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Prognostic Value of Epidermal Growth Factor Receptor in Patients with Glioblastoma Multiforme[1]

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ABSTRACT

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 and EGFR expression by immunohistochemistry using three monoclonal antibodies (EGFR.25 for EGFR, EGFR.113 for EGFRwt, and DH8.3 for EGFRvIII). *EGFR* amplification was detected in 40 (46%) 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%) 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.

INTRODUCTION

GBM[3] is the most common primary malignant neoplasm of the central nervous system in adults. Treatment outcomes even after multimodal therapies, including surgical resection, radiotherapy, and chemotherapy, remain poor; the median survival is ~1 year. Nevertheless, there is considerable variation among GBM patients with respect to survival. 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 (1–7). Advances in molecular biology disclosed the presence of molecular genetic alterations in GBM (8–11). The most frequent alteration of GBM oncogenes consists of amplification of the *EGFR* gene that results in overexpression of EGFR, a transmembrane tyrosine kinase receptor (12–18). *EGFR* amplification is present in 30–50% of all GBM; it occurs more frequently in primary (*de novo*) GBM (12–14, 18–26) and confers advantages of growth and invasiveness and radio- and chemo-resistance on tumor cells (27–32). Furthermore, the majority of GBM with *EGFR* amplification exhibit a considerable variety of qualitative *EGFR* alterations, resulting in different EGFR mutations (16, 17, 21, 33–35). 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 (33, 34, 36, 37). The extracellular ligand-binding domain of EGFRvIII is truncated, and unlike EGFRwt, EGFRvIII displays ligand-independent constitutive activity (38–40) and enhances tumorigenicity *in vivo* (32, 41, 42). There is experimental evidence that *EGFR* amplification may result in a less favorable prognosis; however, clinical studies are inconclusive (6, 13, 18, 43–49). Simmons *et al.* (43) suggested that differences in patient populations may explain the divergent results. To evaluate the prognosis of GBM patients, we examined the relationship between outcome and *EGFR* gene status/EGFR expression in a uniform Japanese population selected from newly diagnosed adult patients with supratentorial GBM enrolled in clinical trials. Feldkamp *et al.* (50) suggested that GBM patients with EGFRvIII may have a shorter life expectancy; however, they were unable to produce statistical evidence for this supposition. Therefore, we also assessed whether EGFRvIII expression plays a determining role in the prognosis of GBM patients. 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. We also document that routine immunohistochemical studies that use combinations of antibodies are useful for the assessment of EGFR expression.

MATERIALS AND METHODS

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 WHO (51, 52); 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

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[3] The abbreviations used are: GBM, glioblastoma multiforme; EGFR, epidermal growth factor receptor; EGFRwt, wild-type epidermal growth factor receptor; OS, overall survival; KPS, Karnofsky performance score; HR, hazards ratio; GTR, gross total resection; PR, partial resection; ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride; PAV, procarbazine, ACNU, vincristine.

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 (53). 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 $\geq$15 years; 8 patients were $\geq$70 years. Patients treated according to protocol 9501 were from 15 to 69 years of age; none were $\geq$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 (54, 55). 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; $P = 0.66$, χ^2 test), and the distribution of surgery (GTR, PR, and biopsy in the analyzable and unanalyzable groups: 43.5, 52.2, and 4.3% *versus* 32.7, 50, and 17.3%; $P = 0.28$, χ^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, $P = 0.5$), 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%, $P < 0.01$; χ^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.

Samples for Gene Analysis. Tumor tissue samples were immediately frozen and stored at -80°C until the extraction of genomic DNA. The mean quantity of tumor sample judged sufficient for subsequent Southern blot analysis was 0.22 ± 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.

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 (56) as the probes. The probes hybridized to all *EGFR* exons and for them to be considered adequate, they had to permit the detection of *EGFRwt* and in-frame deletion mutations, including *EGFRvIII*. 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 (57–59). Genomic DNA (10 μg) digested with *Eco*RI 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$\times$ standard saline citrate, 0.02% (w/v) SDS, 0.1% (w/v) N-laurosylsarcosine NaCl, and 1% (w/v) blocking reagent (Roche)]. 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 was chosen as the threshold. Normalized ratios $\geq$2 were considered indicative of amplification; values $<$2 threshold were recorded as no amplification.

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 (25); 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 (60–62).

