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Doctor’s Thesis
学位論文

Clinical significance of epidermal growth factor receptor
(EGFR) expression in patients with glioblastoma multiforme

{神経膠芽腫患者における上皮成長因子受容体(EGFR)発現の臨床的意義}

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1. Summary

Glioblastoma multiforme (GBM) frequently involves amplification and alteration of the epidermal growth factor receptor (EGFR) gene, resulting in overexpression of varied mutations including the most common mutation, EGFRvIII, as well as wild-type EGFR (EGFRwt). To test the prognostic value of EGFR, we retrospectively analyzed the relationship between treatment outcomes and the EGFR gene in 87 newly diagnosed adult patients with supratentorial GBM enrolled in clinical trials. The EGFR gene status was assessed by Southern blots, EGFR expression by immunohistochemistry using 3 monoclonal antibodies (EGFR.25 for EGFR, EGFR.113 for EGFRwt, and DH8.3 for EGFRvIII). EGFR amplification was detected in 40 (46.0%) of the 87 GBM patients; in 39 (97.5%) of these, EGFR was overexpressed. On the other hand, in 46 of 47 patients without EGFR amplification (97.9%) no EGFR overexpression was present. There was a close correlation between EGFR amplification and EGFR overexpression (P<0.0001). EGFRwt was overexpressed in 27 of the 40 (67.5%) patients with, and in none without EGFR amplification (P<0.0001). Similarly, EGFRvIII was overexpressed in 18 (45.0%) of 40 patients with and in 4 (8.5%) of 47 patients without EGFR amplification (P<0.0001). The finding that 8 (20.0%) of the patients with EGFR amplification/EGFR overexpression manifested overexpression of neither EGFRwt nor EGFRvIII indicates that they overexpressed other types of EGFR. Multivariate analysis demonstrated that EGFR amplification was an independent, significant, unfavorable predictor for overall survival (OS) in all patients (P=0.038, HR=1.67). With respect to the relationship of age to EGFR prognostication, the EGFR gene status was a more significant prognosticator in younger patients, particularly in those < 60 years (P=0.0003, HR=3.15), whereas not so in older patients. EGFRvIII overexpression, on the other hand, was not predictive for OS. However, in patients with EGFR amplification, multivariate analysis revealed that EGFRvIII overexpression was an independent, significant, poor prognostic factor for OS (P=0.0044, HR=2.71).
This finding indicates that EGFRvIII overexpression in the presence of EGFR amplification is the strongest indicator of a poor survival prognosis. In GBM patients, EGFR is of significant prognostic value for predicting survival, and the overexpression of EGFRvIII with amplification plays an important role in enhanced tumorigenicity.
[背景、目的] 最も悪性の脳腫瘍、glioblastoma multiforme (GBM) は放射線治療、化学療法、免疫療法の進歩に関わらず平均生存期間は僅か1年程度と依然短く、その治療成績はここ20年全く変化していない。GBM では癌遺伝子である上皮成長因子受容体 (EGFR) 遺伝子の増幅、EGFR 蛋白の過剰発現、EGFRvIII に代表される種々の変異型 EGFR の発現が高頻度に認められる。多数の基礎実験の報告から, EGFR の質的量的変化が腫瘍の増殖浸潤、治療抵抗性の亢進に大いに寄与しており、この EGFR を標的とした治療の効果が大いに期待されている。その効果を最大限引き出すためには GBM 患者における EGFR 発現の臨床的意義を明らかにすることが必要不可欠であるが未だ結論に至っていない。この問題解明の妨げになっている理由として、対象集団の不均一性の他、EGFR 発現の正確かつ再現性の高い標準的測定法が確立されていないことが挙げられる。これを踏まえ, EGFR が GBM 患者において予後因子になりうるか否か後向きに検討した。

[対象] 母集団を均一にすべて同様の治療を行った第 3 相臨床試験に参加した成人大脳半球発 GBM 患者を対象とし、十分な腫瘍 DNA が得られた 87（男性 54、女性 33）名について解析した。

[方法] EGFR 遺伝子の評価はプローブとして full length EGFR cDNA を用いたサインプロット法で、EGFR 蛋白の評価は3種類のモノクローナル抗体 (EGFR113 は EGFRwt、DH83 は EGFRvIII を特異的に認識し、EGFR25 は両者を含む EGFR を認識する）を用いた免疫染色で行った。生存期間 (OS) を算出し、多変量解析 (Cox hazard regression model) で予後解析を行った。

[結果] EGFR 遺伝子増幅は 40例（46％）で認められ各 EGFR 蛋白発現レベルと有意に相関したが、特に EGFR25 による EGFR 発現レベルとほぼ一致した（P<0.0001）。遺伝子増幅40例のうち8例（20％）は EGFRwt 及び EGFRvIII の高発現が認められなかったが、EGFR25 による EGFR の高発現が確認された。これは他の変異型 EGFR が高発現していることを示唆した結果と考えられた。 EGFR 遺伝子増幅は全症例において OS の有意な独立予後不良因子となった（P=0.038, HR=1.67）。年齢別にみると特に 60 歳未満の若年群でより顕著となったが（P=0.0003, HR=3.15）、逆に 高齢群では有意な予後因子となかった。EGFRvIII 高発現は全症例では予後因子となりなかったが、遺伝子増幅群40例において OS の有意な独立予後不良因子となった（P=0.0044, HR=2.71）。

[結論] EGFR は GBM 患者の生存期間を予測する重要な指標となった。EGFRvIII 高発現例の生存予後が特に不良であることが示唆された。
2. List of published papers

1. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme

Naoki Shinojima, Kenji Tada, Shoji Shiraishi, Takanori Kamiryo, Masato Kochi, Hideo Nakamura, Keishi Makino, Hideyuki Saya, Hirofumi Hirano, Jun-ichi Kuratsu, Koji Oka, Yasuji Ishimaru, and Yukitaka Ushio

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4. Abbreviations and Acronyms

GBM = glioblastoma multiforme
EGFR = epidermal growth factor receptor
EGFRwt = wild-type EGFR
LOH = loss of heterozygosity
PTEN = phosphatase and tensin homology
MAPK = mitogen-activated protein kinase
STAT = signal transducer and activator of transcription
PIP3 = phosphatidylinositol-3,4,5-trisphosphate
PI3K = phosphatidylinositol 3-kinase
OS = overall survival
KPS = Karnofsky performance score
HR = hazards ratio
CI = confidence interval
ns = not significant
GTR = gross total resection
PR = partial resection
ACNU = 3-{(4-amino-2-methyl-5-pyrimidinyl)methyl}-1-(2-chloroethyl)-1-nitrosourea hydrochloride
IA-ACNU = intra-arterial ACNU administration
IV-ACNU = intravenous ACNU administration
PAV = procarbazine, ACNU, plus vincristine
5. Background and Purpose

5.1. Glioblastoma multiforme

5.1.1. Definition

Glioblastoma multiforme (GBM) is the most malignant astrocytic tumor, composed of poorly differentiated neoplastic astrocytes. Histopathologic features include cellular polymorphism, nuclear atypia, brisk mitotic activity, vascular thrombosis, microvascular proliferation and necrosis. Prominent microvascular proliferation and/or necrosis are essential diagnostic features.

5.1.2. Grading

The GBM corresponds to WHO grade IV.

5.1.3. Incidence

The GBM is the most frequent brain tumor, accounting for approximately 12-15% of all intracranial neoplasms (1) and 50-60% of all astrocytic tumors. In most North American and European countries, the incidence is the range of 2-3 new cases per 100'000 population per year (2). On the other hand in Japanese, the incidence of all gliomas including GBM is 2-3 /100'000 population per year, i.e. the incidence of GBM that comprises a part of them is lower than the Caucasian (3, 4).

