EPHA2 mediates PDGFA activity and functions together with PDGFRA as prognostic marker and therapeutic target in glioblastoma

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Platelet-derived growth subunit A (PDGFA) plays critical roles in development of glioblastoma (GBM) with substantial evidence from TCGA database analyses and in vivo mouse models. So far, only platelet-derived growth receptor α (PDGFRα) has been identified as receptor for PDGFA. However, PDGFA and PDGFRα are categorized into different molecular subtypes of GBM in TCGA_GBM database. Our data herein further showed that activity or expression deficiency of PDGFRα did not effectively block PDGFA activity. Therefore, PDGFA might not be necessary for PDGFRα function. To profile proteins involved in PDGFA function, we performed co-immunoprecipitation (Co-IP) and Mass Spectrum (MS) and delineated the network of PDGFA-associated proteins for the first time. Unexpectedly, the data showed that EPHA2 could be temporally activated by PDGFA even without activation of PDGFRα and AKT. Furthermore, MS, Co-IP, in vitro binding thermodynamics, and proximity ligation assay consistently proved the interaction of EPHA2 and PDGFA. In addition, we observed that high expression of EPHA2 led to upregulation of PDGF signaling targets in TCGA_GBM database and clinical GBM samples. Co-upregulation of PDGFA and EPHA2 led to worse patient prognosis and poorer therapeutic effects than other contexts, which might arise from expression elevation of genes related with malignant molecular subtypes and invasive growth. Due to PDGFA-induced EPHA2 activation, blocking PDGFRα by inhibitor could not effectively suppress proliferation of GBM cells, but simultaneous inhibition of both EPHA2 and PDGFRα showed synergistic inhibitory effects on GBM cells in vitro and in vivo. Taken together, our study provided new insights on PDGFA function and revealed EPHA2 as a potential receptor of PDGFA. EPHA2 might contribute to PDGFA signaling transduction in combination with PDGFRα and mediate the resistance of GBM cells to PDGFRα inhibitor. Therefore, combination of inhibitors targeting PDGFA and EPHA2 represented a promising therapeutic strategy for GBM treatment.

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INTRODUCTION
Glioma is the most prevalent brain tumor and pathologically categorized into four grades (I–IV) by the 2016 World Health Organization (WHO) classification of central nervous system tumors. Grade I and II gliomas are considered as low-grade glioma (LGG), assuming slow progression and favorable prognosis, but grade III and IV gliomas are high-grade glioma (HGG) and featured with highly invasive growth and significantly shortened survival. Grade IV glioma, also known as glioblastoma multiforme (GBM), is the most malignant form of glioma and remains intractable despite the progression of surgical and pharmacological therapies. The average survival time of GBM patients is about 15 months and the 5-year survival rate is less than 5%. In clinic, majority of GBM patients (about 90%) are diagnosed with wild-type IDH1/2 and have no glioma history, which is defined as primary or de novo GBM. About 10% GBM patients harbor IDH1/2 mutation and have a history of lower-grade glioma, which is defined as secondary GBM. The Cancer Genome Atlas (TCGA) project have unveiled comprehensive genetic and transcriptomic profiles of GBM through next-generation sequencing, which classifies GBM into four molecular subtypes: Proneural, Neural, Mesenchymal, and Classical.

Platelet-derived growth factor receptor α (PDGFRα) and β (PDGFRβ) belong to receptor tyrosine kinase (RTK) family and function as receptors for platelet-derived growth factor (PDGF). Four PDGF genes (PDGFA, PDGFB, PDGFC, and PDGFD) in mammalian have been identified and encode four peptides (PDGFA, PDGFB, PDGFC, and PDGFD), which form five functional homo- or hetero-dimers: PDGFA-Aβ, PDGFB-BB, PDGFC-CC, and PDGFD-DD. When stimulated with dimeric PDGF peptides, PDGFA and PDGFRβ immediately form homo- or hetero-dimer and undergo autophosphorylation for full activation. Activated PDGFRα and PDGFRβ function as transmembrane receptors and activate downstream signaling cascades.
PDGF/PDGFR axis continue to activate downstream signaling pathways, such as PI3K/AKT/mTOR pathway, RAS/MAPK/ERK pathway, and JAK/STAT3 pathway, which lead to proliferation, survival, and invasion of cancer cells. PDGF ligands and PDGFR receptors have been clarified: PDGFRA homodimer are activated by PDGF-AA, -AB, and -CC, PDGFRB homodimer are activated by PDGF-BB and -DD, and PDGFRA/PDGFRB heterodimer are only activated by PDGF-AB. Therefore, PDGFRA is the only identified receptor to mediate PDGFA function in GBM.

Both PDGFA and PDGFRA have been found to play critical roles in gliomagenesis and tumor progression. On the one hand, PDGFA is one of the core genes related with gain of chromosome 7, which is broadly observed in glioblastoma, and one of the signature genes for the classical subtype GBM; on the other hand, copy number amplification and mRNA overexpression of PDGFRA are typical features of Proneural GBM. Therefore, PDGFRA is the only identified receptor to mediate PDGFA function in GBM.