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 MG.wtEGFR cells (EGFRwt overexpressed); and (*c*) U87 MG.ΔEGFR cells (EGFRvIII overexpressed) kindly gifted by Cavenee *et al.* (41, 63). 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).

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 (64). Slide-mounted sections immersed in 0.01 M sodium citrate buffer (pH 6.0) were placed for 15 min into a 700-W 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 h, 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.

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 h of surgery to easily exclude the effect of time-lapse changes attributable to the surgical procedure (53, 65). 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.

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 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 protocols (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.

RESULTS

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

Table 1 *Clinical characteristics of 87 GBM patients*

Median age yrs (range)	54 (17–78)
No. (%)	
<55	45 (51.7)
55–69	39 (44.8)
≥70	3 (3.5)
Median KPS	
Score (range)	70 (40–100)
Gender	
Male/female ratio	1.64/1
Surgery no. (%)	
GTR	31 (35.6)
PR	48 (55.2)
Biopsy	8 (9.2)
Protocol no. (%)	
8701 (n = 23)	
IA-ACNU[a]	10 (11.5)
IV-ACNU[b]	13 (14.9)
9501 (n = 64)	
PAV	34 (39.1)
PAV plus IFN-β	30 (34.5)
OS	
No. of censored (%)	13 (14.9)
Median, years (range)	1.262 (0.142–7.422)

[a] intra-arterial ACNU.
[b] intra-venous ACNU.

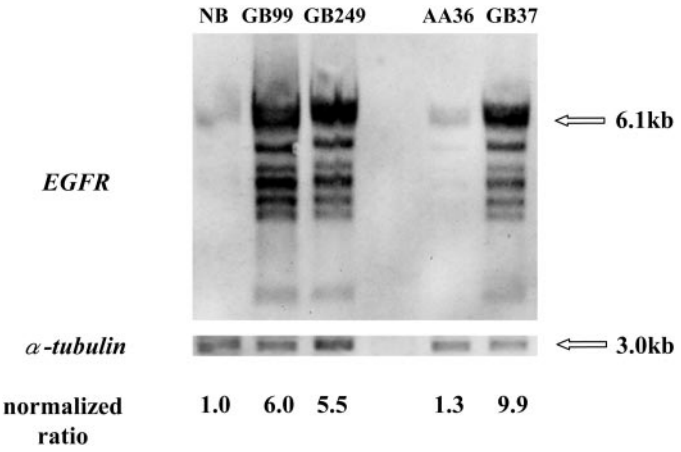

Fig. 1. *EGFR* gene amplification detected by Southern blot analysis in GBM tumor samples. Comparison of the signal intensity of a 6.1-kb *Eco*RI fragment of an *EGFR* gene and a 3-kb fragment of an *α-tubulin* gene in tumor samples (GB99, GB249, AA36, and GB37) and normal brain tissue samples (NB). 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.

(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.

***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 (data not shown); 2 was chosen as the threshold, and normalized ratios ≥2 were considered indicative of gene amplification. When we examined the *EGFR* gene status of tumor samples (Fig. 1), 40 (46%) of 87 patients manifested *EGFR* amplification with normalized ratios ranging from 2.1 to 75.5.

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. 2*A*). On the other hand, EGFR.113 was reactive to the cell surface of only U87 MG.wtEGFR cells (Fig. 2*E*); there was no immunoreactivity with either U87 MG.ΔEGFR− or U87 MG.parental cells (Fig. 2, *B* and *H*). As in other reports (60–62), 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.