5.1.4. Age and gender distribution

The typical median age at diagnosis ranges 55-60 years, and males are significantly more frequently affected (M/F ratio, almost 1.5).

5.1.5. Prognosis and predictive factors
Over the past 2 decades, the treatment outcomes in patients with GBM have remained poor even after multimodal therapies including surgical resection, radiotherapy, and chemotherapy; the median length of survival is approximately one year (5-10). On the other hand only 1-5% of GBM patients survive for more than 3 to 5 years (11-18). Similarly, our institution has met 6 (5.3%) newly diagnosed patients with GBM having the long-term survival at least more than 5 years (19). Many studies, undertaken to improve the clinical management of this lethal tumor by identifying prognostic factors, confirmed that the patient age at diagnosis plays a significant role in the median length of survival and the 5-year survival rate; the younger patients can enjoy better prognosis than the older (5-10, 19, 20). On the other hand evaluation of the preoperative KPS and the extent of surgical resection as prognostic factors has been controversial, but there is some evidence that complete resection and high KPS favor longer survival (11, 12, 14-19, 21, 22).

5.1.6. Primary and secondary GBM

Thus although there is considerable variation with respect to survival among GBM patients, they share histopathologically similar morphologic features and are indistinguishable from one another. From a biological and clinical point of view, Scherer suggested the reclassification subdividing GBM into two subgroups, 'primary and secondary GBM' (23). After a short clinical history, usually of less than 3 months, the primary GBM manifest de novo, i.e. without clinical or histopathological evidence of a pre-existing, less malignant precursor lesion, and account for the vast majority of cases in older patients (mean 55 years). On the other hand the secondary GBM typically develop in younger patients (<45 years), during the long clinical duration due to malignant progression from diffuse astrocytomas WHO grade II or anaplastic astrocytomas WHO grade III. When patients with diffuse astrocytoma WHO grade II
develop a recurrent lesion, the second surgical biopsy often shows histopathologic evidence of increased nuclear atypia, hyperchromasia, mitotic activity (anaplastic astrocytoma WHO grade III) and, eventually, microvascular proliferation and/or necrosis, i.e. features of GBM. The time for progression from diffuse astrocytomas WHO grade II to GBM varies considerably, with time intervals ranging from less than 1 year to more than 10 years (24), the mean interval being 4-5 years (25-27). There is increasing evidence that primary and secondary GBM constitute distinct disease entities, that evolve through different genetic pathways (see 5.2.2), affect patients at different ages, and are likely to differ in response to therapy (28). However the evaluation of the difference in lengths of survival between primary and secondary GBM is controversial. The prognosis of patients with secondary GBM is better than (9) or similar to (29), that of patients with primary (de novo) lesions. Primary GBM is clinically the prevailing type, comprises more than 80% of all GBM (29).

5.2. The role of Epidermal growth factor receptor (EGFR) in GBM

5.2.1. EGFR

EGFR is a transmembrane tyrosine kinase receptor responsible for sensing its extracellular ligands, EGF and transforming growth factor alpha (TGF-α), and for transducing this signal to the cell. Although EGFR is expressed in all of normal tissues’ cells except for blood cells, it has been associated with cancer for three main reasons: (a) it is the cellular homologue of the v-erbB oncogene found in the acutely transforming avian erythroblastosis virus (30-32); (b) when expressed ectopically in cells, it can set up a transforming autocrine loop, so that cellular transformation is ligand-dependent (33); (c) it has been shown to be amplified in several tumor types, with an increased copy number that is directly correlated to an increase in the number of receptors on the cell surface. Normal brain tissues (glial, neuronal, and endothelial cell
lineages) display only a few thousand EGFR molecules/cell, whereas glioma cells often express up to 100,000 EGFR molecules/cell (34). While the gene encoding EGFR maps to chromosome 7, the amplified genes are typically present as double-minute extrachromosomal elements. The wild-type EGFR (EGFRwt) protein is 170 kDa, and is composed of four major domains: the ligand-binding extracellular domain; the transmembrane anchoring domain; the catalytic tyrosine kinase domain; and the carboxyl terminus, which contains both five tyrosines that are target substrates for the kinase, and motifs responsible for ligand-activated endocytosis. Moreover there is also evidence that gliomas express the EGFR ligands, EGF and TGF-α thus suggesting the potential that these tumor cells have autocrine growth stimulatory loops (35). Mechanistically, EGFR-driven mitogenesis appears to encompass: ligand-driven dimerization of receptor monomers; tyrosine kinase activation; tyrosine phosphorylation of the receptor; signaling through various coupling and adaptor proteins, like Shc and Grb2, to signal transduction pathways driven by either phospholipase-C-gamma, PI3K-Akt, Ras-MAPK or STAT; and finally, receptor-ligand internalization and lysosomal breakdown leading to signal attenuation (36). It is well known that the signaling pathway by PI3K-Akt plays important roles in cell proliferation and survival (37, 38) as well as that by Ras-MAPK (39). Moreover PI3K-Akt signaling mediates angiogenesis in tumors through expression of vascular endothelial growth factor (40, 41). These signaling pathways play significant roles in enhanced tumorigenicity (42). In fact, EGFR overexpression confers advantages of growth and invasiveness, and radio- and chemo-resistance on glioma cells in vivo (43-47).

Until the 1980s, it has not been found that EGFR gene amplification that results in overexpression of EGFR frequently occurs in high-grade astrocytomas including GBM (34, 48, 49). EGFR amplification is present in 30-50% of all GBM; it occurs more frequently in primary (de novo) GBM (see 5.2.2), and in <10% of anaplastic astrocytomas (35, 50-57).
5.2.2. Genetic pathways in the evolution of primary and secondary GBM

EGFR amplification was present in 11/28 (39%) of primary, but in none of 22 secondary GBM (58). With respect to protein level, immunoreactivity for the EGFR also prevailed in primary GBM (>60% of cases) vs. 10% in secondary GBM (55). On the other hand, mutations of TP53 tumor suppressor gene are less common in primary GBM (<10%) while secondary GBM have a high incidence of TP53 mutations (26, 55). The frequency of TP53 protein accumulation is also significantly higher in secondary (>90%) than in primary GBM (<35%) (55). Furthermore only one out of 49 GBM showed EGFR overexpression and a TP53 mutation, i.e. EGFR overexpression and TP53 mutations are mutually exclusive events in the evolution of primary and secondary GBM, respectively (55).

Thus the concept that different genetic pathways lead to the GBM as the common phenotypic endpoint is now generally accepted (28, 59, 60). Approximately 60% of patients with secondary GBM manifest TP53 mutations; more than 90% of the mutations are already present in the preceding diffuse astrocytomas WHO grade II or anaplastic astrocytomas WHO grade III. In contrast, patients with primary GBM characterized by EGFR amplification (in approximately 40% of cases) and/or overexpression (60% of cases) often manifest the following other molecular genetic alterations: phosphatase and tensin homology (PTEN) mutations (30% of cases), p16/CDKN2a/INK4a deletion (30-40% of cases), murine double-minute 2 (MDM2) amplification (<10% of cases) and/or overexpression (50% of cases), and loss of heterozygosity (LOH) on the chromosome 10 (50-80% of cases). which maps the gene encoding PTEN (61, 62).

