

Amplification of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* in Gliomas

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Abstract

Receptor tyrosine kinase aberrations are implicated in the genesis of gliomas. We investigated expression and amplification of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* in 87 gliomas consisting of astrocytomas, anaplastic astrocytomas, oligodendrogliomas, or oligoastrocytomas in tumor samples collected at the time of the diagnosis and in samples of the same tumors at tumor recurrence. Gene amplifications were investigated using either chromogenic *in situ* hybridization or fluorescence *in situ* hybridization, and protein expression using immunohistochemistry. In samples collected at glioma diagnosis, *KIT* and *PDGFRA* amplifications were more frequent in anaplastic astrocytomas than in astrocytomas, oligodendrogliomas, and oligoastrocytomas [28% versus 5% ($P = 0.012$) and 33% versus 2% ($P = 0.0008$), respectively]. *VEGFR2* amplifications occurred in 6% to 17% of the gliomas at diagnosis, and *EGFR* amplifications in 0% to 12%. Amplified *KIT* was more frequently present in recurrent gliomas than in newly diagnosed gliomas ($P = 0.0066$). *KIT* amplification was associated with *KIT* protein expression and with presence of *PDGFRA* and *EGFR* amplifications both at the time of the first glioma diagnosis and at tumor recurrence, and with *VEGFR2* amplification at tumor recurrence. Three (4%) primary gliomas and 10 (14%) recurrent gliomas that were evaluable for coamplification of *KIT*, *PDGFRA*, and *VEGFR2* showed amplification of at least two of these genes; the amplicon contained amplified *KIT* in all 13 cases. In conclusion, besides glioblastoma, amplified *KIT*, *PDGFRA*, and *VEGFR* may also occur in

lower-grade gliomas and in their recurrent tumors. It is currently not known whether specific tyrosine kinase inhibitors are effective in the treatment of such gliomas. (Mol Cancer Res 2006;4(12):927–34)

Introduction

Gliomas are the most common brain tumors, with an annual incidence of 5 to 10 cases per 100,000 persons (1, 2). The mean age of adult glioma patients is 40 to 45 years at presentation, and that of glioblastoma patients, 55 to 60 years (3-5). Patients diagnosed with anaplastic astrocytoma have a median survival time of ~3 years after the diagnosis, but the median survival time is only ~1 year following the diagnosis of glioblastoma (3).

Histopathologic classification of gliomas is demanding due to their heterogeneous nature. The WHO classification grading is based on the number of mitoses and presence of nuclear atypia, microvascular proliferation, and tumor necrosis (6, 7). These criteria are to some extent subjective, which may lead to interobserver variation between pathologists in tumor classification (8, 9). Diffuse low-grade astrocytomas tend to progress into anaplastic astrocytomas (WHO grade 3) and eventually to glioblastoma (WHO grade 4). Glioblastomas can be divided into primary glioblastomas, which are thought to arise *de novo*, and secondary glioblastomas, which arise from a lower-grade astrocytoma.

The current treatment of malignant gliomas, based on surgical resection, radiation therapy, and sometimes chemotherapy (2, 3), is usually not curative, and novel therapeutic targets thus need to be identified. Excessive growth factor receptor signaling is essential in the genesis of many malignant brain tumors. Overexpression and amplification of the epidermal growth factor receptor (EGFR) can be identified in a majority of primary glioblastomas, whereas platelet-derived growth factor receptor (PDGFR) pathway aberrations and *TP53* mutations are mainly associated with secondary glioblastomas (2).

Small tyrosine kinase inhibitors, such as imatinib, gefitinib, and erlotinib, have activity in the treatment of some glioblastomas (10-12).

We have recently found that amplification of *KIT*, *PDGFRA*, and *VEGFR2* occurs frequently in primary glioblastomas (13). High-level gene amplification of such tyrosine kinase receptor genes may potentially serve as a biomarker for targeted tyrosine kinase inhibitor therapy. In the

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present study, we investigated whether amplifications of these genes occur in lower-grade gliomas (i.e., astrocytomas, oligodendrogliomas, and oligoastrocytomas) and in their recurrent tumors.

Results

Frequency of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* Copy Numbers in Primary and Recurrent Gliomas

In samples collected at the first presentation of glioma, amplification of *KIT*, *PDGFRA*, and *VEGFR2* was detected in 3% to 14% of astrocytomas, 0% to 6% of oligodendrogliomas, 0% to 11% of oligoastrocytomas, and in 17% to 33% of anaplastic astrocytomas (Table 1; Fig. 1). In samples collected at glioma diagnosis, *KIT* and *PDGFRA* amplifications were more frequent in anaplastic astrocytomas than in astrocytomas, oligodendrogliomas, and oligoastrocytomas [28% versus 5% ($P = 0.012$) and 33% versus 2% ($P = 0.0008$), respectively], whereas no such association was found in the frequency of *VEGFR2* amplification ($P = 0.68$). *EGFR* amplification was rarely present; it occurred in 2 (12%) anaplastic astrocytomas and in 1 (11%) oligoastrocytoma at diagnosis.

