

Differential expression of PDGFRB and EGFR in microvascular proliferation in glioblastoma

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Abstract Glioblastoma (GBM) is the highly malignant glioma and exhibits microvascular proliferation. PCR mRNA arrays and immunohistochemical stains on tissue microarray demonstrated that the expression level of PDGFRB in GBM microvascular proliferation was significantly higher than that in GBM tumor cells while the expression level of EGFR was lower in microvascular proliferation than in GBM tumor cells. PDGFRB protein was selectively expressed in pericytes in GBM microvascular proliferation. By analyzing The Cancer Genome Atlas (TCGA) datasets for GBM, it was found that genomic DNA alterations were the main reason for the high expression of EGFR in GBM tumor cells. Our miRNA microarray data showed that microRNAs (miRNAs) (miR-193b-3p, miR-518b, miR-520f-3p, and miR-506-5p) targeting PDGFRB were downregulated in microvascular proliferation, which might be the most likely reason for the high expression of PDGFRB in GBM microvascular proliferation. The increase of several miRNAs (miR-133b, miR-30b-3p, miR-145-5p, and miR-146a-5p) targeting EGFR in GBM microvascular proliferation was one of the reasons for the lack of expression of EGFR in GBM microvascular proliferation. These findings implicated that miRNAs, such as miR-506, miR-133b, miR-145, and miR-146a, that target PDGFRB or EGFR, might be potential therapeutic agents for GBM. A new

generation of targeted therapeutic agents against both EGFR and PDGFRB might be developed in the future.

Keyword Glioblastoma · Microvascular proliferation · PDGFRB · EGFR · PCR array · Tissue microarray · MicroRNA (miRNA)

Introduction

Glioblastoma (GBM) (WHO grade IV), constituting 50–60 % of all gliomas, is the most common and highly malignant primary brain tumor. The median survival time of GBM is 12–15 months with the combination of chemotherapy and radiation therapy after surgery. Microvascular proliferation distinguishes GBM from lower grade infiltrating astrocytomas [1]. Currently, almost all chemotherapeutic agents target GBM tumor cells but not tumor microenvironments, such as microvascular proliferation. Targeted anti-angiogenic therapy with Avastin, a monoclonal antibody against vascular endothelial growth factor, induces temporary remission of GBM on magnetic resonance imaging (MRI), but does not significantly prolong the overall survival time [2]. Surprisingly, it is found that Avastin can restore the blood–brain barrier in the GBM microvasculature, which reduces the permeability of other chemotherapy agents such as temozolomide [3]. Thorough investigation of the nature of microvascular proliferation is essential for developing more effective targeted therapy. However, the volume of the microvasculature is too low in GBM to identify microvascular specific genes with a standard gene microarray method that starts with RNA isolated from GBM tissue. Rather, it is necessary to start with isolated microvasculature-derived RNA.

In this study, we investigated the difference of messenger RNA (mRNA) expression profiles between GBM

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microvasculature and GBM tumor cells using Human Cancer Drug Targets PCR Arrays. The mRNA array results were validated by immunohistochemistry on tissue microarray. We also explored the possible reasons that cause the high expression of platelet-derived growth factor receptor beta (PDGFRB) in GBM microvasculature and the high expression of epidermal growth factor receptor (EGFR) in GBM tumor cells by genomic DNA analysis using The Cancer Genome Atlas data and microRNA (miRNA) analysis.

Materials and methods

Laser-capture microdissection

This study was approved by the North Shore and Long Island Jewish Health System Institutional Review Board. To obtain microvascular proliferation in GBM (seven cases), laser-capture microdissection (LCM) (Leica LMD7000) was performed on formalin-fixed, paraffin-embedded (FFPE) tissue. Five-micrometer sections of human tissue were cut at room temperature and transferred to PEN-membrane, 4.0 μm slides (Leica) for LCM. About 10,000 cells were laser microdissected from microvascular proliferation in GBM.

Total RNA isolation

Total RNA was extracted from the microdissected microvascular proliferation samples and the GBM tumor tissue after complete microvascular proliferation microdissection using miRNeasy FFPE Kit (Qiagen) and was quantified as previous described [4].

Human Cancer Drug Targets PCR Arrays

Complementary DNA (cDNA) synthesis was performed and cDNA was preamplified using Human Cancer Drug Targets Primer Mix (Qiagen) as primers as previous described [4]. Preamplified cDNA was applied to Human Cancer Drug Targets 384-well format RT² Profiler PCR Arrays (Qiagen). qPCR was performed and Ct values was generated as per the manufacturer's instructions using RT² SYBR[®] Green qPCR Mastermix (Qiagen) on a LightCycler[®] 480 Instrument II (Roche). At the GeneGlobe Data Analysis Center on the Qiagen website, $\Delta\Delta\text{Ct}$ -based fold change and statistical significance analysis were carried out with the Integrated Web-based Software Package for the PCR Array System.