Primary GBM are thought to develop as the result of stepwise accumulations of multiple genetic alterations in a precursor or neural progenitor cell, i.e. it may take the
long periods to become evident as the primary GBM. Therefore the primary GBM may account for the vast majority of cases in older patients. On the other hand the secondary GBM characterized by TP53 mutations develop in younger patients because the tumorigenesis may not need stepwise accumulations of multiple genetic alterations.

LOH on the chromosome 10 mapping the PTEN gene and the mutations of PTEN gene itself frequently occurs in primary GBM. Though PIP3 activates Akt by phosphorylation, PTEN inhibit the signal through PI3K-Akt pathway in a manner that dephosphorylates the PIP3. Therefore the inactivation of PTEN attributable to mutation and LOH 10 makes Akt activity elevated and is thought to play a partial role in the tumorigenesis (63). Surprisingly, Holland et al. reproduced morphological GBM using neural progenitor cells transferred with genes encoding activated forms of Ras and Akt (64). They demonstrated that although neither activated Ras nor Akt alone is sufficient to induce GBM formation, the combination of activated Ras and Akt induces high-grade gliomas with the histological features of human GBMs. Similarly, Sonoda et al. revealed that activation of the Akt pathway is sufficient to morphologically convert human AA model, in which Ras is activated, into human GBM (65). Since EGFR signals both Ras and PI3K, the combination of EGFR amplification and PTEN inactivation may play a partial role in formation of GBM through the signaling pathways driven by both EGFR-Ras-MAPK and EGFR-PI3K-Akt.

5.2.3. Variants of EGFR

The majority of GBM with EGFR amplification exhibit a considerable variety of qualitative EGFR alterations, resulting in different EGFR mutations (34, 49, 66-69). The most common EGFR mutation is EGFRvIII (also known as ΔEGFR and de2-7EGFR), which is characterized by the deletion of exons 2-7 in EGFR mRNA that correspond to cDNA nucleotides 275-1075 encoding amino acids 6-273. This mutation presumably occurs through alternative splicing or gene rearrangements (67, 68,
The expression of the mutant EGFR is also frequently found in other human tumors (71). The EGFRmIII protein whose extracellular ligand-binding domain is truncated is 140 kDa, and unlike EGFRwt the mutant is ligand-independently constitutively autophosphorylated, but at a significantly lower level than is EGFRwt activated by ligand exposure (72-74). However the mutant dramatically enhances tumorigenicity in vivo even when its expression level is low (47, 75, 76). These constitutively active mutant receptors are expressed on the cell surface, but are not downregulated, suggesting that their altered conformation does not result in exposure of receptor sequence motifs required for endocytosis, lysosomal degradation and signal attenuation (75, 77). Consequently, mutant receptors are only internalized at the same low rate as unoccupied wild-type EGFR (78). Mutational analysis showed that the enhanced tumorigenicity conferred by the mutant EGFR was strictly dependent on the intrinsic tyrosine kinase activity and was mediated through the carboxyl terminus, in a similar way to EGFRwt function. However in contrast to EGFRwt, mutation of any single major tyrosine autophosphorylation site abolished its enhanced tumorigenicity. Moreover the mutant receptors are incapable of forming dimmers (72). The mutant EGFR constitutively transduces its signals through the Shc-Grb2-Ras-MAPK pathway in glioma cells (79), or through the PI3K-Akt and JNK pathways in NIH3T3 cells (80, 81) and in glioma cells (82). Moreover the mutant, EGFRmIII bestows enhanced tumorigenicity through both increased cellular proliferation (82) and reduced apoptosis (83). Overexpression of the mutant EGFR in glioma cells also confers resistance to chemotherapeutic drugs such as cisplatin through modulation of Bcl-XL expression and consequent inhibition of induction of apoptosis by the drug treatment (47). In addition, EGFR autocrine signaling induces cell scattering and migration in glioma cells in vitro (84), and glioma cells expressing the mutant EGFR show greater invasiveness when implanted in the mouse brain than control cells (85).
5.2.4. Prognostic value of EGFR

There is experimental evidence that EGFR amplification/overexpression may result in a less favorable prognosis, however clinical studies about relationship of patients’ outcome to EGFR status are inconclusive (20, 52, 56, 86-92). Simmons et al. (86) suggested that differences in patient populations may explain the divergent results.

With respect to relationship of prognosis to EGFRvIII expression, Feldkamp et al. (93) suggested that GBM patients with EGFRvIII may have a shorter life expectancy, however, they were unable to produce statistical evidence for this supposition.

5.2.5 EGFR as a therapeutic target.

Current therapies target tumors in a nonspecific fashion, usually through DNA damage. On the other hand novel target therapies have been developed to specifically block pathways mediating critical tumor phenotypes, including small-molecule tyrosine kinases inhibitors (TKIs), antibodies, immunotoxin conjugates, and antisense oligonucleotides. EGFR pathway represents a particularly attractive therapeutic target in GBM because EGFR is dysregulated through overexpression, amplification, and activating mutations, such as EGFRvIII. Several classes of EGFR inhibitors have been developed, and studied in clinical trials (94-96). Any anti-EGFR targeting therapy down-regulates EGFR signaling pathways, and results in enhancing apoptosis and reducing cellular invasion and angiogenic potential in tumors. One of anti-EGFR targeting therapies is EGFR tyrosine kinase inhibitor, such as AG1478 (97), PKI166 (98) and ZD1839 (Iressa) (99, 100). Phase II trial of ZD1839 has been conducted in patients with GBM at first recurrence, and the TKI may have a clinical response expectancy (95). Monoclonal antibodies to the EGFR have been also produced for potential use for immunotoxin delivery through antibody-mediated internalization of the
5.2.6. Detection methods of EGFR status

There is no established standard method of detecting EGFR status, which is accurate and reproducible.

DNA level: (a) Southern blotting can directly detect gene status and is the most quantitative and reproducible, although it requires adequate lysate samples. (b) Multiplex or quantitative real-time PCR can detect indirectly gene status through amplicons produced from genomic DNA using primers, comparing with reference. (c) FISH (fluorescence in situ hybridization) can directly detect gene status on chromosome.

RNA level: (a) Northern blotting can directly detect gene status and is the most quantitative and reproducible, although it requires adequate lysate samples. (b) RT-PCR can detect indirectly gene status through cDNA amplicons produced from mRNA using reverse transcriptase. (c) DNA microarray (DNA chip) can evaluate transcriptional level by using mRNA in normal tissue samples as reference. However it remains too expensive to be easily available in any institution. Therefore it is inconclusive whether the assay is as accurate and reproducible as conventional methods.

Protein level: (a) Western blotting can detect protein status and is the most quantitative and reproducible. (b) Immunohistochemistry can directly detect the protein at the cellular level. It is frequently used in any institution and the most reproducible method. However, it is difficult to evaluate the staining of EGFR because not the nuclei but the membranes or cytoplasms are stained.

GBM is composed of not only tumor cells but also numerous microvessels, blood cells, and necrotic components. Moreover the GBM cells themselves are genetic heterogeneous, for example, EGFR-amplified cells are often scattered in the tumors
with mutated TP53 (102). Therefore when GBM samples are analyzed, we should select the methods overcoming the intrinsic heterogeneity in the tumor.