Either *KIT*, *PDGFRA*, or *VEGFR2* amplification was present in 19 of the 86 (22%) evaluable gliomas at the time

of the diagnosis and in 27 of their 76 (36%) evaluable recurrences ($P = 0.059$). Amplified *KIT* was more often present at glioma recurrence than at the time of the first diagnosis of glioma ($P = 0.0066$). *PDGFRA* amplifications also tended to be more frequently present in the recurrent tumors than in the newly detected gliomas ($P = 0.061$), whereas *VEGFR2* or *EGFR* amplifications did not ($P = 0.83$ and $P = 0.51$, respectively). *KIT* was amplified in 8 of the 16 (50%) evaluable recurrent anaplastic astrocytomas and in 5 of the 14 (36%) evaluable secondary glioblastomas. *PDGFRA* amplifications were detected in 4 of the 13 (31%) recurrent oligoastrocytomas and in 5 of the 16 (31%) secondary glioblastomas.

Eighty-three (95%) samples taken at the time of the first diagnosis of glioma and 74 (84%) samples collected at tumor recurrence were evaluable for assessment of presence of coamplification of *KIT*, *PDGFRA*, and *VEGFR2*. Amplification of all three genes was present in two tumors at diagnosis and in five tumors at tumor recurrence, and amplification of two of the three genes was present in one tumor at the time of the diagnosis and in five tumors at the time of glioma recurrence. Thus, amplification of at least two of the three genes was present more often in recurrent gliomas (10 of 74, 14%) than in gliomas studied at the time of the diagnosis

Table 1. Frequency of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* Gene Amplifications in Gliomas at Presentation and at Recurrence

	<i>KIT</i> , n (%)	<i>PDGFRA</i> , n (%)	<i>VEGFR2</i> , n (%)	<i>EGFR</i> , n (%)
Gliomas at first diagnosis (n = 87)				
Astrocytoma (n = 38)				
Diploid or aneuploid	34 (94)	34 (97)	30 (86)	33 (100)
Amplification	2 (6)	1 (3)	5 (14)	0 (0)
N.A.*	2	3	3	5
Anaplastic astrocytoma (n = 18)				
Diploid or aneuploid	13 (72)	10 (67)	15 (83)	15 (88)
Amplification	5 (28)	5 (33)	3 (17)	2 (12)
N.A.	0	3	0	1
Oligodendroglioma (n = 20)				
Diploid or aneuploid	18 (100)	19 (100)	17 (94)	19 (100)
Amplification	0 (0)	0 (0)	1 (6)	0 (0)
N.A.	2	1	2	1
Oligoastrocytoma (n = 11)				
Diploid or aneuploid	8 (89)	9 (100)	9 (90)	8 (89)
Amplification	1 (11)	0 (0)	1 (10)	1 (11)
N.A.	2	2	2	2
Recurrent gliomas (n = 87)				
Astrocytoma (n = 17)				
Diploid or aneuploid	11 (85)	11 (92)	13 (93)	11 (85)
Amplification	2 (15)	1 (8)	1 (7)	2 (15)
N.A.	4	5	3	4
Anaplastic astrocytoma (n = 21)				
Diploid or aneuploid	8 (50)	15 (88)	15 (88)	15 (94)
Amplification	8 (50)	2 (12)	2 (12)	1 (6)
N.A.	5	4	4	5
Oligodendroglioma (n = 17)				
Diploid or aneuploid	14 (93)	14 (93)	14 (93)	14 (93)
Amplification	1 (7)	1 (7)	1 (7)	1 (7)
N.A.	2	2	2	2
Oligoastrocytoma (n = 16)				
Diploid or aneuploid	10 (77)	9 (69)	8 (67)	14 (100)
Amplification	3 (23)	4 (31)	4 (33)	0 (0)
N.A.	3	3	4	2
Secondary glioblastoma (n = 16)				
Diploid or aneuploid	9 (64)	11 (69)	14 (88)	12 (86)
Amplification	5 (36)	5 (31)	2 (12)	2 (14)
N.A.	2	0	0	2

NOTE: Amplification analysis of all four genes was uninformative in one case of oligoastrocytoma at first diagnosis of glioma, and in three astrocytomas, four anaplastic astrocytomas, two oligodendrogliomas, and two oligoastrocytomas at tumor recurrence.

*Not available.