### RESULTS

PDGFRA was not necessary for PDGFA function in GBM cells. Kaplan–Meier survival analysis of cases with PDGFAHigh vs. PDGFALow from TCGA_GBM database. PDGFA gene expression in TCGA PanCancer databases. PDGFA-induced temporal expression of indicated proteins in LN18 cells pre-treated with vehicle or IMA. β-actin is used as loading control. PDGFA-induced temporal expression of indicated proteins in LN18 cells transfected with control CRISPR/Cas9 or CRISPR/Cas9 targeting PDGFRA. Enrichment of PDGFA signaling-related genesets for TCGA_GBM cases with PDGFAHigh/PDGFRALow vs. PDGFALow or PDGFAHigh/PDGFRALow vs. PDGFALow. Categorization of PDGFA-associated proteins.

These results suggest critical roles of PDGFRA in GBM and identify PDGFA/PDGFRAX axis as a potential therapeutic target for GBM. Indeed, several anti-tumor agents targeting PDGFA/PDGFRAX have been developed, such as Imatinib (Gleevec®), Sorafenib (Nexavar®), Nilotinib (Tasigna®), and Sunitinib (Sutent®). Although the data in vitro and in vivo support the potent inhibitory effects of targeting PDGFA/PDGFRAX in GBM cells, clinical trials of single PDGFA/PDGFRAX inhibitor have failed to show anti-tumor effects. Therefore, the regulation mechanisms on PDGFA and PDGFRA in GBM need be clarified before clinical application of strategies targeting PDGFA/PDGFRAX signaling axis. In this work, through co-immunoprecipitation (Co-IP) and Mass-Spectrum (MS) identification, we profiled PDGFA-associated proteins and revealed EPHA2 as a new receptor for PDGFA to mediate PDGFA function even without PDGFRAX.

**Fig. 1** PDGFRAX is not necessary for PDGFA function in GBM cells. Kaplan–Meier survival analysis of cases with PDGFAHigh vs. PDGFALow from TCGA_GBM database. PDGFA gene expression in TCGA PanCancer databases. PDGFA-induced temporal expression of indicated proteins in LN18 cells pre-treated with vehicle or IMA. β-actin is used as loading control. PDGFA-induced temporal expression of indicated proteins in LN18 cells transfected with control CRISPR/Cas9 or CRISPR/Cas9 targeting PDGFRA. Enrichment of PDGFA signaling-related genesets for TCGA_GBM cases with PDGFAHigh/PDGFRALow vs. PDGFALow or PDGFAHigh/PDGFRALow vs. PDGFALow. Categorization of PDGFA-associated proteins.
endogenous PDGFRA functions from other cell lines (Supplementary Fig. S1d). Our data showed that recombinant human PDGF-AA (100 ng/ml) stimulation resulted in phosphorylation of PDGFRA without regulation on PDGFRB (Supplementary Fig. S1e). However, PDGF-BB led to activation of both PDGFRA and PDGFRB (Supplementary Fig. S1e). To evaluate the effects of inhibitors on PDGFRA on PDGF signaling, we pre-treated GBM cells with imatinib (IMA), a potent PDGFRA inhibitor, but we
surprisingly found that inhibition on PDGFA activity could not
diminish the activation of AKT, a well-known downstream target
of PDGFA (Fig. 1c). In accordance with this observation, depletion
of PDGFA in LN18 cells (LN18PDGFA−/−) through CRISPR/Cas9
technology did not completely block PDGFA signaling (Fig. 1d).
Interestingly, analysis on TCGA_GBMS and TCGA_GBMLGG data-
bases showed that PDGFA and PDGFR were negatively correlated
with each other and their expression patterns in various molecular subtypes were also inconsistent (Supplementary
Fig. S1f and S1g). Then, we classified 539 GBM cases of TCGA_GBMS
gene expression levels of PDGFA and PDGFR into Subgroup 1 (PDGFAHigh/PDGFRALow, n = 25), Subgroup 2
(PDGFAHigh/PDGFRALow, n = 35), and Subgroup 3 (PDGFALow, n = 67) (Dataset 1). As expected, gene set enrichment analysis (GSEA)31,32 showed that Subgroup 1 significantly enriched genesets of PDGUPV1UP (M2834 from Molecular Signatures Database
v7.4) (ES = 0.4392; P = 0.0008) and WP_PDGF_PATHWAY (M39555
from Molecular Signatures Database v7.4) (ES = 0.5076; P = 0.00403) compared to Subgroup 3 (Fig. 1e). Intriguingly, Subgroup 2 significantly enriched genesets of PDGUPV1UP (ES = 0.4125: P = 0.00499) and WP_PDGF_PATHWAY (ES = 0.5424: P = 0.008) compared
to Subgroup 3 (Fig. 1f). However, PDGUPV1UP and WP_PDGF_PATHWAY genesets was not enriched by Subgroup 1
versus Subgroup 2 (P = 0.2455 and P = 0.9876, respectively) (Data
not shown). These data implied that the expression level of PDGFA
was not decisive for PDGFA signaling and PDGFA might function in a
PDGFA-independent manner in GBM cells.
To understand the regulation network of PDGFA in GBM cells, we profiled PDGFA interactome using LN18 cells. For this purpose, the LN18 cells were treated with 100 ng/ml recombinant human
PDGFA-AA for 15 min and harvested for Co-IP with anti-PDGFA
antibody. Three separated Co-IP samples (triplicate) were sub-
jected to MS identification and 189 proteins were consistently
detected including PDGFA itself (Dataset 2). According to the protein
functional keywords, 157 of 189 proteins could be categorized into six groups, including enzymatic regulation,
protein regulator, protein trafficking, cell mobility, metabolism,
and organelle localization (Fig. 1g and Supplementary Table S1).
Then, we analyzed the interactome via DAVID Bioinformatics
Resources 6.8 (https://david.ncifcrf.gov/). KEGG pathway analysis
indicated that the PDGFA-associated proteins were mainly cancer
and neural system-related (Supplementary Fig. S1h), such as proteoglycans in cancer, pathways in cancer, gloma, and axon
guidance. Moreover, in KEGG pathways, top-ranked eight PDGFA-
involved categories according to P-Value were EGFR transmembrane
kinase inhibitor resistance, Ras signaling pathway, Pathways in
cancer, Gloma, MicroRNAs in cancer, Rap1 signaling pathway,
focal adhesion, and PI3K-AKT signaling pathway (Fig. 1h). GO
analysis showed that PDGFA-associated proteins played important roles in PI3K activity, tyrosine kinase activity, ubiquitin ligase
activity, and protein transport (Supplementary Fig. S1i). In the
kinase category of PDGFA interactome, PDGFR, PIK3R1, PIK3R2,
PIK3R3, PIK3CA, and PIK3CB were highly abundant (Dataset 2 and
Supplementary Table S1). Since PDGFA strongly activated PI3K/
AKT pathway through PDGFR, the interaction of PDGFA with these proteins confirmed the reliability of PDGFA interactome.
Furthermore, we noticed that the kinases consistently contained
SH2-domain (Supplementary Fig. S1)), which are known to be
responsible for RTK activity and trafficking. Together, our study
profiled the regulation network of PDGFA and emphasized the
critical implication of a series of kinases.