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*

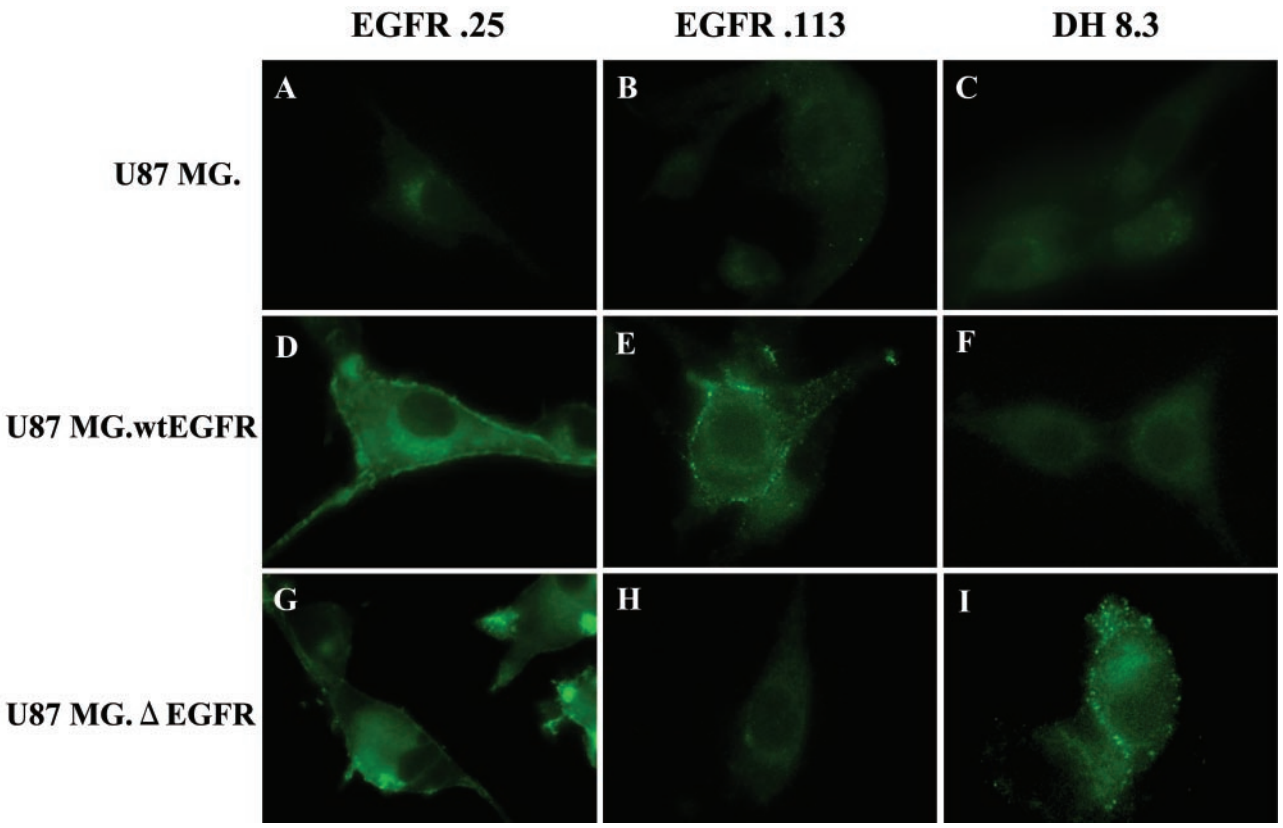

Fig. 2. Immunoreactivity with EGFRwt and EGFRvIII of monoclonal antibodies EGFR.25, EGFR.113, and DH8.3 by fluorescent immunocytochemistry. Shown are cell lines U 87MG. parental cells (*A–C*), U87 MG.wtEGFR cells (*D–F*), and U87 MG.ΔEGFR cells (*G–I*). The cells were treated with the following monoclonal antibodies: EGFR.25 (*left panels*), EGFR.113 (*center panels*), and DH8.3 (*right panels*). EGFR.25 was reactive to both, U87 MG.wtEGFR cells whose cell surface overexpressed EGFRwt, and 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.

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%) 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%) had neither EGFRwt nor EGFRvIII overexpression (Fig. 3, *H* and *I*); however, EGFR.25 showed that all 8 manifested overexpression of EGFR (Fig. 3*G*).

Table 2 *Correlation between EGFR gene status and EGFR expression score in 87 patients with GBM*

	EGFR gene status		
EGFR score	Amplification ($n = 40$)	No amplification ($n = 47$)	*P*
EGFR			<0.0001
0	0 (0)[a]	34.1 (16)	
1	2.5 (1)	63.8 (30)	
2	20.0 (8)	2.1 (1)	
3	77.5 (31)	0 (0)	
EGFRwt			<0.0001
0	20.0 (8)	63.8 (30)	
1	12.5 (5)	36.2 (17)	
2	27.5 (11)	0 (0)	
3	40.0 (16)	0 (0)	
EGFRvIII			<0.0001
0	5.0 (2)	31.9 (15)	
1	50.0 (20)	59.6 (28)	
2	32.5 (13)	8.5 (4)	
3	12.5 (5)	0 (0)	

[a] Percentage with raw numbers in parenthesis.

