

## Amplification and/or Overexpression of Platelet-derived Growth Factor Receptors and Epidermal Growth Factor Receptor in Human Glial Tumors

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### Abstract

Analysis of genomic organization and expression of platelet-derived growth factor receptors (PDGFR) and epidermal growth factor receptor (EGFR) in human malignant gliomas showed amplification and overexpression of both receptors in distinct subsets of tumors. Amplification of the  $\alpha$ PDGFR was detected in 4 of 50 glioblastomas (8%). EGFR was amplified in 9 of the 50 tumors (18%). Western blot analysis showed elevated expression of  $\alpha$ PDGFR and EGFR proteins in 4 (24%) and 3 (18%), respectively, of 17 tumor specimens analyzed. Increased production of  $\alpha$ PDGFR as well as EGFR proteins was observed in the presence or absence of gene amplification. Three of the 4 tumors with elevated levels of  $\alpha$ PDGFR also overexpressed the  $\beta$ PDGFR, which was present as a single copy gene in all 50 tumors analyzed. Our findings suggest that the amplification and/or overexpression either of EGFR or of the  $\alpha$ PDGFR along with the coordinate overexpression of the  $\beta$ PDGFR can contribute to the malignant phenotype of distinct subsets of human glioblastoma.

### Introduction

Glioblastomas, the most common primary intracranial tumors, are among the most malignant forms of human cancer. These tumors are classified according to their histopathology and location. Presently a three-tier classification system has been adopted. According to this, astrocytoma is considered a mildly hypercellular tumor with some nuclear pleomorphism, while anaplastic astrocytoma has moderate hypercellularity, pleomorphism, and no endothelial proliferation. Glioblastoma, the most malignant glial tumor, is an extremely anaplastic and hypercellular tumor with extensive nuclear polymorphism, vascular proliferation, and necrosis (1). This type of classification has prognostic validity in that patients with anaplastic astrocytomas have a 2-year survival rate of 50% compared to about 5% among those with glioblastomas (2).

Genetic analysis has identified several chromosomal abnormalities in glioblastomas. Most common among these are polysomy of chromosome 7 (3, 4) and allelic losses of genes on chromosomes 10 and 17 (5-10). Amplification and/or overexpression of the EGFR<sup>3</sup> has been reported previously in glioblastomas and may contribute to the malignant phenotype by receptor activation through truncation or by an autocrine mechanism (11-13). Overexpression of various growth factors, such as TGF $\alpha$ , TGF $\beta$ , aFGF, and bFGF has also been detected by immunohistochemistry and/or analysis of transcripts in pri-

mary glioblastomas (12-16) as well as cell lines derived from glioblastomas (17, 18).

There is indirect evidence implicating PDGF and its receptors in the etiology of gliomas. PDGF occurs as three isoforms of disulfide-linked A and B chains (PDGF-AA, -BB, and -AB), that bind with different affinities to two cell surface receptors, the  $\alpha$  and  $\beta$  forms of the PDGFR (19). The  $\alpha$ PDGFR binds all three PDGF isoforms with high affinity, whereas the  $\beta$ PDGFR binds only the PDGF-BB dimer (20-22). The *v-sis* oncogene of simian sarcoma virus is a retroviral homologue of PDGF-BB and the intracerebral injection of simian sarcoma virus causes brain tumors in newborn marmosets (23). These experimental tumors share common features with human malignant gliomas including extensive proliferation of vascular endothelial cells (24). Previously, increased expression of B chain of PDGF and  $\beta$ PDGFR, as detected by Northern blotting and *in situ* hybridization, has been reported in proliferating endothelial cells as well as in glioma cells (25, 26). Cultured glioma cells have been reported to secrete mainly PDGF-AA, which is sometimes co-expressed with  $\alpha$ PDGFR (27). These data suggest that an autocrine pathway involving PDGFs and their receptors may be a factor in the neoplastic process of glioblastoma.

To further define the role that aberrant expression of PDGFRs may have in the pathogenesis of glioblastoma, we analyzed amplification and/or overexpression of  $\alpha$ - and  $\beta$ PDGFRs in 50 primary glial tumors. We also analyzed genomic organization and expression of EGFR in the same tumor panel. Our results demonstrate that distinct subsets of glial tumors overexpress both  $\alpha$  and  $\beta$  forms of PDGFR or EGFR and that, in some cases, elevated expression of the  $\alpha$ PDGFR and EGFR is associated with amplification of these genes.