EPHA2 was activated by PDGFA in a PDGFA-independent manner
In the Kinase category, we noticed two RTKs, EPHA2 and AXL, with
high abundance (Dataset 2). Interestingly, treatment of PDGFA-AA
dramatically led to EPHA2 phosphorylation but not AXL in GBM
cells lines with endogenous or exogenous PDGFA expression
(Fig. 2a and Supplementary Fig. S2a). We cultured LN18 GBM cell
line as sphere and similarly observed PDGFA-IA-induced EPHA2
activation (Fig. 2b and Supplementary Fig. S2b). Since EPHA2 has
been reported to be phosphorylated by activated AKT, we blocked
the activation of AKT by MK2206, an AKT inhibitor. Although the
AKT activation was completely blocked by the inhibitor, PDGFA-AA-
induced EPHA2 phosphorylation was not significantly affected
(Fig. 2c and Supplementary Fig. S2c). It was noted that EPHA2
phosphorylation showed a consistent pattern with phosphory-
lated PDGFR, which prompted us to investigate if the
phosphorylation of EPHA2 happened parallelly with or as a result of
PDGFA activation. For this purpose, we treated parental LN18 and
LN18PDGFA−/− with PDGFA-AA. Compared with parental cells,
loss of PDGFA could not diminish PDGFA-AA-induced phosphory-
lation of EPHA2, either (Fig. 2e). Using U251 cells, we found that
the phosphorylation of EPHA2 did not rely on PDGFA protein
or activation (Supplementary Fig. S2d and S2e). Then, we used
inhibitor of EPHA2 (ALW-II-41-27) to pretreat LN18 and U251 cells
followed by PDGFA-AA stimulation. Intriguingly, ALW-II-41-27
resulted in significant decrease of PDGFA-AA-induced activation
of AKT, but AXL inhibitor TP0903, which we used as negative
control, did not alter the phosphorylation trend of AKT
(Supplementary Fig. S2f). We further investigated the PDGFA-AA-
induced spatiotemporal distribution of EPHA2 in GBM cells by
immunofluorescence. Since endosomes are key location for
endoctytic RTKs,33–35 we used EEA1, a marker of endosome, as
a beacon for EPHA2 cellular localization. The results showed that,
without PDGFA-AA, EPHA2 distributed evenly in cells, and
colocalization of EPHA2 and EEA1 were hardly detected (Fig. 2f).
At 15 and 30 min following ligand stimulation, most of EPHA2
colocalized with EEA1, and mainly concentrated around the nuclei,
implying trafficking towards endosome (Fig. 2f). At 60 min,
however, EPHA2 restored distribution as inactivation form and
significantly decreased in accordance with western blotting result
(Fig. 2f). Together, these results indicated that EPHA2 could be
activated by PDGFA in a PDGFA-independent manner and
involved in PDGFA signaling.
Since PDGFA activated EPHA2 in our study, we asked whether
PDGFA could interact with EPHA2. Co-IP-MS analysis using
LN18PDGFA−/− showed EPHA2 could interact with PDGFA (Dataset
3), which was confirmed by Co-IP and western blotting in GBM
cells (Fig. 2g) or through in vitro protein binding assay (Supplementary
Fig. S2g). Then, we simulated the interaction of
PDGFA-AA and EPHA2, and the result showed that homodimer of
mature PDGFA could interact with EPHA2 extracellular domain
(Fig. 2h and Supplementary Fig. S2h). To examine the interaction
between EPHA2 and PDGFA, we determined the binding
thermodynamics of two peptides using Microcal iTC200. The data
showed that recombinant human PDGFA extracellular domain fused
with IgG Fc (rhRA-ECD-Fc), a positive control, could interact
with PDGFA-AW with ΔH = −1066 ± 116.7 (Supplementary Fig. S2i),
indicating spontaneous reaction. Similarly, EPHA2 extracellular
domain tagged with histidine (rhA2-ECD-His) and PDGFA-AA also
produced negative ΔH = −262.3) (Fig. 2i), supporting their
interaction. Proximity ligation assay (PLA), which detects in situ
direct interactions between two proteins that are less than 40 nm
apart,36,37 clearly indicated the interaction of PDGFA-AA with EPHA2
but the interaction could be significantly reduced by rhRA-ECD-Fc
treatment (Fig. 2j and Supplementary Fig. S2j). Thus, EPHA2 might
function as a receptor for PDGFA-AA. Then we asked whether
PDGFA and EPHA2 could function as a complex together.
Co-expression of PDGFA-Flag with PDGFA-HA, EPHA2-Flag with
EPHA2-GFP, or PDGFA-HA with EPHA2-Flag showed that PDGFA
and PDGFR, EPHA2 and EPHA2, or PDGFA and EPHA2 could form
complex in GBM cells (Supplementary Fig. S2k). Indeed, the
activation of PDGFA-AA-induced PDGFA phosphorylation could be
enhanced by overexpression of EPHA2 but reduced by
knockdown of EPHA2 in LN18 cells (Fig. 2K and Supplementary Fig. S2l). With treatment of PDGF-AA, p-AKT(S473) was clearly detected in control LN18 cells but not in LN18 cells with sgPDGFRα and shEPHA2 (Supplementary Fig. S2m). Furthermore, in control LN18 cells, we could detect a temporal increase of PDGF-AA uptake but failed to do so in N18 cells with sgPDGFRα and shEPHA2 (Supplementary Fig. S2n). These data highlighted that EPHA2 might coordinate with PDGFRα to mediate PDGF-AA activity in GBM cells. Together, EPHA2 might function as a novel receptor for PDGFRα and mediate PDGFRα signaling solely or together with PDGFRα.