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, $P = 0.007$; Fig. 4*A*). 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 ($P = 0.42$, $P = 0.8$, 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 ($P = 0.038$,

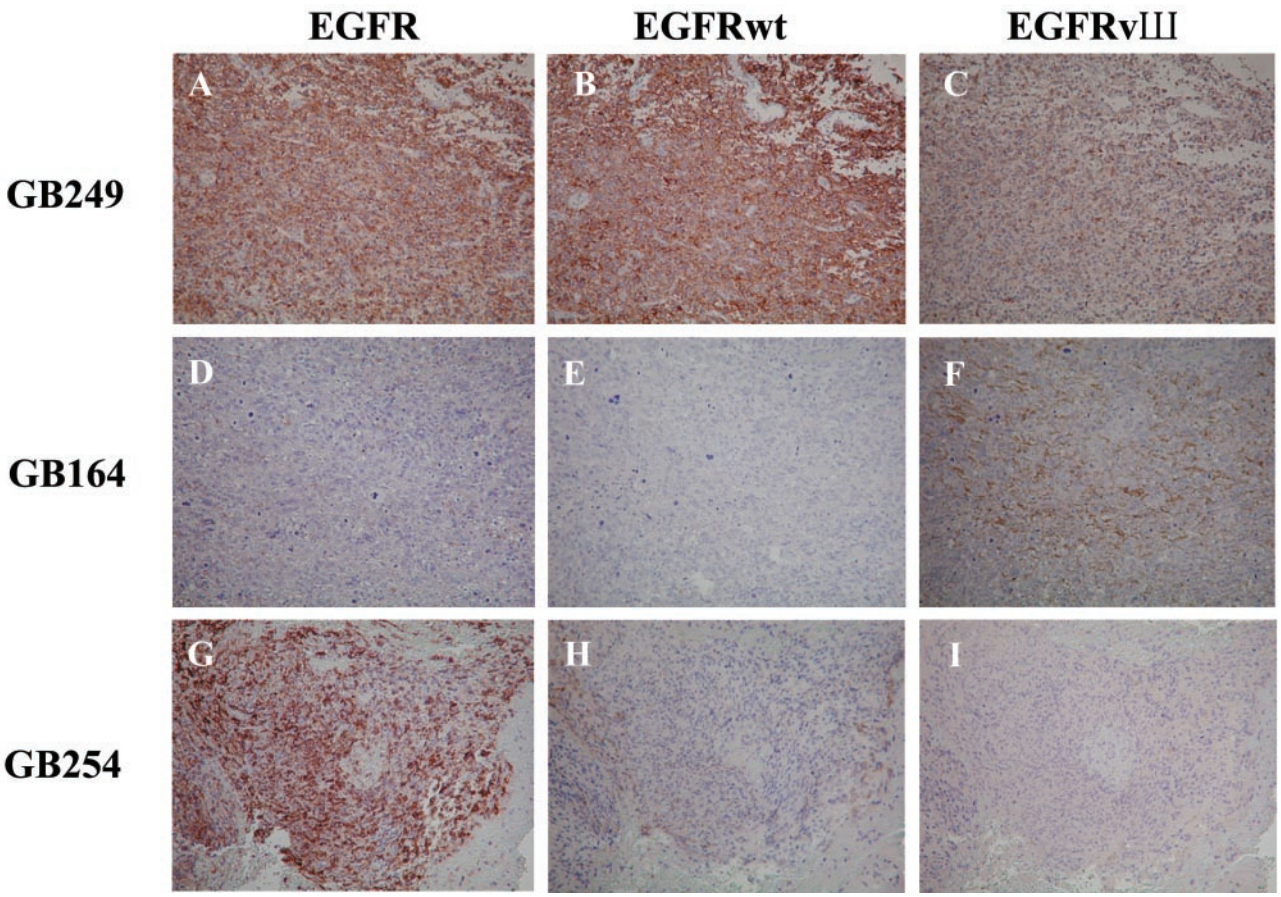

Fig. 3. Expression patterns of each EGFR in three cases with *EGFR* amplification. Shown are three illustrative cases with *EGFR* amplification. The expression of EGFR, EGFRwt, and EGFRvIII is shown in the *left, center,* and *right panels,* respectively. GB249 (*A–C*), the same case as shown in Fig. 1 manifested overexpression of EGFR (*A,* expression score 3), EGFRwt (*B,* expression score 3), and EGFRvIII (*C,* expression score 2) in the same area. GB164 (*D–F*) manifested neither overexpression of EGFR (*D,* expression score 1) nor EGFRwt (*E,* expression score 0). However, there was overexpression of EGFRvIII (*F*) in the same area (expression score 2). GB254 (*G–I*) manifested overexpression of neither EGFRwt (*H,* expression score 1) nor EGFRvIII (*I,* expression score 1). However, there was overexpression of EGFR (*G,* expression score 3) in the same area. Each original magnification: ×100.