5.3. Purpose of this study

Recently several anti-EGFR target therapies for various tumors have been conducted in clinical managements or studied in clinical trials. To make these treatments offer as much therapeutic advantage as possible, it is indispensable to clarify whether EGFR expression is of clinical significance in GBM. Therefore we examined the relationship between outcome and EGFR gene status/EGFR expression in a uniform Japanese population, which was selected from adult patients with supratentorial primary GBM enrolled in clinical trials. We also assessed whether EGFRvIII expression plays a determining role in the prognosis of GBM patients.
6. Materials and Methods

6.1. Patient Population

Our patient population consisted of 87 newly diagnosed adults with histologically verified supratentorial GBM. Histopathological examination was conducted by two neuropathologists (J-i. K. and Y. I.) according to criteria published by the WHO (103, 104): tumors exhibiting prominent microvascular proliferation and/or necrosis, in addition to high cellularity, marked nuclear atypia, and remarkable mitotic activity were diagnosed as GBM. The presence of necrosis was a requisite for a diagnosis of GBM; cases without necrosis were excluded. After surgery, patients enrolled in two prospective randomized phase III trials conducted at Kumamoto University Hospital, Kagoshima University Hospital, and affiliated hospitals received combined radiotherapy and nitrosourea (ACNU)-based chemotherapy. Protocol 8701 (active from December 1987 to June 1995) compared the effectiveness of intra-arterial ACNU administration versus intravenous ACNU administration (105). Protocol 9501 (active from July 1995 to April 2003) compared the effectiveness of PAV versus PAV plus IFN-β. Patients enrolled in protocol 8701 were ≥15 years; 8 patients were ≥70 years. Patients treated according to protocol 9501 were from 15 to 69 years of age: none were ≥70 years. Some of the patients enrolled in these trials were excluded from our study because they did not receive combined radiotherapy and chemotherapy, because they died of other diseases, or because their follow-up was <6 months on the day of analysis or their last known day of life. After histopathological reexamination, also excluded were enrolled patients whose tumors, because they had a significant oligodendroglial component, were reclassified as anaplastic oligoastrocytomas (106, 107). On the day of analysis, 166 patients were eligible to participate in this study (75 of 84 from protocol 8701 and 91 of 99 from protocol 9501). Adequate tumor samples for gene analysis by Southern blots
were obtained from 87 patients (23 enrolled in protocol 8701 and 64 in protocol 9501).

In a comparison of 23 analyzable versus 52 unanalyzable patients enrolled in protocol 8701, there was no statistically significant difference in the median age (53 versus 55 years, respectively. $P=0.36$, Mann-Whitney U test), the median KPS (70 versus 60, respectively, $P=0.07$, Mann-Whitney U test), the gender distribution (analyzable group: 12 males, 11 females; unanalyzable group: 30 males, 22 females; $P=0.66$, $\chi^2$ test), and distribution of surgery (GTR, PR, and biopsy in the analyzable and unanalyzable groups: 43.5, 52.2, and 4.3% versus 32.7, 50.0, and 17.3%: $P=0.28$, $\chi^2$ test). In a comparison of 64 analyzable versus 27 unanalyzable patients enrolled in protocol 9501, there was no statistically significant difference in the median age (55 versus 58 years. $P=0.23$), the median KPS (70 versus 80. $P=0.50$), and the gender distribution (analyzable group: 42 males, 22 females; unanalyzable group: 13 males, 14 females; $P=0.12$). The only characteristic that was significantly different among patients in protocol 9501 was the extent of surgery: of the analyzable group, 32.8, 56.3, and 10.9% underwent GTR, PR, and biopsy, respectively, compared with 11.1, 48.2, and 40.7%, respectively, of unanalyzable patients ($P<0.01$). Among analyzable patients, thus, in many cases, the biopsy procedure yielded an insufficient amount of tumor tissue for gene analysis. In addition, the number of analyzable patients in the earlier protocol (#8701) was smaller than in protocol 9501 (30.7 versus 70.3%. $P<0.01$; $\chi^2$ test). because in many cases, the amount of frozen, stored samples from all of the procedures was insufficient for further analysis. With respect to OS, there was no difference between patients who were assayed and those who were not; the median OS was 1.232 and 1.103 years, respectively ($P=0.67$, Log-rank test), among patients in protocol 8701 and 1.366 and 1.114 years ($P=0.09$) among those in 9501; it was 1.262 and 1.114 years, respectively ($P=0.18$), for all patients in both protocols.

Written informed consent to participate in the clinical trials and in gene analysis was obtained from all patients and/or their family members.
6.2. **Samples for Gene Analysis**

Tumor tissue samples were immediately frozen and stored at \(-80^\circ\text{C}\) until the extraction of genomic DNA. The mean quantity of tumor sample judged sufficient for subsequent Southern blot analysis was \(0.220 \pm 0.118\) gram. Control specimens from 4 patients operated for diseases other than brain tumors consisted of histologically normal brain tissues; these were handled in the identical manner. We verified electrophoretically that there was no oligonucleosomal DNA fragmentation in the tumor samples, confirming that the tumor samples contained no significant necrotic components.

6.3. **Analysis of the EGFR Gene Status**

For quantitative detection of the EGFR gene status, we performed Southern blot analysis using full-length human *EGFR* cDNA (108) as the probes. The probes hybridized to all *EGFR* exons and for them to be considered adequate, they had to permit the detection of *EGFR^{wt}* and in-frame deletion mutations including *EGFR^{III}*. To prepare non-RI, digoxigenin-11-dUTP-labeled random primed DNA probes, *EGFR* cDNA, and full-length human *α-tubulin* cDNA (pEGFP-Tub Vector, BD Biosciences Clontech, Palo Alto, CA) were labeled using a DIG DNA-labeling kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Genomic DNA from frozen tissue samples was extracted by methods described previously (109-111). Genomic DNA (10 µg) digested with *EcoRI* was electrophoretically separated on 0.8% agarose gels, and DNA fragments were then transferred to nylon membranes (Roche). The blots were hybridized with *EGFR* cDNA probes in a hybridization buffer [5 x standard saline citrate, 0.02% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine NaCl, and 1% (w/v) blocking
After overnight incubation at 65°C, the membranes were washed and probed with Anti-Digoxigenin-AP (Roche). The blots were subjected to luminescence reaction using CSPD (Roche). Autoradiographs were taken using x-ray film (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) and intensifying screens. The blots were then stripped and rehybridized with α-tubulin reference probes. Using the Scion Imaging software program (Scion Corp., Frederick, MD), the signal intensities emitted by EGFR fragments from tumor- and normal human brain samples were compared, and the normalized ratio was determined using a reference gene. A cutoff value was set after determining the EGFR gene status of normal human brain tissue samples, and a value of 2.0 was chosen as the threshold. Normalized ratios ≥ 2.0 were considered indicative of amplification; values < 2.0 threshold were recorded as no amplification.

6.4. Monoclonal Antibodies

Mouse monoclonal antibodies were used as primary antibodies: (a) clone EGFR.25 (Novocastra Laboratories Ltd, Newcastle, United Kingdom), which recognizes 200 amino acids of the intracellular domain of the EGFR molecule, excluding the conserved tyrosine kinase domain; (b) clone EGFR.113 (Novocastra), which recognizes the extracellular domain of EGFR molecule (55); and (c) clone DH8.3 (Novocastra), which recognizes only the junctional truncated extracellular domain of EGFRvIII. It has been confirmed that DH8.3 does not cross-react with full-length EGFR (112-114).