Construction of glioma tissue microarray

Compared to hematoxylin and eosin-stained slides, the formalin-fixed paraffin-embedded archival tissue blocks were chosen by a neuropathologist. For GBM cases, areas with

microvascular proliferation were selected. Tissue microarrays (TMAs) were constructed from selected gliomas (29 GBMs and four normal brain tissues) using a microarrayer (Beecher Instruments) as described previously [5]. Each specimen was sampled in duplicate from representative areas of tissue blocks using a 1.5-mm punch.

Immunohistochemistry

After antigen retrieval, antibodies against PDGFRB (Rabbit monoclonal anti-PDGFRB antibody, ABCAM, catalog number: ab32570, clone Y92) (1:50 dilution), EGFR (Ventana, catalog number: 790–2988, clone 3C6, pre-diluted), CD31 (Dako, catalog number: M0823, clone JC70A) (1:40 dilution) or Friend leukemia virus integration 1 (Fli-1) (Cell marque, catalog number 254 M-18, clone MRQ-1, pre-diluted) were added on glioma array tissue sections and incubated overnight at 4 °C. PDGFRB immunohistochemical staining slides were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:500 dilution) at room temperature for 30 min. The Metal-Enhanced DAB Substrate Working Solution (Thermo Scientific) was added to the tissue and incubated until the desired staining was achieved. EGFR, CD31, and Fli-1 immunohistochemical stains were performed on an automated immunostainer (Ventana Autostainer) with DAB Substrate. Immunohistochemical stains for PDGFRB, EGFR, CD31, and Fli-1 were blindly reviewed by a neuropathologist (negative: very weak-to-absent staining, or less than 25 % strongly reacting tumor cells. Positive: strong reaction in ≥ 25 % of tumor cells).

Genomic DNA mutation and amplification analysis using The Cancer Genome Atlas (TCGA) data

To investigate the role of genetic alterations in the differential expression of EGFR and PDGFRB in different components of GBM, we analyzed gene mutation, amplification, and deletion for both EGFR and PDGFRB genes in two sets of TCGA GBM database at Memorial Sloan-Kettering Cancer Center cBioPortal for Cancer Genomics web site (<http://www.cbioportal.org/index.do>) [6, 7].

Human Cancer Pathway Finder 384HC MicroRNA arrays

Five hundred nanograms total RNA from GBM samples were converted to cDNA, 100 ng total RNA from GBM microvascular proliferation were converted to cDNA, and then the cDNA was preamplified as previous described [4]. Real-Time PCR was done with miScript SYBR[®] Green PCR Kit (Qiagen) and miScript miRNA PCR Array for Human Cancer Pathway Finder 384HC (Qiagen) and Ct values was generated as previous described [4]. At the GeneGlobe Data Analysis Center on the

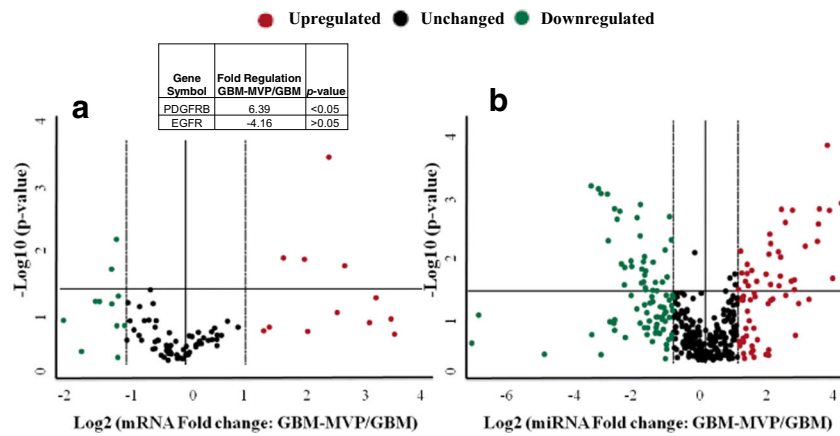


Fig. 1 The volcano plots of PCR mRNA arrays and miRNA arrays. **a** The volcano plot of the signal intensities between GBM microvascular proliferation and GBM tumor cells in Human Cancer Drug Targets PCR mRNA Arrays. **b** The volcano plot of the signal intensities between GBM

microvascular proliferation and GBM tumor cells in Human Cancer Pathway Finder 384HC MicroRNA arrays. Statistical significance and fold changes are displayed on the y- and x-axes, respectively. Red dots and green dots represent outliers beyond the +/-2 fold changes

Table 1 The summary of GBM cases

Case no.	Age (years)	Sex	EGFR in GBM tumor cells	PDGFRB in GBM tumor cells
1	73	Female	Positive	Negative
2	75	Male	Positive	Negative
3	80	Female	Positive	Negative
4	51	Male	Positive	Negative
5	54	Male	Positive	Negative
6	69	Male	Positive	Negative
7	74	Female	Positive	Negative
8	50	Female	Positive	Negative
9	89	Male	Positive	Negative
10	53	Male	Positive	Negative
11	52	Male	Positive	Negative
12	66	Male	Positive	Negative
13	73	Female	Positive	Negative
14	28	Female	Positive	Negative
15	55	Female	Positive	Positive
16	70	Female	Negative	Positive
17	26	Female	Negative	Negative
18	59	Female	Negative	Negative
19	70	Male	Positive	Positive
20	63	Female	Positive	Positive
21	70	Female	Positive	Negative
22	70	Female	Positive	Negative
23	62	Female	Positive	Negative
24	72	Female	Positive	Negative
25	58	Male	Positive	Negative
26	80	Male	Positive	Negative
27	45	Male	Positive	Negative
28	63	Male	Positive	Negative
29	44	Male	Positive	Positive