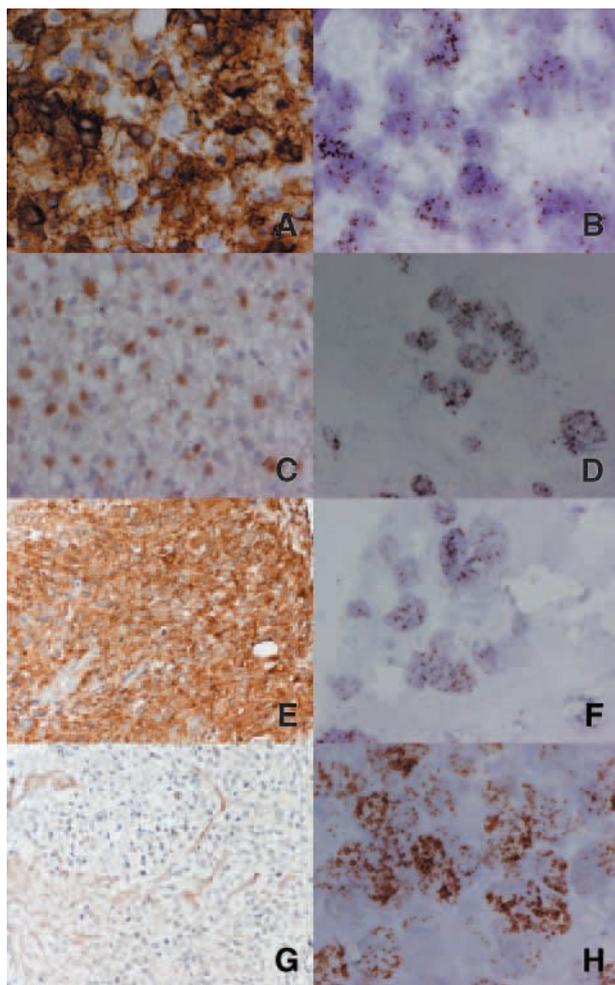


FIGURE 1. **A.** glioblastoma with high (+++) KIT protein expression. **B.** a high-level *KIT* gene amplification in recurrent grade 3 oligodendroglioma. **C.** VEGFR2 expression (+) in secondary glioblastoma. **D.** a high-level amplification of *VEGFR2* in anaplastic astrocytoma. **E.** nestin expression (+++) in glioblastoma. **F.** a high-level *PDGFRA* gene amplification in anaplastic astrocytoma. **G.** a glioblastoma expressing prominin 1 (+). **H.** a high-level amplification of *EGFR* in glioblastoma. Original magnification, $\times 200$ (**A** and **C**); $400\times$ (**B**, **D**, **F**, and **H**); and $\times 100$ (**E** and **G**).

(3 of 83, 4%; $P = 0.025$). All tumors that had amplification of at least two of these three genes at diagnosis were histologically anaplastic astrocytomas, and 7 of the 10 tumors with amplification of at least two of the genes at the time of recurrence were either anaplastic astrocytomas or secondary glioblastomas. Interestingly, *KIT* gene amplification was involved in all 13 tumors with coamplification. All three gliomas that showed coamplification at the time of glioma diagnosis had coamplification also in the recurred glioma. Amplification of only one of the three genes occurred in 15 (18%) samples collected at the time of the diagnosis and in 17 (23%) samples taken from a recurrent glioma; 15 of these 32 (47%) single-gene amplifications were amplifications of *KIT*.

KIT amplifications were significantly associated with presence of *PDGFRA* and *EGFR* amplifications both at the time of the glioma diagnosis and at tumor recurrence, and with

VEGFR2 amplifications at the time of tumor recurrence (Table 2). Presence of *EGFR* amplification was significantly associated with presence of *KIT*, *PDGFRA*, and *VEGFR2* amplification in glioma samples collected at the time of the diagnosis, but only with *KIT* amplifications in recurred gliomas.

Mutation of *TP53* was present at the time of the diagnosis in 11 (29%) astrocytomas, 5 (28%) anaplastic astrocytomas, 4 (20%) oligodendrogliomas, and in 3 (27%) oligoastrocytomas (Fig. 2). *TP53* mutations were not detected more frequently in recurred than in newly detected tumors ($P = 0.25$); they were present in 5 (29%) recurred astrocytomas, 8 (38%) recurred anaplastic astrocytomas, 4 (24%) recurred oligodendrogliomas, 7 (44%) recurred oligoastrocytomas, and in 6 (38%) secondary glioblastomas. A borderline significant association was present between presence of *TP53* mutations and *EGFR* amplifications in the subset of recurred gliomas ($P = 0.075$), but otherwise we found no significant associations between presence of *TP53* mutations and *KIT*, *PDGFRA*, *VEGFR2*, or *EGFR* amplifications either in the subset of newly diagnosed gliomas or among recurred tumors ($P > 0.10$ for all comparisons).

Protein Expression

KIT protein expression was rare in gliomas. Moderate (++) *KIT* protein expression was present in only one of the gliomas at the time of the diagnosis. This was a case of anaplastic astrocytoma that harbored *KIT* gene amplification. Strong (+++) *KIT* protein expression was detected in three recurrent tumors; in two secondary glioblastomas and in one anaplastic astrocytoma. All these three gliomas had *KIT* gene amplification, and *KIT* protein expression was significantly associated with *KIT* amplification in the subset of recurred gliomas ($P = 0.018$). No associations were found between vascular endothelial cell growth factor receptor-2 (*VEGFR*) protein expression and presence of *VEGFR2* amplification ($P = 0.18$), or with *EGFR* protein expression and *EGFR* amplification ($P = 1.00$).