### Materials and Methods

**Tissue Preparation and DNA Extraction.** Surgical biopsy specimens were frozen in liquid nitrogen immediately after removal. All tissue samples were stored frozen at  $-70^{\circ}\text{C}$  until processed. Peripheral blood obtained from all patients at the time of surgery served as a normal control. High molecular DNA was isolated from frozen pulverized tumor tissues and peripheral blood lymphocytes by standard procedure of proteinase K digestion and phenol-chloroform extraction (28).

**Southern Hybridization.** Matching lymphocyte and tumor DNAs were digested with the appropriate restriction enzyme according to the manufacturer's recommendation, electrophoresed on 0.8% agarose gels, and transferred to nylon filters. The complementary DNA probes for the  $\alpha$ - and  $\beta$ PDGFR (22) or EGFR (29) were labeled using the random primer kit (Amersham). Southern hybridization and washing of the filters under stringent conditions were performed according to the standard procedures described (28).

**Western Blot Analysis.** Western blotting of cell lysates was performed as described (22). Briefly, cells were lysed with Staph A buffer (10 mM sodium phosphate, pH 7.5/100 mM NaCl/1% Triton X-100/0.1% sodium dodecyl sulfate/0.5% deoxycholate/0.1% aprotinin/1 mM

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<sup>3</sup> The abbreviations used are: EGFR, epidermal growth factor receptor; TGF, transforming growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor.

phenylmethylsulfonylfluoride) and clarified by centrifugation at 10,000 × *g* for 30 min at 4°C. Lysates (100 μg) were subjected to electrophoresis on 10% sodium dodecyl sulfate/polyacrylamide gels, blotted onto nitrocellulose filters, and treated with antisera specific for either α- and βPDGFRs or the EGFR. Immunocomplexes were identified by using <sup>125</sup>I-labeled protein A and visualized by autoradiography.

**Results**

We initially examined DNA samples isolated from 50 glioblastomas by Southern blot analysis with α- and βPDGFR and EGFR complementary DNA probes. As shown in Fig. 1A, 4 of the 50 tumors exhibited amplification of the αPDGFR gene. Laser scanning densitometry demonstrated 18–35-fold amplification in tumor DNAs from patients 2, 13, 16, and 19 compared to the normal lymphocyte DNAs from the same patients (Fig. 1; Table 1). In contrast, no evidence for genetic rearrangements of either α- or βPDGFR was seen, nor was there any indication of βPDGFR gene amplification in these or any of the other tumors analyzed. The EGFR gene was amplified 4–19-fold in nine tumors, which were distinct from those with αPDGFR amplification (Table 1). Representative examples of four of these tumor DNAs, showing amplification but no evidence of rearrangements, and the corresponding lymphocyte DNAs are displayed in Fig. 1B.

To compare levels of PDGFR proteins expressed in primary glial tumors with those in normal human brain, tissues were lysed and subjected to immunoblot analyses with antibodies specific to α and βPDGFRs. Tissues from 17 of the 50 tumors used for the DNA analysis, including one, tumor 16, with amplification of αPDGFR gene, were available for immunological studies. Initial screening using an antibody that recognizes epitopes shared by α- and βPDGFR showed a significantly elevated level of PDGFR expression in 4 of 17 tumors (data not shown). Analysis of those four positive tumors (tumors 16, 22, 63, and 113) as well as other tumors was then repeated using antibodies specific for α or βPDGFR. Fig. 2A indicates that all

four tumors, which were positive with the common antibody, showed an increased level of αPDGFR expression. With the exception of tumor 22, each of these tumors showed substantially increased levels of βPDGFR protein as well (Fig. 2B).

Previous studies have reported that glioblastomas often exhibit increased EGFR expression in the presence or absence of gene amplification (11, 13). We therefore analyzed EGFR expression levels for comparison in the 17 of 50 tumors from which tissue was available for protein analysis. As shown in Fig. 3, tumors 36, 40, and 44 showed significantly increased levels of EGFR protein the size of which corresponded to that of the normal EGFR protein.