Qiagen website, $\Delta\Delta C_t$ -based fold change and statistical significance analysis were carried out with the Integrated Web-based Software Package for the PCR Array System. At Ferrolab data mining and bioinformatics group (<http://ferrolab.dmi.unict.it/>) and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) websites, a list of miRNAs that target PDGFRB or EGFR was found [8], then the expression level of those miRNAs in our miRNA array data was reviewed.

Results

High mRNA level of PDGFRB in GBM microvascular proliferation and low mRNA level of EGFR in GBM microvascular proliferation

Total RNA was extracted from GBM microvascular proliferation (seven samples) and GBM tissue sections after complete microvascular proliferation microdissection (seven samples). PCR array analysis of the gene expression of 84 actively sought targets for anticancer therapeutics and drug development was done on Human Cancer Drug Targets 384-well format RT² Profiler PCR Arrays with those RNAs. The volcano plots of the signal intensities between GBM microvascular proliferation and GBM tumor cells were produced (Fig. 1a). The mRNA level of PDGFRB (fold change=6.39, p value <0.05) was significantly higher in GBM microvascular proliferation than in GBM tumor cells. The mRNA level of EGFR (fold change=-4.16, p value >0.05) was lower in GBM microvascular proliferation than in GBM tumor cells. Besides PDGFRB was higher in microvascular proliferation, vascular endothelial growth factor receptor 1 (VEGFR-1) (fold change=4.06, p value <0.05), vascular endothelial growth factor receptor 2 (VEGFR-2) (fold change=2.39, p value <0.05) and vascular

endothelial growth factor receptor 3 (VEGFR-3) (fold change = 3.05, p value < 0.05) were also statistically significantly higher in GBM microvascular proliferation compared to GBM tumor cells, which were published previously [9, 10]. On the other hand, PDGFRA (fold change = 2.86, p value > 0.05) was higher in GBM tumor cells than in GBM microvascular proliferation without reaching statistical significance.

Selectively high expression of PDGFRB protein in pericytes of microvascular proliferation in GBM and low expression of EGFR protein in GBM microvascular proliferation

Immunohistochemical stains by anti-PDGFRB or anti-EGFR antibodies were performed on tissue microarray. It was found that in 24 out of 29 GBM cases, tumor cells were negative for PDGFRB while microvascular proliferation in every GBM case was strongly positive for PDGFRB (Figs. 2a–c, and 4a) (Table 1). Normal brain blood vessels were negative for PDGFRB (Fig. 2d). In 26 out of 29 GBM cases, tumor cells were positive for EGFR while microvascular proliferation in every GBM case was negative for EGFR (Figs. 3a–c, and 4b). Interestingly, normal brain blood vessels were positive for EGFR (Fig. 3d). Comparing to the expression patterns of

CD31 and Fli-1, endothelial markers, the PDGFRB immunohistochemical stain mainly highlighted pericytes in microvascular proliferation (Fig. 5).

Genomic DNA alterations were the main reason for the high expression of EGFR in GBM tumor cells but were less likely the reason for the decreased expression of PDGFRB in GBM tumor cells compared to GBM microvascular proliferation

We searched the genetic alterations for both PDGFRB and EGFR genes in two TCGA GBM databases from The Memorial Sloan-Kettering Cancer Center cBioPortal for Cancer Genomics web site [6, 7]. In the GBM (TCGA, 2008) dataset, 45.1 % cases (41/91) had EGFR gene alterations (mutation in four cases, amplification in 26 cases, and multiple alterations in 11 cases) (Fig. 6a). In the GBM (TCGA, 2013) dataset, 53.4 % cases (150/281) had EGFR gene alterations (mutation in 15 cases, amplification in 92 cases, and multiple alterations in 43 cases) (Fig. 6a). In the GBM (TCGA, 2008) dataset, 2.2 % cases (2/91) had PDGFRB mutation (A74T mutation in one and L986F in the other) (Fig. 6b). In the GBM (TCGA, 2013) dataset, 0.7 % cases (2/281) had PDGFRB mutation (S650L mutation in

