ERBB3, IGF1R, and TGFB2 expression correlate with PDGFR expression in glioblastoma and participate in PDGFR inhibitor resistance of glioblastoma cells

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Abstract: Glioma, the most prevalent malignancy in brain, is classified into four grades (I, II, III, and IV), and grade IV glioma is also known as glioblastoma multiforme (GBM). Aberrant activation of receptor tyrosine kinases (RTKs), including platelet-derived growth factor receptor (PDGFR), are frequently observed in glioma. Accumulating evidence suggests that PDGFR plays critical roles during glioma development and progression and is a promising drug target for GBM therapy. However, PDGFR inhibitor (PDGFRi) has failed in clinical trials, at least partially, due to the activation of other RTKs, which compensates for PDGFR inhibition and renders tumor cells resistance to PDGFRi. Therefore, identifying the RTKs responsible for PDGFR resistance might provide new therapeutic targets to synergistically enhance the efficacy of PDGFRi. In this study, we analyzed the TCGA glioma database and found that the mRNA expressions of three RTKs, i.e. ERBB3, IGF1R, and TGFB2, were positively correlated with that of PDGFR. Co-immunoprecipitation assay indicated novel interactions between the three RTKs and PDGFR in GBM cells. Moreover, concurrent expression of PDGFR with ERBB3, IGF1R, or TGFB2 in GBM cells attenuated the toxicity of PDGFRi and maintained the activation of PDGFR downstream targets under the existence of PDGFRi. Thus, ERBB3, IGF1R, and TGFB2 might participate in PDGFR resistance of GBM cells. Consistent with this notion, combination of PDGFRi with inhibitor targeting either ERBB3 or IGF1R more potently suppressed the growth of GBM cells than each inhibitor alone. The positive correlations of PDGFR with ERBB3, IGF1R, and TGFB2 were further confirmed in 66 GBM patient samples. Intriguingly, survival analysis showed that ERBB3 predicted poor prognosis in GBM patients with high PDGFRA expression. Altogether, our work herein suggested that ERBB3, IGF1R, and TGFB2 were responsible for PDGFR resistance and revealed that ERBB3 acted as potential prognostic marker and therapeutic target for GBM with high PDGFRA expression.

Keywords: Glioblastoma, resistance, PDGFR, ERBB3, IGF1R, TGFB2

Introduction

Glioma is a prevalent malignancy in brain and pathologically classified into four grades, i.e. I, II, III, and IV, according to the 2016 World Health Organization (WHO) classification of central nervous system tumors [1]. Grade IV glioma is the most malignant form of glioma and also known as glioblastoma multiforme (GBM) [1]. Despite the progression of surgical and pharmacological therapies, GBM is still an intractable disease and the average survival time of GBM patients is only about one year [2, 3]. The Cancer Genome Atlas (TCGA) project has unveiled critical genetic alterations in glioma [4] and provides important rationales for target therapies. In most of GBM cases, genetic profiling reveals aberrant activation of signaling pathways mediated by receptor tyrosine kinases (RTKs) [4-6], including the platelet-derived growth factor receptor (PDGFR) [5, 6]. PDGFR is a transmembrane receptor with 5 immunoglobulin-like repeats in the extracellular domain and a tyrosine kinase domain in the intracellular domain. Two PDGFR members have been identified: PDGF receptor α (PDGFRA) and PDGF
receptor β (PDGFRB) [7]. Upon the binding of PDGF ligand, PDGFRα and PDGFRB form homo- or hetero-dimer and undergo autophosphorylation to activate downstream targets, including PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)/AKT (protein kinase B) and MAPK (mitogen-activated protein kinases)/ERK1/2 (extracellular signal-regulated kinases 1/2), which results in cell proliferation, survival, migration, and oncogenesis [8]. Remarkably, PDGFRα overexpression has been widely detected in all stages of glioma, and the activation of PDGF/PDGFR signaling pathway is pivotally involved in the initiation and progression of glioma [9, 10].