To examine whether *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* gene amplifications or *TP53* mutations are associated with the putative glioma stem cell marker prominin 1 and nestin expression, we assessed correlations between these variables. In the subset of recurred gliomas, prominin 1 expression was present in 6 of the 17 (35%) gliomas that harbored *KIT* amplification as compared with 7 of the 48 (15%) gliomas that did not have amplified *KIT* ($P = 0.085$). Prominin 1 was expressed in 8 of the 24 (33%) gliomas that contained *TP53*

Table 2. Associations of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* Gene Amplifications in Gliomas

	<i>PDGFRA</i> (<i>P</i>)	<i>VEGFR2</i> (<i>P</i>)	<i>EGFR</i> (<i>P</i>)
Gliomas at presentation			
<i>KIT</i>	0.014	0.23	0.010
<i>PDGFRA</i>	—	0.12	0.0060
<i>VEGFR2</i>	—	—	0.045
Recurrent gliomas			
<i>KIT</i>	0.0005	0.022	0.036
<i>PDGFRA</i>	—	0.015	0.27
<i>VEGFR2</i>	—	—	0.17

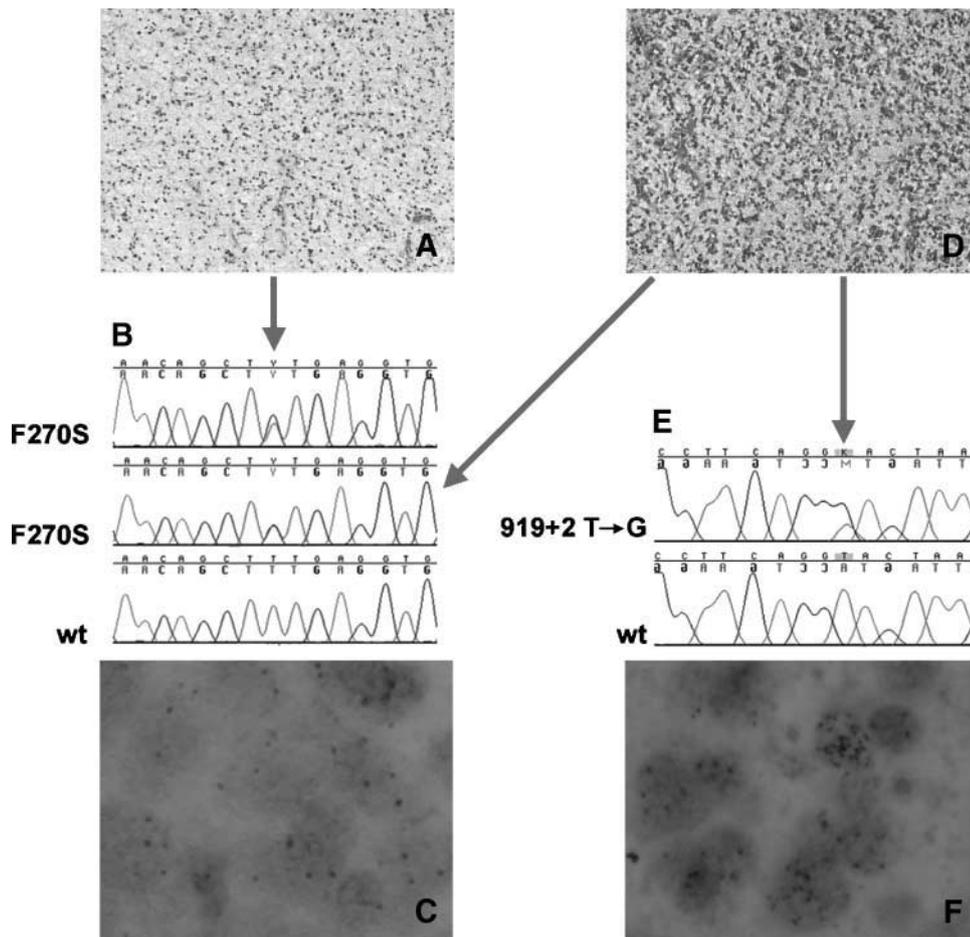


FIGURE 2. A diffuse astrocytoma, H&E staining (A). The tumor harbors a *TP53* gene point mutation F270S (B) and has an aneuploid copy number of *KIT* in CISH (C). Tumor recurrence, classified as anaplastic astrocytoma (D), had acquired a *TP53* splice site mutation (E) and contained high-level *KIT* amplification (F).