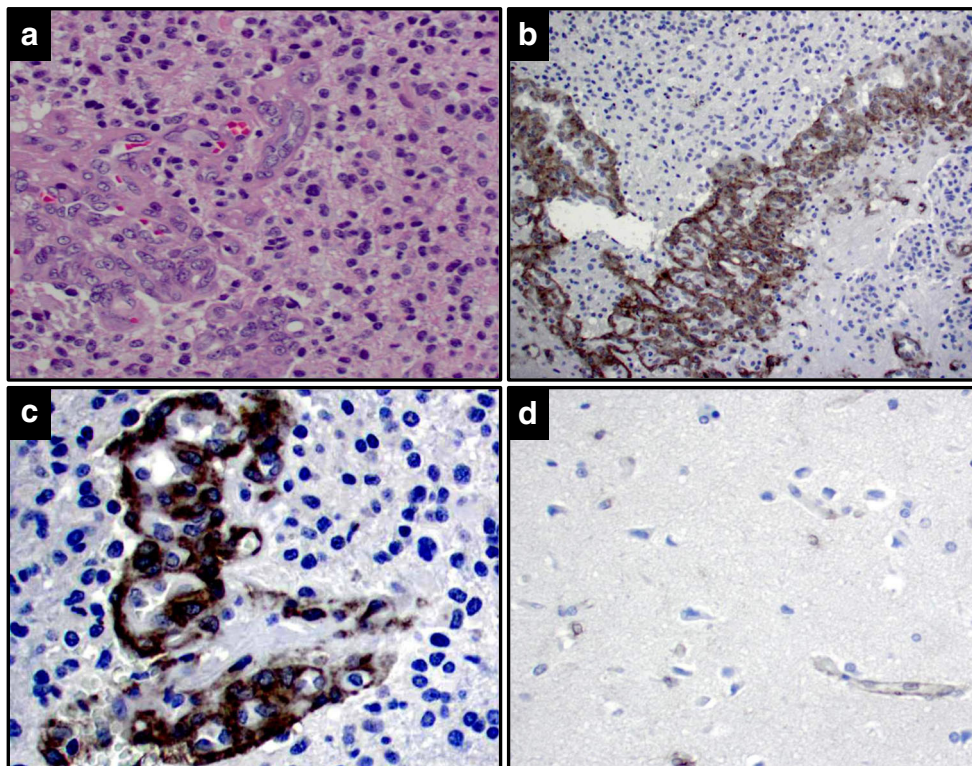


Fig. 2 PDGFRB immunohistochemical stain in glioblastoma. **a** Hematoxylin and eosin stain (H&E stain) shows glioblastoma with microvascular proliferation ($\times 400$). **b, c** Immunohistochemical stain demonstrates that microvascular proliferation in GBM is strongly

positive for PDGFRB while GBM tumor cells are negative (**b** $\times 200$, **c** $\times 400$). **d** Immunohistochemical stain shows that normal brain blood vessels are negative for PDGFRB ($\times 400$)

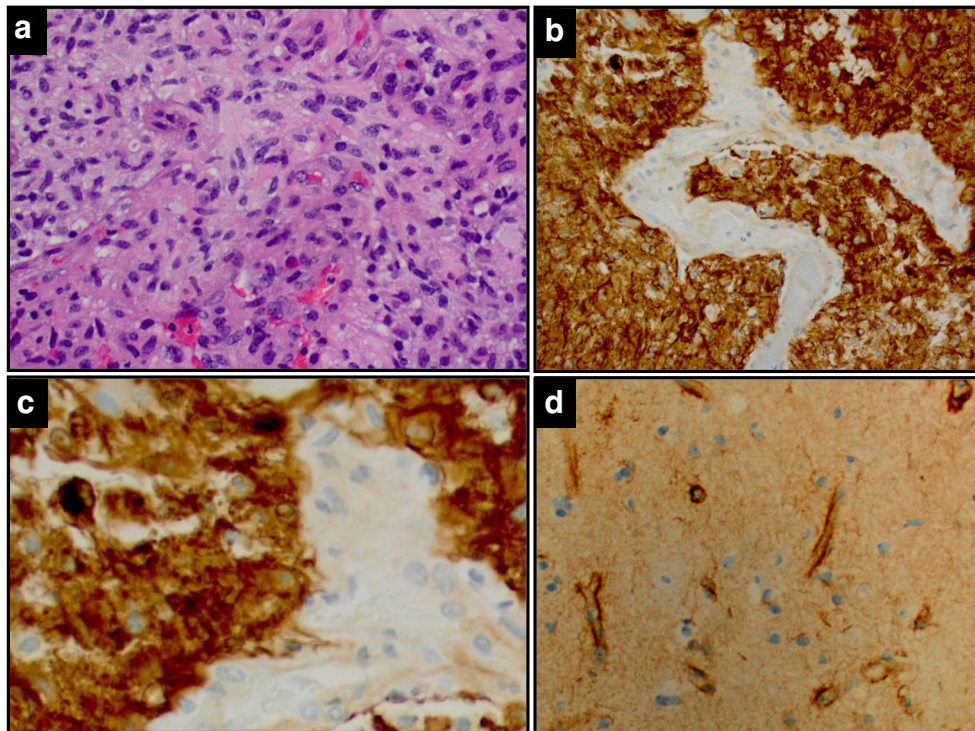


Fig. 3 EGFR immunohistochemical stain in glioblastoma. **a** Hematoxylin and eosin stain (H&E stain) shows glioblastoma with microvascular proliferation ($\times 400$). **b**, **c** Immunohistochemical stain reveals that GBM tumor cells are strongly and diffusely positive for