The critical involvement of PDGFR in glioma makes PDGFR inhibitor (PDGFRi) promising drug to treat glioma, especially PDGFR-dependent GBM. So far, several anti-tumor agents targeting PDGFR have been developed, such as Imatinib (Gleevec®), Sorafenib (Nexavar®), Nilotinib (Tasigna®), and Sunitinib (Sutent®). Although the data from in vitro and animal experiments support the potent inhibitory effects of PDGFRi on GBM cells [11, 12], clinical trials of single PDGFRi have failed to show encouraging anti-tumor effects [13-16], which might result from the rapid emergence of resistance to PDGFRi [17-19]. Multiple mechanisms on resistance to RTK-targeted therapy have been identified, including mutation of the active site, amplification of the targeted RTK, and activation of alternative RTKs [18-20]. Indeed, co-activation of alternative RTKs renders tumor cells resistance to inhibitor targeting original RTK [21]. Additionally, activation of c-MET (MET Proto-Oncogene, Receptor Tyrosine Kinase) and ERBB3 (Erb-B2 Receptor Tyrosine Kinase 3) leads to tolerance of lung cancer cells to Gefitinib, an inhibitor targeting EGFR (Epidermal Growth Factor Receptor) [22]. Similarly, activation of AXL (AXL Receptor Tyrosine Kinase) causes resistance to EGFR-targeted therapy in lung cancer [23]. Therefore, the activation of alternative RTKs might be also responsible for the failure of PDGFRi and identifying these alternatively RTKs could provide new therapeutic targets to synergistically enhance the inhibitory effect of PDGFRi.

So far, few RTKs have been reported to participate in the development of PDGFRi resistance in GBM cells, but TCGA database provides us a powerful tool to systematically evaluate the relationships of PDGFR with other RTKs. In this study, we hypothesized that RTKs concurrently expressed with PDGFR might contribute to PDGFRi resistance with high probability. The analyses on TCGA glioma database and a cohort containing 66 GBM patients revealed tight relationships of PDGFR with three RTKs, i.e. ERBB3, IGF1R, and TGFBR2. Further cellular experiments indicated that the three RTKs were indeed involved in PDGFR resistance and the combination of PDGFRi with inhibitor targeting either ERBB3 or IGF1R might represent a novel therapeutic strategy to treat GBM patients.

Materials and methods

Patient samples

66 GBM samples were obtained from patients diagnosed and treated in Southwest Hospital (Chongqing, China) (Table 1). All patients underwent surgical resection from January 2014 through December 2016. Specimens were fixed in 4% buffered formaldehyde solution after surgical removal, and then paraffin-embedded. Pathohistological diagnoses were independently made by two neuropathologists according to “The 2016 World Health Organization Classification of Tumors of the Central Nervous System” [1]. This study was carried out according to the principles of the Helsinki Declaration and all protocols have been approved by the ethics committee of Southwest Hospital, Third Military Medical University (TMMU).

TCGA glioma database

A group of 669 patient specimens from TCGA_GBMLGG database and 538 patient specimens from TCGA_GBM database (http://gliovis.bioinfo.cnio.es/) were utilized to evaluate the expres-

### Table 1. Clinicopathological information of patients

<table>
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<th>Clinical Feature</th>
<th>Sample Amount</th>
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</tbody>
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RTKs involved in resistance to PDGFR inhibitor

sion correlation, the clinical relevance, and the Gene Set Enrichment assay of RTKs.

Cell culture

LN229 and 293FT cell lines were purchased from ATCC (VA, USA), and primary GBM cells (GBM-1) were established from the tumor specimen of GBM patient treated in Southwest Hospital (TMMU, Chongqing, China) [24]. All cells were grown in Dulbecco’s modified Eagle’s medium (Gibco, NY14072, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, 10270-106, EU-Approved) and 1% (v/v) Penicillin-Streptomycin (HyClone, SV30010, Austria) at 37°C in a humidified incubator with 5% CO₂.

Plasmid constructs, lentivirus production, and transient transfection

Full length cDNAs of PDGFRA and PDGFRB were cloned in pCDH-CMV-MCS-EF1-Puro and pCDH-EF1-MCS-IRES-Neo lentivirus vectors, respectively (System Biosciences, CA, USA) using NheI and NotI sites (New ENGLAND BIOLABS, 6387681, US). The sequences of Flag/Myc were also constructed into lentivirus vectors as empty vector control. The constructs were verified by sequencing (Invitrogen, Shanghai, China). For lentivirus production, 293FT cells were co-transfected with the targeted plasmids and package plasmids (Addgene, MA, USA) with Lipofectamine-2000 (Thermo Fisher Scientific, IL, US). Virus-containing supernatants were collected after 48-72 hr and glioma cells were infected with the purified supernatants and polybrene 8 µg/ml (Sigma, MA, USA). The stable cells were selected in 4 µg/ml puromycin (Invivogen, CA, USA) or 130 µg/ml G418 (Sangon Biotech, A100859, China) and confirmed by western blot assay.