Table 3 *Correlation between EGFRwt and EGFRvIII expression in 40 GBM patients with EGFR amplification*

	EGFRvIII	
EGFRwt	+[a]	−[b]
+	32.5 (13)[c]	35.0 (14)
−	12.5 (5)	20.0 (8)

[a] Overexpression.
[b] No overexpression.
[c] Percentage with raw numbers in parenthesis.

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 ≥ 60 *versus* <60 years and patients ≥ 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. 4*B*). 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,

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

	Mean age (yrs ± SD)	*P*
EGFR gene status		0.085
Amplification	55.1 ± 10.1	
No amplification	49.9 ± 16.2	
EGFR		0.088
Overexpression	55.1 ± 10.0	
No overexpression	50.0 ± 16.3	
EGFRwt		0.13
Overexpression	55.7 ± 8.37	
No overexpression	50.8 ± 15.6	
EGFRvIII		0.14
Overexpression	56.1 ± 13.8	
No overexpression	51.0 ± 13.8	

Table 5 *Univariate analysis for OS in 87 GBM patients*

Variable	No. (%)	Median OS (yrs)	*P*
Age (yrs)			0.0001
≥55	42 (48.3)	0.920	
<55	45 (51.7)	1.621	
Gender			0.42
Male	54 (62.1)	1.336	
Female	33 (37.9)	1.361	
KPS			0.041
70–100	62 (71.3)	1.418	
40–60	25 (28.7)	0.958	
Surgery			0.80
GTR	31 (35.6)	1.366	
PR	56 (64.4)	1.194	
Protocol			0.79
8701	23 (26.4)	1.232	
9501	64 (73.6)	1.366	
EGFR gene status			0.0070
Amplification	40 (46.0)	1.199	
No amplification	47 (54.0)	1.684	
EGFRwt			0.014
Overexpression	27 (31.0)	1.342	
No overexpression	60 (69.0)	1.336	
EGFRvIII			0.081
Overexpression	22 (25.3)	0.966	
No overexpression	65 (74.7)	1.394	

further analysis was carried out. As shown in Fig. 4*C*, 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.

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 (6, 13, 18, 43–50). Using the Log-rank test, Hurtt *et al.* (47) 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.* (13) 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.* (43) 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 (12–14, 20–23). As noted previously (12, 19), 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

