phenotype of the KP fusion increased with prolonged passage in culture, eventually forming distinct foci very similar to typical Ras-transformed cells. In this process, MAPK signaling inversely decreased with prolonged passage, and PI3K signaling became dominant under serum-deprived conditions [data not shown]. Therefore, we performed a series of experiments with the cells of a lower passage number to obtain reproducible results in this study. We grew these cells in soft agar to examine whether cells expressing the KP fusion could proliferate in anchorage-independent conditions. The cells expressing the KP fusion maintained growth and formed colonies in soft agar similar to those expressing PDGFRA<sup>Δ8, 9</sup>,<sup>9</sup> [Fig. 5B, panels b,c,f,g]. In contrast, the cells expressing mock or the K831R mutant could not grow in soft agar with 5% serum conditions [Fig. 5B, panels a,d,e,h].

To investigate the tumorigenic potential of the KP fusion in an allograft model in vivo, we implanted NIH3T3 cells expressing the KP fusion into the flank of nude mice. When 10<sup>5</sup> cells were inoculated, tumors developed as a small nodule within ~2 wk in all mice [Fig. 5C]. NIH3T3 cells [10<sup>6</sup>] expressing vector alone or the K831R mutant of the KP fusion formed small nodules in our experimental model, and grew statistically slower and smaller than NIH3T3 cells expressing the KP fusion [Fig. 5C,D]. These results further support the notion that the KP fusion functions as a potent oncogene similar to PDGFRA<sup>Δ8, 9</sup>,<sup>9</sup>, and that the kinase activity is necessary for oncogenic transformation in NIH3T3 cells. Both PDGFRA mutants functioned as active kinases in primary Ink4/Arf<sup>−/−</sup> glial cells as well as NIH3T3 cells

Figure 5. The KP fusion gene is a transforming oncogene. (A) Cells expressing the KP fusion are morphologically transformed. Cell morphology of NIH3T3 cells expressing mock [panels a,e], the PDGFRA<sup>Δ8, 9</sup>,<sup>9</sup> mutant [panels b,f], the KP fusion [panels c,g], and the kinase-inactive mutant [K831R] of the KP fusion [panels d,h] were photographed at 100× [top panels] or 200× [bottom panels] magnification [bars, 10 μm]. (B) Analysis of anchorage-independent growth. NIH3T3 cell lines described in A were maintained on soft agar with 5% calf serum; colony formation in soft agar is shown with 1× [top, panels a–d] or 100× [bottom, panels e–h] magnification after 4 wk [bars, 10 μm]. (C) Tumor formation in nude mice. Cell lines as described in A were inoculated subcutaneously into the flanks of nude mice. Each tumor was measured weekly and was photographed after 6 wk of inoculation. (D) The growth curve of tumors as described in C. The KP fusion [n = 6] was compared with mock [n = 6] or K831R [n = 4] at the final point, respectively. [* * *] P < 0.001. Tumor formation of cells expressing the KP fusion was reproduced four times in an independent experiment. Representative results are shown in C and D.
Gleevec and PTK787 inhibited the elevated tyrosine phosphorylation of the PDGFRA mutants and the mutant-induced downstream signal transduction activity [Fig. 6A; Supplemental Fig. S5E]. Therefore, to examine whether either MAPK or PI3K activity is required for the transforming potential of PDGFRA mutants, we determined the effect of the MEK inhibitor U0126 and the PI3K inhibitor LY294002 on the morphology of NIH3T3 cells expressing the PDGFRA mutants, respectively [Fig. 6D]. Initially, to confirm the inhibitory effects of individual inhibitors on the PDGFRA mutant-induced downstream signaling pathways, we performed immunoblot analysis on cells after treatment with the individual inhibitors [Supplemental Fig. S5F]. After 72 h of treatment, PI3K inhibition alone could not completely reverse the transformed phenotype of either of the PDGFRA mutant cells, as shown by cluster formation and loss of contact inhibition. The MEK inhibitor alone also gave an incomplete reversal of the transformed phenotype, as shown by the partial relief of cluster formation, although it was more effective than PI3K inhibition alone. In contrast, combined inhibition of both the MAPK and PI3K pathways resulted in complete phenotypical reversion of cells expressing the PDGFRA mutants, yielding the same flat morphology as the NIH3T3 cells expressing mock vector or PDGFRA mutant expressors treated with PDGFR inhibitors [Figs. 5A, 6B,D]. To further dissect the downstream signaling of the PI3K pathway, we used the mTOR inhibitor rapamycin. Neither rapamycin treatment alone nor its combination with LY294002 completely reversed the morphology of NIH3T3 cells expressing the PDGFRA mutants. In contrast, when rapamycin was used together with U0126, the cluster formation of PDGFA mutants was partly decreased, but the spindle-shaped morphology was still maintained, possibly through signaling from the PI3K–Akt pathway rather than via mTOR [Fig. 6D]. These results suggested that the transforming potential of PDGFRA mutants might be mediated by a combination of both MAPK and PI3K pathway activation originating from enhanced PDGFRA signaling.