Cell proliferation and IC₅₀ measurement

Cells were counted and seeded into 96-wells plates at a density of 2000 cells/well with four replicate wells per group. After 6-8 hours, cells were changed by new medium with the gradient RTK inhibitors as needed. Cell proliferation was measured by assay of Cell Counting Kit-8 reagent (CCK-8) (DOJINDO, Japan) and recorded by fluoroanalyzer with OD 450 nm at day 3.

Cell migration and invasion

The upper chamber (Millicell 8.0 µm PET) was coated with 15 µl of Matrigel (CORNING, 354234, Bedford, MA 01730, USA) for invasion assay, and not coated for migration assay. 3×10⁴ cells in 200 µl of serum-free medium were seeded into the upper chamber and the lower chamber was added with 500 µl growth medium supplemented with 10% FBS. After incubation at 37°C with 5% CO₂ for 24 hr for invasion assay and 12 hr for migration assay, the cells were fixed with 4% paraformaldehyde (PFA) followed by crystal violet staining. Non-invading cells were removed with a cotton swab, and the stained cells were counted under a light microscope (Olympus Corporation, Tokyo, Japan).

Co-immunoprecipitation

GBM cells overexpressing PDGFRA-Flag with IGF1R-HA, PDGFRA-Flag with ERBB3-HA, PDGFRB-Myc with TGFR2-HA, or PDGFRB-Myc with FGFR1-Flag were collected in cold PBS using cell scraper (Corning, NY, USA) and lysed with NP40 lysis buffer (Beyotime, P0013F, China) with PMSF (Beyotime, ST505, China), Protease/Phosphatase Inhibitor Cocktail (CST, 5872S, USA), in ice for 60 min. Then, the lysates were centrifuged at 25000×g for 15 minutes at 4°C to remove cell debris. Quantitative lysates were immediately pre-cleared with Protein A/G magnetic beads (GE Healthcare, Beijing, China) for 1 hr. For affinity enrichment, pre-cleared lysates were incubated with beads and antibodies at 4°C overnight. The treated beads were washed three times with 500 µl of cold lysis buffer, and then heated in SDS-PAGE sample buffer for 5 min at 95°C.

Western blotting

Cells were harvested by centrifuged at 1000×g for 5 min at 4°C, then resuspended by RIPA buffer with PMSF, Protease/Phosphatase Inhibitor Cocktail for 30 min in cold ice, centrifuged at 25000×g for 15 minutes at 4°C. The supernatant was measured by BCA (Bio-Rad, CA, USA) and 50 µg of total protein was analyzed by western blot assay as previously described [25]. The antibodies used in this
RTKs involved in resistance to PDGFR inhibitor

**Table 2. Correlation of indicated RTKs in TCGA_GBMLGG database**

<table>
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<th>RTK Name</th>
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<th>PDGFRB</th>
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<td>PDGFRB</td>
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<td>0.000</td>
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<td>ERBB2</td>
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<td>ERBB3</td>
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<tr>
<td>TGFB3</td>
<td>0.021</td>
<td>0.588</td>
</tr>
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</table>

Note: Genes with bold font are RTKs significantly and positively correlated with PDGFRA or PDGFRB. Genes with bold font and covered by grey color are top two correlated RTKs with PDGFRA or PDGFRB.

**Results**

Analysis on TCGA glioma database identified RTKs positively correlated with PDGFR

It has been reported that the activation of alternative RTKs contributes to the resistance of RTK inhibitors, including PDGFRi [17], and we hypothesized that RTKs concurrently expressed with PDGFR might contribute to PDGFR resistance with high probability. To identify the RTKs highly correlated with PDGFR in glioma, we analyzed the TCGA glioma database and evaluated the expression correlations of PDGFA or PDGFRB with several RTK family members critically involved in glioma, including EGFR, ERBB2, ERBB3, IGF1R, FGFR1, FGFR2, FGFR3, FGFR4, IGF1R, IGF2R, TGFB1, TGFB2, and TGFB3 [26-32]. Eight RTK genes were found to be significantly concurrently expressed with PDGFA or PDGFRB (Table 2). Among of them, ERBB3 and IGF1R were top two PDGFA-correlated RTK genes (Figure 1A and 1B), and TGFB2 and FGFR1 were top two PDGFRB-correlated RTK genes (Figure 1C and 1D). Thus, we focused on the four RTKs for the following experiments.
RTKs involved in resistance to PDGFR inhibitor