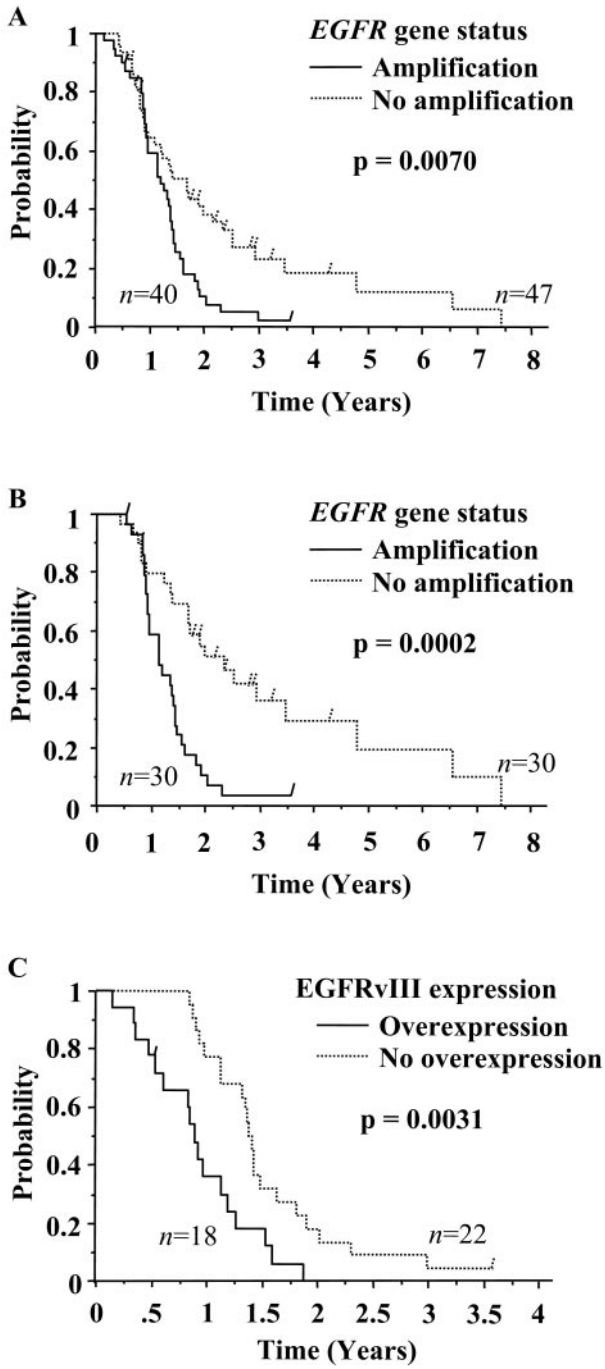

Fig. 4. OS according to *EGFR* gene status and EGFRvIII expression in 87 patients with GBM. Shown are Kaplan-Meier survival curves of OS for all 87 patients, irrespective of the presence or absence of amplification (*A*), 60 patients < 60 years, irrespective of the presence or absence of amplification (*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*, $P = 0.007$). 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*, $P = 0.0031$).

Table 6 *Multivariate analysis by the Cox proportional hazard regression model in a backward manner in 87 GBM patients*

Variable	HR (95% CI)[a]	*P*
Age (yrs)		
≥55 *vs.* <55	2.26 (1.40–3.67)	0.0010
KPS		
40–60 *vs.* 70–100		ns[b]
Surgery		
GTR *vs.* PR		ns
EGFR gene status		
Amplification *vs.* no amplification	1.67 (1.03–2.72)	0.038
EGFRwt		
Overexpression *vs.* no overexpression		ns
EGFRvIII		
Overexpression *vs.* no overexpression		ns

[a] CI, confidence interval.
[b] ns, not significant.

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 (34, 35, 66). 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 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 (26, 43, 46, 48). 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.* (43), 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.* (43) 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. 4*B*; Table 7).

In the small cohort of GBM patients studied by Feldkamp *et al.* (50), 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. 4*C*). 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 (33–37). 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 (25), Okada *et al.* (67), 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.

Our studies included only Japanese patients, *i.e.,* a unique and racially homogeneous population. To develop targeted therapies against tumors expressing EGFR, we must have clinical evidence of the importance of the *EGFR* gene status/EGFR expression in racially

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

Variable	HR (95% CI)[a]	*P*
Age (yrs)		
≥55 *vs.* <55		ns[b]
KPS		
40–60 *vs.* 70–100		ns
Surgery		
GTR *vs.* PR		ns
EGFRwt		
Overexpression *vs.* no overexpression		ns
EGFRvIII		
Overexpression *vs.* no overexpression	2.71 (1.36–5.39)	0.0044

[a] CI, confidence interval.
[b] ns, not significant.

Table 7 *Prognostic value of EGFR gene status by univariate and multivariate analysis in older vs. younger groups according to age thresholds*

	Median OS, yrs (no. of cases)			Multivariate (Amp. *vs.* no amp.)	
Age group	Amp.[a]	No amp.[b]	Univariate *P*	HR (95% CI)[d]	*P*
55 yrs[c]					
Older (n = 42)	0.931 (21)	0.901 (21)	0.71		ns[e]
Younger (n = 45)	1.374 (19)	2.324 (26)	0.0040	2.72 (1.34–5.50)	0.0054
60 yrs[c]					
Older (n = 27)	1.262 (10)	0.865 (17)	0.53		ns
Younger (n = 60)	1.133 (30)	2.324 (30)	0.0002	3.15 (1.69–5.89)	0.0003

[a] Amplification.
[b] No amplification.
[c] Age threshold.
[d] CI, confidence interval.
[e] ns, not significant.

diverse GBM patients. Our results 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.

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