Expression of EPHA2 was correlated with that of PDGFRα signaling targets in GBM
Since the involvement of EPHA2 in PDGFRα signaling, we examined whether EPHA2 had roles in tumor growth and invasion of GBM cells through MTT assay an Matrigel-coated transwell assay in the context of PDGF-AA stimulation, which indicated that EPHA2 knockdown decreased viability (Supplementary Fig. S3a) and invasiveness (Supplementary Fig. S3b). Then, we investigated the potential expression regulation and downstream targets in GBM. In TCGA_GBM database, we observed that EPHA2 expression in classical and mesenchymal GBM was significantly higher than that in neural and proneural GBM (Fig. 3a). We also evaluated the factors involved in EPHA2 expression regulation. Via glivios. bioinfo.cnio.es website, we profiled all genes significantly correlated with EPHA2 (Dataset 4). Through combining these genes with the transcription factor list from Uniprot (Dataset 5), we observed that KLF5 was ranked No.1 in EPHA2-related transcription factors (Fig. 3b and Supplementary Table S2). It has been known that gene transcription is critically regulated by CpG methylation, which promoted us to explore CpG methylation of EPHA2 gene promoter. According to the methylation K450 probe, we noticed that low methylation at 3 of 10 probes in EPHA2 promoter region were corresponding to high expression of EPHA2 mRNA (Fig. 3c and Supplementary Table S3). Consistently, the three probes showed low methylation levels in classical and mesenchymal subtype GBM but high methylation levels in neural and proneural subtype GBM (Supplementary Fig. 5c). To examine whether EPHA2 expression was involved in PDGFRα signaling, we defined geneset of PDGFRα downstream targets through combining several well-described PDGFRα signaling genetses (Dataset 6) followed by heatmap cluster analysis according to EPHA2 expression in TCGA_GBM database. Interestingly, PDGFRα target genes were significantly higher in EPHA2High cases than EPHA2Low cases ($P = 9.446E−59$) (Fig. 3d).

To test the conclusion from TCGA_GBM, we collected four tumor foci from a multifocal GBM patient with four separate lesions at diagnosis60 (Supplementary Fig. S3d). Whole-genome sequencing confirmed the four tumor foci harbored typical genetic features of GBM (Supplementary Fig. S3e), including gain and loss of chromosome 7 and 10, respectively, and loss of key tumor suppressors PTEN and CDKN2A/2B.18,21 In addition, we observed the amplification of EGFR and PDGFRα in all tumor foci and amplification of PDGFRα gene in tumor sample 2 and 3. We then performed RNA sequencing to profile transcriptome of the four tumor foci (Dataset 7) and noticed that KLF5 was also the top 1 transcription factor correlated with EPHA2 in the four samples (Supplementary Fig. S3f and Table S4). We performed whole-genome bisulfite sequencing (Dataset 8) and confirmed that the methylation level in promoter region corresponding to the two critical probes at EPHA2 promoter from TCGA_GBM was negatively related with EPHA2 mRNA level in the four tumor samples (Fig. 3e). Using the transcriptome from the four samples, we performed heatmap cluster analysis on PDGFRα target genes. Similar to TCGA_GBM database, EPHA2 was correlated with the expression of PDGFRα target genes. Moreover, PDGFRα expression seemed not so tightly related to PDGFRα target genes as EPHA2 (Fig. 3f). Together, these data revealed KLF5 and two methylation sites in EPHA2 promoter region as critical regulation elements for EPHA2 expression and confirmed the tight involvement of EPHA2 in PDGFRα signaling pathway.