ERBB3, IGF1R, and TGFBR2 interacted with PDGFR in GBM cells

Generally, interactions between PDGFR and alternative RTKs are required for the alternative RTKs-mediated resistance to PDGFRi treatment [29, 33, 34], which promoted us to examine whether there were interactions between PDGFR with ERBB3, IGF1R, TGFBR2, and FGFR1. For this purpose, we constructed plasmids expressing PDGFRA-Flag, PDGFRB-Myc, ERBB3-HA, IGF1R-HA, TGFBR2-HA, or FGFR1-Flag, and transfected them into a GBM cell line (LN229) and a primary human GBM cell line (GBM-1) [24] to ensure the successful expression of the plasmids (Figure 2A and 2B). Then, we performed co-immunoprecipitation (co-IP) assay and clearly detected the interactions between PDGFR and IGF1R (Figure 2C), PDGFR and ERBB3 (Figure 2D), and PDGFRB and TGFBR2 (Figure 2E), but no obvious interaction between PDGFRB and FGFR1 (Figure 2F). Therefore, the data revealed novel interactions between PDGFR and concurrently-expressed RTKs, i.e. ERBB3, IGF1R, and TGFBR2.

ERBB3, IGF1R, and TGFBR2 contributed to the resistance of GBM cells to PDGFRi

Next, we explored whether ERBB3, IGF1R, TGFBR2, and FGFR1 affected the sensitivity of GBM cells to Imatinib, a small-molecule PDGFRi. At first, we calculated IC$_{50}$ of Imatinib on growth of LN229 cells (16.94 µM) and GBM-1 cells (12.3 µM) through CCK-8 assay [35] (Figure 3A and 3B). Then, we treated the GBM cells with Imatinib at concentrations around the IC$_{50}$ (Figure 3C and 3D) and identified 15 µM as appropriate concentration to treat GBM cells in the following assays. To easily assess the inhibitory effect of Imatinib on

![Figure 1. Correlation analysis of indicated genes using TCGA_GBMLGG database. (A-D) Pearson correlation analysis of PDGFR and IGF1R expressions (A), PDGFR and ERBB3 expressions (B), PDGFRB and TGFBR2 expressions (C), or PDGFRB and FGFR1 expressions (D).]
RTKs involved in resistance to PDGFR inhibitor

![Co-IP experiment of PDGFR with PDGFR-correlated RTKs.](image)

GBM cells, we used CCK-8 assay to calculate the Relative Growth Rate (RGR) of Imatinib versus vehicle (RGR = OD value at day-5 under Imatinib treatment/OD value at day-5 under vehicle treatment). In GBM-1 cells, forced expression of PDGFR, IGF1R, ERBB3, or PDGFRA with IGF1R, or PDGFRA with ERBB3 produced comparable growth curves under vehicle treatment (Figure 3E). Interestingly, although Imatinib treatment potently inhibited the growth of GBM-1 cells expressing PDGFR (RGR = 0.45), IGF1R (RGR = 0.46), or ERBB3 (RGR = 0.4), it just mildly reduced the growth of GBM-1 cells transfected with PDGFR plus IGF1R (RGR = 0.73) or PDGFR plus ERBB3 (RGR = 0.79) (Figure 3E). In addition, we observed similar growth curves for GBM-1 cells transfected with PDGFRB, TGFBR2, FGFR1, PDGFRA plus TGFBR2, or PDGFRA plus FGFR1 under vehicle treatment (Figure 3F). With Imatinib treatment, GBM-1 cells with PDGFRA plus TGFBR2 seemed insensitive to Imatinib (RGR = 0.8) compared to GBM-1 cells with other transfections (RGR = 0.41–0.64) (Figure 3F). We performed the same set of experiments in LN229 cells, and consistently observed Imatinib tolerance in LN229 cells transfected with PDGFR plus IGF1R (RGR = 0.88), PDGFR plus ERBB3 (RGR = 0.82) (Figure 3G), or PDGFRA plus TGFBR2 (RGR = 0.78) (Figure 3H) compared to PDGFR (RGR = 0.8), IGF1R (RGR = 0.54), ERBB3 (RGR = 0.51), PDGFRA (RGR = 0.33), TGFBR2 (RGR = 0.34), FGFR1 (RGR = 0.47), or PDGFRA plus FGFR1 (RGR = 0.53) (Figure 3G and 3H). Together, these data suggested that ERBB3, IGF1R, and TGFBR2, but not FGFR1, were potentially responsible for Imatinib resistance in GBM cells.