Discussion

Although the common internal deletion of the EGFR gene, EGFRvIII, has been frequently identified and shown to contribute to glioma progression, the PDGFR gene rearrangement has been thought, to date, to be a rare event. The functionality between aberrant PDGFR signaling and gliomagenesis remains to be clarified despite the fact that it is the second most frequently amplified gene in GBMs (Sugawa et al. 1990; Wong et al. 1992; The Cancer Genome Atlas Research Network 2008). In the present study, we investigated two activating PDGFA gene rearrangements in gliomas. First, we identified the first gene fusion in GBM surgical specimens, between KDR and PDGFA, by aCGH, detecting them as intragenic copy

The transforming activity of both the MAPK and PI3K signaling pathways is essential for the transforming potential of both PDGFRA mutants

Oncogenic PDGFR rearrangements in GBMs

PDGFRA

Gleevec completely inhibited the KP fusion-induced trans-

age-independent growth of NIH3T3 cells expressing the

complete (Fig. 6B). PTK787 also inhibited anchor-

completely inhibited them at 10

morphology, and 10

induced transformed phenotype to the untransformed

PTK787 treatment partially reverted the KP fusion-

require binding of their respective ligands for activation.

possible that the KP fusion interacts with RTKs that

acts in a ligand-independent fashion. However, it is

S5D). On the surface, the data suggest that the KP fusion

The transforming activity of the PDGFRA mutants

is reversed by the PDGFR kinase inhibitors

Gleevec and PTK787

Consistent with the observation that the KP fusion local-

izes mainly to the cytoplasm [Supplemental Fig. S4C], a

neutralizing antibody to the extracellular domain of KDR

did not affect tyrosine phosphorylation and colony forma-

tion in soft agar of NIH3T3 cells expressing the KP fusion

[Supplemental Fig. S5B,C], while this same antibody

treatment blocked phosphorylation of WT-KDR [Supple-

mental Fig. S5A]. VEGF stimulation showed a slight in-

crease in the tyrosine phosphorylation of the KP fusion;

possibly a small percent of the KP fusion protein is capable

of reaching the cytoplasmic membrane and binding with

the ligand for activation of the receptor [Supplemental Fig.

S5D]. On the surface, the data suggest that the KP fusion acts in a ligand-independent fashion. However, it is possible that the KP fusion interacts with RTKs that

require binding of their respective ligands for activation.

The KP fusion has an intact protein kinase domain of

PDGFR and may require TK activity of PDGFR for its

transforming potential, as shown in Figure 5. Therefore,

we addressed the effect of two RTK inhibitors with known

activity against PDGFR: Gleevec and PTK787 [Druker

et al. 1996; Wood et al. 2000]. Both PTK787 [Fig. 6A;

Supplemental Fig. S5E, left panel] and Gleevec [Fig. 6A;

Supplemental Fig. S5E, right panel] effectively reduced the

level of phosphotyrosine together with active downstream

pathways in NIH3T3 cells expressing the KP fusion in a concentration-dependent manner. Furthermore, 1 μM