High expression of PDGFRα and EPHA2 enriched oncogenic genesets in GBM cells
To understand the overall effects of co-upregulation of PDGFRα and EPHA2 on GBM cells, we profiled transcriptomes of LN18 cells with forced expression of EGFR as control, PDGFRα only, or PDGFRα with EPHA2, respectively (Dataset 9).

Compared with either PDGFRα or EPHA2 individual expression, co-upregulation of the two genes could significantly enrich mesenchymal and classical signature genes (Fig. 4a), but failed to enrich Proneural and Neural signature genes (Supplementary Fig. S4a).

Similar conclusion was drawn from analysis on TCGA_GBM database (Fig. 4a and Supplementary Fig. S4a). We noticed that PDGFRαHigh/EPHA2High in LN18 cells enriched genesets of ANASTASSIOU MULTICANCER INVASIVENESS SIGNATURE and SCHUETZ BREAST CANCER DUCTAL INVASIVE UP (Fig. 4b), which was observed in TCGA_GBM database (Fig. 4b).

Moreover, analysis on TCGA_GBM indicated that PDGFRαHigh/EPHA2High enriched hallmark genesets of EPITHELIAL MESENCHYMAL TRANSITION, ANGIogenesis, HARRIS BRAIN CANCER PROGENitors, and CORDEnOSI YAP CONSERVED SIGNATURE (Supplementary Fig. S4b), further supporting the oncogenic roles of PDGFRα and EPHA2. G-CIMP subtype of GBM shows low expression of some oncogenes due to DNA methylation-related gene silence and assumes better prognosis than Non-G-CIMP subtype of GBM.18,41 GSEA indicated that PDGFRαHigh/EPHA2High transcriptome significantly enriched genes silenced in G-CIMP GBM compared with that of Non-PDGFRαHigh/EPHA2High in both LN18 cells and TCGA_GBM database (Fig. 4c).

We specifically analyzed consistently altered genes in both LN18 cells and TCGA_GBM database in the context of PDGFRαHigh/EPHA2High vs. Non-PDGFRαHigh/EPHA2High. 50 genes were significantly altered in both datasets ($P < 0.01$) with 139 upregulated and 11 downregulated (Fig. 4d and Supplementary Table S5). Eight genes (SCG2, TMEM45A, SLC2A3, CHI3L1, PLAT, PTX3, ICAM1, STT3A) were upregulated more than 1.5-fold. Next, we analyzed the relationships of eight significantly upregulated genes with expression of EPHA2, PDGFRα, and PDGFRα in TCGA_GBMMLGG database via GEPIA 2 (http://gepia2.cancer-pku.cn). The result showed that each of eight genes were positively correlated with combined expression of EPHA2, PDGFRα, and PDGFRα (Supplementary Fig. S4c). Forced expression of PDGFRα and EPHA2 together in LN18 cells with PDGFRα-AA treatment could significantly upregulate seven of eight target genes with exception of CHI3L1 (Supplementary Fig. S4d), confirming the regulation of these genes by EPHA2 and PDGFRα. Analysis on the consistently upregulated proteins via David software showed that cell-extracellular matrix interaction-related categories were top ranked in GO and KEGG categories (Fig. 4e), which well supported the effect of PDGFRα and EPHA2 on invasive growth of GBM cells. In addition, angiogenesis and PI3K-AKT signaling pathway were significantly enriched (Fig. 4e), which were consistent with known functions of PDGFRα and EPHA2. Among the top-ranked 8 genes, SCG2, SLC2A3, CHI3L1, PLAT, PTX3, and ICAM1 were found as markers for poor survival of GBM patients using the TCGA_GBM database (Supplementary Fig. S4e). High expression of TMEM45A or STT3A also showed worse prognosis than low expression despite no statistical significance (data not shown). Thus, these significantly upregulated genes might contribute to increased invasive growth of GBM cells induced by PDGFRα and EPHA2 co-expression.
PDGFRA and EPHA2 were promising pharmaceutical targets for GBM.

To evaluate protein expression of PDGFRA and EPHA2 using clinical samples of glioma, we collected a 180-case glioma cohort (Cohort-180). Kaplan–Meier survival analysis on Cohort-180 showed that patients with higher-grade gliomas had significantly shortened survival time compared to those with lower-grade gliomas (supplementary Fig. S5a), confirming the reliability of the cohort.

We stained the Cohort-180 with antibodies of anti-PDGFRA and anti-EPHA2 antibodies with definition of high expression and low expression according to staining percentage and intensity (supplementary Fig. S5b). Cases with PDGFRAHigh/EPHA2Low showed dramatically improved prognosis compared to those with PDGFRAHigh/EPHA2High (Fig. 5a, b), which was confirmed by TCGA_GBM database (supplementary Fig. S5c). It has been reported that Proneural subtype showed better survival in comparison with the other three subtypes in TCGA_GBM dataset.15 Interestingly, the survival curve of PDGFRAHigh/EPHA2High similar to those of Mesenchymal, Classical, and Neural (Fig. 5c), but the survival curve of PDGFRAHigh/EPHA2Low matched with that of Proneural (Fig. 5d), which implied that both proteins might be used as pathological markers to predict prognosis without testing the subtypes of patients.