6.5. Immunofluorescence Microscopy

To confirm the specificity of monoclonal antibodies used in this study for each
EGFR, fluorescence immunocytochemistry was performed on stable cell lines: (a) U87 MG parental cells; (b) U87 MGwtEGFR cells (EGFRwt overexpressed); and (c) U87 MGΔEGFR cells (EGFRvIII overexpressed) kindly gifted by Cavenee et al. (75, 85). The cells were grown on a 35-mm Petri dish, fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min. The cells were blocked with 5% goat serum/PBS for 60 min at room temperature and then incubated with each primary antibody (1:300 dilution in 0.2% BSA/PBS) at room temperature for 60 min. The primary antibodies were visualized with fluorescein-conjugated goat anti-mouse IgG (1:300 dilution; Biosource, Camarillo, CA) for 45 min. The stained cells were mounted with 2.5% 1,4-diazabicyclo [2.2.2] octane/glycerol and analyzed under a fluorescent microscope (BX 52; Olympus Optical Co., LTD., Japan).

6.6. Immunohistochemical Technique to Determine EGFR Expression

Paraffin-embedded tissue sections (3 μm) were placed on glass slides and dried. After routine deparaffinization, rehydration, and blocking of endogenous peroxidase activity, microwave-enhanced antigen retrieval was performed (115). Slide-mounted sections immersed in 0.01 M sodium citrate buffer (pH 6.0) were placed for 15 min into a 700W microwave oven at maximum power. After blocking nonspecific protein binding with 3% BSA/PBS, the sections were incubated with primary antibodies: EGFR.25 (dilution 1:100), EGFR.113 (dilution 1:100) at room temperature for 1 hr, and DH8.3 (dilution 1:20) at 4°C overnight. In subsequent steps, we used the Vectastain ABC kit and 3,3'-diaminobenzidine as the chromogen (Vector, Burlingame, CA). The sections were lightly counterstained with hematoxylin. Positive and negative controls were included with each batch of sections to confirm the consistency of the analysis. Sections were examined for immunoreactivity of each EGFR by at least one independent
neuropathologist who was unaware of the patients' outcomes or clinical features. The membrane and/or cytoplasm of cells were typically stained for EGFR. EGFR expression was scored according to the intensity of staining and number of stained tumor cells as 0 (no staining), 1 (light or focal), 2 (moderate), and 3 (strong). For statistical analysis, scores of 0 or 1 were defined as no overexpression; scores of 2 and 3 as overexpression.

6.7. Clinical Details

Clinical details, including the patient's age at entry into the trial, gender, preoperative KPS score, extent of surgical resection, protocol number, and the recorded date of disease progression or death, were notated. The goal of the operation was to remove as much tumor as possible. Except for the deep-seated lesions, such as thalamus and basal ganglia, craniotomy and surgical resection were carried out. To identify the extent of resection, contrast-enhanced neuroimaging data, i.e., computed tomograms or magnetic resonance images, were obtained within 1-2 weeks; starting in 1994, these were obtained within 72 hr of surgery to easily exclude the effect of time-lapse changes attributable to the surgical procedure (105, 116). GTR was recorded when there were no contrast-enhanced lesions, subtotal resection when < 10% of the preoperatively contrast-enhanced lesion remained, and PR when ≥ 10% of the contrast-enhanced lesion was noted. Subtotal resection and PR were subsumed into the PR classification. When the lesion was deep seated and considered inaccessible for direct removal, biopsy was performed by stereotactic surgery techniques using the Leksell apparatus. To harvest diagnostic tissue specimens, we selected one or two targets in the enhanced lesions on 3-mm-thick contrast-enhanced magnetic resonance image.
6.8. Statistical Analysis

For outcome analysis, patients were classified according to the presence or absence of EGFR amplification or EGFR overexpression. OS was calculated as the interval between trial entry and the day of death attributable to tumor recurrence. Patients whose day of death was uncertain were censored on the last known day of life; patients alive on the day of analysis were censored on April 30, 2003. Other potential prognostic variables were age (≥55 versus <55 years), gender, surgery (GTR versus PR), preoperative KPS score (40-60 versus 70-100), and enrollment protocol (8701 versus 9501); there was no significant difference in survival time between the two treatment arms of the protocol's (data not shown). The Log-rank test was used for univariate analysis to estimate differences in survival times for these variables. To plot survival curves, we used the Kaplan-Meier method. Using the Cox proportional hazards regression model, multivariate analysis was performed in a backward manner. Possible correlations between patient age and EGFR gene status/EGFR expression were based on the unpaired t test and the correlation between the EGFR gene status and EGFR expression score on the Mann-Whitney U test. All calculations were performed with commercially available software (Statview, Version 5.0; Abacus Concepts, Inc., Berkeley, CA). A probability value of < 0.05 was considered statistically significant. This study was approved by The Committee for the Development of Advanced Medicine at Kumamoto University Hospital.
7. Results

7.1. Assessment of Clinical Characteristics

Table 1 shows the clinical characteristics of the 87 GBM study subjects. All 87 patients (54 males and 33 females; ratio 1.64:1) were Japanese. Their median age was 54 years (range 17-78 years); 45 patients (51.7%) were < 55, 39 (44.8%) were from 55 to 69 years, and 3 (3.5%) were ≥70 years. The number of patients in protocol 8701, which enrolled subjects ≥70 years, was small (23 of 87: 26.4%). The median preoperative KPS score was 70 (range 40-100): 31 (35.6%) patients underwent GTR. 48 (55.2%) underwent PR. Because the number of biopsied patients (n=8; 9.2%) was small, biopsy was subsumed into the PR classification in statistical analyses. The median OS was 1.262 (range 0.142-7.422) years. On the day of analysis, 74 patients (85.1%) were dead; 13 (14.9%) were alive and censored.

7.2. EGFR Gene Status

To determine the cutoff value for EGFR amplification, the EGFR gene status of normal human brain tissue samples was examined. The normalized ratio of EGFR varied between > 0.5 and < 2.0 (data not shown); 2.0 was chosen as the threshold, and normalized ratios ≥2.0 were considered indicative of gene amplification. When we examined the EGFR gene status of tumor samples (Fig. 1), 40 (46.0%) of 87 patients manifested EGFR amplification with normalized ratios ranging from 2.1 to 75.5.

7.3. Specificities of Primary Antibodies

To confirm the specificity of the primary antibodies for each EGFR, fluorescent
immunocytochemistry was performed using the following cell lines: (a) U87 MG parental cells; (b) U87 MG wtEGFR cells whose cell surface overexpressed EGFRwt, and (c) U87 MG ΔEGFR cells whose cell surface overexpressed EGFRvIII. Because U87 MG parental cells express little endogenous EGFR, it is impossible to detect EGFR. However, it is possible to detect EGFR if cells with exogenous overexpression of EGFR are used and cells with EGFR overexpression are suitable for evaluation of the specificity of anti-EGFR antibodies. As shown in Fig. 2, EGFR.25 was reactive to the cell surface of both U87 MG wtEGFR- and U87 MG ΔEGFR cells (Fig. 2, D and G), but not U87 MG parental cells (Fig. 2A). On the other hand, EGFR.113 was reactive to the cell surface of only U87 MG wtEGFR cells (Fig. 2E): there was no immunoreactivity with either U87 MG ΔEGFR- or U87 MG parental cells (Fig. 2, B and H). As in other reports (112-114), the specificity of DH8.3 for U87 MG ΔEGFR cells expressing EGFRvIII was confirmed (Fig. 2, C, F and I). Therefore, we used EGFR.25 for the evaluation of EGFR, including EGFRwt and EGFRvIII, EGFR.113 for the evaluation of EGFRwt, and DH8.3 for the evaluation of EGFRvIII.