mutation whereas only 7 of the 46 (15%) gliomas that did not contain *TP53* mutation expressed prominin 1 ($P = 0.080$). Associations between *KIT* amplification, *TP53* mutation, and prominin 1 expression were not found in the subset of newly diagnosed gliomas, and no associations were present between prominin 1 expression and *PDGFRA*, *VEGFR2*, or *EGFR* gene amplifications ($P > 0.1$ for all comparisons). Nestin expression was associated with presence of *TP53* mutations in the subset of recurrent gliomas. Twenty-five of the 28 (89%) recurrent gliomas that harbored *TP53* mutation expressed nestin as compared with 30 of the 45 (67%) recurrent gliomas that did not contain *TP53* mutation ($P = 0.029$). Nestin expression was not associated with *TP53* mutations in the subset of newly diagnosed gliomas, and no significant associations were detected between presence of *EGFR*, *KIT*, *PDGFRA*, or *VEGFR2* amplifications and nestin expression.

Association of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* Amplification with Survival

Presence of either *KIT*, *PDGFRA*, or *EGFR* gene amplification in glioma at the time of the first diagnosis was associated with poor overall survival in univariate survival analyses, and also presence of *VEGFR2* amplification tended to be associated with an unfavorable outcome (Table 3). None of the four gene amplifications was associated with gender. Patients who had

glioma with *PDGFRA* amplification were older than those with glioma without *PDGFRA* amplification at the time of the diagnosis (41.1 versus 35.4 years, respectively; $P = 0.041$). Similarly, patients with glioma with *EGFR* amplification at diagnosis were older than those without amplification (49.4 versus 35.7 years, respectively; $P = 0.032$), but neither *KIT* nor *VEGFR2* amplification was associated with age at presentation ($P = 0.48$ and $P = 0.63$, respectively).

Because gene amplifications tended to be associated with a low grade of glioma differentiation, we added *KIT*, *PDGFRA*,

Table 3. Association of *KIT*, *PDGFRA*, *VEGFR2*, and *EGFR* Amplification with Survival

Gene	n	5-y survival (%)	P
<i>KIT</i>			
Amplification present	8	38	0.015
Not present	67	64	
<i>PDGFRA</i>			
Amplification present	6	33	0.047
Not present	66	63	
<i>VEGFR2</i>			
Amplification present	10	40	0.097
Not present	65	63	
<i>EGFR</i>			
Amplification present	>3	0	0.0003
Not present	69	62	

VEGFR2, and *EGFR* gene amplification as a cofactor in a multivariate model that contained the histologic diagnosis of glioma as another cofactor. Presence of *KIT* amplification tended to be associated with unfavorable survival in this multivariate model that accounted for the histologic type of glioma (risk ratio, 2.0; 95% confidence interval, 0.9-4.4; $P = 0.075$), whereas presence of *PDGFRA* and *VEGFR2* amplification was not associated with outcome in the model ($P = 0.58$ and $P = 0.11$, respectively).

Discussion

The present results indicate that amplified *KIT*, *PDGFRA*, and *VEGFR2* may occur in astrocytomas, anaplastic astrocytomas, oligodendrogliomas, and oligoastrocytomas and in their recurrent tumors. Approximately one third of primary glioblastomas harbor amplified *PDGFRA*, *KIT*, and *VEGFR2* receptor tyrosine kinase genes (13). The three genes are located on adjacent positions on chromosome 4q12. In the present series, amplified *KIT* and *PDGFRA* were more frequently present in anaplastic astrocytomas than in astrocytomas, oligodendrogliomas, and oligoastrocytomas, and amplified *KIT* was more often found in recurrent gliomas than in gliomas at the time of their first diagnosis, suggesting that these genes may be involved in glioma progression. *KIT* amplifications were associated with *KIT* protein expression and with presence of *PDGFRA*, *VEGFR2*, and *EGFR* amplifications. In cases where simultaneous amplification of *KIT*, *PDGFRA*, and *VEGFR2* was present, the amplicon always contained *KIT*. Taken together, these findings suggest that amplification of *KIT*, in particular, may be implicated in the molecular pathogenesis of some gliomas.

A role for *KIT* in the pathogenesis of gliomas has not been suggested earlier. The earliest alterations in the molecular pathogenesis of low-grade astrocytomas have been suggested to include overexpression of PDGF ligand and the PDGFRs that cause an autocrine growth factor stimulation loop and inactivation of *TP53* (2). Anaplastic astrocytomas and glioblastomas have been found to accumulate further genetic alterations, such as deletions of *P16/CDKN2A*, amplification of *CDK4* (cyclin-dependent kinase 4), mutations of *RB* (retinoblastoma), and loss of chromosome 10q, which eventually leads to an uncontrolled progression of the cell cycle (14). Primary glioblastomas that arise *de novo* often show amplification and overexpression of *EGFR* (>50% of glioblastomas), whereas *TP53* mutations are rare. The *EGFR* gene, located on 7p12, is frequently rearranged in primary glioblastoma encoding a truncated, constitutionally activated receptor tyrosine kinase, named *EGFRvIII*. Glioblastomas may also express the endogenous ligands of *EGFR*. Loss of heterozygosity on chromosome 10q is present in 80% to 90% of glioblastomas. This region contains *PTEN* (phosphatase and tensin homologue) gene, which is mutated in 20% to 30% of primary or secondary high-grade astrocytomas (2). Interestingly, we found that amplification of *KIT*, *PDGFRA*, and *VEGFR2* was associated with presence of *EGFR* amplification in gliomas at the time of the diagnosis, but only *KIT* amplification was associated with *EGFR* amplification in recurrent gliomas.