To simplify the potential diagnostic application of the two proteins, we compared PDGFRAHigh/EPHA2High cases with all non-PDGFRAHigh/EPHA2High cases. In Cohort-180, cases with PDGFRAHigh/EPHA2High had worse prognosis than those with non-PDGFRAHigh/EPHA2High cases. In Cohort-180, cases with PDGFRAHigh/EPHA2High had worse prognosis than those with non-PDGFRAHigh/EPHA2High (Fig. 5e). PDGFRAHigh/EPHA2High showed increased percentage with glioma progression: 4% (1 in 25 cases) for grade I glioma, 6.3% (5 in 80 cases) for grade II glioma, 11.8% (6 in 51 cases) for grade III glioma, and 12.5% (3 in 24 cases) for GBM (Fig. 5f). In TCGA_GBM database, the percentage of cases with PDGFRAHigh/EPHA2High in Classical (30.3%) and Mesenchymal

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**Fig. 3** EPHA2 expression regulation and relationship with PDGF downstream targets. a EPHA2 expression in different molecular subtypes of GBM in TCGA_GBM database. b Pearson correlation of EPHA2 and KLFS in TCGA_GBM database. c Methylation levels of EPHA2 promoter region measured with methylation K450 probes. d Heatmap cluster analysis of PDGF signaling target genes with EPHA2High vs. EPHA2Low in TCGA_GBM database. e The methylation level in promoter region corresponding to two critical probes at EPHA2 promoter from TCGA_GBM and the EPHA2 mRNA level in four tumor foci from a multifocal GBM patient. f Upper: Heatmap cluster analysis of PDGF signaling target genes in the four tumor foci; Lower: mRNA expression of EPHA2, PDGFA, and PDGFRA in the four tumor foci.
(24.1%) was much higher than Proneural (12.9%) and Neural (11.5%) (Supplementary Fig. S5d). We then analyzed TCGA_GBM database on the expression of PDGFRA and EPHA2. The data consistently showed that patients with PDGFRAHigh/EPHA2High had shorter survival time than those with PDGFRAHigh/EPHA2Low in all grades and HGGs with statistical significance (P < 0.0001 and P = 0.0006) and in low-grade glioma without statistical significance (P = 0.1179) (supplementary Fig. S5e). In addition, the number of cases with PDGFRAHigh/EPHA2High in HGG was much higher than that in low-grade glioma (90 vs. 38), but the number of cases with PDGFRAHigh/EPHA2Low in HGG was lower than that in low grade glioma (101 vs. 121) (Supplementary Fig. S5f). We also performed IHC staining using anti-PDGFA and anti-phospho-AKT (S473) antibodies (Supplementary Fig. S5g). The $\chi^2$ test indicated that high expression of PDGFA was positively related with high levels of p-AKT(S473) (Supplementary Fig. S5h). It was noted most of PDGFAHigh/p-AKT(S473) high cases (74 in 78) had high expression of PDGFRA and EPHA2, either individually or together (Supplementary Fig. S5h). In addition, correlation between PDGFA and p-AKT (S473) was much more significant in cases with high expression of PDGFA and/or EPHA2 than those with low expression of PDGFA and EPHA2 ($P = 2.7e-8$ vs. $P = 0.018$) (Supplementary Fig. S5h). Thus, the distribution feature of PDGFA and EPHA2 implied that the two proteins were related with malignant phenotype of glioma. We further evaluated the significance of PDGFA and EPHA2 expression for treatment effects. The result showed that single regimen treatment (radiation or chemotherapy) did not show benefit on all patients, and radio-chemo therapy mildly prolonged patient survival with PDGFRAHigh/EPHA2High (Fig. 5g). However, both single regimen and radio-chemo therapy significantly improved survival of patients with non-PDGFRAHigh/EPHA2High (Fig. 5h). In addition, clinical therapy prolonged median survival time in patients with non-PDGFRAHigh/EPHA2High much more than those with PDGFRAHigh/EPHA2High (224 days vs. 137.5 days for single regimen and 402 days vs. 296 days for radio-chemo therapy) (Fig. 5i). Therefore, our clinical analysis confirmed that concurrent expression of PDGFA and EPHA2 could be promising prognostic markers and therapeutic targets.

Simultaneously targeting EPHA2 and PDGFRA suppressed growth of GBM cells in vitro and in vivo. Since PDGFA could activated EPHA2 bypassing PDGFRA, we speculated that GBM cells with high EPHA2 might be resistant to IMA but GBM cells with low EPHA2 might be sensitive to IMA. As expected, we found that IC50 of LN18EPHA2 to IMA was higher than that of LN18 and IC50 of LN18shEPHA2 to IMA was lower than that of LN18 (Fig. 6a). Similarly, IC50 of U251EPHA2 to IMA was higher than that of U251 and IC50 of U251shEPHA2 to IMA was lower than that of U251 (Fig. 6a). Growth curve measurement through MTT indicated that the decreased expression of EPHA2 sensitized GBM cells to IMA (Supplementary Fig. S6b). GSEA on TCGA_GBM database consistently showed that high expression of EPHA2 significantly enriched genes upregulated in IMA resistant patients (GSE155800) and cells (MAHADE-VAN GIST MORPHOLOGICAL SWITCH) (Supplementary Fig. S6c). To evaluate whether there was coordination of PDGFA and EPHA2

![Fig. 4 Transcriptomic analyses on PDGFRA and EPHA2 co-upregulation in GBM cells.](image-url)
EPHA2 upon PDGF-AA stimulation, we used IMA and ALW alone or in combination to treat GBM cells. Antibody array result showed that the combination of two inhibitors exerted significantly stronger inhibition on the activation of PDGFA downstream targets than each inhibitor alone (Fig. 6b). Furthermore, MTT assay confirmed that the two inhibitors showed synergetic effects in several GBM cell lines (Fig. 6c). Colony formation assay consistently proved that the combination of IMA and ALW more potently suppressed expansion of GBM cells than either one of the two inhibitors (Supplementary Fig. S6d). Thus, our results revealed that EPHA2 might coordinate with PDGFRA to augment PDGF-AA effects in GBM cells.