7.4. **EGFR Expression**

Table 2 shows the correlation between the **EGFR** gene status and the EGFR expression level in 87 GBM patients. Of 40 patients with **EGFR** amplification, 39 (97.5%) manifested EGFR overexpression, *i.e.*, scores ≥2, whereas 46 (97.9%) of 47 patients without **EGFR** amplification had no EGFR overexpression; there was a close correlation between the presence of the **EGFR** gene and EGFR expression (*P*<0.0001). In one case (GB164) with **EGFR** amplification but no EGFR overexpression (expression score 1), DH8.3 revealed overexpression of EGFRvIII (Fig. 3, D-F). Of 40 patients with **EGFR** amplification, 27 (67.5%) manifested EGFRwt overexpression; no tumors without **EGFR** amplification overexpressed EGFRwt (*P*<0.0001). EGFR.25 showed
that all tumors with EGFRwt overexpression overexpressed EGFR (Fig. 3, A and B). Irrespective of the presence or absence of EGFR amplification, 70 of the 87 (80.5%) patients manifested EGFRvIII expression: overexpressed EGFRvIII was found in 18 (45.0%) of 40 patients with and 4 (8.5%) of 47 patients without EGFR amplification ($P<0.0001$). There were 18 amplification-positive, EGFRvIII-overexpressing tumors; EGFR.25 revealed that all but one (GB164) manifested EGFR overexpression (Fig. 3, A and C); 4 amplification-negative tumors with EGFRvIII overexpression had EGFR expression scores of 1, i.e., no overexpression, by EGFR.25 (data not shown). As shown in Table 3, of 40 EGFR amplification-positive patients, 8 (20.0%) had neither EGFRwt nor EGFRvIII overexpression (Fig. 3, H and I); however, EGFR.25 showed that all 8 manifested overexpression of EGFR (Fig. 3G).

7.5. Statistical Analysis

We next examined the possibility of a correlation between patient age and the EGFR gene status/EGFR expression (Table 4). Although the mean age of patients with EGFR amplification or EGFR overexpression tended to be higher than that of patients without, no statistically significant difference was found ($P=0.085$ versus 0.088).

The results of univariate analysis for OS are shown in Table 5. The median OS of patients with EGFR amplification was significantly shorter than in those without (1.199 versus 1.684 years, $P=0.0070$, Fig. 4A). Similarly, compared with patients without overexpression, OS was significantly shorter in patients with overexpressed EGFRwt ($P=0.014$). However, EGFRvIII overexpression did not have a significant negative impact on OS ($P=0.081$). Higher age and a worse preoperative KPS also had a significant negative impact on OS ($P=0.0001, 0.041$, respectively). Gender, the extent of surgery, and the protocol did not have a significant negative impact on OS ($P=0.42, P=0.80$, and $P=0.79$). Gender and protocol were excluded as covariates in
subsequent multivariate analysis.

To test the prognostic value of the $EGFR$ gene status and of EGFR expression, we performed multivariate analysis for OS on the 87 GBM patients (Table 6). We found that $EGFR$ amplification was an independent, significant, poor prognostic factor for OS ($P=0.038$, $HR=1.67$). The prognostic value of EGFRwt and EGFRvIII expression was not sufficient to reach statistical significance. Age was the only other independent significant predictor for OS ($P=0.001$, $HR=2.26$). KPS was eliminated as a significant variable, although it was an independent significant factor, unless EGFR was also included in this model as a covariate (data not shown).

We next tested the relationship of age to EGFR prognostication in GBM patients. As shown in Table 7, we chose the typical median age of 55 years and 60 years as the thresholds and divided the patients into two groups, i.e., an older versus a younger age group (patients $\geq 60$ versus $<60$ years and patients $\geq 55$ versus $<55$ years, respectively). Then, the prognostic significance of the $EGFR$ gene status in individual groups was tested by univariate and multivariate analysis. Multivariate analysis by the Cox regression model in a backward manner included adjustments for KPS, surgery, EGFRwt, and EGFRvIII. In each younger age group, especially in patients $<60$ years, $EGFR$ gene amplification played a stronger role in survival than in all 87 patients (1.133 versus 2.324 years, $P=0.0002$; Fig. 4B). Similarly, by multivariate analysis, the prognostic significance of the $EGFR$ gene status was more pronounced in each younger age group ($P=0.0054$, $HR=2.72$; $P=0.0003$, $HR=3.15$). On the other hand, in neither of the two older age groups was the $EGFR$ gene status/EGFR expression of prognostic significance. With respect to other significant variables, KPS was the only independent significant variable in each older age group; there was no significant variable except for the $EGFR$ gene status in each of the younger groups (data not shown). The prognostic value of EGFRvIII expression was not sufficient to reach statistical significance in any of the groups categorized by age.
To determine the clinical significance of EGFRvIII expression, further analysis was carried out. As shown in Fig. 4C, of 40 patients with EGFR amplification, those with EGFRvIII overexpression manifested significantly shorter OS than did patients without (median OS 0.893 versus 1.374 years, \( P=0.0031 \)). In addition, multivariate analysis on the 40 amplification-positive patients revealed that EGFRvIII overexpression was an independent, significant, poor prognostic factor for OS (\( P=0.0044 \), HR=2.71; Table 8). Age was eliminated as a significant variable.
8. Discussion

There is experimental evidence that in GBM, *EGFR* amplification resulting in EGFR overexpression may signal an unfavorable prognosis. However, the results of clinical studies are currently inconclusive or inconsistent (20, 52, 56, 86-93). Using the Log-rank test, Hurtt *et al.* (90) demonstrated that in supratentorial GBM, *EGFR* amplification was significantly associated with shorter survival. However, their study lacked confirmation by multivariate analysis of the prognostic value of the *EGFR* alteration. On the other hand, Waha *et al.* (52) reported that multivariate analysis attributed no statistical value to *EGFR* amplification in terms of the survival of patients with grade 2-4 astrocytic gliomas. It is not clear from their report whether patient populations with the different tumor grades were clinically uniform. In fact, Simmons *et al.* (86) suggested that differences in the studied patient populations may explain the divergence in reported results. For our multivariate analysis of the prognostic value of the *EGFR* gene/EGFR protein status, we selected a uniform population among Japanese GBM patients enrolled in clinical trials carried out at our institutions.

We found that the frequency of *EGFR* amplification/EGFR overexpression was consistent with data reported by others (35, 51-54, 66, 117). As noted previously (35, 50), there was a close correlation between the *EGFR* gene status determined by Southern blots and the EGFR protein expression levels assessed by immunohistochemical analysis, especially when EGFR.25, which recognizes the intracellular domain of the EGFR molecule, was used (Table 2). Because full-length *EGFR* cDNA and EGFR.25 were able to widely detect EGFR, including EGFRwt and EGFRvIII, the gene status and expression level of EGFR was thought to be closely correlated. Interestingly, 8 (20%) of 40 GBM with *EGFR* amplification manifested neither EGFRwt nor EGFRvIII overexpression; however, EGFR.25 detected EGFR overexpression (Fig. 3, G- I; Table 3). The incidence of 20% was too high for attribution to differences in the affinity of the
different antibodies we used. This observation led us to suspect that these eight tumors expressed EGFR type(s) other than EGFRwt and EGFRvIII. EGFR.25 recognizes 200 amino acids of the intracellular domain of the EGFR molecule excluding the conserved tyrosine kinase domain. This antibody is thought to recognize the receptor internalization domain and the kinase inhibitory domain that are located nearer to the COOH-terminal tails than the tyrosine kinase domain. Our finding suggests the existence of other types of mutations that conserve the cytoplasmic domain near the COOH-terminal tails reported by others (68, 69, 118). EGFR.25 revealed that in 1 of 47 (2.1%) amplification-negative tumors, there was a distributed pattern of EGFR-overexpressed regions with an expression score of 2. This minimal inconsistency between the gene status and the protein expression level may be explicable by sampling errors attributable to regional heterogeneity in these tumors.