**Figure 2.** Co-IP experiment of PDGFR with PDGFR-correlated RTKs. (A and B) Western blotting evaluation on transfection efficiency of indicated genes in GBM-1 (A) and LN229 cells (B). Actin (β-actin) is used as loading control. (C-F) Co-IP assay between Flag-tagged PDGFR and HA-tagged IGF1R (C), Flag-tagged PDGFR and HA-tagged ERBB3 (D), Myc-tagged PDGFRA and HA-tagged TGFBR2 (E), as well as Myc-tagged PDGFRA and Flag-tagged FGFR1 (F).

**Figure 3.** Western blotting evaluation on the activation of PDGFR downstream targets with Imatinib treatment.

To further evaluate the effects of IGF1R, ERBB3, TGFBR2, and FGFR1 on PDGFR downstream targets in GBM cells, we examined the activation of AKT, as well as S6, a typical AKT downstream target (Figure 3E). Interestingly, although Imatinib treatment potently inhibited the growth of GBM-1 cells expressing PDGFR (RGR = 0.45), IGF1R (RGR = 0.46), or ERBB3 (RGR = 0.4), it just mildly reduced the growth of GBM-1 cells transfected with PDGFR plus IGF1R (RGR = 0.73) or PDGFR plus ERBB3 (RGR = 0.79) (Figure 3E). In addition, we observed similar growth curves for GBM-1 cells transfected with PDGFRB, TGFBR2, FGFR1, PDGFRA plus TGFBR2, or PDGFRA plus FGFR1 under vehicle treatment (Figure 3F). With Imatinib treatment, GBM-1 cells with PDGFRA plus TGFBR2 seemed insensitive to Imatinib (RGR = 0.8) compared to GBM-1 cells with other transfections (RGR = 0.41–0.64) (Figure 3F). We performed the same set of experiments in LN229 cells, and consistently observed Imatinib tolerance in LN229 cells transfected with PDGFR plus IGF1R (RGR = 0.88), PDGFR plus ERBB3 (RGR = 0.82) (Figure 3G), or PDGFRA plus TGFBR2 (RGR = 0.78) (Figure 3H) compared to PDGFR (RGR = 0.8), IGF1R (RGR = 0.54), ERBB3 (RGR = 0.51), PDGFRA (RGR = 0.33), TGFBR2 (RGR = 0.34), FGFR1 (RGR = 0.47), or PDGFRA plus FGFR1 (RGR = 0.53) (Figure 3G and 3H). Together, these data suggested that ERBB3, IGF1R, and TGFBR2, but not FGFR1, were potentially responsible for Imatinib resistance in GBM cells.
RTKs involved in resistance to PDGFR inhibitor

substrate. In GBM-1 cells, transfection of PDGFR, PDGFR plus IGF1R, or PDGFR plus ERBB3 resulted in comparable activation of AKT marked by the level of phosphorylated AKT5473 (p-AKT) (Figure 4A, lane 1-lane 3). As expected, Imatinib treatment obviously depressed AKT activation in GBM-1 cells with PDGFR (Figure 4A, lane 4), but failed to do so.
RTKs involved in resistance to PDGFR inhibitor

in GBM-1 cells with PDGFRA plus IGF1R or PDGFRA plus ERBB3 (Figure 4A, lane 5 and lane 6). Consistently, the phosphorylation of S6 (p-S6) was resistant to Imatinib treatment in GBM-1 cells with PDGFRA plus IGF1R or PDGFRA plus ERBB3 (Figure 4A, lane 5 and lane 6), but reduced by Imatinib in GBM-1 cells with PDGFRA (Figure 4A, lane 4). Unexpectedly, Imatinib treatment seemed ineffective for GBM-1 cells with exogenous expression of PDGFRB (Figure 4B, lane 4), but co-transfection of PDGFRB and TGFBR2 further increased p-AKT and p-S6, especially under existence of Imatinib (Figure 4B, lane 5), which was not obvious for the co-transfection of PDGFRB and FGFR1 (Figure 4B, lane 6). Supporting the data in GBM-1 cells, we acquired similar results using LN229 cells (Figure 4C and 4D). Therefore, the western data indicated that ERBB3, IGF1R, and TGFBR2, but not FGFR1, maintained the activation of PDGFR downstream targets under the existence of PDGFRi.