We detected *KIT*, *PDGFRA*, and *VEGFR2* gene amplifications only infrequently in oligodendrogliomas. Oligodendrogliomas are characterized by chromosomal deletions of 1p and 19q. Although the putative tumor suppressor genes on 1p and 19q are unknown, these deletions are considered important events in the tumorigenesis (15-18). Oligoastrocytomas are clonal tumors that can be roughly subdivided into astrocytoma-related tumors that contain *TP53* mutations and oligodendroglioma-related tumors with 1p and 19q deletions (2). Amplification of *KIT*, *PDGFRA*, and *VEGFR2* was found frequently in recurrent oligoastrocytic tumors (in 23-33% of the cases), suggesting that these receptor tyrosine kinases may be implicated in tumor progression of oligoastrocytomas.

Glioma genesis and progression may be driven in part by cancer stem cell populations, which have self-renewal and differentiation potential (19). Some gliomas may arise from a cell with neural stem cell-like properties (20). The present findings may also reflect the cancer stem cell element in the molecular pathogenesis of gliomas because *KIT* protein is the receptor of the stem cell factor. *KIT* is expressed in human bone marrow progenitor cells and in glial progenitor cells of rodents, but its expression is lost when the glial progenitor cells differentiate into post-mitotic oligodendrocytes (21). Stem cell factor is up-regulated in high-grade human gliomas, it promotes angiogenesis, and its expression is associated with short survival (22). *KIT* autophosphorylation is also induced in gliomas. The stem cell factor and *KIT* activation may thus have a role in glioma development and progression.

Amplification of *KIT*, *PDGFRA*, and *EGFR* was associated with poor survival in a univariate survival analysis. However, only one marginally significant association between presence of *KIT* amplification and survival was found in a multivariate survival analysis that included also tumor histology as a cofactor. *EGFR* amplification has not been found to be associated with unfavorable outcome in glioblastoma, and it seems to be only weakly or not at all associated with survival in anaplastic astrocytoma (23, 24).

A minority (9-20%) of glioblastoma patients respond to the combination of imatinib and hydroxyurea (10, 11). The molecular mechanisms to explain the treatment responses are not known, but imatinib is a specific inhibitor of *KIT* and the PDGFRs. Hypothetically, amplifications of *KIT* and *PDGFRA* might serve as biomarkers for those gliomas that respond to imatinib or to other *KIT* and PDGFR inhibitors such as sunitinib, sorafenib, vatalanib, nilotinib, or dasatinib. Clinical trials with these agents in patients diagnosed with glioblastoma, anaplastic astrocytoma, or recurrent lower-grade glioma containing amplified *KIT* or *PDGFRA* may be warranted.

In conclusion, the present findings indicate that receptor tyrosine kinase genes *KIT*, *PDGFRA*, and *VEGFR2*, which are juxtaposed on chromosome 4 in the human genome, may be amplified not only in glioblastomas but in lower-grade gliomas as well. Their amplification is more often present in anaplastic astrocytomas than in lower-grade astrocytomas and oligodendrogliomas. Amplification of *KIT*, *PDGFRA*, and *VEGFR2* is associated with *EGFR* amplification in newly diagnosed gliomas, and amplified *KIT* is associated with *KIT*

protein expression. At present, it is not known whether glioblastomas or lower-grade gliomas with amplified *KIT*, *PDGFRA*, or *VEGFR2* are responsive to specific inhibitors of these receptor tyrosine kinases.

Materials and Methods

Patients

Formalin-fixed paraffin-embedded tumor tissue from 119 patients who had craniotomy for a primary glioma in 1979 to 2000, and who had brain tumor tissue available for analysis both from the primary tumor and its recurrence, were retrieved from the archives of the Department of Pathology, Helsinki University Central Hospital. The tumors were originally diagnosed either as diffuse astrocytoma (grades 2-4), oligodendroglioma (grade 2 or 3), or oligoastrocytoma (grade 2 or 3). The histologic diagnoses were reviewed by two professional pathologists (O.T. and A.P.) based on the WHO criteria (6). We excluded from this series 14 patients who had either glioblastoma or pediatric glioma (ages of <17 years) at the time of the diagnosis. Further 18 patients were excluded because all attempted gene amplification analyses were uninformative both from the sample taken at diagnosis and from the sample taken at tumor recurrence, which left 87 patients in the final analysis. At presentation, 38 (44%) tumors were grade 2 astrocytomas, 18 (21%) anaplastic astrocytomas, 20 (23%) oligodendrogliomas, and 11 (13%) oligoastrocytomas.