It has been documented that EGFR amplification/EGFR overexpression is significantly more frequent in older patients (57, 86, 89, 91). In our series, the mean age of patients with EGFR amplification and EGFR overexpression was 55.1 ± 10.1 years; however, their age was not significantly different from amplification- and overexpression-negative patients (Table 4). This may be attributable to the fact that only 3 of our 87 patients (3.5%) were ≥70 years (Table 1).

Simmons et al. (86), who also studied GBM patients enrolled in clinical trials, demonstrated that immunohistochemically confirmed EGFR overexpression was an independent, unfavorable prognostic factor in only a limited subgroup, i.e., a cohort whose age was less than the median age of 55 years of their study population and whose TP53 status was normal. On the other hand, our multivariate analysis confirmed that EGFR amplification was an independent, unfavorable predictor for survival in our study population (Table 6). However, we cannot rule out the possibility that our study carried an age bias because 96.5% of our patients (84 of 87) were < 70 years. In fact, the median survival time for our 87 patients was 1.262 years. similar to that of patients
enrolled in protocol 9501, which excluded patients ≥70 years (1.194 years, data not shown) and longer than that reported in other GBM series that included patients ≥70 years. We recognize that our study carries a bias for the younger group of GBM patients, and our results support the suggestion of Simmons et al. (86) that EGFR is most negatively prognostic in younger patients with GBM. In fact, in our patients younger than the typical median age of 55 - 60 years, the prognostic significance of the EGFR gene status was more pronounced than in all 87 patients; this was not true for the older age group (Fig. 4B: Table 7).

In the small cohort of GBM patients studied by Feldkamp et al. (93), those with EGFRvIII -positive tumors appeared to have shorter survival periods than did those with EGFRvIII -negative tumors. However, no statistical documentation was presented. We carried out multivariate analysis and found that the predictive value of EGFRvIII overexpression for survival was not sufficient to reach statistical significance (Table 6). However, among our GBM patients with EGFR amplification, those manifesting EGFRvIII overexpression had significantly shorter survival periods than those who did not (Fig. 4C). Multivariate analysis confirmed that EGFRvIII overexpression was an independent, unfavorable predictor for survival (Table 8). On the basis of the results presented here, we suggest that the overexpression of EGFRvIII in the presence of EGFR amplification is the strongest indicator of a poor survival prognosis.

It is controversial whether EGFRvIII occurs through alternative splicing or by gene rearrangements after amplification (67-71). In our series of 87 cases, 32 (36.8%) manifested EGFRvIII expression (scores ≥ 1) in the absence of EGFR amplification detected by Southern blots (Table 2). Although EGFR and TP53 are reportedly mutually exclusive in GBM (55), Okada et al. (102), who used fluorescence in situ hybridization, recently demonstrated that GBM with mutated TP53 frequently manifested EGFR gene amplification at the cellular level. It is possible that EGFRvIII occurs through gene rearrangements after low-level amplification of the EGFR gene in scattered
cells and that lysate-based approaches, such as Southern blot analysis, fail to detect this phenomenon.
9. Conclusions

We subjected our data to multivariate analysis and now present clinical evidence that EGFR amplification and EGFR overexpression including EGFRvIII play a significant role in the prognosis of GBM patients.

The present studies included only Japanese patients, i.e., a unique and racially homogeneous population. To develop target therapies against tumors expressing EGFR, we must have clinical evidence of the importance of the EGFR gene status/EGFR expression in racially diverse GBM patients. The results in this study are a step toward the development of therapies to treat GBM patients with EGFR amplification and/or EGFR overexpression. They also indicate that routine immunohistochemical studies that use combinations of antibodies are useful for assessing the EGFR expression status in GBM patients.
10. References


19. Shinojima N. et al. Gender and the presence of giant cells affect the postoperative
long-term survival in adult supratentorial glioblastoma multiforme. submitted.


28. Kleihues, P. and Ohgaki, H. Primary and secondary glioblastomas: from concept to


35. Ekstrand, A. J., James, C. D., Cavenee, W. K., Seliger, B., Pettersson, R. F., and Collins, V. P. Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in


growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing tumor cells


108. Okutani, T., Okabayashi, Y., Kido, Y., Sugimoto, Y., Sakaguchi, K., Matsuoka, K.,


114. Hills, D., Rowlinson-Busza, G., and Gullick, W. J. Specific targeting of a mutant,


# TABLES

Table 1. *Clinical characteristics of 87 GBM patients*

<table>
<thead>
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<th>Variable</th>
<th>Value</th>
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<td>Age (yrs)</td>
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<tr>
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<tr>
<td>No. (%)</td>
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<td>&lt;55 yrs</td>
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<td>Median, years (range)</td>
<td>1.262 (0.142 - 7.422)</td>
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</table>

<sup>a</sup> intra-arterial ACNU

<sup>b</sup> intra-venous ACNU
Table 2. Correlation between EGFR gene status and EGFR expression score in 87 patients with GBM

<table>
<thead>
<tr>
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</tr>
<tr>
<td>0</td>
<td>5.0 (2)</td>
<td>31.9 (15)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50.0 (20)</td>
<td>59.6 (28)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32.5 (13)</td>
<td>8.5 (4)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>12.5 (5)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* Percentage with raw numbers in parenthesis
Table 3. Correlation between EGFRwt and EGFRvIII expression in 40 GBM patients with EGFR amplification

<table>
<thead>
<tr>
<th>EGFRwt</th>
<th>+(^a)</th>
<th>-(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>32.5 (13)(^c)</td>
<td>35.0 (14)</td>
</tr>
<tr>
<td>-</td>
<td>12.5 (5)</td>
<td>20.0 (8)</td>
</tr>
</tbody>
</table>

\(^a\) Overexpression; \(^b\) No overexpression; \(^c\) Percentage with raw numbers in parenthesis

Table 4. Correlation between age and EGFR gene status/EGFR expression in 87 GBM patients