Co-expression of PDGF with ERBB3, IGF1R, or TGFBR2 promoted migration and invasion of GBM cell in vitro

One of the typical characteristics of GBM cells is highly invasive growth, which is derived from the increased migration and invasion ability [36-38]. Moreover, the development of resistance is usually accompanied with the increased mobility and invasibility of GBM cells [39]. Thereby, we asked whether the PDGFR-correlated RTKs could regulated the migration and invasion of GBM cells. In GBM-1 cells, we found that co-expression of PDGFRA with ERBB3 or IGF1R effectively enhanced the migration and invasion as indicated by chamber assay without Matrigel and with Matrigel,
RTKs involved in resistance to PDGFR inhibitor
respectively (Figure 5A and 5B). Additionally, co-expression of PDGFRB with TGFBR2 also significantly promoted the migration and invasion of GBM-1 cells (Figure 5C and 5D). Interestingly, we observed that among the three combinations, PDGFRB and TGFBR2 exhibited the strongest promoting effect on migration and invasion. In accordance with this observation, GSEA [40, 41] on TCGA_GBM database further confirmed that concurrent upregulation of both PDGFRB and TGFBR2 dramatically enriched the genes responsible for cell migration and invasion in the context of GO_CELLULAR_EXTRAVASATION geneset (Accession G0:0045123) (Figure 5E) and ANASTASSIOU_MULTICANCER_INVASIVENESS_SIGNATURE geneset [42] (Figure 5F). Thus, co-expression of PDGFR with ERBB3, IGF1R, or TGFBR2 played positive roles for the migration and invasion of GBM cells.

*Simultaneous inhibition of PDGFR and IGF1R or ERBB3 potently dampened the growth of GBM cells*

It has been known that proneural subtype of GBM is featured with PDGFRA overexpression and insensitive to radio-chemotherapy in comparison with other subtypes [5, 6]. Although PDGFRA is considered as a therapeutic target for proneural GBM, PDGFRAi did not produce benefit for the patients in clinical trials, at least partially, due to the resistance to PDGFRAi [17-19, 43]. Our data revealed that the ERBB3 and IGF1R were interacted with PDGFR and contributed to PDGFRi resistance, and we speculated that combination of Imatinib with ERBB3 inhibitor (Sapitinib) or IGF1R inhibitor (AZD3463) would have more potent inhibitory effects on the growth of GBM cells than application of each inhibitor alone. To test this hypothesis, we examined whether the combination of Imatinib with Sapitinib or AZD3463 could effectively reverse the Imatinib resistance. We first performed growth assays using AZD3463 (Figure 6A and 6B) or Sapitinib (Figure 6C and 6D) and found that 2 µM AZD3463 or 20 µM Sapitinib did not show high cytotoxicity on cell growth (Figure 6A-D) and were appropriate to test combination with Imatinib. Western data showed that Imatinib (10 µM) treatment eliminated the activation of AKT in PDGFR overexpression cells (Figure 6E), while overexpression of PDGFR with IGF1R or ERBB3 maintained the activation of AKT under the existence of Imatinib (Figure 6E). As expected, combination of Imatinib (10 µM) with AZD3463 (2 µM) or Sapitinib (20 µM) effectively blocked the activation of AKT (Figure 6E). Growth assay from two GBM cell lines further showed that application of Imatinib, AZD3463, or Sapitinib alone partially reduced the cell growth, but combination of Imatinib with AZD3463 or Sapitinib almost completely inhibited the cell growth (Figure 7A-D).