Table 1 summarizes the results of analysis of gene amplification and overexpression of PDGF and EGF receptors in glial tumors with their histopathology and clinical status. Among those tumors for which molecular genetic and/or immunological results were available, 19 were grade IV glioblastoma multiforme and 7 were grade III anaplastic astrocytoma. There were roughly equal numbers of primary and recurrent tumors. An 18–35-fold amplification of the αPDGFR was detected in four tumors. One of these, tumor 16, was a recurrent tumor which overexpressed both α- and βPDGFR proteins. Tissues from the three other αPDGFR amplified tumors, tumors 2, 13, and 19, were not available for the receptor analysis. Three other tumors, tumors 22, 63, and 113, expressed significantly elevated levels of the α-receptor protein compared to adult brain in the absence of gene amplification. All αPDGFR overexpressor tumors, except for tumor 22, also expressed increased levels of βPDGFR protein.

EGFR amplification and/or overexpression was detected in a subset of glioblastomas distinct from that with the PDGFR abnormalities. Of the nine tumors exhibiting amplification of EGFR gene, tumors 8, 20, and 36 could be analyzed for the expression of the EGFR protein. Tumor 36 expressed very high levels of the EGFR protein compared to other tumors, whereas

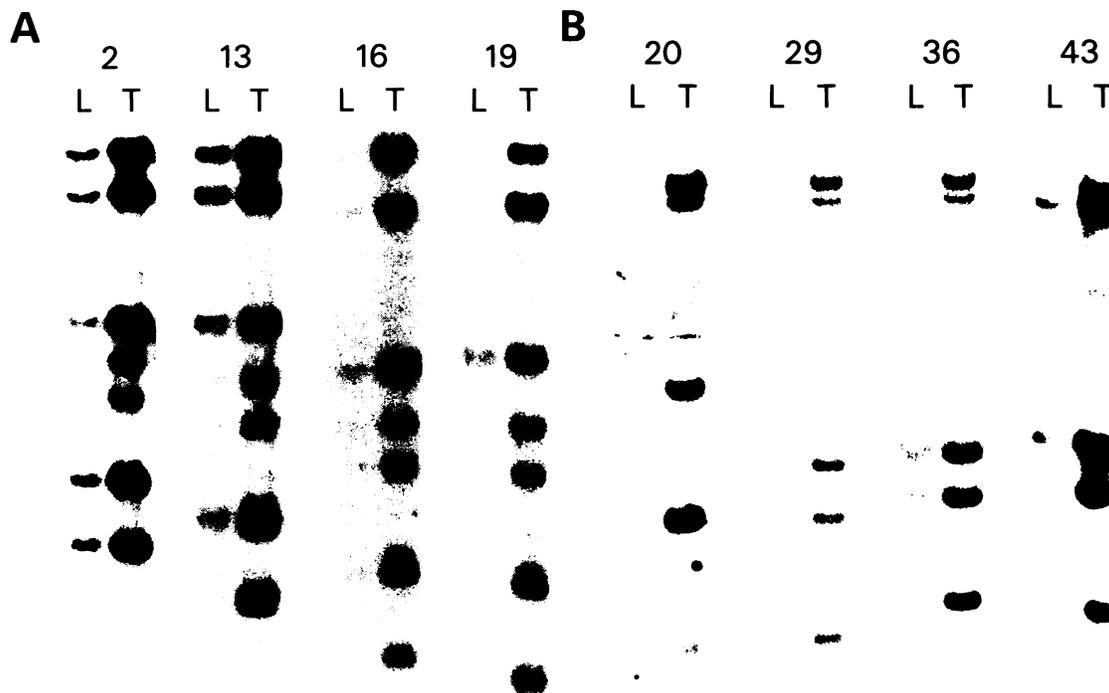


Fig. 1. Amplification of αPDGFR (A) and EGFR (B) genes in glial tumors. Southern blots of Taq1-digested lymphocyte and tumor DNAs hybridized with the αPDGFR and EGFR complementary DNA probes. L, lymphocyte DNA; T, tumor DNA.