PTK787 treatment partially reversed the KP fusion-

induced transformed phenotype to the untransformed

morphology, and 10 μM treatment reversed this pheno-
type completely [Fig. 6B]. PTK787 also inhibited anchor-
age-independent growth of NIH3T3 cells expressing the

KP fusion beginning at 1 μM concentration and com-

pletely inhibited them at 10 μM [Fig. 6C], while 1 μM

Gleevec completely inhibited the KP fusion-induced trans-

formed phenotype [Fig. 6B,C]. In addition, we addressed

the inhibitory effect of both PDGFR inhibitors on the

PDGFRΔ8,9 mutant, which has an intact protein kinase

domain of PDGFR. Both Gleevec and PTK787 showed an

inhibitory effect on the elevated tyrosine phosphoryla-
tion of the mutant itself and the active downstream signals

[Fig. 6A; Supplemental Fig. S6E], and on the transformed

phenotype of the PDGFRΔ8,9 mutant, similar to cells

expressing the KP fusion [Fig. 6B,C]. These results further

support the idea that both the KP fusion and PDGFRΔ8,9

mutants behave as constitutively active PDGFRAs, and that the transformed phenotype is dependent on the TK activity of both mutant receptors.
number changes in an individual gene. In addition, we demonstrated that the previously identified PDGFRA D8,9 is a recurrent gene rearrangement occurring in 40% of GBMs with amplified PDGFRA, a rate nearly similar to that found for EGFRvIII in the subset of EGFR-amplified gliomas. Other PDGFRA gene rearrangements may exist in GBMs that have not yet been identified.

PDGF signaling contributes to tumor formation in a subset of GBMs, some of which are driven by the PDGF ligand, while others are driven by genetic alterations of the PDGFRA gene, such as gene amplification, intrachromosomal deletion, and activating point mutation. Our data indicate that the KP fusion and PDGFRA D8,9 represent two examples of these genetic mechanisms in this subset of PDGFRA-driven GBMs.

Several observations suggest that these PDGFRA mutations can contribute to the formation or progression of these particular tumors: First, these rearrangements appear to have been selected for in these tumors; second, both the KP fusion and the PDGFRA D8,9 mutant are experimentally oncogenic; and, finally, dysregulated PDGF signaling is causal for glioma formation in mice (Uhrbom et al. 1998; Dai et al. 2001). We examined the effect of PDGFR inhibitors on cells expressing PDGFRA mutants. These inhibitors effectively reverse the mutant receptor-mediated cellular transformation of these cells. Although PDGFR inhibition alone has not been successful in the treatment of unselected GBM patients, such an approach may have had beneficial effects in cases of patients having gliomas with similar rearrangements at this locus. However, such
Oncogenic PDGFRA rearrangements in GBMs

optimism should be tempered by the feeble response of EGFR mutant GBM patients to EGFR inhibition, as well as the capacity for other coactivating RTKs to swap in upon extinction of any one RTK [Stommel et al. 2007].

Expression of constitutively active versions of EGFR by themselves does not form gliomas in experimental mouse models; these mutations must be combined with additional genetic lesions, such as Ink4/Arf deletion, for EGFR-induced glioma formation [Holland et al. 1998; Zhu et al. 2009]. Moreover, all GBMs with EGFR gene rearrangement have amplified wild-type EGFR genes as well, suggesting that the wild-type allele of EGFR may contribute to the oncogenic effect of the mutant allele [Mellinghoff et al. 2005]. The same pattern is seen with rearrangements in PDGFR, most of which were associated with the amplification of the wild-type PDGFR gene as well. It is possible that amplification and elevated expression of wild-type receptors may be essential for the oncogenic potential of such mutant receptors.