Interestingly, analysis on TCGA database suggested that both PDGFR and ERBB3 were highly expressed in proneural-subtype GBM compared with classical- and mesenchymal-subtype GBM, but the expression of IGF1R was similar in all three subtypes of GBM (Figure 7E). Through GSEA on TCGA_GBM database and using geneset of MAHADEVAN_IMATINIB_RESISTANCE [44] as background, we found that the genes upregulated in Imatinib-resistant cells were significantly enriched in GBM samples with PDGFRA<sup>high</sup>/ERBB3<sup>low</sup> versus PDGFR<sup>high</sup>/ERBB3<sup>low</sup> (Figure 7F), which strongly supported the participation of Erbb3 in Imatinib resistance. Therefore, the combination of inhibitors targeting PDGFR and related-RTKs was a novel strategy to suppress high-PDGFR GBM cells, and in particular, PDGFR and ERBB3 were hopeful therapeutic targets for proneural GBM.

The correlations of PDGFR with ERBB3, IGF1R, and TGFBR2 were confirmed in human GBM tissues

The cellular analysis showed expression and function correlations of PDGFR with ERBB3,
PDGFRA with IGF1R, and PDGFRB with TGFBR2 in GBM cells, which promoted us to evaluate their expressions in a cohort containing 66 human GBM tissues (Cohort-66) \((\text{Table 1})\) through IHC. For scoring the IHC staining, five images of each sample were taken and the average integrated optical density (IOD) was measured by image pro plus 6.0 software. Immunostaining on successive slides (\(\text{Figure 8A}\)) clearly showed positive correlations of PDGFRA with ERBB3, PDGFRA with IGF1R, and PDGFRB with TGFBR2 (\(\text{Figure 8B}\)). To assess the prognostic significance of IGF1R or ERBB3 in the context of high PDGFRA expression and TGFBR2 in the context of high PDGFRB expression, we used quartile of all IOD values as cutoff to define high and low expressions of interested proteins (\(\text{Figure 9A}\)). Kaplan-Meier survival analysis showed that in the context of high PDGFRA expression (PDGFRA\(^{\text{high}}\)), high ERBB3 expression (ERBB3\(^{\text{high}}\)) predicted poor survival in com-
RTKs involved in resistance to PDGFR inhibitor

**Figure 7.** Cell growth assay of GBM cells under treatment of inhibitors. A. Cell growth assay of GBM-1 cells with exogenous expression of RA plus I1R under indicated treatment. B. Cell growth assay of GBM-1 cells with exogenous expression of RA plus EB3 under indicated treatment. C. Cell growth assay of LN229 cells with exogenous expression of RA plus I1R under indicated treatment. D. Cell growth assay of LN229 cells with exogenous expression of RA plus EB3 under indicated treatment. Single inhibitor: A3463 2 µM, SAP 20 µM, IMA 15 µM; Combination of inhibitors: A3463 2 µM, SAP 20 µM, IMA 10 µM. The data are shown as mean ± SD, *P < 0.01 vs. control, **P < 0.001 vs. control, n = 3 per group. E. Box-plots showing the level of PDGFR, ERBB3, and IGF1R in patients with different molecular classifications using TCGA_GBM database. F. Geneset enrichment assay of PDGFR<sup>high</sup>/ERBB3<sup>high</sup> versus PDGFR<sup>high</sup>/ERBB3<sup>low</sup> in MAHADEVAN_IMATINIB_RESISTANCE geneset using samples in TCGA_GBM database.

**Discussion**

In this work, we reported ERBB3, IGF1R, and TGFBR2 concurrently expressed with PDGFR in...
RTKs involved in resistance to PDGFR inhibitor

A

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B

- $r = 0.4777, P < 0.0001$
- $r = 0.6418, P < 0.0001$
- $r = 0.5139, P < 0.0001$
RTKs involved in resistance to PDGFR inhibitor

Figure 8. Correlation analysis between the expression of PDGFR with IGF1R, ERBB3, or TGFBR2 in human GBM tissues. A. Representative immunohistochemical images for PDGFRA/IGF1R, PDGFRA/ERBB3, and PDGFBRB/TGFBR2 in successive slides of human GBM tissues, respectively. Scale bar = 100 µm (original image) and 50 µm (inset). B. Pearson correlation analysis of indicated proteins in the cohort containing 66 human GBM patients.