Table 1 Amplification and/or overexpression of  $\alpha$  and  $\beta$ PDGFR genes in glial tumors

Quantitative estimation of  $\alpha$ PDGFR and EGFR amplification was carried out by laser scanning of the top band (Fig. 1) from lymphocyte and tumor DNAs from each patient (similar results were obtained with any other band). The copy number was calculated relative to the marker D10S4 (35), D17S28, or D17S34 (36), which was heterozygous in the tumor DNA and was arbitrarily assigned a copy number of 1. The amplification was calculated relative to the lymphocyte DNA from the same patient after normalizing the signals for any one of the control probes.

Tumor	Diagnosis	Gene amplification		Overexpression		
		$\alpha$ PDGFR	EGFR	$\alpha$ PDGFR	$\beta$ PDGFR	EGFR
1	GBM <sup>a</sup> (R)	-	-	-	-	-
2	GBM (P)	35	-	ND	ND	ND
5	GBM (R)	-	-	-	-	-
8	GBM (P)	-	6	-	-	-
9	GBM (P)	-	-	-	-	-
13	AA (P)	22	-	ND	ND	ND
16	GBM (R)	22	-	+++	+++	-
18	GBM (P)	-	10	ND	ND	ND
19	GBM (P)	18	-	ND	ND	ND
20	GBM (P)	-	16	-	-	-
22	GBM (P)	-	-	+++	-	-
29	GBM (P)	-	5	ND	ND	ND
31	GBM (R)	-	-	-	-	-
36	GBM (P)	-	8	-	-	+++
37	GBM (R)	-	5	ND	ND	ND
38	GBM (R)	-	18	ND	ND	ND
39	GBM (R)	-	-	-	-	-
40	GBM (P)	-	-	-	-	++
43	GBM (R)	-	19	ND	ND	ND
44	AA (R)	-	-	-	-	++
63	AA (P)	-	-	+++	+++	-
70	GBM (R)	-	7	ND	ND	ND
73	AA (R)	-	-	-	-	-
75	AA (R)	-	-	-	-	-
112	AA (P)	-	-	-	-	-
113	AA (P)	-	-	++	+++	-

<sup>a</sup> GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; P, primary tumor; R, recurrent tumor; ND, not done; -, negative; +++, highly positive; ++, moderately positive.

no overexpression was detected in tumors 8 and 20. Two other tumors, tumors 40 and 44, expressed moderately high levels of EGFR in the absence of gene amplification.

Discussion

Growth factors and their cell surface receptors are important mediators of the signal transduction pathways in normal cells. Abnormalities in the expression of these growth factors and/or their receptors perturbing the complex mechanisms of controlled mitogenesis and differentiation are often encountered in a variety of human tumors (for review see Ref. 30). For example, coordinate overexpression of bFGF and its high affinity receptor *flg* or PDGF A and B chains and  $\alpha$  and  $\beta$  forms of PDGFR have been reported in cell lines derived from glioblastomas (17, 18). Elevated expression of TGF $\alpha$  and EGFR as well as PDGF B and  $\beta$ PDGFR was also detected in primary glial tumors suggesting the involvement of autocrine loops in human glioblastomas. These findings together with the observation that simian sarcoma virus, encoding the *v-sis* oncogene (the homologue of the B chain of PDGF), induces glioblastomas in experimental animals (23) imply that EGF and PDGF pathways may be important in the pathogenesis of glioblastomas.

Analysis of 50 gliomas for possible genetic alterations of the  $\alpha$  and  $\beta$  forms of PDGFR revealed amplification of the  $\alpha$ PDGFR gene in one recurrent and three primary glioblastoma multiforme tumors. Recently amplification and rearrangement of the  $\alpha$ PDGFR has been reported in one glial tumor (31). In the present study, there was no evidence of rearrangements of the amplified  $\alpha$ PDGFR gene in the 4 tumors and none of the 50 tumors revealed amplification or rearrangement of the  $\beta$ PDGFR gene. Amplification of  $\alpha$ PDGFR gene may be indic-