EGFR while microvascular proliferation in GBM is negative (**b** $\times 200$, **c** $\times 400$). **d** Immunohistochemical stain demonstrates normal brain blood vessels are positive for EGFR ($\times 400$)

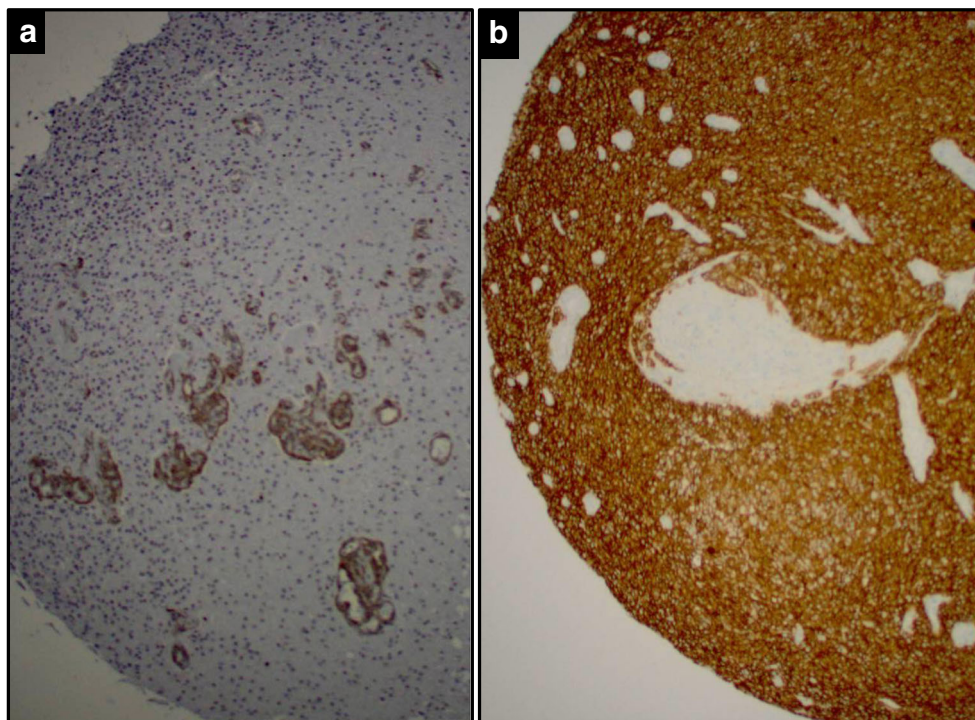


Fig. 4 PDGFRB and EGFR immunohistochemical stains in glioblastoma at low power view. **a** Microvascular proliferation in GBM is strongly positive for PDGFRB while GBM tumor cells are negative

($\times 100$). **b** GBM tumor cells are strongly and diffusely positive for EGFR while microvascular proliferation in GBM is negative ($\times 100$)