<table>
<thead>
<tr>
<th></th>
<th>Mean age (yrs ±SD)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR gene status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>55.1 ±10.1</td>
<td>0.085</td>
</tr>
<tr>
<td>No amplification</td>
<td>49.9 ±16.2</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td>0.088</td>
</tr>
<tr>
<td>Overexpression</td>
<td>55.1 ±10.0</td>
<td></td>
</tr>
<tr>
<td>No overexpression</td>
<td>50.0 ±16.3</td>
<td></td>
</tr>
<tr>
<td>EGFRwt</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Overexpression</td>
<td>55.7 ±8.37</td>
<td></td>
</tr>
<tr>
<td>No overexpression</td>
<td>50.8 ±15.6</td>
<td></td>
</tr>
<tr>
<td>EGFRvIII</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>Overexpression</td>
<td>56.1 ±13.8</td>
<td></td>
</tr>
<tr>
<td>No overexpression</td>
<td>51.0 ±13.8</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Univariate analysis for OS in 87 GBM patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (%)</th>
<th>Median OS (yrs)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥55</td>
<td>42 (48.3)</td>
<td>0.920</td>
<td>0.0001</td>
</tr>
<tr>
<td>&lt;55</td>
<td>45 (51.7)</td>
<td>1.621</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>Male</td>
<td>54 (62.1)</td>
<td>1.336</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>33 (37.9)</td>
<td>1.361</td>
<td></td>
</tr>
<tr>
<td>KPS</td>
<td></td>
<td></td>
<td>0.041</td>
</tr>
<tr>
<td>70-100</td>
<td>62 (71.3)</td>
<td>1.418</td>
<td></td>
</tr>
<tr>
<td>40-60</td>
<td>25 (28.7)</td>
<td>0.958</td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
<td>0.80</td>
</tr>
<tr>
<td>GTR</td>
<td>31 (35.6)</td>
<td>1.366</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>56 (64.4)</td>
<td>1.194</td>
<td></td>
</tr>
<tr>
<td>Protocol</td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>8701</td>
<td>23 (26.4)</td>
<td>1.232</td>
<td></td>
</tr>
<tr>
<td>9501</td>
<td>64 (73.6)</td>
<td>1.366</td>
<td></td>
</tr>
<tr>
<td>EGFR gene status</td>
<td></td>
<td></td>
<td>0.0070</td>
</tr>
<tr>
<td>Amplification</td>
<td>40 (46.0)</td>
<td>1.199</td>
<td></td>
</tr>
<tr>
<td>No amplification</td>
<td>47 (54.0)</td>
<td>1.684</td>
<td></td>
</tr>
<tr>
<td>EGFRwt</td>
<td></td>
<td></td>
<td>0.014</td>
</tr>
<tr>
<td>Overexpression</td>
<td>27 (31.0)</td>
<td>1.342</td>
<td></td>
</tr>
<tr>
<td>No overexpression</td>
<td>60 (69.0)</td>
<td>1.336</td>
<td></td>
</tr>
<tr>
<td>EGFRvIII</td>
<td></td>
<td></td>
<td>0.081</td>
</tr>
<tr>
<td>Overexpression</td>
<td>22 (25.3)</td>
<td>0.966</td>
<td></td>
</tr>
<tr>
<td>No overexpression</td>
<td>65 (74.7)</td>
<td>1.394</td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Multivariate analysis by the Cox proportional hazard regression model in a backward manner in 87 GBM patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥55 vs. &lt;55</td>
<td>2.26 (1.40 - 3.67)</td>
<td>0.001</td>
</tr>
<tr>
<td>KPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40-60 vs. 70-100</td>
<td>-</td>
<td>ns</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTR vs. PR</td>
<td>-</td>
<td>ns</td>
</tr>
<tr>
<td>EGFR gene status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification vs. No amplification</td>
<td>1.67 (1.03 - 2.72)</td>
<td>0.038</td>
</tr>
<tr>
<td>EGFRwt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpression vs. No overexpression</td>
<td>-</td>
<td>ns</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpression vs. No overexpression</td>
<td>-</td>
<td>ns</td>
</tr>
</tbody>
</table>

*aCI, confidence interval; bns, not significant.
Table 7. Prognostic value of EGFR gene status by univariate and multivariate analysis in older vs. younger groups according to age thresholds

<table>
<thead>
<tr>
<th>Age group</th>
<th>Median OS, yrs (no. of cases)</th>
<th>Univariate</th>
<th>Multivariate (Amp. vs. no amp.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amp. a</td>
<td>No amp. b</td>
<td>P</td>
</tr>
<tr>
<td>55 yrs c</td>
<td>Older (n=42)</td>
<td>0.931 (21)</td>
<td>0.901 (21)</td>
</tr>
<tr>
<td></td>
<td>Younger (n=45)</td>
<td>1.374 (19)</td>
<td>2.324 (26)</td>
</tr>
<tr>
<td>60 yrs c</td>
<td>Older (n=27)</td>
<td>1.262 (10)</td>
<td>0.865 (17)</td>
</tr>
<tr>
<td></td>
<td>Younger (n=60)</td>
<td>1.133 (30)</td>
<td>2.324 (30)</td>
</tr>
</tbody>
</table>

a Amplification.; b No amplification.; c Age threshold.; d Cl. confidence interval.; e ns, not significant.

Table 8. Multivariate analysis by the Cox proportional hazard regression model in a backward manner in 40 GBM patients with EGFR amplification

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI) a</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>≥55 vs. &lt;55</td>
<td>-</td>
</tr>
<tr>
<td>KPS</td>
<td>40-60 vs. 70-100</td>
<td>-</td>
</tr>
<tr>
<td>Surgery</td>
<td>GTR vs. PR</td>
<td>-</td>
</tr>
<tr>
<td>EGFRwt</td>
<td>Overexpression vs. No overexpression</td>
<td>-</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>Overexpression vs. No overexpression</td>
<td>2.71 (1.36 - 5.39)</td>
</tr>
</tbody>
</table>

a Cl. confidence interval.; b ns, not significant.
Figure 1. *EGFR* gene amplification detected by Southern blot analysis in GBM tumor samples. Comparison of the signal intensity of a 6.1-kb EcoRI fragment of an *EGFR* gene and a 3.0-kb fragment of an *α-tubulin* gene in tumor samples (GB99, GB249, AA36, GB37) and normal brain tissue samples (NB). The normalized ratio of the signal intensity of the *EGFR* fragment was determined by that of *α-tubulin*. The numbers at the bottom of the figure are the normalized ratios. *EGFR* amplification was found in GB99, GB249, and GB37.
Figure 2. Immunoreactivity with EGFRwt and EGFRvIII of monoclonal antibodies EGFR.25, EGFR.113, and DH8.3 by fluorescent immunocytochemistry.

Shown are cell lines U87MG.parental cells (A-C), U87 MG.wtEGFR cells (D-F), and U87 MG.ΔEGFR cells (G-I). The cells were treated with the following monoclonal antibodies: EGFR.25 (left panels), EGFR.113 (middle panels), and DH8.3 (right panels). EGFR.25 was reactive to both, U87 MG.wtEGFR cells whose cell surface overexpressed EGFRwt, and to U87 MG.ΔEGFR cells whose cell surface overexpressed EGFRvIII. EGFR.113 was reactive to only U87 MG.wtEGFR cells, and DH8.3 was reactive to only U87 MG.ΔEGFR cells.
Figure 3. Expression patterns of each EGFR in 3 cases with EGFR amplification.

Shown are 3 illustrative cases with EGFR amplification. The expression of EGFR, EGFRwt, and EGFRvIII is shown in the left, middle, and right panels, respectively. GB249 (A, C, E), the same case as shown in Fig. 1, manifested overexpression of EGFR (A, expression score 3); EGFRwt (B, expression score 3), and EGFRvIII (C, expression score 3) in the same area. GB164 (D, F) manifested neither overexpression of EGFR (D, expression score 0), nor EGFRwt (F, expression score 0). However, there was overexpression of EGFRvIII (F, expression score 3) in the same area. GB254 (G, H, I) manifested overexpression of neither EGFRwt (H, expression score 0) nor EGFRvIII (I, expression score 0). However, there was overexpression of EGFR (G, expression score 3) in the same area. Each original magnification: x100.
Figure 4. OS according to EGFR gene status and EGFRvIII expression in 87 patients with GBM. Shown are Kaplan-Meier survival curves of OS for all 87 patients, irrespective of the presence or absence of amplification (A), 60 patients < 60 years, irrespective of the presence or absence of amplification (B), and 40 amplification-positive patients with or without EGFRvIII overexpression (C). Among all 87 patients, those with EGFR amplification (n=40, solid line) had significantly shorter survival periods than did patients without (n=47, dotted line) (A, P=0.0070). Among 60 patients < 60 years, those with EGFR amplification (n=30, solid line) had significantly shorter survival periods than did patients without (n=30, dotted line) (B, P=0.0002). Among 40 patients with EGFR amplification, those with EGFRvIII overexpression (n=18, solid line) had significantly shorter survival periods than did patients without (n=22, dotted line) (C, P=0.0031).