Figure 9. Survival analysis of IGF1R, ERBB3, and TGFBR2 in the context of high PDGFR expression in human GBM tissues. (A) Protein expression intensity above and below the quartile of all samples were categorized as high and low, respectively. (B-D) Kaplan-Meier curves of overall survival rate for GBM patients with PDGFRA<sup>high</sup>/IGF1R<sup>high</sup> versus PDGFRA<sup>low</sup>/IGF1R<sup>low</sup> (B), PDGFRA<sup>high</sup>/ERBB3<sup>high</sup> versus PDGFRA<sup>low</sup>/ERBB3<sup>low</sup> (C), and PDGFBRB<sup>high</sup>/TGFBR2<sup>high</sup> versus PDGFBRB<sup>low</sup>/TGFBR2<sup>low</sup> (D).

glioma, including GBM, through analysis on TCGA database and a cohort of GBM patients. Further experiments revealed novel interactions between PDGFRA with ERBB3 or IGF1R, and between PDGFBRB and TGFBR2. Moreover, co-expression of PDGFRA with ERBB3, PDGFRA with IGF1R, or PDGFBRB with TGFBR2 not only enhanced the Imatinib tolerance but also promoted the migration and invasion in GBM cells.

It has been well-known that RTKs pivotally modulate important pathological properties of tumor cells, such as proliferation, resistance to apoptosis, and cell motility. Aberrant activation of RTKs has been linked to the initiation, maintenance, and progression of many different tumor types including GBM [45-47]. A wide range of inhibitors targeting RTKs, also known as tyrosine kinase inhibitors (TKI), has been tested in gliomas [4-6]. These inhibitors, predominantly targeting PDGFR and EGFR, showed preclinical benefit, but finally failed with insufficient therapeutic efficacy. For example, Imatinib showed promising anti-tumor activities in preclinical studies but could not lead to survival improvement in patients with recurrent GBM, partially due to the resistance to the inhibitors [15, 16]. One of the mechanisms underlying the resistance is the activation of alternative RTKs, and thereby, simultaneous inhibition of the originally targeted RTK and the alternative RTKs could attenuate, even eliminate, the resistance and suppress the tumor growth. Our work herein revealed ERBB3, IGF1R, and TGFBR2 as alternative RTKs related with PDGFR. Moreover, we treated the GBM cells using the combination of Imatinib with ERBB3 inhibitor or IGF1R inhibitor, and indeed
observed that the combination almost completely repressed the growth of GBM cells, which confirmed that the alternative RTKs were functionally involved in the PDGFRi resistance. Actually, ERBB3, IGF1R, and TGFB2 have also been reported to function in GBM. For example, ERBB3 is coordinated with other ERBB members to promote growth of GBM cells [27], and also plays a role in the resistance to EGFR-targeted therapy for GBM [48]. IGF1R plays important roles in the development and progression of GBM [28, 49], and has been found to participate in the resistance to EGFR and PDGFR-targeted treatment [29, 30]. In addition, TGFB2 is a mediator for TGF-β-induced signaling pathway in GBM and functions as oncogene in glioma cells as well as glioma stem cells [31, 32, 50]. Accordingly, our data linked these RTKs with PDGFR and expanded the network of RTKs in GBM.

Transcriptomic analysis classified GBM into four clinically relevant subtypes: proneural, neural, classical, and mesenchymal, and each of these subtypes is defined by a specific molecular signature [5]. For example, proneural GBM is featured with overexpression of PDGFRA and enhanced activation of PDGF/PDGFR signaling pathway [5, 6]. Interestingly, proneural GBM is insensitive to radio- and chemo-therapy in comparison with other subtypes [5, 6], which implies that PDGFRA is a hopeful drug target in proneural GBM. Our work indicated that ERBB3 was tightly correlated and interacted with PDGFRA, and moreover, ERBB3 was identified as an indicator for poor survival in GBM patients with high PDGFR expression. Intriguingly, simultaneous inhibition of PDGFRA and ERBB3 showed potent inhibitory effects on cell growth, which emphasized that inhibitors targeting PDGFRA and ERBB3 might act as a novel independent regimen or adjuvant agents for standard treatment in proneural GBM.

Altogether, this study identified candidate RTKs responsible for the development of PDGFRi resistance via a TCGA database-based analysis, which we thought might be applicable to other RTK inhibitors. Clinical data further emphasized that PDGFRA<sup>agonist</sup>/ERBB3<sup>agonist</sup> predicted poor prognosis for GBM patients. Moreover, the identification of PDGFRi-related alternative RTKs, i.e. ERBB3, IGF1R, and TGFB2, not only provided new insights on PDGF/PDGFR signaling pathway, but also implied novel combination targets for PDGFRi-based therapy in GBM, especially in proneural subtype.

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**Disclosure of conflict of interest**

None.

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