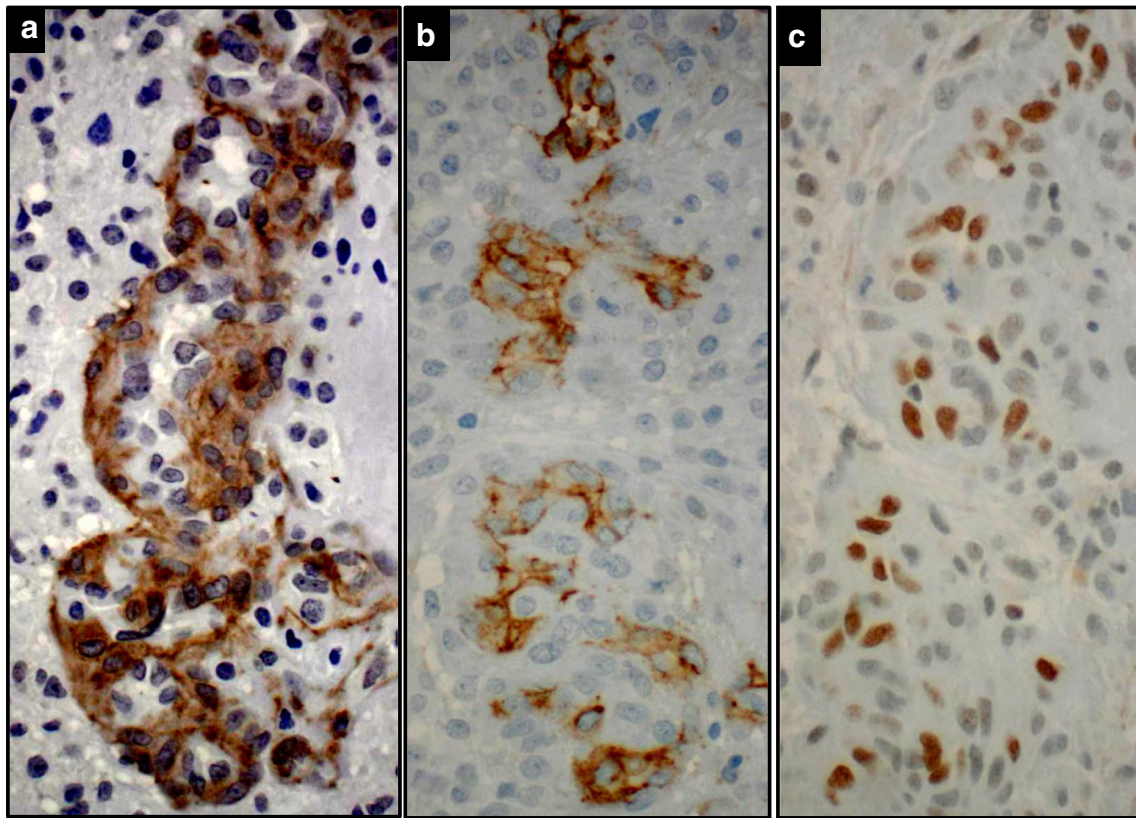


Fig. 5 PDGFRB, CD31 and Fli-1 immunohistochemical stains in glioblastoma microvascular proliferation. **a** Pericytes in microvascular proliferation are positive for PDGFRB ($\times 400$). **b** Endothelial cells in

microvascular proliferation are positive for CD31 ($\times 400$). **c** Endothelial cells in microvascular proliferation are positive for Fli-1 (nuclear staining) ($\times 400$)

both) (Fig. 6b). Using the GBM (TCGA, 2013) dataset, the correlation between the EGFR mRNA expression and EGFR gene copy number changes and mutations was showed in Fig. 7a. It was very convincing that EGFR amplification and mutations probably were the main reason for the high expression of EGFR in GBM tumor cells. In contrary, PDGFRB gene mutations and copy number changes were unlikely the reason for the decreased expression of PDGFRB in GBM tumor cells compared to GBM microvascular proliferation (Fig. 7b).

Multiple miRNAs that target PDGFRB in GBM microvascular proliferation were significantly decreased while multiple miRNAs targeting EGFR in GBM microvascular proliferation were significantly upregulated

Total RNA from the same specimens used for mRNA array (seven samples of microdissected GBM microvascular proliferation and seven samples of GBM tissue sections after complete microvascular proliferation microdissection) was used for profiling 372 miRNAs differentially expressed in tumors versus normal tissue with Human Cancer Pathway Finder 384HC MicroRNA array. The volcano plots of the signal

intensities of different miRNAs between GBM microvasculature and GBM tumor cells were produced (Fig. 1b). At Ferrolab data mining and bioinformatics group (<http://ferrolab.dmi.unict.it/>) and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) website, a list of miRNAs that target PDGFRB or EGFR was generated [8] and then the expression level of those miRNAs from our Human Cancer Pathway Finder 384HC MicroRNA array data was reviewed. Four miRNAs (miR-193b-3p, miR-518b, miR-520f-3p, and miR-506-5p) targeting PDGFRB mRNA were significantly downregulated in GBM microvascular proliferation (ranging from -2.58 to -7.24) compared to GBM tumor cells ($p < 0.05$) (Table 2). Four miRNAs (miR-133b, miR-30b-3p, miR-145-5p, and miR-146a-5p) targeting EGFR mRNA were significantly increased in GBM microvascular proliferation (ranging from 2.52 to 10.56) compared to GBM tumor cells ($p < 0.05$) (Table 3).

Discussion

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in the elderly population. The genomic DNA alterations (mutation, amplification, or deletion) of