We then examined the inhibitory effects of the two inhibitors using orthotopic mouse model. The SCID mice was orthotopically inoculated with U251 cells together with PDGFA virus, which could effectively promote in vivo growth of GBM cells in brain (Supplementary Fig. S6e and S6f). One week later, the mice were treated with PBS, IMA (25 mg/kg), ALW (10 mg/kg), or IMA + ALW via intraperitoneal injection. The data showed that IMA or ALW alone could reduce the size of GBM but the effects was not statistically significant (P > 0.05). Combination of the two inhibitors, however, significantly inhibited the tumor growth compared with PBS or ALW treatment (Fig. 6d, e) and the inhibitory effects were supported by Ki67 staining (Fig. 6f). Parallel animal experiments for survival analysis suggested that combination of ALW and IMA could improve survival of mice with GBM (Supplementary Fig. S6g). IMA (STI571, CGP-57148B) is known as a selective blood-brain barrier-permeable PDGFR antagonist.42,43

Fig. 5 Clinical significance of EPHA2 and PDGFRA in GBM. a Representative immunohistochemistry images of EPHA2 and PDGFRA proteins on continuous tissue sections. Scale Bar = 200 μm (upper) and 50 μm (lower). b Kaplan–Meier survival analysis on cases with PDGFRA High/EPHA2 High vs. PDGFRA High/EPHA2 Low from our glioma cohort. c Survival curve comparison between cases with PDGFRA High/EPHA2 High and different molecular subtypes according to TCGA_GBM mRNA expression dataset. d Survival curve comparison between cases with PDGFRA High/EPHA2 Low and different molecular subtypes according to TCGA_GBM mRNA expression dataset. e Kaplan–Meier survival analysis on cases with PDGFRA High/EPHA2 High vs. all other cases from our glioma cohort. f Case count with different protein expression patterns from our glioma cohort according to tumor grades. g, h Kaplan–Meier survival analysis of different treatment ways under specific gene expression patterns according to TCGA_GBM database. i Therapeutic effects of treatment ways on survival time of patients with specific gene expression patterns.
We stained p-EPHA2 in xenograft with or without ALW, and the data revealed marked decrease of p-EPHA2 with treatment of ALW (supplementary Fig. S6h), implying the permeability of ALW towards BBB. Thus, the tumor suppression was resulted from inhibitors and simultaneously targeting PDGFRA and EPHA2 could effectively repress GBM growth in vivo.

PDGFA, but not EFNA1, endowed oncogenic roles of EPHA2 in GBM cells

It has been known that EFNA1 is a major cognate ligand of EPHA2 in vivo, but the function of EFNA1 is thought to impair EPHA2 activity in glioma cells, which promoted us to investigate the difference of PDGFA/EPHA2 axis and EFNA1/EPHA2 axis. To avoid the interference of PDGFA on PDGFA-EPHA2 axis, we treated PDGFRA−/− GBM cells with PBS as control, recombinant soluble human EFNA1 withFc-tag, and recombinant human PDGFA homodimer followed by RNA sequencing to profile transcriptomes (Dataset 10). The RNA sequencing data showed that genes altered by PDGFA and EFNA1 were few overlaid (Fig. 7a,b). David analysis showed that genesets enriched by PDGFA-upregulated genes (Cutoff: P < 0.05 and Fold Chang > 1.25) were obviously distinguished from those enriched by EFNA1-upregulated genes (supplementary Table S6). The former included cell mobility and PDGF-related genesets, but the latter included metabolism, apoptosis, and lysosome-related genesets. In addition, PDGFA could enrich EMT genesets, but EFNA1 failed to do so (Fig. 7d). Thus, EFNA1 could not induce EPHA2 oncogenic roles in GBM, which was consistent with previous reports.

DISCUSSION

It has been documented that PDGFA/PDGFRα is highly expressed in GBM and plays critical roles in gliomagenesis, but targeting PDGFRα by small molecule inhibitors do not show therapeutic significance in clinic. In this study, we found that, besides PDGFRα, EPHA2 could also mediate PDGFA signaling pathway in a PDGFRα-independent manner. In addition, EPHA2 and PDGFRα could also function together to enhance PDGFA functions. Therefore, inhibition of PDGFRα or EPHA2 only was not sufficient to block PDGFA function but concurrent suppression of the two kinases could be promising regimen for GBM treatment (Fig. 7e). EPHA2 belongs to the largest RTK subfamily—EPH receptor family, and is the most frequently altered EPH members in...
cancers. In GBM, EPHA2 functions as a mitogen and high expression of EPHA2 is correlated with poor survival of patients. Our data clearly showed that EPHA2 was required for viability and invasiveness of GBM cells and significantly upregulated genes by EPHA2 were also involved in malignant phenotype of GBM. Interestingly, oncogenic roles of EPHA2 in GBM are independent of its cognate ligand—type-A ephrin, implying other unidentified proteins may act as ligands of EPHA2. In this work, we found that PDGFA interacted with and activated EPHA2 in GBM cells, and furthermore, in vitro assay and three-dimension structure simulation indicated PDGFA as a EPHA2 ligand. The identification of PDGFA as a ligand of EPHA2 might explain the functional regulation of EPHA2 in GBM. Moreover, EPHA2 has been known to play important roles in glioblastoma stem cells or stem-like cells. We also observed that higher protein level of EPHA2 in GBM cells cultured in neurosphere medium than in attachment medium (Data not shown). Thus, targeting EPHA2 not only reduced glioblastoma stemness but also suppressed PDGF-AA-induced tumor growth.