The median age at the time of the diagnosis was 36.4 years (range, 17.3-62.1 years) and 47 (54%) were male. The primary therapy consisted of surgery with or without postoperative external beam radiation therapy. The median age at glioma recurrence was 41.1 years (range, 18.3-66.7 years). Seventeen (20%) of the recurrent gliomas were grade 2 astrocytomas, 21 (24%) anaplastic astrocytomas, 17 (20%) oligodendrogliomas, 16 (18%) oligoastrocytomas, and 16 (18%) cases that had progressed into glioblastomas. Nine (10%) of the patients were alive at the time of the analysis; six patients were lost from follow-up. The median follow-up time of the patients still alive as calculated from the first diagnosis of glioma was 11.8 years (range, 2.8-22.2 years). The median survival time was 70 months as calculated from the first diagnosis of glioma.

The study was approved by the Ethical Committee of the Hospital District of Helsinki and Uusimaa. We obtained a permission to use tumor tissue for the study from the Ministry of Social Affairs and Health, Finland.

Immunohistochemistry

Tissue microarray blocks were prepared using a 0.6-mm-diameter core biopsy needle. Five-micrometer sections were cut and examined for *KIT*, *VEGFR2*, *EGFR*, *TP53* (p53), nestin, and prominin 1 (CD133) expression using immunohistochemistry. *KIT* was stained with a rabbit polyclonal anti-CD117 antibody (dilution, 1:300; A 4502, DAKO, Glostrup, Denmark), *EGFR* with a mouse monoclonal anti-EGFR antibody (1:150; NCL-EGFR, Novocastra Laboratories Ltd., Newcastle, United Kingdom), and *TP53* with a mouse monoclonal anti-TP53 antibody (1:500; NCL-P53-D07) as described in details elsewhere (13). The *KIT* and *EGFR* stainings were graded

either as negative (–), weakly positive (+; <10% of tumor cells expressed the protein), moderately positive (++; 10-50% of tumor cells were positive), or strongly positive (+++; >50% of the tumor cells expressed the protein). *TP53* was graded as positive when >20% of tumor cell nuclei were stained.

To examine *VEGFR2* expression, we did antigen retrieval by heating the sample to 120°C in an autoclave for 2 min in a 10 mmol/L sodium citrate buffer (pH 6.0). An epitope-specific rabbit anti-*VEGFR2* antibody (Flk-1 Ab-1, NeoMarkers, Lab Vision Corp., Fremont, CA), diluted in a PowerVision pre-antibody blocking solution, was incubated with the sample for 1 hour at room temperature. The binding of the primary antibody was detected with a PowerVision+ Poly-HRP histostaining kit (DPVB+110DAB, ImmunoVision Technologies Co., Daly City, CA). The tissue sections were counterstained with hematoxylin. The immunostainings were graded as either negative (–), faintly positive (+), moderately positive (++; or markedly positive (+++)) by a pathologist (O.T.) using a consultation microscope (Nikon Eclipse E600, Nikon Instech Co., Ltd., Kanagawa, Japan).

Nestin expression was assessed by immunohistochemistry with a mouse monoclonal anti-nestin antibody (dilution, 1:500; clone 10C2, Chemicon International, Inc., Temecula, CA), and prominin expression with a mouse anti-human CD133 antibody (dilution, 1:10; CD133/1, AC133 pure, Miltenyi Biotec, Bergisch Gladbach, Germany). Before immunostaining for prominin, the tissue samples were treated in an autoclave for antigen retrieval as described for *VEGFR2* staining above. Anti-nestin and anti-prominin antibodies were diluted in a PowerVision pre-antibody blocking solution and were incubated with the sample for 1 hour at room temperature (nestin) or at 4°C (prominin) overnight. Bound antibodies were detected using a PowerVision+ Poly-HRP histostaining kit as described above. Both stainings were graded semiquantitatively either as negative (–), faintly positive (+), moderately positive (++; or strongly positive (+++).

Tissue sections containing histologically normal breast, lung, liver, heart muscle, skeletal muscle, cerebrum, and cerebellum were stained as controls. Strong *KIT* expression of tissue mast cells served as a positive control for CD117 (*KIT*) stainings. Dermal wound tissue was used as a positive control for *VEGFR2* immunostainings. Tissue samples consisting of histologically normal breast tissue, breast cancer, melanoma, or glioblastoma were used as negative and positive controls in immunostainings for nestin, prominin, p53, and *EGFR*.