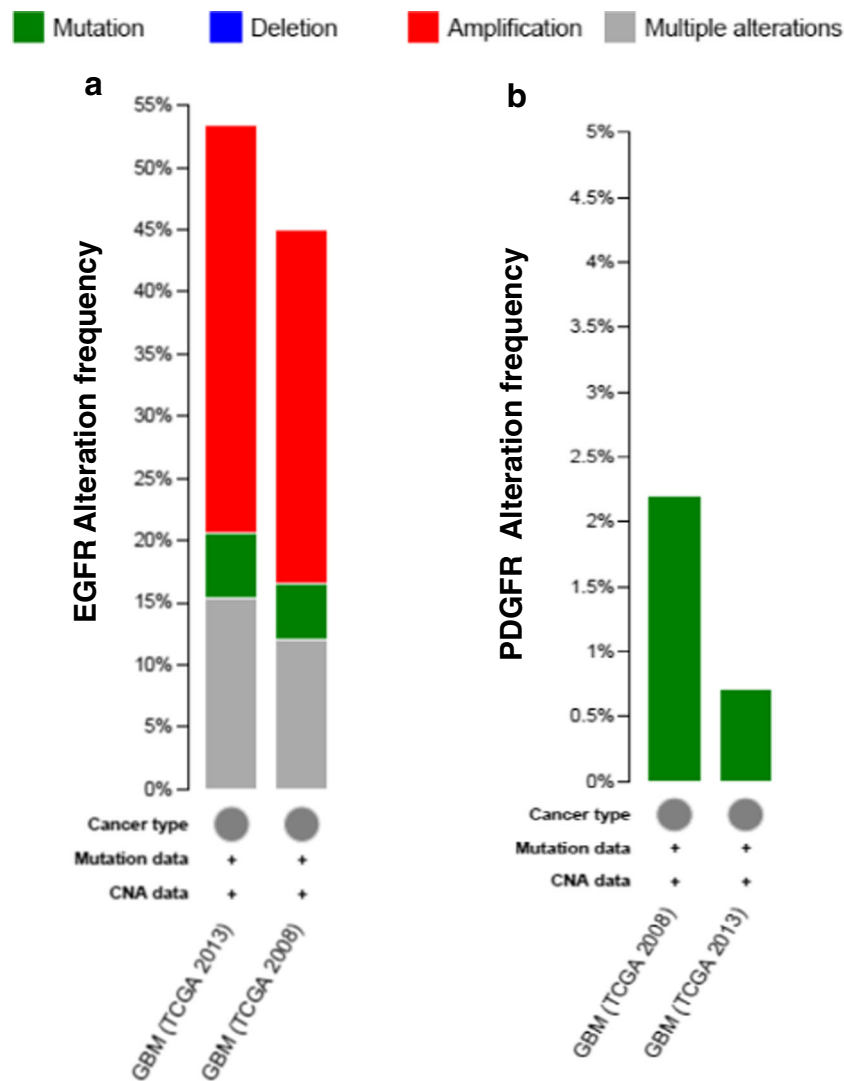


Fig. 6 EGFR and PDGFRB genomic DNA alterations in GBM. **a** EGFR Genomic DNA alterations in The Cancer Genome Atlas (TCGA) GBM databases at cBioPortal for Cancer Genomics. **b** PDGFRB genomic DNA

alterations in The Cancer Genome Atlas (TCGA) GBM databases at cBioPortal for Cancer Genomics

EGFR gene are commonly present in GBM [11]. EGFRvIII is the most frequent variant of EGFR, which has a deletion of 267 amino acids in the extracellular ligand-binding domain and constitutively activates EGFR pathway in GBM [12]. Activation of EGFR pathway increases cellular proliferation and reduces apoptosis [12]. PDGFR family has four ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) and two receptors (PDGFRA and PDGFRB) [13]. Our study shows that there is a statistically significant increase in the expression of PDGFRB in GBM microvascular proliferation at mRNA and protein levels compared to GBM tumor cells, while there is a significant decrease in the expression of EGFR in GBM microvascular proliferation at mRNA and protein levels compared to GBM tumor cells. One characteristic feature of GBM compared to other lower grade infiltrating astrocytomas is microvascular proliferation. The differential expression of PDGFRB on tumor cells and microvascular proliferation

implicates that PDGFRB pathways may be involved in GBM angiogenesis, and enable microvascular proliferation to develop in the surrounding tissue, which facilitate tumor cell proliferation and migration.

It is very common that gene mutation and/or amplification of genomic DNA can cause high expression of certain oncogenes in several different cancer cells including GBM. Two sets of glioblastoma database from The Memorial Sloan-Kettering Cancer Center cBioPortal for Cancer Genomics website show that 45–53 % of GBMs have EGFR gene alterations at genomic DNA levels including mutation, amplification, and multiple alterations. However, only a few GBM cases (less than 3 %) have PDGFRB mutation without other genomic DNA alterations. The correlation between the EGFR mRNA expression and EGFR gene copy number changes and mutations from GBM dataset shows EGFR amplification and mutations probably are the main reason for the high

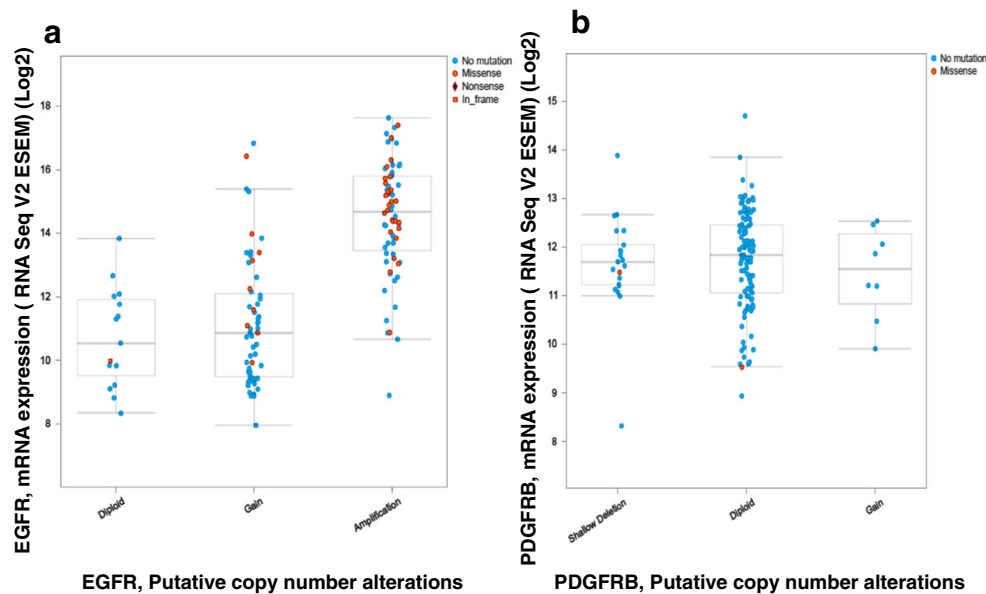


Fig. 7 The correlation of mRNA expression level with genomic DNA alterations. **a** The correlation of EGFR mRNA expression level with EGFR genomic DNA alterations at cBioPortal for Cancer Genomics. **b**