Although four PDGF ligands have been identified, the frequency of genetic alteration (gain of copy number) and mRNA increase of PDGFA was significantly higher than those of PDGFb, C, and D in GBM, indicating the critical involvement of PDGFA in GBM. Interestingly, we did not detect interaction between EPHA2 with PDGFb, PDGFC, or PDGFd (Data not shown), implying that interaction between EPHA2 with PDGFA might be specific in GBM cells. The only identified receptor of PDGFA is PDGFRα, which is one of the most typical features of Proneural GBM, and interestingly, all subtypes are thought to evolve from proneural-like glioma precursor and most secondary GBMs highly resemble proneural phenotype. PDGFRα functions as a putative driver gene during glioma development induced by intracranial radiation. These findings reveal PDGFA/PDGFRα as a potential therapeutic target, but targeting the signaling axis has failed in clinical trials. Since EPHA2 could be activated by PDGFA even without PDGFRα activation (IMA treatment) or PDGFRα expression (PDGFRα−/−), the cells with co-upregulation of PDGFA and EPHA2 might be insensitive to PDGFRα inhibitor alone, but could be sensitive to combination of PDGFA inhibitor and EPHA2 inhibitor. Thus, our current results provided an explanation for the failed clinical trials targeting PDGFA.
In the study, we used four tumor foci from a multifocal GBM patient to examine the regulation and function of EPHA2. About 10–20% of GBM patients are diagnosed with more than one tumor lesion or multifocal GBM. Patients with multifocal GBM have shortened overall survival compared to those with one GBM mass or unifocal GBM and are resistant to current therapeutic measures. Various tumor foci from same multifocal GBM patient are actually evolved from monoclonal origin. Therefore, analysis on tumor foci of same multifocal GBM patient could accurately profile evolution difference among these tumor foci without interference of individual genetic and epigenetic difference. Using this model together with TCGA_GBM database, we revealed transcription factor KLF5 and two methylation sites in EPHA2 promoter region as potential regulation elements of EPHA2 transcription, which needed further pursuit in following work. Moreover, our findings suggested that EPHA2 high expression was correlated with high expression of PDGF signaling targets, confirming the tight involvement of EPHA2 in PDGFα function.

In combination with TCGA_GBM database, we noticed that co-expression of PDGFα and EPHA2 significantly enriched genesets of mesenchymal and classical but not proneural and neural, and moreover, several invasive growth-related genesets were also enriched by concurrent expression of the two proteins. We collected a panel of potential target genes upregulated by EPHA2 and PDGFα and found that the functions of these genes were mainly in the extracellular matrix, cell adhesion, angiogenesis, and PI3K-AKT, which were tightly correlated with malignant phenotypes of GBM with high expression of PDGFα and EPHA2. Further analysis on clinical treatment efficacy using TCGA_GBM database showed that GBM with PDGFα and EPHA2 was insensitive to radiation or/and chemotherapy but GBM without PDGFα and EPHA2 responded well to clinical treatment strategy. Therefore, pathological examination of PDGFα and EPHA2 might be valuable for the prediction of survival and clinical treatment efficacy.

In addition, our study for the first time profiled the regulation network of PDGFα-associated proteins, including kinases, protein modifying growth factor-induced activation of RTKs, including EGFR, FGFR, IGF1R, PDGFα, and PDGFRβ, are dependent on receptor internalization, trafficking, and endocytosis. Extensive researches on EGFR endocytosis through high-resolution proteomics depict endocytosis-related temporal interactomes of EGFR, which form a dynamic regulation network for EGFR activation. From the EGFR-induced EGFR interactomes, several novel EGFR regulators have been identified as potential therapeutic targets for cancers driven by EGFR, and hence blocking the interaction between RTK with its regulators represents a novel strategy to target the overactivated RTK.

Altogether, our study for the first time profiled the interactome of PDGFα in GBM cells and revealed a critical interaction between PDGFα and EPHA2, which provided new insights on PDGFα/PDGFRα and PDGFα/EPHA2 signaling axes in GBM. Moreover, our work implied that EPHA2 and PDGFα might be therapeutic targets for GBM with high expression of both proteins, emphasizing that the molecular mechanisms underlying PDGFα signaling activation by new binding partners need to be clarified in detail for application of PDGFα-related therapeutic strategies on GBM treatment.

**DATA AVAILABILITY**

All data are available in the manuscript and its supplemental materials.

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**AUTHOR CONTRIBUTIONS**

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**ADDITIONAL INFORMATION**

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