Chromogenic In situ Hybridization and Fluorescence In situ Hybridization

Tissue sections mounted on glass slides were deparaffinized and incubated in 0.1 mol/L Tris-HCl (pH 7.0) in a temperature-controlled microwave oven (at 92°C for 10 min), followed by cooling down for 20 min at room temperature. After a wash with PBS, enzymatic digestion was carried out by applying 100 µL of digestion enzyme onto the slides for 10 to 15 min at room temperature (Digest-All III solution, Zymed, Inc., South San Francisco, CA). Digoxigenin-labeled BAC probes were applied onto the slides, sections were denatured, and hybridization was done overnight at 37°C (13). After

hybridization, the slides were washed with $0.5\times$ SSC (5 min at 75°C), followed by three washes in PBS (20°C). The probes were detected with a mouse anti-digoxigenin antibody (diluted 1:300; Roche Biochemicals, Mannheim, Germany), an anti-mouse-peroxidase polymer (Powervision+, ImmunoVision Technologies), and diaminobenzidine chromogen according to the manufacturer's protocol. The tissue sections were counterstained with hematoxylin. Gene amplification was considered to be present when six or more signals were detected per nucleus. Cases with three to five signals per nucleus were classified as aneuploid and those with two copies as diploid (the normal copy number).

Fluorescence *in situ* hybridization (FISH) analysis was done from 13 (15%) primary tumors that showed weak or absent chromogenic *in situ* hybridization (CISH) signals for *KIT*, *PDGFRA*, and *VEGFR2*. Information on the BAC-clones, their processing, and methods is described elsewhere (13). All FISH, CISH, and immunohistochemistry analyses were carried out on coded slides without knowledge of other data. In cases where the red and green hybridization signals showed different intensities, the gene copy number was verified by interchanging the labeling of the probes.

PCR

The genomic DNA was extracted from formalin-fixed paraffin-embedded tissue using standard methods. Fifty nanograms of the genomic DNA were amplified in a PCR reaction containing 0.6 $\mu\text{mol/L}$ Platinum PCR Buffer (Invitrogen, Carlsbad, CA), 1.4 to 2.4 mmol/L MgCl_2 , 160 $\mu\text{mol/L}$ deoxynucleotide triphosphates (Clontech, Palo Alto, CA), 0.3 $\mu\text{mol/L}$ forward and reverse primers, DNA polymerase AmpliTaq Gold (1.25 units; Applied Biosystems, Branchburg, NJ), and Platinum Taq (1.25 units; Invitrogen) in a volume of 50 μL . The forward and reverse oligonucleotide primers used to amplify *TP53* exons 6 to 9 were Ex6F, AGGGTCCCCAGGC-CTCTGAT; Ex6R, CCATGACAACCACCTTAA; Ex7F, TGCTTGCCACAGGTCTCC; Ex7R, TGTGCAGGGTGG-CAAGTGGC; Ex8-9F, AGTAGATGGAGCCTGGTTTTT; and Ex8-9R, AGAAAACGGCATTGAGTG. The PCR cycling conditions consisted of an initial denaturation step at 94°C for 14 min, followed by 35 cycles at 94°C for 30 s, annealing at 56°C for 45 s and 2 min at 72°C , and final extension for 10 min at 72°C . Heteroduplex formation was created by denaturing the PCR products for 5 min at 95°C , and then allowing the samples to reanneal by decreasing the temperature at $1^{\circ}\text{C}/\text{min}$ from 95°C to 40°C .

Denaturing High-Performance Liquid Chromatography

Five to 10 μL of the PCR product were injected on the Helix DNA HPLC Column 50×3.0 mm (Varian, Inc., Walnut Creek, CA), and eluted at a flow rate of 0.45 mL/min within a linear acetonitrile gradient consisting a mixture of buffer A (100 mmol/L triethylammonium acetate and 0.1 mmol/L EDTA; Varian) and buffer B (100 mmol/L triethylammonium acetate, 0.1 mmol/L EDTA, and 25% acetonitrile; Varian). The annealing temperature was 58°C for *TP53* exon 6, 64°C for exon 7, and 63°C for exons 8 and 9. The elution temperature was 62°C for exons 6 and 7 and 63°C for exons 8 and 9.

DNA Sequencing

Samples with an abnormal elution profile in denaturing high-performance liquid chromatography as compared with a normal noncancerous control sample were subjected to automated sequencing. The PCR products were first purified using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Direct bidirectional sequencing of the PCR products was done using BigDye3 termination chemistry (Applied Biosystems) and an ABI 3100 Genetic Analyzer (Applied Biosystems) according to the instructions provided by the manufacturer.

Statistical Analysis

Frequency tables were analyzed by the χ^2 test or Fisher's exact test. Survival was analyzed using the Kaplan-Meier method, and groups were compared using the log-rank test. Overall survival was computed from the date of the diagnosis to death, and the patients still alive were censored on the last date of follow-up. Multivariate analyses were done using the Cox proportional hazards regression model. All *P* values are two sided.

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