The correlation of PDGFRB mRNA expression level with PDGFRB genomic DNA alterations at cBioPortal for Cancer Genomics

expression of EGFR in GBM tumor cells; PDGFRB gene mutations and copy number changes are unlikely the reason for the decreased expression of PDGFRB in GBM tumor cells compared to GBM microvascular proliferation.

It is well-known that miRNAs can act as potential oncogenes or tumor suppressor genes during the initiation and progression of cancer [14]. Recently, we find that miR-7-5p is downregulated in glioblastoma microvasculature and inhibits vascular endothelial cell proliferation by targeting RAF1 [15]. Human Cancer Pathway Finder 384HC MicroRNA array analysis shows that the expression levels of miR-193b-3p, miR-518b, miR-520f-3p, and miR-506-5p, which target PDGFRB mRNA, are significantly downregulated in GBM microvascular proliferation compared to GBM tumor cells ($p < 0.05$) (Table 2). The expression levels of miR-133b, miR-30b-3p, miR-145-5p, and miR-146a-5p, which target EGFR mRNA, are significantly increased in GBM microvascular proliferation compared to GBM tumor cells ($p < 0.05$) (Table 3). The findings indicate that the downregulation of the miRNAs targeting PDGFRB is the most likely reason for the high expression of

PDGFRB in GBM microvascular proliferation as shown in the present study; the upregulation of the miRNAs targeting EGFR in GBM microvascular proliferation is one of the reasons for the lack of expression of EGFR in GBM microvascular proliferation. One study shows that miR-193b targets Smad 3 and induces cell proliferation in human glioma [16]. Several studies reveal that miR-506 inhibits epithelial-to-mesenchymal transition in gastric cancer, epithelial ovarian cancer and breast cancer cell lines, and suppresses the angiogenesis in gastric cancer [17–19]. MiR-506 also inhibits the proliferation of ovarian cancer, clear cell renal cell carcinoma, breast cancer and hepatocellular carcinoma cells [20–23]. MiR-133b contributes to arsenic-induced apoptosis in U251 glioma cells by targeting the hERG channel [24]. By targeting EGFR, the combination of miR-133b and cetuximab can inhibit the growth and invasion of colorectal cancer cells [25]. By targeting EGFR as well as other targets such as connective tissue growth factor, ABCG2, ADAM17, Sox9, adducin3 or NUDT1, miR-145 inhibits the proliferation, invasion, and migration of glioma and suppresses cell proliferation of lung adenocarcinoma [26–30].

Table 2 miRNAs targeting PDGFRB are downregulated in GBM microvascular proliferation (GBM-MVP) compared to GBM tumor cells (GBM)

miRNAs targeting PDGFRB	Up- and downregulation GBM-MVP/GBM	<i>p</i> value
hsa-miR-193b-3p	-2.58	<0.05
hsa-miR-518b	-4.45	<0.05
hsa-miR-520f-3p	-5.92	<0.05
hsa-miR-506-5p	-7.24	<0.05

Table 3 miRNAs targeting EGFR are upregulated in GBM microvascular proliferation (GBM-MVP) compared to GBM tumor cells (GBM)

miRNAs targeting EGFR	Up- and downregulation GBM-MVP/GBM	<i>p</i> value
hsa-miR-133b	6.06	<0.05
hsa-miR-30b-3p	2.52	<0.05
hsa-miR-145-5p	10.56	<0.05
hsa-miR-146a-5p	3.55	<0.05

MiR-146a acts as a tumor suppressor in prostate cancer and pancreatic cancer cells by targeting EGFR [31, 32]. It is very clear that miR-506, miR-133b, miR-145, and miR-146a function as tumor suppressors in glioma or other cancers by negatively regulating different genes.

During last decade, several EGFR-targeted therapies, including humanized monoclonal antibodies and small-molecule inhibitors, have been developed [11]. Gefitinib and erlotinib, small-molecule EGFR inhibitors, have been thoroughly tested and do not show promising treatment effect [33]. Because GBM tumor cells have abundant expression of EGFR and PDGFRB is selectively expressed in GBM microvasculature, a new generation of targeted therapy against both EGFR and PDGFRB at RNA and protein levels that aims to effectively destroy both tumor cells and microvascular proliferation in GBM might be developed in the future. MiRNAs targeting PDGFRB or EGFR, such as miR-506, miR-133b, miR-145, and miR-146a, might be potential therapeutic agents for GBM.

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Compliance with ethical standards This study was approved by the North Shore and Long Island Jewish Health System Institutional Review Board.

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Conflicts of interest None

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