Dysregulated PDGFB ligand expression induces oligodendroglioma-like gliomas in mouse models [Uhrbom et al. 1998; Dai et al. 2001]. However, the glial cells expressing either the KP fusion or PDGFA5,9 did not transform even if they had an Ink4/Arf+/− genetic background, for unknown reasons [data not shown]. This discrepancy is consistent with the fact that, although the PDGF ligand is overexpressed in gliomas of all grades, these receptor mutations are only late in the progression of the tumors in humans. In mouse models, the PDGF ligand is fundamentally different from a constitutively active PDGFRA because the PDGF ligand is capable of activating not only glia by autocrine stimulation, but also other cell types, such as endothelia, by paracrine stimulation. The discrepancy between the oncogenic effect on NIH3T3 cells and glial cells suggests that the efficiency of these PDGFR mutants as transforming agents is cell type-specific.

Histologic analysis of the tumors expressing PDGFR mutants showed characteristic features of oligodendroglioma to various degrees, possibly corresponding to the GBM subtype “GBM with oligodendroglioma component” in the World Health Organization (WHO) classification. Genomic analysis of this subtype revealed that there were no distinguishable genetic alterations from GBMs without an oligodendroglioma component [Homma et al. 2006]. Our present study suggests that abnormal PDGFR signaling is likely to contribute to histological features of oligodendroglioma, a notion supported by the induction of similar gliomas by forced PDGFB ligand in mouse models [Uhrbom et al. 1998; Dai et al. 2001]. However, from a therapeutic standpoint, the histological characteristics of oligodendroglioma with PDGFR gene rearrangement may be useful as a marker in the selection of PDGFR inhibitor treatment in GBMs.

The KP fusion formed a heterodimer with wild-type PDGFR or wild-type KDR and, to various degrees, colocalized with them in the cytoplasm, possibly contributing to enhanced wild-type receptor activity in that location. It is known that RTKs can form heterodimers with other RTK family members at the cell surface, and such heterodimers can activate signaling pathways [Saito et al. 2001; Faraone et al. 2006]. In particular, PDGFR and VEGFR belong to the type III RTK family [the immunoglobulin superfamily] characterized by Ig-like domains in their extracellular region necessary for receptor–receptor interaction. Therefore, heterodimer formation of PDGFR or KDR with the KP fusion might be similar to the heterodimer formed between PDGFRB and FGFR1, which also belong to the immunoglobulin superfamily [Faraone et al. 2006]. In addition, given that the seventh Ig-like domain of KDR is required for inhibition of receptor dimerization [Tao et al. 2001], the deletion of this sequence in the KP fusion may also relieve the inhibition, resulting in increased dimerization between the KP fusion and itself or other RTKs.

The KP fusion localized predominantly in the cytoplasm, as indicated by the lack of effect of anti-KDR antibody treatment of these cells. The PDGFRA5,9 mutant also shows a predominantly cytoplasmic localization [Clarke and Dirks 2003]. This mutant has an in-frame deletion in the extracellular region, resulting in loss of approximately half of the fourth Ig-like domain and half of the fifth Ig-like domain. Thus, the defect of the fourth or fifth Ig-like domain of PDGFRAs as well as the KP fusion may affect its subcellular localization differently than the plasma membrane-localized EGFRvIII mutant or other RTK family members that do not have Ig-like domains. A majority of the KP fusion appears not to reach the cell surface and sequesters the wild-type receptor in the cytoplasm, as shown by heterodimerization with predominantly phosphorylated forms of the wild-type receptor.

EGFRvIII functions as an activated monomer and uses an altered spectrum of signaling cascades for oncogenic transformation relative to the wild-type EGFR dimer [Chu et al. 1997]. In contrast, the KP fusion exists in a dimeric form and shows the same activation pattern of downstream signaling as wild-type PDGFR, implying the possibility that heterodimer formation between the KP fusion and endogenous PDGFR might enhance PDGFR signaling and drive cellular transformation of NIH3T3 cells. It is possible that wild-type PDGFR may be needed for the transformed phenotype of NIH3T3 cells that normally express endogenous PDGFRs.

Gliomas are a molecularly heterogeneous disease. The identification and analysis of these PDGFR rearangements illustrates how common phenotypic behaviors, such as PDGF receptor-driven gliomagenesis, can arise from a variety of genomic events. From a therapeutic standpoint, the similarities in the biology of the members of these tumor subtypes may outweigh differences in the details of how the pathways are driven, allowing this class of gliomas to respond similarly to therapeutic intervention.