ative of a selective advantage favoring its expression in glioblastoma. In total, 17 tumors (11 glioblastomas and 6 astrocytomas) could be analyzed for the expression of PDGFRs and EGFR proteins. Antibodies specific for PDGFR isoforms demonstrated that glial tumors generally overexpressed both  $\alpha$ - and  $\beta$ PDGFRs. These findings are in agreement with earlier reports of increased expression of both  $\alpha$  and  $\beta$  forms of PDGFR as detected by Northern analysis of glioma cells *in vitro* (32) or by immunohistochemistry on glioma tissue (33). Overexpression of the  $\alpha$ PDGFR was detected in two anaplastic astrocytomas and two glioblastomas, including the recurrent glioblastoma 16, which contained high copy number of the  $\alpha$ PDGFR gene. The other overexpressors contained only a single normal copy of the  $\alpha$ PDGFR gene. Overexpression of the  $\beta$ PDGFR also occurred in the absence of amplification at the gene level. The mechanism responsible for overproduction of PDGFRs in glial tumors without gene amplification remains to be elucidated.

Overexpression of the EGFR protein with or without amplification and/or rearrangement of the gene in grade III anaplastic astrocytoma and grade IV glioblastoma multiforme has been reported previously (11, 13). We observed amplification and/or overexpression of EGFR in grade IV glioblastoma multiforme. Two tumors, tumors 8 and 20, were amplified (8- and 5-fold, respectively) for the EGFR gene, but overexpression at the protein level was not detected. This implies that EGFR may have been initially overexpressed in these tumors, but later elevated levels of the protein were no longer required. Alternatively, the EGFR gene may have been fortuitously included in

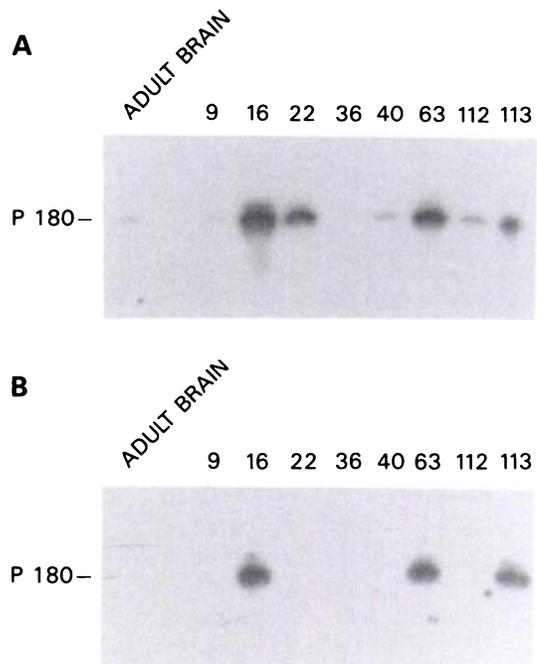


Fig. 2. Overexpression of  $\alpha$  and  $\beta$  forms of PDGFR protein in glioblastomas. Total cell lysates (100 mg) were analyzed by immunoblotting with a monoclonal antibody specific either for  $\alpha$ PDGFR (A) or for  $\beta$ PDGFR (B). Normal adult brain was used as a control. The mature form of PDGFR is indicated by the p180 band.

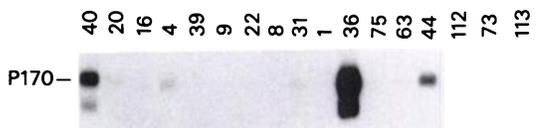


Fig. 3. Overexpression of EGFR in glioblastomas. Total cell lysates (100 mg) were analyzed by immunoblot using a peptide antibody specific for the carboxy terminus of the EGFR. The p170 band indicates the mature form of the EGFR.

the amplicon carrying another gene the expression of which was relevant to the malignant progression of gliomas.

The amplification and/or overexpression of PDGFRs and EGFR occurred in distinct subsets of glial tumors. This is consistent with the concept that a variety of genetic mechanisms contribute to the emergence of the neoplastic phenotype. Various autocrine loops of the growth signaling pathways may be involved, individually or in concert, in a particular tumor type depending on the microenvironment. Previous studies have demonstrated that the PDGFR activation can be abrogated at the cell surface (34) suggesting that the PDGF autocrine pathway is amenable to intervention by reagents that can interfere with the ligand/receptor interaction. Our findings support the concept that intervention with PDGF and EGF pathways may be logical therapeutic approaches to the treatment of glioblastoma.

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