Materials and methods

Glioma specimens

Formalin-fixed paraffin-embedded [FFPE] and frozen archival human specimens were obtained from the following institutions:

Materials and methods

Glioma specimens

Formalin-fixed paraffin-embedded [FFPE] and frozen archival human specimens were obtained from the following institutions:
aCGH analysis and identification of the KP fusion transcript

aCGH detection and mapping of intragenic copy number aberration (iCNA) was performed in two stages. A screening array was designed to cover 98 TKs, each with 75 oligonucleotide probes of 60-bp length directed to three regions: the 5′ coding region, the coding region 3′ to the kinase domain, and the 3′ promoter region (Agilent Technologies). Candidate iCNA events were detected as a shift in log2 ratio between the 5′ and 3′ probe sets (Student’s t-test). The paired iCNA events in KDR and PDGFRA were verified and fine-mapped on a second array with tiling coverage through the PDGFRA-KIT-KDR locus: 12,487 probes covering 54.92–55.85 Mb on chromosome 4 with a median interprobe interval of 48 bp and 2874 reverse-complement replicate oligonucleotide probes. Probes were optimized for Tm by adjusting the length (45–60mer). For breakpoint mapping, the tumor profile was normalized by control profiles from tumors with uniform amplification through the PDGFRA and KDR genes in order to account for variation in individual probe performance (nonspecific hybridization). All results are presented using human genome build hg18.

A 4 × 3-matrix RT–PCR panel was designed to analyze all of the possible in-frame exon combinations between KDR and PDGFRA around their potential intragenic breakpoint regions. Information about forward primers in KDR exons 3, 9, and 13 and reverse primers in PDGFRA exons 7, 9, and 12 is listed in the Supplemental Material. RT–PCR was performed using the Qiagen one-step RT–PCR kit. PCR products were electrophoresed on agarose gels. The promising RT–PCR products were purified with a Qiagen gel purification kit and sequenced with PDGFRA-S7 or PDGFRA-S8 primers on an Applied Biosystem automated sequencer, or were cloned into pGEM-T vector (Promega) and then sequenced as well. The information about the primers is listed in the Supplemental Material.

PDGFRA gene status was determined by q-PCR analysis in the 87 tumors with significant PDGFRA mRNA overexpression, which was examined with the PDGFRA-S3/A52 primer pair. MSK259 and KNZ15 were excluded from this analysis because no DNA samples were available. DNA was extracted from human frozen brain tumor tissues or paraffin sections using the DNeasy Blood and Tissue Kit (Qiagen) and quantitated on a NanoDrop spectrophotometer (Thermo Scientific). Selected areas containing at least 85% of the tumor were macroscopically and manually dissected from paraffin sections. Reference DNA was obtained from lymphocytes from a total of four healthy volunteers. q-PCR was performed in triplicate using each PDGFRA and 18S gene-specific primer set as described previously [Martinho et al. 2009], reagents, and protocols from Applied Biosystems in a 7900 HT Fast Real-Time PCR System. The PCR amplification was performed in a 10-μL reaction volume under the following conditions: 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μM primers, and 20 ng of DNA. The ΔΔCt method was used to calculate PDGFRA gene copy number in the tumor DNA sample normalized to the reference gene [18S] and calibrated to normal DNA. Gene amplification was defined as average DNA quantities greater than four, corresponding to more than eight copies.

Statistical analysis

All values are expressed as means ± SD. Statistical significance was determined using a two-tailed Student’s t-test using GraphPad Prism software, and a p-value of P < 0.05 was considered significant. For the analysis of tumor growth on nude mice, P-values were determined at the final point.

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References


Campbell PJ, Stephens PJ, Pleasance ED, O’Meara S, Li H, Santarius T, Stebbings LA, Leroy C, Edkins S, Hardy C,
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PDGFRA gene rearrangements are frequent genetic events in PDGFRA-amplified glioblastomas

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References
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